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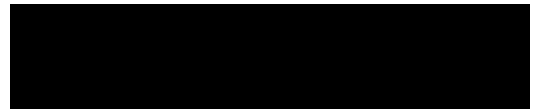
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Sarah Anne Walsh

EXOSOMES AS COURIERS OF INFLAMMATORY MEDIATORS FOLLOWING
ACUTE TRAUMATIC INJURY

by

Sarah Anne Walsh

Dissertation submitted to the Faculty of the
Molecular and Cell Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2021



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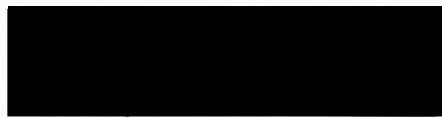
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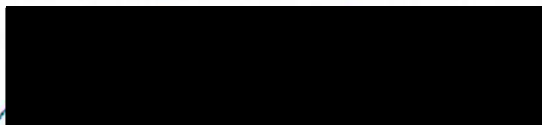
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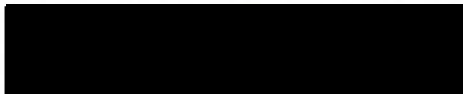
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DEDICATION

I want to dedicate this work to my parents, Peter and Gabriell Walsh, who always believed in me.

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ABSTRACT

“Exosomes as couriers of inflammatory mediators following acute traumatic injury”

Sarah Anne Walsh, Molecular and Cell Biology PhD Program, 2021

Thesis directed by: Dr. Thomas Davis, PhD, Professor, Department of Surgery

Complex polytraumatic injuries induce a robust innate immune response that can lead to systemic inflammatory complications and end organ damage. Early identification of at-risk patients is essential for appropriate management and intervention. This research was aimed at exploring proinflammatory signaling in models of acute traumatic injury by investigating the kinetics of secretion and biodistribution-compartmentalization of proinflammatory mediators found associated with serum exosomes versus freely circulating forms.

An exhaustive review of the available trauma, innate immunity, inflammation, and exosome literature was conducted to synthesize the extent of the literature, identify gaps in knowledge, and provide a foundation for the investigative approach using the animal models in the research studies described herein. Limited investigations regarding the role exosomes play in driving trauma-induced inflammatory complications were found.

Using well-established rat models of combat-related blast trauma, the biodistribution of proinflammatory mediators in serum compartments was determined at

various timepoints post-injury. Depending on the physiological state of the animal (healthy versus type and extent of injury), significant differences were observed in the spacio-compartmentalization (freely circulating versus exosome-membrane associated, exosome encapsulated), pattern of release, and concentrations of proinflammatory cytokines like IL-1 β , IL-6 and TNF α . A significant number and high levels of “hidden cytokines” were shown to be encapsulated within the lipid bilayer membrane of exosomes and thus were not detectable by conventional ELISA multiplex immunoassays. Packaging of these cytokines into exosomes indicates the importance of early innate proinflammatory cytokine signaling and delivery post injury, as the exosome lipid bilayer shields internal cargo from the microenvironment, leading to stability of the bioactive material within, and increased efficiency of delivery to effector targets. Collectively, these results demonstrate that serum proinflammatory cytokines involved with acute innate immune responses are differentially compartmentalized in the naive state with shifts in distributions and cytokines levels given the severity of traumatic injury. Importantly, these findings highlight that conventional ELISA-based assessments underestimate the full immunoinflammatory response to trauma; therefore, detection of both free and exosome-associated proinflammatory cytokines provides a more accurate and clinically useful strategy to identify, diagnose, and monitor patients at highest risk for post-traumatic complications.

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LIST OF ABBREVIATIONS

ALIX	programmed cell death 6-interacting protein
AKI	acute kidney injury
AKT	protein kinase B
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
ALT	alanine aminotransferase
ALP	alkaline phosphatase
ATP	adenosine triphosphate
B	blast
BCA	bicinchoninic acid assay
Bim	B-cell lymphoma-2-like protein 11
BUN	blood urea nitrogen
CABG	coronary artery bypass graft
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
CI	confidence interval
CINC	cytokine-induced neutrophil chemoattractant type-1
CLMAT	colorectal liver metastasis-associated transcript 3
cm	centimeter
COI	complex orthopaedic injury
CLP	cecal ligation and puncture
Cr	creatinine
CRP	C-reactive protein
Ctnn1	catenin alpha 1
CXCL1	C-X-C motif chemokine ligand 1
DAMP	damage associated molecular pattern
dHLA	delayed hind limb amputation
DIC	disseminated intravascular coagulation
DC	dendritic cell
dL	decaliter
DLS	dynamic light scattering
DoD	United States Department of Defense
EC	exosome encapsulated
EDS	exosome depleted serum
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
FC	freely circulating
g	gram
GCS	Glasgow coma score
HLA	hind limb amputation
HO	heterotopic ossification
HOTAIRM1	homeobox antisense intergenic ribonucleic acid myeloid 1

HMGB1	high-mobility group box 1
HIF-1 α	hypoxia-inducible factor 1-alpha
hr	hour
HSP	heat shock protein
IACUC	Institutional Animal Care and Use Committee
ICU	intensive care unit
IED	improvised explosive device
IFN- γ	interferon-gamma
IGEPAL CA-630	Octylphenoxy poly(ethyleneoxy)ethanol, branched
IL	interleukin
ILV	intraluminal vesicle
IP-10	interferon gamma-induced protein 10
IRAK	interleukin receptor associated kinase
IRI	ischemia-reperfusion injury
ISS	injury severity score
ITGA2B	integrin alpha-2b
ITGB3	integrin beta-3
KC/GRO	keratinocytes-derived chemokine/growth-regulated oncogene
kDa	kilodalton
kg	kilogram
KIA	killed in action
kPa	kilopascal
L	liter
lncRNA	long non-coding ribonucleic acid
LPS	lipopolysaccharide
MA	exosome membrane associated
MALAT1	metastasis-associated lung adenocarcinoma transcript 1
MCP-1	monocyte chemoattractant protein-1
MDSC	myeloid-derived suppressor cell
mg	milligram
MIP	macrophage inflammatory protein
miRNA	micro-ribonucleic acid
MHC	major histocompatibility complex
ml	milliliter
mmHg	millimeters of Mercury
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
MOI	mechanism of injury
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
mtDAMP	mitochondrial damage associated molecular pattern
mTOR	mammalian target of rapamycin
MVB	multivesicular body
NaCl	Sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NATO	North Atlantic Treaty Organization

NF- κ B	nuclear factor kappa beta
NK	natural killer
NLR	nucleotide-binding oligomerization domain-like receptors
NLRP3	nucleotide-binding oligomerization domain-like receptor protein-3
nm	nanometer
NO	nitric oxide
PAMP	pathogen associated molecular pattern
pg	picogram
PI3K	phosphoinositide 3-kinase
PRR	pattern recognition receptor
PSI	pounds per square inch
RAGE	receptor for advanced glycation end products
RANTES	regulated on activation, normal T-cell expressed and secreted
RNS	reactive nitrogen species
ROS	reactive oxygen species
ROUT	robust regression followed by outlier identification
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SOFA	sequential organ failure assessment
TBI	traumatic brain injury
TBS	Tris buffered saline
TCCC	Trauma Combat Casualty Care
TGF- β	tumor growth factor-beta
TNF- α	tumor necrosis factor-alpha
TEM	transmission electron microscopy
Th	T helper
TLR	toll-like receptor
TRAF	tumor necrosis factor associated factor
Treg	T regulatory
TREM-1	triggering receptor expressed on myeloid cells-1
Tris-HCl	Tris-hydrochloric acid
TSG101	tumor susceptibility gene 101
U	units
μ g	microgram
μ L	microliter
US	United States
Vsig1	V-set and immunoglobulin domain containing 1
x g	times gravity, relative centrifugal force
ZOI	zone of injury

CHAPTER 1: INTRODUCTION

1.1 COMBAT INJURY PATTERNS

The prolonged period of conflict in the Middle East (2001-2021) has borne witness to vast improvements in battlefield medicine. The advent of new body armor and battlefield interventions has caused a reduction in servicemen and women killed in action (KIA) and created a need to address now survivable injuries (29; 31). Introduction of the Trauma Combat Casualty Care (TCCC) guidelines has led to a reduction in preventable deaths in the pre-hospital setting (22). One of the core components of TCCC is to prevent life-threatening hemorrhage (33). The widespread use of the tourniquet has decreased deaths due to exsanguinating injuries and led to a higher number of servicemen and women surviving traumatic amputations (12; 23; 34). The 75th Ranger Regiment demonstrated a significant reduction in both soldiers KIA and those who died of wounds by training all deployed personnel in TCCC. Between 2001 and 2010, this Regiment reported 43% of injuries due to non-improvised explosive device (IED) explosives, and 24% due to IEDs. Combined, this represents a 67% blast-related mechanism of injury (MOI). This is reflective of MOI across North Atlantic Treaty Organization (NATO) forces, which is reported to be at 72% for explosives (18). Adversaries have expanded their use of IEDs since the onset of conflict, and this is associated with higher numbers of major limb amputations reported from military actions involving NATO forces (19).

The United States Department of Defense (DoD) defines IEDs as “A device placed or fabricated in an improvised manner incorporating destructive, lethal, noxious, pyrotechnic, or incendiary chemicals and designed to destroy, incapacitate, harass, or

distract” (1). Use of these weapons can cause a variety of different injury patterns more complex than traditional blast injuries. The injuries experienced followed by exposure to an explosion can be separated into four injury groups: primary, secondary, tertiary, and quaternary. Primary injury encompasses those injuries caused by the pressure waves, which is mostly confined to hollow organs, but also causes traumatic brain injuries (TBI). Secondary injury includes those injuries resulting from projectiles caused by the blast, mainly penetrating and blunt force injuries. Tertiary injury is caused by collision of the body with fixed objects or the ground, as the individual is propelled by the force of the blast. Quaternary injuries are mostly related to the chemical effects of the explosion, including heat and toxic chemical related injury (21). The rise in the use of IEDs has contributed to complex extremity injuries resulting mostly from primary, secondary, and tertiary blast-related injuries. Due to pre-hospital treatment methods, these are a common survivable injury pattern in combat personnel (5; 28). In deployed US service members from 2005-2009, approximately 3 out of 1000 experienced a musculoskeletal casualty, with the majority of these being caused by an explosive MOI (5). These injuries are no longer limited to combat personnel, as terrorist activity has seen a rise in the use of IEDs in civilian settings (13; 20).

In the deployed setting, military surgeons perform extremity amputations through the zone of injury (ZOI) as a method of limb sparing. Paradoxically, this introduces another pattern of injury on top of the trauma to which the extremity has already been exposed (7). The traumatic nature of complex orthopedic injuries has led to an increase in the appearance of co-morbid pathologies such as delayed wound closure, wound

infection, systemic inflammatory response syndrome (SIRS), sepsis, multi-organ dysfunction/failure and heterotopic ossification in combat-wounded personnel (30).

1.2 POST-TRAUMATIC INJURY INFLAMMATION IN THE COMBAT WOUNDED

Advancements in evacuation procedures and critical care have made it so that deployed personnel are able to reach a definitive care center as early as 24-48 hours from point of injury, depending on combat conditions (15). Currently, the focus on addressing long term sequelae does not begin until later in the injury cycle, after the patient has landed stateside and undergone critical care procedures (4). At this point, the effects of inflammatory dysregulation have already begun to disrupt the microenvironment of the wound as the immune system attempts to transition from acute inflammatory response around 72 hours after initial insult (25). Outcomes of acute inflammation usually follow one of three paths: complete resolution, healing by connective tissue replacement, or progression to chronic inflammation (25). Complex injuries can follow a different path, leading to a prolonged state of “acute” inflammation.

During this process, it is important that the body is able to overcome the initial inflammatory phase. While this is important for the clearance of damaged tissue and potential infections, failure to properly transition to the proliferative phase results in impaired wound healing and abnormal tissue repair. Further, it can result in a systemic response to injury, and ensuing dysregulation can cause pathologic states such as SIRS (25).

Monitoring patients following traumatic injury can be accomplished in many ways. However, it is important to detect early changes that affect patient trajectory, and traditional measurements such as clinical parameters and clinical chemistries often do not identify at risk patients early enough for prevention. In assessing post-traumatic injury patient inflammatory status, measuring interleukin-6 (IL-6) levels improved the prognosis timeline as release of this key inflammatory cytokine precedes the previously used c-reactive protein (CRP) (11; 27). While other signaling molecules such as IL-1 β and tumor necrosis factor- α (TNF- α) are released earlier than IL-6, capturing these inciting signals is more difficult due to their shorter half-lives (10; 24; 26; 27).

1.3 EXOSOMES AS EFFECTORS OF THE INNATE IMMUNE RESPONSE TO TRAUMA

Exosomes are small (30-200 nm), endosomal-derived extracellular vesicles that are released from a variety of cells both constitutively and in response to cell stress, cellular activation, senescence, and pathological conditions (2; 3; 6; 8; 9; 16; 17; 32). The role of exosomes in both the innate and adaptive immune responses has been well-described. However, the potential role of exosomes as effectors of the innate immune response to trauma is unclear. Therefore, an initial goal of this project was to perform a comprehensive literature review to understand and report on the known role of exosomal cargo, particularly proinflammatory mediators, in inflammation and regulation of immunological responses to injury insults. Next, this knowledge was applied to working models of traumatic injury to identify potential deficiencies in current methods of monitoring post-traumatic inflammatory status.

Our group has an established and well characterized combat-related polytrauma rat model, which incorporates critical components of combat-mediated polytraumatic limb injuries – specifically blast, fracture, crush, tourniquet use, and amputation. The model recapitulates many of the severe traumatic injury patterns often observed in the combat injured which predispose patients to systemic and wound healing complications resulting from a dysregulated immune response. Previous studies in this model revealed marked changes in the level of inflammatory cytokines, especially at six hours post-injury, with similar cytokine changes identified in clinical patients. Exosomes are known to carry cytokines, and it was previously identified that cytokines encapsulated within exosomes may evade detection using conventional immunoassays (14). We hypothesized that there may be differences in the biodistribution, profile, and quantity of inflammatory mediators found associated with exosomes in comparison to what is detected freely circulating in serum using a commercially available multiplex immunoassay across time and injury patterns. To investigate this, we characterized the serum compartmentalization profiles of key pro-inflammatory mediators collected from naïve animals under the normal steady-state condition, and several rat models of acute trauma. We then compared kinetic changes in compartmentalization and levels of key pro-inflammatory mediators at several timepoints following acute traumatic injury to identify the impact of traumatic injury on exosomal cargo and the role these nanovesicles may have as effectors of the innate immune response to trauma.

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CHAPTER 2: ALARMING CARGO: THE ROLE OF EXOSOMES IN TRAUMA-INDUCED INFLAMMATION

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2.1 ABSTRACT

Severe polytraumatic injury initiates a robust immune response. Broad immune dysfunction in these patients with such injuries has been well-documented; however, early biomarkers of immune dysfunction post-injury, which are critical for comprehensive intervention and can predict the clinical course of patients, have not been reported. Current circulating markers such as IL-6 and IL-10 are broad, non-specific, and lag behind the clinical course of patients. General blockade of the inflammatory response is detrimental to patients, as a certain degree of regulated inflammation is critical and necessary following trauma. Exosomes, small membrane-bound extracellular vesicles, found in a variety of biofluids, carry within them a complex functional cargo, comprised of coding and non-coding RNAs, proteins, and metabolites. Composition of circulating exosomal cargo is modulated by changes in the intra- and extracellular microenvironment, thereby serving as a homeostasis sensor. With its extensively documented involvement in immune regulation in multiple pathologies, study of exosomal cargo in polytrauma patients can provide critical insights on trauma-specific, temporal immune dysregulation, with tremendous potential to serve as unique biomarkers and therapeutic targets for timely and precise intervention in trauma patients.

2.2 INTRODUCTION

Trauma is a leading cause of morbidity and mortality worldwide (58; 98; 116; 133; 143). Polytrauma occurs daily in the civilian population as a result of motor vehicle collisions, falls from height, firearm discharges, mass transit collisions and derailments, industrial workplace injuries, terrorist attacks, and natural disasters (25; 42; 63; 64; 96; 147; 170; 190). Polytraumatic injuries are complex and involve a combination of

concomitant insults to multiple body regions and organs including thermal injuries, large open wounds and major tissue loss, traumatic amputation and eye and nervous system injuries, often resulting in hemorrhage, shock, prolonged systemic inflammation, major organ dysfunction/failure, psychiatric issues, and death.

Blast-related polytraumatic injuries caused by explosive munitions are responsible for 72% of North Atlantic Treaty Organization (NATO) coalition combat casualties in the Global War on Terror (104). Often during their hospital course, these patients develop severe life-threatening inflammation-mediated complications, to include systemic inflammatory response syndrome (SIRS), compensatory anti-inflammatory response syndrome (CARS), acute respiratory distress syndrome (ARDS), pneumonia, sepsis, multi-organ dysfunction syndrome (MODS), and/or multiple organ failure (MOF). These complications are associated with prolonged stays in the intensive care unit (ICU), increased in-hospital mortality, and long-term physical, cognitive, and psychosocial comorbidities (17; 19; 57; 107; 143; 166; 167; 175; 187; 192; 221; 238; 245).

The body's innate immune response after trauma is triggered by the detection of damage-associated molecular patterns (DAMPs) released by cell stress, injured and/or dying cells, or through detection of pathogen-associated molecular patterns (PAMPs) in the event of injury-associated infection (108; 153; 178; 182; 193). Activation of endothelium results from both mechanical stress and the detection of DAMPs and PAMPs, leading to platelet activation and leaky barriers allowing for leukocyte extravasation (91). DAMPs, PAMPs and inflammatory cytokines activate neutrophils and macrophages which play a central role in pathogen immune surveillance, tissue inflammation, clearance of debris and necrotic tissue, and in the regulation of successive

homeostatic processes involved in tissue repair, healing, and tissue regeneration (107). Under normal conditions, the immune response is predictable and transient, and well-regulated by a controlled balance of pro- and anti-inflammatory mediators. Trauma-induced inflammation can be double-edged sword. A “cytokine storm” resulting from the exaggerated production and dysregulation of pro-inflammatory cytokines can result in deleterious systemic inflammation causing further tissue damage, end organ injury, and increased mortality (119; 133; 143; 167). Therefore, identification of reliable biomarkers of aberrant inflammation early post-injury to predict clinical outcomes and guide treatment is critical. This requires a thorough understanding of the complex local and systemic communication network between cells, molecular mediators, various signal pathways, and feedback loops that initiate and regulate the trauma-induced inflammatory response.

Exosomes are the smallest form of extracellular vesicles (EVs) released by cells that play key roles in facilitating intercellular communication, both locally and systemically, under both homeostatic physiological and pathophysiological conditions (13; 18; 27; 28; 47; 51; 89; 102; 111; 200). These small (30-200 nm diameter) endosome-derived membrane vesicles are found in a variety of biofluids, and contain critical cellular messengers (e.g., cytokines, growth factors, RNA, transcription factors, adhesion molecules) (8; 33; 47; 60; 62; 220). Exosomes can affect target cells either by stimulating them directly via surface expressed ligands, or by transferring biologically active molecules between cells (47). The cell types, insult and severity of injury, and cellular microenvironment influence the complex payload-content (cargo) of exosomes shed (33; 47; 54; 61; 72; 232; 235). Both localized and distant exosomal-mediated delivery systems

have been shown to be critical drivers in the initiation, progression, and resolution of inflammation (4; 27; 33; 48; 67; 114). Importantly, the cargo of circulating exosomes is now considered to contain important early molecular signatures and surrogate diagnostic markers of inflammation and disease states in multiple pathologies, especially cancer (13; 18; 27; 40; 48; 77; 121; 136; 145; 154; 191; 214; 240).

Here, we provide a brief overview of exosomes and the inflammatory response to trauma to establish a foundational base in order to identify research gaps and opportunities to bridge the two fields. Specifically, we highlight the role of exosomes in the response to severe trauma and discuss the possibility of bioactive molecules contained within exosomes to serve as biomarkers in the diagnosis and prognosis of trauma-associated inflammation and complications.

2.3 BIOLOGICAL CHARACTERISTICS OF EXOSOMES

2.3.1 Biogenesis and intercellular communication

Extracellular vesicles are small, non-replicative membrane-bound structures released from cells, that serve as a vehicle for biomolecular transfer between cells, both locally and systemically. These vesicles can be isolated from a variety of biofluids, including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile, and semen (47). There are several types of small circulating vesicles, including exosomes, microvesicles (also termed ectosomes), and apoptotic bodies. Aside from their function in biological processes, the major difference between these EV subpopulations is their intracellular origin. Exosomes are derived from endosomes, whereas microvesicles are derived by the budding of the plasma membrane, and apoptotic bodies are vesicles

released into the extracellular space via plasma membrane blebbing during the disassembly of dying cells (100; 128; 156). The EV subtypes also vary in size, with overlapping diameter ranges, spanning from 30 – 200 nm for exosomes, 100 – 1000 nm for microvesicles, and 50 – 5000 nm for apoptotic bodies (8; 33; 60; 171). Microvesicles and exosomes function similarly in the transport of cargo biomolecules, and can be released under similar conditions, whereas apoptotic bodies are only released from dying cells (47). Both microvesicles and exosomes are released constitutively and in response to cell stress, cellular activation, senescence, and pathological conditions; therefore size, origin of vesicle release, and composition are important in discriminating between these EVs (47; 151; 199; 215).

