Dynamic Changes of Monocytes and Chemokine Pathway Signaling During Wound Healing Post-Burn Injury

by

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Abstract

Background:

There are over 11 million people hospitalized for burns annually according to the World Health Organization, resulting in painful skin scar contractures and restricted movements, as well as mental and physical stresses. Up to 70% of deep dermal injury result in hypertrophic scars, which currently have no standard treatment or reliable outcome. Monocytes and cytokines play a significant role in wound healing, and when dysregulated can cause abnormal healing. This research investigated cytokine and chemokine pathway signaling at different stages after burn injury in patients with varying severities of injury. The aim of this research was an in-depth observational study to enhance our understanding of cytokines and their differential response in wound healing after post-burn injury, to help guide the search for targeted therapeutic treatments.

Methods:

Our research involving burn patients follows the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, and is approved by the Health Research Ethics Board at the University of Alberta. Burn patients treated in the Firefighter's Burn Treatment Unit at the University of Alberta Hospital were recruited for this study. Healthy individuals were recruited as controls. Burn patients were stratified by burn total body surface area (TBSA) into minor (≤20% TBSA), moderate (21-50% TBSA), and severe (>50% TBSA). Peripheral blood samples were taken at 48 hours, 1 week, 1 month, and more where applicable post-burn in patients, and once in controls. Serum was isolated from blood to determine chemokines of stromal cell-derived factor 1 (SDF-1), monocyte chemoattractant protein-1 (MCP-1), and regulated upon activation normal T cell expressed and presumably secreted (RANTES), chemokines and other

cytokines by enzyme-linked immunosorbent assay. Peripheral blood mononuclear cells were separated from the blood and stained for monocyte and chemokine receptors CXCR4, CCR2, and CCR5, and monocyte populations were quantified by flow cytometry. Statistical analysis was done by one-way ANOVA with a Tukey correction, and regression analysis was performed using Pearson's Correlation analysis.

Results:

Burn injury increased the number of CD14+CD16+ expressing monocytes in burn patients compared to controls, with an earlier peak in severe burns. The monocytes expressing CD14+CD16- were significantly higher in mild burns at 4-7 days than that in controls ($p<0.05$). Burn injury increased CXCR4, CCR2, and CCR5 expression in CD14+CD16+ monocytes, with these cell populations responding differently to different cytokines, time, and burn severity. MCP-1 had a positive correlation 0-3 days after burn injury in severe burns ($r^2 = 0.7388$). IL-6, IL-8, RANTES, and MCP-1 significantly increased with increasing burn severity ($p<0.01$). IL-10 and IL-1RA increased significantly at 0-3 days in severe burns compared to controls ($p<0.0001$), while BCA-1 increased significantly for 7-30 days in severe burns ($p<0.0001$).

Conclusions:

Circulating monocytes expressing CD14+CD16+ increased immediately after severe burn injury, and monocytes expressing CD14+CD16- increased early in minor burns. Burn injuries increased the chemokine receptor expression in the Mo expressing CD14+CD16+. Increasing cytokine levels of IL-6 with burn severity is also shown to promote a pro-fibrotic response, as well as IL-8 being beneficial but after passing a threshold in severe burns becomes detrimental. IL-10 and IL-1RA showing increases in the first 3 days post-burn injury, combined with previous research,

suggest that antagonism immediately after burn injury may be therapeutic. The significant increases in BCA-1 in severe burns may suggest a novel impact of this cytokine in hyper-angiogenesis and further pathogeneses of fibrotic scarring post-burn injury. RANTES should be further explored in its aggregated or disaggregated forms as it has been suggested to have different effects, which could explain a later time point increase in mild burns representing tissue remodeling and an increase in severe burns early after injury as a pro-inflammatory role. The monocytes and the chemokine receptors expressed, serum cytokines, and other molecules involved in wound healing of burn patients and scar development need further study to find a therapeutic treatment and progress our understanding of the abnormal wound healing after burn injury.

Preface

The University of Alberta Research Ethics board approved the collection of blood from burn and control patients. Support for this project has been made possible by the Firefighters Burn Trust Fund at the University of Alberta Hospital.

This thesis is the original work of Lindy Schaffrick, who was responsible for data collection, performing all experiments, analysis, and manuscript composition. Edward E. Tredget, Jie Ding, and Peter O. Kwan assisted with design of experiments, analysis of results, and manuscript edits. Antoinette Nguyen assisted with the statistical regression analysis. My committee provided suggestions for final manuscript of revisions and submission.

Dedication

I would like to dedicate this thesis to my mom, Karen Schaffrick, in her memory. Also to my dad, David, and brother, Miles Schaffrick, for their continuous support.

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First, I would like to thank my primary supervisor, Dr. Edward Tredget for the opportunity to be a part of this research group. It has been a privilege to learn directly about the issues burn patients face in improving their treatment outcomes, fibrotic scarring, wound healing, and guidance in academia. A big thanks to Dr. Jie Ding, for always being in the lab to help guide with any questions, mentorship, guidance through a range of research techniques, always assisting with editing, and her kindness and support.

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List of Abbreviations

ARISE	Alberta Research Information Services
AD-MSC	Adipose derived-mesenchymal stem cells
AP-1	Activating protein-1
APC	Antigen presenting cell
BCA-1/CXCL13	B cell-attracting chemokine 1/ C-X-C motif ligand 13
bFGF	Basic fibroblast growth factor
CD	Cluster of Differentiation
CHOP	C/EBP homologous protein
CO_2	Carbon dioxide
CREB	cAMP response element binding protein
CTGF	Connective tissue growth factor
CCL2	C-C Motif Chemokine Ligand 2
CCL5	C-C Motif Chemokine Ligand 5
CXCR4	C-X-C Motif Chemokine Receptor 4
CCR2	C-C Motif Chemokine Receptor 2
CCR5	C-C Motif Chemokine Receptor 5
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle's Medium
Ds	Double stranded
ECM	Extracellular matrix
EDA-FN	Extra Domain A Fibronectin

FBS	Fetal Bovine Serum
HSCs	Hematopoietic stem cells
HTS	Hypertrophic scars
IFN-α2b	Interferon-alpha 2b
IFN-$\boldsymbol{\gamma}$	Interferon-gamma
IL	Interleukin
IL-1RA	Interlekin-1 Receptor Antagonist
JAK/STAT	Janus Kinase and Signal Transducer and Activator of Transcription
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LSM	Lymphocyte Separation Medium
MCP-1/CCL2	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
NkRF	Nuclear factor κB repressing factor
P13/Akt	Phosphatidylinositol 3/Protein Kinase B
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PDL	Pulsed-dye laser
PMN	Polymorphonuclear
RANTES/CCL5	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted

SDF-1	Stromal cell-derived factor-1
SGS	Silicone gel sheeting
Ss	Single stranded
TARC	Thymus and activation-regulated chemokine
TBSA	Total body burn surface area
TGF-β	Transforming growth factor-beta
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-alpha
TRAIL	Tumor necrosis factor (TNF)-related apoptosis inducing ligand
Treg	Regulatory T-cells
USD	United States Dollars
VEGF	Vascular endothelial growth factor
α-SMA	Alpha-smooth muscle actin

Chapter 1 -

Introduction

1.1 Hypertrophic Scars

Hypertrophic scars (HTS) are classified as a human dermal fibroproliferative disorder which occurs following burns, trauma, surgery, or inflammation (5). Patients who develop HTS suffer from a reduced functional range of motion due to joint contracture as well as disfigurement (6). HTS are raised, painful, pink or red in color, hard, inelastic, and are confined to the boundaries of the wound (7). Apart from functional or cosmetic problems, patients with HTS may suffer psychiatric disorders, such as depression, reduced self-esteem, anxiety, or posttraumatic stress (8). HTS show a rapid growth curve for the first 6 months post-injury, after which they will usually decrease over a period of a few years (3). Although the molecular and cellular events leading to HTS formation have been frequently studied, the exact pathogenesis, as well as treatment, remains elusive. The outcome of HTS remains highly variable, as patients receive a wide range of treatments with unpredictable results.

1.1.1 Epidemiology

Skin is the largest organ of the human body, continuously exposed to the external environment. Its functional role is to provide protection against bacteria, and it has cosmetic factor due to its visibility. The epidemiology of HTS demonstrates an equal sex distribution of scar occurrence, with 70% to 91% of full thickness burns resulting in HTS (9, 10). HTS occurs across all races, apart from an extremely low incidence in albinos, while those with darker skin are more susceptible to keloid formation, another dermal form of fibroproliferative disorders. (11). There is a much higher incidence of HTS in children and young adults (12). This observation is partially attributable to young people being more prone to activities that may result in trauma, the skin containing more elastic fibers (resulting in greater tension), and collagen synthesis occurring at a higher rate (13). It has also been observed that the First Nations Indigenous racial background is associated with an increased risk of HTS development (14).

Anatomic sites with a high risk of HTS developing typically experience high tension, such as the shoulders, neck, joints, or anywhere that has experienced deep abrasion (15). HTS have an indefinite duration, and commonly result in abnormal pigmentation over the wounded site (9). In a previous study it was reported that the most common stressors and complications in burn patients who developed HTS was their altered appearance (75.2%), pruritus (73.3%), surface texture (70.5%) and pain (67.6%) (16). Although pruritus, or itching, is a very common symptom in patients, it may be reduced slightly by antihistamines, which has been recently found to be evoked after activation of opioid receptors (17). This forms a basis for the future direction and exploration to treat one of the symptoms of HTS including pruritus. Current treatments are not always effective or individualized, but this is a direction for further research.

1.1.2 Burn Injury

Besides affecting patients' quality of life, the expenses linked to wound treatments and recovery are difficult for the average patient to manage. In 2004, nearly 11 million individuals world-wide had a burn injury severe enough to require hospitalization (18), although the numbers have likely increased. Some patients may choose more reconstructive treatment after the acute phase of burn injury, which would increase the expense; however the market for wound care products is expected to expand by 5.37% from 2018 to 2023, leading to a total global revenue of $23.2 billion (USD) by 2023 (Research and Markets, 2018). The demand for advanced wound care is anticipated to increase in the coming years, suggesting the strong benefit to understanding the pathogenesis and mechanism of hypertrophic scarring, which could lead to novel treatments in the future. Realizing the scope of problems that arise from disability of HTS in the burn patient including unpredictable expenses, lost wages, variability in treatment results,

and decreased productivity, underscores the importance of maximizing the efficacy of future procedures and understanding the biological mechanism of HTS.

1.2 Wound Healing

Physiological wound healing following skin injuries can be separated into three stages: inflammation, proliferation, and tissue remodeling, summarized in Figure 1 (3). Within these stages, there is a complex network of pro- and anti-fibrotic molecules, chemokines, growth factors, blood cells, mesenchymal stem cells (MSC), and extracellular matrix (ECM) proteins regulating the outcome of the dynamic wound healing process.

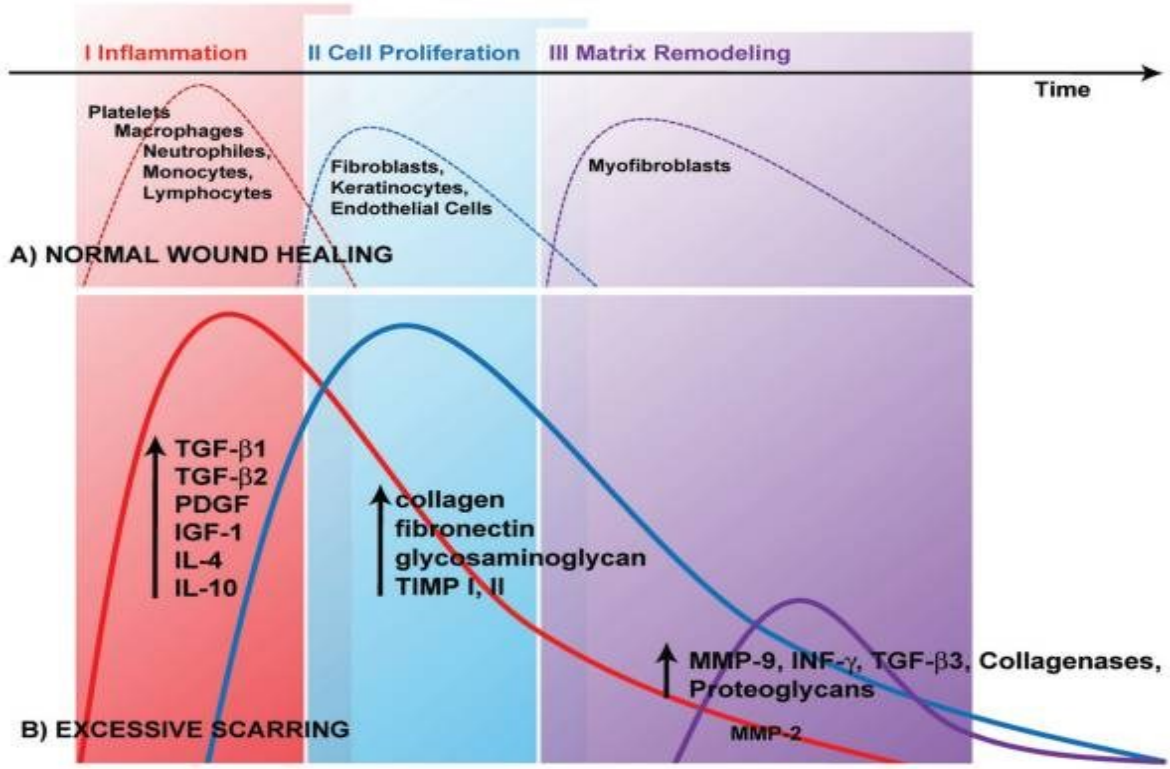

Figure 1 — The molecular and cellular changes in normal wound healing and excessive scarring (3).

1.2.1 Inflammation

The first stage of physiological wound healing begins as a vascular response, hemostasis, takes place immediately after injury and occurs within hours to days. The injured skin causes

clotting factors to form a clotting cascade to create a blood clot. The blood clot is composed of cross-linked fibrin molecules and ECM proteins such as fibronectin, vitronectin, and thrombospondin (19). The clot stops the bleeding, functioning as a temporary shield to protect the wounded tissue and provides a scaffold for cytokines and growth factors to attach and proliferate (20). The platelet thrombus and the fibrin clot have been shown to capture the endotoxin lipopolysaccharide (LPS) (21), which acts as an inflammatory signal to initiate the next stage in wound healing. LPS interacts with its main exogenous ligand, Toll-like receptor 4 (TLR4), accelerating the resolution of proinflammatory cytokines and altering the secretion in mediators involved in the wound healing process (22).

An early mediator in the hemostatic stage of wound healing is thrombin, which plays a role in both clot formation and inflammation (23). Thrombin signals for the release of pro-inflammatory cytokines, including monocyte chemoattractant protein-1 (MCP-1/CCL2), interleukin (IL)-6, and IL-8 from endothelial cells, inducing monocyte chemotaxis (24). The up-regulation of these pro-inflammatory cytokines may be seen in peripheral blood monocytes. When macrophages are activated during inflammation they release cytokines, which stimulate lymphocytes to cause a delayed, hypercellular response, and disrupting mechanisms that regulate inflammation, leading to fibrosis (25). Excessive wound healing will result in hypertrophic scarring, which may be prevented by an antagonist in a pathway creating less intense stimuli from cytokines and chemokines.

The next stage of the wound healing process is the inflammatory phase, which establishes an immune barrier against microorganisms and creates a hypercellular response. The inflammatory phase is not a distinct phase, as it begins during the first stage, hemostasis, with neutrophil recruitment and shows monocyte activity at the end of the inflammation phase.

Polymorphonuclear neutrophils (PMNs) become entrapped in the blood clot and are present for the first 2-5 days (unless the wound site becomes infected), which may amplify the inflammatory response by releasing several mediators such as tumor necrosis factor-alpha (TNF-α), IL-1β and IL-6 (6). Aside from releasing a variety of factors to amplify the clotting and inflammation process, PMNs use phagocytosis and proteinases to remove necrotic tissue and kill local bacteria, initiate a coagulation cascade, and are shown to release chemoattractants for other cells involved in the inflammatory phase (26). Chemokines and their receptors are crucial mediators for recruitment during repair, some of which include IL-8 and MCP-1 (27). Leukocyte subsets such as neutrophils, macrophages, mast cells, and lymphocytes are necessary to serve as immunological effector cells, and are tightly regulated through chemokines during wound repair (28). Circulating fibrocytes from the blood migrate to the wound site during the inflammatory phase, and are able to secrete proinflammatory cytokines, chemokines, growth factors, and change the ECM (29). Thus, fibrocytes are strongly implicated in wound healing and tissue repair in HTS development (30).

The later part of the inflammatory phase occurs within 2-3 days after injury when neutrophil infiltration ceases, and monocytes differentiate into macrophages after migration from the blood into the tissue of the wound site under the influence of chemokine pathway signaling (26). Macrophage infiltration is heavily regulated by its microenvironment producing a gradient of specific chemotactic factors, such as chemokine macrophage inflammatory protein 1α (MIP-1α) (31). Macrophage functions include aiding in host defense and amplifying and resolving inflammation through immunologic functions, producing antigen-presenting cells (APCs) and phagocytosis during wound healing (32). Macrophages also synthesize growth factors including: transforming growth factor-beta (TGF-β), TGF-α, basic fibroblast growth factor (bFGF),

platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), to regulate cell growth, division, and assist in building the ECM (33). VEGF induces the proliferation and migration of vascular endothelial cells and is essential in wound healing, for both physiological and pathological angiogenesis, and in addition binds to their cognate tyrosine kinase VEGF receptors in endothelial cells to create downstream signaling effects (34). In a recombination therapy study, PDGF subunit b was mixed with stem cells, and was shown to promote the proliferation of the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) (35), which plays an important role in secreting collagen type I in tendon repair and in the tissue remodeling phase of wound healing. Adipose-derived mesenchymal stem cells (AD-MSCs) have also been shown to efficiently secrete and provide an appropriate microenvironment required to increase FGF1, which in turn stimulated angiogenic proliferation (36), which decreases the likelihood of tissue defects and aid in the wound healing process. These cellular factors released during the inflammatory phase move the wound healing process toward the next stage, proliferation.

1.2.2 Proliferation

The proliferation stage contributes to wound healing by re-epithelialization, angiogenesis, and granulation tissue formation. This stage reduces the wound size by contraction and fibroplasia, and stimulates keratinocytes into their active form (37) to ensure covering of the wound surface by providing a epithelial barrier. HTS may result from abnormal keratinocyte function, causing a shortage of its secretion of cytokine IL-1α causing Langerhans cell existence to prolong on the epidermis (38). Endothelial cells become activated at nearby uninjured blood vessels surrounding the wound site, and release growth factors such as VEGF to undergo differentiation into new arteries and venules from recruited pericytes and smooth muscle cells

(39). Pericytes differ substantially from vascular smooth muscle cells, fibroblasts, or other cell types in that they are mural support cells, which belong to the mesenchymal cell lineage and are believed to help stabilize the vessel wall and prevent vascular leakage (40). Cytokines like interferon gamma (IFN-γ) and TGF-β regulate the synthesis of collagen, fibronectin and other tissue to generate the new matrix of connective tissue and assist in closing the wound gaps and restore strength to the site (41). Necrotic tissue releases breakdown products, such as apoptotic signals that are not fully understood, locally capable of either amplifying or maintaining the inflammatory response (42). In HTS, there is a balance of both ECM synthesis and ECM degradation; with the synthesis of collagen increasing while the proliferation of fibroblasts is reduced significantly (43). Signals from damaged tissue and inflammation stimulate the process of epidermal renewal which relies on a network of signaling cascades, triggered by keratinocyte receptor interactions through attraction from cytokines, chemokines, and antimicrobial peptides (44). If uncontrolled, this also results in severe skin lesions and a hyperinflammatory response.

The proliferative phase of wound repair begins with the migration of fibroblasts into the wound site, replacing the fibrin-based provisional matrix built in the hemostatic or inflammatory stage with a collagen enriched granulation tissue (45). Fibroblasts are recruited to the periphery of the wound and are responsible for breaking down the fibrin clot, creating a new ECM, secreting collagen in the matrix to support other migratory cells in the wound healing, and also wound contraction (46). Hypertrophic and keloid scars in the skin have a large number of myofibroblasts during wound contraction, showing their significance in driving fibrotic diseases and scarring (47). Myofibroblasts express alpha smooth muscle actin (α-SMA), which is a marker for HTS (48). MSCs are progenitor cells in the dermal sheath around hair follicles that are facing epithelial stem cells, are heavily involved in wound healing and can also differentiate

into myofibroblasts (49), increasing collagen production and thus contributing to HTS formation. Bone marrow MSCs are hematopoietic precursor cells involved in HTS development, which contribute to maintenance and regeneration of connective tissue and the proliferation of cells.

Angiogenesis during the proliferative phase of wound repair rebuilds existing blood vessels and forms new vessels using paracrine and autocrine signaling. The migration and stimulation of endothelial cells in the ECM, through an integrins-collagen I interaction, causes angiogenesis to occur, accompanied by neovascularization (50). This is essential in wound healing, to provide oxygen and nutrients to the tissue and immune cells to the wound site. As angiogenesis continues, the granulation tissue formation begins as the last step in the proliferation phase, while the tissue is hypervascularized. It replaces the fibrin-based ECM and produces a scar by maturation. It is characterized by a high number of fibroblasts, granulocytes, elastin, proteoglycans, hyaluronic acid, macrophages, procollagen bundles and vessels (51). Fibroblasts are the highest cell concentration during this phase, contributing to collagen production and the ECM, providing a supporting, dynamic scaffold for cell adhesion and regulation of cell movement and differentiation (52). After some time, angiogenesis diminishes, blood flow decreases, and the cell density dwindles, which is the beginning of tissue remodeling.

1.2.3 Tissue remodeling

Tissue remodeling is the last phase in wound healing, characterized by cessation of granulation tissue production and a change in collagen present from type III to a stronger type I. This is the longest phase, occurring from day 21 to upwards of 1 year after injury (51). The collagen bundles are orientated parallel to the epidermal surface in HTS, unlike healthy dermal tissue, which contains normal and relaxed collagen orientation, which are “basket-weave-shaped”, and randomly orientated bundles (Figure 2). There are features of the dermal layer that do not have potential to fully recover after wound closure, such as hair follicles or sweat glands

in post-natal humans. However, it has previously been observed that fetal wound healing may occur after the 20th week of gestation with no signs of scarring (53).

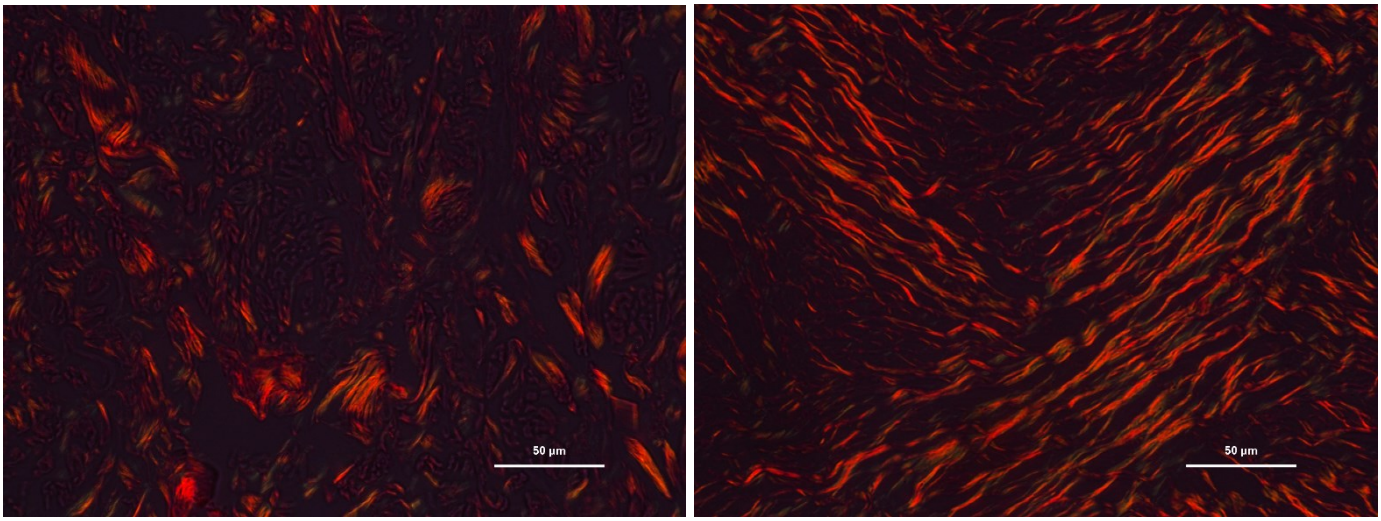

Figure 2– Picrosirius red staining of normal and hypertrophic scar tissues. Normal tissue (left) shows a collagen orientation with basket-weaved bundles, while hypertrophic scar shows a signature parallel, wavy collagen orientation. Original images by author, published in *Advances in Wound Care* (1, 2).

1.3 Hypertrophic Scar Pathogenesis

The HTS formation is a conserved, complex, and multicellular process that involves the coordinated efforts of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages, cytokines, chemokines, platelets, growth factors, and many other cells (54). HTS morphology includes a defined, red, elevated and visible scar on the patient's body (55). Excessive deposition of ECM proteins and other up-regulated chemokines and cells over long periods results in an increased duration of the inflammation response, which contributes to fibrosis. The histopathological findings observed in HTS include the replacement of the papillary and reticular dermis with scar tissue containing increased vertically oriented blood vessels (56).

In HTS formation, a chronic inflammatory response is activated, resulting in a dysregulated response and pathological wound repair, causing permanent scar tissue at the injury site where the tissue may not return to normal function (57). Removal of the inflammatory trigger is believed to be the most direct way to block the excessive tissue production and allow the normal scar healing to be restored after injury, such as has been observed in hepatitis B infection (58). As soon as the epithelial layer is damaged, there is a disruption of the keratinocytes, which respond by releasing IL-1, and is the first signal that alerts the surrounding cell niche of the recent tissue damage (59). Platelets respond by releasing factors, which include epidermal growth factor (EGF), PDGF, and TGF-β, that plays a role in attracting neutrophils to the injured site to remove bacteria and prevent infection (60). These factors also stimulate epithelial cell migration and proliferation. In uninjured epidermis, keratinocytes are attached to each other via desmosomes and the ECM of the basement membrane by hemidesmosomes, however tissue damage occurs, the disassembly of this barrier allows for keratinocytes to migrate to the wound edges to participate in the re-epithelialization process and closure of the wound (61). Activated keratinocytes are a source of many cytokines involved in inflammation, and abnormalities in the basal membrane promote basal keratinocytes to adopt a proliferative phenotype in HTS (62).

Dermal fibroblasts from the deeper dermis in burn injury express ECM proteins differently than other layers of skin, such as differing ratios of collagenase type I and III (63). Both the papillary and reticular zones of human skin show unique populations of fibroblasts, which differ in epidermal cell support and interaction with keratinocytes (64). Thus, it is suggested that HTS formation occurs in response to fibrogenic cytokines causing selective proliferation of profibrotic deep dermal fibroblasts (65). While there is support for a unique

subpopulation of cells in HTS, the particular cells recruited during burn injury have yet to be identified.

1.3.1 Hypertrophic Scars vs Keloids

It is important to understand the clinical differentiation between HTS and keloids, as incorrect identification could lead to inappropriate treatment and contribute to further surgical procedures. The distinction between HTS and keloid treatments is often left undescribed in articles and reports, making it difficult to study HTS treatment. Both scars are raised above the epithelial surface level, however HTS will remain within the original wound boundaries, while keloids will extend beyond these margins (66). The histological differences include increased amounts of fine, organized, primarily parallel type III collagen bundles with numerous nodules containing excess myofibroblasts in HTS (3); whereas, keloids contain thick, disorganized, large type I and III collagen bundles with a minimal numbers of myofibroblasts and no nodules (67). Keloids have a higher collagen index and a 45-100% chance of recurrence following excision, while HTS recur less commonly (10%) (68). HTS have a rapid growth phase that usually occur 4-8 weeks following wound infection or skin injury, while keloids may develop after several years or occur spontaneously and is accompanied by hyperpigmentation (69). Clinical treatments for both HTS and keloids, however, have much room for improvement to achieve a higher clinical efficacy and better treatment outcomes for patients. A deeper understanding of the pathophysiology underlying HTS is needed for better treatment and therapeutic strategies.

1.4 Cellular and Molecular Factors Significant to Hypertrophic Scarring

HTS tissue has a significantly higher number of fibroblasts and myofibroblasts when compared to uninjured tissue (70). Downregulating myofibroblasts shows the potential therapeutic treatment of HTS. Inhibition of fibroblast proliferation and ECM deposition would likely improve HTS outcomes. This suggests that severe burn injury causes up-regulation of fibroblasts and proinflammatory cells from the blood to the wound site, causing excessive collagen deposition and inflammation. The primary function of fibroblasts is to remodel the ECM after injury and maintain the physical integrity of connective tissue (71). A specialized form of fibroblasts, myofibroblasts, are responsible for the dense collagen matrix seen in fibrosis, which express α-SMA, primarily producing collagens I, II, IV, and V in the ECM (72). Fibrotic tissue is characterized by the excessive deposition of ECM proteins and hypercellularity as compared to a normal tissue microenvironment.

1.4.1 Protomyofibroblasts

The cause of HTS development is strongly attributable to the excessive ECM production and the hypercellular response, causing the dense collagen matrix during the maturation period as fibroblasts differentiate into myofibroblasts (73). In the differentiation from fibroblasts to myofibroblasts there are transient cells involved, termed the protomyofibroblasts (74). The most notable difference in the protomyofibroblasts is that they lack the α-SMA compared to myofibroblasts, but both contain actin stress fibers and intracellular fibronectin (75). Protomyofibroblasts also have small fibronexi (fibrous extensions) while myofibroblasts contain large fibronexi (76). It is generally agreed that differentiation from fibroblasts to protofibroblasts is mainly a result of mechanical tension produced by the fibroblasts, but PDGF may also play a role (77). Protomyofibroblast to myofibroblast maturation is mainly attributable to a synergistic

action involving mechanical tension, TGF-β1, and extra domain-A fibronectin (EDA-FN) (76). Myofibroblasts synthesize ECM components to replace the normal matrix, and have contractile properties regulated by α-SMA organized in compact microfilament bundles or fibers (78). As the wound closes and evolves into HTS, the later stages of wound healing show an increase in myofibroblasts and vascular cell apoptosis (79). This regulation of myofibroblasts via apoptosis may also be important in scar development in HTS.

