Characterization of Blast-Induced Activation of Human Immune Cells

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science, in the Department of
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ABSTRACT

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Abstract

Blast related injuries have become a common occurrence among soldiers and civilians serving in Iraq and Afghanistan, and minor traumatic brain injuries associated with such incidents have increased correspondingly. Advances in protection and treatment have allowed many individuals to survive what would have previously been deadly blasts but there is a concern that there are additional negative side effects associated with such exposure. This study hypothesizes that human T leukocytes and promyelocytes respond to blasts by initiating cell death processes and releasing microparticles that could lead to further systemic inflammation. It was found that there was a significant ($p < 0.05$) increase in lactase dehydrogenase activity and microparticle release in HL-60 cells blasted using a shock tube (with an incident blast overpressure of either 1000 or 1300 kPA and a duration of 2 ms) compared to control populations after 24 hours. There were no corresponding increases in Jurkat cells exposed to similar conditions.

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Acknowledgements

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Introduction

Recent events on the international stage have resulted in a large increase in exposure to blast events by both soldiers and civilians (Elder, Risling, Saljo, Warden), such as those experienced by proximity to an IED. Because of the nature of these conflicts, it is often impossible to avoid such exposure. Advances in medical care and protective equipment have reduced the mortality of such exposures (Okie), but that has been accompanied by an apparent increase in chronic conditions associated with the blast, including traumatic brain injury (Gondusky). Compared with blunt trauma injuries, the clinical course of such injuries represents a conundrum with symptoms initially improving but worsening over long time scales (Bass 2011; Bass 2012). One hypothesis for this behavior is immune activation causing referred brain injury or systemic inflammation that causes similar symptomology, perhaps originating in extremities exposed to blast.

One potential source of this immune activation is microparticles - a class of small, membrane-bound vesicles containing cellular information. They have been implicated in cerebral inflammation and subsequent damage, and may possibly be a contributing factor in the development of autoimmune conditions such as rheumatoid arthritis (Distler). They are released from cells and enter circulation during apoptosis or as a response to cell damage (Horstman, Reich). Literature has suggested that inflammation may be a major pathological process associated with blast-related traumatic brain injury

(Arun). The goal of this research was to characterize the response of human T leukocytes and promyelocytes to blast exposure, particularly the expression and release of microparticles.

Exposure to blasts is also associated with post-traumatic stress disorder (PTSD) (Sundin), a neurological state characterized by chronic disruptions in the stress response systems of the central nervous system (Pace). Interestingly, there is an increased rate of comorbidity with somatic disorders that involve immune and inflammatory processes among patients with PTSD (Pace). These symptoms include cardiovascular disease (Boscarino 2008, Kibler), rheumatoid arthritis (Boscarino 2010, Qureshi), psoriasis, and thyroid disease (Boscarino 1999).

Exposing the cells of interest (in this case human T lymphocytes and promyeloctyes) to blast overpressures in a carefully-controlled environment may separate their response to primary blast injury from other conditions typically associated with explosive detonations. A careful observation of the behavior of cells following such exposure may provide new insight into the pathophysiology of blast injury with respect to immune activation and inflammation, and perhaps might offer clues toward a novel medical intervention that could prevent or treat such conditions.

Problem Statement

There is currently an incomplete understanding of the mechanisms of chronic and delayed brain damage that can result from exposure to blast trauma. It is the goal of this set of experiments to determine if aspects of the inflammatory processes can be initiated by blast in the hope of developing a better comprehension of the mechanisms that can lead to brain damage and chronic inflammatory conditions in blast victims.

Background

Blast Incidents

A blast is characterized by a sudden and rapid increase in pressure (shock) that quickly decays to a partial vacuum phase, then returns to ambient conditions and dissipates. The energy of this pressure travels radially outward from the source of the disruption (e.g. the detonation of an IED) and is transmitted by local fluid molecules, typically air or water. For the wave to qualify as a blast, the pressure front must expand at supersonic speeds, which can have disruptive effects on objects in their path, including human tissue (cf. Bass 2012).

Since the early days of World War I, there has been an interest in characterizing the effects of blast exposure on the body (Carver). In the earliest times, neurological symptoms were associated with blast exposure or "shell shock." It was unclear at the time whether the symptoms were the result of a psychological reaction to the stress and intensity of modern combat (Crocq) or physical neurotrauma (e.g. Carver). However, many of the symptoms (amnesia, headache, dizziness, confusion, and tinnitus) were consistent with acute brain trauma, though there was often no sign of external injury (Mott, Jones 2007). It was not until World War II that further efforts were made to understand the mechanisms of blast-related traumatic brain injury (Myers 1940, Krohn, Zuckerman 1940, Zuckerman 1941, Jones 2007). Starting from the end of World War II

blast research was largely concerned with studying the pulmonary consequences of blast waves (Bass 2008, Bass 2012, Martinez).

The character of recent conflicts has changed. The proportion of injuries caused by explosive mechanisms is higher in the modern warzone than has ever been seen in large-scale conflict (Owens). The dramatic recent increase in mild traumatic brain injury has led to it being dubbed "the signature injury of the wars in Iraq and Afghanistan," (Elder, Okie) with 10 to 20% of veterans returning from service suffering from such an injury (Goldstein, Schneiderman, Terrio). There is limited evidence of pulmonary injuries resulting from blast trauma (DePalma). However, modern body armor protects pulmonary organs against blast (Wood) increasing the relative risk to the extremities and other organs, such as the brain. In recent conflicts, it has been estimated that 97% of injuries to Marine units involved in Operation Iraqi Freedom II are the result of blasts; at least two thirds of these come from incidents involving improvised explosive devices (IEDs) (Gondusky).

Moreso than in any conflict in the past, soldiers who are exposed to explosions are surviving the initial incident, due to both improved protective equipment and better battlefield and medical treatments (Bellamy, Elder). Although the usual clinical course of mild TBI is complete recovery with no ongoing clinical signs or symptoms—indeed around 82% of soldiers experiencing blast are able to return to active duty after three days (Taber)—some of the soldiers exposed are permanently plagued by the effects of

this injury. As a result, they can suffer emotionally, personally, and financially (Owens). Thus, it has become increasingly necessary to develop an understanding of the conditions associated with such exposures to provide better long-term care for our veterans.

Many soldiers are exposed to blast trauma in the form of a roadside bomb detonation as their transport passes by. The improvised explosive device (IED) typically employed in these attacks is a 105 or 155-mm Howitzer shell packed with an explosive load equivalent to 2.4 or 7.3 kg of TNT, respectively (Panzer 2012a). Most convoys drive down the center of the road, so soldiers are typically five to ten meters away from a shell at the moment of detonation (Nelson). Soldiers threatened by these IED blasts can be exposed to peak incident overpressures of 50 to 1000 kPa with a 2 to 10 ms duration (Panzer 2012a).

The threshold for fatal blast-induced injuries to the pulmonary or gastrointestinal systems has been found to be lower than would produce death by brain injury; however it is held that with the torso protected by modern ballistic armor, it is possible for a human to sustain blast-induced brain injury (Bass 2012). It is estimated that a blast characterized by an 500 - 800 kPa overpressure and a duration of 5 ms can be expected to result in death by traumatic brain injury in 50% of cases (Rafaels, Ramasamy) (Figure 1). Even in cases where the head and torso are adequately protected, however, the extremities frequently remain vulnerable (Ramasamy).