Exosomes are generated from intraluminal vesicles (ILVs) within late endosomes, which are also known as multivesicular bodies (MVBs) (Fig 1). In brief, ILVs form via invagination of the endosome during a process known as microautophagy, therefore containing cell-specific cargo of proteins, lipids, and nucleic acids. A lipid bilayer, similar to a cell's plasma membrane, makes up the outer membrane of exosomes (8). The formation of ILVs and packaging of their contents can be endosomal sorting complex required for transport (ESCRT) dependent or ESCRT independent (223; 253). MVBs are either trafficked to lysosomes for fusion and ILV content degradation, or to the plasma membrane, where ILVs are released as exosomes into the extracellular space via exocytosis upon fusion with the plasma membrane (117; 199). While several molecules have been identified in the trafficking of MVBs to the plasma membrane for exosome release, including the ESCRT proteins, the exact mechanism of this process is still unknown (101; 253). Proteins and genetic material from the cell of origin are delivered

via exosomal binding to the plasma membrane of target cells, or by cellular phagocytosis of exosomes by target cells (27; 47). Uptake of exosomes by target cells is mediated by the interaction between exosomal ligands and receptors of recipient cells wherein they play a pivotal role in local and distant intercellular communications in modulating and coordinating the cellular functional activities of adjacent target cells, as well as those in distant tissues and organs (95). The lipid bilayer of exosomes makes them unique cellular communication vehicles. Exosomes are stable in circulation and able to shield their cargo from the microenvironment, leading to stability of the bioactive material within, and increased efficiency of delivery to effector targets (82; 154; 180; 189; 196).

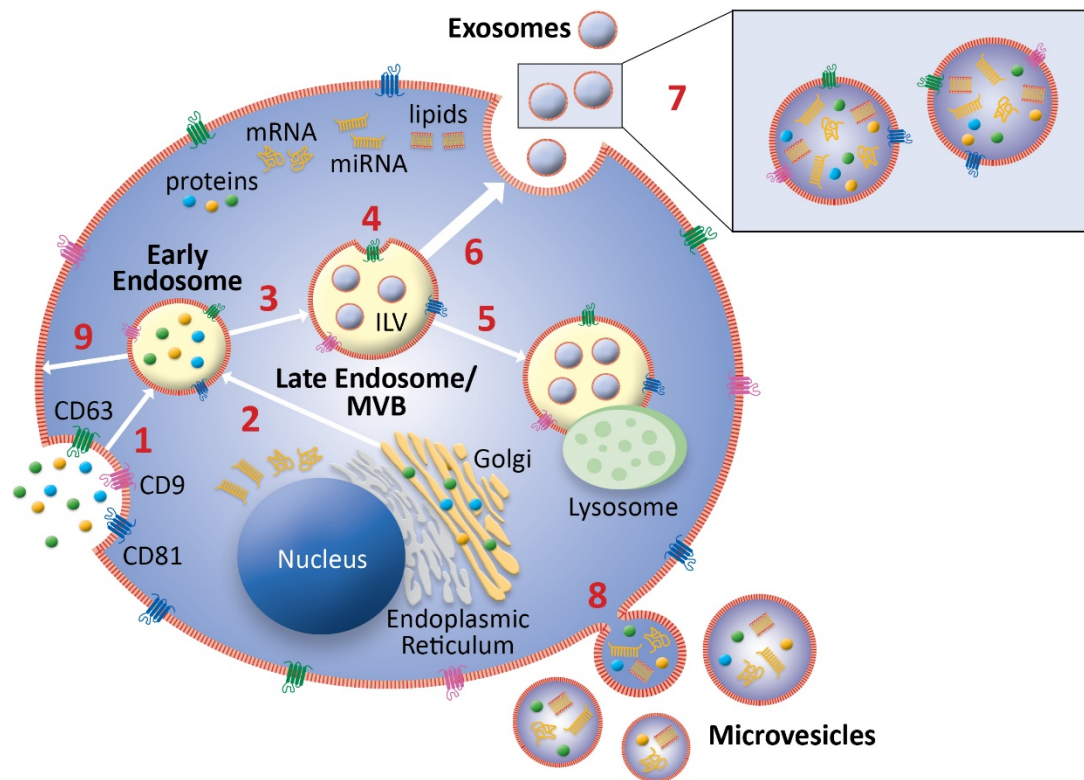


Figure 1. Exosome biogenesis.

1. Invagination of the plasma membrane of the cell collects extracellular material and generates a membrane bound endosome within the cell which retains plasma membrane proteins such as tetraspanins.
2. Golgi network packaging and transport of cellular material to the endosome.
3. Endosomal sorting complex required for transport (ESCRT) machinery packaging of biomaterial.
4. Invagination of the endosome forms intraluminal vesicles (ILVs). The collection of ILVs within an endosome forms a multivesicular body (MVB). There is cellular content from the cytosol contained within the ILVs as a result of microautophagy.
5. Trafficking of the MVB to the lysosome. MVB fusion with the lysosome results in ILV degradation.
6. Exosomes are released when the ILVs are released following MVB fusion with the plasma membrane.
7. Close-up of exosomes at release, with tetraspanins and bioactive cargo.
8. Budding of the plasma membrane forming microvesicles. These extracellular vesicles (EVs) are distinguishable from exosomes by nature of their biogenesis, and size.
9. Recycling of early endosome content to the plasma membrane.

2.3.2 Exosomal cargo overview

Exosomal cargo is dependent on the cell of origin and its state of activation (26; 46; 223). Exosomes contain a variety of bioactive molecules, to include cytosolic proteins (e.g. cytokines, growth factors), integrins, vesicle transport associated molecules, cytoskeletal proteins, heat shock proteins (HSPs), enzymes, lipids, signaling molecules, RNAs, and glycosylated transmembrane scaffold proteins called tetraspanins which mediate diverse functions including cell motility, protein trafficking, cell adhesion, and intracellular signaling coordinating ligand-receptor interactions (60; 62; 101; 220). Exosomal cargo contains both proteins and their mRNA transcripts, suggesting a redundancy in the loading of exosomes, as well as the importance of their cargo (84).

The nature of the biogenesis of exosomes results in variations in protein content based on the parent cell and the sorting mechanism. Proteomic analysis has revealed several different types of protein modifications involved in the processing of proteins for packaging into exosomes, including phosphorylation, ubiquitination, ISGylation, SUMOylation, myristoylation, glycosylation, and oxidation (8). Membrane scaffold proteins called tetraspanins have emerged as a unique protein identifier of exosomes in enrichment and isolation processes. Further, tetraspanins may have roles in exosome biogenesis, packaging of cargo, binding and uptake by target cells, and exosomal antigen-presenting functions in the immune response (9). CD63, considered a specific marker for exosomes, indicates endosomal origin, and is involved in the formation of ILVs (9). CD63 is found in late endosomes and contains an internalization motif that promotes rapid endocytosis. CD81 and CD9 are structurally similar surface membrane proteins with broad distribution on diverse cell types where they play a critical role in cell-cell

fusion, especially in macrophages (34). EVs found to contain both CD63 and CD81 are classically considered to be exosomes. The identification of exosomal tetraspanins can vary based on the parent cell, and various combinations of the markers CD9, CD63, and CD81 indicate successful exosomal isolation in the laboratory (9).

Two main functional forms of RNA are found in exosomes: coding RNAs like messenger RNA (mRNA), and non-coding RNAs, which include both micro-RNA (miRNA) and long non-coding RNA (lncRNA). The mRNA found in exosomes is functional, and able to be translated to proteins when exosomes are taken up by target cells (84). However, size analysis of these mRNAs shows they are between the length of miRNAs and full-length mRNAs, suggesting that exosomes carry primarily truncated, but functional, post-transcriptional mRNAs (16). miRNAs are short (20-25 nucleotides) non-coding RNAs that participate in post-transcriptional modifications, specifically with the end result of silencing target mRNAs. This is accomplished either by complementary binding with target mRNAs causing decreased expression of proteins, or by partial binding resulting in mRNA degradation. Thus, the effects of changes in miRNA expression can be observed both in terms of target mRNA levels or in target protein output (14). The cleavage of pre-miRNAs by the Dicer endonuclease also produces antisense miR* passenger strands, which are less stable than their mature miRNA counter-parts, but may be biologically active (93). These antisense miRNAs can also be found in exosomes (110; 256). miRNAs are differentially expressed across cell and tissue types, and their loading into exosomes is reflective of cellular activation, making them a valuable target for monitoring of disease (15; 92; 198; 207). lncRNAs include a diverse and dynamic population of non-coding RNAs longer than 200 nucleotides and can be a

source of miRNA biogenesis (244; 247). Similar to miRNAs, lncRNAs modulate protein expression via post-transcriptional mRNA modifications, but are also able to interfere with mRNA transcription, and modulate expression of other noncoding RNAs (36; 169). Given the diversity of exosomal content, the isolation of exosomes and analysis of their cargo may provide critical insights to the development and progression of various disease states.

2.4 INFLAMMATION AND THE ROLE OF EXOSOMAL CARGO

2.4.1 Inflammatory response to severe trauma

The body's response to severe traumatic injury can be characterized by three acute phases: survival, hyperinflammation, and repair (Fig 2). Death after traumatic injury is classically described in accordance with a trimodal mortality model, wherein death occurs immediately (< 1 hour), early (< 24 hours), and late (> 24 hours) following injury (203). Patients who sustain insurmountable injuries to major organ systems, including catastrophic hemorrhage from great vessel disruption or severe intra-cranial injuries, will typically die during the survival phase in the immediate to early stages of care (173; 212). Patients that initially survive often face severe hypercoagulable, hyperinflammatory, and infectious complications, principally during the inflammatory phase of the acute response. Patients who sustain polytraumatic injuries have a greater risk of developing these complications, as they experience a more prolonged and robust inflammatory response than patients sustaining a singular injury. As a result of a prolonged and heightened inflammation phase, the repair phase is ultimately delayed, as it cannot be initiated until the successful resolution of hyperinflammation. Over-activation of the immune system also interferes with other components of the response to

severe trauma, including hemostasis and anti-microbial defenses (29; 75; 143).

Importantly, an exaggerated and lengthy hyperinflammatory response can increase the risk of developing tissue repair complications, MODS, MOF, sepsis, and death.

The underlying pathophysiology of trauma-induced inflammation remains elusive, and the mechanisms are complex and multifactorial. Identification of early biological signatures to predict development, progression, and clinical outcome of severe inflammation-mediated immune dysfunction is critical for timely intervention and mitigation of downstream effects. Prior research efforts have largely focused on individual or small numbers of classic systemic inflammatory markers as biological signatures (2; 44; 55; 56; 68; 73; 83; 87; 124; 133; 206). A combinatorial signature involving genome-wide multi-plexed analyses of interlinked pathways may have a greater predictive value at any given point. Importantly, much of the underlying inciting biomolecular signaling that contributes to early immune dysregulation may be overlapping and/or distinct in the activation of the innate immune system preceding the development of a measurable systemic response. Additionally, the use of exosome-associated biomarkers found in circulation may serve as an early stage, non-invasive “liquid biopsy” companion to facilitate real-time diagnosis and monitor clinical progression, recovery, pathological complications, and therapeutic responses.

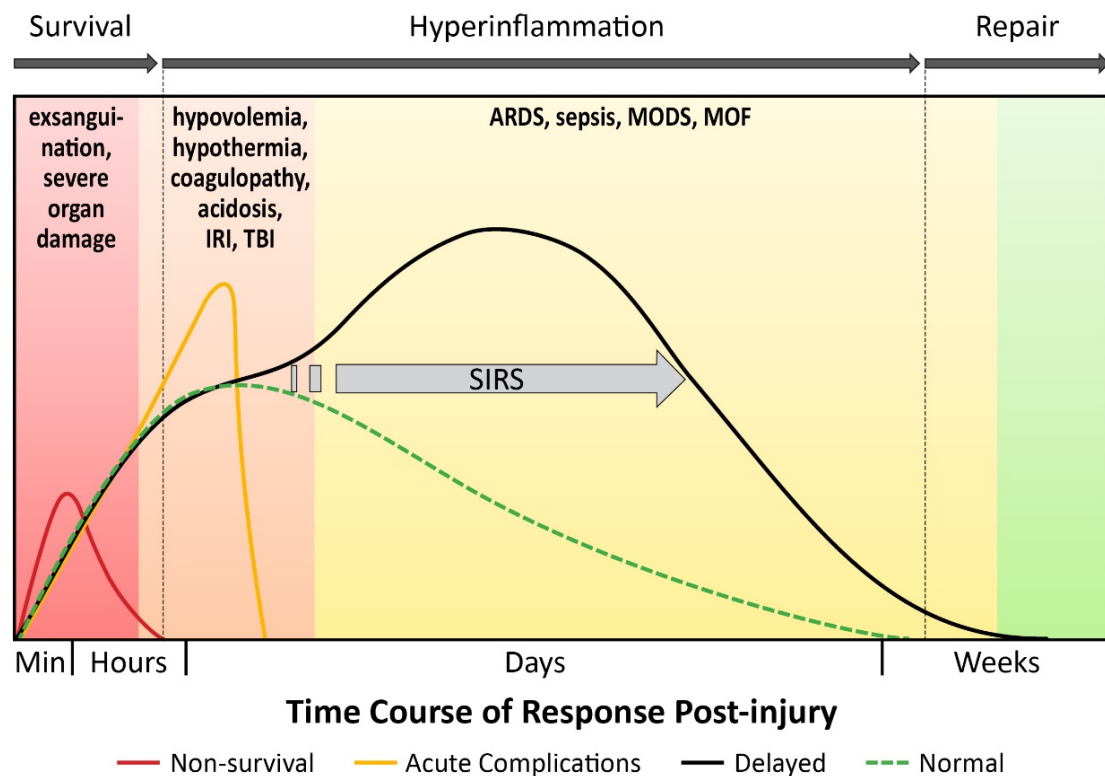


Figure 2. Response to traumatic injury.

The three phases following injury are identified by the arrows at the top of the timeline: survival, hyperinflammation, and repair. The longer the hyperinflammation phase is extended, the more delayed the initiation of the repair phase, contributing to late term healing complications. The text beneath the response phase arrows lists various complications associated with increased mortality during the course of the body's response to trauma – the majority of these conditions after the survival phase are caused by or related to inflammation. The dotted green line shows a “normal” appropriate, restrained inflammatory response that begins to resolve a few days after injury. The red curve represents non-survival – patients that will expire immediately following injury or within the earliest phases of care due to insurmountable injuries and exsanguination. These patients will fail to survive long enough to mount a robust inflammatory response. The orange curve represents patients that will be lost in the first few days of care, usually due to complications related to resuscitation, including ischemic reperfusion injury (IRI), or injury severity, especially in the setting of traumatic brain injury (TBI). The black curve encompasses the patient population that will experience delayed trauma-associated hyperinflammation-induced complications, beginning several days after the initial injury. Dysregulation of the inflammatory response or further insults will cause a patient to deviate from normal recovery, making them more susceptible to the development of systemic inflammatory response syndrome (SIRS), or infection that may cause further complications or death.

The innate immune system is activated within minutes after acute injury (107; 143). Following trauma, there is an uncontrolled local and systemic release of endogenous mediators into the circulation as a result of tissue damage and cellular necrosis associated with hemorrhage, ischemia-reperfusion injury (IRI), musculoskeletal injuries, burns, surgical procedures, subsequent secondary tissue injuries, and other insults (88; 134; 152; 178; 217; 232). These tissue-derived DAMPs include high-mobility group box 1 (HMGB1), the S100 proteins, HSPs, and mitochondrial DAMPs (mtDAMPs) (23; 108; 193; 251). In severe trauma, HMGB1, a DNA binding protein and key amplifier of inflammation, is actively secreted by immune inflammatory cells and passively released by injured and necrotic cells into the extracellular microenvironment to modulate the activation, metabolic activity and migration of immunocompetent cells and other cells at the site of injury (44; 125; 194; 219). The S100 proteins participate in calcium homeostasis intracellularly and are passively released in the case of cell necrosis, but also play a role in cytokine release and influencing cell migration in inflammation (23; 243). While HSPs normally provide functions as chaperone proteins, they are also stress responsive proteins upregulated in traumatized tissues (21). Similar to the other DAMPs, mtDAMPs are released by cells in response to damage, particularly mechanical and oxidative trauma. mtDAMPs participate in the trauma-induced immune response, and their activity has also been linked to specific injury patterns, including fracture (137; 195; 251). Invading pathogens contain biochemical signatures known as PAMPs, with the most recognized being endotoxin, also known as bacterial lipopolysaccharide (LPS), found on Gram negative bacteria. The innate immune system senses the diverse biochemical signatures of DAMPs and PAMPs via pattern recognition receptors (PRRs),

including extracellular toll-like receptors (TLRs), cytosolic nucleotide oligomerization domain (NOD)-like receptors (NLRs), and the receptor for advanced glycation end products (RAGE), allowing these danger signals to contribute to inflammation by activating and recruiting immune cells.

Detection of DAMPs and PAMPs propagates a cascade of early innate intracellular signaling, leading to the activation of key transcription factors, such as nuclear factor kappa beta (NF- κ B), that regulate diverse cellular responses and the transcription of pro-inflammatory mediators to include cytokines, chemokines, interferons, and anti-microbial peptides (74; 108; 193; 251). Endothelial activation, and local inflammatory mediators like histamine, kinins, and arachidonic acid cause leaky barriers and edema, allowing extravasation of neutrophils, monocytes, and macrophages into the tissue (91; 133). Monocytes are recruited from the circulation or the splenic reservoir to the site of inflammation where they infiltrate the tissue and undergo marked phenotypic and functional differentiation into macrophages or dendritic cells (DCs) to join and/or replace tissue-resident macrophages, all of which have been shown to play distinct roles in resolving inflammatory responses and promoting tissue repair-regeneration. (53; 103; 112; 209). Macrophage polarization is a key part of this process, explained by the M1/M2 paradigm. The M1 phenotype is pro-inflammatory, while the M2 phenotype is pro-reparative and healing (1; 112). Classically activated M1 macrophages activated by DAMPs or in response to pathogens release a milieu of early pro-inflammatory mediators (interleukin 1 beta (IL-1 β), IL-6, IL-8, IL-12, tumor necrosis factor alpha (TNF- α), macrophage inflammatory protein 2 alpha (MIP-2 α /CXCL2), interferon gamma-induced protein 10 (IP-10/CXCL10), regulated on activation, normal

T-cell expressed and secreted (RANTES/CCL5), reactive oxygen species (ROS), and reactive nitrogen species (RNS)). At the site of inflammation, these mediators propagate the pro-inflammatory cascade by activating endothelial cells and inducing the recruitment of other immune cells into the in-flamed tissue by activating more macrophages and inducing sustained inflammatory responses (193).

The binding of DAMPs to PRRs on innate immune cells triggers both intense pro-inflammatory, and concomitant anti-inflammatory responses. The initial anti-inflammatory response plays a critical role in balancing the hyperactive inflammatory state in the setting of excessive injury to the host following severe trauma (230). However, both uncontrolled pro- and anti-inflammatory responses may lead to pathological consequences. While excessive inflammation promotes the development and propagation of secondary tissue injuries beyond the initial site of injury/damage, the anti-inflammatory response can often lead to host defense impairment and sepsis, frequently leading to severe complications, MODS, and death. DAMPs and PAMPs bind similar cellular receptors, and post-traumatic cellular stimulation with DAMPs can lead to receptor tolerance upon pathogen exposure, such that cells fail to mount an appropriate response when binding to pathogen-triggered PAMPs, leading to infection (226). This impaired infection response is compounded in a state of systemic inflammation by reduced human leukocyte antigen-DR expression (HLA-DR) on peripheral blood mononuclear cells, which may pre-dispose patients to sepsis (212).

