The Immune Response of a Burn Injury Compared to an Excisional Injury in a Murine Model

Volume 1

Samantha Maria Valvis

Submitted in accordance with the requirements for the degree of Doctor of Medicine

The University of Leeds
School of Medicine

March 2018

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

All chapters formed the base of a jointly authored publication; Valvis, S. M., Waithman, J., Wood, F. M., Fear, M. W., & Fear, V. S. (2015). The Immune Response to Skin Trauma Is Dependent on the Etiology of Injury in a Mouse Model of Burn and Excision. *Journal of Investigative Dermatology*.

I was involved with murine injury and monitoring; sample collection; lymph node preparation; whole blood and serum preparation; cytokine assay and data analysis. Dr Jason Waitman assisted in splenic assays. Prof Fiona Wood was involved with experimental design. Ass Prof Mark Fear was involved with experimental design, manuscript editing. Dr Vanessa Fear supervised data collection; flow cytometry analysis; data interpretation and analysis and manuscript writing.

Acknowledgements

This research has been carried out by a team that has included; Dr Mark Fear, Prof Suzanne Rea, Dr Vanessa Fear and Prof Fiona Wood. My own contributions, fully and explicitly indicated in the thesis, have been; murine injury and monitoring; sample collection; lymph node preparation; whole blood and serum preparation; cytokine assay; data analysis & thesis writing.

The other members of the group and their contributions have been as follows:

Ass Prof Mark Fear was involved with experimental design, ethical approval, experimental supervision; data interpretation and thesis editing.

Dr Vanessa Fear supervised data collection and lymph node preparation; provided flow cytometry analysis; assisted in data interpretation and analysis and thesis editing.

Ass Prof Hilary Wallace assisted with statistical analysis.

Dr Janine Duke assisted with statistical analysis.

Prof Suzanne Rea was involved in supervising animal injury procedure.

Prof Fiona Wood was involved with experimental design and thesis editing.

I would like to thank all my supervisors Assoc/Prof Mark Fear, Assoc/Prof W/Prof Fiona Wood and Prof Petros Giannoudis. To Mark, thank you for your support and feedback. Vanessa, thank you for your teaching and

patience. Prof Wood, thank you for your endless motivation and encouragement. Thank you Prof Rea for helping with the animal work and for your support and feedback over these years. Hilary and Janine, thank you for all your advice and support, and your help with the understanding of statistical analysis.

Andy and Dulharie, thank you for your friendship and teaching me how to be a ‘scientist’, I couldn’t have done this without you. Thank you to my colleagues at BIRU, Vetri, Uliya and Mansur for all your support.

Thanks to Mary and at Cell Central and Leah for all your help in the Pre-Clinical Facility (PCF).

To Sean my husband, thank you for your love & support, relentless patience, and coping with my general absence; I couldn’t have done this without you by my side. To my parents, sister, brother and friends, thank you for your support and encouragement. I couldn’t have achieved anything without you all.

Finally, I would like to extend my gratitude to the Fiona Wood Foundation and the Cynthia Banham Surgical research scholarship for the support they have given me during the course of my studies.

Abstract

Cutaneous injury triggers a significant immune response. Cells of the innate and adaptive systems are recruited to coordinate repair, prevent infection and limit damage. It remains unclear if different aetiologies of injury influences the characteristics of the immune response as well as the long-term impact post injury.

A murine model was used to characterise the immune response. A 8% TBSA full thickness dorsal burn or excision injury was used. At days 1,3,7,14,28 and 84 post injury mice were euthanased; whole blood was collected for haematology and cytokine profiling, and inguinal lymph nodes were harvested for immune cell populations including dendritic and T cells.