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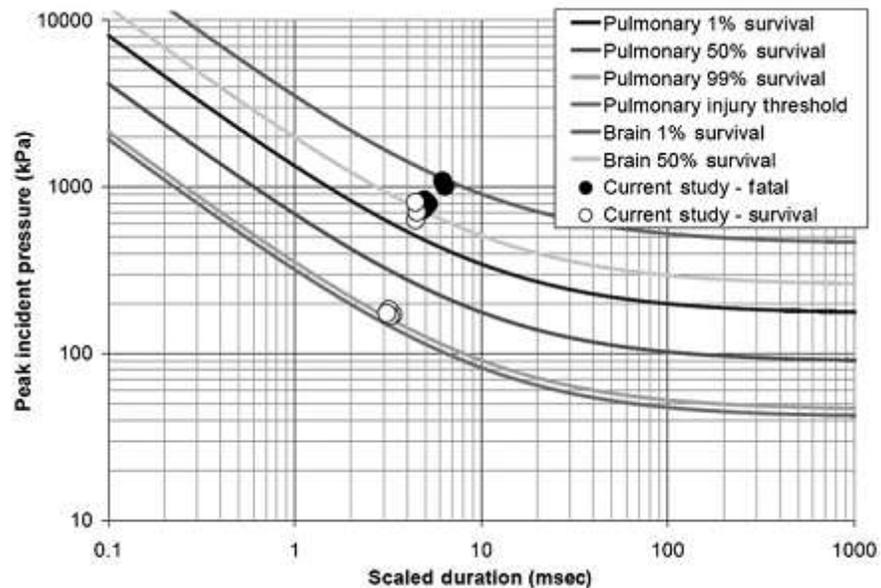
Figure 1: "Brain and pulmonary injury criteria across various overpressures and durations." (Rafaels)

Blast Exposure

Injuries from a blast fall into four broad categories (White). Primary blast injuries are caused by direct exposure to the extreme overpressures characteristic of a blast. Secondary blast injuries are caused by the effect of projectiles accelerated by the blast, such as shrapnel or flying debris. Tertiary injuries occur as a result of body translation. That is, injuries such as whiplash or broken bones that happen when the body is thrown by the blast. Quarternary injuries are any injuries not covered by the previous categories, and typically include exposure to heat, flame, or toxins involved in the explosion (Bass 2008).

Immune Cells

White blood cells, formally known as leukocytes, are a family of cells involved with the immune system. They circulate throughout the blood taking up about 1% of the total serum volume -- there are approximately 8000 white blood cells per microliter of whole blood (Othoro). There are five types of white blood cells, each associated with a certain immune function. Lymphocytes are the second most common type of blood cell and are further separated into three subclasses (Hoffman, Horne). Natural killer cells target and destroy cancerous and virus-infected cells. B cells "memorize", through a process akin to natural selection, the patterns of specific antigens and assist in the activation of T (thymus) cells. Only large, complex molecules may bind selectively to B cell antibodies, and so the system is tuned to detect harmful proteins, cells, and organisms (Rajewsky 1987, Rajewsky 1996). This is known as the adaptive immune response and it is complemented by the less specific, more "primitive" innate immune response (Medzhitov).

The innate immune response was for many years considered to act completely independently from the adaptive immune response (Medzhitov). It is "always on" and responds to cell wall components from bacterial or fungal organisms, or to components released from tissue damage. It is regulated by inflammatory mediators released from macrophages, peripheral immune cells, and microglia. These mediators include cytokines interleukin-1beta, interleukin-6, and TNF-alpha (Jones 2012). IL-1beta is

considered the master cytokine as its release triggers the release of other important components of the acute immune response (Dinarello, Goshen). IL-6, the release of which is triggered by IL-1beta, signals tissue injury systemically (Goshen). Neutrophils, the most abundant type of white blood cell, play an important role in the first response to injury or infection by flocking to a site marked by initiator cytokines released from damaged cells and releasing cytokines to amplify the inflammatory reaction in other cells (Hickey).

It is important to note that IL-1beta and TNF-alpha can enter the brain by active transport (Skinner, Gutierrez) where they are capable of inducing their own synthesis in a pathway that can lead to prolonged inflammation of the brain (Pitossi). In certain disease states, this inflammatory response can actually become a part of the pathological process (Jones 2012, O'Byrne, Anisman, Maes, Sluzewska).

Neutrophils are the most abundant class of mammalian white blood cells. They are a type of granulocyte (white blood cells characterized by cytoplasmic granulocytes) and are "first-responders" in the innate immune system. Neutrophils are recruited to the site of inflammation by chemotaxis toward cytokines (notably interleukin-8) released by damaged tissue or other immune activators. Once there, they release granules of antimicrobial enzymes and can eliminate, by phagocytosis, antigens that have been marked for destruction (Burg). The promyelocytes involved in this study (HL-60 cells) are undifferentiated precursors to neutrophils; though they are found in the bone

marrow rather than the blood, they still exhibit some phagocytic activity and granulation (Collins, Gallagher).

T cells are responsible for the bulk of the cell-mediated immune response (Ross). Once activated by B cells that have sensed an appropriate antigen, they reproduce and mobilize to bind to and destroy (through phagocytosis) the antigen-presenting cells or proteins (Frei). Delta-gamma T cells are considered to act as a link between the innate and adaptive immune responses, depending on how they are activated (Bachmann, Holtmeier).

Apoptosis

Apoptotic processes occur as part of normal tissue function and lead to programmed cell death and destruction. A careful balance between cell proliferation and cell death is required to maintain homeostasis, particularly with respect to lymphocytes (Rathmell). Many apoptotic events take place during growth and differentiation (Strasser), but may also be triggered to reduce the spread of disease or in response to less immediately-fatal cell damage (Thompson). Studies suggest that blast-like trauma to certain types of cells may be sufficient to activate surface receptors on the cell membrane and initiate the apoptotic self-destruct cascade (Alford, Hemphill).

It is known that apoptosis can be initiated internally, through the mitochondrial pathway, or externally, in response to cell damage (Elmore). For many years, these two mechanisms were thought to be distinct, but recent research has indicated that there is a

degree to which the molecules in each pathway may influence the other (Igney). Additionally, cytotoxic T-lymphocytes and natural killer cells may induce the apoptotic process (Martinvalet); there is thus a concern that damage to the T-cells may initiate a cascade effect which can result in the activation or destruction of many more immune cells and surrounding tissues (O'Byrne).

Apoptosis begins with a noticeable shrinkage of the cell. Chromatin within the nucleus and cytoplasm begins to contract and condense; this process is known as pyknosis and is one of the most characteristic features of apoptosis. This shrinkage is accompanied by the formation of apoptotic bodies known as blebs, in a process known as zeiosis, described in greater detail below (Elmore).

To initiate the structural changes that occur within the cell, many biochemical pathways are altere. Caspases (a class of enzymes that degrade proteins) are widely expressed and can initiate a proteolytic cascade, accelerating the cell death process as proteins are cleaved at aspartic acid residues. There are ten such caspases involved in the apoptotic process; they are categorized as either initiatiors, executioners, or inflammatory caspase (Elmore, Kanduc).