Regulation of innate immune cell mediated pro-inflammatory response at sites of tissue injury/damage is orchestrated through the early release of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α (Fig 3). The development and severity of an

inflammatory response can be monitored by tracking systemic levels of IL-1 β , IL-6, IL-8, TNF- α , and MIP-1 α indicative of the body being in a hyper-inflammatory state (143; 206). This array of soluble mediators directly evokes further immune cell activation in different cell types through the stimulated synthesis and release of a plethora of cytokines, chemokines, and other pro-inflammatory mediators used to communicate with other cells that initiate and constrain inflammatory response to pathogens and injury. These highly regulated, sequentially, and temporally orchestrated pleiotropic proteins initiate a cascade of signaling events that regulate host defenses against pathogens, leukocyte chemotaxis and migration, vascular endothelial permeability (vascular leak), production of acute phase proteins, and tissue damage repair. TNF- α is one of the first cytokines released following injury, peaking 1-2 hours after injury, with a short serum half-life of about 20 minutes (56; 133). TNF- α contributes to endothelial activation and vascular leak while also stimulating natural killer (NK) cells, monocytes, and macrophages. IL-1 β induces the production of cytokines (IL-6 and TNF- α), and chemokines (monocyte chemoattractant protein 1 (MCP-1)/CCL2 and MIP-1 α /CCL3) from immune cells, and the production of acute phase proteins including C-reactive protein (CRP) from the liver (59; 133). IL-1 β has a longer half-life at 90 minutes, but this is still a small window in the setting of acute trauma care, making fluctuations of the cytokine itself more difficult to monitor, and pointing towards downstream effectors as signs of activity (130; 133). IL-1 β is critical for the acute development of inflammation, and inability to produce IL-1 β results in an impaired systemic inflammatory response to stimuli, likely due to the resulting failed induction of IL-6 (70). Without IL-6, the immune system is unable to mount a normal inflammatory response (70). Both TNF- α

and IL-1 β promote the production of IL-6 by macrophages. IL-6 activates NK cells, inhibits apoptosis of neutrophils, and mediates the release of acute phase proteins from hepatocytes (133; 183). The interlinked pathways and redundancy of pro-inflammatory activation between TNF- α , IL-1 β , and IL-6 allows for general monitoring of inflammation using any one of the cytokines as a reference, instead of each separately (71). Prolonged and elevated increases in circulating levels of IL-6 post-injury correlates with a high Injury Severity Score (ISS), and is a reliable predictor of MODS, MOF, and mortality in polytrauma patients (56; 87). Levels of IL-6 can peak as early as four hours post-injury in major trauma and can estimate trauma severity up to six hours post-injury (83). However, the predictive value of IL-6 for major secondary complications like ARDS and MODS, and mortality peaks around 72 hours (206). The half-life of IL-6 is longer than TNF- α and IL-1 β , around 15 hours, making it easier to monitor in serum or plasma using commercially available immunoassay detection platforms (131). This makes IL-6 a more reliable indicator of the progress of inflammation and immune response, because while TNF- α can induce SIRS on its own and IL-6 cannot, the early window of release and short half-life make TNF- α an unsuitable marker. Circulating IL-6 levels often serve as strong predictors to guide escalation of treatment in patients with high risk of developing hyper-inflammatory syndrome (56; 133). IL-8 (CXCL8) is a major inflammatory chemokine released early post-injury by macrophages, at which point it plays a key role in the recruitment of neutrophils and other immune cells to the site of injury (133; 183). In addition, IL-8 plays a pivotal role in other wound healing processes including keratinocyte/fibroblast proliferation, angiogenesis, and tissue remodeling (115; 135). It can remain persistently elevated following trauma, up to a

period of weeks after the initial inflammatory response (56; 133). Elevations of IL-8 are correlated with patients developing ARDS and MOF, as well as increased mortality when significantly elevated at 24 hours. This is due to the chemotactic function of the cytokine, attracting neutrophils to organs where they then exhibit destructive effects through degranulation and the release of nitric oxide (NO) and ROS (56; 57). Prolonged inflammation increases the time of neutrophil extravasation, and this increases damage to the tissue, resulting in a self-propagating cycle of tissue destruction and response, and delaying wound healing and tissue repair (Fig 3) (143; 238).

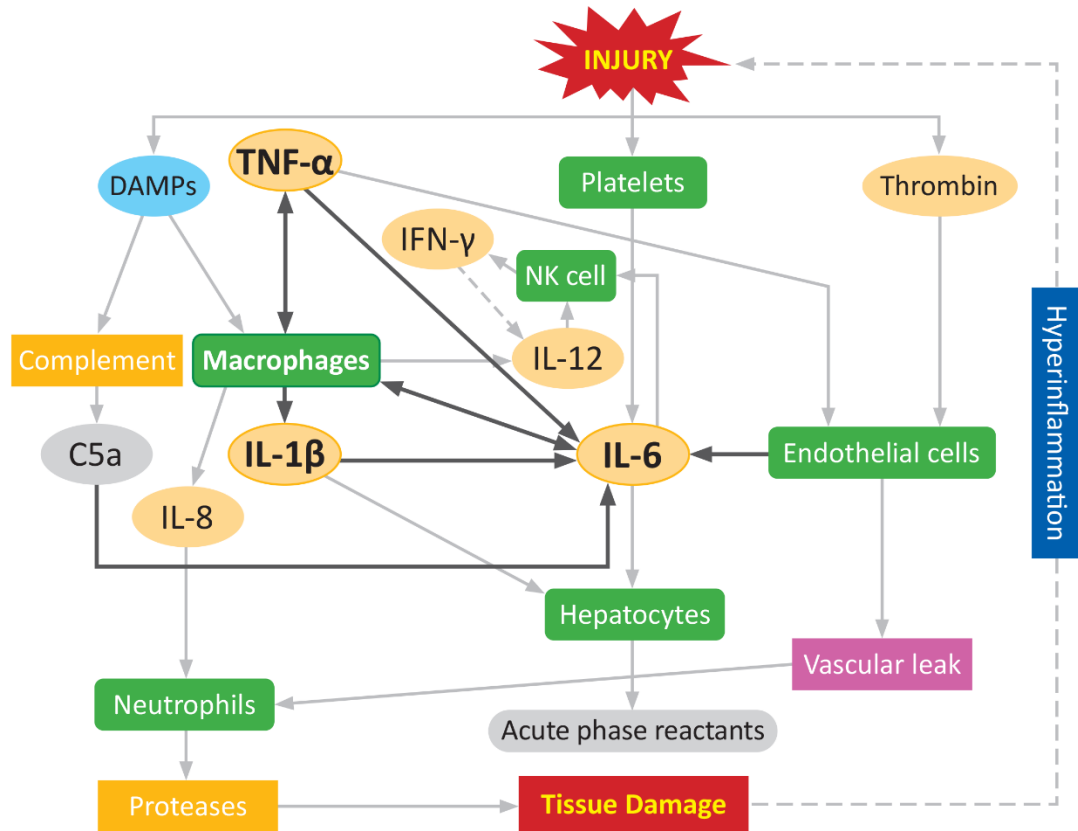


Figure 3. Inflammatory cytokines and the self-propagating trauma cycle.

This diagram is a simplified representation of the complex web of pro-inflammatory cytokines and how continued activation leads to further tissue damage. The release of damage associated molecular patterns (DAMPs), platelets, and thrombin in response to injury activates macrophages and endothelial cells to mount a response. Cytokines, including interleukins (IL), and tumor necrosis factor (TNF)- α are key early messengers in the inflammatory response, acting to activate natural killer (NK) cells, macrophages, neutrophils, and hepatocytes. Hyperinflammation from an unrestrained response further contributes to tissue damage, engaging the inflammatory response again.

Severe trauma not only induces pro-inflammatory reactions, but also anti-inflammatory reactions to reverse this process via CARS. Once the inflammatory response is established, control is critical. The inability of IL-4, IL-10, and other anti-inflammatory cytokines like tumor growth factor beta (TGF- β) to deactivate the inflammatory cascade results in a transition to a persistent inflammatory state. IL-10 is a cytokine that signals for attenuation of the immune response (143; 206). This is a rapid response, with IL-10 being released within 60 minutes of trauma (56). In SIRS, high levels of IL-10 are indicative of the body attempting and failing to control an inflammatory response (143). The interplay of IL-6 and IL-10 is an indicator of patient prognosis. In the final peak of the trimodal polytrauma mortality model, patients with a low ratio of IL-6 to IL-10 24 to 72 hours after injury have a higher incidence of ARDS, MODS, and death (Fig 2) (206). TGF- β works to dampen the immune response by inhibiting pro-inflammatory cytokine production and suppressing macrophages, DCs, and NK cells, in addition to its effects on the adaptive immune response (246).

The innate immune system, while providing the robust inflammatory response to trauma, is not the only responder. While the innate immune response provides a rapid and non-specific response, adaptive immunity is delayed, specific, cell-mediated, and humoral. Similar to the response of the innate immune system, the adaptive immune response can be a double-edged sword – necessary to clear dead, necrotic tissue and pathogens, but with the potential to become dysregulated and potentiate systemic inflammation and further tissue damage. While SIRS is primarily mediated via the innate immune response, CARS is mediated by the adaptive immune response (163). Briefly, the adaptive immune system begins to respond days following initial insult, as opposed to

the minutes it takes to activate the innate immune response, with DCs acting as a conduit between the two systems to control T-cell responses, specifically that of CD4⁺ T cells in inflammation. DCs are antigen presenting cells that act as sentinels, sensing the presence of microbes and tissue damage. Outside of sentinel functions, DCs can be matured by activated NK cells. Together, these cell types engage in immunoregulatory crosstalk via cytokines and cell-to-cell contact (39; 202). Once activated, DCs induce naïve CD4⁺ T cell activation and proliferation, including differentiation into T helper (Th) subsets Th1, Th2, and Th17, as well as T regulatory (Treg) phenotypes. The inflammatory Th1 cells produce interferon gamma (IFN- γ), which results in classical activation of M1 macrophages and the production of TNF- α , while anti-inflammatory Th2 cells produce IL-4, IL-5, and IL-13 causing alternative M2 macrophage activation (1; 213). Th17 production of IL-17 aids in neutrophil recruitment, a key aspect of the response to infection (249). While infection stimulates Th1 and Th17 responses, trauma is known to suppress CD4⁺ T cell responses (208). Studies have reported both the elevation of levels of Th2 associated cytokines leading to decreased survival in trauma patients with sepsis and decreases in both Th1 and Th2 responses following trauma, pointing to the importance of Th1 and Th2 balance in effective injury response (230). Tregs are responsible for restraining the activity of the Th subtypes mediated primarily via IL-10 and TGF- β (225). Injury-induced activation of Tregs appears to be responsible for controlling the CD4⁺ T cell response in trauma, with Treg depleted mice showing appropriate Th1 inflammatory response following injury (162). The presence of high levels of IL-10 in SIRS is linked to CARS, with IL-10 being a powerful immunosuppressive cytokine produced by multiple adaptive immune cells, including

DCs, Th2, and Treg cells (142; 163; 225; 228). This is consistent with CARS being associated with increased Th2 and Treg activity (208). Appropriate engagement of the adaptive immune system is necessary for a balanced immune response, but escalation to CARS, like SIRS, can be detrimental.

2.4.2 Role of exosomes in regulating inflammation

Damage associated molecular patterns contained within exosomes have been shown to activate the innate immune system, and therefore have the potential to contribute to the development of SIRS following traumatic injury (72; 227). While not yet identified specifically in exosomes isolated from patients during the early phase after severe trauma, many DAMPs are locally and systemically detectable following several different injury patterns, including fracture, hemorrhage, burn, and IRI (88; 134; 152; 178; 217; 232). Circulating DAMPs of interest in the study of trauma and inflammatory disease that have been identified in exosomes are HMGB1, the S100 proteins, HSPs, and mtDAMPs (23; 30; 45; 72; 84; 94; 113; 159; 188; 216). Physiologic stressors can modulate the location of HSPs in relation to exosomes, with acute stress increasing Hsp72 expression on plasma exosomes, and decreasing intravesicular miRNAs miR-142 and miR-203 (20; 72).

Macrophages release exosomes that activate, and induce pro-inflammatory responses in naïve immune cells, including differentiation of mononuclear cells into active macrophages releasing pro-inflammatory cytokines (32; 48). M1 macrophage-derived exosomes containing miR-155 are able to regulate macrophage polarization, decreasing the anti-inflammatory M2 phenotype (5). Through TLRs, DAMPs, PAMPs, and other inflammatory signaling increases miR-155 expression in macrophages (5; 144;

164). Upregulation of miR-155 increases inflammatory cytokines including IL-6, IL-1 β , TNF- α , and IL-8, and decreases anti-inflammatory cytokines, namely IL-10, and TGF- β (5; 144). Exosomes also carry inflammasome proteins, which are necessary to generate the biologically active form of IL-1 β , a key cytokine in immune system activation responsible for fever and leukocyte recruitment (54; 122; 177). Exosomes derived from NK cells contain cytotoxic proteins and are able to mediate cytotoxic functions similar to their donor NK cells (138). Once the inflammatory response is established, exosomes can propagate inflammation and impair healing, as well as inducing production of inflammatory cytokines and chemokines (12; 229). Macrophages co-cultured with mesenchymal stromal cell (MSC)-derived exosomes exhibit higher rates of oxygen consumption, and translocation of NF- κ B, a key component of cellular regulation of inflammatory factor transcription (174). Differentially expressed proteins in exosomes under inflammatory conditions are linked to other components of the physiologic response to trauma including the complement and coagulation cascades, such as complement C3, complement C4b, fibrinogen gamma chain, fibrinogen beta chain, and plasminogen activator inhibitor 1 (48; 234).

Exosomes post-injury contain important regulatory cytokines, growth factors, repair mediators and functional RNAs, such as mRNA and miRNA, that play important roles in inflammation and coordinating wound repair-healing (52). A deficiency in exosome secretion results in a persistent inflammatory phenotype and inappropriate response to inflammatory stimuli (4). TGF β -1 is an important activator of MSCs, which play a key role in the healing and repair process. Exosome associated TGF β -1 exerts a more potent effect on MSC migration and phenotype than the freely circulating cytokine

(197). miR-146 is an anti-inflammatory mediator and associated with the NF- κ B signaling pathway (24; 132; 164; 239). IL-1 β stimulates production of miR-146, and its packing into exosomes. Exosomal miR-146 has been reported to enhance M2 polarization of macrophages (204). Within the miR-146 family, miR-146a targets TNF receptor associated factor (TRAF) 6, IL receptor associated kinase (IRAK) 1, and IRAK2, resulting in negative regulation of IL-8 and CCL5 (184). miR-146a has both pro- and anti-inflammatory effects, whereas miR-146b is only anti-inflammatory (161). Myeloid-derived suppressor cell (MDSC)-derived exosomes, and miR-126 from endothelial-derived exosomes also promote M2 polarization (30; 224). Given the role of miRNAs in modifying cytokine expression, changes in miRNA expression and transport mediated by exosomes in response to acute trauma could provide insights into subtle changes in the innate response that may potentially lead to systemically devastating inflammatory cascades, prior to clinical detection of IL-6. The ability to load miRNAs into exosomes makes them a unique delivery vehicle, especially in the context of combating an inflammatory condition like sepsis with an anti-inflammatory miRNA like miR-146 (3).

Research regarding the mechanistic role of exosomal lncRNAs in the innate immune inflammatory response to injury and infection is limited, as many studies have focused on the utility of exosomal lncRNAs as disease biomarkers (120). Many immunomodulatory lncRNAs have been identified in exosomes derived from cancer cells, evidenced by their frequent tumor-associated naming conventions, but must be interpreted in the context of the unique tumor microenvironment (35; 139; 158). In a murine model of acute lung injury, the reduction of the lncRNA termed colorectal liver metastasis-associated transcript 3 (CLMAT3) found in monocyte-derived exosomes was

reported to upregulate NF- κ B activation, resulting in increased IL-1 β , IL-6, and TNF- α production that contributed to pathogenesis (37). In a controlled cortical impact traumatic brain injury model, Patel et al demonstrated the immunomodulatory impact of therapeutic exosomal delivery of lncRNAs. Infusion of human adipose-derived stem cells (hASC)-derived exosomes containing the lncRNA termed metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) modulated both coding and non-coding inflammatory RNAs, reduced neural lesion volume, and attenuated behavioral and motor symptoms, indicating importance of future studies for development of lncRNA exosome-based diagnostic and therapeutic tools (169).

Exosomes also play a role in the response of the adaptive immune system. DC-derived exosomes exchange major histocompatibility complex molecules between DCs and activate naïve CD4⁺ T-cells (31; 211). DC-derived exosomes also participate in the transfer of miR-155 and 146a, key immunomodulatory miRNAs (3). Bystander DCs undergo maturation when bound by DC-derived exosomes presenting TLR-ligands, amplifying the pro-inflammatory response through increased Th1 polarization (202). T cell receptor triggering increases the number of exosomes released from CD4⁺ T cells (222). Exosomal Hsp70 from DCs is able to modulate T cell differentiation (254). “Lethal” exosomes containing membrane-Fas Ligand released from T lymphocytes are able to trigger Fas-dependent apoptosis, a key mechanism of immune homeostasis in controlling over-active or auto-reactive T cells (7).

The cargo contained in exosomes has important functions in controlling immune cell function acting across all aspects of the inflammatory response. The extracellular communication system has been investigated more in depth in cancer and the tumor

environment but is critical across a multitude of pathologies. Identifying and understanding these roles will not only aid in the comprehensive understanding of how the body reacts to trauma, but also may serve as early indicators for immune dysregulation leading to delayed complications following traumatic injury.

2.5 EXOSOMES AND THEIR CARGO IN TRAUMA-ASSOCIATED COMPLICATIONS

The delayed effects of trauma manifest days to weeks post-injury. These issues can be compounded by interventions such as aggressive fluid resuscitation and surgery that further stress the body and contribute to inflammation. Identifying early signs of immune dysfunction following acute injury is critical for intervention and prevention of clinical sequelae. While the clinical symptoms can develop rapidly, activation of the innate immune system and the inflammatory response can potentially be identified on a biomolecular level preceding the development of a systemic response. Multifactorial, yet poorly defined, cellular, genomic, and proteomic inflammatory immune responses in patients with severe trauma, severe burns, or bacterial infection are well-documented (50; 99; 238). These characterizations have increased understanding of the physiologic response to trauma; however, further investigation is necessary for how these changes correlate with injury, and the timeline of response to injury. Current characterizations have been performed on samples taken several hours to days after injury, demonstrating the prolonged inflammatory impact, but not capturing the inciting biomolecular signaling. The ability of exosomal cargo such as miRNAs to modify expression of proteins in target cells and manipulate cytokine production make them valuable potential targets for early identification of inflammatory complications. In this section, we examine the consequences of trauma-associated inflammatory complications and the exosomal cargo

identified in the setting of their development, including IRI, ARDS, and sepsis, as well as highlighting the potential for future investigation into MODS and MOF (Table 1).

Table 1. Summary table of bioactive cargo of exosomes in trauma-associated inflammatory complications. This table specifically highlights micro-RNA (miRNA), messenger RNA (mRNA), long non-coding RNA (lncRNA), and proteins found in exosomes associated with ischemic reperfusion injury (IRI), acute respiratory distress syndrome (ARDS), and sepsis.

Trauma associated condition	Bioactive material	Exosomal cargo	Literature
IRI	miRNA	miR-23a	Crouser et al, 2021 (49)
		miR-24	Minghua et al, 2018 (150)
		miR-1, miR-133a, miR-24, miR-210, miR-133b	Emanuelli et al, 2016 (66)
		miR-16-5p, miR-24-3p, miR-200c-3p, miR-9a-5p, miR-141-3p, miR-200a-3p, miR-429	Sonoda et al, 2019 (205)
		miR-1, miR-208, miR-499, miR-133	Chen et al, 2019 (38)
	mRNA	Upregulated: Vsig1, Top1, Ccbp2, 0610010K06Rik, Krit1, D230019N24Rik, Amy2a1, Lba1, Zfp385c, 2700057C20Rik, Ptar1, Smad3, 2810002D19Rik, Phf6, Hsd17b11, 6720457D02Rik, Yipf7, Mep1a, Sox15, 4930473M17Rik	Eldh et al, 2010 (65)
		Downregulated: Cttna1, Pigq, Cct2, Rfc4, Gnas, Ttc3, Laptm5, Gabarapl1, Ipo4, Dnpep, Lmna, Ssr3, Qars, Gsn, Arap3, Med22, Csnk1d, Coro7, Laspl1, Ric8	
	Protein	C3 propeptide, PK-120 precursor, alpha amylase one precursor, beta-enolase isoform 1, adenylosuccinate synthetase isozyme 1	Yang et al, 2015 (242)
		Hsp70	Zheng et al, 2018 (254)

ARDS	miRNA	Upregulated: miR-146a, miR-27a, miR-126, miR-155	Wu et al, 2019 (237)
		Downregulated: miR-223, miR-181b	
		miR-155	Jiang et al, 2019 (114)
	lncRNA	Upregulated: AOC4P, BCAR4	Chen et al, 2020 (37)
		Downregulated: CLMAT3, MIAT	
	Protein	caspase 12, caspase 9, RIP3, microtubule associated proteins 1A/1B light chain B3	Kim et al, 2019 (123)
caspase 3		Moon et al, 2015 (155)	
Sepsis	miRNA	miR-155, miR-146a	Alexander et al, 2015 (3)
		miR-27a	Hashemian et al, 2020 (97)
		miR-19a, miR-21a, miR-22, miR-27a, miR-103-2, miR-107, miR-126a, miR-146b, miR-182, miR-200b, miR-203, miR-762	Appiah et al, 2020 (11)
		miR-16, miR-17, miR-20a, miR-20b, miR-26a, miR-26b	Wu et al, 2013 (236)
		Upregulated: let-7b-5p, let-7c-5p, miR-122-5p, miR-1227-3p, miR-125b-5p, miR-1260a, miR-1262, miR-1267, miR-1290, miR-1298-5p, miR-1300, miR-140-3p, miR-16-5p, miR-1825, miR-192-5p, miR-193a-5p, miR-194-5p, miR-195-5p, miR-19a-3p, miR-25-3p, miR-30a-5p, miR-320a, miR-320b, miR-363-3p, miR-486-5p, miR-518d-3p, miR-519b-3p, miR-520d-3p, miR-532-3p, miR-548a-3p miR-548c-3p, miR-597-5p, miR-618, miR-625-3p, miR-636, miR-645, miR-720, miR-	Real et al, 2018 (179)

	758-3p, miR-770-5p, miR-885-5p, miR-886-5p, miR-92a-3p, miR-99b-3p	
	Downregulated: miR-127-3p, miR-146a-5p, miR-151a-3p, miR-186-5p, miR-18a-5p, miR-199a-3p, miR-221-3p, miR-26a-5p, miR-28-5p, miR-301a-3p, miR-328, miR-331-3p, miR-335-5p, miR-339-3p, miR-340-5p, miR-340-3p, miR-590-3p, miR-628-5p, miR-744-5p	
	miR-27b, miR-125b, miR-21-5p, miR-30a-5p, miR-100-5p, miR-122-5p, miR-193a-5p	Reithmair et al, 2017 (181)
lncRNA	HOTAIRM1	Alkhateeb et al, 2020 (6)
	NADPH, NO synthase	Gambim et al, 2007 (79)
Protein	IL-1 β , IL-2, IL-6, TNF- α , IL-4, IL-10, CCL2, CCL3	Gao et al, 2019 (80)

2.5.1 Resuscitation and ischemia-reperfusion injury

Polytrauma frequently involves major vessel disruption and hemorrhage, which can rapidly develop into hemorrhagic/hypovolemic shock. Volume loss and shock-induced vasoconstriction leads to insufficient oxygen delivery to cells throughout the body and ischemic tissue injury. This ischemic environment is locally worsened in distal extremities when prolonged tourniquet use is necessary to prevent exsanguination (217; 218). Fluid resuscitation, whether by blood or other substitutes, is necessary to improve oxygen delivery (185). However, while this fluid resuscitation can improve initial survival, it was noted in the Vietnam War that overly aggressive fluid resuscitation resulted in increased deaths due to ARDS as a late complication (129). Early endothelial damage from reperfusion results in leaky barriers and increased leukocyte extravasation (185).