Both the innate and adaptive immune responses differed between the two aetiologies. The burn injury generated a rapid and greater acute cytokine response displaying both Th1 and Th2 cytokine profiles resulting in changes to the innate cells, with a drive towards monocyte to macrophage differentiation following burn injury. The dendritic cell profile differed between the two injury groups, with reduced dendritic cell maturation that persisted to day 84 post-injury. The cytokine and dendritic cell profiles appeared to impact the adaptive response, with reduced T cell activation after burn injury which was sustained to the later time points.

The data suggests there are significant differences in the immune response, which is dependent on injury aetiology. The burn injury had sustained an immunological change that was not apparent following excisional injury. This may explain in part the long-term implications of burn injury, such as increased incidence of infection and malignancy, and provides a basis for further investigation to guide clinical intervention after burn injury.

Table of Contents

Appendix

A. University of Western Australia Animal Ethics Committee Application & Approval (Perth, Western Australia; Animal Ethics Approval Number RA/3/100/1155)

B. Post Mortem Results; Burn Day 28 group

C. Post Mortem Results; Excision Day 84 group

D. Valvis, S. M., Waithman, J., Wood, F. M., Fear, M. W., & Fear, V. S. (2015). The Immune Response to Skin Trauma Is Dependent on the Etiology of Injury in a Mouse Model of Burn and Excision. *Journal of Investigative Dermatology***.**

E. Fear, V.S., Wee-Peng, P., Valvis, S.M., Waithman, J., Foley, B., Wood, F.M., Fear. M.W., (2016). Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. *Burns,*

List of Tables and Figures

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Abbreviations

APC	Antigen presenting cells
APC	Allophycocyanin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
B.D	Bis Die (Twice a daily)
BSA	Bovine serum albumin
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of differentiation
cDC	Conventional dendritic cells
CDP	Common dendritic cell precursor
CSF1	Colony stimulating factor 1
CWC	Cotton wool column
DALYs	Disability adjusted life years
DAMP	Danger associated molecular patterns
DC	Dendritic cells
dDC	Dermal dendritic cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGP	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead P3

G-CSF	Granulocyte colony-stimulating factor
GKN	Glucose potassium and nitrate solution
GM-CSF	Granulocyte macrophage colony stimulating factor
H_2O	Water
H&E	Haematoxylin and eosin
HLA-DR	human leukocyte antigens-DR
HMGB-1	High mobility group box 1 protein
HSP	Heat shock proteins
iDC	Immature dendritic cell
IGF	Insulin-like Growth Factor
IgG	Immunoglobulin G
ICU	Intensive care unit
IL-	Interleukin
I.M	Intramuscular
INF-γ	Interferon gamma
I.P	Intraperitoneal
ISS	Injury severity score
KGF	Keratinocyte growth factor
KC	Keratinocyte derived cytokine
KCl	Potassium chloride
LC	Langerhans cells
LN	Lymph node
mAB	Monoclonal antibodies
MafB	musculoaponeurotic fibrosarcoma oncogene homolog B
MCP-1	Monocyte chemoattractant protein-1

MDP	Macrophage-dendritic cell progenitor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MR	Mannose receptor
NaCl	Sodium chloride
Na_2HPO_4	Disodium phosphate
NHMRC	National Health and Medical Research Council
NLR	NOD-like receptor
NK	Natural killer cells
NO	Nitric oxide
nTreg	Natural T regulatory cell
PBS	Phosphate buffered solution
pDC	Plasmacytoid dentritic cell
PAMP	Pathogen associated molecular patterns
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PMN	Polymorphonuclear cells
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T expressed and secreted
RBC	Red blood cells
RLR	RIG-I-like receptors
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
TBSA	Total body surface area

TCR	T cell receptor
T.D.S	Ter Die Sumendum (Three times a day)
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
Th	T-Helper
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-α
Treg	T regulatory cell
WCC	White cell count

Chapter 1

Introduction

1.1: Introduction

Burn injuries have a significant global impact medically and socio-economically. The World Health Organisation has estimated that 195,000 burn related deaths occur worldwide per year. Furthermore, burn injury is ranked 34th of all causes of disability-adjusted life years (DALYs) from 21 different worldwide regions; where DALYs are defined as the sum of years of life lost secondary to premature mortality and the years lived with disability (disability defined as short and long-term health loss aside from death, and health defined in terms of function, including mobility, pain affect and cognition) [1]. The loss of economic contribution following the initial recovery post burn is linked to the age at the time of injury, to local scar outcome limiting physical and psychological function [2] and the widespread systemic impact [3-7].