Proteins are prepared for destruction by extensive cross-linking mediated by tissue transglutaminase. Endonucleases begin to break down internucleosomal DNA, resulting in fragments around 180-200 base pairs in length, which can be visualized through electrophoresis as a characteristic DNA ladder. It is thought that this

breakdown of DNA plays a major role in reducing the inflammatory response following apoptosis (Bortner).

Cell surface markers are modified to become more recognizable to phagocytes by the movement of phosphatidylserine from the inner membrane of the lipid bilayer to the exposed outer layer. With the help of surface-expressed Annexin I and calreticulin, the cell is prepared for easy digestion by neighboring cells to minimize damage to the surrounding tissue (Elmore). It is dramatically important that the components of apoptotic cells are digested and removed early and quickly (Nauta).

Necrosis

Necrosis is the process by which cells *prematurely* die following some sort of fatal external insult. It has been described as "an acute, non-apoptotic form of cell death." (Syntichaki) The cell membrane and its contents are disrupted to such a degree that normal function becomes impossible and the cell dies. Necrosis is highly disruptive and, left unchecked, can result in widespread tissue damage that can ultimately be fatal. Toxic chemicals can be released from the compromised cell and organelles normally dedicated to destroying cell waste can be unleashed into the intercellular fluid where their caustic interiors can wreak havoc on neighboring cells. Gangrene is an extreme example of the outcome of this dangerous cascade of cell death (Syntchaki).

For a long time, it was assumed that necrosis was completely unregulated, occurring as a natural chain reaction of cellular breakdown. However, it is now

becoming understood that even in the throes of death, the cellular system breaks down

in an orderly, predictable fashion under certain conditions (Beyer, Festjens, Kanduc).

There are several mechanisms by which necrosis can proceed, likely depending on the

nature and extent of damage. ATP is consumed to thrust these pathways forward

(though the energy requirements are much less prohibitive than those of apoptosis), and

they are all heralded by the production of tumor necrosis factors, followed by a cascade

of enzyme activations ultimately resulting in cell necrosis (Proskuryakov). Because of

the reduced energy dependence of necrosis compared to apoptosis, there are situations

in which the apoptotic process can be initiated and then aborted before it is completed in

favor of necrosis; this is known as aponecrosis, necrapoptosis, or secondary necrosis

(Chen).

Despite this control, the necrosis generally held to be more harmful to

surrounding tissues than the apoptotic process. During necrosis, there is an incomplete

phagocytosis of cellular debris, typically resulting in inflammation of neighboring tissue

(Nauta, Kanduc) triggered by exposure to cytokines (such as IL-1beta, TNF-alpha, and

IFN-gamma) depending on the current biochemical phenotype of the activated cells

(Proskuryakov).

During cell activation and death, particularly during the later stages of necrosis

and apoptosis, high mobility group box 1 (HMGB1) proteins are released into the

intracellular media. These are non-histone, chromatin-binding proteins known to be

novel mediators of the inflammatory process, inducing pro-inflammatory cytokine expression by binding to DNA and regulating transcription. Elevated levels of HMGB1 have been reported in the synovia of patients suffering from rheumatoid arthritis (Ardoin). Thus, it can be seen that improperly regulated cell death can have a direct impact on the development of rheumatoid arthritis and other similarly debilitating autoimmune conditions (Distler, Eguchi, Reich).

Zeiosis

Blebbing (or zeiosis) is a process by which part of the plasma membrane protrudes from the cell surface and becomes decoupled from the cytoskeleton by activation of the contractile cortex, allowing a small vesicle to detach and move away from the cell (Charras). Aminophospholipid translocase, floppase, scramblase, and calpain are enzymes responsible for the maintenance of a dynamic asymmetry in the phospholipid chemistry, and the disruption of this steady state is reported to initiate blebbing (Piccin), as the membrane morphs to a lower-energy state and releases the hairpin deformations that will become microparticles. This is commonly associated with apoptosis, causing the cell to fall apart into small units for digestion by phagocytes, but because the blebs contain some of the originating cell's membrane proteins, they have been implicated in several intercell communication processes, including immune activation, even in the absence of full apoptosis (Distler).

Microparticles

Microparticles are a class of small (100 to 1000 nm in diameter (Mause)), membrane-bound vesicles that are used by cells to transmit information about the cell. They have been repeatedly found to act as very important mediators in fundamental physiological processes, both in the maintenance of homeostasis and in pathological responses (Piccin, Reich). Microparticles bleb off from the cell membrane during normal activation, and their release is also thought to be triggered by certain traumatic events, particularly those in which apoptosis is initiated. Notably, microparticle release has also been measured in cases of high shear stress at the cellular surface (Nomura).

The content of microparticles varies as a function of the cell composition, state of the cell membrane, and release trigger. The membrane of a microparticle may be studded with proteins and enzymes that engage tissue factors or initiate clotting; integrins and GPI-linked proteins to enable targeting, interaction, and adhesion properties; and major histocompatibility complexes to stimulate local cells (Hugel). The complexities of their membrane enable them to act as highly efficient transmitters of cellular information (Mesri, Owens, Faure).

The membrane of a microparticle may be studded with aminophospolipids, phosphatidylserine, and phosphatidylethanolamine that initiate apoptosis in other cells or trigger inflammation cascades coagulation cascades (Morel). Shed microparticles have been observed to contain interleukin-1beta, a cytokine that acts as an important

mediator of the inflammatory response. They have also been shown to be a source of proinflammatory mediator and platelet agonist aminophospholipids (Distler, Mallat, Fourcade). Microparticles that arise from leukocytes membranes are known to stimulate endothelial cytokine release and upregulate tissue factor expression through the JKN1 pathway (Mesri).

Phophatidyl-studded microparticles derived from platelets or endothelial tissues have a short half-life in the body, on the order of approximately ten minutes (Dasgupta). However, it has been shown by Brodsky, et al and Owens that circulating endothelial microparticles increased significantly in patients following liver surgery, and remained elevated in the body for as long as one week after. Thus, it can be understood that a non-fatal insult to the leukocytes or other peripheral tissue, such as might be experienced after exposure to a blast, could result in significant delayed pathophysiological effects (Bauman).

Brain Injury

Mild traumatic brain injury is typically accompanied by an initial and temporary period of reduced brain function (Management of Concussion/mTBI Working Group). There is also concern that exposure to blasts may provoke long-term brain conditions that have severe negative effects on health and quality of life (Hoge, Faul, Finkelstein, Taber, Trudeau).