Ischemia-reperfusion injury is a unique injury pattern characterized by a period of hypoxia, followed by sudden hyperoxia, gradually returning to the normoxic state. Tissue damage is induced by cellular ATP depletion and an intense pro-inflammatory process. The latter is initiated by the ischemic period (hypoxia), but occurs mainly during the reperfusion phase, and is characterized by a large neutrophil recruitment (innate immune response). NF- κ B is a redox sensitive translational factor, which upregulates TNF- α production, initiating the pro-inflammatory cytokine cascade, including production of IL-1, IL-6, and IL-8 (186). This inflammatory response is an appropriate reaction to ischemia-induced cellular necrosis. Upon reperfusion however, neutrophils (innate immune response) and CD4⁺ T cells (adaptive immune response) are recruited to the site of injury, amplifying the inflammatory response, resulting in accumulation of free radicals, thereby potentiating systemic effects of local IRI (201). The local inflammatory

response as a result of IRI includes the simultaneous production/release of cytokines (TNF- α , IFN- γ , IL-1, IL-6), chemokines (IL-8), danger signals, ROS, and other mediators resulting in complex aberrant activation of multiple signaling pathways and increased apoptosis, leading to the temporary or permanent functional impairment of an organ or tissue, having implications either locally or at a distant site. IRI can lead to serious clinical manifestations to include SIRS, MODS/MOF, extremity amputations, and even death.

Kojima et al used a rat model of trauma and hemorrhagic shock to demonstrate modulations in the concentration of circulating exosomes in mesenteric lymph during normostasis, hemorrhagic shock, and in the resuscitation phase (126). The concentration of exosomes increased during shock and decreased during resuscitation. While the cargo of these exosomes was not profiled, the exosomes isolated during the resuscitation phase induced higher levels of monocyte NF- κ B expression and increased intra-cellular TNF- α levels in macrophages (126).

Hypoxic conditions and the induction of HIF-1 α increases the release of exosomes from various cell types, including renal proximal tubular cells, and cardiomyocytes (248; 252). Cancer cells under hypoxic conditions are known to release exosomes with increased miR-23a, but Crouser et al recently identified elevated exosomal miR-23a in patients with myocardial ischemia (22; 49; 105). miR-23a impairs NK cell cytotoxicity and is associated with TGF- β and angiogenesis in the tumor microenvironment (22; 105). Knockdown of miR-23a causes suppression of the NF- κ B pathway, and results in downstream reduction of IL-6 and TNF- α production (172). Exosomes released from cardiomyocytes cultured under hypoxic conditions contain

functional TNF- α and induce apoptosis of other cardiomyocytes (248). The mRNA cargo in exosomes released from cells under oxidative stress is significantly different than the exosomal cargo from untreated cells and is able to confer oxidative stress tolerance to other cells (65). Eldh et al reported 40 differentially expressed mRNA transcripts in exosomes derived from cells undergoing oxidative stress. The most upregulated was Vsig1, which codes for a cell adhesion protein, and the most downregulated was Ctnn1, which codes for beta-catenin, an adherens junction protein (65). Circulating serum exosomes in a model of skeletal muscle IRI induced by clamping of the femoral artery contained increased levels of complement component C3 prepropeptide, PK-120 precursor, alpha-amylase one precursor, beta-enolase isoform 1, and adenylosuccinate synthetase isozyme 1 (242). In another model of skeletal IRI induced by tourniquet, plasma exosomes contained significantly upregulated levels of miR-24 compared to sham. When these exosomes were added to cells cultured under oxidative stress, the transfer of miR-24 resulted in reduced apoptosis and decreased Bim expression in these cells, suggesting a protective role for the cargo of IRI-induced exosomes (150). Zheng et al showed that DC-derived exosomal delivery of Hsp70 to naïve T cells stimulated the PI3K/mTOR axis leading to increased Treg differentiation and decreased Th17 differentiation in a model of hepatocyte IRI (254).

Emanuelli et al found increases in five exosomal miRNAs (miR-1, miR-133a, miR-24, miR-210 and miR-133b) induced by ischemic injury following human coronary-artery-bypass-graft (CABG) surgery. These miRNAs positively correlated with cardiac troponin levels, but the appearance of elevated concentrations of circulating plasma exosomes appeared earlier than troponin, a gold standard in the clinical identification of

cardiac injury and ischemia, particularly in cases of myocardial infarction. Increases in circulating exosomes were seen immediately post-operatively, whereas troponin elevations were not detectable until 24-48 hours post-operatively (66). In a mouse model of acute myocardial infarction, Cheng et al demonstrated that plasma exosomes containing miR-1, miR-208, miR-499, and miR-133 were preferentially taken up by mononuclear cells within the bone marrow compartment within 6 to 12 hours post-injury. The net effect was mobilization of bone marrow hematopoietic stem/progenitor cells with endogenous repair capacities from the bone marrow to the site of ischemic injury (38). Sonoda et al, using a model of kidney IRI induced by clamping the renal arteries, showed that urinary exosomal cargo could act as a biomarker of acute kidney injury progression. Urinary exosomes contained unique differentially expressed miRNAs in the injury phase (post-injury days 1 and 2), in the initial recovery phase (day 3-7), and in the extended recovery phase (day 7-14), allowing for discrimination between IRI and sham groups after injury resolution. One day after IRI, exosomal miRNAs with targets related to decreasing NF- κ B activity (miR-16-5p, miR-24-3p, and miR-200c-3p) were increased (41; 141; 205; 255). On day 3, miRNAs with targets related to TGF- β signaling (miR-9a-5p, miR-141-3p, miR-200a-3p, miR-200c-3p, miR-429) were increased, with active TGF- β signaling manipulating the expression profile of these miRNAs in the extended recovery phase (205).

2.5.2 Systemic inflammatory response syndrome

The onset of SIRS has been used a surrogate predictor of outcome in polytrauma. Clinical assessments such as ISS correlate a higher degree of polytraumatic injury with the onset of SIRS (149). The clinical diagnosis of SIRS is based on changes in vital signs

(temperature, heart rate, blood pressure, and respiratory rate) accompanied by a laboratory assessment of a change in blood leukocytes (leukocytosis, leukopenia, or bandemia) (146). These inflammatory changes can also be seen post-operatively in patients undergoing major surgery (10; 86). Studies of trauma patients have created a “two-hit” model in correlation with IL-6 levels, where major reconstructive procedures or continued under-resuscitation in the early hyper-inflammatory post-trauma period causes patients to develop dangerously high levels of inflammatory cytokines, predisposing them to SIRS (87). Systemic inflammatory mediators are monitored as a metric for tracking patient progress following severe injury, and progression to SIRS and other delayed complications (68; 99; 134; 241). Patient development of SIRS is a clinical indication of immune dysregulation, and a warning of potential patient decompensation and development of other inflammation-related complications, which have likely already been set into motion on a molecular level, including exosomal intracellular communication.

There is a lack of literature regarding the specific role of exosomes and their cargo in the development and resolution of SIRS. Closely related to SIRS is sepsis, and exosomes and their cargo have been more extensively investigated in that setting. While the role of exosomal cargo in immune communication, onset, and resolution has been investigated, and patient development of SIRS is directly related to immune control, exosomal changes have not been studied. Future investigation of how exosomes and their cargo relate to ISS and SIRS offer the potential for earlier identification of at-risk patients and may unravel a biomarker better suited clinically than the most commonly utilized

current marker, IL-6, which often follows clinical signs that the patient is decompensating.

2.5.3 Acute respiratory distress syndrome

Acute respiratory distress syndrome is a complex pulmonary state of acute lung injury (ALI) that can result in pulmonary edema, lung fibrosis, pulmonary hypertension, and catastrophic respiratory failure, with the leading cause of death being development of MOF. In the setting of trauma, the immune response, resulting in overwhelming systemic inflammation such as in SIRS, causes NF- κ B activation and upregulation of pro-inflammatory mediators. This leads to the recruitment of neutrophils which release ROS and proteases that inappropriately damage the alveoli. Histological analysis of lung tissue shows hyaline membranes with diffuse neutrophil and macrophage invasion, protein-rich fluid in the alveolar spaces, disruption of the alveolar epithelium, and capillary injury (175; 231). The development of ARDS is well documented in critically ill polytrauma patients, particularly in those with ISS>25, traumatic brain injury, blood transfusion requirements, and significant orthopedic trauma such as long bone fractures (127; 187). Frequently, ARDS patients must be placed on prolonged mechanical ventilation with pressure support to prevent alveolar collapse, which itself can further contribute to issues of hyperoxia, nosocomial infection, and destruction of lung architecture in ventilator-induced lung injury. Gaining control of systemic inflammation is a key factor in improving patient outcomes in ARDS (148).

Neutrophil activity is a key aspect of ARDS, and their release of exosomes with pathogenic payloads has been demonstrated in other lung pathologies such as chronic obstructive pulmonary disease and bronchopulmonary dysplasia (85; 175). These

pathogenic exosomes contain higher levels of surface-bound neutrophil elastase, which normally provides an antimicrobial role, but in aberrant expression, degrades lung architecture (85). In patients with community acquired pneumonia that went on to develop ARDS, serum exosomes had increased expression of miR-146a, miR-27a, miR-126, and miR-155, and decreased miR-223 and miR-181b compared to patients that did not develop ARDS (237).

In a model of LPS-induced ALI, Jiang et al showed that serum exosomes contained increased levels of miR-155, which stimulated NF- κ B in macrophages, resulting in enhanced TNF- α and IL-6 production. When exosomes from ALI mice were infused into naïve mice, the disease state exosomes activated lung macrophages, expanded the M1 population, and induced pathological pulmonary inflammation (114). Chen et al identified 29 differentially expressed lncRNAs as part of the cargo from circulating monocyte-derived exosomes collected from ALI patients. The authors demonstrated that the co-culture of the human monoblastic cell line, U937, with the ALI exosomes augmented the secretion of IL-1 β , IL-6, IL-15, IL-18, and TNF- α , suggesting M1 type macrophage activation. Moreover, they demonstrated that ALI monocyte-derived exosome preparations contained decreased levels of the lncRNA CLMAT3 which activates the CtBP2-p300-NF- κ B transcriptional complex to induce robust levels of proinflammatory cytokines IL-1 β , IL-6, and TNF- α . Overexpression of CLMAT3 reversed this effect, indicating the importance of the lncRNA in NF- κ B modulation and inflammation in the setting of ALI (37).

Exosomes in the bronchoalveolar lavage fluid of ARDS patients contain proteins associated with apoptosis (caspase 12, 9), necroptosis (RIP3), and autophagy

(microtubule-associated proteins 1A/1B light chain 3B) (123). In the setting of hyperoxia-induced lung trauma, increased numbers of exosomes were released from lung epithelial cells in bronchoalveolar lavage fluid, containing increased levels of caspase-3. When the hyperoxia-induced exosomes were administered to naïve mice, it caused pro-inflammatory effects, evidenced by increased numbers of alveolar macrophages and neutrophils in their bronchoalveolar lavage fluid. The lung epithelium-derived exosomes were also detected circulating in the serum, and peritoneal macrophages exposed to the serum exosomes expressed elevated levels of TNF- α and MIP-2 (155).

2.5.4 Sepsis

Sepsis, like SIRS, is diagnosed when the patient is experiencing a systemic inflammatory response but is differentiated by the presence of a known infection. The severity of the immune response to trauma, and concomitant CARS can place patients at risk for developing a severe infection. In an immunocompetent patient, infections are swiftly dealt with by the immune system with an appropriate inflammatory reaction ridding the body of the inciting pathogen. However, overstimulation of the immune system by both trauma and microbes creates the setting for an infection, leading to sepsis (160). Increased ISS, decreased Glasgow Coma Score (GCS), and pre-existing conditions place trauma patients at higher risk for developing sepsis (166). If allowed to proliferate, local infections may progress to systemic infections attacking multiple organ systems. The systemic infection attacks the endothelial and epithelial barriers, leading to tissue damage and organ dysfunction, including ARDS and acute kidney injury (90). A fatal complication of sepsis is disseminated intravascular coagulation (DIC), as the inflammatory cascade and inappropriate clot formation deplete coagulation factors,

leading to spontaneous, and uninhibited bleeding (165). Decreased systemic vascular resistance leads to low blood pressure and perfusion issues and can cause septic shock (90). These perfusion issues contribute to lactate being a recognized biomarker of sepsis since anaerobic glycolysis produces high levels of lactate. However, single biomarkers have proven less effective in predicting patient progression and outcome than combined panels of both pro- and anti-inflammatory targets (69).

The role of exosomes in sepsis has been previously reviewed, highlighting their cargo as more precise biomarkers for patient status and progression, and as therapeutic targets (168; 210). In sepsis, exosomes carry critical mediators of the inflammatory response. Inhibition of exosome biogenesis and release with GW4869 (a pharmacological agent that blocks the ceramide-mediated inward budding of MVBs and mature exosome release) decreased the production of pro-inflammatory cytokines in a combined surgical trauma and endotoxin model, and improved survival (67). Platelet-derived exosomes from septic patients carry active NADPH oxidase and NO synthase, contributing to the disruption of the endothelial barriers in the vasculature (79). In an LPS model of sepsis, Alexander et al were able to demonstrate functional transfer of exosomal miR-155 and miR-146a both in vitro and in vivo. The infusion of exosomes containing miR-155 in miR-155 $-/-$ mice challenged with LPS resulted in increased levels of serum TNF- α and IL-6, whereas infusion of exosomes containing miR-146a in miR-146a $-/-$ mice caused decreased serum levels of TNF- α and IL-6 (3). Several other miRNAs are related to the pathogenesis of sepsis, particularly miR-27a, which induces an inflammatory response, is correlated with the developed of septic shock, and is differentially expressed in the plasma exosomes of sepsis patients (97). Appiah et al investigated intestinal epithelium-

derived exosomes in the intestinal lavage fluid of a cecal ligation and puncture (CLP) sepsis model. Isolated exosomes from injured mice contained 360 differentially regulated miRNA and 190 unique miRNAs compared collections obtained from healthy controls. Injection of septic exosome preparations into the lumen of healthy ileum resulted in downregulation of TNF- α , IL-1 β , IL-6, IL-17A, and IL-22, while treatment of inflamed ileum resulted in suppressed TNF- α and IL-17A activity, suggesting immunosuppressive activity (11). Using a CLP model of sepsis, Wu et al identified six miRNAs, miR-16, miR-17, miR-20a, miR-20b, miR-26a, and miR-26b, with increased expression in serum exosomes eight hours after injury (236). Also in a CLP model, Alkhateeb et al found an 18-fold increase in the lncRNA HOXA transcript antisense RNA, myeloid-specific 1 (HOTAIRM1) in late sepsis ($\geq$ day 5) Gr1+CD11b+MDSC-derived exosomes in a CLP model. In early sepsis ($<$ day 5), the protein S100A9 was mostly confined to the cytosol, however, HOTAIRM1 causes a nuclear translocation of S100A9 in late sepsis, resulting in an immunosuppressive phenotype of Gr1+CD11b+MDSCs. Assessment of plasma exosomes in human sepsis patients also showed increased Hotairm1 (6). Real et al identified 28 differentially expressed exosomal miRNAs in sepsis patients both at the time of enrollment in the study and seven days later. Additionally, 35 differentially expressed plasma exosomal miRNAs were linked to patient outcome, and analysis of the pathways influenced by these miRNAs identified the targets as involving the inflammatory and immune response, particularly IL-6 signaling, acute phase response signaling, NF- κ B signaling, and IL-10 signaling (179).

In a CLP model of sepsis, Gao et al demonstrated that serum exosomes from septic mice obtained 2 hours post injury contain significantly elevated levels of pro-

inflammatory cytokines IL-1 β , IL-2, IL-6, and TNF- α whereas the lymphocyte chemoattractants CCL2 (MCP-1) and CCL3 (MIP-1 α) and anti-inflammatory cytokines IL-4 and IL-10 were not increased until 12 hours and 24-48 hours, respectively. The elevations of cytokines and chemokines in exosomes had different dynamics than their soluble counterparts in serum, suggesting a unique role for exosomal cargo in sepsis (80). Reithmair et al found a similar pattern in the miRNA cargo of exosomes, where intracellular, exosomal, and serum sepsis-associated miRNAs were compartment specific. While miR-27b was increased in blood cells, serum exosomes, and unfractionated serum, miR-125b elevations were unique to serum exosomes. The only other similarity existed in the upregulation of five miRNAs, miR-21-5p, miR-30a-5p, miR-100-5p, miR-122-5p, and miR-193a-5p, in both serum exosomes and unfractionated serum. Elevation of three exosomal miRNAs, miR-30a, miR-125b, and miR193a, correlated with disease severity and survival prediction (181).

2.5.5 Multi-organ dysfunction syndrome and multi-organ failure

Advances in trauma care have resulted in increased survival of patients after initial insults. However, these patients can experience delayed complications resulting from infection, hemorrhage and reperfusion injury, and dysregulated inflammation (57). Multiple organ failure is a leading cause of delayed mortality in trauma patients (108; 159). The development of MOF occurs days to weeks after an inciting event (Fig 2) (55). Major risk factors for the development of MOF in trauma patients includes ISS ≥ 16 , age >45 years, and male sex (76). Injury patterns and interventions associated with a high ISS and increased risk of MOF are severe abdominal trauma, multiple fractures, severe thermal injuries, infection, and massive blood transfusions (17; 55). Transient multiple

organ dysfunction may be present post-injury and during the resuscitation phase out to 48 hours due to a hyperinflammatory response but does not always progress to MOF (43). The models for the development of MOF progression from SIRS are either “one hit” or “two hit.” In the “one hit model,” patients experience such severe trauma that SIRS rapidly progresses and leads to early MOF. In the “two hit” model, initial injury and under-resuscitation prime injured patients in a SIRS-like state to progress to MOF when exposed to a secondary insult such as surgery or other procedural resuscitation techniques (86; 87). This phenomenon can complicate surgical decision-making attempting to balance the resuscitative benefits of early appropriate care against the deleterious effects of surgery-induced inflammatory reactions. The development of SIRS, MODS, and MOF exist on a continuum, where recognition of these conditions and timely intervention can prevent progression to the next stage. The use of anti-cytokine and immunomodulation therapies in prevention of post-operative SIRS has not achieved success, and prevention of patient progression to MOF is regarded as the most effective treatment (2; 86; 192). The clinical parameters for differentiating between the stages are similar, and progression may be subtle, necessitating identification of specific markers (17).

Research into the role of exosomes and their cargo specifically as they relate to the development of MODS and MOF post-injury are limited. Im et al report an association between the level of plasma exosomes and Sequential Organ Failure Assessment (SOFA) in the prognosis of critically ill patients with sepsis but did not profile the contents of the exosomes (109). Murao et al reviewed the impact of exosomes in end-organ damage in sepsis contributing to MODS, emphasizing the impact of exosomal delivery of sepsis-induced inflammatory mediators on the lungs, kidneys, liver,

cardiovascular system, and central nervous system (157). Examining the systemic inflammatory response to sepsis offers a potential framework for examining the impact of exosomal communication in traumatic injury, as PAMPs and DAMPs act on similar receptors. The study of MODS and MOF is largely a study of overwhelming and uncontrolled inflammation. As highlighted in the previous section, exosomal cargo has a significant role in intercellular communication of immune cells and the development and resolution of inflammation. Hemorrhage necessitates massive transfusions, and a major consequence of uncontrolled infection is sepsis, which has been discussed, but fracture and thermal injuries are also associated with unique exosomal cargo that may portend inflammatory progression and MODS or MOF development.