In the United Kingdom approximately 250,000 people sustain a burn injury each year. The burn patient population cohort is heterogenous, 30% of admissions are paediatric which includes over half of the severe burns reported [8]. Of the 175,000 patients attending medical services 13,000 require hospital admission, of which 1000 require formal fluid resuscitation due to the extent of the injury, and approximately 300 deaths are reported per year [8]. The remainder of burn injury admissions are non-severe injuries but may require a range of interventions to achieve expedient wound healing. The incidence of burn injury in the UK has remained fairly constant over the last

decade with an average of 20.6 per 100,000 (12,400 based on population of 62 million) in England and Wales between 2005 and 2009 admitted to the National Health Services for burn injury [9]. Similar burn injury admission rates are seen in other developed countries, with a single centre In Western Australia (WA) reporting that the majority (86%) of burn injuries seen over a 25 year period were non major injuries [10], with 60% of the total patients having burns of less than 1% total body surface area (TBSA) [11]. This strongly suggests a large proportion of health care service demands are secondary to what can be termed as a non-severe burn injury (<10% TBSA without inhalation injury) [12]. The linkage of the hospital data in WA has reported an impact on the life time risk of a range of subsequent pathologies despite the majority of the burn injuries being non-severe [3, 5, 6, 13, 14].

Following burn injury, patients have a high incidence of infection and sepsis compared to other categories of trauma [15, 16]. This has been attributed to multiple factors including disturbances in the wound microflora (initially sterile with progressive colonisation within 7 days) [17-19]. In addition, a persistent systemic inflammatory response syndrome (SIRS) predisposes the development of subsequent compensatory anti-inflammatory response syndrome (CARS), which renders these patients vulnerable to infectious challenge [20]. The burn patients have a differing cytokine profile (increased IL-6 & IL-8) with increased incidence in the development clinical complications associated with this in comparison to other trauma of the same standard measure (Injury Severity Score (ISS) [21, 22]. Standardised trauma outcome scoring systems such as the Injury Severity Scoring (ISS) system are supportive of this disparity in the immune response to different injury as burns

have a poorer than predicted outcome which is not observed in other forms of trauma [23]. The cytokine profiles observed in burn injury correlate clinically with the presence of SIRS, characterised by hypo or hyperthermia, tachycardia, tachypnoea and leucocytosis. In consequence a suppressed T cell response may follow SIRS, characterised clinically by cutaneous anergy, hypothermia, leukopenia, infection susceptibility and failure to clear infection (CARS) [20, 24-27].

In response to the clinical differences observed between burn and non-burn patients The National Institute of General Medical Sciences (NIGMS) embarked on a large collaborative research program known as “Inflammation and the Host Response to Injury”. This program supported research into the immune response to different injury types which involved profiling immune cells during the acute phase response. This included transcriptome profiles which demonstrated that although there are many changes to gene expression that are common between different injury aetiologies there are also some distinct signatures that appear to be related to the different aetiologies of injury [28].

There is also evidence that a sexual dimorphism exists in the immune response following trauma. At present the exact interactions between the sex hormones and the immune systems are unclear. Clinically, male burn injury patients have a lower morbidity and mortality in both the acute and long-term settings, whereas female mortality rates are over twice that of males [5, 29, 30]. In contrast the opposite is seen in non-burn injury with females having a lower morbidity and mortality in both trauma and surgical injury [31, 32]. Most literature reports that the differences seen between genders cease after the

ages of 50-65 years, coinciding with female menopause [30, 32, 33]. The gender disparity following burn and non-burn further supports that there is a difference in the cell-mediated immunity following different injury [34, 35].