1.4.2 Mast cells

Mast cells are bone marrow-derived cells that circulate as immature hematopoietic progenitors that mature after reaching resident tissue (80). Mast cells have the potential to promote or suppress immune responses (81). They are also known to act as a mediator with diverse functions due to their ability to secrete cytokines and growth factors, which significantly influence inflammation and tissue remodeling (80). In HTS, there are neuropeptides released from damaged dermo-epidermal nerve endings, which initiate the vessel's response to neurogenic inflammation and thus, directly activating local mast cells (82, 83). When tissue injury occurs, the mast cells undergo degranulation, releasing mediators necessary to trigger the inflammation response, and influencing local endothelial cells (84). Mast cells are found to increase fivefold at the wound borders, which shows a positive correlation with the chemokine MCP-1 (85). The increase in mast cell numbers also correlates with an increase in TGF-β, acting as a wound healing cytokine and a very potent chemoattractant for mast cell stimulation (86). Mast cells function in microbiological cleansing of injured tissue by undergoing degranulation, releasing soluble factors (87). The proliferation phase of wound healing is dominated by specific events including angiogenesis, fibroplasia, re-epithelialization, all of which can be modulated by mast cells producing differential biological mediators (Figure 3) (88). Studies have also

demonstrated that mast cells are present in HTS with active expression, playing an important role in the signaling pathway (89).

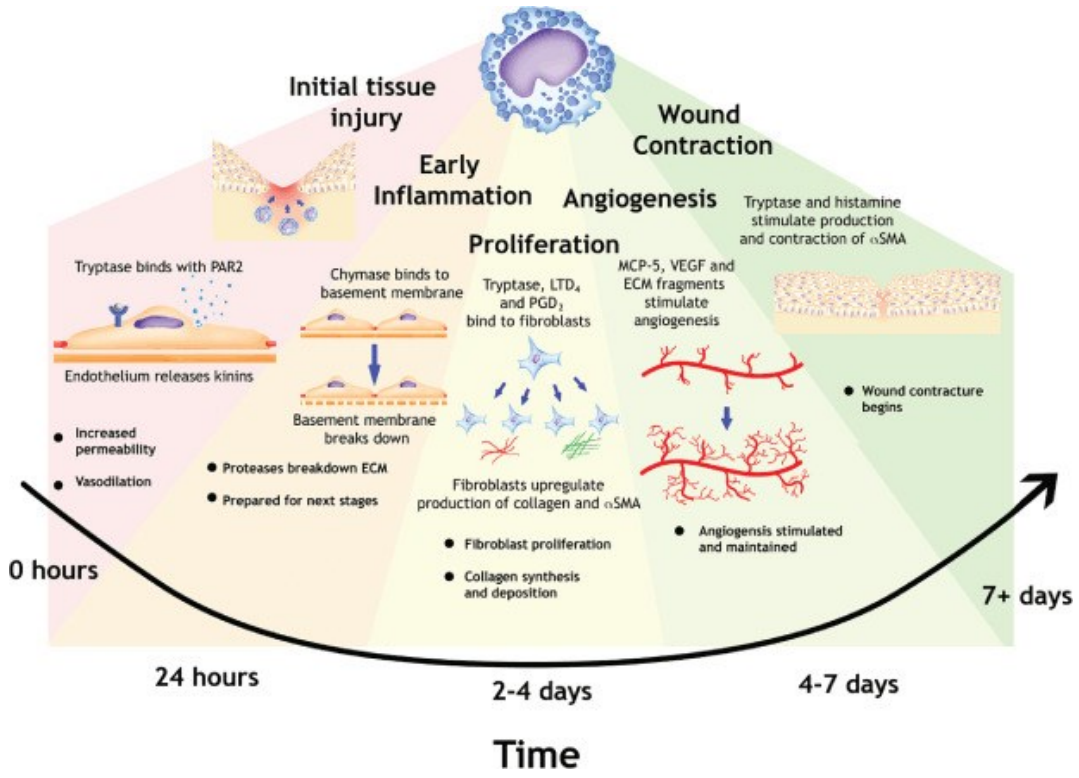

Figure 3 – The roles of mast cells in wound repair, image taken from (2), with open access.

1.4.3 Fibroblasts and Decorin

Mast cells can also stimulate fibroblasts, which are active during the proliferative phase of wound healing to repair the dermal layer (90). During wound healing, the formation of a provisional ECM containing fibrin, fibrinogen, and fibronectin is occupied by fibroblasts, which proliferate in response to cytokines produced by neutrophils and macrophages in the injured tissue (91). In the remodeling phase of wound healing, decorin is produced, which regulates collagen fibrillogenesis by presenting a "C"-shaped protein that is bound between collagen fibrils (92). Decorin binds and neutralizes the effects of TGF-β, which stimulates the proliferation of the ECM (93). After burn injury, myofibroblasts eventually become surrounded in collagen, which can exert adverse effects on surrounding cells, such as causing arrest in the G1 phase of

their cell cycle (94). The degradation of collagen also reduces the ability of myofibroblast adhesions (95, 96), which furthermore causes the cells to undergo apoptosis (96). The accumulation of myofibroblast apoptosis results in a collagen-rich HTS because of recruited cytokines and hypercellularity to replace degraded tissue (91). Myofibroblasts in HTS are found to have an increased resistance to apoptosis, further inducing cell cycle arrest or apoptosis, resulting in their accumulation and prolonged existence (97), playing a further role in HTS development.

1.4.4 Neutrophils

As discussed thus far, efficient wound repair requires the coordinated effort of a number of different cell types. In HTS and chronic wounds, active neutrophils are recruited for a prolonged period of time, causing proteases to be released and additional tissue destruction, thus resulting in chronic inflammation and increased tissue damage (98). PMNs demonstrate an up-regulation of anti-apoptotic genes, cytokines and chemokines critical for chemotaxis of macrophages, T-cells, and other receptors involved in the inflammatory response (99). Apoptotic neutrophils become signaled for phagocytosis of macrophages for degradation, with the uptake of apoptotic cells providing a strong signal for the inflamed tissues to return to homeostasis (100). A reduced clearance by macrophages can prolong inflammation and further aid in the inflammatory development of HTS (41). Therefore, neutrophils are found to produce mediators in immune responses to contribute to tissue repair and influence autoinflammatory responses (101).

1.4.5 TGF-β

HTS shows increased numbers of growth factors, including PDGF and TGF-β (102), when compared to normal tissue. TGF-β has been strongly linked to HTS and is known to play an important role in wound healing and tissue remodeling, regulating the expression of many ECM proteins. TGF-β maintains homeostasis in the tissue by regulating various cell types—including epithelial, endothelial, immune cells, and stromal fibroblasts in their microenvironment (4) (Figure 4). The three isoforms, TGF-β1, TGF-β2 and TGF-β3, share 60-80% homology and appear to have important functions in wound healing that mediate their effects through cell-surface threonine kinase receptors to intracellular mediators, known as Smads. TGF-β1 has been found to accelerate acute wound healing, secreted by keratinocytes, platelets, monocytes, macrophages, and fibroblasts (103). Other aspects to understand about the TGF-β family include knowing the downstream mediators, which are connective tissue growth factor (CTGF), decorin, and binding protein 311 (12), which may underlie the pathogenesis of fibrotic scarring after burn injury.

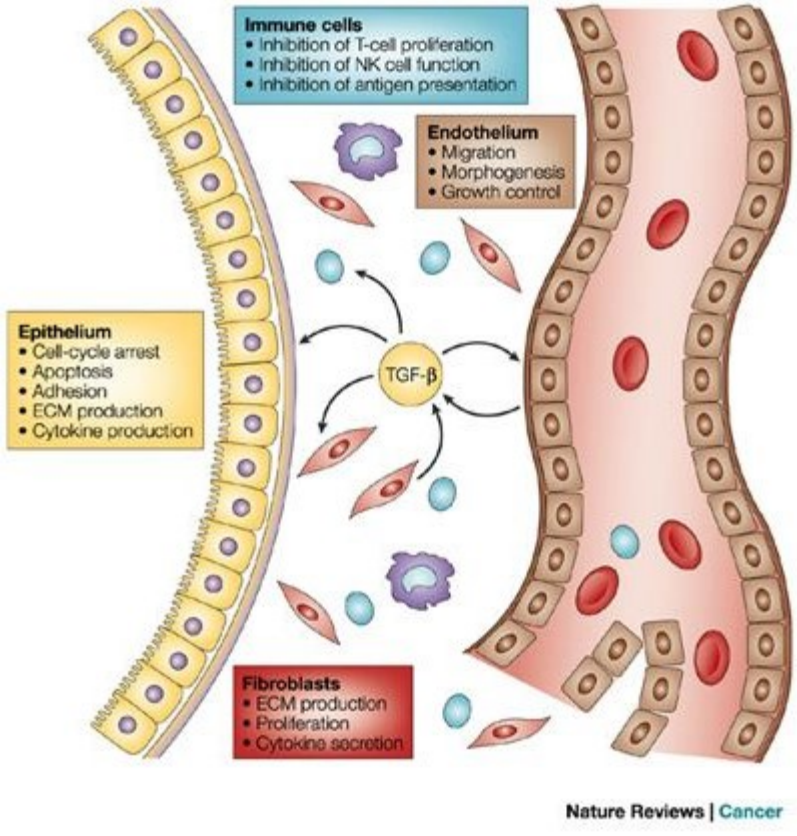

Figure 4 –Cell regulation by TGF-β (4). TGF-β is involved in many processes in wound healing, such as fibroblast proliferation, immune response, endothelial control, and epithelium regulation.

Decorin is known to regulate collagen fibrillogenesis, and interacts with other growth factors including CTGF (104) and shows a decreased or absent level in post-burn HTS (105). The Smad pathway is a downstream signal-transduction pathway that acts as a mediator for TGF-β when phosphorylated. Smad 7 prevents phosphorylation of receptor-regulated Smads, while studies have shown Smad 7 overexpression and Smad 3 and 4 inhibition to be potential targets for HTS treatment (3). TGF-β up-regulates the components of the ECM, including fibronectin, collagens, matrix proteoglycans, stimulates angiogenesis, fibroblast proliferation, and induces inhibitors of matrix metalloproteinases (MMPs) and plasminogen-activator inhibitor (106). HTS have previously been shown to express five-fold more TGF-β1 mRNA than normal skin per unit of weight (107) demonstrating the role of TGF-β1 in the development of HTS.

1.4.6 Monocytes

Monocytes are of significant importance in relation to HTS formation. Monocytes originate from hematopoietic stem cells in the bone marrow, which may later differentiate into macrophage/dendritic progenitors, the immediate precursors of monocytes (108). The role monocytes play in circulating blood vessels carrying a chemokine receptor and migrating via attraction of a chemogradient to its ligand is given in Figure 5 for reference. Monocytes circulate in the blood, while their more differentiated forms, macrophages and dendritic cells, are tissue residents and predominantly immobile (109). There are three subpopulations of monocytes, in which the first distinguished one is the "classical" monocytes expressing $CD14^{++}CD16^{-}$, and accounts for 90-95% of the total monocytes in a healthy person (110). The "nonclassical" monocytes are considered proinflammatory, expressing $CD14^{+}CD16^{+}$, only consisting of 5-10% the total monocytes in healthy blood, which are shown to express increased TNF-α protein in response to TLR4 and TLR2 agonists (110, 111). The "intermediate" monocytes are considered

the most important in wound healing due to their high expression of surface markers including endothelial growth factors I and II and the chemokine SDF-1 receptor, C-X-C chemokine receptor type 4 (CXCR4) (112). Chemokine receptors C-C chemokine receptor type 5 (CCR5) is expressed on $CD14^{+}CD16^{+}$ cells, while CCR2 is characteristic of the classical $CD14^{hi}CD16^{-}$ cells (113). The differential level of specific chemokine receptors and adhesion molecules has an important implication in the susceptibility to infection (114) and is likely specific to certain monocyte subpopulations preferentially (115). Immediately following acute injury to the tissue, the inflammatory response is buffered by mobilizing monocytes infiltrating the site of injury from reservoirs in the bone marrow and spleen (116). Tissue damage is a trigger for the body's reservoir of monocytes leading to rapid accumulation, however such an aggressive response to inflammatory events may not be desired for full tissue restoration (116). However, the role of each subset in burn injury remains unclear.

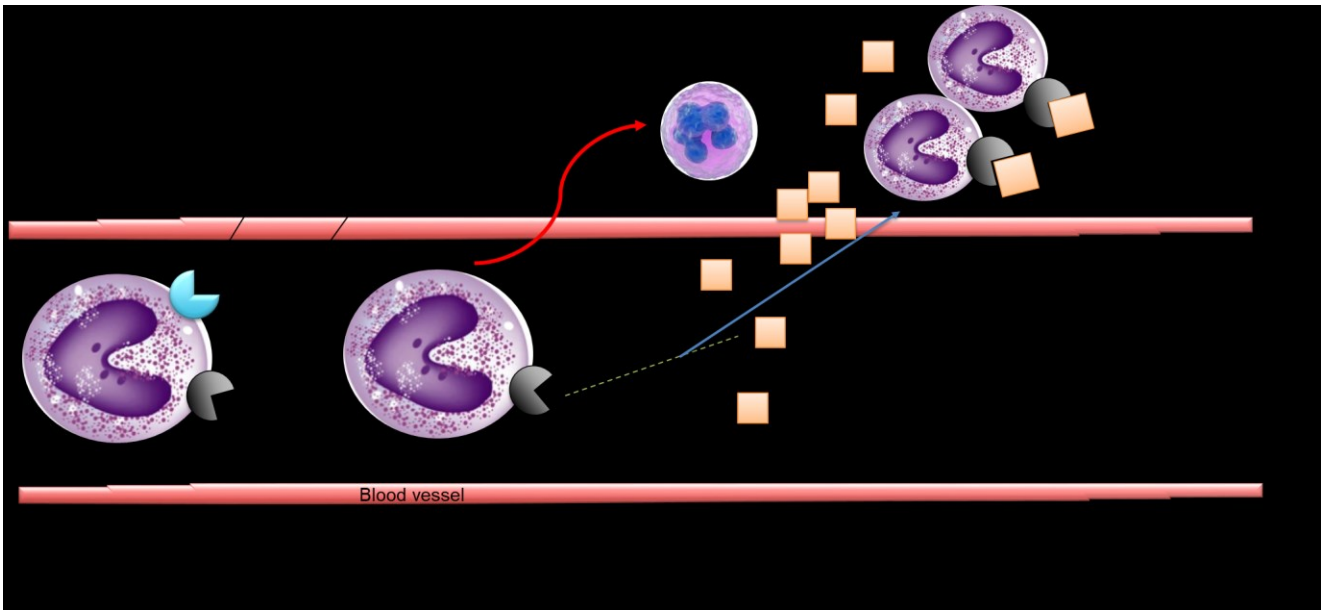

Figure 5. Chemokine receptors expressed on monocytes that migrate to its ligand via a gradient stimulating differentiation.

1.4.7 Macrophages

Macrophages can be classically activated, M1 macrophages being inflammatory, or alternatively activated M2 which aid in tissue repair and decreased inflammation (117). As M2 macrophages are involved in wound healing, it is expected to see them at elevated levels in the circulation, and a subpopulation of peripheral blood mononuclear cells (PBMCs) has been found in the blood in burn patients within 2 weeks post-injury (118). Knowing which chemokines are capable of up-regulating monocytes and fibrosis-inducing fibrocytes post-burn injury will provide a greater window into new therapeutic treatments to inhibit HTS formation. Macrophages are involved in both inflammation and tissue remodeling in HTS. In wound healing, the microenvironment may induce a change of macrophage phenotype from M1 to M2 (119). The various macrophage subtypes carry distinct surface markers and secrete distinct cytokines (120). Because of their proinflammatory function, M1are believed to be in the inflammatory phase of wound healing and later, in tissue remodeling, switch to the M2 phenotype (23). While M2 macrophages are involved in tissue remodeling, and fibrocytes induce scarring, they share the same marker, CD163 (121). Currently their functional relationship is unclear, but further understanding of chemokines inducing these cell types and receptors or possible cytokine-induced switching may enable the manipulation of cell phenotypes in lessening scar development in burn patients.

1.5 Current Treatments, Biologic Mechanisms, and Effects

Today, there is no clear or reliable treatment for preventing or reducing scarring. Previous clinical studies have not shown much progress due to the insufficient sample size and lack of robust controls and outcome measures. There have been some potential therapies studied, however, directed towards the molecular healing including apoptosis, ECM, inflammation,

growth factors, and mechanical support. Potential therapies for HTS include silicone, laser therapy, radiotherapy or cryotherapy. The relevance of previous studies is also put into question when comparing animal models to human models, since there is no widely agreed upon animal model of human HTS. Human studies pose ethical challenges and may be difficult to conduct without significant supporting evidence for a prospective outcome, which usually comes from animal studies. Although there has been a substantial amount of research done on hypertrophic scarring, the understanding of the mechanisms remains vague, resulting in non-specific treatments with unpredictable outcomes for patients, that fall short of full recovery and healing. With the large number of patients, especially younger ones at risk of scarring and burn injury, completely understanding the mechanism behind scarring is necessary for further scientific progress, and effective clinical treatment for HTS.

1.5.1 Compression Therapy and Corticosteroids

The first hypothesized therapeutic mechanism to treat HTS mentioned was regulation of apoptosis or reducing the proliferation of the hypercellular response. This may be done using combination treatment, compression, fibrostat (122), intralesional corticosteroids, or systemic interferon alpha 2b (IFN-α2b). Traditionally, compression therapy was used to treat burn injuries, but further analysis discovered that compression therapy failed to show efficacy in treating HTS (9). The mechanism for compression therapy is poorly understood, however there has been clear improvement in collagen orientation patterns following pressure intervention in scarring (123). Corticosteroid injections taken individually or in combination are very widely used in HTS treatment. They are mainly administered intralesionally, performed every four to six weeks or until the symptoms of pain subsides and the scar has reduced in size vertically. The response rates vary for corticosteroid injection, with a recurrence rate of 9-50% (124), therefore, they are not without limitations. Improved scar reduction has been shown from intralesional

injection with IFN-α2b, IFN-γ, mitomycin-C, bleomycin, and 5-fluorouracil used alone or in combination with other treatments for HTS (125).

1.5.2 IFN-α2b

IFNs are a family of cytokines with antiproliferative activity, so they show potential for regulation of fibrotic conditions (126). Clinical studies have suggested that IFN-α2b improves scar quality in patients with HTS post-treatment, which may be associated with a decrease in angiogenesis (127). Prevention of primary vascular abnormality and new blood vessel formation in fibrosis may be one of the more promising therapeutic strategies (128, 129). IFN-γ demonstrated thinning of the suprapapillary plates in the epidermis, diminished thickness of collagen bundles in the dermis, and increased inflammatory cells, suggesting their use in abnormal fibrosis (130). Patients with severe hypertrophic scarring show a significant clinical improvement in scar quality during IFN-α2b therapy, which reduced the IFN and histamine levels (131). TGF-β stimulates collagen synthesis, but is antagonized by IFN-α or IFN-γ, and additive effects follow (132). There has also been reported improvement of HTS in post-burn patients, which may be a result of decreased fibrocytes after IFN-α2b treatment (133). Therefore, burn patients with HTS treated with IFN-α2b have shown decreased angiogenesis, endothelial cell proliferation and VEGF-proliferation (127), decreasing the pathway contributing to HTS.

1.5.3 Topical Creams

The formation of HTS occurs as a result of the imbalance of synthesis and degradation of collagen, containing the same ECM as the tissue they replace but with a higher ratio of collagen type III:I. Compared to normal tissue, HTS has an ECM synthesis seven times higher than normal, as well as fibronectin, laminin, and decreased hyaluronic acid and decorin (73, 91). There is a misconception in the general population that vitamin E will modulate wound healing and scar formation and downregulation of collagen synthesis. However, in 90% of cases in a

study, topical vitamin E either had no effect or worsened the cosmetic appearance of scars (134). Therefore, vitamin E used as a single agent is not recommended for the treatment in improved scar appearance. Imiquimod 5% cream has been shown to improve the cosmetic outcome in HTS, however there have been some uncommon, but severe side effects (135). Imiquimod cream shows minimal recurrence of scars, and works by decreasing the density of the ECM by downregulating TNF-α, IL-1, IL-4, IL-5, IL-6, IL-8, and IL-12 and up-regulating IFN-α (73). Additional studies are necessary to determine if imiquimod cream is an effective treatment of HTS and to ensure safe administration.

1.5.4 Taping

The local mechanical forces in the skin are believed to be a major contributor to the cellular behavior that develops scarring (136). Thus, extended mechanical support using tapes, sheets, or garments is recommended to prevent hypertrophic scarring. Silicone tape is gentler than paper tape, as it prevents epidermal injury caused by repeated taping (137). Paper tape significantly decreases scar volume, however in patients with sensitive skin there can be a localized red rash beneath the tape (138). Taping is also used for convenience, as the patients do not need to change after a bath or shower as they will stay in place for 1-2 weeks (139). Flurandrenolide tape (Cordran® tape), deprodone propionate tape (Eclar® plaster), and fludroxycortide tape (Drenison® tape) are available worldwide, but the appropriateness of each tape may differ on a case-by-case basis (139). Adhesive zinc tape used to treat HTS has also shown significantly reduced scarring and decreased redness and itching (140). Kinesio Tape has also been introduced into the US after use in Japan for 10 years, it is made from cotton, allowing the skin to be exposed to oxygen through the porous fibers. Surgery or radiation is not recommended in children (less than 18 years), so steroid tapes/plasters are a reasonable first-line therapy for pediatric patients.

1.5.5 Silicone gel

Silicone gel or a silicone sheet (SGS) is another therapeutic modality used in HTS treatment. Silicone gel contains polysiloxanes, which are made of silicone dioxide and a volatile component. Silicone gel forms an extremely thin sheet that self-dries in minutes, and has been reported to be highly effective for both texture, colour, and height reduction in scars (141). Other benefits from silicone gel includes: increasing hydration of the stratum corneum, regulating fibroblasts and reducing collagen deposition, while exposing the skin to partial oxygen; protecting injured tissue from bacteria; modulating growth factors FGF-β (normalizing collagen synthesis) and TGF-β (stimulates fibroblasts); and reducing discomfort associated with HTS such as itching (142). Self-drying silicone is very convenient for patients and easy to use, as lotion or other products may be applied over it once the gel has dried. The advantages of using the silicone gel sheets are that it is a noninvasive treatment and can be applied at home by the burn patient. The disadvantages is that to achieve maximal results, the gel must be worn continuously for 6-12 months (143). Although the mechanism of therapy for SGS is not fully understood, the key physical modes of action known are the sanitary conditions provided for wound healing and hydration of the scar site, which likely blocks signals for keratinocytes to produce cytokines, thus preventing excess collagen production (143, 144). Although there are signs of improvement in HTS using SGS, there is little significant improvement in severe cases and the mechanism must be further explored to optimize its potential for use in HTS treatment.

1.5.6 Laser Therapy

Laser therapy is a common treatment for HTS caused by burn injury, which can improve the color of the scar, pain, skin contraction, and reduce the thickness of the scar. Lasers differ in their radiation sources, being ablative and non-ablative lasers. The pulsed dye laser (PDL) is an example of a non-ablative laser, at 532-585 nm, and is useful for improved colour pigmentation,

shown to be especially beneficial for patients prone to develop severe lesions (145). Carbon dioxide (CO_2) laser, at 16,000 nm, uses fractional photothermolysis to re-stimulate the wound healing process, being an ablative and more aggressive treatment, but often with significantly improved results compared to non-ablative laser treatments (146, 147). Fractional photothermolysis rearranges the dense, parallel collagen fibers to replace with new, basket-weaved shaped collagen bundles and reduced abnormal pigmentation (148). Radiation inhibits fibroblast proliferation over six sessions, which are restricted to adults only due to the linked possible risk of induced carcinogenesis (149). Although radiotherapy shows improvement in the healing of scars, evidence is subjective over long-term follow up. Lastly, cryotherapy is a more recent development using a lumen needle placed at the center of the hypertrophic scar to release nitrogen vapor, rapidly freezing the scar from the core to its periphery. Cryotherapy is lacking in large and well-conducted studies; therefore, a larger meta-analysis is needed to confirm its efficacy. Previous studies demonstrate this technique is a safe treatment for HTS, showing significant scar reduction within a few treatments, although adverse effects may include recurrence, depigmentation, and pain (150), and reactions may differ among patients.

1.6 Chemokine Pathways

Chemokines are named for their ability to induce chemotaxis on effector/responsive cells (151). These signaling pathways may either recruit immune cells to a wounded site or control the migration of cells during normal tissue development (152). Chemokines are involved in the hemostasis phase of wound healing using stromal cell derived factor-1 (SDF-1), also known as CXCL12 and formerly known as PF-4 (153). They are involved in the inflammatory phase as well, predominantly, the CXC cytokines stored in blood platelets are released after activation in

response to tissue injury—including MCP-1 and CCL2, or RANTES/CCL5 (151). Chemokines play a significant role in wound healing, notably in the inflammatory and proliferative phases (154). They have been found to play a crucial role in leukocyte migration and recruitment, activation and effector functions, T cell helper type 1 (Th1) and Th2 responses, infections, metastasis, B-cell and T-cell development, and regulation of angiogenesis and localization of myofibroblasts (155). Chemokines are part of a large family of cytokines, which are 8-10 kDa in size, that are mediated by 7 transmembrane G protein-coupled receptors by binding to them and containing four highly conserved cysteine residues (156). The effect of chemokines downstream on their target cells is mediated by the G-protein-coupled receptors, such as studies done on leukocyte chemoattractant protein and receptor, which is involved in erythrocyte function and microbial pathogenesis during the inflammatory response (157). Chemokines are necessary for wound healing, and hyperactivation of them likely leads to HTS (158, 159). Chemokines variety of roles in abnormal wound healing after burn injury has many unknowns. Understanding the mechanisms leading to HTS will aid in finding therapeutic treatments to prevent and reduce HTS.

1.6.1 Increased Chemokine Response

A mechanism thought to contribute to HTS is the up-regulation of chemokines during inflammation, which include IL-6, IL-8, SDF-1, MCP-1, and CCL5. Major inflammation is caused by cytokine release, however the period of resolution and the extend of these cells involved is not fully understood. The first pathway we will look at is the regulation of the immune responses by fibroblasts through TLR pathway. The cytokine receptor TLR3 controls the anti-inflammatory response, while TLR4 stimulates a pro-inflammatory response (160), as seen in burn injuries. TLR4 is activated by LPS, leading to cytokine gene transcription, activated B cells, and other co-stimulatory molecule expression to enhance the inflammation response in

HTS (161). Furthermore, this study showed increased TLR4 expression in HTS fibroblasts compared with normal fibroblasts, including significant increases in mRNA and protein concentrations for MyD88, IL-6, IL-8, and MCP-1. TLR signaling is activated by PAMPs of bacteria in injured tissue and damage associated molecular patterns (DAMPs) to produce cytokines (162), showing the potential for TLR signaling modulation to improve treatment outcomes for HTS in patients. In a large study of >20% total body surface area (TBSA) burns, the leukocyte count was significantly increased, as well as upregulation of 8 out of 10 human TLRs, with the two down-regulated TLRs being TLR3 and TLR7 which function in double-stranded (ds) RNA and single stranded (ss) RNA recognition (163). Controlling inflammation via the TLR4 signaling in the epithelium has been found to be a potential treatment for HTS and other fibrotic disorders (164, 165).

1.6.2 Interleukin-6

IL-6 is involved in inflammation and maturation of B-cells, and is known to be an inflammatory marker. IL-6 is a necessary checkpoint regulator for neutrophil trafficking during the inflammatory response through chemokine synthesis and leukocyte apoptosis driven by the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, specifically STAT3 but not STAT1, limiting the inflammatory recruitment of neutrophils (166). It is suggested that IL-6 does not directly promote fibrosis, but it is IL-6's up-regulation of IFN-γ and STAT1 signaling that accounts for hyperinflammatory tissue damage. It has also been shown IL-6 is essential for T-cell survival and increases Th1 cell quantity as well as aids in maintaining their effector characteristics (166). IL-1β and TNF-α lead to increased IL-6, showing significant autocrine growth-factors in fibroblasts and in fibrosis and after CCL4 exposure (58). These observations suggest that IL-6 down-regulation interventions may be useful in the treatment of damaged tissue and fibrosis.

Mild burn injuries have reported lower concentrations of IL-6, increasing with severity of burn injury (167). Severe burns cause hypercatabolism of muscle, inducing an extended hypermetabolic response and is correlated with severity of injury (168). Burn serum-stimulated cells with IL-6 antibodies showed improved mitochondrial membrane potential, suggesting this cytokine plays a role in muscle degradation after systemic injury (169). Alcohol presence in burn patients greatly exaggerates immune dysfunction, has increased risk of infection, and increases pro-inflammatory cytokines, such as IL-6 (170). The knockout of IL-6 has shown to decrease the inflammatory response to the same level as the base burn injury, rather than the hyper-exaggerated response seen with alcohol (171). These are examples of links for IL-6 and its effects in the inflammatory response during burn injury.

1.6.3 Interleukin-8

IL-8 contributes to the increase in neutrophil numbers during inflammation. IL-8 is a chemokine encoded by the CXCL8 gene, which may be inhibited by nuclear factor-κB repressing factor (NkRF) or activated by transcription factors NF-κB, activating protein (AP-1), C/enhancer binding protein-beta (EBPβ) or NF-IL-6, C/EBP homologous protein (CHOP) and cAMP response element binding protein (CREB) (172). Inflammation is highly regulated by chemokines after burn injury in the tissue and skin epithelium, controlling trafficking of leukocytes using their transmembrane receptors. One of the chemokines that plays this role in the inflammatory response is IL-8. Once neutrophils arrive at the wound site, they are st5mulated further by IL-8 to carry out phagocytosis and increase the productivity of wound healing in hypertrophic scarring. Neutrophils are also involved in bacterial destruction (through phagocytosis), tissue healing, angiogenesis, degranulation, regulation and production of chemokines and production of VEGF (173). Secreted IL-8 also functions as a paracrine factor to

activate macrophages in the cellular response during fibrosis, and has been shown to expand the mesenchymal stem cell population to active wound sites (174).

1.6.4 SDF-1/CXCR4 Pathway

One of the chemokines that is involved in inflammation and immunity during wound healing is SDF-1; via a chemoattractant gradient to attract cells to the injured area. SDF-1, also known as CXCL12, belongs to the CXC family, meaning its structure is comprised of N-terminal cysteines (C), separated by one amino acid (X), with its receptor attached, CXCR4. The SDF-1 gradient attracts circulating cells expressing CXCR4, particularly in the inflammatory and proliferative stages, into the peripheral tissue where cellular adherence can occur (175). SDF-1/CXCR4 causes hemopoietic stem cells (HSCs) derived from the bone marrow to migrate into the blood, a process that can be observed in the peripheral blood (176). Bone-marrow derived PBMCs may mature into epithelial-like cells, endothelial cells, monocytes, macrophages, myofibroblasts, and protomyofibroblasts (intermediate form of myofibroblasts) (175).

Myofibroblast differentiation is classically known to be induced by TGF-β (177), however more recent research reports that the SDF-1/CXCR4 pathway cross-talks with the TGF-β/Smad signaling which induces myofibroblast differentiation (178). It has been shown that higher protein levels of α-SMA, which is a phenotypic marker for myofibroblasts, also correlate increased SDF-1 levels (179). This shows the role of the SDF-1/CXCR4 pathway may play in fibroblast differentiation and maturation into myofibroblasts, and hypertrophic scarring and fibrosis.