One of the most disruptive and debilitating forms of brain injury is diffuse axonal injury (DAI) (Adams) -- damage that occurs over a widespread area of the cerebral tissue, mostly defined by extensive lesions of the white matter (Davenport). Hypothesized to be important in certain blast-related traumatic brain injury scenarios (Taber), DAI is characterized by long term disruption of pyschological or cognitive functions, such as confusion, difficulty concentrating, amnesia, or loss of coordination (Levi, Wallesch). Diffuse brain damage may be accompanied or exacerbated by associated hypoxic brain damage, brain swelling, and vascular injury and the damage can be difficult to ascertain immediately without magnetic resonance imaging (Adams, Paterakis). It is "recognized as the most common structural abnormality underlying severe neurological disability and the vegetative state," (Blumbergs), present in about one third of unselected cases of fatal head trauma (Pilz). Degrees of DAI are classified mainly by the duration of unconsciousness (Gennarelli) and it is generally understood that a longer period of unconsciousness or coma correlates to a more dire prognosis (Jennett, Perel, Yuan). It is worth noting that in one third of the DAI fatalities studied by Pilz, the patient was not permanently unconscious - they recovered consciousness and in some cases survived for more than four weeks after (Pilz, Blumbergs).

Some DAI may additionally be incurred as a result of processes secondary to the initial insult, such as hypoxia or posttraumatic edema (Jellinger). The sum result of neural inflammation is almost always a disruption in normal nervous system function.

Reflex pathways may be blunted by inflammation, leading to the failure of homeostatic and peripheral functions (Andersson, Barnes).

Chronic inflammation of the brain is known to be correlated with Alzheimer's symptoms in affected patients (Colton). Particles of damaged tissue, cytokines, and other inflammatory mediators released during the sustained inflammation response may trigger further upregulation of the immune response (Hanisch), creating a positive feedback loop that can accelerate degeneracy (Akiyama, Griffin, Lucin). Thus, there may be a danger that an improperly-initiated immune response (as from a primary blast injury) might have severe, long-lasting effects on the health and wellbeing of a patient.

Post-traumatic stress disorder has an 8% prevalence in the United States (Pace) and as many as 31% of military personnel active in Iraq or Afghanistan may experience some form of PTSD by the time they return home (Sundin, Sayer). Sufferers of PTSD may experience anxiety, depression, psychosis, nightmares, emotional flattening, or increased arousal and hypervigilance (Yehuda, Helzer, Sayer). It is estimated that the annual loss in productivity resulting from PTSD symptoms could be as much as $3.8 billion (Kessler, Pace 2011). PTSD is associated with disorders in the brain's stress response systems: the hypothalamus-pituitary-adrenal axis and the sympathetic-medullary-adrenal axis (Pace); the anxiety symptoms of PTSD correlate positively with increased blood levels of dopamine and epinephrine (Yehuda).

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Additionally, recent literature has suggested that PTSD may be linked to systemic immunochemical changes. PTSD symptoms are associated with increased circulating inflammatory markers such as C-reactive proteins and IL-6 (Pace) and chronic immune activation (Wilson). Combat-associated PTSD has been correlated to higher natural cytotoxicity, either due to an increased number of natural killer cells or an increased ratio of lysis events per natural killer (Laudenslager). PTSD patients have been found to be more sensitive to lipopolysaccharide-induced cytokine production (Rohleder) and to have a generally elevated level of serum cytokines (Spivak).

Overall, PTSD patients have an increased comorbidity with cardiovascular disease (Boscarino 2008, Kibler), rheumatoid arthritis (Qureshi, Boscarino 2010), psoriasis, and thyroid disease; it is held that these conditions are the result of chronic overexpression of inflammatory mediators (Boscarino 1999) combined with a reduced circulation of anti-inflammatory corticoids (Kunz-Ebrecht, Greaves).

Delayed Pathophysiology

It has been suggested that the immune cell activation resulting from exposure to blast trauma can result in brain damage and chronic inflammatory diseases such as rheumatoid arthritis (Pace, Koliatsos). Research suggests that certain classes of microparticles are capable of traversing the blood-brain barrier naturally (Faille). If the barrier's integrity has been compromised by the blast injury or by local necrosis (Combes), microparticles could be responsible for initiating a harmful inflammation

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response within the brain (Fitch). Damage to the ependymal cells that make up the thin epithelial membrane protecting the brain and spinal cord has been observed to persist for as long as a week after exposure to blast (Readnower).

In addition to the mechanical insults sustained by exposure to the blast pressure, a recent study found that cell damage might be characterized by a widespread disruption of integrins, protein receptors that mediate communication and attachment among neighboring cells (Hemphill). Though the integrity of the cell membrane or the organelles within may resist the sudden change in pressure, vibrations introduced by the blast wave could cause unwanted signals to propagate throughout the cellular communication network (Alford). These signals could drive forward changes in cellular function that damage the entire system, including the initiation of apoptotic pathways culminating in cell death and inflammation of surrounding tissue (Reich).

The primary concern is that a compromise in the integrity of the blood-brain barrier may lead to further injury by permitting inflammatory molecules (such as cytokines) access to otherwise protected nervous system tissue. Once there, the apoptotic processes initiated by the inflammatory molecules may continue unchecked and result in a number of inflammation-linked brain disorders (Pace, Rohleder, Pitossi, Jones 2012, Goshen).

Materials and Methods

Cells

Two types of human immune cells were chosen to be the subject of the experiments: Jurkat and HL-60. Jurkat cells come from an immortalized line of T lymphocytes and are commonly used to study cell signaling (Abraham). HL-60 (Human promyelocytic leukemia cells) are a line of leukemic neutrophil precursors (Collins, Gallagher) and were chosen for their increased susceptibility to activation (Reich). Both cell types can be generally understood to act as mediators, activators, and initiators of the innate immune system. T lymphocytes are found normally in the blood. Promyelocytes are neutrophilic precursors normally found in the marrow.

Jurkat cells (grown in the Duke University Health System Cell Culture Facility) and HL-60 cells (ATCC Catalog No. CCL-240) were seeded in regular growth medium at 2×10^6 cells/mL, supplemented with a 25 mM HEPES buffer (Life Technologies) to balance the pH of the media.

Both types of cells were cultured and tested at roughly half the density of leukocytes naturally found in whole blood to minimize the potentially harmful effect of "cross talk" among damaged or disturbed cells (Ardoin, O'Byrne).

In the majority of the tests, 3 samples were used for each control and each experimental condition. The final test employed 6 samples for each condition. Each sample consisted of 1 mL of the cell solution inserted into a UV-sterilized 30 mm x 30

mm plastic FoodSaver® bag, which was sealed using a heat clamp. After blasting,

samples were transported back to the VA Medical Center and carefully removed from

the packets. The cells and media were transferred to a labeled 24-well plate and stored in

an incubator, after which the supernatants were isolated through centrifugation and

prepared for analysis.