Recent epidemiological studies have demonstrated that the long term sequelae observed following burn injury is more severe than in other cutaneous trauma. This includes an increased lifetime risk of cardiac dysfunction, an increased incidence of malignancy and increased age adjusted mortality in burns injury patients. The data demonstrating an association of burn injury to long-term sequelae has been consistently observed in several population-based studies [3, 5, 6, 14, 36, 37]. While it is clear the burns cohort differ to the general population with regards to increased lifestyle risk factors, a higher incidence of lower socioeconomic status as well as a higher number of indigenous patients when compared to, data analysis in these papers used well accepted methods to adjust for these co-variances that are known to have an impact on health outcomes [3, 5, 6, 14, 36, 37]. Furthermore, studies of paediatric patients have also observed similar increases in post burn injury sequalae, including an increase in age adjusted mortality, minimising the likely impact of lifestyle factors on the observed differences [37]. The differences in the long-term impact post burn and non-burn trauma have also been identified in population based studies, demonstrating a greater impact in burn injury when compared to other trauma types [3].

The cardiac changes have been observed in both animal and human scientific studies and population-based studies [3, 38]. With regards to the malignant change, it has been observed that there is a greater prevalence for non-

cutaneous malignant change in females [39-43]. This supports the literature surrounding the gender dimorphism that occurs in the inflammatory response post trauma suggesting the presence of a long-term impact and alteration to the immune system following burn injury.

In summary, there is clear evidence from both clinical and population based studies of differences in the impact following trauma of different aetiology, both in the acute and long-term setting. However, to date there has been limited investigation into why the aetiology matters. There is also paucity in the literature with regards to the impact of non-severe burn injury, with the focus of most studies confined to major/severe burn injury. Therefore the work presented in this thesis has focused on understanding differences in the immune response to different injury types to investigate whether the impact on the immune response drives the differences seen in the clinical consequences for patients. The work has also focused on non-severe burn injury as this represents the majority of the clinical caseload in developed countries. Further to this, there is a need to understand the systemic impact of the burn injury with respect to the inflammation and the immune response. The understanding of the underlying mechanism will drive therapeutic opportunities into the future.

The following chapters provide the context by exploring the structure of the skin, the relevant immune systems, the sequelae of injury and outlining the current knowledge on which the experimental design was established.

1.2: The Skin

The skin is the largest organ of the body and accounts for up to 15% of total body weight and on average covers an area of 1.7m^2 [44]. The skin is host to a number of cell types which each plays a role in enabling the skin to perform a multitude of functions, including; protection from physical, chemical and pathological insults, thermoregulation, electrolyte and fluid balance. The function of the skin is related to its architecture and the presence of appendage structures. In consequence significant injury to the skin and disruption to these structures can result in circulatory shock, ARDS and multi-organ failure [45, 46].

The skin is broadly divided into 2 layers, the epidermis and dermis. The epidermis primarily contains keratinocytes, as well as melanocytes, Merkel cells and scattered Langerhans cells (LCs). Langerhans cells are antigen presenting cells (APCs) unique to the epidermis. The dermis primarily consists of fibroblasts, which are responsible for the secretion of collagen, elastic fibers and the production of ground substance [47]. In addition there are dendritic cells (DCs) that are the APCs of the dermis. The dermis contains the capillary and lymphatic networks that support these structures and the epidermis. Other immune cells of the dermis include natural killer cells and gamma delta T cells (see section 1.3.3).