2.5.6 Fracture healing

While exosomes regularly participate in bone homeostasis and remodeling, they also participate in resolution and potentiation of disease states in the skeletal system (18; 51; 78; 81). In a CD9 ^{-/-} mouse model, fracture healing was delayed, but rescued by injection of MSC-derived exosomes. Within these exosomes, miR-21, which is known to promote osteogenic differentiation of MSCs, was highly expressed (78). Consistent with some degree of inflammation necessary in the setting of injury, miR-21 is also an NF- κ B-dependent miRNA that is upregulated in response to peripheral blood mononuclear cell stimulation with LPS (257). Currently, research on exosomes and bones outside of the setting of osteoporosis is limited, but since exosomal cargo is known to have a role in the setting of fracture, additional studies in the setting of fracture in patients with healthy bone density may yield more benefits to the study of severe trauma.

2.5.7 Thermal injury

Large surface area burns drive a systemic inflammatory response from massive tissue injury accompanied by major fluid loss through local edema and barrier disruption, liberated tissue breakdown products, and hypovolemia-induced cardiac dysregulation. While catecholamines and corticosteroids are the primary mediators of metabolic dysfunction in burn patients, inflammatory mediators such as IL-1, IL-6, and TNF have also been implicated, particularly in contributing to the severity of dysfunction and catabolism (118; 233). Muscle catabolism impairs the immune response and wound healing, and places patients at risk for complications and death (233). Qin et al found that there was differential expression of 31 proteins in the serum exosomes of burn patients. Of particular note in their findings was the upregulation of ITGA2B and ITGB3 in the burn group, as these proteins act in the PI3K/AKT signaling pathway, which promotes cell proliferation and growth (176). Wound healing, especially cutaneous, is critical in a burn patient, to re-store the body's barrier to fluid loss and infection. Persistence of inflammation can delay wound healing, but anti-inflammatory miRNAs such as miR-124a and miR-125b found in exosomes have the ability to manipulate expression of inflammatory cytokines in the wound microenvironment to create a more favorable setting for repair (106). The role of exosomal cargo as a therapeutic option, especially MSC-derived exosomes, in the setting of burn has been more extensively studied than the profiles of exosomes in injured burn patients. Li et al determined in a rat burn model that human umbilical cord MSC-derived (hucMSC) exosomal delivery of miR-181c was able to attenuate the immune response to burn by decreasing TLR4 expression and NF- κ B/p65

activation (140). In a rat burn model, Zhang et al demonstrated that hucMSC-derived exosomes carrying Wnt4 enhanced cutaneous wound healing (250).

2.6 CONCLUSIONS

Previous research has demonstrated the role of exosomes and their cargo in the development, control, and resolution of inflammation. Characterization of their role in inflammatory immunological processes indicates they are pivotal in many pathological states. Information is lacking in how these extracellular messengers contribute in the context of severe trauma, and the subsequent uncontrolled inflammation that follows. Further investigation into the exosome profiles following polytraumatic injuries may provide valuable insight into the early stages of severe systemic inflammation and provide opportunities for early intervention. Identification of inciting biomolecular events for conditions like SIRS is critical for preventing delayed patient deterioration to ARDS, sepsis, or MOF after initial resuscitation. While systemic markers of inflammation, such as IL-6 are already widely used, the unique cargo of exosomes may help to differentiate the effects of trauma upon the body, rather than using broad markers of systemic dysfunction. Secondly, indication that markers of inflammation are detected earlier within circulating exosomes compared to their free, non-exosomal counterparts in circulation, provides potential opportunity of earlier intervention than in current practices. Exosomal cargo appears to reflect local tissue and systemic pathology, thereby effecting remote organ function, and potentially functioning as a critical read-out of post-traumatic damage. Put together, these circulating, membrane-bound vesicles offer a new method of characterizing trauma-related “fingerprint molecular signatures,” with the opportunity to enhance the efficacy of clinical decision-making tools and precision medicine. The

observed association between inflammation and change in nature or expression levels of specific exosomal cargo is the fundamental step for identifying possible novel biomarkers of inflammatory-based pathologies, diseases, and clinical sequelae.

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**CHAPTER 3: KEY EARLY PROINFLAMMATORY SIGNALING
MOLECULES ENCAPSULATED WITHIN CIRCULATING
EXOSOMES FOLLOWING TRAUMATIC INJURY**

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3.1 ABSTRACT

Assessment of immune status in critically ill patients is often based on serial tracking of systemic cytokine levels and clinical laboratory values. Exosomes are extracellular vesicles that can be secreted and internalized by cells to transport important cellular cargo in the regulation of numerous physiological and pathological processes. Here, we characterize the early compartmentalization profile of key proinflammatory mediators in serum exosomes in the steady state and following trauma. Adult male Sprague-Dawley rats (91 including naïve) were divided into one of four traumatic injury model groups incorporating whole-body blast, fracture, soft-tissue crush injury, tourniquet-induced ischemia, and limb amputation. Serum was collected at 1, 3, 6, and 24 hr, and 3- and 7-day post-injury. Electrochemiluminescence-based immunoassays for 9 key proinflammatory mediators in whole serum, isolated serum exosomes, and exosome depleted serum were analyzed and compared between naïve and injured rats. Serum clinical chemistry analysis was performed to determine pathological changes.

In naïve animals, substantial amounts of IL-1 β , IL-10, and TNF- α were encapsulated, IL-6 was completely encapsulated, and CXCL1 freely circulating. One hour after blast injury alone, levels of exosome encapsulated IFN- γ , IL-10, IL-6, IL-13, IL-4, and TNF- α increased, whereas freely circulating and membrane-associated levels remained undetectable or low. Rats with the most severe polytraumatic injuries with end organ complications had the earliest rise and most pronounced concentration of IL-1 β , IL-10, TNF- α , and IL-6 across all serum compartments. Moreover, CXCL1 levels increased in relation to injury severity, but remained almost entirely freely circulating at all timepoints.

These findings highlight that conventional ELISA-based assessments, which detect only freely circulating and exosome membrane-bound mediators, underestimate the full immunoinflammatory response to trauma. Inclusion of exosome encapsulated mediators may be a better, more accurate and clinically useful early strategy to identify, diagnose, and monitor patients at highest risk for post-traumatic inflammation-associated complications.

3.2 INTRODUCTION

Trauma is a leading cause of morbidity and mortality worldwide (1). Complex polytraumatic injuries occur due to motor vehicle collisions, falls from height, firearm discharges, mass transit collisions and derailments, industrial workplace injuries, terrorist attacks, and natural disasters (5; 11; 16; 17; 24; 34; 38; 48). Injury immediately triggers the body's complex innate immune inflammatory response, essential for immune surveillance, clearance of debris and necrotic tissue, and preparation for healing and regeneration (27). However, if unchecked, an overwhelming aberrant immune response at the site of injury can spread systemically to distant organs leading to other life-threatening inflammatory complications, to include systemic inflammatory response syndrome (SIRS), compensatory anti-inflammatory response syndrome (CARS), acute respiratory distress syndrome (ARDS), pneumonia, sepsis and/or multiple organ dysfunction syndrome (MODS) resulting in collateral tissue damage, end organ injury, and increased mortality (15; 27; 33; 37; 39; 46; 49; 56; 58).

The innate acute phase immune response to traumatic injury is initially measured by the systemic release of both pro and anti-inflammatory mediators, particularly interleukin-6 (IL-6) (21; 32; 53). However, IL-6 levels peak hours after initial injury, and

IL-6 is downstream of other mediators such as tumor necrosis factor-alpha (TNF- α) and IL-1 β , limiting early prediction and diagnosis of a patient's hyperinflammatory state, complications, and outcome (14; 22; 53). Extensive reports have shown that exosomes, small extracellular vesicles (EVs) with a diameter of 30-200 released by all cell types to maintain their regular cellular homeostasis, transfer their molecular cargo to target cells wherein they play a critical role in facilitating intercellular signaling under both homeostatic physiological and pathophysiological conditions (3; 4; 6; 9; 12; 13; 23; 26; 28; 52; 57). Further, exosome-encapsulated cytokines have been reported to be protected from environmental degradation and exert more potent effects than freely-circulating cytokines (51). Differences in the distribution of free-circulating and EV-encapsulated or EV-associated cytokines have been identified and these differences may be stimulus-dependent (18).

Little is known about exosomes in relation to the steady-state and the early trauma-induced inflammatory immune response. Our objective was to characterize the proinflammatory mediator cargo of serum exosomes in several models of traumatic injury. Specifically, we determined the timing and quantitative level of critical proinflammatory mediators, as well as changes based on insult, and location in the serum compartment, identifying which are freely circulating or exosome membrane-associated, versus those encapsulated in exosomes and undetectable using standard immunoassay methods. We show detection of both free and exosome-associated proinflammatory mediators may be a better, more accurate, and clinically useful early strategy to identify, diagnosis and monitor patients at the highest risk for post-traumatic complications.

3.3 METHODS

3.3.1 Animals

Pathogen-free adult male Sprague-Dawley rats (*Rattus norvegicus*; 400-550 g; 91 animals) were purchased from Taconic Farms (Germantown, New York, USA). All rats were paired and housed in clean standard plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow), fresh water, and appropriate enrichment. Rats were allowed to acclimate to the vivarium conditions for at least three days before any handling or experimental procedures. After injury, rats were returned to clean home cages with soft bedding. All experimentation involving rats was performed in accordance with institutional standard guidelines and approved (SUR-20-997) by the Uniformed Services University Institutional Animal Care and Use Committee (IACUC) in compliance with all applicable Federal regulations governing the protection of animals in research.

Rats were divided into one of four experimental groups (n=21/group) (Fig 4) consisting of (1) blast overpressure exposure only (B); (2) complex orthopaedic injury consisting of right femur fracture and soft tissue crush injury (COI) with 3 hr of tourniquet-induced hind limb ischemia and reperfusion injury with tourniquet release (IRI) followed by limb amputation (HLA) through the zone of injury (ZOI); (3) B and COI followed by a 1 hr delayed hind limb amputation (dHLA); (4) B and COI with IRI followed by dHLA. Additionally, samples from normal, healthy naïve rats (n=7) were included in the study for comparison.

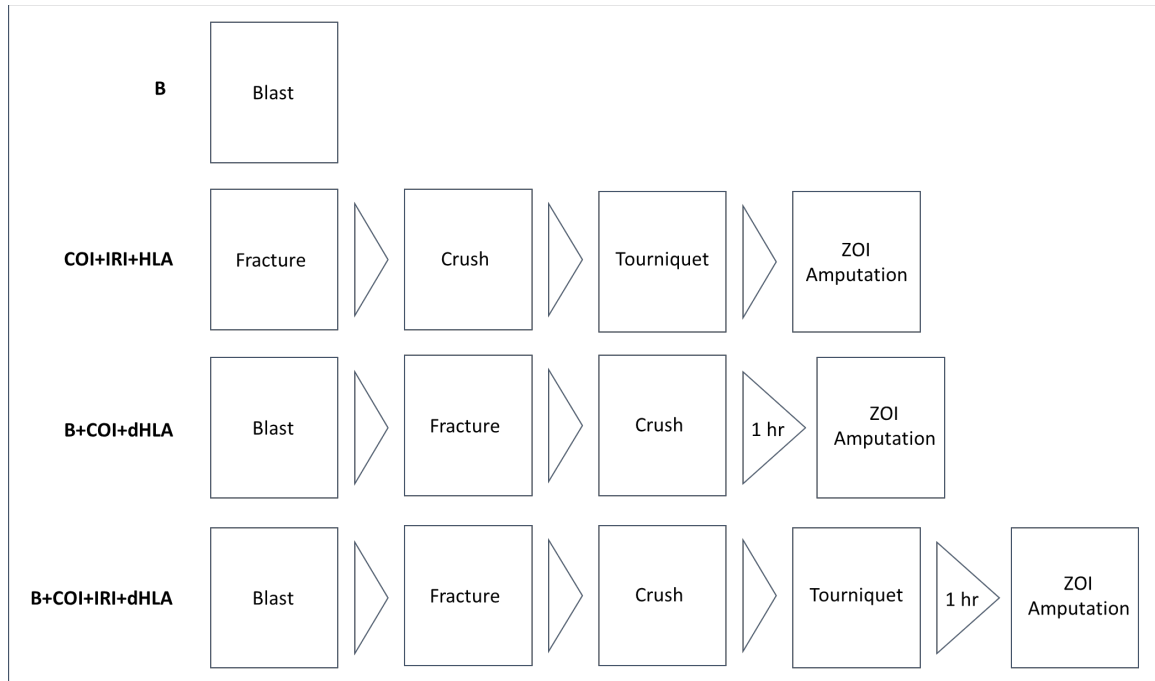


Figure 4. Diagram of experimental groups and sequence of trauma injury patterns. Rats were randomly assigned to undergo blast (B), complex orthopaedic injury and ischemia reperfusion injury followed by hind limb amputation through the zone of injury (ZOI) (COI+IRI+HLA), B+COI+1 hour delayed (d) HLA, or B+COI+IRI+dHLA.

3.3.2 Trauma injury patterns

3.3.2.1 Anesthesia

Rats were initially anesthetized with 4% isoflurane and administered a ketamine-xylazine (75mg/kg ketamine (Henry Schein Animal Health, Dublin, Ohio, USA), 10 mg/kg xylazine (Akorn, Inc., Lake Forest, Illinois, USA)) mixture, with appropriate re-dosing as necessary for the duration of the procedure.

3.3.2.2 Blast overpressure exposure (B)

To induce a whole-body blast injury, rats were secured and placed in an Advanced Blast Simulator overpressure shock tube (ORA Inc., Fredericksburg, Virginia, USA), and exposed to a blast overpressure of 125 ± 10 kPa as previously described (40).

3.3.2.3 Complex orthopaedic injury (COI)

Animals undergoing extremity injuries first had the right hind limb shaved. A Lateral Long Bone Ballistic System (University of Alabama-Birmingham, Birmingham, Alabama, USA) was used to create a comminuted femur fracture in a similar fashion previously described (7). The right hind limb of the rat was internally rotated and placed between the two anvils on the support stage, then a weight was dropped from a height of 88 cm to reliably produce a mid-shaft comminuted femur fracture. Immediately following the fracture, the anvils and support stage were adjusted to apply a soft tissue crush injury to the fracture site. A pressure of 20 psi was applied for one minute, as determined using a Chatillon DF series force gauge (AMETEK Inc., Berwyn, Pennsylvania, USA).

3.3.2.4 Tourniquet-induced ischemia/reperfusion (IRI)

For pneumatic tourniquet application, a pneumatic cuff (Hokanson, Bellevue, Washington, USA) inflated to 300 mmHg was applied as proximally as possible on the

right hind limb to induce prolonged ischemia for 3 hr, then released to cause reperfusion injury (44).

3.3.2.5 Hind limb amputation (HLA)

Amputation of the right hind limb was performed through the fracture site with appropriate hemostasis and debridement of devitalized tissue and bone fragments, followed by hamstring and quadriceps myoplasty over the exposed residual femur in a manner similar to that previously described (40; 42).

3.3.3 Post-operative monitoring

All rats received sustained released buprenorphine (1.2 mg/kg; Zoopharm, Windsor, Colorado, USA) for post-operative pain management, with repeat dosing three days post-injury. Rats were assessed twice daily for three days post-injury using IACUC approved pain charts.

3.3.4 Serum collection

Whole blood was collected at various timepoints post-injury (n=5-7 rats/injury group). Lateral tail vein sampling occurred at 1 hr, 3 hr, and 3-day post injury. At 6 hr, 24 hr, and 7-day post-injury, blood was collected through exsanguination via cardiac puncture. Serum was separated by centrifugation (2000x g, 10 min, room temperature), then aliquoted and stored at -80°C.

3.3.5 Clinical chemistry

Whole serum chemistries were measured using an Element DCSX Veterinary Chemistry Analyzer (Heska, Loveland, Colorado, USA). Kidney function was assessed by measuring blood urea nitrogen (BUN), creatinine (Cr), and the BUN:Cr ratio. Liver

function was assessed by measuring alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin levels.

3.3.6 Isolation and characterization of exosomes

Serum exosomes were isolated from 250 μ L of freshly isolated whole serum according to manufacturer recommendations using the ExoQuick ULTRA EV Isolation Kit for Serum and Plasma (System Biosciences, LLC, Palo Alto, California, USA). Exosomes were pelleted, and the supernatant was aspirated and saved as the exosome-depleted serum (EDS). The exosome pellet was then re-suspended and subject to column purification for depletion of highly abundant serum proteins such as albumin and immunoglobulin. EDS and exosome samples were aliquoted and stored at -80°C .

Size of isolated exosomes was characterized by dynamic light scattering size analysis using a DynaPro NanoStar and Dynamic 7.9 software (Wyatt Technology Corporation, Santa Barbara, California, USA). The acquisition time of each exosome preparation (4 μ L) was five seconds with 10 acquisitions performed in triplicate, and a specific refractive index increment (dn/dc) of 0.185.

Imaging of exosomes was performed by transmission electron microscopy (TEM). Re-suspended exosomes were applied to formvar-coated carbon-stabilized 3 mm copper grids (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) for one minute and then the excess was wicked away with filter paper. Grids were rinsed gently and briefly in nanopure water to remove buffer salts and 2% aqueous uranyl acetate was then applied for one minute before the excess was wicked away. Grids were allowed to air dry and then were examined in a JEOL JEM-1011 transmission electron microscope

(JEOL USA, Inc., Peabody, Massachusetts, USA). Images were recorded on an AMT XR50 digital camera (Advanced Microscope Techniques, Woburn, Massachusetts, USA).

Protein analysis of whole serum and serum exosomes was performed using SDS-PAGE under reducing and denaturing conditions with protease inhibitors (Promega, Madison, Wisconsin, USA) according to manufacturer instructions (NuPAGE 4-12% Bis-Tris gradient gels; Invitrogen, Carlsbad, California, USA). Sample protein concentrations were determined using a BCA Protein Assay (Pierce BCA Protein Assay kit; Thermo Scientific, Rockford, Illinois, USA).

Exosomal markers were assessed by Western blot analysis of tetraspanins CD9 and CD81, tumor susceptibility gene 101 (TSG101), and programmed cell death 6-interacting protein (ALIX) in total protein preparations of matched serum (10µg) and serum exosomes (5µg). Proteins were transferred to a nitrocellulose membrane for 30 minutes using NuPAGE Transfer Buffer (Invitrogen, Carlsbad, California, USA) with a Bio-Rad TransBlot Turbo machine (Bio-Rad, Hercules, California, USA). Nitrocellulose membranes were blocked for one hour at room temperature in blocking buffer (5% BSA (Sigma-Aldrich Co., St. Louis, Missouri, USA) in 0.05% Tween 20 in TBS (Invitrogen, Carlsbad, California, USA)). The membranes were quickly washed with washing buffer (0.05% Tween 20 in TBS), followed by two consecutive washes, then were incubated with primary antibodies in the blocking buffer. Rabbit anti-rat monoclonal antibodies against CD9 (ab109201; Abcam Inc., Cambridge, Massachusetts, USA), CD81 (ab92726; Abcam Inc., Cambridge, Massachusetts, USA), TSG101 (ab133586; Abcam Inc., Cambridge, Massachusetts, USA), and ALIX (ab86429; Abcam Inc., Cambridge, Massachusetts, USA) expression were used at 1:1000 dilution. After overnight incubation

at 4°C and washing, the membranes were incubated with secondary antibody (goat anti-rabbit IgG/HRP conjugate, ab6721; Abcam Inc., Cambridge, Massachusetts, USA) at 1:10,000 dilution at room temperature for one hour, followed by washing. The protein bands were developed using chemiluminescence kit reagents (Immobilon Western Chemiluminescent, Millipore Sigma, St. Louis, Missouri, USA) and visualized on BioRad ChemiDoc (Hercules, California, USA). The intensity of specific bands was quantified by densitometry and Image Lab software (version 6.0.1; BioRad, Hercules, California, USA).

Total protein visualization was performed using a silver nitrate stain of equal total protein preparations of matched serum and serum exosomes. The gel was rinsed in de-ionized water, then placed in a fixative (10% acetic acid, 40% methanol). After fixing, the gel was washed in de-ionized water and sensitized in 12.5% glutaraldehyde. The gel was then washed again in de-ionized water followed by a wash in 20% ethanol. Next, the gel was stained using a silver stain composed of 0.4% silver nitrate, 0.25% ammonium hydroxide, 0.2% sodium hydroxide, and 18.6% ethanol. Following staining, the gel was washed in 20% ethanol, then protein bands were developed using a solution of 20% ethanol, 0.04% formaldehyde, and 0.0006 M citric acid.