Shock Tube

The simulated blasts central to this study were performed out using a custom-

built steel "shock tube" device designed by Panzer et al (Panzer 2012a,b) to deliver

controlled, repeatable blast overpressures without exposing cells to the heat or debris of

a traditional explosion (Figure 2). It consists of a 3-inch inner diameter hollow

aluminum tube topped by a flanged chamber into which a number of thin (0.254 mm)

polyethylene terephthalate membranes are secured in place by bolts. The chamber is

filled with high-pressure helium gas and the membranes burst outward when the

chamber pressure exceeds their tensile strength; the pressure achieved is thus dependent

on the number of membranes used. The released pressure wave travels down the shock

tube, past another protective silicone rubber membrane (used to shield the samples from

debris) and into the test column proper. The sample is submerged in water to simulate

blast wave conditions within a depth of human flesh, and a FIZO™ transducer measures

the final change in pressure just below the sample. Because there is very little

momentum associated with the pressure wave, the pressure measured below the sample

should be identical to that experienced by the sample itself (Panzer 2012b).
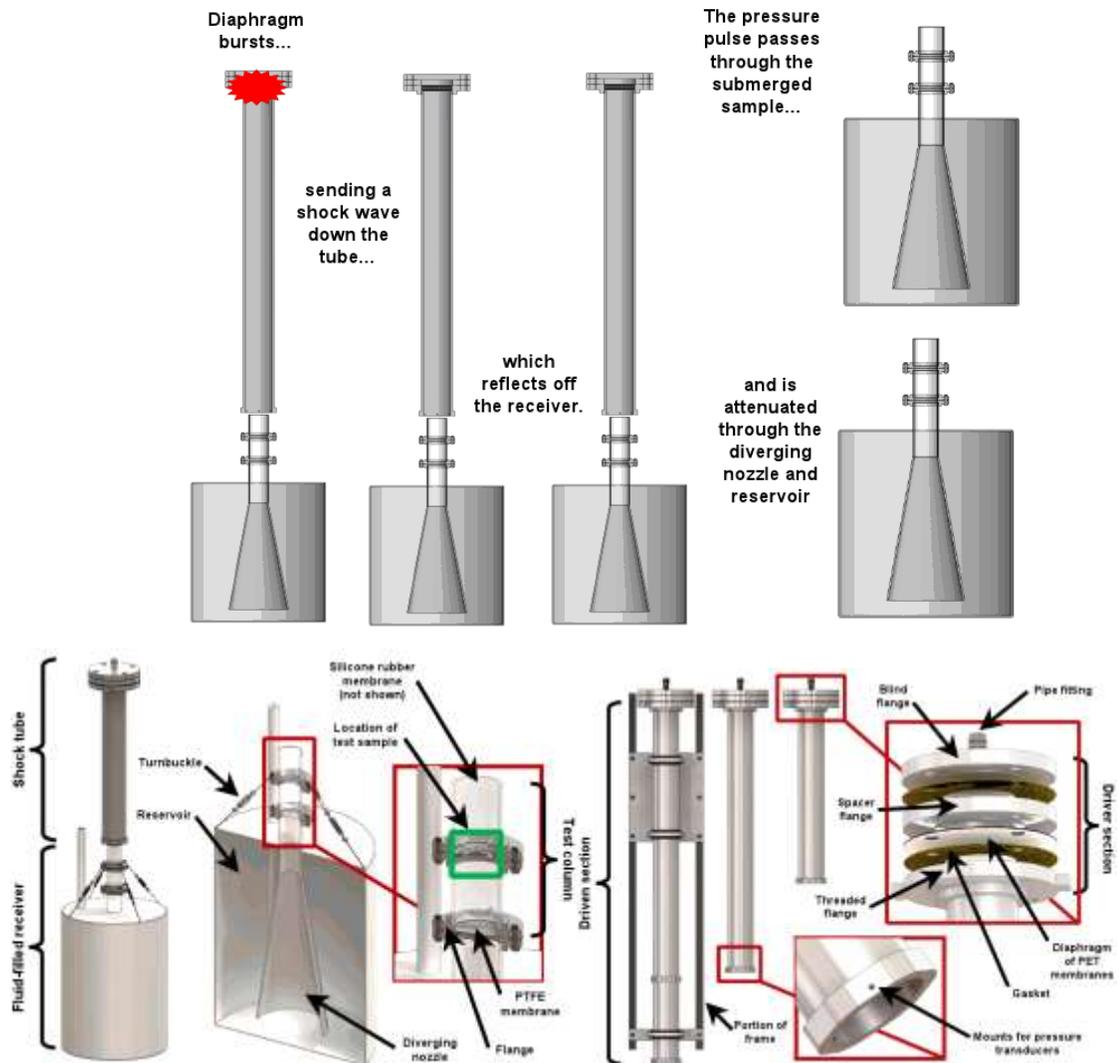

Figure 2: Diagram of construction and operation of shock tube developed to deliver blast waves to samples. Image and accompanying text reproduced with permission. (Panzer 2012a)

The bagged samples were transported in an insulated plastic container to the nearby blasting facility. Cell packets were loaded into the blast chamber one at a time for testing, and each packet was exposed to a single blast. Blasts were generated using the following membrane thicknesses: 0 mil (sham), 20 mil, 40 mil, and 60 mil (see Figure 3 for characterization of incident overpressure). Either three or six iterations were performed at each degree of thickness.
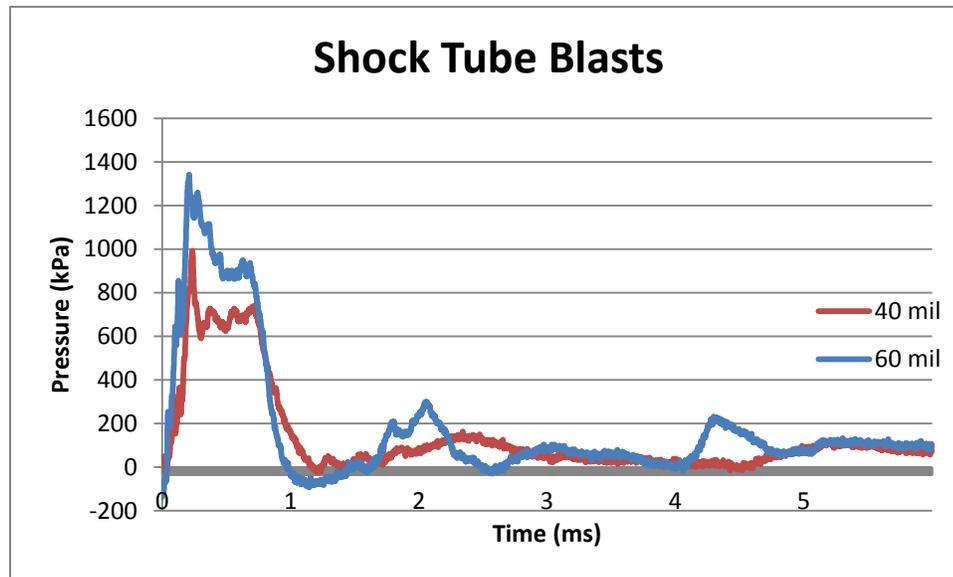

Figure 3: Representative traces of the reflected pressure wave at the sample by shock initiated using a diaphragm of either four or six 10 mil-thick membranes.

Harvest

Following the blasts, the samples were carefully removed from the plastic packets and placed directly into a 96-well culture plate. Initial tests were performed after both 1.5 and 24 hours of incubation to investigate the time-dependence of injury

mechanism; the 1.5 hour time point was deemed insignificant with regards to our hypothesis, but the results are still reported below.

The plate was incubated at 37 °C for 24 hours, allowing the apoptotic and necrotic mechanisms to process for a time. The samples were transferred to a series of autoclaved Eppendorf flasks and centrifuged for 5 minutes at 5 G to isolate the supernatant. 200 μL of supernatant from each sample was transferred to a new set of Eppendorf flasks for storage and further analysis; these were stored in a sample refrigerator at 20 °C.