During burn injury, monocytes are found to express both CD14 and CXCR4, while fibrocytes express only CXCR4 (180). This study also showed that deep-dermal fibroblasts have a higher SDF-1 expression than superficial fibroblasts, showing a positive correlation between burn injury and SDF-1 levels. It is also suggested that there is more than one source of SDF-1

production, including bone marrow-derived stromal cells, inflammatory cells, and locally activated fibroblasts at the wounded site. CXCR7 may also form heterodimers with CXCR4, with CXCR7 being recycled back into the cell membrane and CXCR4 being degraded in the cells (181). The CXCR7 interaction with the SDF-1/CXCR4 pathway is complex, but it appears that the CXCR7 removes any excess extracellular SDF-1 and causes CXCR4 endocytosis and degradation, regulating the downstream levels of any cells attracted along this gradient (182). SDF-1 is known to be induced by proinflammatory factors, such as LPS, IL-1, and TNF-α (183) from the bone marrow into the blood, and then further into the injured tissue. The direct role of SDF-1 on monocyte populations during burn injury and wound healing is not known. The aim of this research will be to follow SDF-1/CXCR4 pathway along time gradients after burn injury.

1.6.5 MCP-1/CCR2 Pathway

The MCP-1 and its receptor CCR2 pathway has a dominant role in attracting bone marrow monocytes into the peripheral blood circulation under both homeostatic and stressful conditions (184, 185). The MCP-1/CCR2 pathway is also implicated in fibrotic and inflammatory conditions following tissue and burn injury (186). The MCP-1 is expressed by pro-inflammatory and stromal cells, functioning as a chemokine that controls gene transcription. MCP-1 is produced by several cell types, mainly monocytes/macrophages, but also endothelial cells, fibroblasts, epithelial cells, pericytes, astrocytes, mesangial cells, monocytes, and microglial cells (186). The mediator MCP-1 attracts circulating blood cells into the site of injury (187), while locally interacting and activating other resident tissue cells. MCP-1 induces angiogenesis by inducing VEGF (188). It has been observed that interruption in the MCP-1/CCR2 axis delays macrophage up-regulation, and reduces the influx of cytokines, including TNF-α, IL-1β, and TGF-β (189), however this pathway has mainly been studied in cardiovascular research. Further exploring the cells recruited by the MCP-1/CCR2 pathway

during HTS will help identify novel therapeutic targets in clinical application, which will be further explored in this research.

1.6.6 RANTES/CCR5 Pathway

CCR5 is expressed in lymphoid organs, platelets, smooth muscle, endothelial and peripheral blood leukocytes, such as macrophages and T-cells (190, 191). CCR5 is also found to be involved in a broad range of human immune diseases, including multiple sclerosis, rheumatoid arthritis, asthma, HIV, inflammatory diseases, allograft rejection, and fibrosis (192)—suggesting a CCR5 antagonist may be beneficial as a pharmacological intervention. CCR5 is closely related to CCR2B, sharing 71% of the same amino acid residues and having the gene encoding CCR5 downstream by only 18-kB pairs from CCR2 on chromosome 1p21 (193), suggesting the receptors share an ancestral gene. In a study involving cardiac remodeling; their findings suggests that chemokine signaling via CCR5 prevents uncontrolled inflammation, thus having adverse effects on cardiac remodeling (194). CCR5 may also regulate the immune response by controlling the regulation of foxp3-expressing regulatory T (Treg) cells, a $CD4^+$ subpopulation of T-cells that plays a critical role in immune homeostasis and self-tolerance (195). The CCL5/CCR5 chemokine axis has been observed to recruit Treg cells, preventing increased severity of chronic inflammation in the intestine (196). CCL5, or RANTES (Regulated upon Activation, Normal T Cell Expression and Secretion), has also been found to promote VEGF-dependent angiogenesis, by decreasing the PI3K/Akt signaling pathway, which is known for facilitating tumor growth (197). Therefore, it appears that the CCL5/CCR5 chemokine pathway is important for regulating the inflammatory response through the recruitment of T cells, but no significant study of these theories in relation to HTS have been conducted. However, due to its involvement in the inflammatory process, a better knowledge of the

mechanism appears promising for future treatment. The RANTES/CCR5 pathway will be followed as well at various time points and TBSA's during wound healing in our study.

1.7 Gaps in Research

A recent study observed 20 inflammatory mediators in burn patients 0-21 days after burn injury (167), however in this research we will follow up long term where possible, up to 6 months, as well as looking at 71 cytokines and monocyte populations. The exact mechanism for what causes worse scarring outcomes in burn patients is unknown. The aim of this research is to observe over time and burn severity the degree of cytokine involvement, monocyte subpopulation, and the outcome of the severity of scarring. Further knowledge of the molecular mechanisms involved in burn scarring will allow for growth of new, targeted, preventative and therapeutic treatments to improve patient's quality of life in the future.

1.8 Summary

The chemokine pathways including MCP-1/CCR2, SDF-1/CXCR4 and RANTES/CCR5, as well as many other cytokines all have little known about which specific cells they attract to HTS through chemotaxis by the immune reaction after burn injury. The hypercellular response at the site of epithelial injury mobilizes many cells into the inflamed tissue, which produces the excessive deposition of the ECM, growth factors, chemokines, antigens, angiogenesis, and all the other responses of the wound healing process. In these chemokines, the recruited blood-borne cells assemblages particular to each pathway responding to varying injury severities have yet to be discovered. Further knowledge of their functional relationship may enable the manipulation of cell pathways in future therapeutic treatments for burn injury, allowing for a normal scar healing

process. It is the aim of this study to find the cells both upregulated and downregulated by cytokines and monocyte subpopulations during various time points and severity of burn injury. The main chemokine pathways of interest to be studied in this research are MCP-1/CCR2, RANTES/CCR5, and SDF-1/CXCR4 at various time points after burn injury.

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Chapter 2:

The dynamic changes of monocytes and chemokine pathway signaling during wound healing post burn injury

2.1 Background

Burns are among the most devastating injuries, representing a major global public health crisis (198-200), especially in middle- and low-income countries (201). There are at least 40,000 cases of hospitalization related to burn injuries in the U.S. each year (200). Hypertrophic scars (HTS) are a fibroproliferative response, occurring in up to 70% of patients after burn injury (202). HTS are characterized by redness in colour, raised morphology, contracted, and within the wound boundary, which causes patients significant physical and emotional discomfort (198, 203). There is much support in research to utilize molecular evidence for cytokine therapies to manage wound healing (204, 205), however there remains considerable unresolved understanding of the pathophysiology of the cellular factors and cytokine signaling in HTS, and as a result, effective treatment of HTS is suboptimal.

Cytokines are known to have a significant role in wound healing at all stages, but most notably during inflammation (206). Cytokine and chemokines are responsible for pushing the wound healing process into the next stage, involved in regulation of normal healing and the dysregulation seen in HTS (158). BCA-1, or CXCL13, has not been researched in burns at varying levels, however has been shown to upregulated collagen type I mRNA expression in osteoarthritic patients (207). BCA-1 also attracts B-cells and pro-inflammatory cytokines (208), and has been found in the blood monocytes of severely burned patients (209), thus we hypothesized that it would also have a role in burn patients during wound healing. In our previous study, we described the role of SDF-1/CXCR4 signaling in the formation of HTS following burn injury. This cytokine pathway appears to be upregulated in the serum and CD14+CXCR4+ cells in peripheral blood mononuclear cells (PBMCs) (210); however, this study was conducted during the remodeling phase of wound healing when patients were

rehabilitating from burn injury and the number of patients studied did not allow correlations to be made for the severity of burn injury. Thus, this study investigated a larger number of burn patients at earlier time points post-injury to further understand the role and correlation of monocyte populations and the cytokines they produce in development of HTS.

Cytokines act as chemoattractants, recruiting blood-borne cells such as monocytes, which may function as pro- or anti-inflammatory cells to the site of injury (211, 212). Circulating monocytes are recruited by chemokines into wound sites, differentiate into various subtypes of macrophages in the tissue, and play an indispensable role in wound healing (186, 213). Monocytes express chemokine receptors, which bind their ligands to carry out specific immune responses, playing roles in both normal healing and fibrotic scarring. Although chemokines are involved during all stages of wound healing (154), little research has been done in the burn population. It is our hypothesis that after burn injury, activated monocytes play a role in abnormal wound healing and scar formation via cytokines. We investigated changes in circulating monocytes and their specific subpopulations, as well as chemokine receptor expression and chemokine and cytokine levels in the blood of burn patients with varying severity of injury at multiple time points post-burn injury, to provide insight into potential therapeutic opportunities.

2.2 Methods

2.2.1 Burn Patients and Control Participants

Twenty seven patients with 3-70% total body burn surface area (TBSA) burns treated in the Firefighter's Burn Treatment Unit at the University of Alberta Hospital were enrolled in this study (Table 1). The burn severity was categorized in three groups, minor with TBSA ≤20% (n=18), moderate with TBSA 21-50% (n=6), and severe with TBSA >50% (n=3). The research

and ethics protocol was approved by Human Research Ethics by Alberta Research Information Services (ARISE) at the University of Alberta. Informed consent was obtained from the burn patients and healthy volunteers before they participated in the study.

Blood samples were collected from the burn patients for the isolation of peripheral blood mononuclear cells (PBMC) at 2, 7, 30 days, and longer post injury. A brief study design is included in Figure 1. Blood samples from 13 healthy individuals were collected once as controls.

2.2.2 Isolation of PBMCs and Serum

PBMCs were isolated from heparinized blood by a density gradient using lymphocyte separation medium (LSM), following standard protocol (214) (Mediatech, Inc, Manassas, VA). Blood samples were diluted with Phosphate-buffered saline (PBS) at a 1:1 ratio, before LSM was added at the bottom of 50ml-centrifuge tubes and diluted whole blood was carefully loaded on top at a ratio of 1:2 (215). The PBMCs were isolated by centrifugation without brake at 2,000 rpm for 20 minutes. The PBMC layer was collected and washed with PBS and spun at 1,000 rpm and isolated again for the PBMC pellet. The cell pellet was counted and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 1% antibiotic-antimycotic (Gibco, Life Technologies, Grand Island, NY) (80% of freezing medium), 10% Dimethyl sulfoxide (DMSO) and 10% fetal bovine serum (FBS). The PBMC were and stored in liquid nitrogen until further analysis. All PBMC's were counted and labeled before storage and also after thawing to ensure viability and same cell count in all tubes.

Serum was isolated from non-heparinized blood by allowing the blood to clot for 30 minutes at room temperature, followed by centrifugation for 10 minutes at 2,000x g. The supernatant was collected and stored at -80℃ for chemokine and cytokine analysis.

2.2.3 Flow Cytometry

Cryopreserved PBMCs were thawed following standard protocol, by placing briefly in a water bath at 37℃ and spinning in a centrifuge at 1200 rpm for 7 minutes followed by two washes with PBS to extract the pure PBMCs, followed by counting the live cells to ensure viability. The PBMCs were put in tubes of 1 x 10^6 live cells each and stained for monocyte markers CD14 and CD16, and chemokine receptors CCR2, CXCR4 and CCR5. The antibodies and the volume per test used include 20 µl fluorescein isothiocyanate [FITC] conjugated mouse anti-human CD14, 5 µl allophycocyanin [APC]-Cy^7 conjugated mouse anti-human CD16, 5 µl brilliant violet [BV]-421 conjugated mouse anti-human CD192/CCR2, 20 µl APC conjugated mouse anti-human C184/CXCR4, and 20 µl phycoerythrin [PE] conjugated mouse anti-human CD195/CCR5. To correct for non-specific binding, the correct Ig subclass and fluorophore were used as isotype controls for each antibody, which include 20 µl FITC mouse IgG2a, 5 µl APC mouse IgG2 aκ, 20 µl PE mouse IgG2aκ, 20 µl APC-Cy^7 mouse IgG2aκ, and 5 µl BV421 mouse IgG2bκ. Because our main populations being studied used CD14 and CD16 to determine monocyte subpopulation, and CD16 contains an Fc receptor, an Fc receptor block was unable to be used. Each condition comprised 1 x 10^6 cells each, and set up as unstained, isotype, compensation, and burn samples were included. The PBMC were incubated with antibodies for 30 minutes at room temperature. Room temperature was chosen over refrigeration or on ice as there is studies showing higher subset PBMC yield and distribution, however there is further work to determine which method is officially a more viable protocol (216). After incubation, the cells were washed with 3 mL of PBS twice, the cells were fixed with 200 µl of 1% paraformaldehyde in PBS. Flow cytometry counting was limited to 10,000 events using the same gating on all imaging using Attune Nxt (ThermoFisher Scientific, Waltham, MA), and done in batches to ensure all time points per patient were done at the same time (190). An example of the

gating used and results for unstained, isotype control, and antibody populations are included using a 20% TBSA burn patient in Figure 2. Gating was done to collect white blood cells including monocytes and further specify to correct for doublets and background staining, with some novel alternatives for gating as recommended in newer research (217). Each sample was counted for chemokine receptor expression and displayed as a percentage of each monocyte population (CD14+CD16+ or CD14+CD16-). All antibodies were purchased from BD Bioscience, San Jose, CA.

2.2.4 Multiplex Fluorescent Bead Assay

Multiplex fluorescent bead assay services were provided by Eve Technologies, Calgary, AB. Fluorescence intensity reflects the amount of protein in the serum samples, by utilizing colour-coded polystyrene or superparamagnetic microparticles coated with antibodies that recognize the specific target analytes. The assay for human cytokines utilizes 150 µl of undiluted serum per analysis. The assays are controlled by running a control sample with every assay run to ensure assay signals fall within a small window of variability, as described previously (218-220).

2.2.5 Statistical Analysis

Samples were groups by severity of burn injury, including minor ≤20% TBSA, moderate 21-50% TBSA, and severe >50% TBSA. Statistical analysis was performed using Graphpad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA). Data was analyzed by one-way ANOVA within groups of similar severity of injury (TBSA) using Tukey to correct for multiple comparisons, and by a Greenhouse-Geisser correction for within subject differences. Data is expressed as the means ± standard error (SE) of the means, where $p \leqslant 0.05$ was considered

statistically significant. Linear regression analysis was performed using STATA 13.0 software, (StataCorp, College Station, TX), and reported with the adjusted R-squared value.

2.3 Results

2.3.1 Monocyte Subpopulations after Burn Injury

0-3 days post-burn, we observed an increase in the circulating monocytes expressing CD14+ CD16+ in the patients compared to controls. By 30-60 days post-burn injury, minor burns (⩽20% TBSA) showed both populations of monocytes (CD14+CD16+ and CD14+CD16-) reduced over time during wound healing, approaching the level in controls. In moderate burns (21-50%), these CD14+ CD16+ populations were reduced from the highest level at 0-3 days to the lowest level at 4-7 days. Thereafter, they gradually recovered to the level of healthy normal volunteer controls at 30 days. In the patients with severe burns, monocyte levels were high in the first three days, lowest at 4-7 days, higher at 8-14 days, and low again at 15-30 days after burn injury. In minor burn injury, the monocytes expressing CD14+CD16- was significantly higher than the control at 4-7 days ($p<0.05$) (Figure 3).

2.3.2 Chemokine Receptor Expression on Monocyte Subpopulations

Burn injury shows a higher CXCR4 expression on CD14+CD16+ monocytes than CD14+CD16-, similar to controls, which were sustained throughout the study as seen in Figure 2A. Expression of CXCR4 in the CD14+CD16- monocytes was lower in comparison, which was more dynamic with varying severity of burn injury. In all burn patients, both populations remained at the same level until 60 days. In minor burn patients, there was a drop in the CD14+CD16+ monocytes that returned to the previous levels by 120 days after injury. In moderate and severe burns, the CD14+CD16+ population remained as seen in minor burns at the

same time points; however, in moderate burns, the CD14+CD16- population dropped slightly at 8-14 days and increased at 8-30 days, while severe burns dropped slightly at 4-7 days and peak at 8-14 days, with a delayed drop at 15-30 days.

After burn injury, patient's expression of CCR2 increased more on the CD14+CD16+ monocytes than the CD14+CD16- monocytes. In minor burns in both of these subpopulations, the CCR2 expression dropped at 4-7 days, followed by an increase at 30 days, followed by another decline. In moderate burns, on the CD14+CD16- monocytes, CCR2 expression was the highest during the first three days after burn injury followed by a decrease. In CD14+CD16+, CCR2 was high at the initial 0-3 days, with a drop at 4-7 days, followed by an increase until after one month. In severe burns, the CCR2 expression of CD14+CD16+ monocytes is lower than the controls except at 8-14 days, which rose in a similar fashion in minor and moderate burn patients of different TBSA. On the CD14+CD16- monocytes, CCR2 expression had much more exaggerated rise and drop, with an increase at 8-14 days and a drop at 15-30 days follow up in severe burn patients (Figure 4B). Overall, CCR2 seemed to show the same pattern between the two subpopulations of monocytes within the same burn severity.

In minor burn patients, the CCR5 expression on both monocyte subpopulations showed an increase late at 31-60 days after burn injury. In moderate burns, CCR5 expression had higher levels on the CD14+CD16+ monocytes, peaking at 4-7 days, with a drop at 15-30 days, whereas on the CD14+CD16- monocytes, CCR5 expression dropped at 8-14 days, and increased past the CD14+CD16+ monocytes at 15-30 days. In severe burn patients, CCR5 expression rose at 4-7 days on the CD14+CD16+ monocytes, with a decrease at later time points. The CD14+CD16- monocytes had lower CCR5 expression in patients with higher TBSA after the first week, and overall much lower than the CD14+CD16+ monocytes (Figure 4C).

2.3.3 Serum Chemokines Correlate with Burn Size

Monocyte chemoattractant protein-1 (MCP-1), or CCL2 levels in the serum of minor burn patients was very constant over time; however in the most severe burn patients, MCP-1 significantly increased as compared to minor burns at 0-3 days post injury ($p<0.0001$). This increase in the severe burns also remained significantly different at 4-7 days, as compared to the minor and moderate burns ($p<0.001$). MCP-1 level in patients with severe burns was significantly higher than controls at 0-3 days ($p<0.0001$) and 4-7 days ($p<0.001$) (Figure 5A). MCP-1 early after burn injury was positively correlated with increasing TBSA ($r^2=0.7388$, Figure 5B). Serum levels of MCP-1 is significantly higher in patients with severe burns compared to patients of minor and moderate burns, and controls.

Serum RANTES, or CCL5, significantly increased during the first three days ($p<0.01$) in severe burn patients compared to minor burns (Figure 6). Moderate burn patients increased serum RANTES at 4-7 days, and decreased at 8-14 days. Severe burn patients showed a significant decrease of serum RANTES from the initial 0-3 days to 4-7 days ($p<0.01$) (Figure 6A). There is a positive correlation at 0-3 days between RANTES and burn severity ($r^2=0.4326$, Figure 6B).

Early after burn injury, serum interleukin (IL)-6 increased with the severity of burn injury in the patients with moderate and severe burns which was very significant compared to minor burns or normal controls (Figure 7A). In patients with minor burn injuries, the levels of serum IL-6 was low throughout and comparable to the normal healthy controls. In the moderate burns, the increase of serum IL-6 was not sustained over time; however, severe burn injury had significant sustained increases in their serum IL-6 at each time point throughout the study ($p<0.0001$) (Figure 7A). There was a positive correlation between serum IL-6 and severity of

burn injury at all time points after injury, 0-3 days (r^2=0.4818, Figure 7B) and 4-7 days (r^2=0.6272, Figure 7C), and 8-30 days (r^2=0.6786, Figure 7D).

In patients with minor burns, the levels of serum IL-8 was increased in all burn patients after injury, 4-7 days, and remained elevated at 4-7 days in moderate and severe burns as compared to minor burns ($p<0.0001$, Figure 8A). The increase in the level of serum IL-8 was reflected by the correlation of its serum levels with the severity of burn injury (r^2=0.3478, Figure 8B). Severe burns at 4-7 days showed a very significant increase of IL-8 compared with controls ($p<0.0001$), whereas minor burn patients showed relatively sustained levels throughout. In patients with moderate and severe burns, IL-8 level peaked significantly at 4-7 days, before declining thereafter; however, still above the level in minor burns or controls. Patients with severe burn injury had significant increases at each time point, with a peak of IL-8 at 4-7 days, which declined at 8-30 days, and remained higher than minor and moderate burns and controls (Figure 8A).

The anti-inflammatory cytokine IL-10 was significantly increased in moderate and severe burns at early time points but not in patients with minor burn injuries as compared to controls. In both moderate and severe burns, the high levels of IL-10 in serum samples was not sustained beyond three days after injury. At 0-3 days and 4-7 days in moderate, and 0-3 days severe burns, the IL-10 was significantly higher compared to controls ($p<0.0001$) (Figure 9A). There was a positive correlation between IL-10 and burn severity at 0-3 days (r^2=0.4711, Figure 9B).

In patients with minor burns, serum IL-1RA levels were comparable to controls at all time points. As burn severity increased, the IL-1RA increased significantly in moderate and severe burns as compared to the minor burns and controls ($p<0.001$). Moderate and severe burns

both showed a sudden drop in serum IL-1RA levels after 0-3 days ($p<0.05$), as seen in Figure 10A. IL-1RA shows a positive correlation with burn severity at 0-3 days, with an $r^2=0.4038$ (Figure 10B).

Serum B cell-attracting chemokine 1 (BCA-1), or CXCL13, in patients with minor burns remained low throughout and similar to controls. A significant increase in the serum levels of BCA-1 was found from 4-7 and 8-30 days in severe burns compared to controls ($p<0.0001$), while minor and moderate burns remained lower. There was also a significant increase from the first three days to 4-7 and 8-30 days in the severe burns ($p<0.01$). In severe burns at 4-30 days BCA-1 was significantly higher than controls ($p<0.001$) and moderate burns at 4-7 and 8-30 days ($p<0.01$) (Figure 11).

2.4 Discussion

It has been suggested that there is a novel subpopulation of PBMCs, which modulates the functions of cytokines and pro-inflammatory factors and contributes to HTS development (118). In this study, two subpopulations of monocytes expressing chemokine receptors CCR2, CXCR4, and CCR5, and cytokines in the serum were investigated in burn patients with varying TBSA. Higher levels of CD14+CD16+ monocytes are suggested to be associated with a more pro-inflammatory subtype of monocytes, by expressing less IL-10 (221). This supports our results showing an increase of this population initially after burn injury on the first three days, as well as the much more pronounced fluctuation in severe burns with TBSA >50% and showing another large spike at 14 days. The findings suggest that the CD14+CD16+ monocytes may be up-regulated with greater severity of burn injury. High numbers of CD14+CD16+ monocytes

correspond with high levels of IL-6 in patients with rheumatoid arthritis (222), however our results show no similar correlation between IL-6 and CD14+CD16+ levels in burn patients.

CD14+CD16+ monocytes express CCR2, CCR5, and CXCR4 (223). Stromal-cell derived factor-1 (SDF-1) which is responsible for homing mesenchymal and multipotent stem cells to wound sites, has been found to be up-regulated by our group previously; however, this increase in SDF-1 was correlated with the severity of TBSA but examined dermal fibroblasts and PBMCs, at much later time points than in this study (210). Our results in this study showed no significant differences between CXCR4 compared with controls at any of the earlier time points up to 30 days post injury. This may be due to differences in the severity of burns but also more importantly the period of time after injury, where SDF-1 may peak at much later time after acute burn injury, rather than in the acute early period (<30 days). This high expression of CXCR4 at a later time point after burn injury suggests that down regulation by an antagonist for the receptor could possibly reduce the recruitment of CXCR4+ monocytes into the wound limiting their profibrotic effects during a critical period in burn patients, which appears after wound closure but before formation of HTS (158, 224). In a nude mouse model of skin grafting and HTS healing, a CXCR4 antagonist injections daily for the first 2 weeks and weekly thereafter reduced scar thickness, cellularity, and vascularity (224). This therapeutic value of long-term SDF-1/CXCR4 chemokine pathway antagonism to control scarring would require human investigation at carefully selected time points after burn injury.

CCR5 is reported as a human immunodeficiency virus (HIV) coreceptor (225). While there are many studies pertaining to the RANTES/CCR5 chemokine pathway in this population, studies in the burn population are lacking. In pediatric burn patients greater than 3 years post-injury with mild severity of injury (<20% TBSA), no significant differences were found in CCR5

(226). In our study, CCR5 expression did show a large increase at 7 days after injury in severe burns, but was not found to be statistically significant due to our small sample size in the severe burn patients (n=3). CCR5 is reported to be detected nearly exclusively in macrophages and endothelial cells in the wound tissue where it may be more beneficial to study this chemokine in healing burns (227). CCR5 has been suggested to be important for recruitment of endothelial progenitor cells in healing wounds in mice (228); however, further studies on burn wound healing in humans would be required to understand its role.

Previously, our group has found a significantly increased novel population of PBMCs in patients with severe burn injury (about 5 days post injury), who later developed severe HTS (210). Classical monocytes make up the CD14+CD16- population, and have an unknown role in wound healing after burn injury. However, a recent study has suggested that CD14+CD16- monocytes produce higher levels of anti-inflammatory cytokines (229). Minor burn patients have significantly higher levels of CD14+CD16- monocytes than controls, which suggests that more severe burns may benefit from a higher level of this subpopulation to promote a higher anti-inflammatory and normal wound healing response.

MCP-1, or CCL2 binds to its receptor, CCR2, and is a monocyte chemoattractant which comes from inflamed tissue (230), recruiting monocyte populations during the wound healing response after injury and induces angiogenesis (231). Our research group has studied a CCR2 antagonist in a mouse model of human HTS, and found significant reductions in scar thickness, vascularity, macrophage recruitment and myofibroblast differentiation (224). In burn patients, CCR2 shows less expression in monocytes at more severe injury compared with its ligand, MCP-1 which increases with burn severity. Together, these findings suggest that there may be a dysregulation in the ligand-binding mechanism, or perhaps another receptor competing for

binding at greater severity of burn injury. MCP-1 is shown to promote a profibrotic response while degrading collagen in vitro, by increasing expression of tissue inhibitor of metalloproteinase 1 in human dermal fibroblasts (232). MCP-1 has been studied after burn injury in mice (224, 232), and an older mRNA study (233). A previous study has also found that MCP-1 was significantly increased in the blister fluid after burn wounds at 16 hours post-injury (234). This resembles our results from moderate and severe burns where significant positive correlation exists between MCP-1 levels and burn severity.

RANTES, or CCL5, is involved in a range of inflammatory disorders, acting by up-regulating leukocytes to sites of inflammation (235). CCL5/RANTES stimulates the migration of adipose stem cells and dermal stromal cells, and may be a possible contributor to inflammatory diseases (236, 237). Higher levels of CCL5 are associated with normal wound repair (31). MCP-1 and CCL5 recruit macrophages to the site of injury (173). RANTES has been shown to be significantly elevated in younger burn patients wounds compared to the elderly (238); however our study found no strong positive correlation with age of the patient. This may be due to a difference in time points between studies. There was a significant positive correlation between TBSA and RANTES, suggesting that in part, RANTES level correlates to burn severity. Blocking the CCL5/CCR5 pathway in a mouse model of HTS using a CCR5 antagonist, Maraviroc, demonstrated down-regulated fibrotic gene expressions, reduced macrophage and myofibroblasts recruitment, scar thickness, and reduced collagen deposition in scars (224).

Other investigators have found that serum IL-6 levels positively correlates with severity of injury (TBSA), and are associated with a higher risk of sepsis in burn patients (239) as well as the hyper-inflammatory state leading to HTS. Our results support previous studies (240), with a significant positive correlation between IL-6 and burn severity. Thus, IL-6 may account for the

prolonged inflammatory process leading to HTS and abnormal wound healing in fibrotic scarring. This increase appears to extend throughout the acute phase of burn injury, where there is a stronger correlation between TBSA and IL-6 levels as time progresses during the early post burn period (<30 days, by Pearson's correlation, data not shown). The higher levels of IL-6 in severe burn patients compared to minor burn patients, is associated with the elevated inflammatory response of IL-6 with prolonged periods of time post burn, identifying it as potential candidate for antagonists to reduce scarring and inflammation after burn injury (241).

IL-8 is markedly increased in burn patients and septic patients (242, 243). IL-8 has also been used as a biomarker for sepsis and infection in burn patients, as an indication of the heightened immune response (243), as well as having significantly higher levels in adults than in children (244). Our results show a significant positive correlation between IL-8 and burn severity, suggesting IL-8 may be another cytokine to contribute to the hyper-inflammatory response seen in severe burn injuries. Previous studies identified IL-8 as a marker for increased inflammatory response, because of more pronounced increases in more severe burn injuries. In an animal study, IL-8 was used as a topical treatment for wound healing, increasing rates of re-epithelization and contraction (245); however, no human studies have been conducted after burn injury in humans. Thus, future studies to explore the role of IL-8 as a topical treatment for burns of varying severity after injury is required. However, in our study the significant increase in moderate and severe burns suggests antagonizing IL-8 after severe injury at specific times may be beneficial to curb excessive inflammation and scarring.

Increased IL-6 and IL-10 levels in burn patients have been associated with an increase in acute respiratory distress syndrome and a greater risk for mortality (246). However, long-term studies at different times after injury and varying severity of injury are rare. The significant

continuous increase in IL-6 in severe burns group coupled with the reductions in IL-10 shortly after the initial peak suggests that in severe burns these cytokine levels are regulated differently from patients with smaller wounds. High IL-6 and IL-10 levels have been found to be correlated with septic burn patients, and low IL-10 levels were reported in survivors (247). Our research found a very significant increase at 0-3 days in moderate and severe burns compared with minor burn and control patients, which likely contributes to the development of sepsis and excessive inflammation in the wound healing response. Increases in IL-10 in patients with sepsis has been suggested as an immune response to counteract the initial inflammatory response to infection (248). Mice treated with anti-IL-10 antibody at day 1 showed a significant improvement in recovery from sepsis, possibly due to restoration of T-cell and cytokine function (249). The dosage, therapeutic value and side effects, as well as administration of IL-10 as a specific target treatment for wound healing post-burns is being heavily researched a potential in clinical practice (250). Based on our observations of a significant increase initially in IL-10 after burn injury, current clinical trials with IL-10 in scarring may prove a valuable therapeutic opportunity.

IL-1 receptor antagonist (IL-1RA) inhibits the pro-inflammatory responses induced by IL-1, and may play a role in serious infections and inflammatory conditions (251). In a comparative study of cytokine profiles in burns >20% TBSA, in adults and children, IL-1β and IL-1α showed significantly higher levels in pediatric patients than adult burn patients (244). Another study with >20% TBSA with burn patients found that plasma levels of IL-1RA and TBSA had a significant correlation after burn injury, and rose when infection or sepsis occurred (252). This is consistent with our results showing higher serum levels of IL-1RA with increasing severity of burn injuries. A study with 80 patients admitted to the burn intensive care unit who were evaluated on a smoke inhalation and pulmonary inflammatory response scale, found that

higher plasma IL-1RA remained significantly associated with mortality and increased with greater severity of inhalation injury (253). The strong positive correlation between IL-1RA and burn severity support the role of IL-1RA in activation by pro-inflammatory mediators to combat the severe inflammatory response seen in severe burns. To date, there have been no human studies in burn patients to establish an appropriate dose in severe burns, which levels of overstimulation becomes detrimental or may prove therapeutic. More cytokine regulation in relation to HTS development and burn injury outcomes are necessary to discover if a potential treatment lies in regulating such cytokines, such as IL-1RA.