The use of murine models in the study of the skin and the immune system have been used for many years and has provided significant insight into skin function, wound repair and the immune response. However, despite many

structural and functional similarities, some key differences must be acknowledged [48]. Human skin is thicker & stiffer than murine skin due to more epidermal layers and a thicker dermis. Mice have more densely packed hair follicles and a different transition point between epidermis and dermis known as the dermal-epidermal junction, which is relatively flat with the exception of the hair follicles causing multiple involutions [49, 50]. In contrast in humans there are fewer hair follicles and the dermis abuts the epidermis [49, 50]. Mouse skin is more prone to wound contraction due to the presence of the panniculous carnosum that is mostly absent in humans [50]. Despite these differences the mouse remains an important model to investigate skin and the immune response to injury [50, 51].

1.2.1: The Epidermis

The epidermis consists primarily of keratinocytes, other cells present include melanocytes, Langerhans cells and Merkel cells. The epidermis is subdivided into stratified layers (Figure 1.1).

The basal cell layer is the deepest layer of the epidermis at the interface between the epidermis and dermal layers (the dermoepidermal junction). These cells attach to the basement membrane via hemidesmosomes and anchoring filaments [44]. As a consequence of the basement membrane the immune cells within the epidermis (Langerhans cells) take longer to reach the draining lymph nodes [52]. The stratum spinosum layer contains larger amounts of keratin [53]. The stratum granulosum is the intermediate layer with

the stratum lucidum also found in thicker skin such as the glabourous skin on the palms and soles; it is considered a sub-division of the stratum corneum and enhances the barrier function in these areas. The stratum corneum follows and is the most superficial layer containing the now fully keratinised cells [44]. Keratinocytes comprise almost 95% of the epidermis. Their main function is the production of keratin, they facilitate in cellular adhesions and create the water tight layer that is essential for protection, control of electrolyte balance and thermoregulation [44].

1.2.2: The Dermis

The dermis provides the structural support of the skin, and contains many of the cells and structures that relate to its multiple functions. Cells present include fibroblasts, dendritic cells, tissue resident T cells, and mast cells. In addition to these cell types dermal appendages such as the sebaceous glands, apocrine glands and hair follicles are also present [44]. The dermal appendages play a role in wound healing as they are enveloped and closely associated with keratinocytes thus facilitating re-epithelialisation [50].

Fibroblasts make up the majority of the cells in the dermis and are responsible for production of the extracellular matrix (ECM), including collagen and elastin fibres which occurs in the acellular deeper reticular layer [47, 54]. The collagen provides structural support and the elastin allows for recoil. It is predominantly these proteins and other aspects of the ECM that change

following injury and contributes to the loss of function that is observed in scarred skin.

Dendritic cells are a type of antigen presenting cell (APC) that regulate immunity, their position in both the dermis and epidermis allows them to encounter foreign antigens and inflammatory signals in the skin, after which they undergo a series of changes to stimulate local immunity and/or migrate to the draining lymph nodes of the region to promote the immune response [55].

The dermal plexus and cutaneous nerves are found in the superficial layer of the dermis [44, 47]. These draining lymphatic vessels begin in the dermis and traverse through the deeper layers and eventually access the local lymph nodes (LNs). The LNs are connected to the peripheral circulation via an artery and vein as well as the afferent and efferent lymphatic vessels and are arranged in a series of chains [47]. It is at these primary and secondary lymphatic structures where immune cells communicate with peripheral and circulatory cells, thus connecting the innate and adaptive systems.