Following the initial viability study (Figure 8), it was noted that there was a consistent increase in cell death among all samples exposed to the bagging and transport process, regardless of whether or not they were actually hit with a blast, particularly after the cells had been left to incubate for 24 hours. It was hypothesized that the cells were responding negatively to ethanol used to sterilize the bags in which they were contained for blasting, so a sub-study was performed to evaluate the effects of different modes of sterilization, namely ethanol and UV light. See Figure 8 in Results for more.

Flow Cytometry

15 μL of each of the supernatant samples were transferred to a set of MiniFACS tubes containing 285 μL of 1x phosphate-buffered saline (PBS) solution. The remaining samples were frozen for later assay processing. The samples were sequentially processed at 1 μL/s (Orozco) in a FACScan flow cytometer. The microparticle counts per

second were observed until they became visibly steady and then the data was recorded

for 30 seconds. Using side-scatter fluorescence, the software (FlowJo CE, version

7.5.105.0) was able to generate a report on the density of cells fitting a given profile

(corresponding to that known to be associated with microparticles), enabling a

comparison of microparticle release among samples exposed to different conditions.

Assays

The frozen supernatant samples were thawed and then analyzed for cell death

and DNA release using the Cytotox 96 Non-Radioactive Cytotoxicity Kit (Promega) and

Quant-iT PicoGreen dsDNA Reagent (Life Technologies). All assays were run in

duplicate to minimize the effect of experimenter error.

The Cytotox 96 Non-Radiocative Cytotoxicity kit is a colorimetric assay that

quantitatively detects the presence of lactate dehydrogenase (LDH), a stable enzyme

released from cells upon lysis. Thus, LDH can be correlated to the number of cells in a

given sample that have undergone necrosis or apoptosis. LDH in the sample reacts with

an enzymatic assay, converting tetrazolium salt into a red formazan salt, with the

amount of red color formed after a 30-minute reaction corresponding to the proportion

of lysed cells in the sample. This can be measured precisely in a spectrometer (using

Softmax Pro v2.6.1 and a Molecular Devices microplate reader) set to detect absorbance

at 490 nm. However, due to the nature of the methods used in this research, it is

impossible to quantitatively measure the percent of cytotoxic cells within a sample; only the relative cytotoxicity of samples can be ascertained.

Similarly, Quant-iT PicoGreen dsDNA Reagent is an assay kit that is used to detect the amount of double stranded DNA (dsDNA) in a sample using quantitative fluorescence. dsDNA in the sample reacts with the enzymatic assay to form a green-fluorescing stain, the intensity of which (measured at 260 nm using Magellan v3 software and Tecan GENios fluorescent plate reader) corresponds to the concentration of double-stranded DNA according to the standard curve established by the kit. Since dsDNA is typically digested during normal apoptotic processes, this assay provides a measure of necrosis separate from normal lytic cell death (Beyer). Both assays were performed at a 1:20 dilution.

Statistical Analysis

Quantitative comparisons of the cell death markers collected by flow cytometry microparticle analysis and the respective assays were carried out using ANOVA with Tukey-HSD ($p < 0.05$), performed with JMP v10 (SAS Institute, Inc).

Results

Vertical bars in all following figures correspond to one standard deviation in each direction. A parameter estimation performed on the least squares regression of the HL-60 data indicated that the test date (correlating directly to the number of samples) was a significantly confounding factor, so the results are presented separately below as n=3 and n=6.

Exposing HL-60 cells to blast conditions in the shock tube produced increased levels of markers associated with cell death. Increased microparticle release was observed in samples exposed to blast (Figure 4). In the 3-sample run, there was a significant increase from the sham to the 60 mil conditions and a significant increase from the 40 mil condition to the 60 mil condition. There was no significant difference between the sham and 40 mil condition. In the 6-sample run, there was a significant difference between the sham and 40 mil conditions and between the sham and 60 mil condition, but no significant difference between the 40 mil and 60 mil conditions.
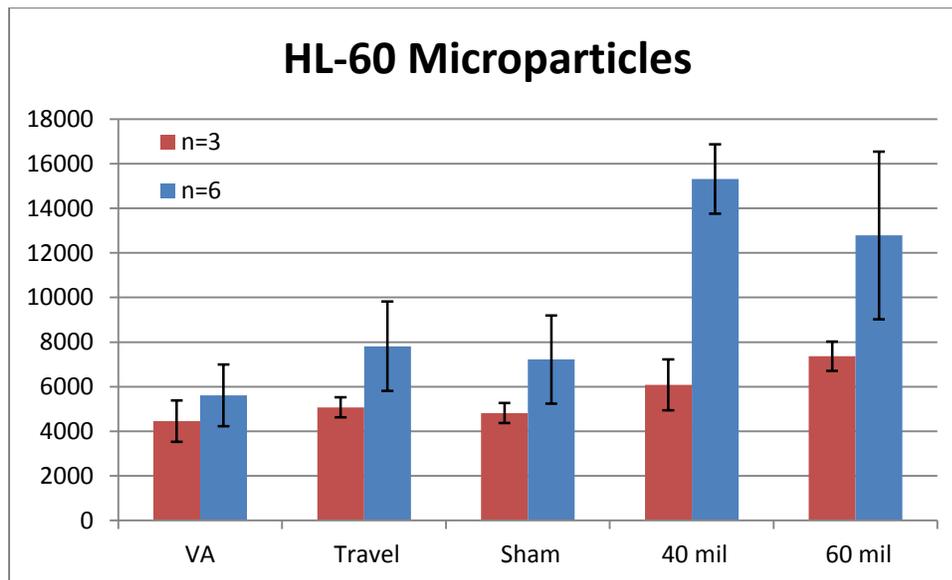
Figure 4: Average microparticle counts over 30s for a blast sequence performed on HL-60 cells.

The cells released more lactate dehydrogenase, consistent with an increased proportion of lysed cells, under blast conditions compared with control conditions (Figure 5). In the three-sample run, there was a significant increase in cytotoxicity between the sham and 60 mil conditions, as well as a significant increase between the 40 mil and 60 mil conditions, but no significant difference between the sham and 40 mil conditions. In the six-sample run, there was a significant difference between the sham and 60 mil conditions, but no difference between the sham and 40 mil conditions or the 40 mil and 60 mil conditions.
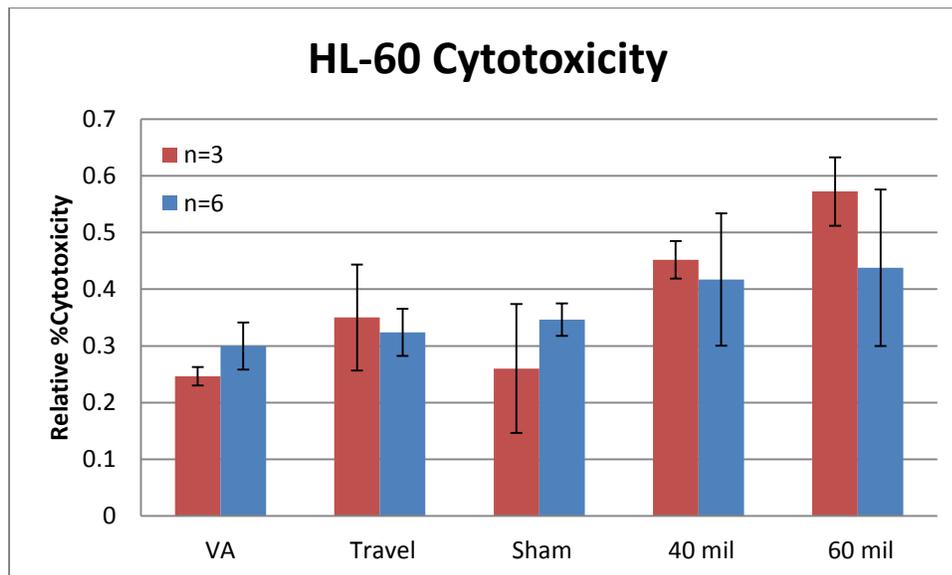
Figure 5: Average LDH assay resultss for a blast sequence carried out on HL-60 cells.