BCA-1, or CXCL13 and its receptor CXCR5 have been found to attenuate chronic immune responses (254). BCA-1 attracts B-lymphocytes, and is believed to support chemotaxis of T-cells (255). BCA-1 is angiostatic (256), which may suggest a role in abnormal wound healing seen in the patients with higher TBSA after wound closure. However our results appear to be novel as no study of the role of BCA-1 during burn injury appears to exist to date. BCA-1 increasing in greater burn injury at later time points may account for some of the dysregulation in wound healing leading to HTS, as well as inflammation as suggested by other studies in non-burn patients (257, 258). Further research on the possible association of BCA-1 and burn injury is necessary to have a greater understanding of BCA-1/CXCR5 signaling axis in burn patients.

2.5 Conclusions

In this study, an increased population of CD14+CD16+ monocytes was found in the first three days after burn injury, which shifted to a more anti-inflammatory CD14+CD16- monocytes after the first week, except in severe burn injury. Chemokine pathways appear to be deregulated after burn injury, where MCP-1 increases significantly with greater severity of burn injury with less expression of CCR2. RANTES, IL-6, IL-8, and BCA-1 are significantly higher in the

patients with severe burn injury, suggesting they contribute to the inflammatory response seen in HTS. IL-6 has the strongest correlation to burn severity, which supports previous research suggesting that antagonism of IL-6 may have therapeutic effect. Chemokines MCP-1 and RANTES increase in greater burn severity, where previous studies with receptor antagonists support their importance in response to burn injury and HTS development. Similar to previously reported findings, significant increases in IL-10 and IL-1RA early after burn injury in the patients with more severe burn injuries, suggests that a higher need for control of inflammatory response during this period. Further studies are needed to determine which cytokine dosage is beneficial or detrimental in the wound healing response to burn injury.

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2.7 Figures

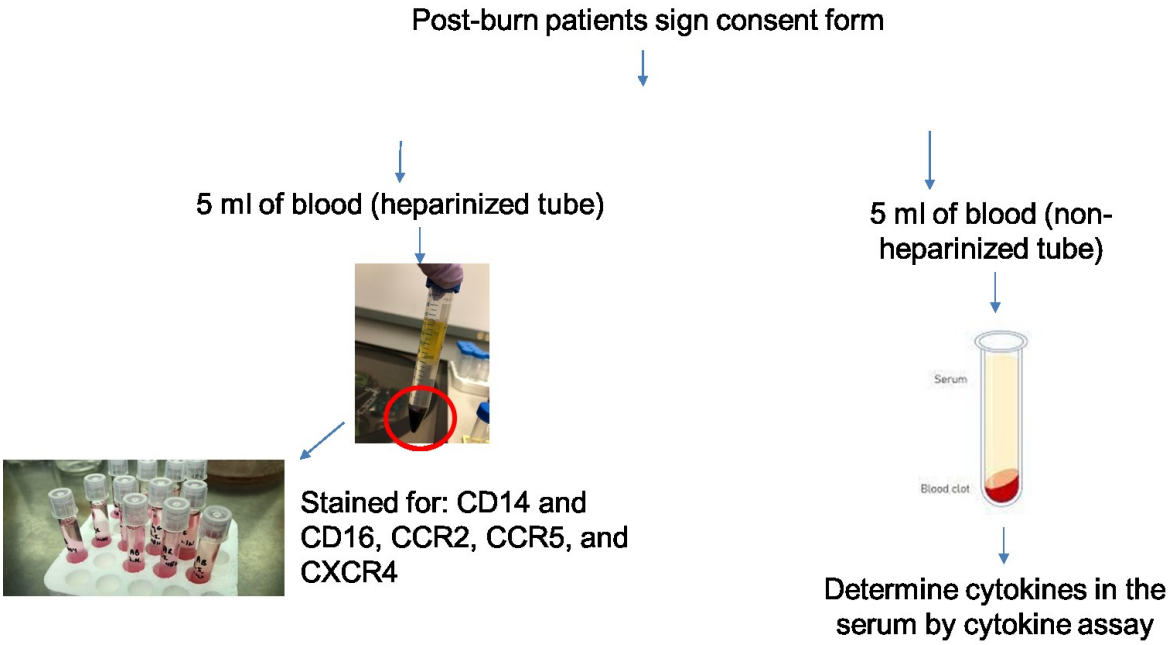
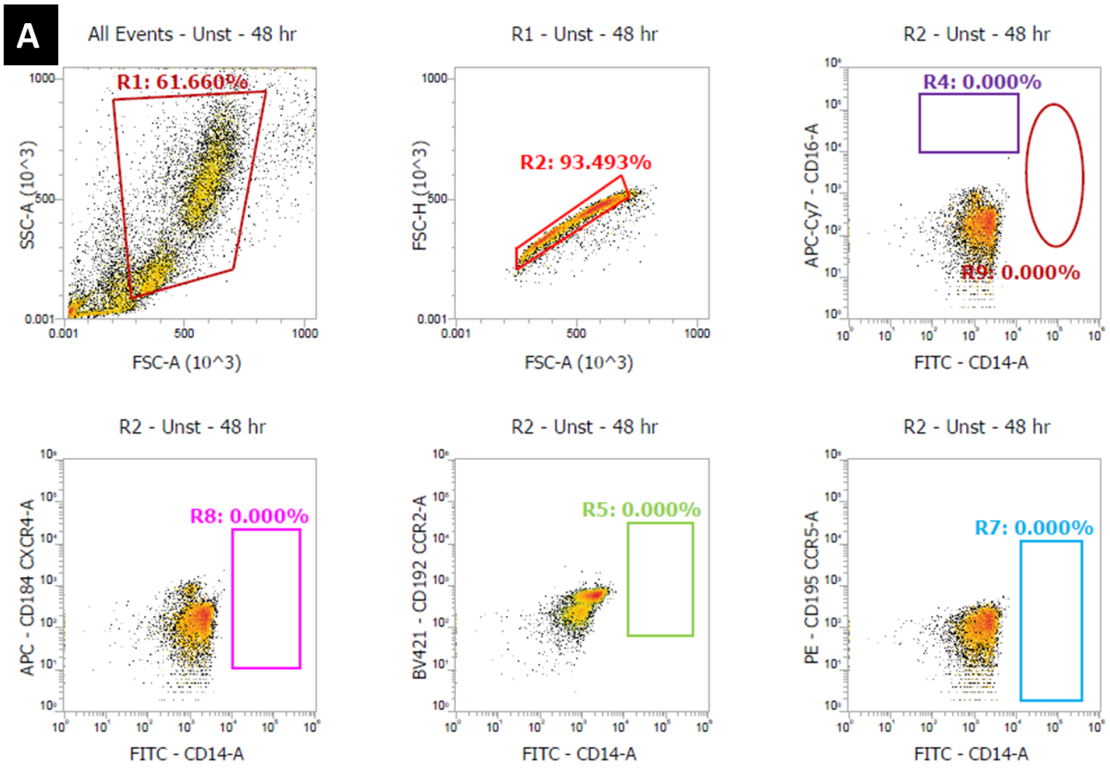


Figure 1. Study Design.



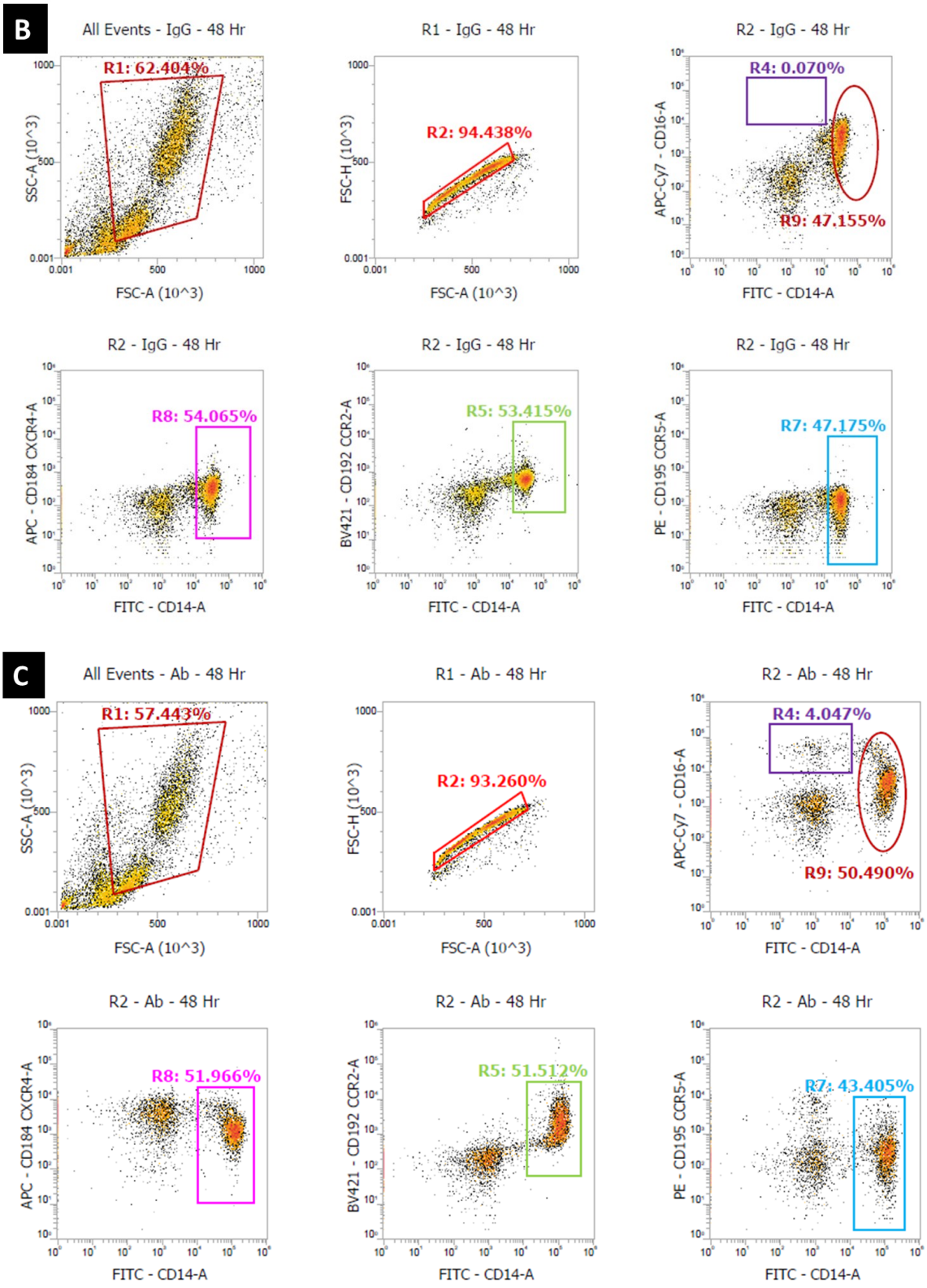

B
All Events - IgG - 48 Hr
R1: 62.404%
SSC-A (10^3)
FSC-A (10^3)
R1 - IgG - 48 Hr
R2: 94.438%
FSC-H (10^3)
FSC-A (10^3)
R2 - IgG - 48 Hr
R4: 0.070%
R9: 47.155%
APC-Cy7 - CD16-A
FITC - CD14-A
R2 - IgG - 48 Hr
R8: 54.065%
APC - CD184 CXCR4-A
FITC - CD14-A
R2 - IgG - 48 Hr
R5: 53.415%
BV421 - CD192 CCR2-A
FITC - CD14-A
R2 - IgG - 48 Hr
R7: 47.175%
PE - CD195 CCR5-A
FITC - CD14-A
C
All Events - Ab - 48 Hr
R1: 57.443%
SSC-A (10^3)
FSC-A (10^3)
R1 - Ab - 48 Hr
R2: 93.260%
FSC-H (10^3)
FSC-A (10^3)
R2 - Ab - 48 Hr
R4: 4.047%
R9: 50.490%
APC-Cy7 - CD16-A
FITC - CD14-A
R2 - Ab - 48 Hr
R8: 51.966%
APC - CD184 CXCR4-A
FITC - CD14-A
R2 - Ab - 48 Hr
R5: 51.512%
BV421 - CD192 CCR2-A
FITC - CD14-A
R2 - Ab - 48 Hr
R7: 43.405%
PE - CD195 CCR5-A
FITC - CD14-A

Figure 2. Flow cytometry gating at 48 hours in a 20% TBSA burn patient. A) Unstained PBMC results of CD14, CD16, CXCR4, CCR2, and CCR5, B) Isotype control IgG results of CD14, CD16, CXCR4, CCR2, and CCR5, and C) Antibody results of CD14, CD16, CXCR4, CCR2, and CCR5.

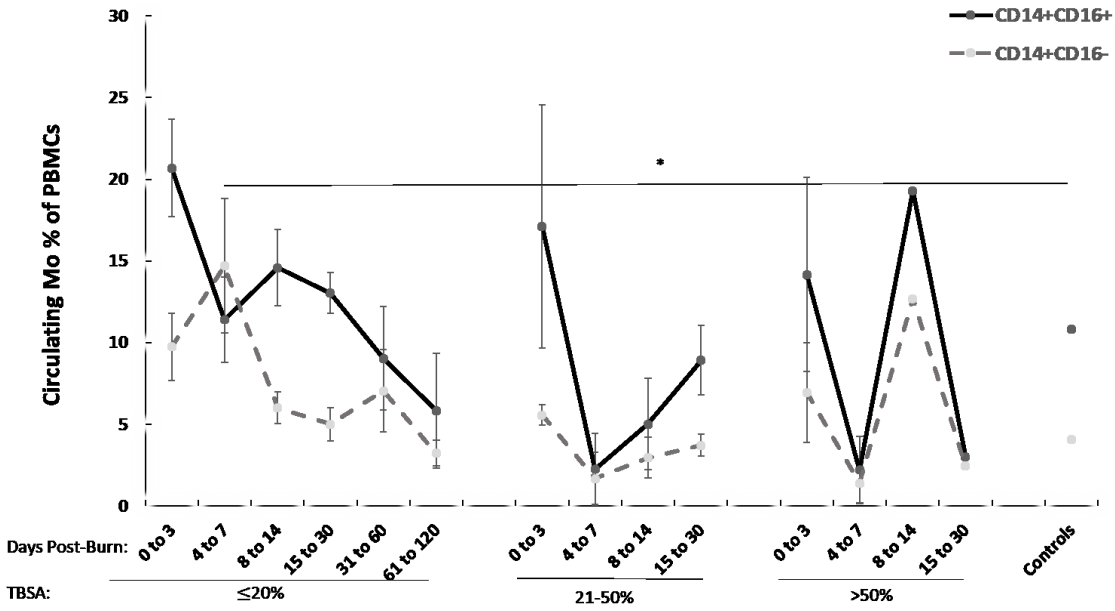
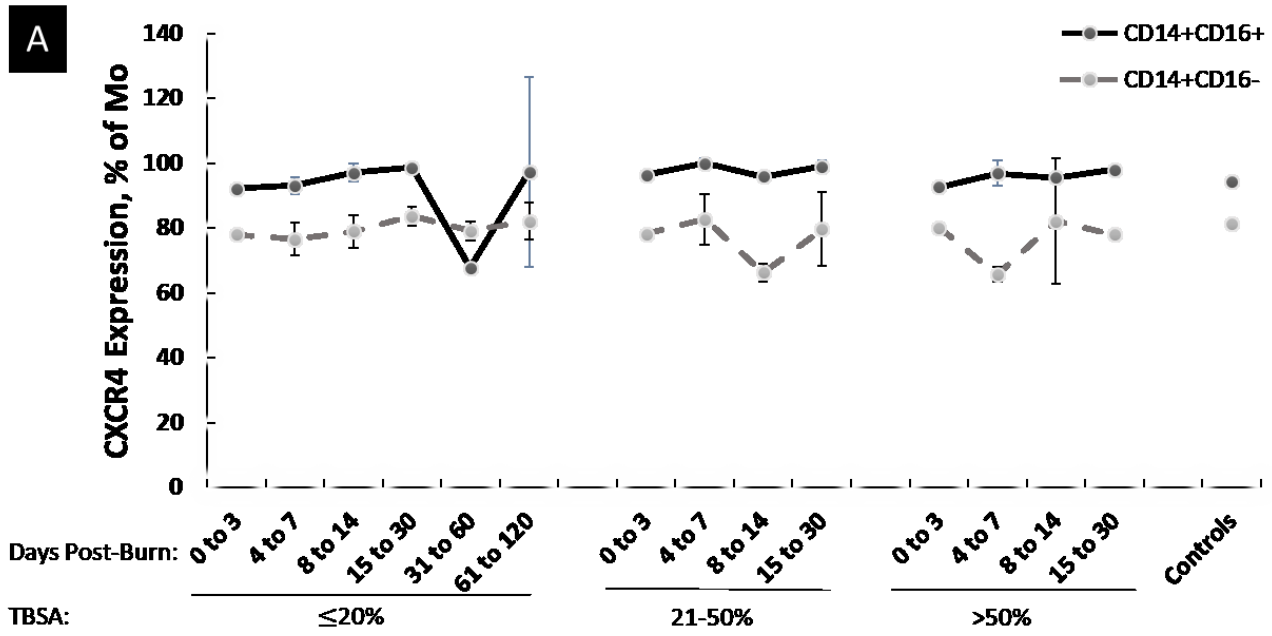
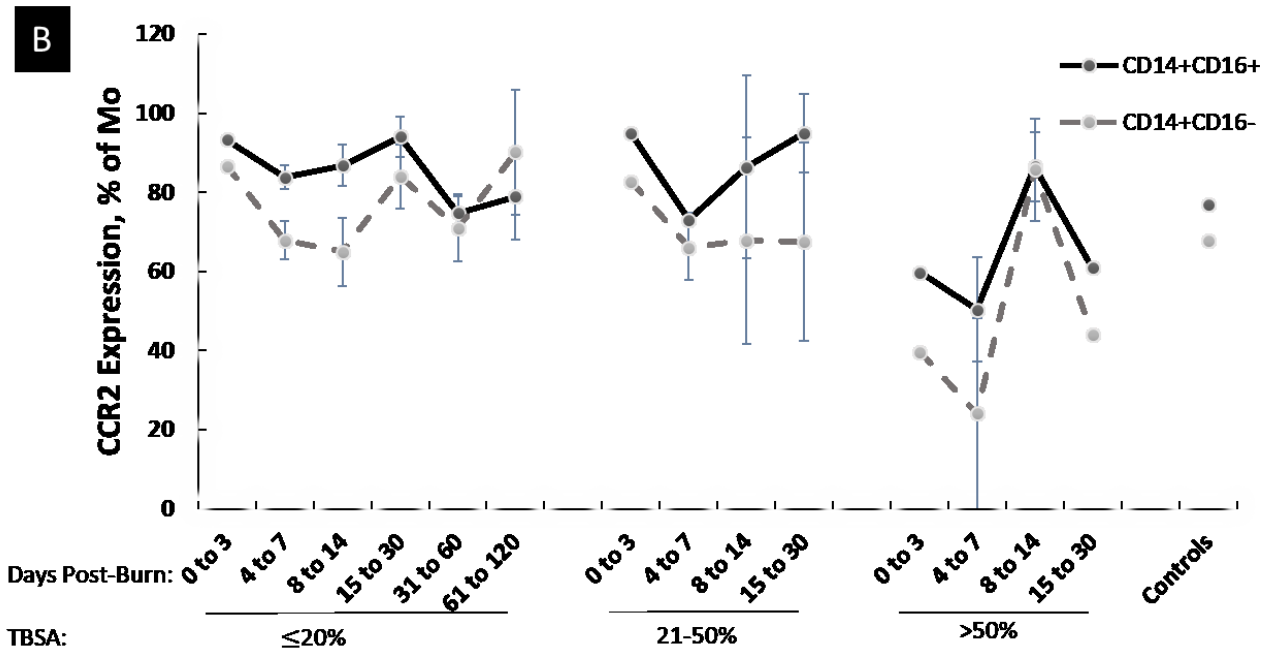


Figure 3. Percentage of total Monocytes and monocyte subpopulations in burn patients. PBMCs were isolated from the blood following a standard protocol and stained for CD14 and CD16. Two subpopulations of circulating monocytes expressing CD14+CD16+ and CD14+CD16- were identified at various time points in patients of various degrees of burn. Burn patient sample size at each time point included minor burns with an n of 12, moderate burns with an n of 3, and severe burns with an n of 3. Significance is denoted by a p value of <0.05 = *, <0.01 = **, <0.001 = ***, and <0.0001 = ****.


A
CXCR4 Expression, % of Mo
140
120
100
80
60
40
20
0
CD14+CD16+
CD14+CD16-
Days Post-Burn:
0 to 3
4 to 7
8 to 14
15 to 30
31 to 60
61 to 120
0 to 3
4 to 7
8 to 14
15 to 30
0 to 3
4 to 7
8 to 14
15 to 30
Controls
TBSA:
≤20%
21-50%
>50%


B
CCR2 Expression, % of Mo
120
100
80
60
40
20
0
CD14+CD16+
CD14+CD16-
Days Post-Burn:
0 to 3
4 to 7
8 to 14
15 to 30
31 to 60
61 to 120
0 to 3
4 to 7
8 to 14
15 to 30
0 to 3
4 to 7
8 to 14
15 to 30
Controls
TBSA:
≤20%
21-50%
>50%

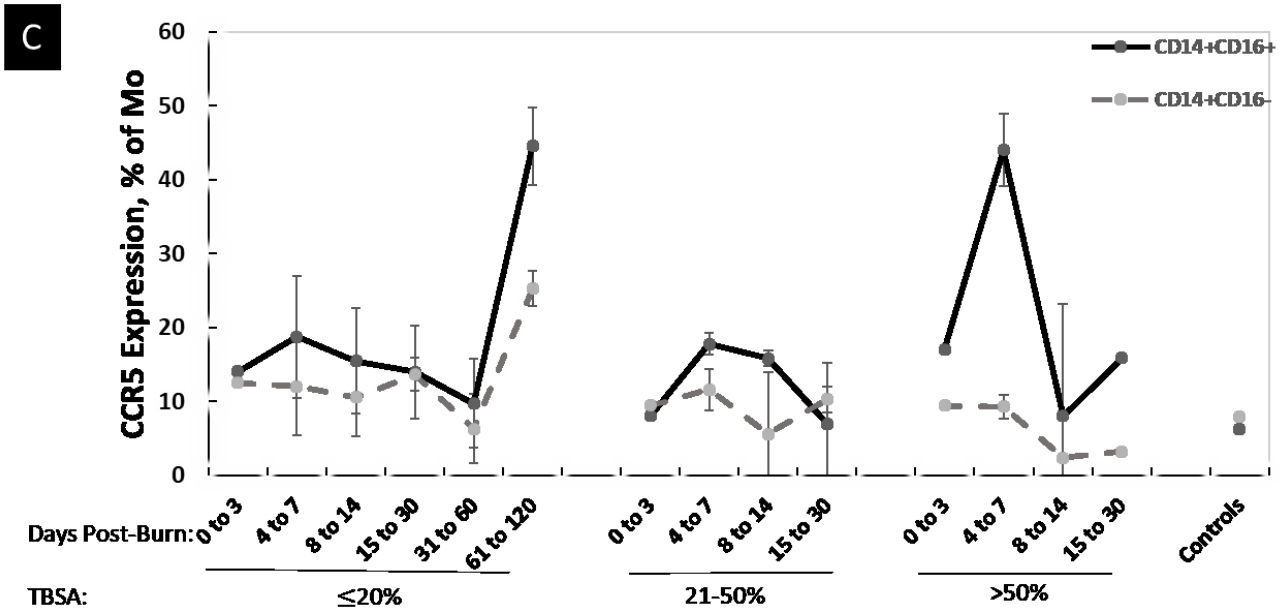
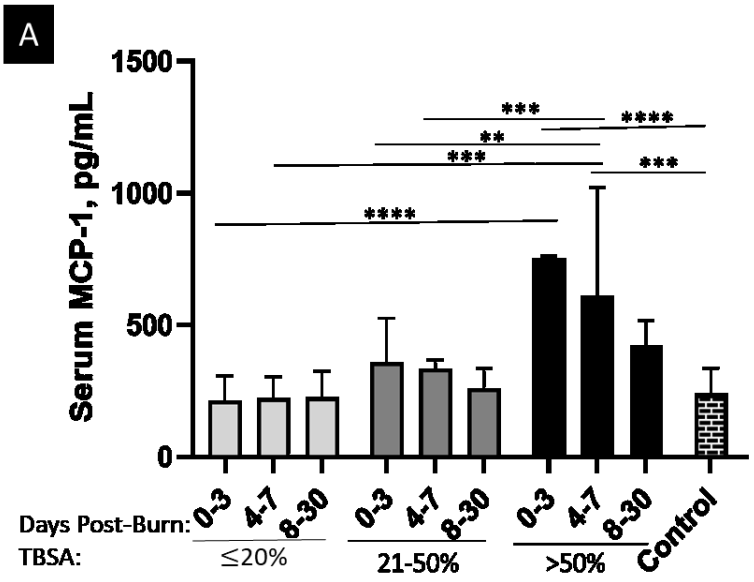


Figure 4. CXCR4, CCR2, and CCR5 expression of monocyte subpopulations in burn patients at various time points. A) CXCR4 expression in monocyte subpopulations, B) CCR2 expression in monocyte subpopulations, and C) CCR5 expression in monocyte subpopulations, with no statistical significance found between groups compared. Burn patient sample size at each time point included minor burns with an n of 12, moderate burns with an n of 3, and severe burns with an n of 3. Significance is denoted by a p value of <0.05 = *, <0.01 = **, <0.001 = ***, and <0.0001 = ****.



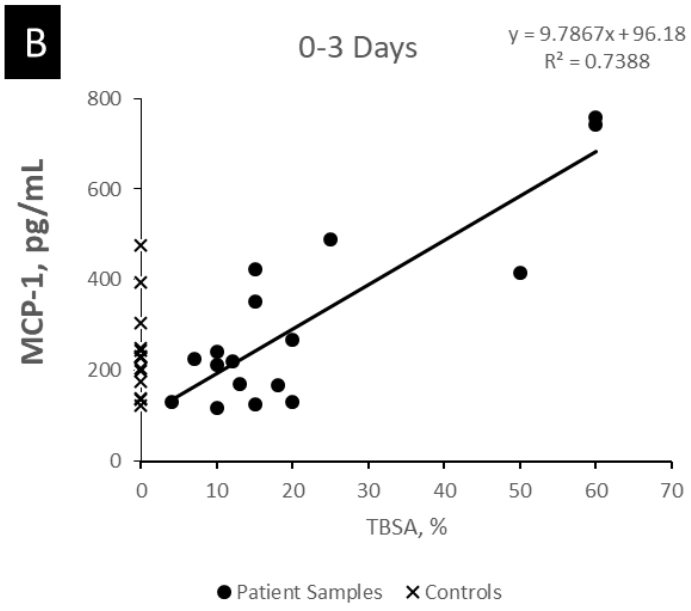
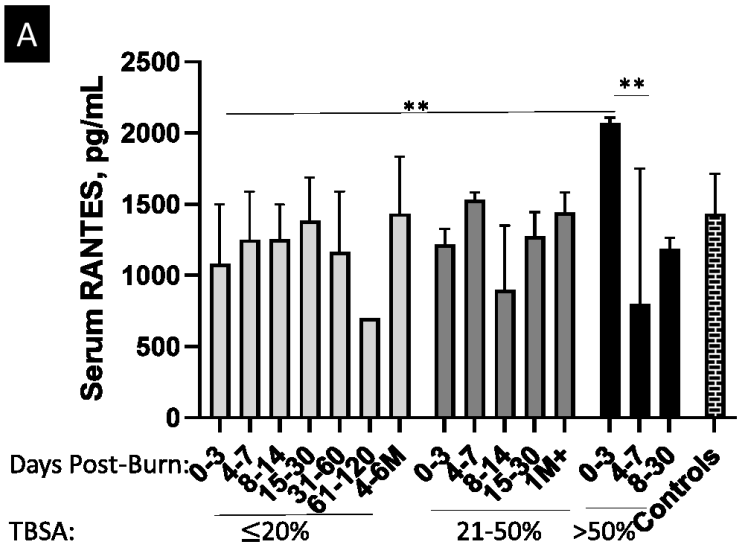


Figure 5. MCP-1 serum levels and correlation in burn patients. (A) MCP-1 in the serum of burn patients, (B) shows the positive correlation of serum MCP-1 and TBSA at 0-3 days. Burn patient sample size at each time point included minor burns with an n of 13, moderate burns with an n of 3, and severe burns with an n of 2. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.



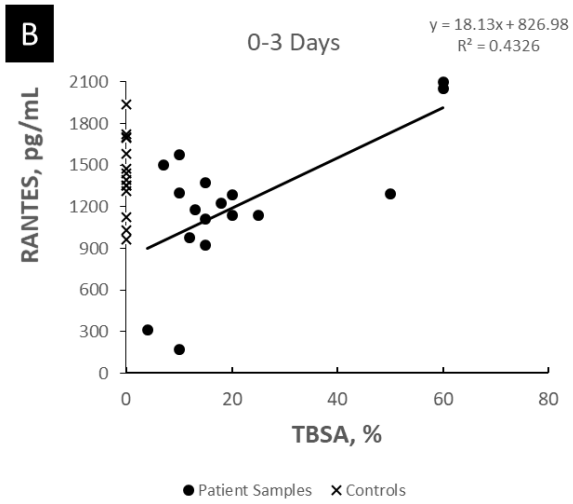
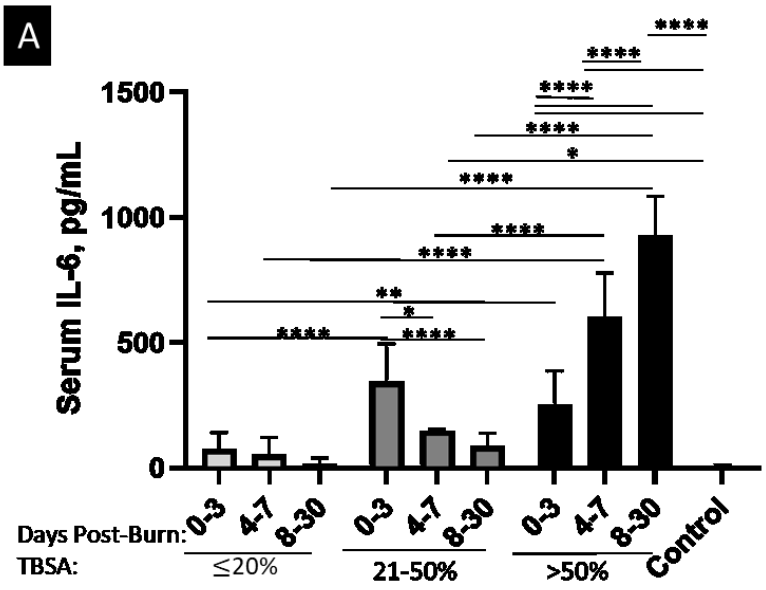


Figure 6. (A) RANTES/CCL5 in the serum of burn patients and (B) shows the positive correlation of serum RANTES and TBSA at 0-3 days. Burn patient sample size at each time point included minor burns with an n of 13, moderate burns with an n of 4, and severe burns with an n of 2. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.