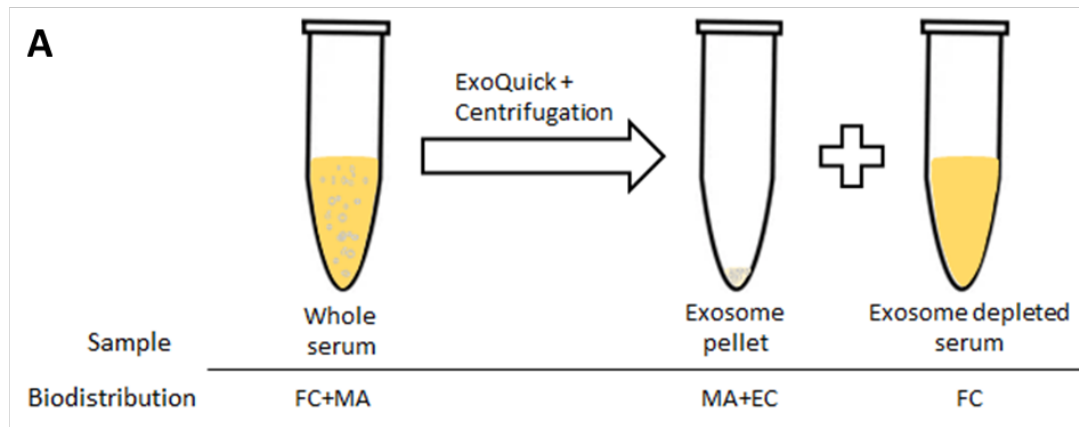
3.3.7 Proinflammatory mediatory analysis

At various timepoints post-injury, proinflammatory mediator levels (IFN- γ , IL-1 β , IL-10, IL-4, IL-5, IL-6, KC/GRO (CXCL1), IL-13, and TNF- α) in whole serum, isolated serum exosomes, and EDS samples were simultaneously measured using a commercial 9-plex multiplex protein array (V-PLEX Proinflammatory Panel 2 Rat Kits; catalog K15059D; Meso Scale Diagnostics, Rockville, Maryland, USA). Prior to assaying,

exosome samples were lysed using a non-ionic lysis buffer (50 mM Tris-HCl pH 7.4, 1% IGEPAL-CA630, 1 mM EDTA, 150 mM NaCl). Samples were analyzed in duplicate, and assays were performed per manufacturer kit instructions. Data acquisition was performed using a Meso Sector S600 (Meso Scale Diagnostics, Rockville, Maryland, USA) and quantitative results were generated using Methodical Mind software (version MMPR 1.0.27; Meso Scale Diagnostics, Rockville, Maryland, USA). Exosome and EDS samples were corrected to account for dilution effects in the isolation process. Quality control and data visualization was accomplished using the Workbench Software (version LSR_4_0_13; Meso Scale Diagnostics, Rockville, Maryland, USA).

In determining the compartmentalization of proinflammatory mediators, the following assumptions were made regarding the detection of cytokines in each of the serum fractions (Fig 5):

1. The measured whole serum proinflammatory mediators include freely circulating (FC) and exosome membrane-associated (MA) proinflammatory mediators: **Whole Serum = FC + MA**
2. After precipitation of serum exosomes, measured proinflammatory mediators in the EDS are, by definition, FC. **EDS = FC**
3. The measured serum exosome proinflammatory mediators include both encapsulated (EC) and membrane-associated (MA) proinflammatory mediators: **Serum Exosomes = EC + MA**
4. MA and EC fraction cytokine levels must then be calculated from the Whole Serum, Serum Exosome, and EDS measured values (Fig 5B).



B

$$EC = \text{Serum Exosomes} - (\text{Whole Serum} - EDS)$$

$$MA = \text{Whole Serum} - EDS$$

$$FC = EDS$$

Figure 5. Calculation of concentrations of proinflammatory mediators within each serum compartment.

A) Diagram of serum fraction and associated proinflammatory mediators detected. The gray circles represent exosomes and are not to scale. B) Equations for calculating proinflammatory mediators in each compartment.

3.3.8 Statistical analysis

Graphing and statistical analysis were performed using GraphPad Prism (version 9.0.2, GraphPad software, San Diego, California, USA). Outliers were determined using the GraphPad Prism ROUT analysis (Q=1%). Welch's t-test was used when comparing two groups. Statistical significance was defined as p-value < 0.05.

3.4 RESULTS

3.4.1 Evidence of systemic injury measured by clinical chemistry

We evaluated the levels of clinical chemistry analytes to assess severity of injury in relation to systemic end organ impact, particularly acute kidney injury and acute liver injury (Fig 6). Blast injury alone had no major effect on serum Cr or BUN levels at any timepoint. As expected, all polytrauma injury patterns caused elevations of BUN and Cr, as well as the BUN:Cr ratio beginning at 6 hr post-injury, with significant sustained elevations noted in the B+COI+IRI+dHLA group at 6 and 24 hr post-injury ($p < 0.0001$ and $p = 0.185$, respectively). Serum levels of ALT were increased from the normal physiological range at 6 hr post-injury in all injury groups, and this increase was significant in groups with IRI (COI+IRI+HLA $p = 0.0049$, B+COI+IRI+dHLA $p = 0.0007$). AST was also increased in all injury groups at 6 hr post-injury, but this increase was only significant in the polytrauma groups (COI+IRI+HLA $p = 0.0027$, B+COI+dHLA $p = 0.0109$, B+COI+IRI+dHLA $p = 0.0032$). Hypoalbuminemia was observed in all groups at all timepoints, with the lowest level compared to baseline noted in the B+COI+IRI+dHLA group at 6 hr post-injury ($p < 0.0001$). Based on these results, physiological impact of injury patterns, in decreasing severity, is B+COI+IRI+dHLA > B+COI+dHLA > COI+IRI+HLA > B.

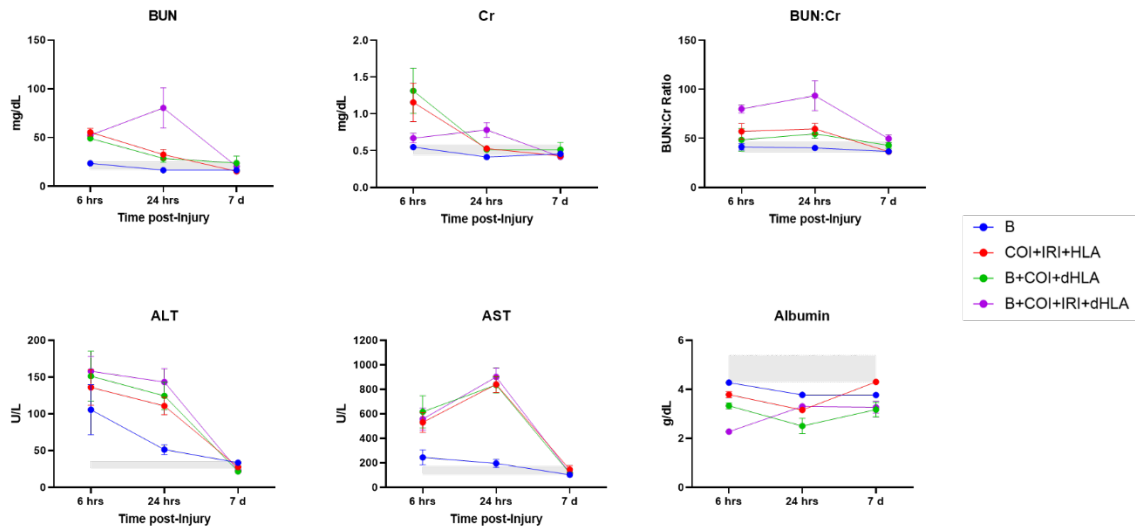


Figure 6. Severity of injury corresponds to acute kidney and liver injury. Serum creatinine (Cr), blood urea nitrogen (BUN), BUN/creatinine ratio (BUN:Cr), alanine transaminase (ALT), aspartate aminotransferase (AST), and albumin levels were analyzed using commercial kits at 6 hr, 24 hr and day-7 post-injury. Data are mean values $\pm$ SEM from n=6-7 rats/timepoint. The shaded gray region represents the reference range for normal values (95% CI) from age/weight matched naïve rats (n=7).

3.4.2 Exosome characterization

The combination of particle size, biochemical, and imaging techniques (DLS, TEM, silver stain, Western blot) indicated successful enrichment and isolation of serum exosomes (Fig 7). The exosomes had diameters between 30 and 200 nm, with an average size of 164 nm, and contained exosome marker proteins CD9, CD81, TSG101, and ALIX.

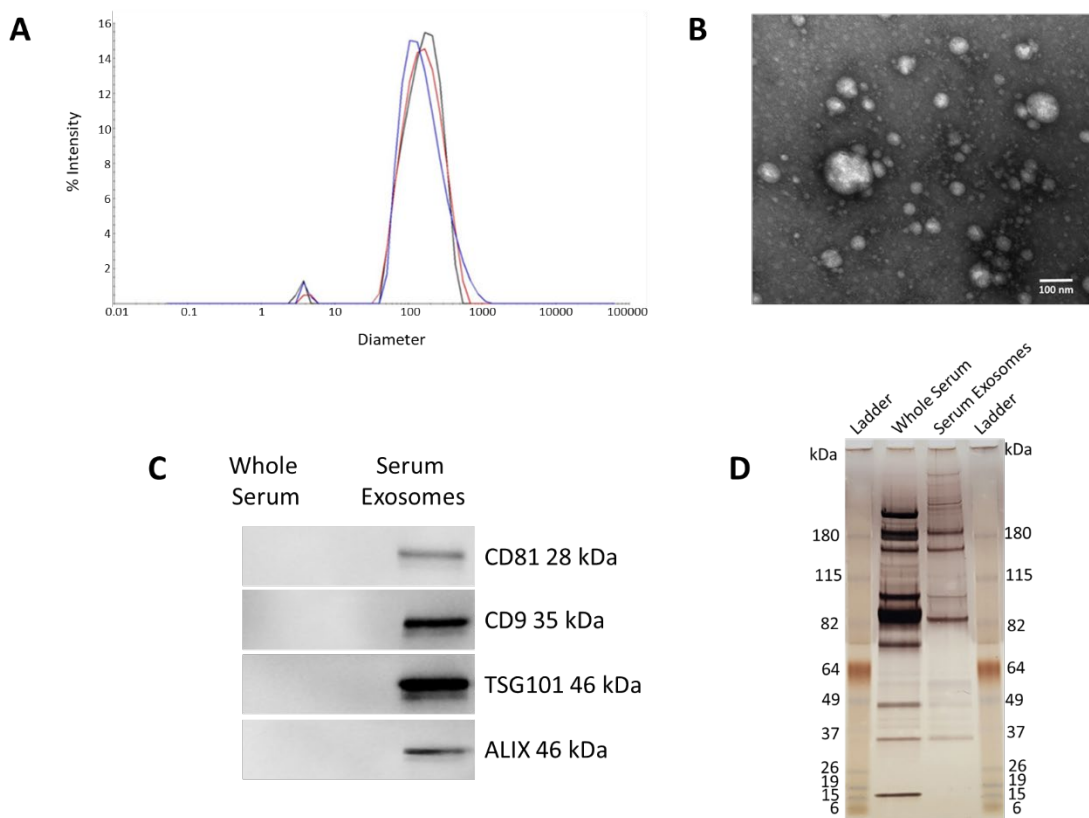


Figure 7. Characterization of serum exosomes.

A) Dynamic light scattering (DLS) results performed in triplicate indicating an average diameter of 164 nm. B) Transmission electron microscopy (TEM) demonstrating a heterogeneous size mixture of discrete vesicles ranging from about 30 – 200 nm. C) Western blot results showing intense CD9, CD81, TSG101, and ALIX (exosome protein markers) signals in the serum exosomal fraction, but not in the whole serum. D) SDS-PAGE analysis of whole serum and serum exosome protein patterns visualized by silver staining.

3.4.3 Exosome encapsulated proinflammatory mediators are not detected using conventional immunoassay techniques

Using whole serum and isolated serum exosomes collected from healthy, naïve, uninjured rats, we first investigated baseline serum levels and the compartmentalization of IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-3, IFN- γ , TNF- α , and CXCL1, which play important roles in driving early innate proinflammatory responses to trauma. We hypothesized that some proinflammatory mediators are encapsulated by the lipid bilayer membrane of exosomes and not detectable by conventional ELISA and multiplex immunoassays. Therefore, in assessing compartmentalization we expected FC and membrane associated MA mediators to be detected in whole serum, EDS to contain only FC analytes, and the serum exosome samples to contain both MA and EC mediators. We developed compartmentalization groupings based on calculated FC, MA, and EC distributions. Mediators detectable after removal of exosomes were classified as “Primarily Freely Circulating.” Mediators not detected in EDS or whole serum were designated “Primarily Exosome Encapsulated” whereas mediators were detected in both whole serum and serum exosomes, but not EDS were classified as “Primarily Exosome Associated” to include both MA and EC. Mediators detected across all compartments were classified as having a “Mixed Distribution.” Similarities in the quantitative levels of mediators between whole serum and EDS for FC mediators indicated that mediators did not co-precipitate in the exosome isolation process (Fig 8).

CXCL1 and TNF- α were detected in both the whole serum and EDS (Fig 8). Exosome depletion, removing exosome MA analytes, had no significant effect on CXCL1 assay measurements, indicating that this chemokine is predominantly FC. TNF- α

levels in whole serum and EDS were similar, suggesting predominantly FC form. IFN- γ , IL-10, IL-13, IL-1 β , IL-4, and IL-5 were detected in whole serum, but not EDS, suggesting that these mediators are MA, but not FC. IL-6 was not detected in either whole serum or EDS (Fig 8).

We next isolated exosomes from fixed serum volumes, then treated the exosome preparations with a non-ionic lysis buffer to disrupt the exosomal membranes and release encapsulated mediators (Fig 6). Of the 9 proinflammatory mediators tested, an abundance of CXCL1 was detected as only FC. Low to modest levels of MA IL-1 β , IL-4, IL-5, IL-10, IL-13 and IFN- γ were detected. We found that there were relatively high levels of proinflammatory mediators encapsulated in exosomes which were not detected in the standard immunoassay procedure in the steady state (Fig 6). Importantly, an abundance of IL-6 was detected only in the EC compartment in naïve animals. We observed equal or greater amounts of EC IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-3, and IFN- γ versus FC or MA, demonstrating these mediators were undetectable using this immunoassay platform without exosomal lysis. After evaluating the exosome preparations, TNF- α was revealed to be equally distributed as a FC and EC cytokine, with a smaller amount in MA form. Our data suggests that most mediators are exosome associated, either encapsulated or membrane-bound, in the steady-state.

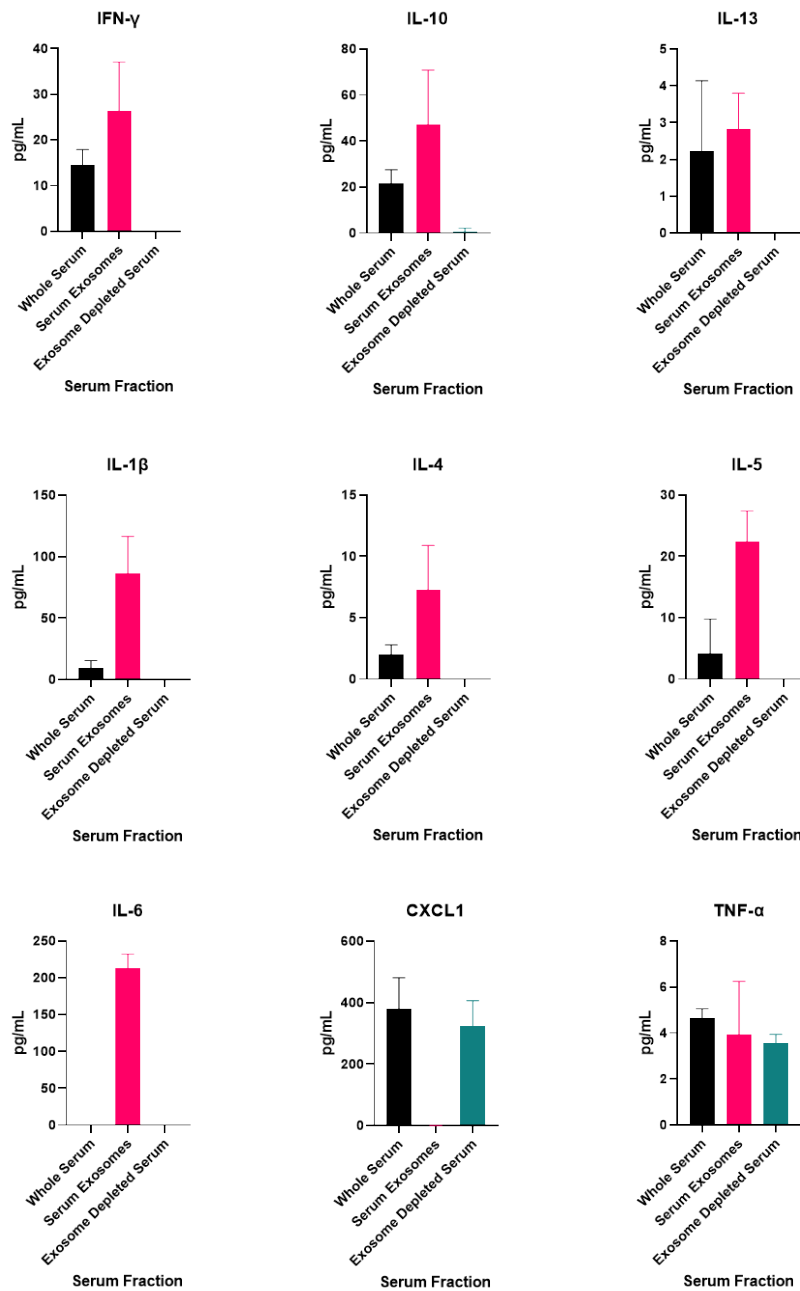


Figure 8. Proinflammatory mediators are predominately exosome-associated in the steady state.

Levels of mediators in matched samples from naïve, uninjured rats, of serum, serum exosomes, and exosome depleted serum measured on an electrochemiluminescence multiplex immunoassay. Results represent the mean $\pm$ SEM from n=7 rats.

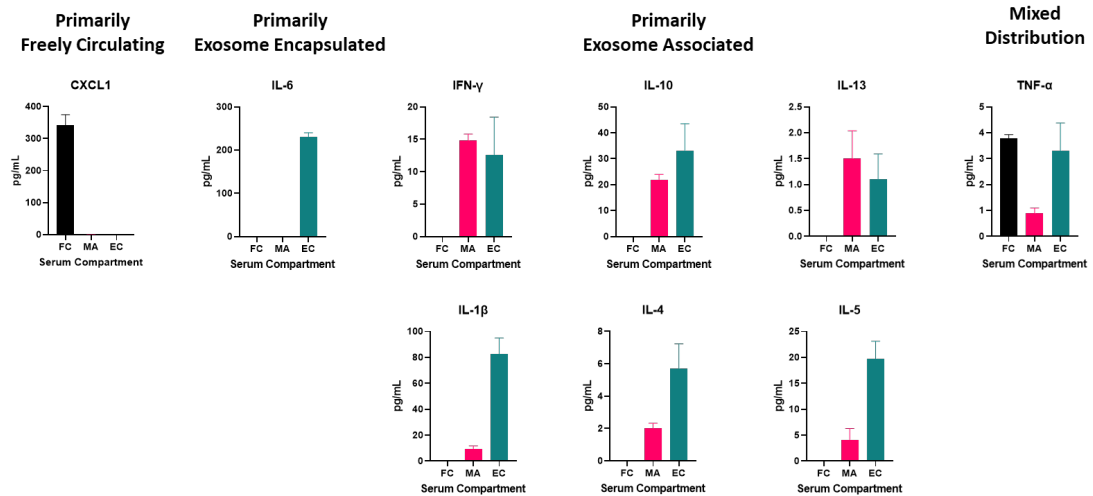


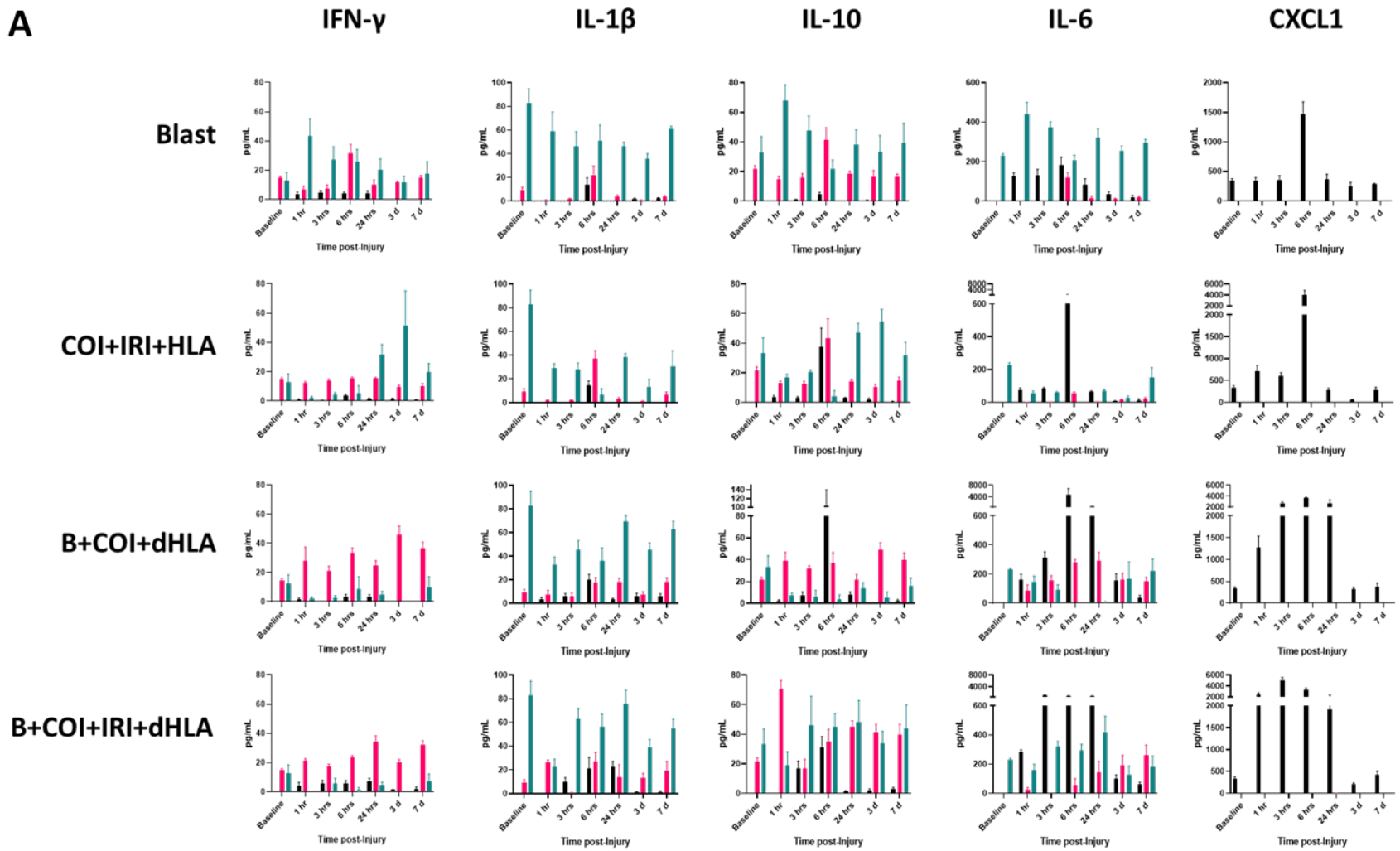
Figure 9. A large proportion of encapsulated proinflammatory mediators in the steady-state evade detection with conventional immunoassays. Serum compartmentalization of proinflammatory mediators in adult male naïve rats according to calculations of freely circulating (FC), exosome membrane-associated (MA), and exosome encapsulated (EC) levels. Results represent the mean $\pm$ SEM from n=7 rats.