Blast exposure elicited no consistent response with respect to release of double-stranded DNA (Figure 6), indicating no increased necrotic response compared to controls. In the initial three-sample run, there was a significant increase in dsDNA release at the 60 mil condition compared to the sham, with the 40 mil samples not differing significantly from the sham condition. However, when the experiment was repeated with six samples per condition, no significant differences were detected among the samples.
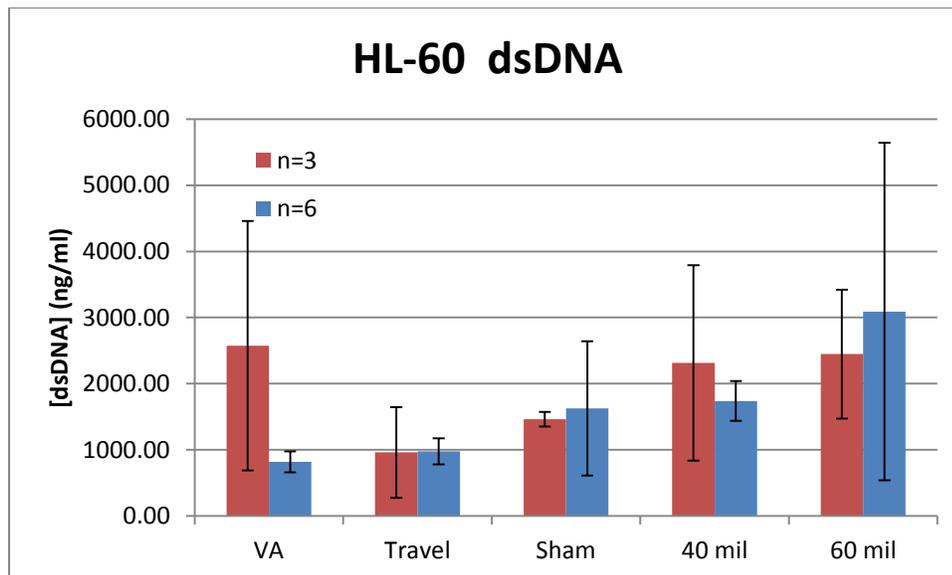
Figure 6: Average dsDNA assay results for a blast sequence carried out on HL-60 cells.

Though the Jurkat line is commonly used in experiments to characterize cell signalling and activation, the cells proved to be resistant to the blast pressures that were investigated in this study. An initial study suggested a promising immediate damage response to blast exposure (in the form of cytotoxicity measured by LDH assay) when the cells were collected and analyzed 1.5 hours after the blast, but the significance of these results vanished when they were analyzed again at 22.5 hours (Figure 7).
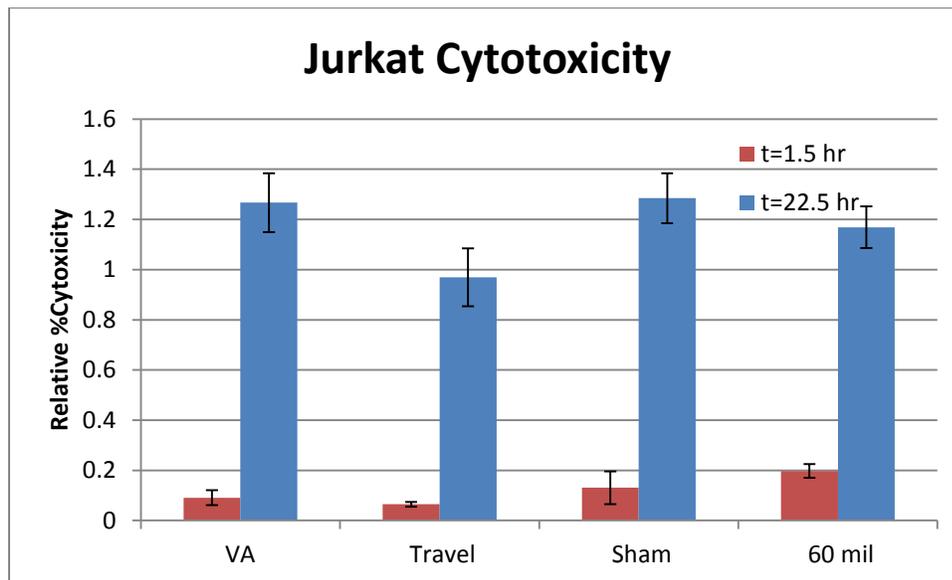
Figure 7: Average LDH assay readings for an exploratory blast of Jurkat cells, with samples harvested at 1.5 hours and 22.5 hours after blasting.

It was hypothesized that the lack of response was due to a cell death saturation effect initiated by exposure to the ethanol used as a disinfectant. A viability study was performed to evaluate if ultraviolet (UV) light could be used as a replacement sterilization method (Figure 8). There was a significant increase in cytotoxicity (measured using the LDH assay) in all samples exposed to ethanol. UV exposure did not cause any significant changes in cytotoxicity compared to controls.
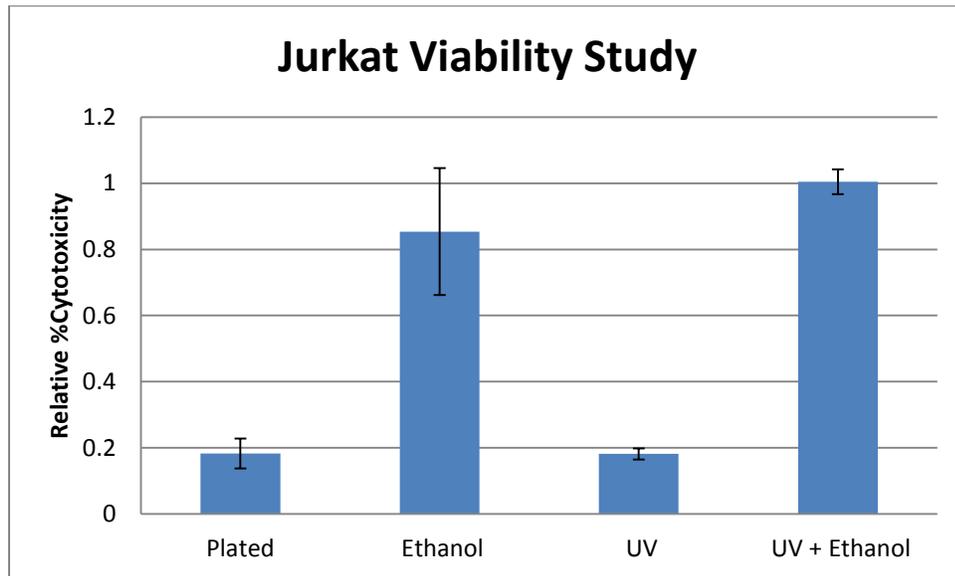
Figure 8: LDH assay readings for a Jurkat cell viability study to determine the ideal way to sterilize the bags used to contain the cells for blasting.