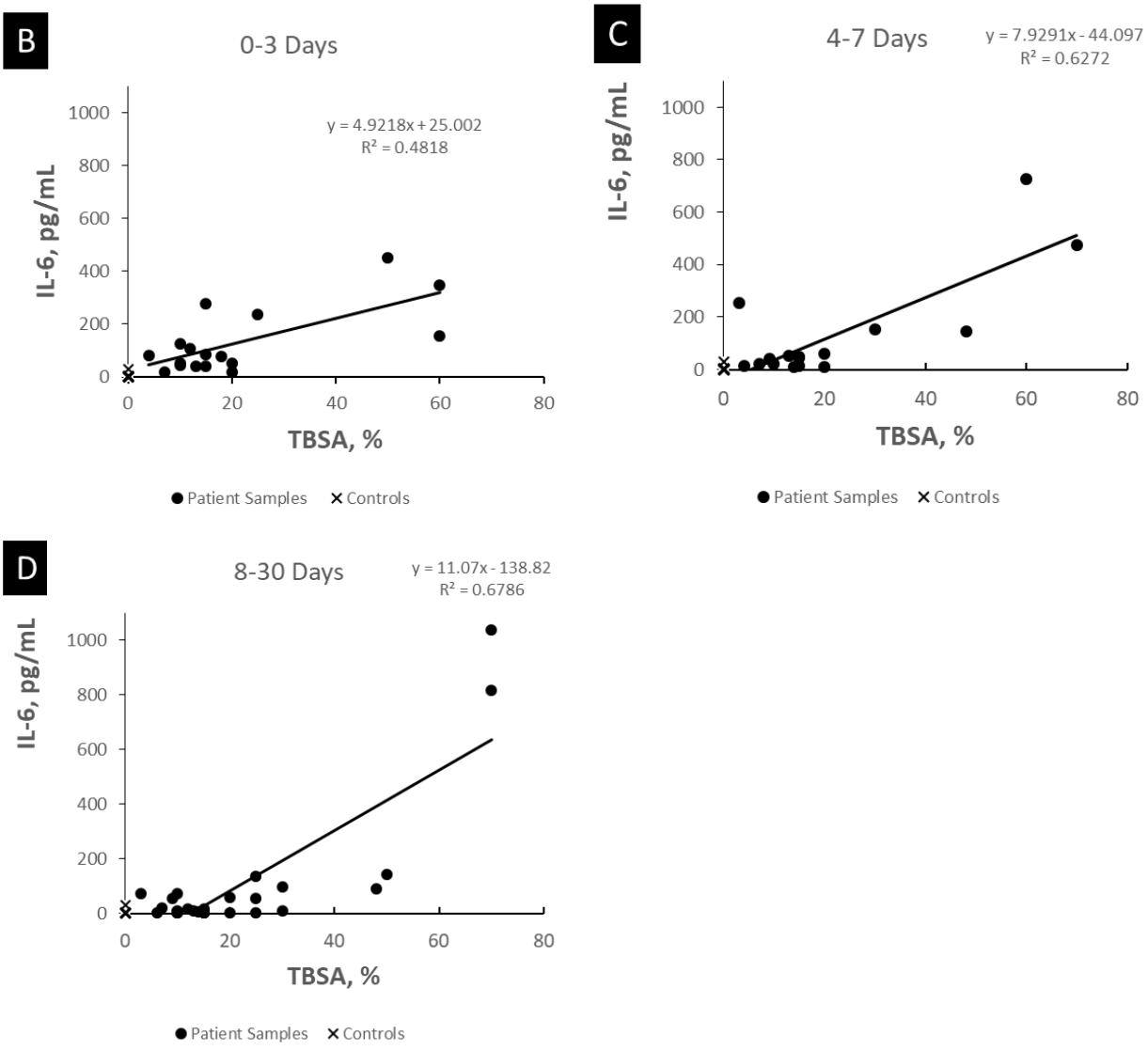


Figure 7. Serum levels of IL-6 and correlation with severity of burn. (A) IL-6 in the serum of burn patients, (B) positive correlation of serum IL-6 and TBSA at 0-3 days, and (C) at 4-7 days, and (D) 8-30 days. Burn patient sample size at each time point included minor burns with an n of 14, moderate burns with an n of 3, and severe burns with an n of 2. Significance is denoted by: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, and ****=$p<0.0001$.

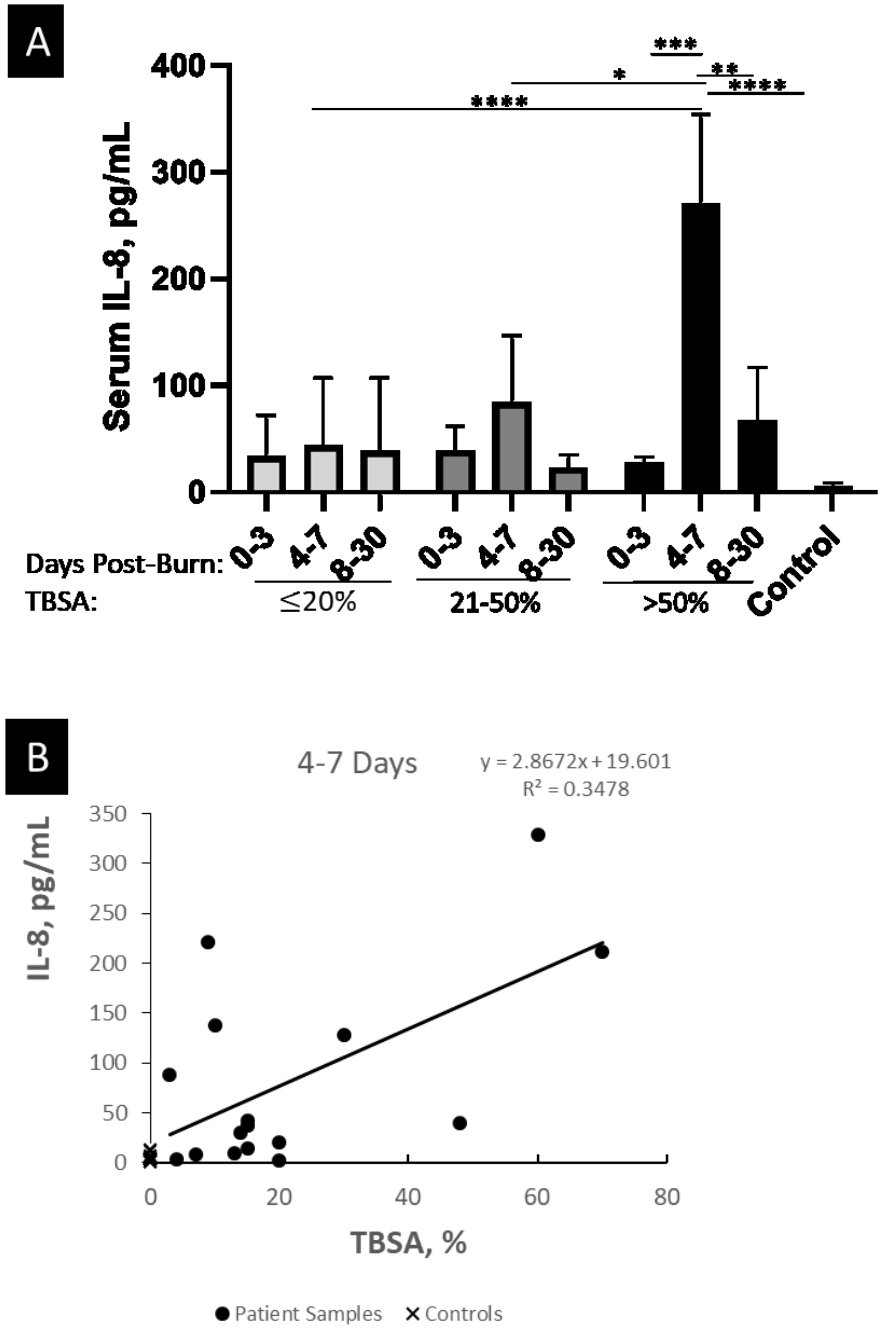


Figure 8. Serum levels of IL-8 and correlation with burn severity at various time points. (A) IL-8 in the serum of burn patients and (B) positive correlation of serum IL-8 and TBSA at 4-7 days. Burn patient sample size at each time point included minor burns with an n of 14, moderate burns with an n of 4, and severe burns with an n of 2. Significance is denoted by: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, and ****=$p<0.0001$.

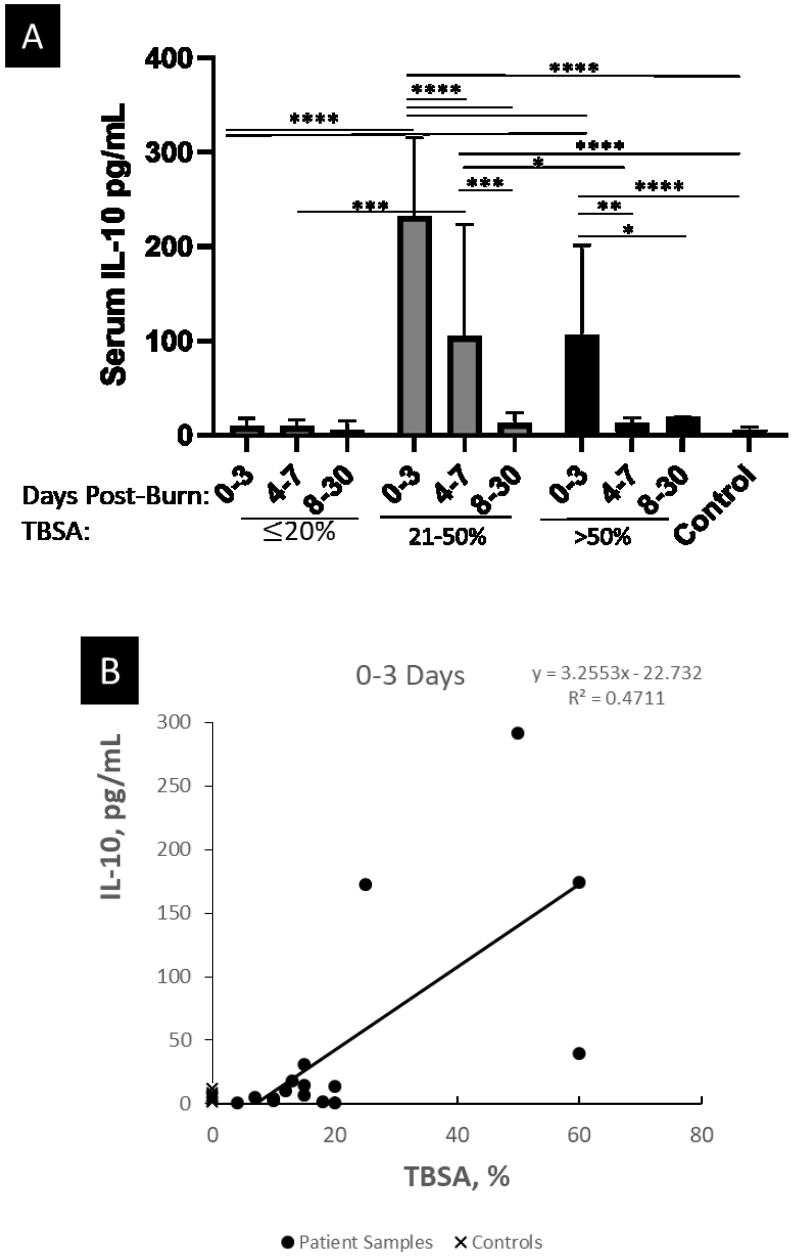


Figure 9. Serum levels of IL-10 and correlation by burn severity at various time points. (A) IL-10 in the serum of burn patients and (B) positive correlation of serum IL-10 and TBSA at 0-3 days. Burn patient sample size at each time point included minor burns with an n of 12, moderate burns with an n of 4, and severe burns with an n of 2. Significance is denoted by: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, and ****=$p<0.0001$.

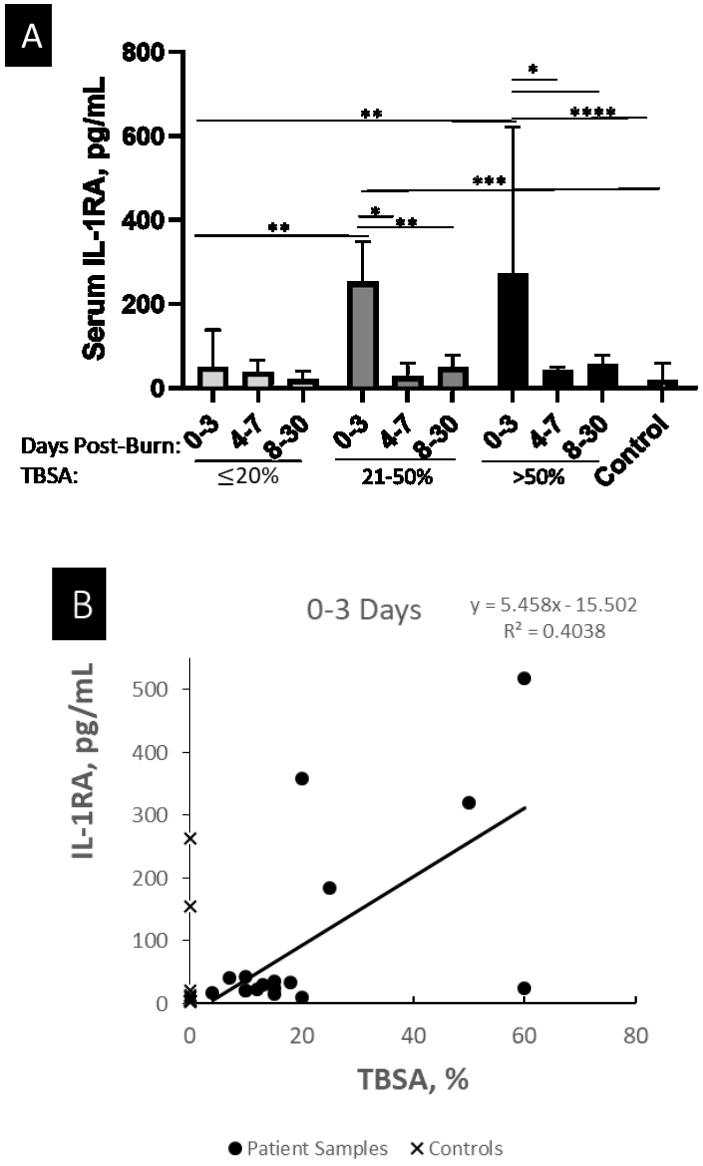


Figure 10. Serum levels of IL-1RA and correlation by burn severity at various time points. A) IL-1RA in the serum of burn patients and B) positive correlation of serum IL-1RA and TBSA at 0-3 days. Burn patient sample size at each time point included minor burns with an n of 14, moderate burns with an n of 4, and severe burns with an n of 2. Significance is denoted by: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, and ****=$p<0.0001$.

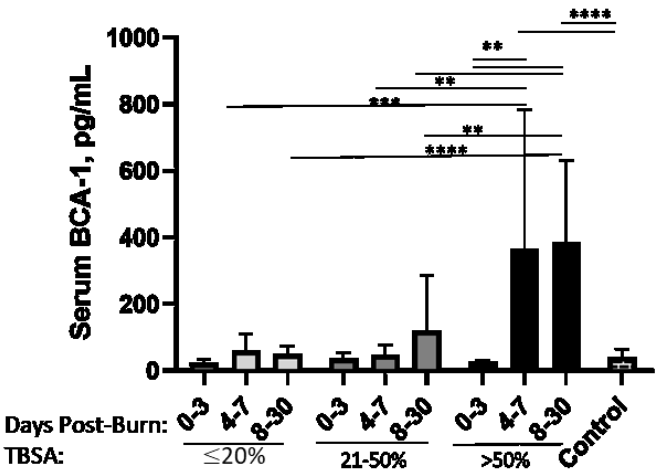


Figure 11. Serum levels of BCA-1 by burn severity at various time points. Burn patient sample size at each time point included minor burns with an n of 13, moderate burns with an n of 4, and severe burns with an n of 2. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

Table 1. Burn and Control Participant Demographics (B = Burn patient, C = Control, M = Male, F = Female).

Pati-ent	**Age** (years)	**Gender**	**% TBSA**	**Area of burn injury**	**Burn degree**	**Sepsis**	**Staph Infection**	**Antibiotics/ Dosage(s)**	**Pre-existing conditions or other medications**
B1	57	M	7	Back, left hip, thighs, calves, hands	5% 2nd, 2% 3rd	No	3+ Virdans group streptococci, 1+ Bacillus cereus group	Cephalexin 500 mg TID	None
B2	52	M	50	Hands and arms, face, shoulder, back, hips, thighs, legs	13% 2nd, 37% 3rd	Yes	1+ Methicillin Resistant Staphylococcus aureus	Vancomycin, TMP/SMX, and meropenem Tazocin	ADHD, Anxiety, Hypertension (HTN), Other Depressive Disorder, Other Agitated Delirium Oculogyric Crisis and Dyskinesias, Wound

									Infection Hyponatremia
B3	46	M	15	Right hand and wrist, thighs and knees, right ankle and foot	13% 2nd, 2% 3rd	No	1+ Bacillus cereus group, 1+ Coagulase negative Staphylococcus	Cefzolin 2 gms IV	Previous pneumothorax: Appendectomy. Daily ETOH consumption
B4	76	F	70	Neck, right hand and wrist, chest, shoulders, arms, legs	10% 2nd, 60% 3rd	Yes/multi-organ Failure (deceased)	1+ Coagulase negative staphylococcus, 4+ Stenotrophomonas maltophilia, 2+ Lactose-fermenting Gram negative bacilli, 1+ Enterococcus species abnormal	Ceftazidime and Ciprofloxacin Tazocin	Increased intraocular pressure adult respiratory distress syndrome (ARDS), hypoxia atrial fibrillation, right pneumothorax lschemia of R foot
B5	57	M	20	Lower extremities	18% 2nd, 2% 3rd	No	1+ Coagulase-negative staphylococcus, 2+ Methicillin-resistant staphylocccus aureus + pseudomonas stutzeri	None	COPD, CVA, Dyslipidemia myocardial infarction (MI) with PCI, HTN
B6	38	M	70	Upper and lower	70% 3rd and 4th	Yes (deceased)	1+ Lactose-fermenting gram negative	Meropenum Ancef	Multi-organ failure, acute kidney injury,

				extremities			bacilli, 3+ pseudomonas putida abnormal, 3+ enterococcus species abnormal, 2+ bacillus cereus group abnormal, 3+ acinetobacter species abnormal		dilated SB loops with pneumatosis on jejunum, diffuse mesenteric edema
B7	26	M	7	Hands, face, and neck	7% 2nd	No	No Staph Aureus 2+ Coagulase-negative Staphylococcus	None	Healthy
B8	24	F	18	Hands, forearms, thighs	10% 2nd, 8% 3rd	No	1+ Staphylococcus aureus Abnormal, 1+ Pseudomonas aeruginosa, 1+ Enterococcus species	Ancef 500 mg tid	Healthy
B9	28	M	15	Lower legs, abdomen, thigh	5% 2nd, 10% 3rd	No	3+ Staphylococcus aureus, 4+ Streptococcus agalactiae (Group B), 3+ Streptococcus pyogenes (Group A), 3+ Enterococcus species Abnormal	Ancef TMP-SMX	Burn wound cellulitis

B10	41	M	25	Chest, abdomen, thighs, back	15% 2nd, 10% 3rd	No	2+ Coagulase-negative Staphylococcus, 1+ Coryneform bacilli, 1+ Bacillus cereus group, 1+Candidadubliniensis, 4+ Staphylococcus aureus, 3+ Staphylococcus lugdunensis	Ancef, Ceftriaxone, Vancomycin, metronidazole	Asthma (severe), previous testicular resection, MVA
B11	23	M	50	Scalp, face, buttocks, upper body	17% 2nd, 33% 3rd	Yes	4+ Methicillin-resistant Staphylococcus aureus, 3+ Coagulase-negative Staphylococcus Abnormal, 3+ Candida albicans	Vancomycin, ciprofloxacin, TMP-SMX DS	Acute renal failure, leukocytosis, respiratory failure, keratitis
B12	26	M	15	Lower extremities and face	15% 3rd	No	1+ Coagulase-negative Staphylococcus, 3+ Pseudomonas aeruginosa, 2+ Enterococcus species, 3+ Candida dubliniensis, 1+ Viridans group streptococci, 1+ Staphylococcus aureus	Cefotaxime, ciprofloxacin	Healthy

B13	75	M	15	Lower legs, calf, right hand	14% 2nd, 1% 3rd	No	Pseudomonas, enterococcus, and coag negative staph	Cefazolin, ciprofloxacin	Hemothorax [J94.2], multiple rib fractures [S22.49]
B14	36	M	12	Lower back, hands, face, arms	12% 2nd	No	1+ Coliform-like Gram-negative bacilli Abnormal, 1+ Coagulase-negative Staphylococcus	Clindamycin	Obesity, HTN amlodlPine (10 mg oral), daily bacitracin-polymyxinB, topical, daily enoxaparin (40 mg subcutaneous, q12h), SCH mafenide, topical, bid multivitamin with minerals (1 tablet oral daily with breakfast), perindopril (4 mg oral, daily)
B15	55	M	13	Left face, left upper extremity, bilateral lower extremities	13% 2nd	No	2+Staphylococcus aureus Abnormal, 1+ Bacillus cereus, 1+ Aerobic spore-bearing bacilli group, 1+ Coagulase-negative Staphylococcus, 2+ Pseudomonas fluorescens Abnormal	Ancef	HTN, DM, DLD, active smoker -Tramacet, polysporin, metformin 1000 daily

B16	48	M	30	Neck, chest, arms, face	30% 3rd	Yes	1+ Coagulase-negative Staphylococcus Methicillin-resistant Staphylococcus aureus Abnormal, 2+ Candida parapsilosis	Ciprofloxacin, septra, vancomycin	MVA requiring splenectomy and nephrectomy, spinal fusion (T4-5), drug induced psychosis, Hep C+, IVDU, EtOH abuse
B17	39	M	6	Feet and right arm	6% 3rd	No	2+ Coagulase-negative Staphylococcus, 2+ Bacillus cereus group	None	Healthy
B18	44	F	24	Face, neck, chest, abdomen, thighs	22% 2nd, 2% 3rd	No	2+ Pseudomonas aeruginosa, 1+ Lactose-fermenting Gram-negative bacilli, 2+ Enterococcus species, 1+ Coagulase-negative Staphylococcus	Pip tazo, septra, ancef	Asthma, previous shoulder and knee surgeries for torn meniscus -Gabapentin (300 mg capsule), Prazosin (1 mg tablet)
B19	33	M	11	Bilateral arm and back	6% 2nd, 5% 3rd	No	1+ Coagulase-negative Staphylococcus, 1+ Cutibacterium (Propionibacterium)-acnes, 1+ Stenotrophomonas maltophilia	Ciprofloxacin, septra, pip-tazo, ancef	Major depressive disorder, generalized anxiety DO with panic attacks/Tx - Desvenlafaxine (long active 100 mg)

B20	38	M	4	Hands, face, bilateral ankles	4% 2nd	No	2+ coagulase-negative staphylococcus	None	Chronic back pain, dyslipidemia
B21	41	F	15	Abdomen and lower extremities	10% 2nd, 5% 3rd	No	2+ Coagulase-negative Staphylococcus l, 1+ Aerobic spore-bearing bacilli, 1+ Glucose non-fermenting Gram-negative bacilli	Ancef	Endometrial polyp-GERD/Chronic gastritis tramadol-acetaminophen (37.5 mg, 325 mg per tablet)
B22	60	M	15	-	n/a	No	-	-	-
B23	23	M	6	Bilateral hands and face	6% 2nd	No	1+ Pseudomonas stutzeri, 1+ Lactose-fermenting Gram-negative bacilli, 1+ Enterococcus species Aspergillus nidulans	Ciprofloxacin	Healthy
B24	59	M	70	Upper and lower extremities	51% 2nd, 19% 3rd	Yes	2+ Klebsiella (Enterobacter) aerogenes, 1+ Pseudomonas aeruginosa, 1+ Candida tropicalis, 1+ Bacillus cereus group, 1+ Glucose non-fermenting Gram-negative bacilli, 1+	TMP-SMX Ceftazidime (2g), fluconazole (400 mg V q8hrs, pip-taz, micafungin, vancomycin	Scheduled: Cetirizine (10mg PO Daily), Codeine (15mg PO QID), Enoxaparin (30mg SC BID), Melatonin (3mg PO qHS), Multivitamin with minerals,

							Candida albicans		Quetiapine (25mg PO qHS), Ranitidine (150mg PO BID), Vitamin D (1000U PO Daily) PRN Meds: Eucerin cream to lips, Dilaudid (0.5-2mg IV q2h), Dialudid (1mg PO q3h), PEG 3350 (17g PO Daily), Quetiapine (25-50mg PO q8h for agitation), Sennekot (10ml PO qHS), Trazadone (12.5-25mg PO q8h for sleep)
B25	22	M	10	Upper and lower extremities, abdomen	10% 2nd	No	2+ Pseudomonas aeruginosa Abnormal, 1+ Methicillin-resistant Staphylococcus aureus, 3+ Streptococcus pyogenes (Group A), 3+ Staphylococcus aureus, 3+	Vancomycin, septra, ancef	Healthy

							Corynebacterium striatum, 1+ Coliform-like Gram-negative bacilli Abnormal		
B26	62	M	3	Left chest and right thigh	3% full thickness	No	2+ Proteus mirabilis, 2+ Coagulase-negative Staphylococcus, 1+ Non-lactose fermenting Gram-negative bacilli	Cephalexin Aripiprazole, (2 mg oral daily), cefazoline (3 g intravenous)	DVT (deep venous thrombosis), HTN, wheelchair bound OSA, bipolar/depression
B27	60	F	10	Back, abdomen, arm, and hands	7% 2nd, 3% 3rd	No	1+ Pseudomonas aeruginosa, 1+ Coagulase-negative Staphylococcus, 2+ Enterococcus gallinarum	Ciprofloxacin, septra, pip-tazo	Rectal prolapse, transfusion reaction, polyneuropathy, splenic infarct, anemia, stable angina, STEMI (ST elevation myocardial infarction), osteoarthritis, scleroderma, HTN
C1	68	M							
C2	57	F							
C3	24	F							
C4	53	F							
C5	24	M							
C6	24	M							
C7	41	F							
C8	23	F							
C9	25	M							
C10	53	M							
C11	21	M							
C12	33	F							

C13	24	F							

Chapter 3:

Conclusions and Future Directions

3.1 Conclusions

The severe and prolonged inflammatory response after burn injury leads to HTS, thus we studied circulating monocyte subpopulations and serum levels of 71 cytokine levels during wound healing in burn patients. Many of the cytokines in this study have never been observed over time in burn patients, as well as control participants, which provides a new scope at the impact that they may have which is being overlooked in the inflammatory and anti-inflammatory regulation that leads to fibrotic scarring. It is observed however that each cytokine and monocyte population has differential responses over time, depending on the severity of burn injury. These specific observations and analysis can help direct where our focus and more directed research can be aimed for further understanding of the molecular mechanism of burn healing and HTS development. Our results suggest that there is an increased pro-inflammatory CD14+CD16+ population of monocytes at greater burn injuries. Classical monocytes are the CD14+CD16- population, which have had an unknown role in wound healing in burns, however a suggested anti-inflammatory phenotype supports our results of lower circulating levels with increasing burn injury.

Based on previous research, we can conclude that SDF-1 peaks at much later time points in burn patients than was followed in this study, therefore time is an important factor to consider its impact. SDF-1's receptor CXCR4's peak at later time points and previous research using an antagonist show this may also be a therapeutic target for HTS prevention and better wound healing. Higher levels of RANTES was correlated with increasing TBSA, supporting its recent use as an antagonist in improving wound healing and reducing scar thickness. The strong correlation between IL-6 and burn severity, and its pro-inflammatory role combined with other studies support its role in the hyper-inflammatory response characteristic of fibrotic scar

development. Based on our results, IL-8 is likely a cytokine that contributes to the hyper-inflammatory response as well based on its significant increase with higher severity of burns. IL-10 has a large peak at early time points, increasing with greater burn severity suggesting its role in burn wound healing. IL-1RA increases with higher burn injury, which likely has a similar effect in wound healing to IL-10, as they are both anti-inflammatory. BCA-1 showing a peak at later time points supports its role in burn wound healing as an angiostatic cytokine, and suggests that it has more of a role in burns and wound healing than has been previously known.

3.2 Future Directions

Future studies could focus on correlating the levels of circulating peripheral blood cytokine levels to their levels in the wound site, and follow the accumulation of collagen and myofibroblasts, which are characteristics of HTS tissue. Along with histological patterns, following long-term results in scar outcomes would be beneficial to come to an understanding of which cytokines may be correlated to more severe scar outcomes. Further on, regulating the levels of CD14+CD16+ monocyte population for optimal burn healing and controlling the inflammatory response as prevention for HTS, as well as developing a better understanding for the CD14+CD16- monocyte population. The SDF-1/CXCR4 pathway appears to peak at later time points, in which future studies on the appropriate time for an antagonist may lead to a new therapy for HTS prevention. SDF-1 however being significantly lower after burn injury compared with controls supports a biological, observational basis for earlier usage of an SDF-1 therapy to improve healing outcomes. The significant increase of MCP-1 in peripheral blood and decrease of its receptor, CCR2, may suggest its being activated elsewhere (i.e. tissue in macrophages), thus a study of its levels in the wound site would give a wider understanding of its role in scar development and outcomes in burn patients. The RANTES/CCR5 pathway is

involved in the immune response, but future research would provide more information in the burn population if its levels were observed in the tissue. RANTES has also been suggested to have multiple functions depending on if it is in its aggregated or disaggregated forms, which should be taken into account in future research to distinguish its formation and the differential response this cytokine has in wound healing. Future studies may use IL-6 antagonist therapy to curb the hyper-inflammatory response that leads to HTS, as well as utilizing findings to support IL-8 as contributing to the cytokine storm and exploring this in the future as a topical treatment for burns. Figure 1 shows an image of the cytokines increased and their potential use as therapy for future research. The rapid increase in IL-10 post-injury, which is greater with increasing burn severity. Future research may find that a very early antagonist is helpful to allow the immune response to have full effect at early time points, and become therapeutic afterwards to prevent a hyper-inflammatory response. IL-1RA although beneficial in effect as a pro-inflammatory mediator to antagonize its effects, may have a point where it becomes too high and detrimental, which may be explored by further research on antagonism immediately after injury, with success seen in IL-10 as a therapy in previous research. BCA-1's levels in burn wound healing are novel to our study, and the significantly higher rates at later time points suggest that further research is needed to further understand its impact in hyper-angiogenesis and role in fibrotic scarring in burn-injured patients.