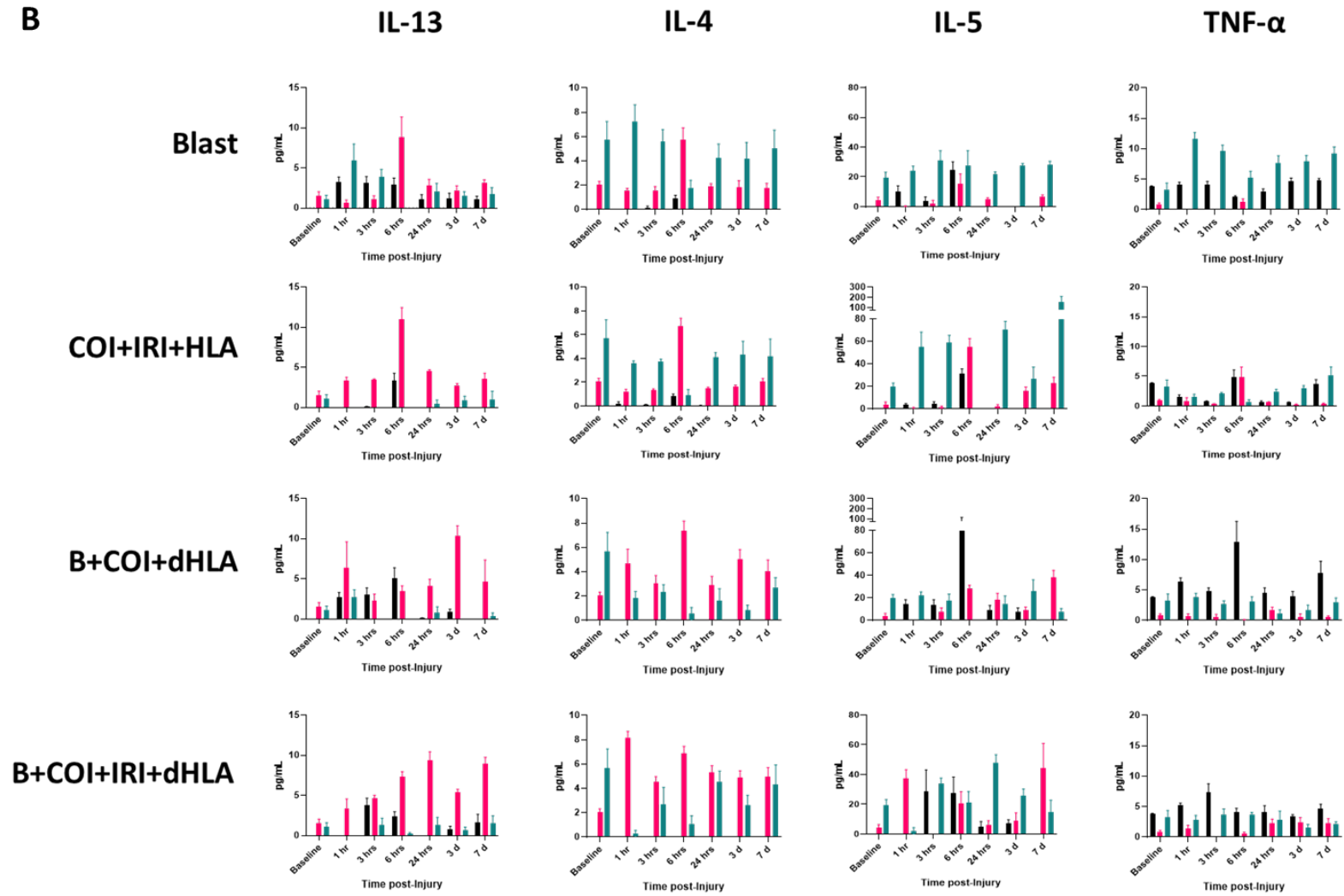
3.4.4. Unique proinflammatory mediator compartmentalization changes following blast overpressure exposure

After characterizing compartmentalization of proinflammatory mediators in naïve animals, we repeated this procedure in four different models of traumatic injury across six timepoints from 1 hr to 7-days post-injury to compare changes in the serum profile as well as the timing and compartmentalization of the same key proinflammatory mediators. After blast exposure alone, serum multiplex analysis demonstrated IFN- γ (1-24 hr), IL-1 β (6 hr), IL-6 (1-24 hr), IL-10 (6hr) CXCL1 (6 hr), IL-13 (1-24 hr), IL-4 (6 hr) and IL-5 (1-6 hr) levels were low to modestly increased as FC mediators (Fig 10). At 6 hr post-injury, a transient increase in MA IFN- γ , IL-1 β , IL-10, IL-6, IL-13, IL-4, and IL-5 was observed. EC IFN- γ , IL-10, IL-6, IL-13, and TNF- α rose sharply at 1 hr ($p=0.0484$, 0.0441 , 0.0163 , 0.0640 , 0.0002 , respectively) then decreased gradually and returned to near baseline levels at 3 days post injury (Fig 10). In general, the number of mediators and the percent EC during the first 24 hr post-injury was the greatest in the blast alone group.

Figure 10. The kinetics of circulating proinflammatory mediator levels and their biodistribution in serum compartments.

Compartmentalization values over time post-injury were based on calculation of freely circulating (FC) (black), exosome membrane-associated (MA) (pink), and exosome encapsulated (EC) (green) levels following blast (B), complex orthopaedic injury and ischemia reperfusion injury followed by hind limb amputation (COI+IRI+HLA), B+COI+1 hour delayed (d) HLA, and B+COI+IRI+dHLA. Panel-A depicts interferon-gamma (IFN- γ), interleukin-1 β (IL-1 β), IL-10, IL-6, and chemokine (C-X-C motif) ligand 1 (CXCL1) levels in serum compartments. Panel-B depicts IL-13, IL-4, IL-5, and tumor necrosis factor-alpha (TNF- α) levels in serum compartments. Data are mean values $\pm$ SEM from $n=4-7$ rats/timepoint. Baseline values are from age/weight matched naïve rats ($n=7$).

A

B

3.4.5 Time course changes of proinflammatory mediator compartmentalization and secretion patterns following polytraumatic injury

During the early phase of recovery from polytrauma (1-24 hr post-injury) we observed significant changes in the repertoire of FC mediators, specifically higher detectable levels of FC cytokines to include IL-1 β , IL-5, IL-6, IL-10, and IL-13 (Fig 10). The inflammatory chemokine CXCL1, which plays a key role in the innate immune response by recruiting neutrophils, was remarkably measurable only in the FC compartment and was significantly increased within 1 hr post injury in all polytrauma groups, with the highest concentrations in the B+COI+IRI+dHLA group, where CXCL1 concentrations peaked by 3 hr (4991 ± 1307 pg/ml vs. 340.9 ± 88.7 pg/ml in naïve controls; $p=0.0013$) (Fig 10). IFN- γ and IL-4 remained primarily exosome associated across all injury patterns and timepoints (Fig 10). By day-7, all mediators returned to similar baseline compartmentalization profile patterns, albeit with a tendency towards higher levels than at baseline, with the noted exception of persistent low levels of FC IL-6 across all polytraumatic injury patterns. Interestingly, there was an increase in EC IL-6 similar to that of the blast only data noted in the B+COI+IRI+dHLA, beginning at 3 hr (323.4 ± 71.5 pg/mL vs 229.9 ± 23.6 pg/mL in naïve controls; $p=0.0402$) (Fig 10).

3.4.6 Utilization of calculated total cytokine levels increases detection over standard whole serum measurements

We have demonstrated that a significant fraction of the assayed proinflammatory mediators is carried as exosomal bound encapsulated cargo both in the steady-state and following traumatic injury. Using Venn diagrams, we illustrate the profile and early compartmentalization changes of these key proinflammatory mediators following

traumatic injury (Fig 11). Therefore, conventional ELISA-based immunoassays no doubt provide an underestimate of the actual biological response. To illustrate this point, we graphed our findings based on measurements obtained using the immunoassay kit versus a calculated total measurement (EC+MA+FC) when encapsulated mediators are considered. In addition, we plotted the proportion of each mediator that is found in the encapsulated form at various time points post injury (Fig 12).

We observed a re-distribution of IL-6, IFN- γ , IL-4, IL-13 in the first 24 hr post-injury, with a less robust response noted for IL-1 β and IL-10. The early decrease in proportion of encapsulated mediators corresponded with a delayed increase in detected levels of FC mediators. These changes corresponded in magnitude to the severity of injury. While certain trends were consistent across trauma patterns, such as the sharp decline in proportion of EC IL-1 β , IL-6, IL-10, TNF- α , and IFN- γ at 6 hr, there were important kinetic differences between trauma patterns. In the blast group, the proportion of EC mediator increased for all mediators except CXCL1, an observation unique to this injury pattern. The combination of blast and COI, without considering IRI status, caused sustained depression of the proportion of EC IL-1 β , IL-6, IL-10, TNF- α , and IFN- γ , while blast only or COI+IRI+HLA rebounded to higher than pre-injury levels by day-7. IL-13 had a sharp decrease at 1 hr post-injury in trauma patterns including IRI, but a more gradual decline in trauma patterns without IRI.

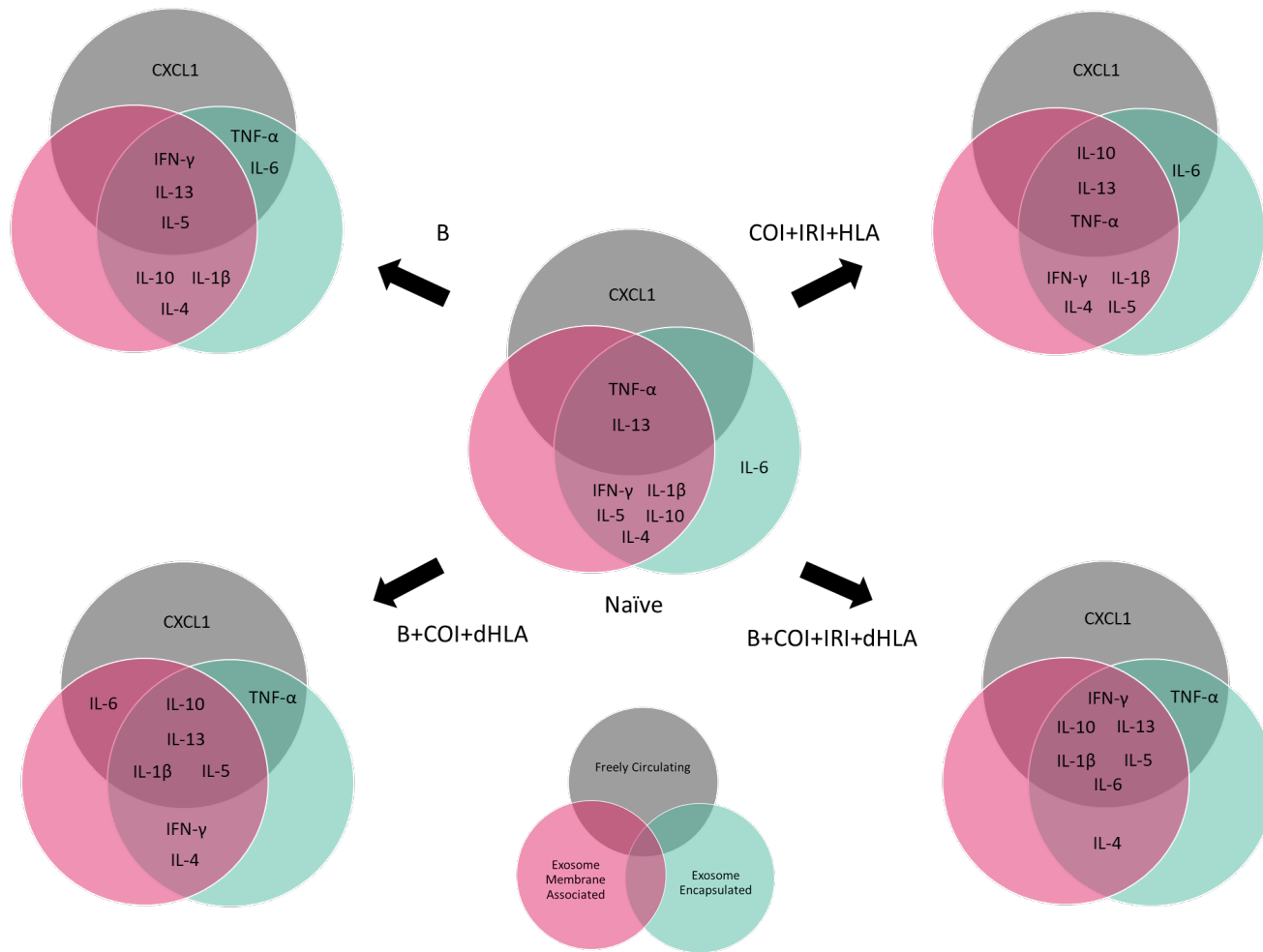
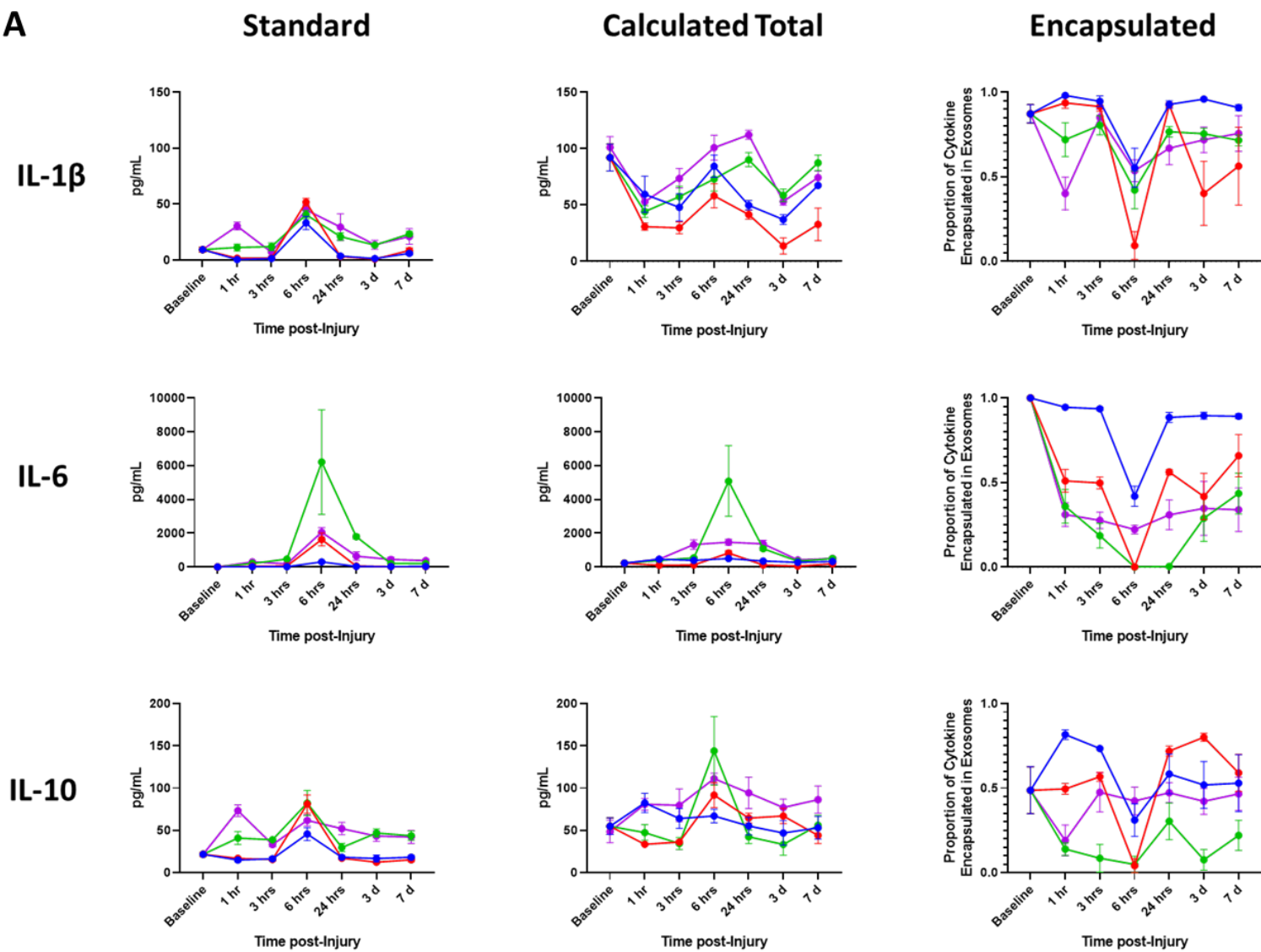
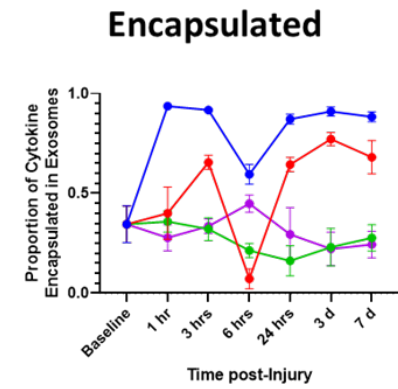
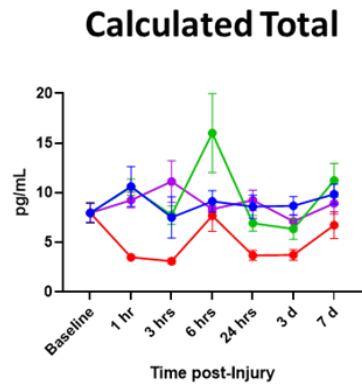
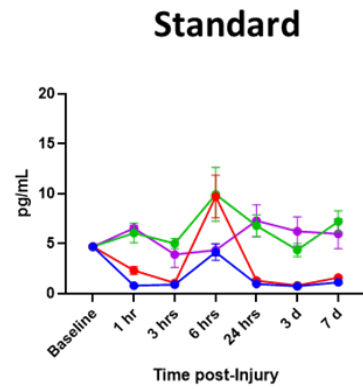
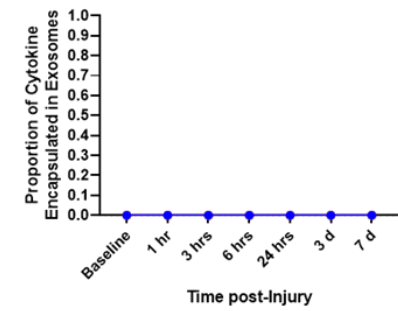
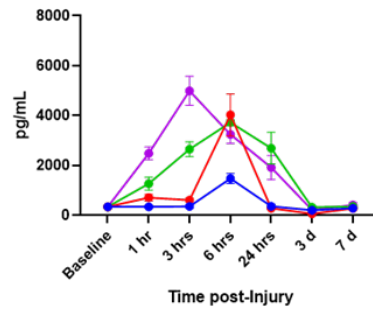
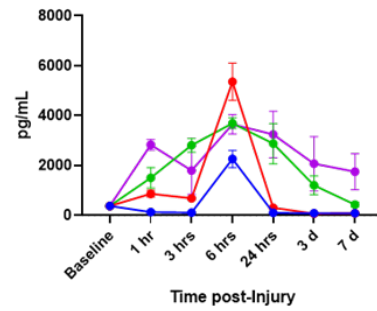
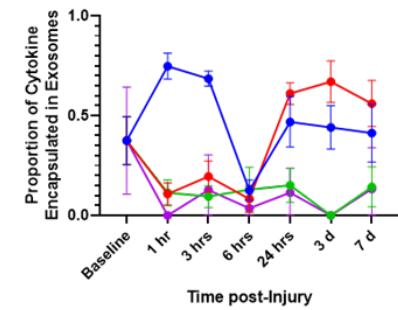
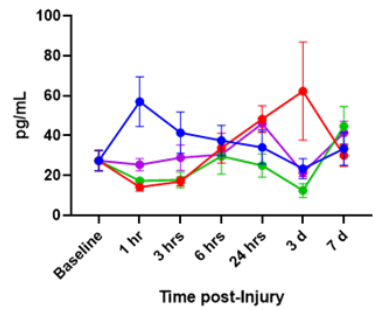
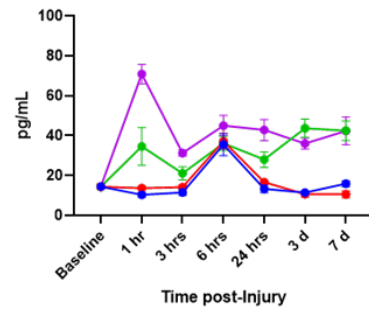