However, even with the revised procedure to isolate cell damage as a function of

blast exposure, the cells did not exhibit any significant response (via dsDNA release) to

blast trauma (Figure 9).
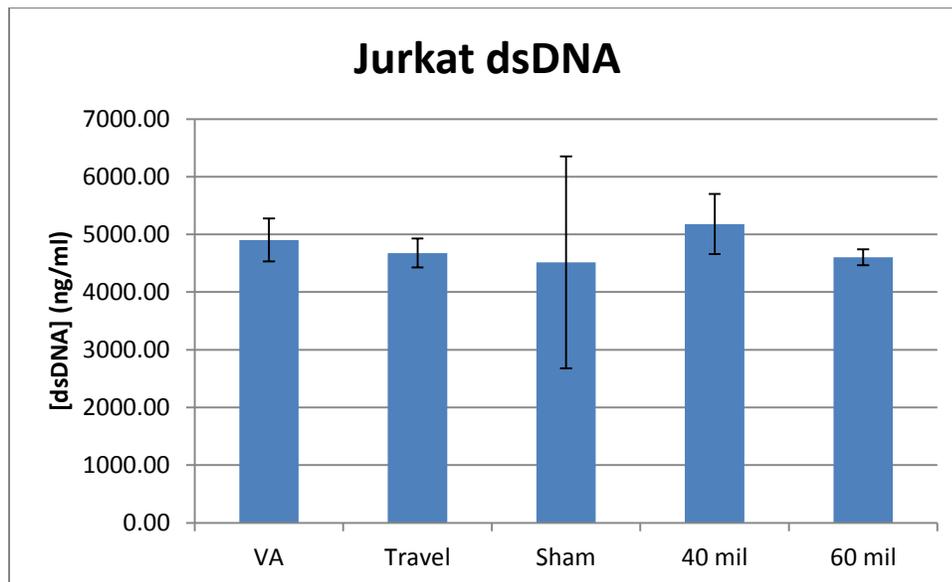
Figure 9: Average picogreen assay results for a blast sequence carried out on Jurkat cells.

The same lack of response was mirrored in the microparticle release, with no significant difference between the control and blast conditions (Figure 10). With these results in mind, on the advice of Dr. David Pisetsky, LDH analysis of cytotoxicity was not performed and the experiment was expanded to evaluate HL-60 cells.

Figure 10: Average microparticle counts over 30s for a blast sequence performed on Jurkat cells.

Overall, HL-60 cells demonstrated a significant increase in both mortality and microparticle release when exposed to blasts generated with 40 and 60 mil membranes. However, it was also discovered that Jurkat cells experienced no significant increase in mortality or microparticle release following similar exposure.

Discussion and Conclusion

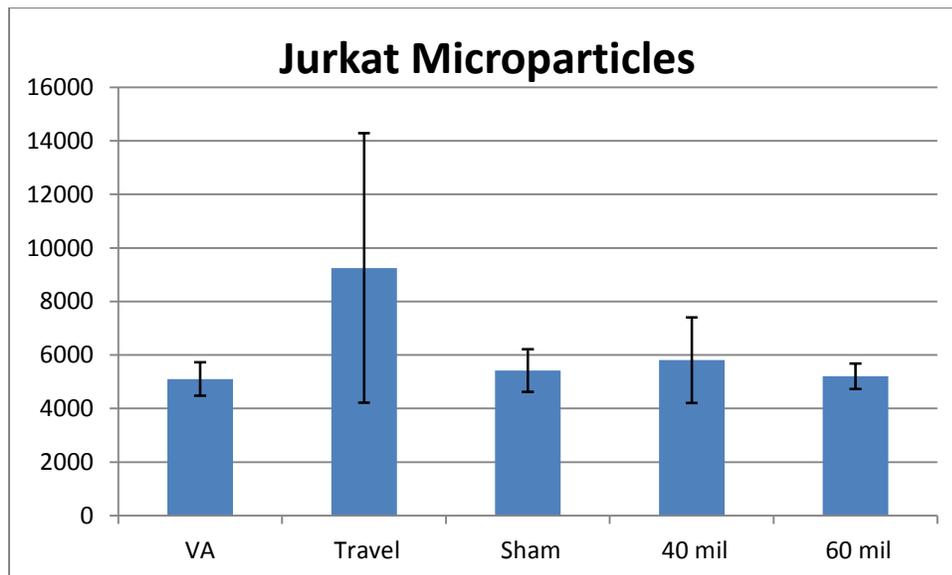The results of these experiments suggest that blast exposure may provide a mechanism that can activate immune response in humans. HL-60 cells in conditions that may be analogous to those found within the blood stream are vulnerable to damage incurred by blast overpressures and respond by demonstrating an increased amount of cell lysis and increased microparticle release, without a corresponding increase in the release of double stranded DNA; this response is uniquely characteristic of apoptotic cell death. Jurkat cells exposed to the same conditions did not demonstrate any significant response; this could be an indication of a resistance to blast injury or an artifact of the experimental methods employed. Similarly, the difference in effects observed between the 3-sample and 6-sample experiments suggest that there was a confounding variable that was not accounted for in the controls. It is possible that these were caused by a differences in the cell concentration in the cultures from which the samples were drawn or an effect arising from the extended handling time required to process more samples.

The scope of this study was limited by the cell types used; though they are regularly used in the study of human immune response they are, on account of their immortal status, not completely representative of normal leukocytes. Additionally, HL-60 cells *in vivo* might not respond as strongly to blast injury since the neutrophilic promyelocytes are normally found only in the bone marrow; the more rigid and dense

tissue of the bone could have an attenuating effect on the incident overpressure experienced by the cells.

Still, because these cells are also important in activating and maintaining immune response, particularly when microparticles are involved, it is possible that widespread damage to or activation of these cells could trigger a systemic inflammation that could, in some cases, become self-propagating and result in a number of delayed inflammatory auto-immune conditions. Note that these results only confirm the hypothesized immune cell activation by blast; they should not be taken as absolute measures of the magnitude potential immune response.

Since the pressure wave generated by our shock tube loaded with a 60 mil membrane is roughly comparable to protected exposure to an IED detonation at a standoff distance of approximately five meters (Rafaels), individuals exposed to such events – an increasingly common occurrence on the modern battlefield – may be at risk for developing conditions associated with inflammation, such as mild traumatic brain injury or rheumatoid arthritis, even if no initial symptoms are detected. Further research to better determine the potential for blast activation of such immune activating events *in vivo* could lead to novel treatments for our veterans.

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