Future studies should focus on cytokines with significant differences between burn severity and controls, which may further prove to be extremely beneficial in wound healing treatment with a targeted approach to assist in an ideal immune response. This observation will further our understanding of the mechanism of HTS and profibrotic scarring, and what both monocyte subpopulations and cytokines may be contributing factors to the abnormal wound

healing response. Burn injuries is a difficult patient dynamic to study due to limitation of samples and population, but continued research on these patients will aid in finding a more accurate understanding and be closer to discovering an ideal treatment therapy for burn injuries and their outcome including HTS development.

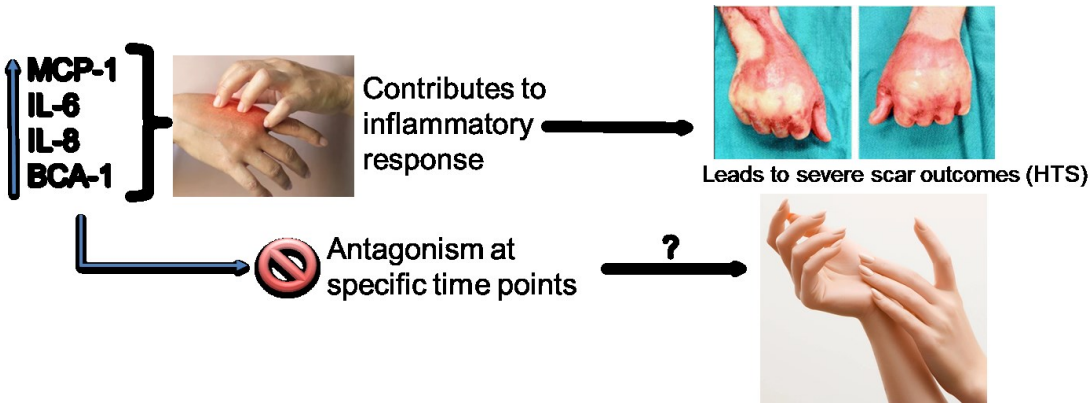


Figure 1. MCP-1, IL-6, IL-8, and BCA-1 increased in burn patients and potential therapy.

Appendix A: Supplemental Cytokine Data

The cytokine data included in chapter 2 were results we found most significant and publishable, however there were 71 cytokines observed and analyzed overall. During wound healing, there is a massive "cytokine storm". We believe that a mosaic of cytokines contribute to the abnormal wound healing seen in burn injuries that lead to HTS. In our supplemental data, there is a results and discussion on the remaining cytokines with significance, as well as the data and charts for non-significant cytokines. The remaining cytokines include SDF-1, sCD40L, IL-7, IL-18, IL-27, TRAIL, TARC, LIF, PDGF-AA, PDGF-AB/BB, VEGF-A, TGFα, IL-2, EGF, Eotaxin, FGF-2, FLT-3L, Fractalkine, GM-CSF, IFN-α2, IFNγ, GROα, IL-1α, IL-1β, IL-4, IL-5, IL-9, CTACK, Eotaxin-3, MDC, CXCL9, IL-12p40, IL-12p70, IL-17A, IL-15, IL-25, IL-17F, IL-22, IP-10, MCP-3, M-CSF, MIP-1α, MIP-1β, TNFα, MCP-2, TNFβ, Eotaxin-2, I-309, MCP-4, IL-16, and 6CKine.

Results

SDF-1 serum cytokine levels were significantly lower than controls in mild burns at 0-3 days ($p<0.01$) and 8-14 days ($p<0.05$), as seen in Figure 1. SDF-1 had low correlation observed at all time points except for 15-30 days ($r^2=0.7601$, Table 2). sCD40L serum levels significantly increased in mild burns from 0-3 to 8-14 days, which was also significantly higher than controls ($p<0.01$) (Figure 2). IL-7 serum levels were significantly increased in moderate burns at 0-3 days compared to mild burns and controls ($p<0.05$), with another increase at 15-30 days in moderate burns compared to controls ($p<0.05$) (Figure 3). IL-18 had a significant increase at 8-30 days from 0-3 days ($p<0.05$) in severe burns, 15-30 days in mild burns ($p<0.001$), and controls ($p<0.0001$) (Figure 4). IL-27 had a significant increase at 4-7 days compared to controls ($p<0.05$) (Figure 5). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) significantly increases in mild burns from 0-3 days to 8-14 days ($p<0.05$) and 4-6 months ($p<0.01$) (Figure 6).

Thymus and activation regulated chemokine (TARC) showed a significant increase from 0-3 days in mild burns to 8-14 ($p<0.01$) and 15-30 ($p<0.05$) days, as shown in Figure 7. Leukemia inhibitory factor [LIF] serum levels were significantly increased in moderate burns at 0-3 days compared to 4-30 days ($p<0.05$), mild burns ($p<0.01$), severe burns ($p<0.05$), controls ($p<0.01$) (Figure 8). PDGFA platelet derived growth factor (PDGF) subunit A (PDGF-AA) serum levels significantly increased in mild burns from 0-3 days to 8-14 days ($p<0.05$), shown in Figure 9. PDGF-AB/BB serum levels were significantly higher at the same points in mild burns, at 8-14 days from 0-3 days ($p<0.05$), but were also found to be significantly lower than controls at 0-3 days ($p<0.01$) (Figure 10). Vascular endothelial growth factor (VEGF-A) had a significant increase at mild burns at 8-14 days from 0-3 days ($p<0.01$), as shown in Figure 11.

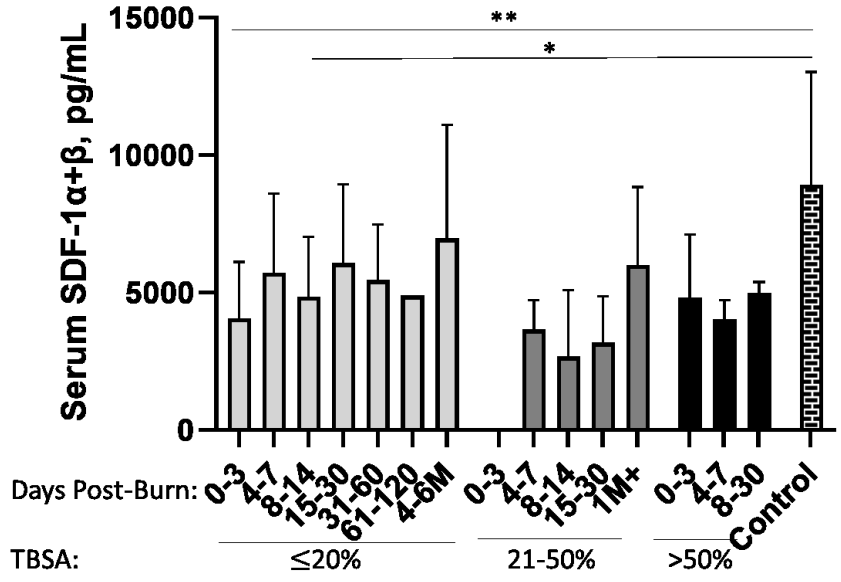


Figure 1. Serum levels of SDF-1 isoforms alpha and beta at varying levels of burn injury and time points. Mild burns shows to be significantly lower at 0-3 days and 8-14 days than controls. Significance is denoted by: *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

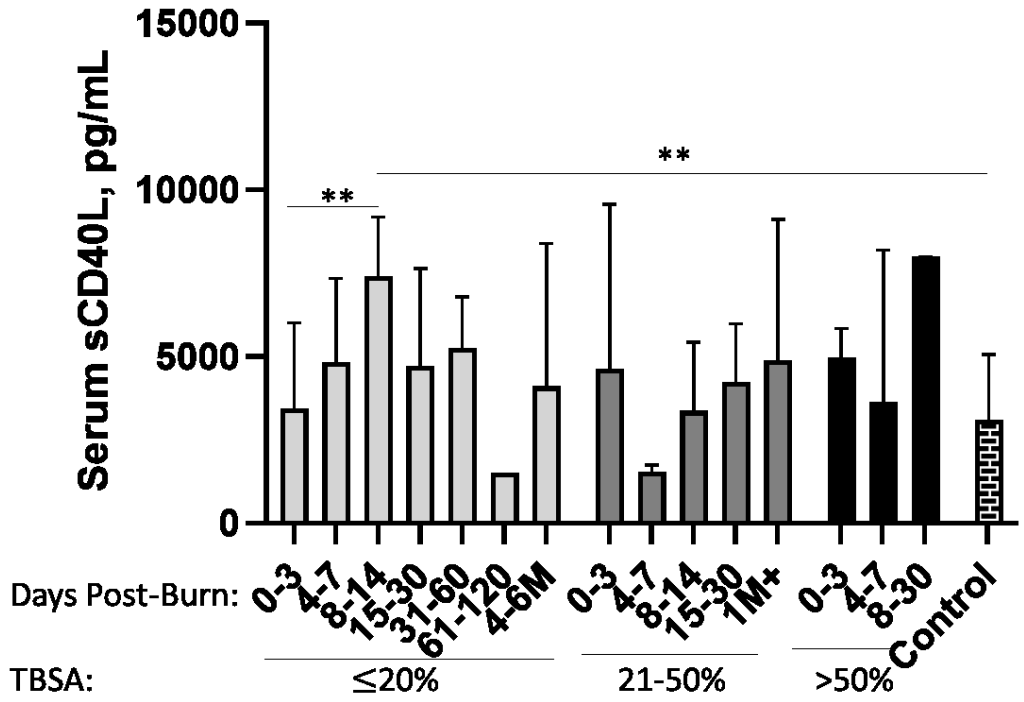


Figure 2. Serum levels of sCD40L at varying levels of burn injury and time points. Mild burns show a significant increase at 8-14 days from 0-3 days post-burn, which is also significantly increased compared to controls. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

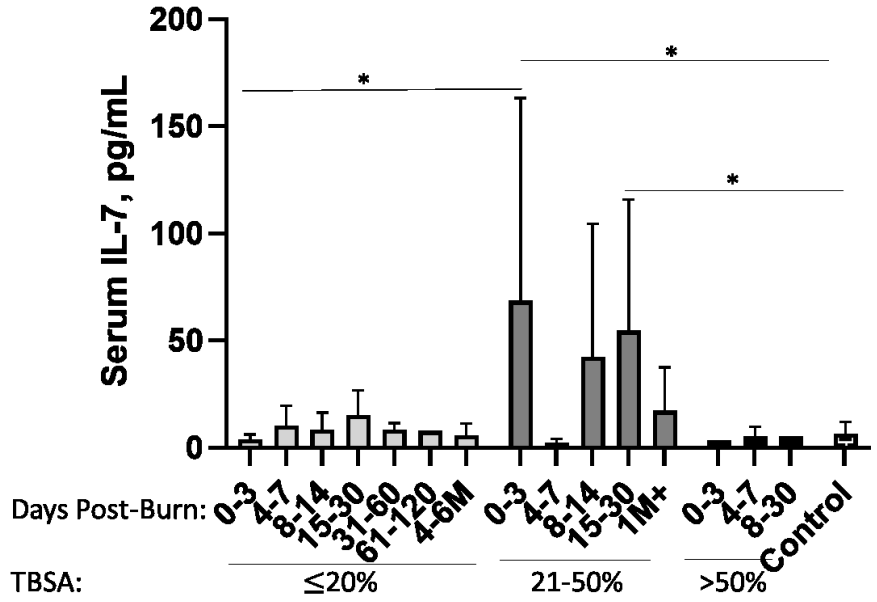


Figure 3. Serum levels of IL-7 at varying levels of burn injury and time points. Moderate burn levels show a significant increase initially after burn injury (0-3 days) compared to mild burns and controls, and are significantly higher than controls at 15-30 days. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

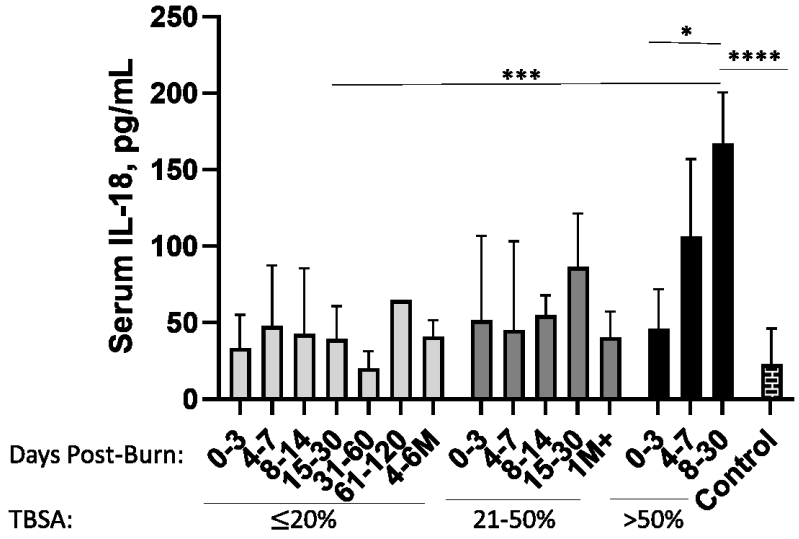


Figure 4. Serum levels of IL-18 at varying levels of burn injury and time points. Severe burns significantly increase at 8-30 days compared to 0-3 days, 15-30 days in mild burns, and controls. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

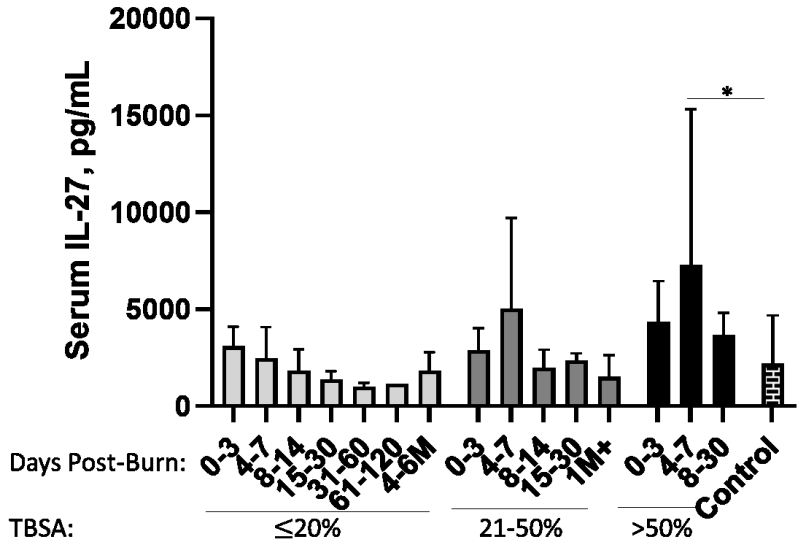


Figure 5. Serum levels of IL-27 at varying levels of burn injury and time points. Severe burns show a significant increase in IL-27 at 4-7 days compared to controls. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

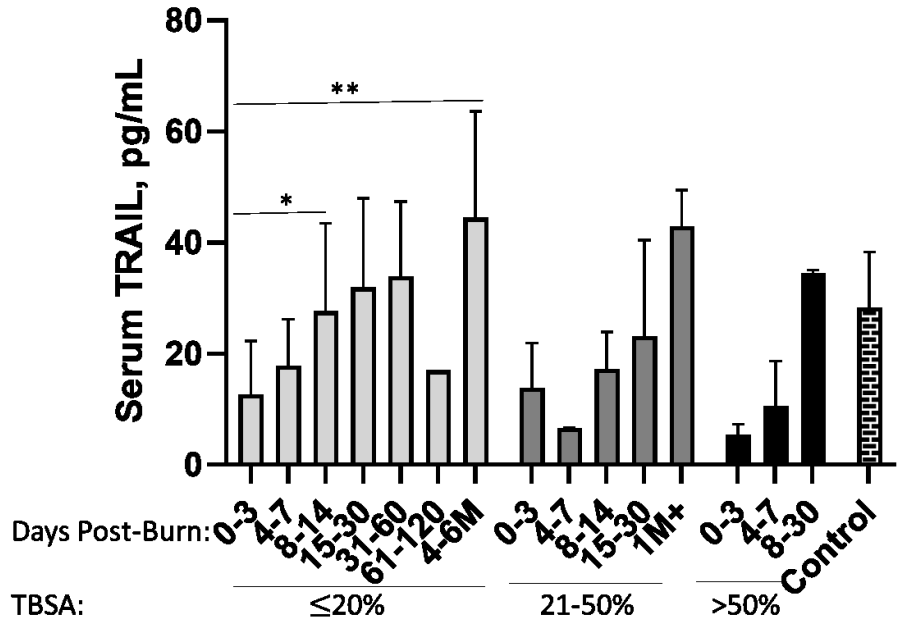


Figure 6. Serum levels of sCD40L at varying levels of burn injury and time points. TRAIL significantly increases at 4-7 days and 4-6 months compared to 0-3 days in mild burns. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

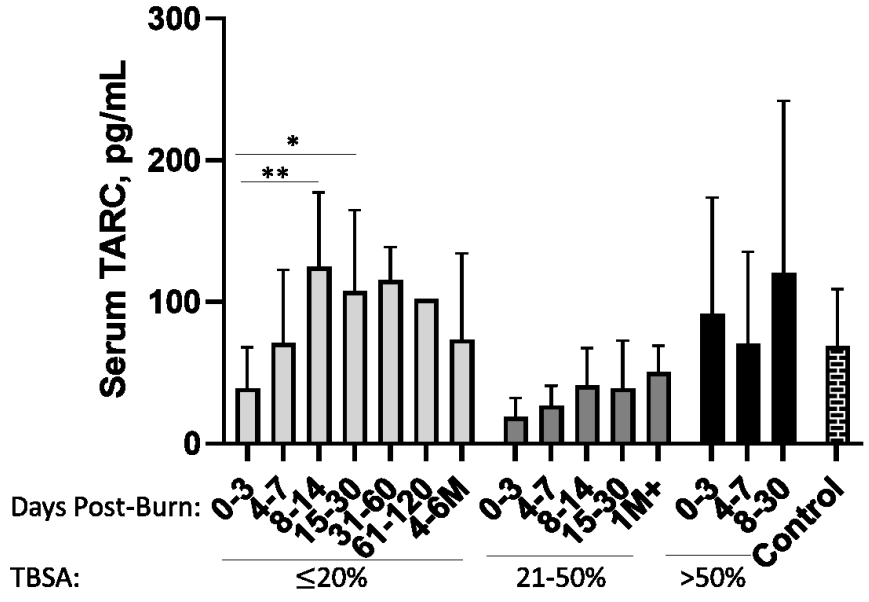


Figure 7. Serum levels of TARC at varying levels of burn injury and time points. In mild burns, TARC significantly increased at 8-14 days and 15-30 days compared to 0-3 days. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

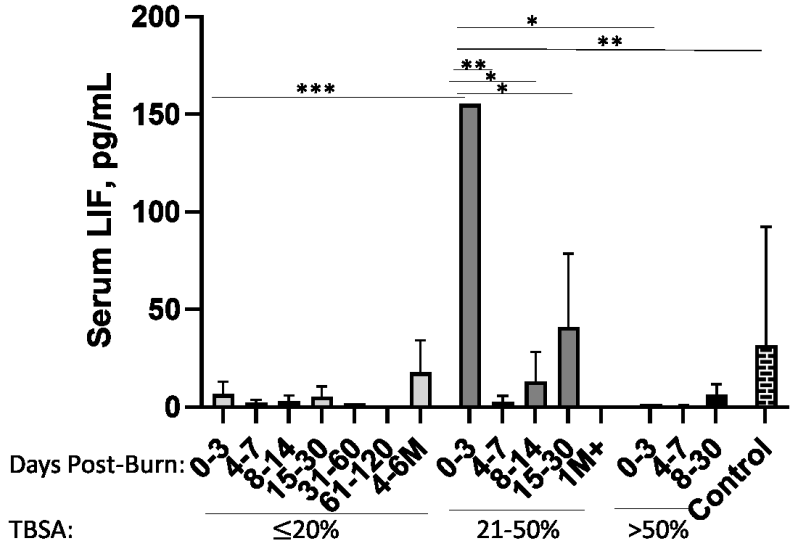


Figure 8. Serum levels of LIF at varying levels of burn injury and time points. Moderate burns significantly increased at 0-3 days compared to 0-3 days in mild and severe burns, all other time points in moderate burns, and controls. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

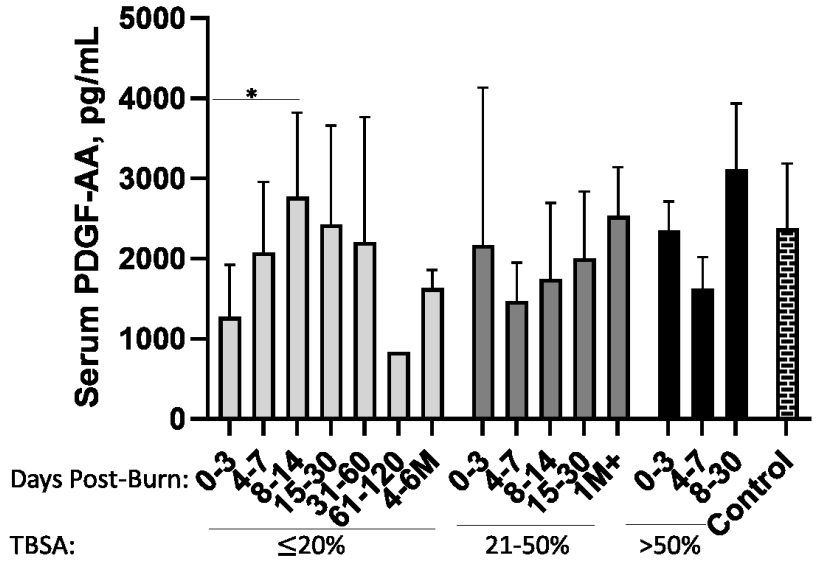


Figure 9. Serum levels of PDGF-AA at varying levels of burn injury and time points. Mild burns had a significant increase at 8-14 days from 0-3 days. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

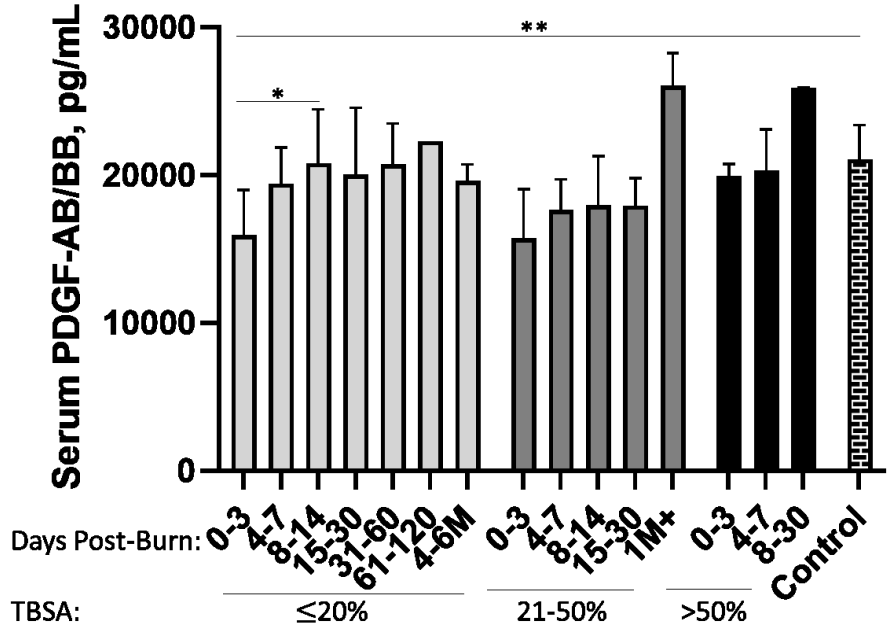


Figure 10. Serum levels of PDGF-AB/BB at varying levels of burn injury and time points. Mild burns are significantly lower at 0-3 days compared to 8-14 days and controls. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

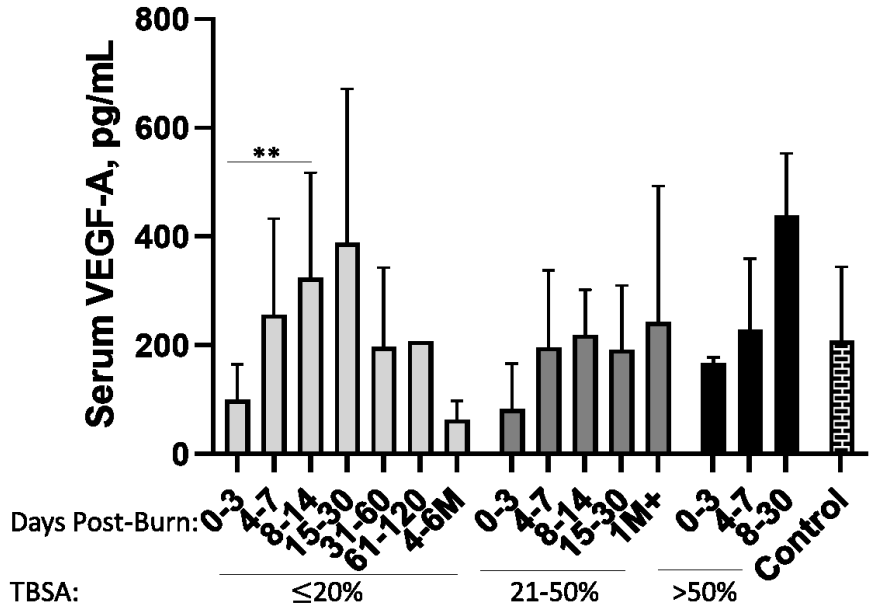


Figure 11. Serum levels of VEGF-A at varying levels of burn injury and time points. Mild burns significantly increase at 8-14 days compared to 0-3 days. Significance is denoted by: *=p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001.

Serum levels of cytokines TGFα, IL-2, EGF, Eotaxin, FGF-2, FLT-3L, Fractalkine, GM-CSF, IFN-α2, IFNγ, GROα, IL-1α, IL-1β, IL-4, IL-5, IL-9, CTACK, Eotaxin-3, MDC, CXCL9, IL-12p40, IL-12p70, IL-17A, IL-15, IL-25, IL-17F, IL-22, IP-10, MCP-3, M-CSF, MIP-1α, MIP-1β, TNFα, MCP-2, TNFβ, Eotaxin-2, I-309, MCP-4, IL-16, 6CKine (Figures 12-51) showed no significant differences. These figures are found at the end of this chapter.

Discussion

Previously, our research group has studied SDF-1 and its receptor CXCR4 in burn and HTS. SDF-1 levels in serum in this study were found to be up-regulated in burns compared with controls, and that this chemokine pathway is involved with the migration of CD14+CXCR4+ cells from the blood stream into wound sites, possibly contributing to HTS (210). This study found that in peripheral serum, SDF-1 was only significantly lower than controls in mild burns, and overall controls appear to have higher levels at earlier time points. The difference in outcomes can be explained by the difference in time points, with this study having stratified and grouped TBSA and times, while the other was grouped together for both to create a mean, with PBMC's having a time point from 14 days-35 months, and serum from 1-17 days (210). SDF-1 has also had higher levels in burn deep dermal fibroblasts (259), which suggests a close relationship to fibrotic scarring and macrophage levels in tissue. It was found by our group that blocking the SDF-1/CXCR4 pathway by CTCE-9908 (an analog of SDF-1) in a mouse model minimized HTS formation and improved collagen remodeling (259). AMD3100 is a CXCR4 antagonist, which has also been administered in mice scar skin grafting, which was found to improve scar contraction, thickness, collagen deposition, vascularity, and macrophage recruitment (224). Based on these studies, the SDF-1/CXCR4 pathway appears to play a role in scarring outcomes and HTS formation. Finding the best time point and burn levels to administer an antagonistic effect may have a significant therapeutic value.

Soluble CD40 ligand (sCD40L) has shown a large proinflammatory effect, with continuously higher serum levels associated with mortality and sepsis (260). sCD40L is a platelet-derived cytokine that is involved in inflammation and hemostasis (261), playing a role in wound healing, however its role in burn injury has not been discovered. Mild burns had

significantly higher levels of sCD40L than controls. This increased at 8-14 days, with a sharper and delayed increase in more severe burns; however, the lower number of burn patients available at specific time points is likely why there is non-significance. The higher sCD40L levels later on in more severe burns does support the extended inflammatory response that leads to HTS, suggesting this cytokine could have more involvement than previously been noticed in burns injury outcomes.

IL-7 is required for T-cell development, and is produced by stromal tissues, having been found to enhance viral clearance in acute and chronic infections in animals (262). Correlation alone in burns and other cytokines and the level of IL-7 has been observed, having $r>0.7$ correlation with IL-12p70 and IFNγ (263). Pediatric burn patients have also been shown to have sustained elevation of circulating cytokines up to 3 years post-injury, and in burns, IL-7 remained 1.63 fold higher >3 years after burn injury when compared to time of injury (226). The previous research suggests IL-7 therapy useful for chronic infections, by significance in the cytokine profile during burn injury, and pediatric burns showing a much higher and lasting change to the immune profile overall, making IL-7 a cytokine of interest. The results of following this cytokine over time in a more observational and stratified study is the first to show serum levels at all burn TBSA' and earlier time points. Our results showed interestingly similar responses in earlier time points in mild and severe burns, while moderate burns were significantly increased at 0-3 and 15-30 days compared with controls and appear higher overall. Moderate burns may have increased IL-7 in attempt to reduce the inflammation from burns, however the levels remaining low in severe burns is somewhat of a mystery that future studies could resolve. However, there remains very little research on burns making IL-7 difficult to come to a conclusion.

IL-18 enhances IFN-γ production, and is a strong pro-inflammatory cytokine. In burn-injured mice given IL-18 treatment, there was an increase in IgM levels in the serum and improved post-burn survival, which has an important role in fighting infections when presented with an antigen (264). A later study also showed IL-18 treated, burn-injured mice improved neutrophil functions, phagocytic activity, production of reactive oxygen species, and ultimately increased survival from post-burn infection (265). Although IL-18 appears to have therapeutic value, it has not had its levels observed post-burn injury at varying time points and severity of burn condition. IL-18 had a steep climb upwards in severe burns, being significantly higher than controls at 8-30 days. This suggests that a time-specific dosage of IL-18 would be important to consider, as it likely contributes to the hyperinflammatory response seen in fibrotic wounds in more severe burns.