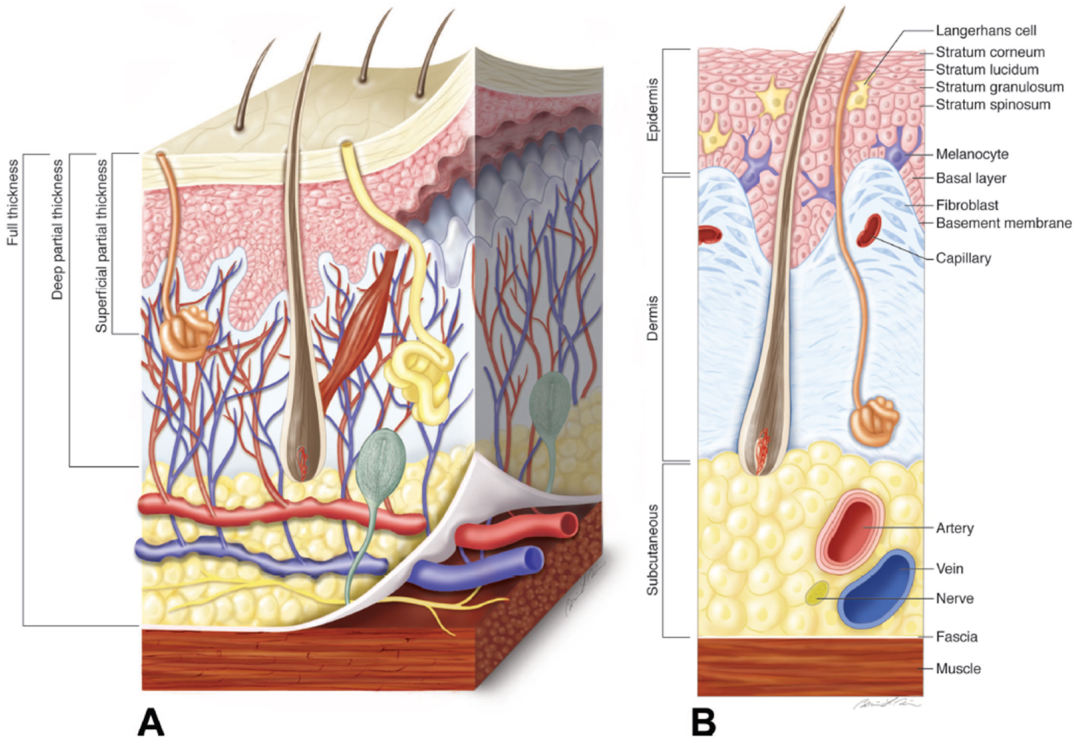


Figure 1.1: Skin Structure: *A) Anatomy and histology of the skin; B) Important histological zones and location of different cell types including the relationship of the Langerhans cells, melanocytes and fibroblasts to the layers of the skin (Figure adapted from [56]).*