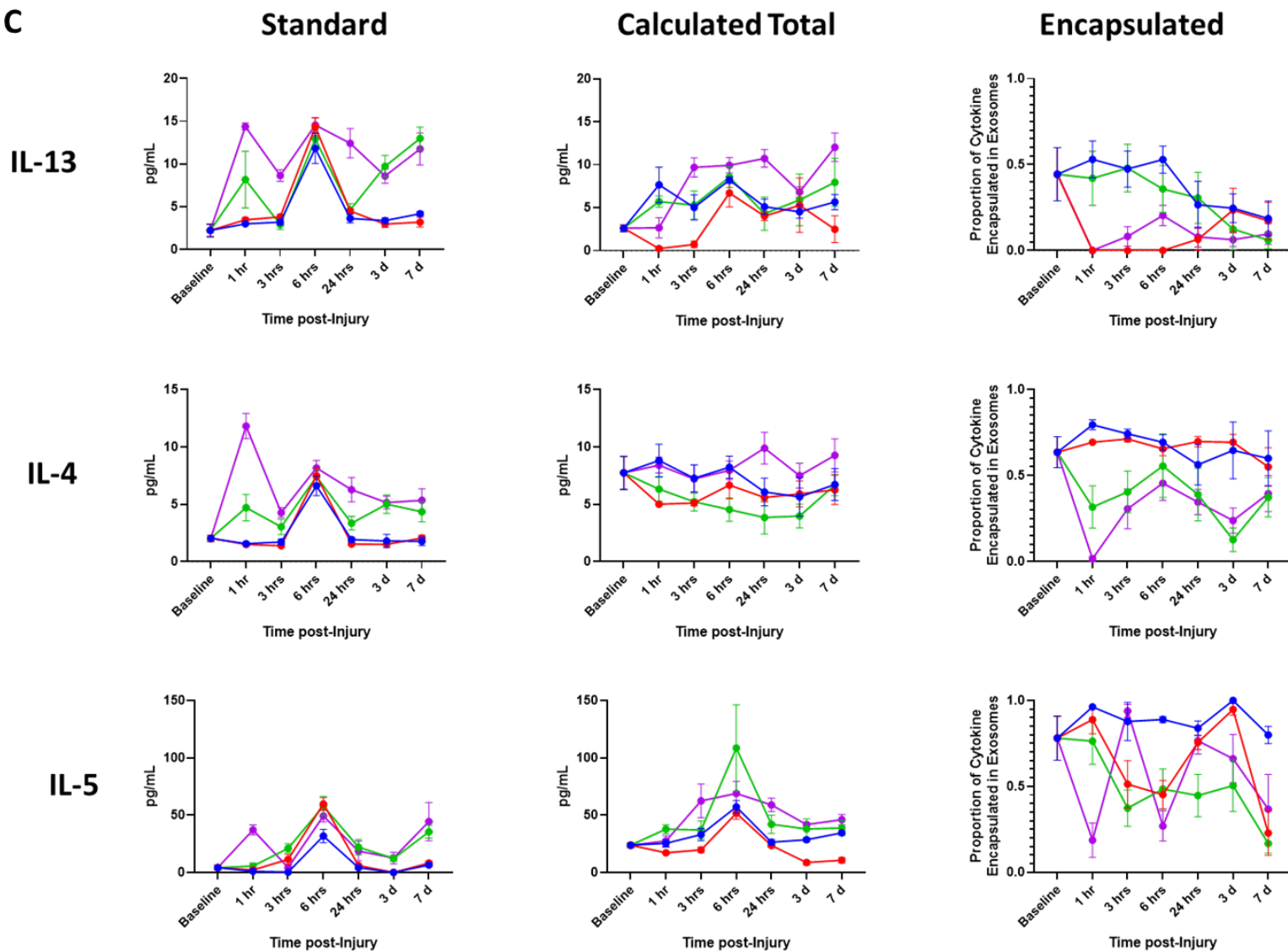
Figure 11. Summary of early proinflammatory mediator compartment changes following traumatic injury.

Figure 12. Conventional immunoassay measurements underestimate total proinflammatory mediator levels as they fail to take into account an abundance of encapsulated factors.

Comparison of measurements of whole serum and calculated total mediator levels accounting for encapsulated mediators, as well as proportion of encapsulated mediator relative to total mediator level over time after Blast (B) (blue), complex orthopaedic injury followed by ischemia reperfusion injury, then hind limb amputation (COI+IRI+HLA) (red), B+COI+1 hour delayed(d)HLA (green), and B+COI+IRI+dHLA (purple) injury patterns. Panel-A interleukin-1 β (IL-1 β), IL-6, and IL-10. Panel-B depicts tumor necrosis factor-alpha (TNF- α), chemokine (C-X-C motif) ligand 1 (CXCL1), and interferon-gamma (IFN- γ). Panel-C depicts IL-13, IL-4, and IL-5. Results represent the mean $\pm$ SEM from n=4-7 rats/timepoint. Baseline values are from age/weight matched naïve rats (n=7).

A

B**TNF- α** **CXCL1****IFN- γ** 

C

3.5 DISCUSSION

This study was aimed to better understand the local and systemic immune response to trauma. While commercially available ELISA and multiplex immunoassays are conventionally used to measure the innate immune response to trauma by assessing the concentrations of proinflammatory mediators such as cytokines in peripheral blood, our results indicate that these measured parameters are underestimated, inaccurate, and fail to provide a true picture of the early post-traumatic inflammatory response to trauma. To our knowledge, this is the first qualitative and quantitative profile study demonstrating differential serum compartmentalization of key proinflammatory mediators prior to and following traumatic injury. Depending on the physiological state of the animal (healthy versus type and extent of the injury), significant differences were observed in the compartmentalization (freely circulating versus exosome-associated), pattern of release, and concentrations of proinflammatory cytokines like IL-1 β , IL-6 and TNF- α . Our findings indicate that a significant number and high levels of “hidden cytokines” are encapsulated within the lipid bilayer membrane of exosomes and thus are not detectable by conventional ELISA and multiplex immunoassays. We speculate that these exosome-encapsulated mediators play an important yet ill-defined role in early post-traumatic inflammatory response cytokine delivery-signaling and are critical in the regulation of differential physiological and pathological responses. Packaging of cytokines into exosomes may indicate importance of cytokine signaling and delivery, as the lipid bilayer of exosomes gives them the ability to shield their cargo from the microenvironment, leading to stability of the bioactive material within, and increased efficiency of delivery to effector targets (20; 35; 36; 43; 47; 50). Therefore, monitoring total cytokine levels

should be done routinely as a new approach to assess patient status and earlier diagnosis of severe post-traumatic complications.

In assessing the serum compartmentalization of proinflammatory mediators in naïve animals in a normal physiological state, we identified a set of encapsulated mediators, undetected using a conventional multiplex immunoassay, at moderate to high levels. The encapsulated mediators are likely sequestered and presumably not immediately active while circulating in the serum, whereas freely circulating and membrane-associated mediators have direct access to receptors expressed on cell membranes. The identification of high levels of sequestered IL-6 suggests exosomes may serve a role as a reservoir for immediate release in activation of the innate immune response, and this is supported by the re-distribution phenomenon noted in the polytraumatic injury patterns. IL-6 is not the first mediator released in response to trauma; induction of IL-6 is dependent on TNF- α and IL-1 β , therefore, a sequestered ready reserve would be valuable in the response to injury, especially to activate hepatocytes for early release of acute phase proteins (32). Similarly, we found IL-1 β is mostly encapsulated, but a small proportion is immediately accessible, albeit membrane bound. Since TNF- α is a more immediate responder with a short half-life, it is conceivable that it would be found across all compartments at low levels. CXCL1 is a critical chemoattractant involved in recruitment and activation of neutrophils and macrophages during the innate immune response, but also has been reported to function in NLRP3 inflammasome activation, reactive oxygen species formation, and neutrophil extracellular trap formation, suggesting a need for easily accessible basal levels for immediate response (29).

Systemic blast overpressure wave exposure has myriad effects. In our model, the pressure is sufficient to induce mild traumatic brain injury (TBI), primary blast injuries to hollow organs such as the lungs and increase the complexity of associated extremity injuries through endothelial activation (2; 10; 30; 54; 55). Our investigation of the encapsulated levels of proinflammatory mediators following blast injury reveals an important observation regarding the acute inflammatory status following exposure to a blast overpressure wave. To our knowledge, this study is the first to identify that while conventional immunoassay measurements reveal non-significant early changes in levels of proinflammatory mediators, there is a profound undetected response occurring in exosomes that is not detectable by conventional assays that measure soluble, circulating biomarkers. A study of pre- and post-deployment serum cytokine measurements in military members meeting qualifications for blast-related TBI showed no significant differences in a majority of inflammatory mediators, despite meeting the clinical requirements for TBI (45). As demonstrated clinically, and in previous studies using our model, the addition of blast increases delayed inflammatory-related healing complications such as heterotopic ossification, suggesting an important role of blast in activation of inflammatory signaling (8; 40). Furthermore, we show that the addition of blast prior to polytraumatic injuries results in heightened inflammatory responses leading to increased end organ damage. Results from these studies indicate that blast injury induced exosome associated mediators may play a critical role in the early activation of the immune response and could possibly be earlier drivers of down-stream neuroinflammatory events.

Our polytraumatic models recapitulate complex combat injuries with documentation of increased complications related to post-traumatic inflammation (19; 25; 41). Management of the inflammatory response is critical for appropriate resolution, prevention of secondary complications, and transition to a pro-healing phenotype (33; 57). In relation to the mediators we assessed, previous clinical characterizations of post-traumatic injury inflammatory status using conventional immunoassays rely heavily on IL-6, IL-10, and TNF- α (14). In polytrauma these mediators are predictive of the development of MODS and MOF. IFN- γ , IL-1 β , and IL-4 are reported to have inconsistent value, or minimal data available to assess value in predicting outcome (14). IL-6 elevations precede other markers of injury severity such as the hepatic acute phase protein c-reactive protein (CRP) (21). IL-6 levels are noted to have an early increase, followed by a steady decrease in the first 24 hr following trauma (21). Specifically in rats, IL-6 and CINC-1 (CXCL1) are reported as appropriate surrogate inflammatory markers in acute inflammation, peaking in the first 24 hr, consistent with our data, where magnitude correlates with severity of injury (31). An important observation made in assessment of cytokines for predicting patient outcome is that the interplay of different cytokines (IL-6 and IL-10) and ratios of specific cell-associated signaling molecules (Th1-associated vs Th2-associated) are important factors in stratification of patient risk for development of secondary inflammation-induced complications (53). This observation is particularly relevant to our data which shows increased total levels of IL-10 and TNF- α when accounting for encapsulated cytokines.

The improved prognostic value for added consideration of EC mediators in our model appears to be about 3 hr using IL-6 as a surrogate marker of inflammatory status.

By 6 hr, the majority of IL-6 is found in the FC form and captured using conventional methods. The molecular re-distribution phenomenon precludes the 1 hr data being improved by EC IL-6 inclusion, possibly because the cytokine has been delivered to target cells for consumption. Based on the clinical chemistry results, B+COI+IRI+dHLA was deemed the most severe injury pattern, but using conventional parameters, the level of IL-6 is highest in the serum of the B+COI+dHLA cohorts, whereas B+COI+IRI+dHLA is similar to COI+IRI. When the EC IL-6 is taken into account, there is an earlier significant increase in the B+COI+IRI+dHLA that is sustained for a longer period of time. Whereas the COI+IRI and B+COI+dHLA groups show decreases in levels of EC IL-6, the B+COI+IRI+dHLA groups show increasing EC IL-6 compared to baseline in addition to increased FC IL-6, suggesting a potential role for EC IL-6 in sustained inflammatory response and systemic injury in severe polytrauma. This prognostic window may be improved to 1 hr post-injury when using EC IL-1 β as a prognostic indicator. IL-1 β levels are much higher in the calculated total cytokine measures compared to standard at all timepoints, due to a consistently large population of EC IL-1 β . Our data may offer returned value to measuring IFN- γ and IL-4 in post-traumatic injury inflammatory status, if decreased encapsulation levels can be consistently quantified across patient populations. The more severe injury patterns demonstrate a consistent, and sustained decrease in the proportion of encapsulated mediator. Expanding the number of reliable mediators for assessing patient status would also give a more complete picture of the immune and inflammatory response.

The post-injury molecular re-distribution from EC to FC/MA in the polytrauma models suggests that there is a stimulus dependent change in exosome transport of

proinflammatory mediators following traumatic injury. Given the differences in this change across injury pattern, this effect appears to go beyond the binary “injured” vs “not-injured” and linked to specific trauma patterns. Increasing encapsulated mediators may indicate patient predisposition to a larger inflammatory response in subsequent insults, based on the molecular re-distribution phenomenon found in the polytrauma models including blast. Further, B+COI+IRI+dHLA is the most severe injury pattern, and interestingly shows a rebound increase in encapsulated mediators compared to COI+IRI+HLA and B+COI+dHLA, suggesting an undefined role for exosome encapsulation and transport of circulating mediators in severe, extended inflammatory responses.

There were some study limitations. The exosome isolation process is not 100% efficient and calculations were necessary to account for dilutional effects, but likely do not fully compensate for the differences between true and observed values. The total mediator levels reported rely on normalized calculated values. Based on these considerations we believe that the true levels of exosome associated mediators are underestimated, and that the actual impact may be greater than what we are reporting. The freely circulating levels may be overestimated in some cases due to the potential for lysis of exosomes to occur as part of the freeze/thaw cycle of serum. At this time, we are unable to make definitive statements regarding whether observed changes in the levels of exosome encapsulated mediators are due to changes in cellular production and packaging or an overall change in cellular release of exosomes. Regardless of these limitations, our results indicate that standard immunoassay measurements are not capturing the complete picture of the post-traumatic inflammatory status. Another important consideration is that

we are reporting circulating serum mediator levels, and these do not directly reflect the local wound environment, which may be more appropriately characterized using wound effluent and/or tissue biopsies. Further study limitations were a result of IACUC considerations. We used the minimal number of animals necessary to achieve statistical power analyses and relied on data pooled from cohorts rather than serial longitudinal sampling in individual animals. Because of these constraints, we were also limited in how many sampling timepoints we could accommodate.

3.6 CONCLUSION

Exosomes, as couriers of proinflammatory mediators, appear to play a role in orchestrating the progression and outcomes of trauma. Selective encapsulation of mediators and temporal changes in serum compartmentalization suggests a dynamic balance in delivery of inflammatory signaling molecules for modulation of response, and roles for exosomes as effectors of the immune response to trauma. Further studies are warranted to investigate additional timepoints post-injury, and to elucidate the mechanics of mediator packaging and exosome release following traumatic injury.

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CHAPTER 4: CONCLUSION

4.1 SUMMARY

Understanding the development of severe, uncontrolled inflammatory responses to trauma is critical to developing clinical decision-making tools and intervention strategies. To expand this knowledge, we identified gaps in knowledge regarding exosomes in the immune response to trauma and sought to address these gaps through a study characterizing the biodistribution-compartmentalization of proinflammatory mediators in serum exosomes in several models of traumatic injury. A literature review was conducted to understand current knowledge, in addition to gaps, in the fields of trauma, innate immunity, and inflammation regarding the role of exosomes in the immune response to trauma. We found a severe lack of studies investigating the role of exosomes in trauma-induced inflammation, despite numerous studies describing trauma-induced inflammatory complications and exosomes as mediators of inflammation in other pathologies. Based on these findings, we hypothesized that exosomes had a critical role as transport vehicles for proinflammatory mediators following traumatic injury.

We are the first laboratory to describe the profile, kinetics, and the differential biodistribution-compartmentalization of nine key proinflammatory mediators pre- and post-trauma. Proinflammatory mediators were classified as exosome-encapsulated, exosome-membrane associated, or freely circulating. We showed that conventional ELISA-based assays underestimate true proinflammatory mediator levels, and thus inflammatory status, due to significant levels of encapsulated mediators undetected without disruption of the lipid bilayer membrane. In the trauma models evaluated, the profile and inclusion of exosome encapsulated levels of mediators served as better, and

potentially alternative, earlier surrogate predictors of systemic inflammation, which clinically may allow earlier diagnosis and intervention, which may lead to improved patient outcomes.

We showed that several key proinflammatory mediators are exosome associated in the steady-state, supporting the importance of exosomes as couriers of inflammatory signaling molecules, and suggesting a role for exosomes as inflammatory reservoirs for immediate signaling response. Compartmentalization patterns were not ubiquitous, supporting the conclusion that injury is not a binary state for the immune response, and different injury patterns result in different responses. Blast overpressure exposure increased exosome production and/or packaging of cytokines within exosomes, a process which we speculate may prime the immune system for attenuating or augmenting bidirectional communication signaling between immune and non-immune cells. While trauma generally increases the freely circulating form of cytokines, increasing severity and complexity of injury results in increased encapsulation of mediators, suggesting a role for exosomes in the protracted and uncontrolled systemic inflammation that leads to delayed complications.

This research is the first report of serum compartmentalization patterns of proinflammatory mediators in the steady-state and after traumatic injury. These results describe the importance of exosomal transport of cytokines in the innate immune response to trauma and highlight weaknesses in the current ELISA-based detection methods used to assess patient inflammatory status. This is a key foundational study for further investigation into defining the role of exosomes as couriers of inflammatory signaling molecules following traumatic injury.

4.2 FUTURE DIRECTIONS

We characterized exosomal transport of mediators in blast and musculoskeletal injury models, and these results need to be compared in other models of trauma such as blunt force injuries, thermal injuries, TBI, and spinal cord injuries. Localized traumatic injuries are often associated with the development of systemic inflammation and determining cellular origin of circulating exosomes containing proinflammatory mediators is necessary to better understand how immune signaling progresses following injury. Proximity barcoding or proximity extension assay technology for identification of cell surface markers can be used to determine cellular origin and tissue engagement in inflammatory signaling (1; 2). Additional studies investigating the intricate crosstalk that exists between exosomal mediators as well as the protective role of exosomes in attenuating or stimulating inflammatory and/or tissue repair-regenerative responses within the local wound environment are warranted, including investigation of exosomes in wound effluent, and will provide greater insight into how inflammatory signaling spreads from the site of injury to cause systemic impact. Effluent is a result of extravasation of fluids due to localized swelling following injury, and examination of exosomes in effluent offers the opportunity to potentially sample a high concentration of these signaling vehicles prior to emergence in circulation through serum or plasma sampling. We observed significant changes in serum exosome cargo occurring in the acute phase post-injury (up to 3-days post-injury). Extensive longitudinal sampling at early timepoints, including within minutes following injury, would permit a better assessment of the exosomal cytokine cargo biodistribution patterns and their development over time, at the individual level, as well as their correlation to clinical outcomes. We

identified injury insult-based changes in exosome cytokine cargo, however the effector cells, and the packaging-release-secretion mechanics of exosomes are still not well understood. Quantification studies using acetylcholinesterase assays or ELISA-based assays directed at exosomal surface proteins of exosomes pre- and post-injury would yield greater insight into whether changes in the level of proinflammatory mediators is due to changes in production, cargo packaging of, or overall changes in the release of exosomes.

In our pivotal studies, we focused on nine proinflammatory protein signaling molecules, and exosomal cargo includes a heterogenous variety of bioactive molecules. We have defined a protocol for investigating the compartmentalization of mediators using commercial ELISA-based multiplex assays. Further knowledge must be obtained regarding the roles that exosomes play in delivering a more comprehensive set of bioactive proteomic inflammatory mediators, including a broad spectrum of chemokines, pro- and anti-inflammatory cytokines, as well as DAMPs. Genomic cargo, including miRNA and lncRNA should be investigated using NextGen sequencing, high density throughput microarrays, and RT-PCR. Unique miRNA biomolecular signatures within exosomes may exist based on injury pattern or extent of injury, presenting an opportunity for novel biomarker discovery in trauma-induced inflammatory complications and end-organ damage. miRNA expression is highly variable in response to stimuli, suggesting it is more sensitive than mRNA expression, and may be more specific than currently identified genomic signatures.

Further exploration of the role of exosomes in the transport of inflammatory signaling molecules will undoubtedly expand our knowledge of the development of

severe systemic inflammation. Defining the relationship between exosomes and the vast array of critical signaling molecular cargo linked to the activation, propagation, and progression of acute innate inflammatory responses following traumatic injury may provide opportunities for early intervention and improved clinical decision making.

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