IL-27 has been shown to have both anti-inflammatory and pro-inflammatory functions, thus interpreting the results of this cytokine may be difficult unless testing specific subunits and their functions (266). Using a protein folding switch linked to disulfide bonds, a group was able to create a human-like IL-27 system in mice, which would prove beneficial in future studies (267). Observations of IL-27 in the burn population has not been researched to date. Serum IL-27 shows a peak in moderate and severe burns at 4-7 days, with severe burn injury being significantly higher than controls at this time. The differences in IL-27 levels between severities of burn injury suggests there is a role it plays, however the mechanism remains unknown.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in many cells, resolving inflammation (268), which is a key stage that is essential to occur to promote normal wound healing. However, in a study of asthma patients and resolution, increased TRAIL was positively associated with lower lung function, and characterized more severe

asthma (269). After ischemia, TRAIL has been found to promote pro-inflammatory cytokine secretion and macrophage migration (270). Like many cytokines, TRAIL appears to have a dynamic function depending on the type of cells that are measured, with burn injuries being another group and niche that needs further exploration. Our results show in all burns a rise in TRAIL as time progresses. In mild burns, there was a significant increase, likely due to higher patients available in this group at time points to show significance as well as an increased differential. This suggests TRAIL is being activated later on in burn patients to resolve inflammation, however a side by side comparison of normal wound healing and burn patients would provide further knowledge on the function of TRAIL in wound healing.

Thymus and activation regulated chemokine (TARC) is constitutively expressed in the thymus and is a ligand for the receptor CCR4, in which serum levels have been found to sharply reflect severity of atopic dermatitis (271). Thus, it may also play a role in other inflammatory skin conditions, such as HTS, but has not been studied in a burn population. In mild burns, TARC significantly increased from initial injury to 8-30 days, which may reflect an increase in the burn severity. This would also support increased levels of TARC earlier on and throughout in moderate and severe burns. There is very little research on TARC in inflammation and burns, so further studies would need to occur to determine further its contribution to wound healing in burn severity and outcomes.

Leukemia inhibitory factor, or LIF, belongs to the IL-6 superfamily, is highly pleiotropic, and is recently believed to be a promising strategy to increase the proangiogenic potential of mesenchymal stem cells to aid in tissue repair (272). LIF is involved in maintaining self-renewal of embryonic stem cells (273), thus may be involved in wound healing in burns. In burns, LIF appears to show an increase in its levels in the serum at later time points, and was significantly

higher at the beginning of moderate burns and throughout compared to other severities of burn injury. This suggests there may be a range in which LIF is more activated in correlation with severity of injury and inflammation, which may not reach the threshold in mild burns, but may be beyond recovery or inhibited by another factor in severe burns. This is the first observational study of LIF in burns, so there remains a large unknown in its involvement in scar development.

Platelet-derived growth factor (PDGF) isoforms AB and BB have been found to be potent chemoattractants for lung fibroblasts (274). Asthma is another form of chronic inflammation, in which PDGF enhances collagen synthesis and lung fibroblasts (275), which may be a promising mediator in therapy. There was a randomized control trial of 50 patients with burn injuries, with a platelet dressing of PDGF which showed enhanced epithelialization and granule tissue formation (276). Our results show significantly lower levels of both PDGF-AA and PDGF-AB/BB in the first few days after injury in mild burns, with a similar pattern of lower levels after burn injury in all severities and increasing at a later time point. Combining the previous platelet dressing study and our results, this suggests that the best time to administer a topical agent including PDGF would be early on in the wound healing stages, in which future studies could lead to an appropriate therapy for better burn recovery and outcomes.

Vascular endothelial growth factor-A (VEGF-A) is a pro-angiogenic factor that is secreted by cells and endothelial cells (277). In diseases with abnormal growth of blood vessels, VEGF-A plays an important role in wound healing for vascular regeneration and remodeling (278, 279). By inhibiting VEGFs with a specific peptide, VGB4, this led to decreased angiogenesis and induction of apoptosis (280). Previously, VEGF serum levels are found to be significantly increased after burn injuries, and has been suggested that an antagonist may have beneficial effects for severe burn patients (281). A more observational study showing the levels

of VEGF-A over time and burn severity has not yet been done. Our results showed a significant increase from initial injury in mild burns to 8-14 days, and while levels dropped in mild burns, in severe burns the levels increasingly rose up to 30 days. As hypothesised by other researchers, there may be a benefit to eliciting an antagonist for VEGF in severe burn injuries, to aid in wound closure and normalize wound healing.

Supplemental Cytokine ANOVA Figures

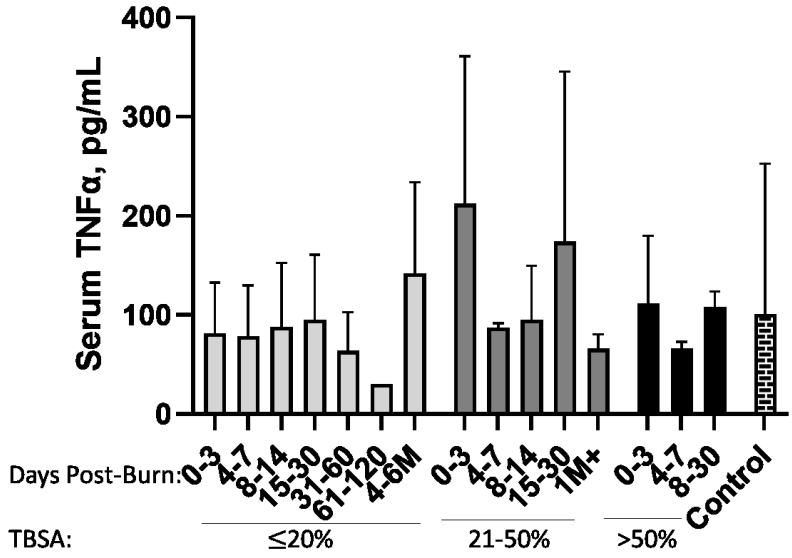


Figure 12. Serum levels of TGFα at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

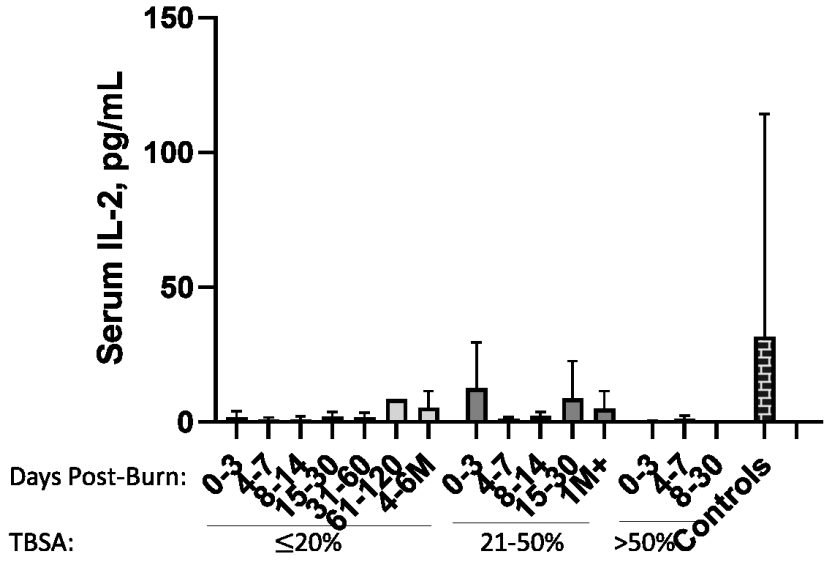


Figure 13. Serum levels of IL-2 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

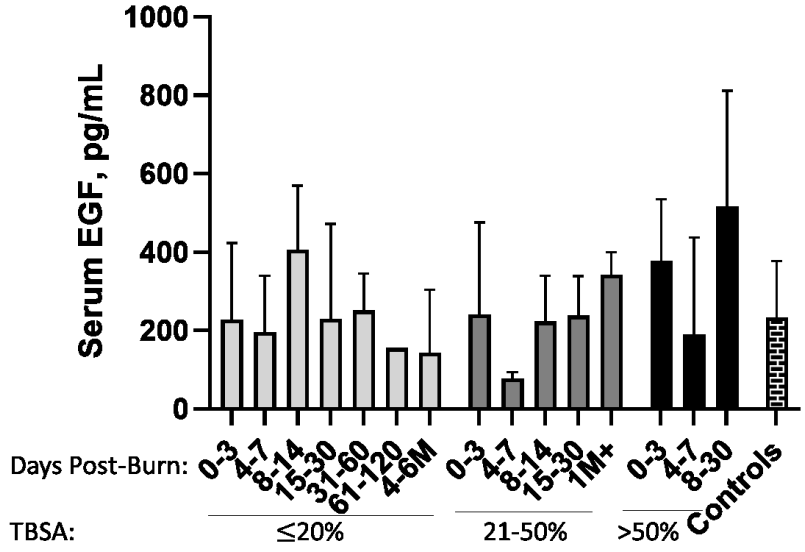


Figure 14. Serum levels of EGF at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

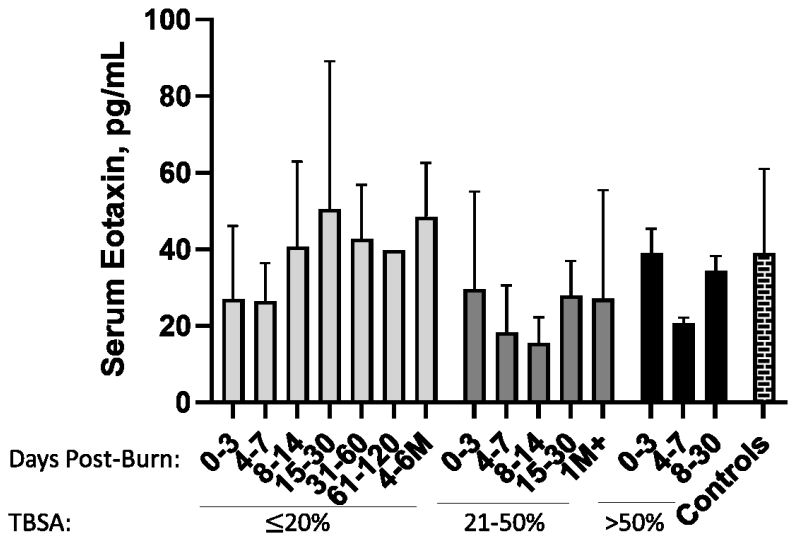


Figure 15. Serum levels of Eotaxin at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

Figure 16. Serum levels of FGF-2 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

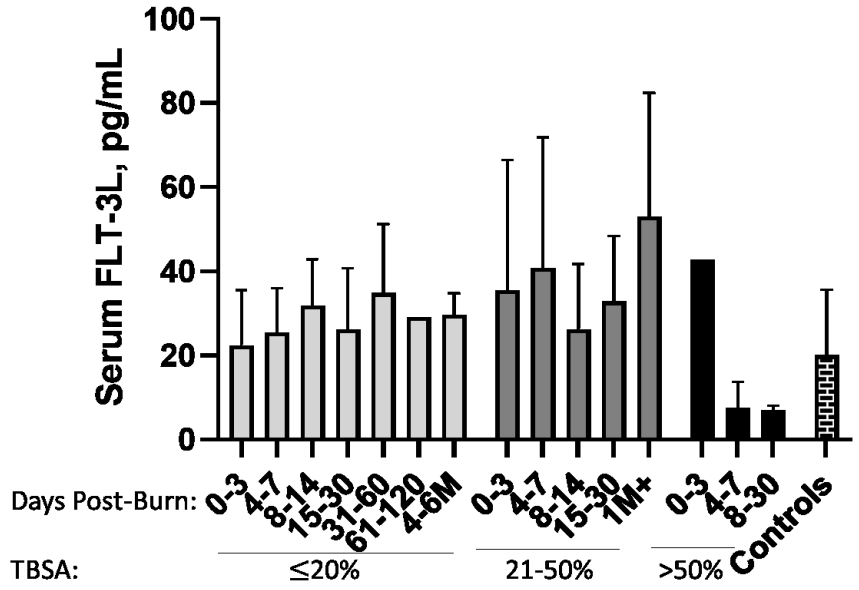


Figure 17. Serum levels of FLT-3L at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

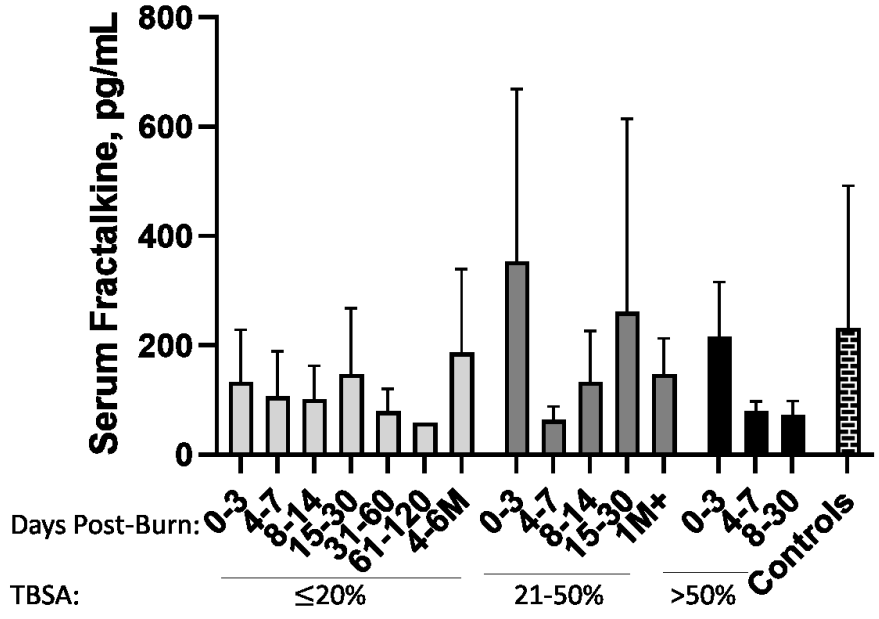


Figure 18. Serum levels of Fractalkine at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

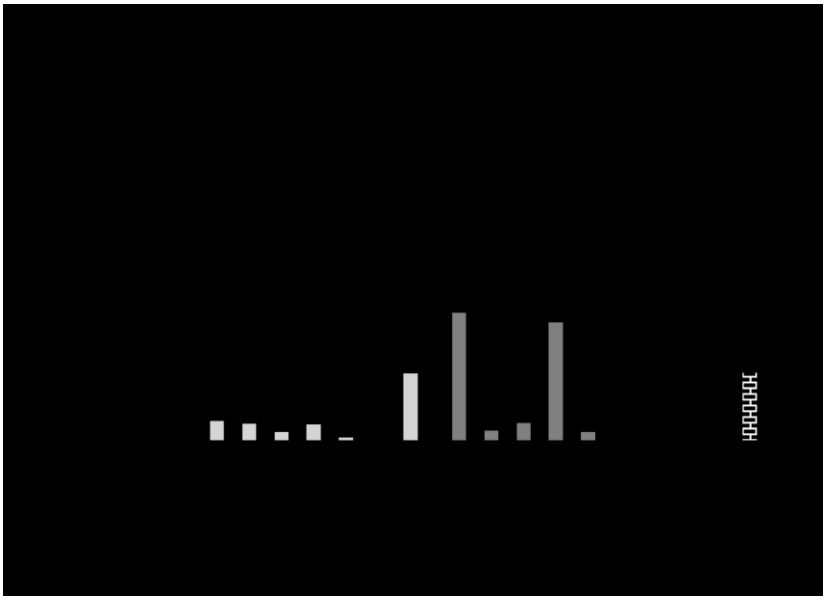


Figure 19. Serum levels of GM-CSF at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

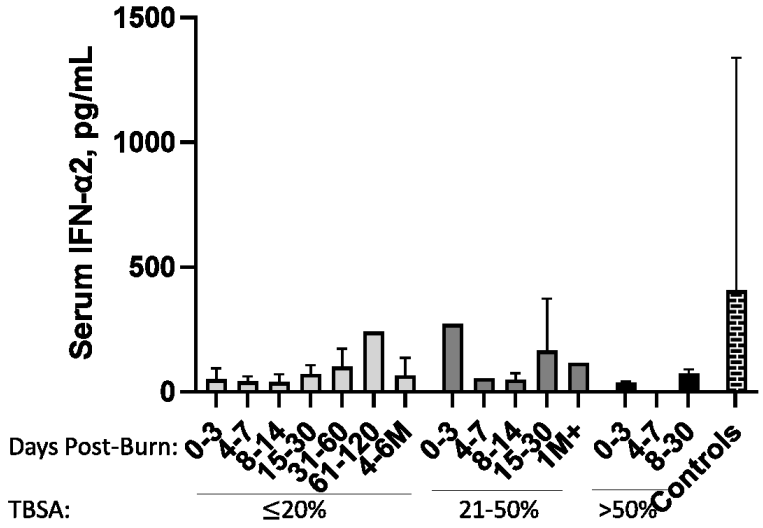


Figure 20. Serum levels of IFN-α2 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

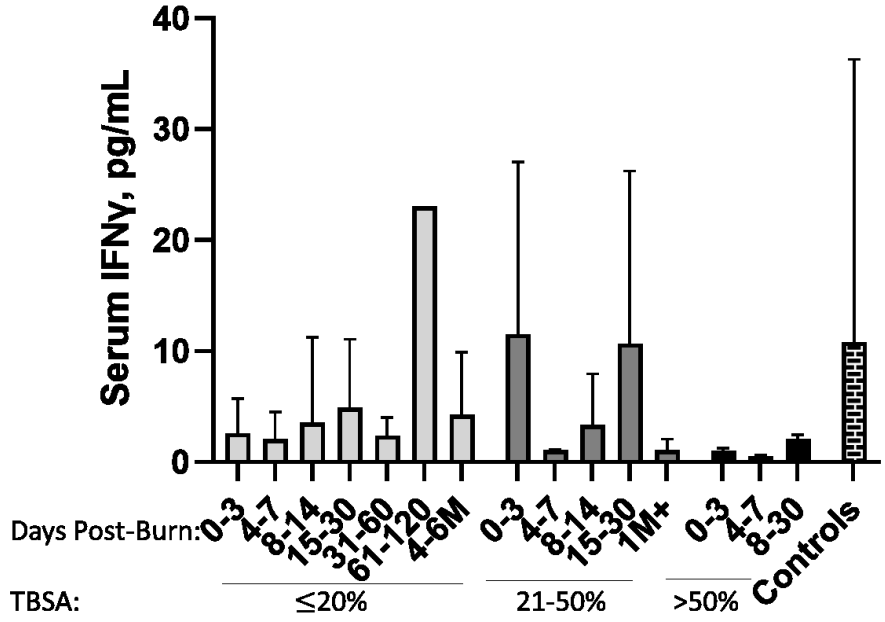


Figure 21. Serum levels of IFNγ at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

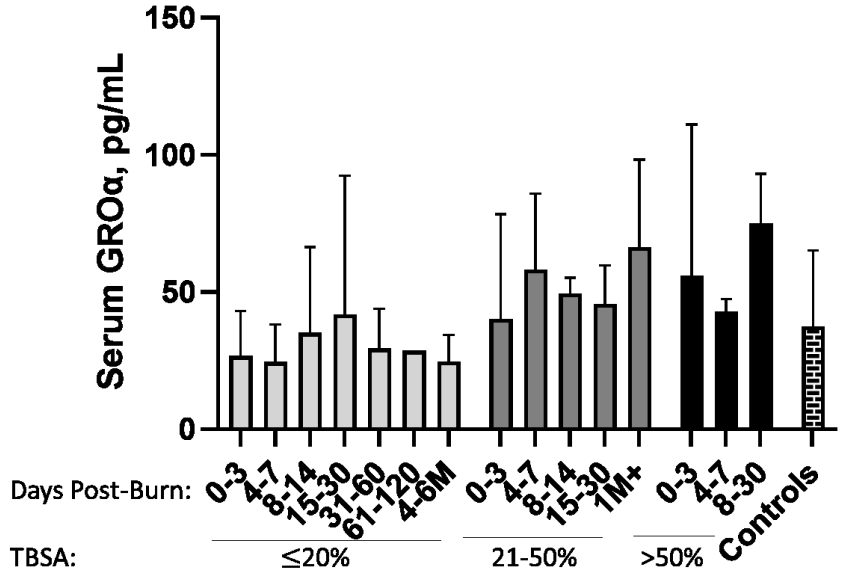


Figure 22. Serum levels of GROα at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

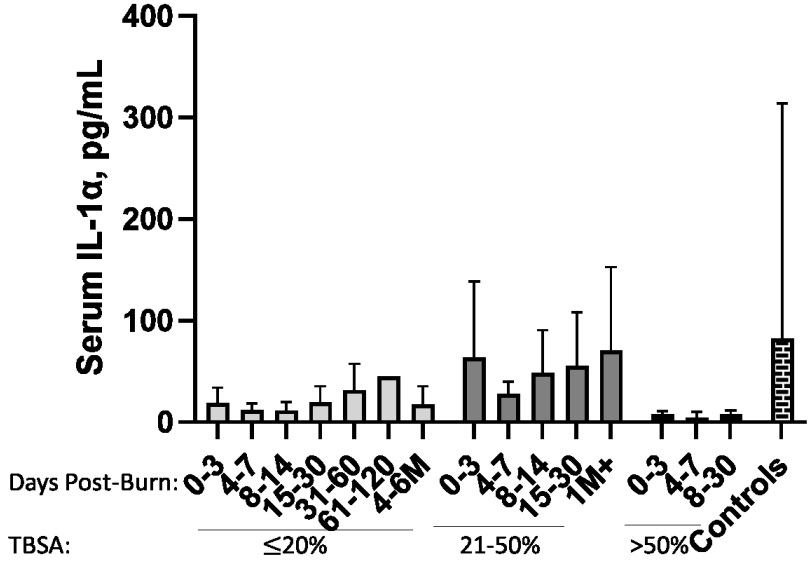


Figure 23. Serum levels of IL-1α at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

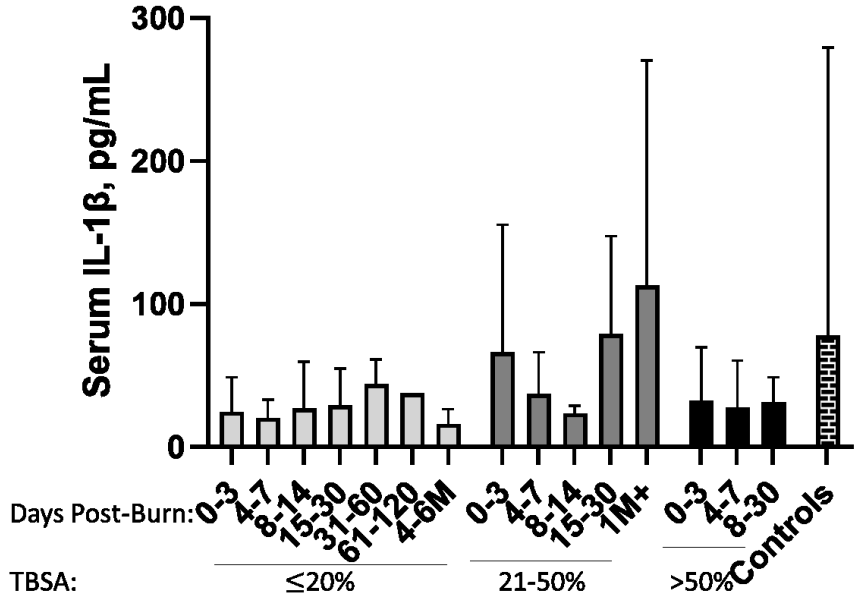


Figure 24. Serum levels of IL-1β at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

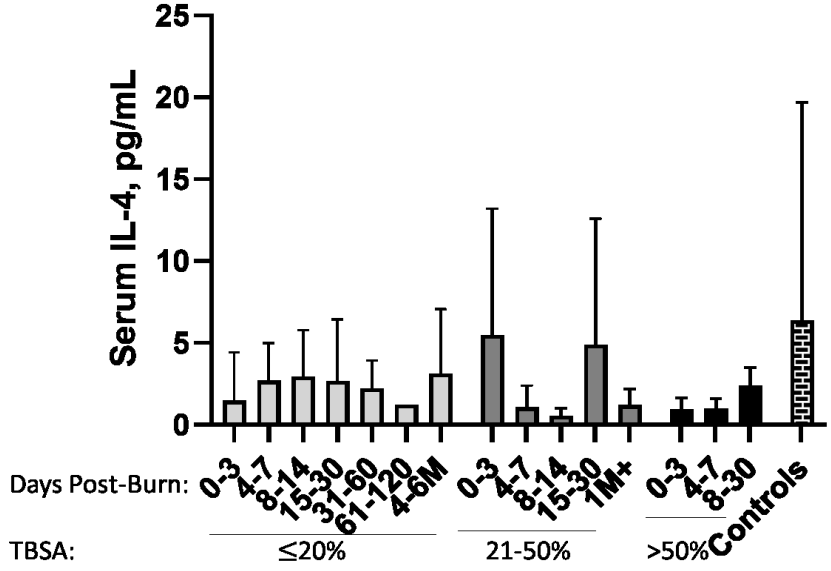


Figure 25. Serum levels of IL-4 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

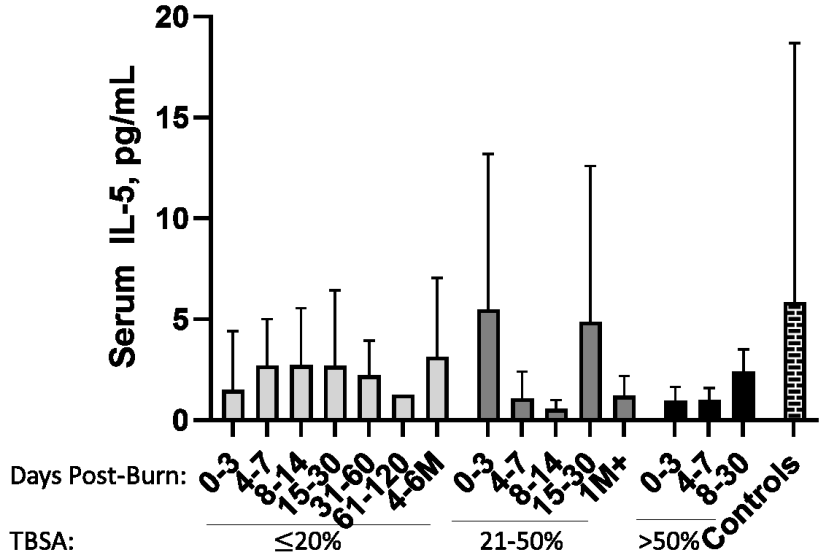


Figure 26. Serum levels of IL-5 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

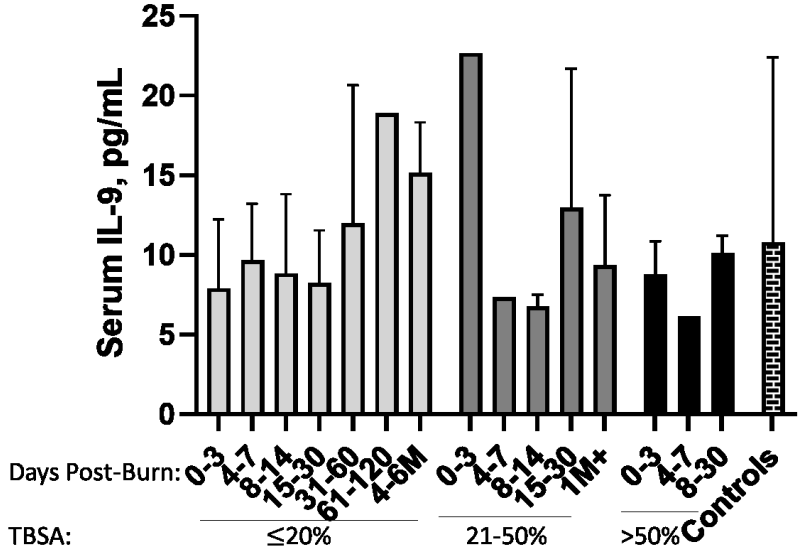


Figure 27. Serum levels of IL-9 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

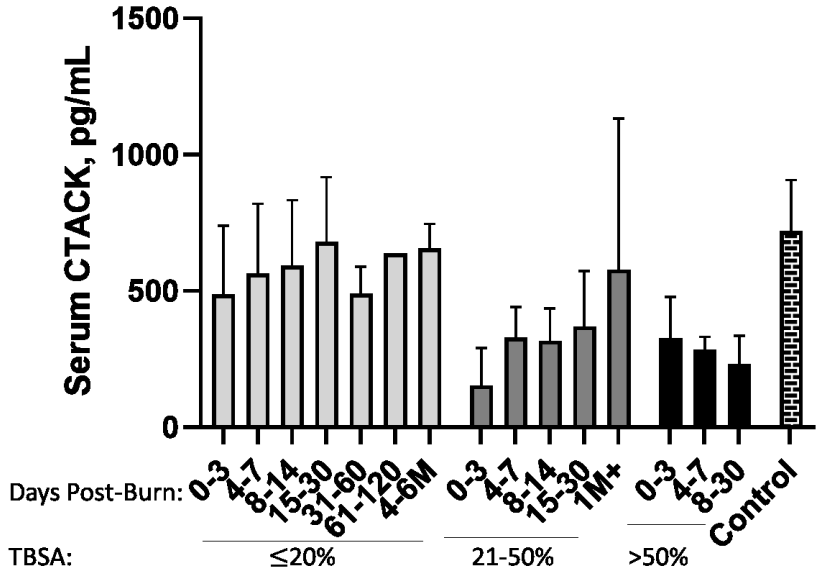


Figure 28. Serum levels of CTACK at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

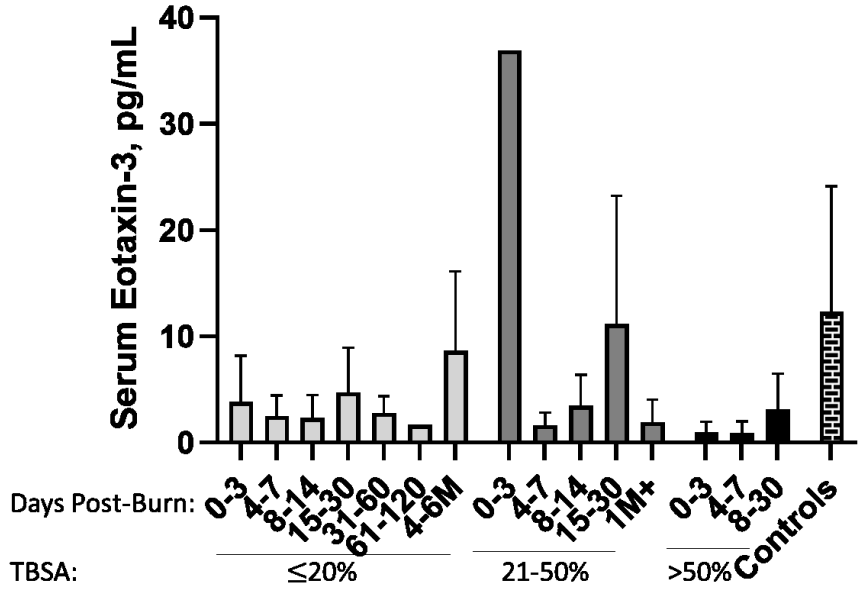


Figure 29. Serum levels of Eotaxin-3 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