1.2.3: Normal skin regeneration

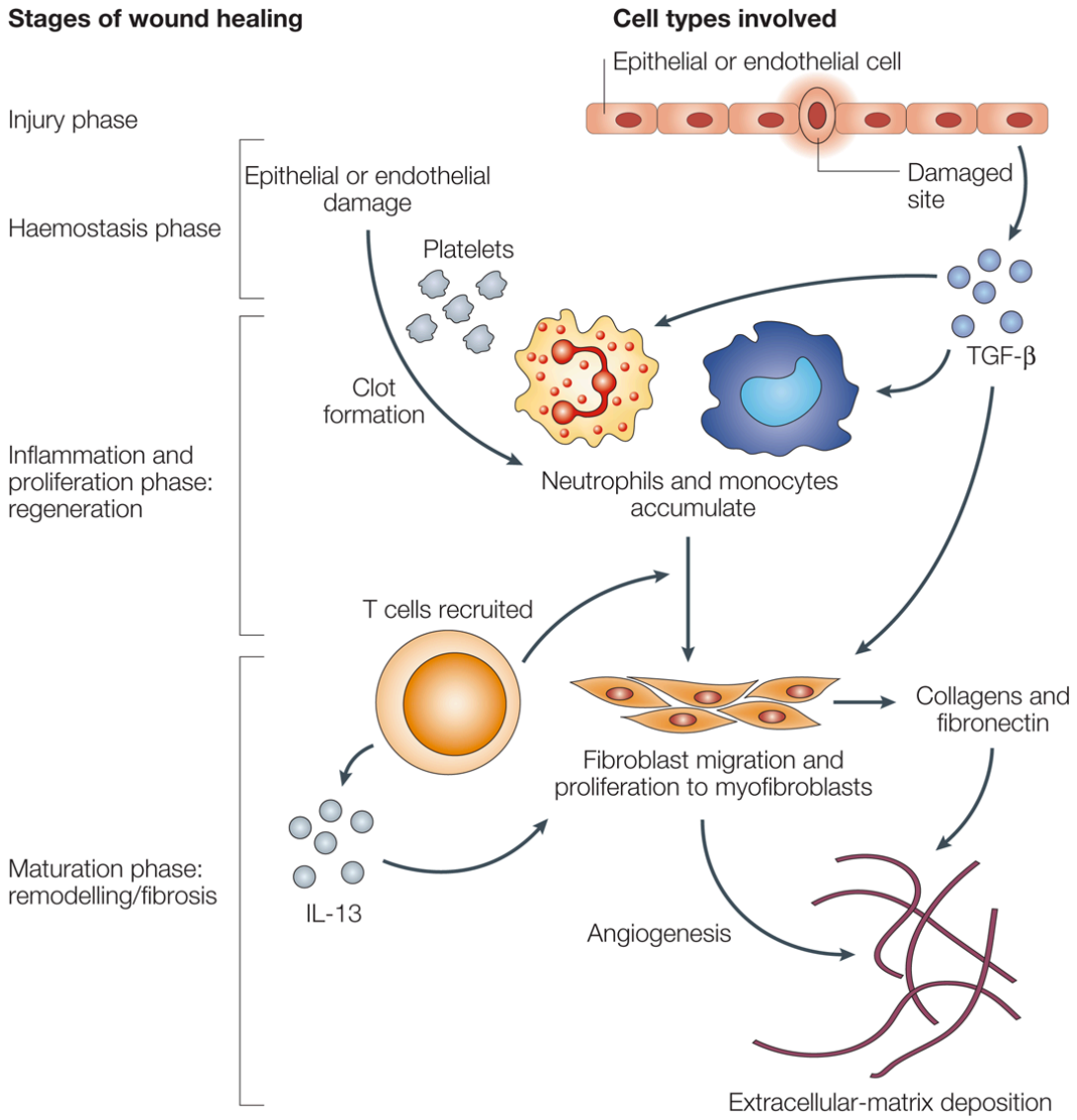
The keratinocytes found in the epidermis originate from the basal cell layer and differentiate as they migrate through the epidermal layers where they eventually become flattened enucleated squames that are keratinised forming the impermeable stratum corneum. This process of epidermal turnover from the basal cell layer to the desquamation takes approximately 2-4 weeks [57].

As previously discussed, the fibroblast is the primary cell of the dermis, which is a spindle shaped cell that produces collagen and elastin. Unlike keratinocytes found in the epidermis this cell infrequently replicates and

dermal turnover, defined as degradation remodelling and collagen production is slow in normal skin [57].

1.3: Cutaneous Injury

A full thickness injury to the skin is thus named as it inflicts damage through all skin layers, epidermis to sub-dermis. Whether elective surgery or traumatic injury this initiates a complex multifactorial cascade of cellular events which involves both the innate and adaptive immune response (Figure 1.2) [58, 59]. The described stages of wound healing comprise of three major overlapping phases: inflammation; re-epithelialisation and granulation tissue formation; remodelling [60]. These phases can all be influenced by the local and systemic immunological environment and the cytokine milieu and as such alter the wound healing process. The immune response generated is critical to the wound repair process and if disrupted, wound healing may be severely compromised, resulting in chronic wound formation, delayed healing and/or sepsis. In conjunction with genetic predisposition, these alternate pathways of wound healing can result in the formation of hypertrophic scarring which can impact on patient function, leading to disability associated with their scar [61].