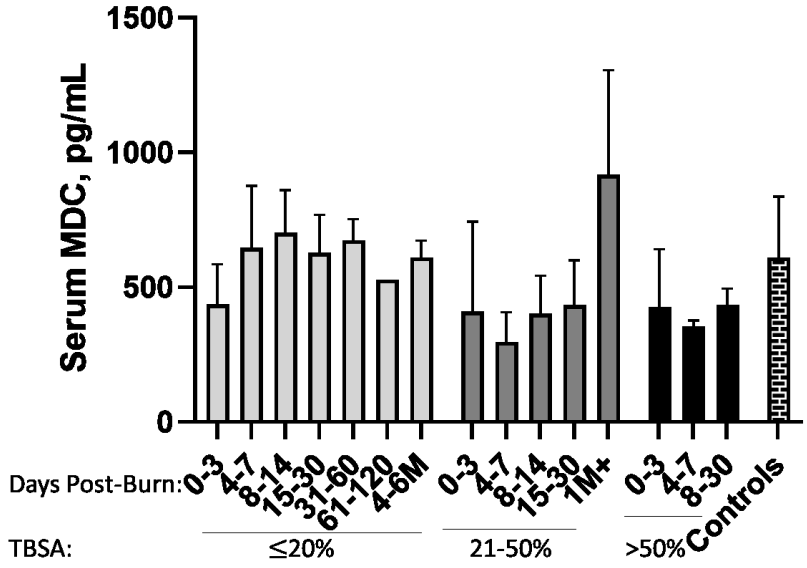


Figure 30. Serum levels of MDC at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

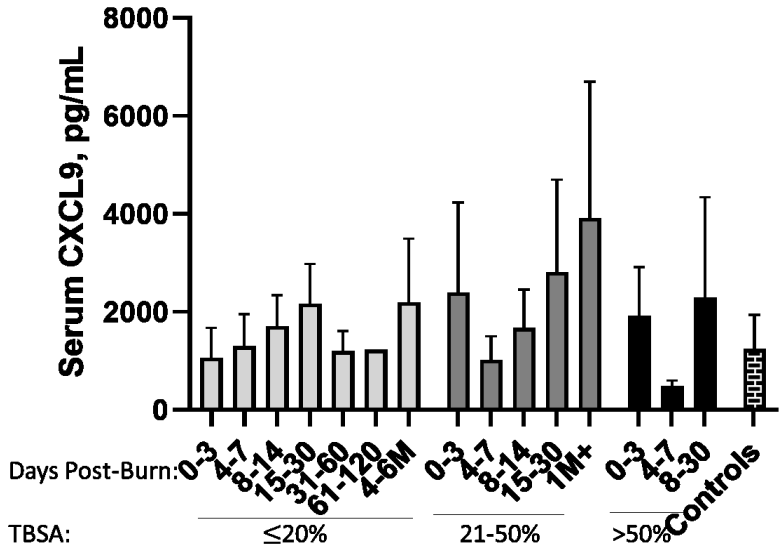


Figure 31. Serum levels of CXCL9 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

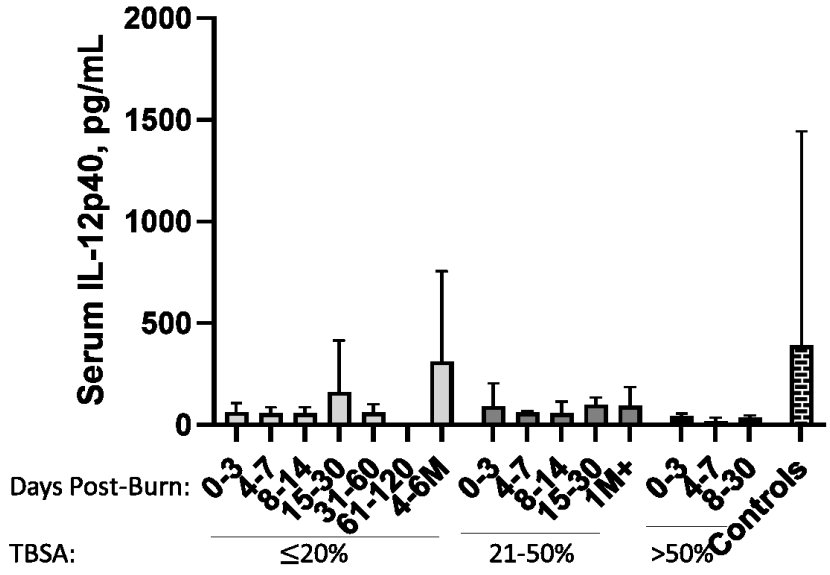


Figure 32. Serum levels of IL-12p40 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

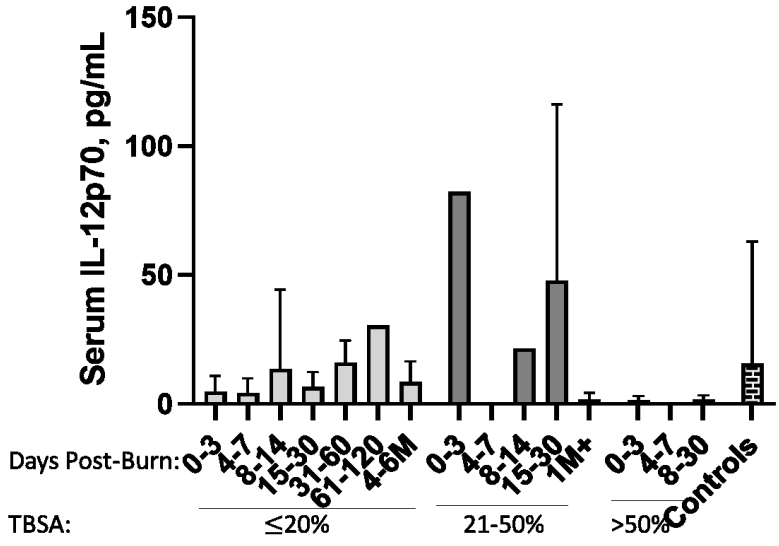


Figure 33. Serum levels of IL-12p70 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

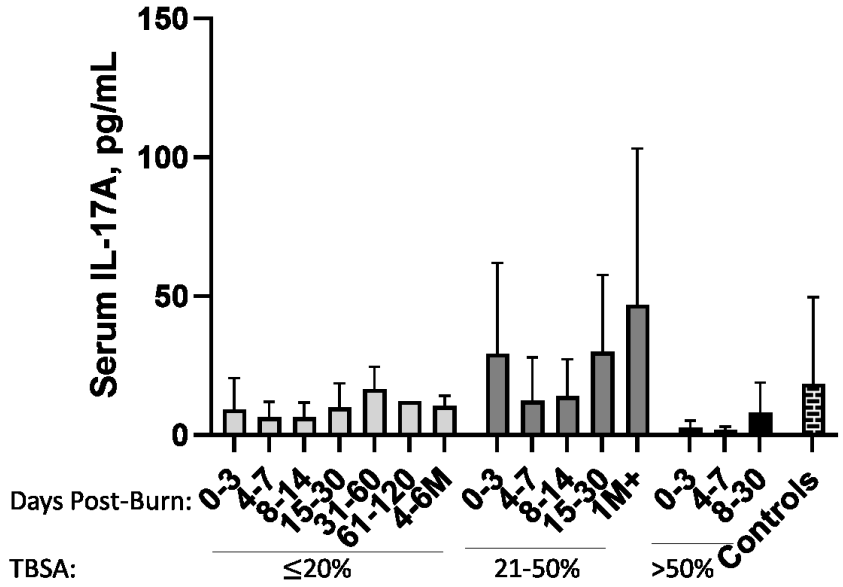


Figure 34. Serum levels of IL-17A at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

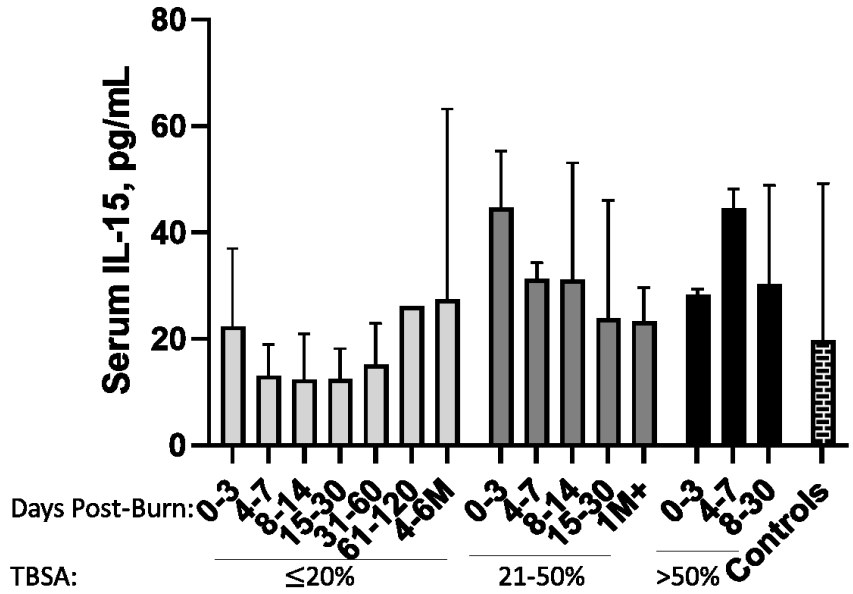


Figure 35. Serum levels of IL-15 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

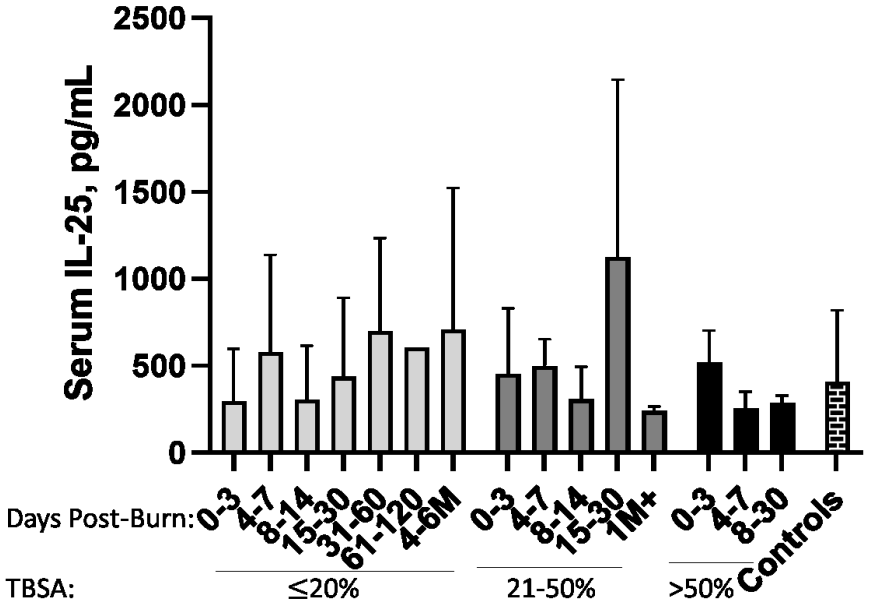


Figure 36. Serum levels of IL-25 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

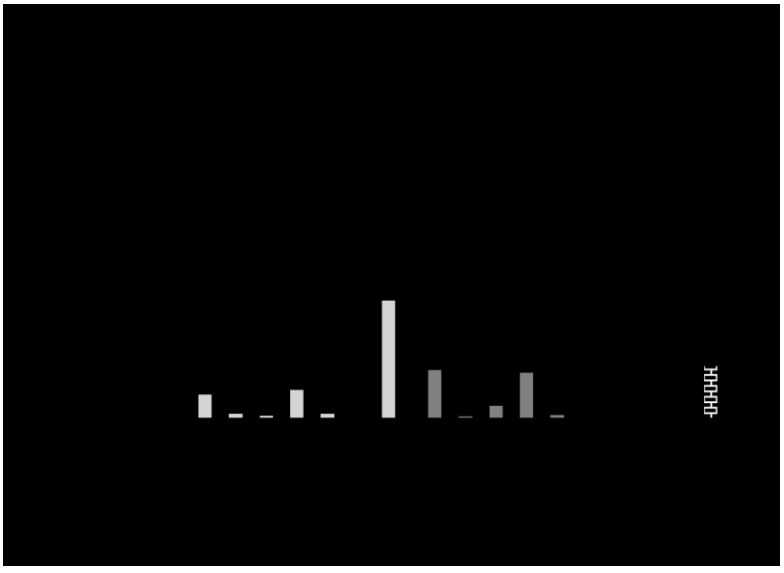


Figure 37. Serum levels of IL-17F at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

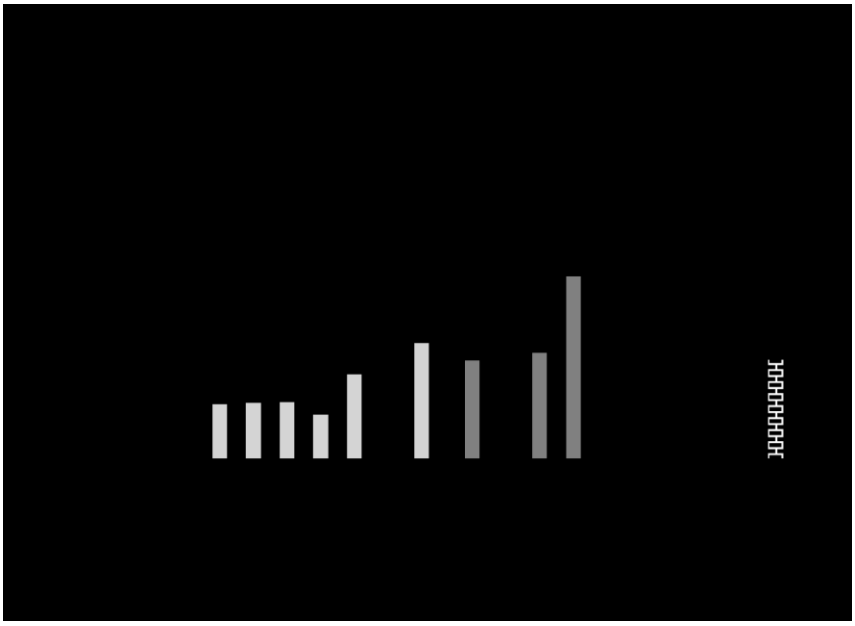


Figure 38. Serum levels of IL-22 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

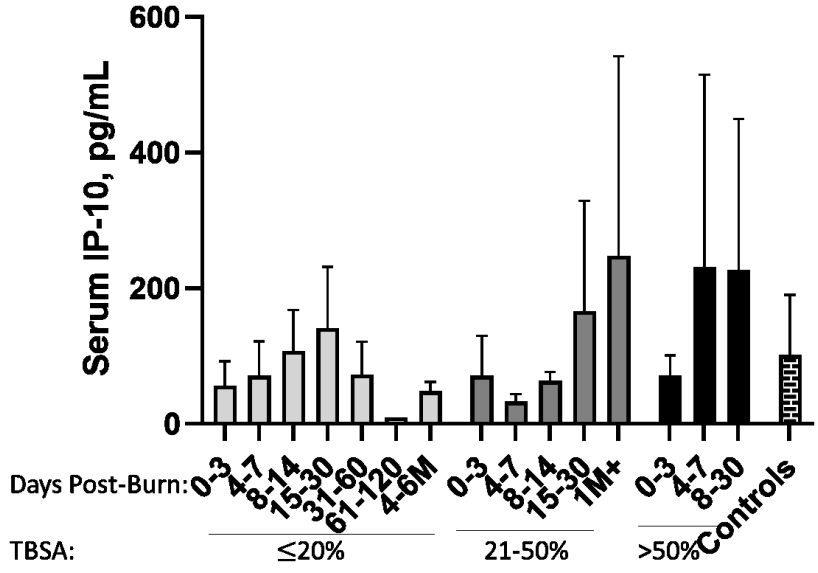


Figure 39. Serum levels of IP-10 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

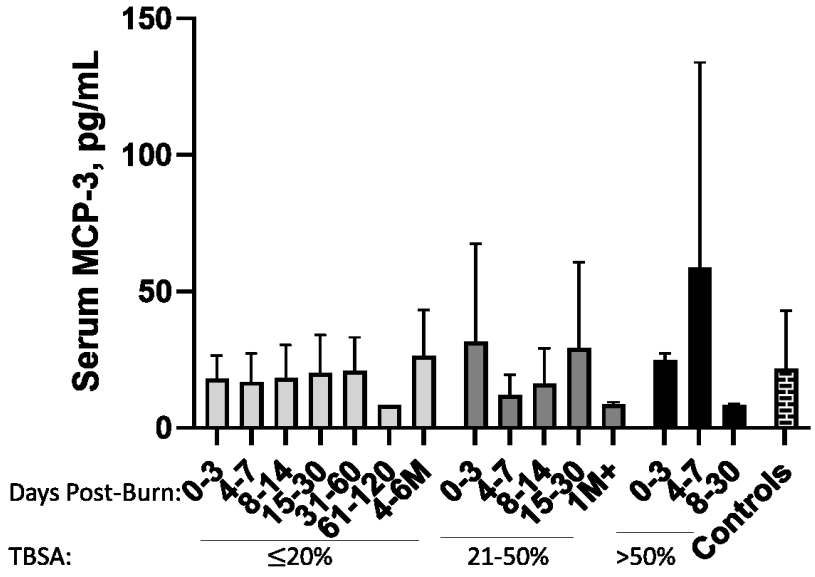


Figure 40. Serum levels of MCP-3 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

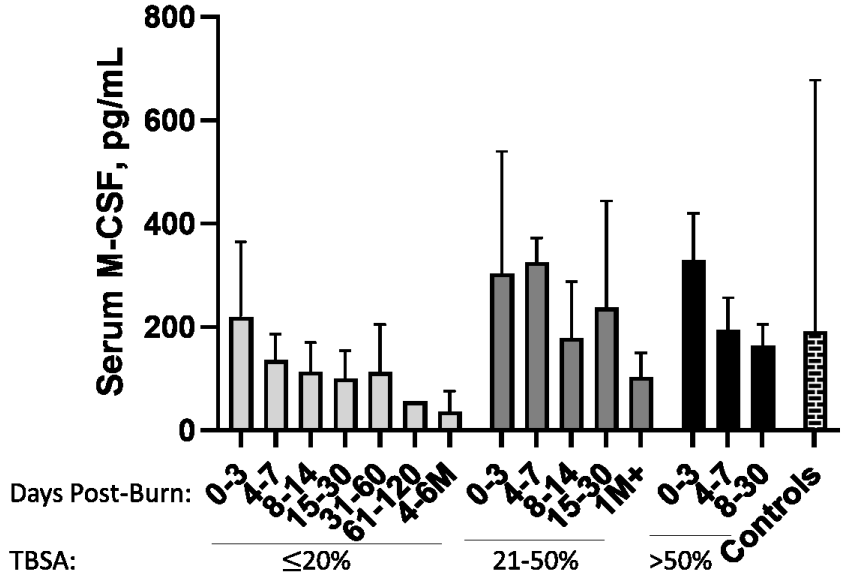


Figure 41. Serum levels of M-CSF at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

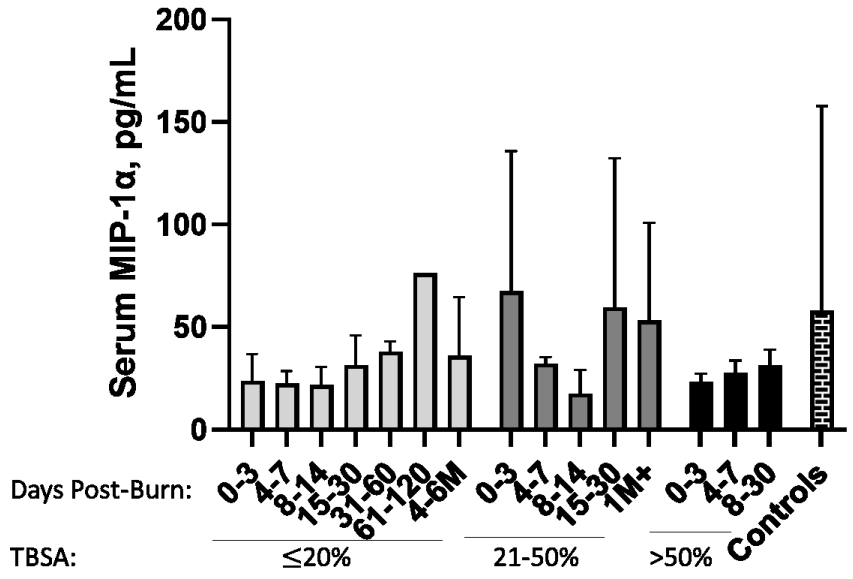


Figure 42. Serum levels of MIP-1α at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

Figure 43. Serum levels of MIP-1β at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

Figure 44. Serum levels of TNFα at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

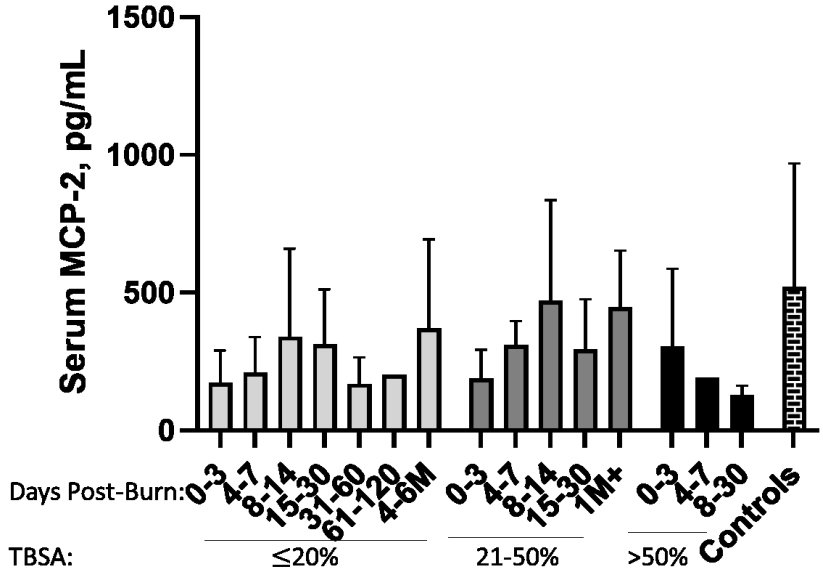


Figure 45. Serum levels of MCP-2 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

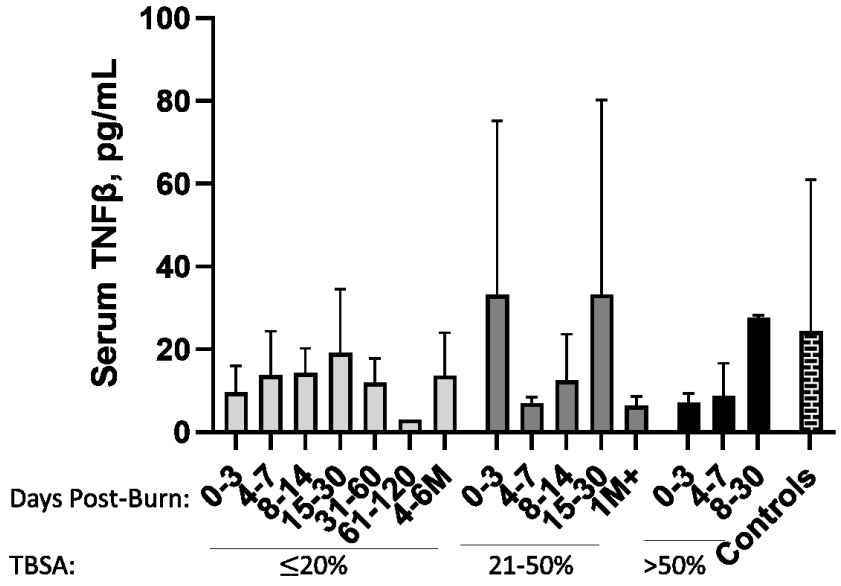


Figure 46. Serum levels of TNFβ at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

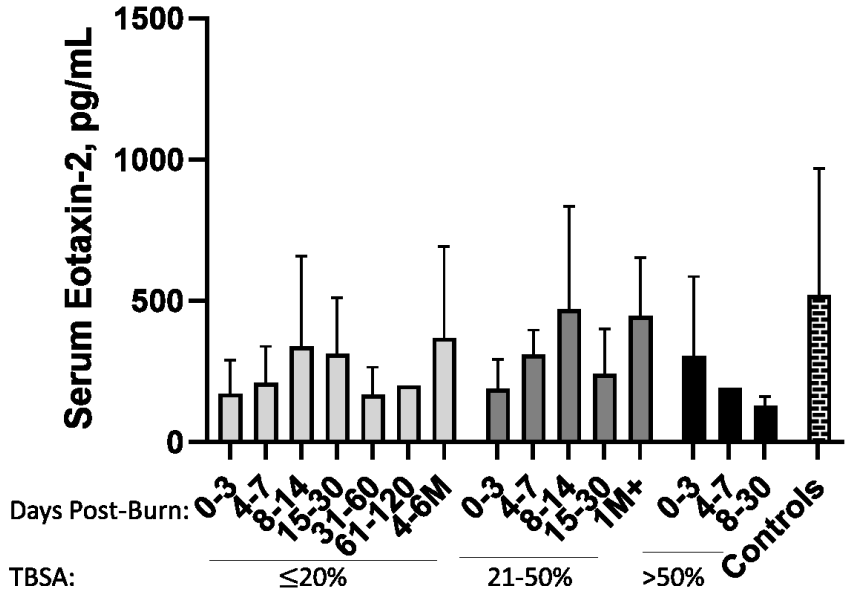


Figure 47. Serum levels of Eotaxin-2 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

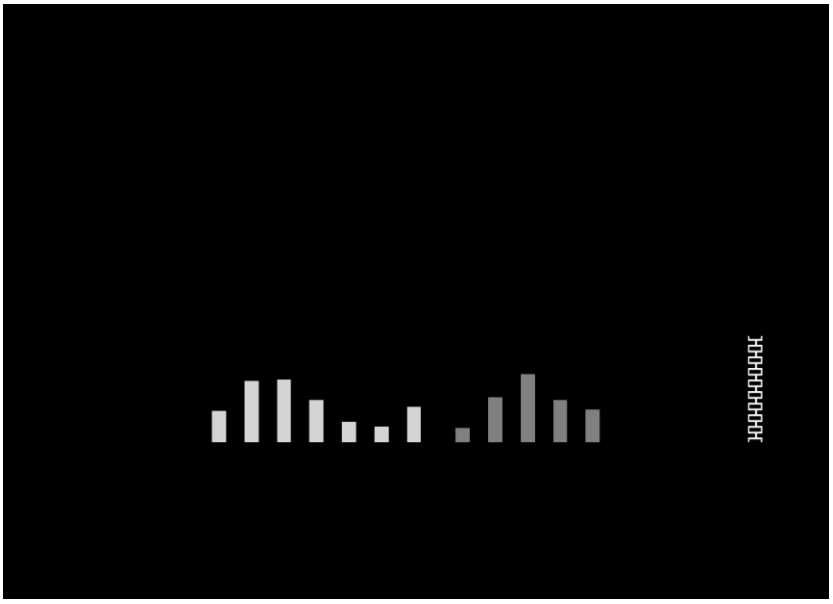


Figure 48. Serum levels of I-309 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

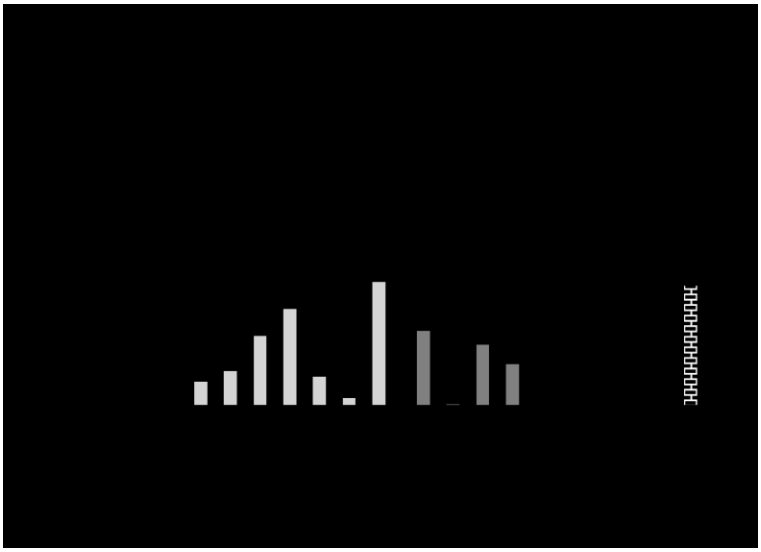


Figure 49. Serum levels of MCP-4 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

Figure 50. Serum levels of IL-16 at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

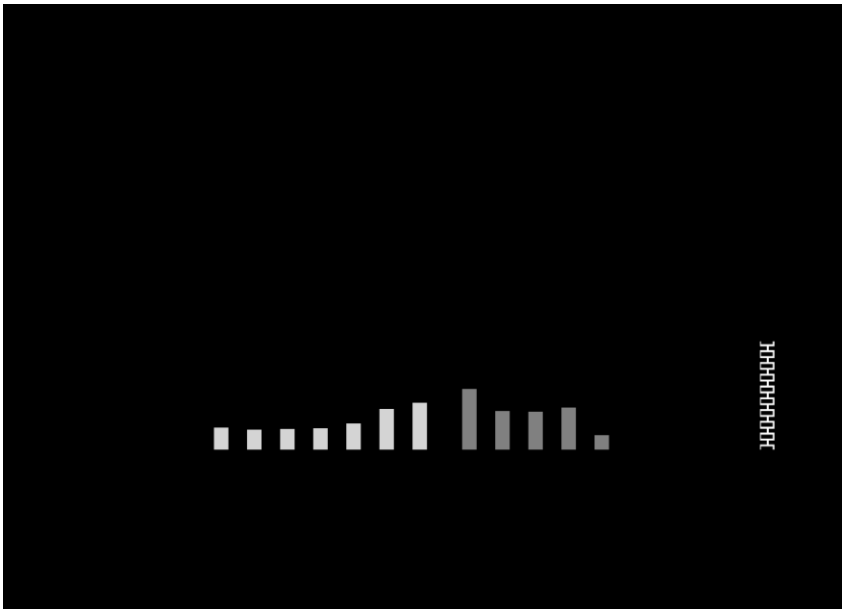


Figure 51. Serum levels of 6CKine at varying levels of burn injury and time points. There was no significance in this data set between groups or time points.

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