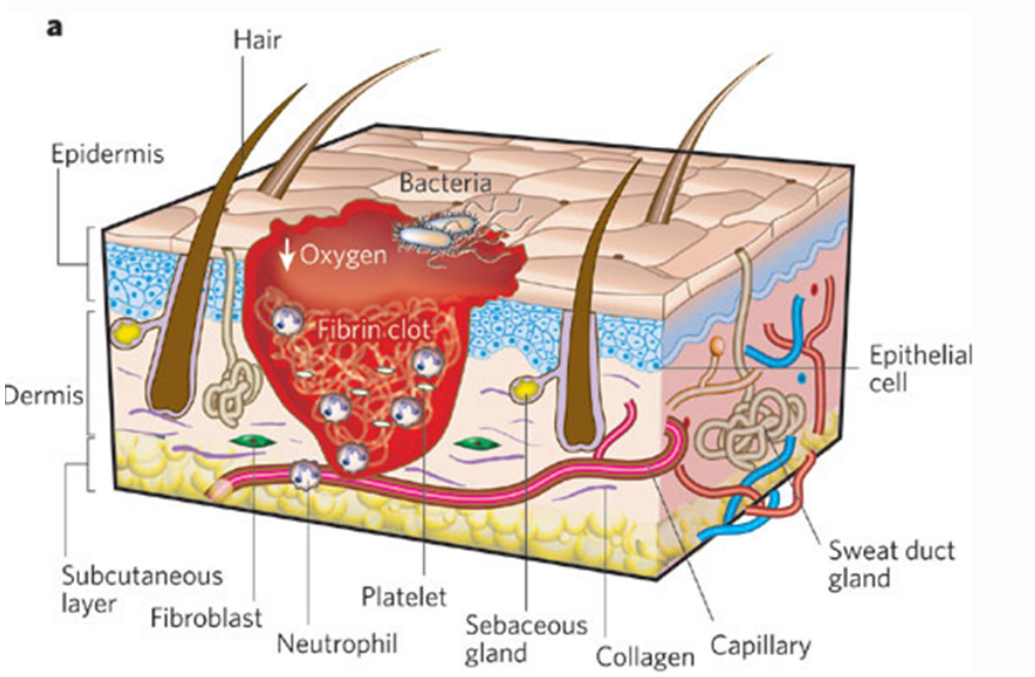
[62]

Figure 1.2: Stages of cutaneous wound healing. *Within hours the inflammatory phase begins which involves haemostasis and the influx of inflammatory cells to the wound. The cytokines and growth factors release during this time include TGF-b, IL-1α, IL-6 and TNFα. During inflammatory cell recruitment chemokines such as MIP1α, MIP1β and MCP1 are released. Within days the proliferation phase begins as keratinocytes begin re-epithelialisation and fibroblasts facilitate the formation of granulation. T cells are recruited and coordinate the transition from inflammation through to proliferation until wound closure occurs. The remodeling phase begins and the scar matures and collagen deposition occurs.*

1.3.1: Inflammatory phase of wound healing

The initial response to injury commences with haemostasis to limit blood loss and initiate vessel repair. In addition, this initial response aims to reduce exposure to the external environment and microbes to limit risk of sepsis and stimulate repair of damaged structures (Figure1.3).

Following trauma and resultant injury, damaged vascular endothelial cells initiate haemostasis. Platelets are recruited to the damaged tissue site and subsequent interaction between clotting factors and collagen activates the platelets resulting in aggregation. Activation of the coagulation cascade occurs and results in the formation of a platelet plug [63]. At the end of the haemostasis phase the clot serves as a barrier to microorganisms, provides a matrix for recruited cells and acts as a reservoir of growth factors such as platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) which initiates inflammatory cell infiltrate (neutrophils and monocytes) (Figure 1.3) [64][65]. In addition, the complement cascade is activated and aids in the recruitment of neutrophils and monocytes to the site of injury as well as initiating mast cell, basophil and eosinophil degranulation [66]. Clinically this stage post injury is evident by the 4 cardinal signs of inflammation; dolor (pain); calor (heat), rubor (erythema), tumour (swelling) as described by described by Celsus (ca 30 BC–38 AD). Later Virchow added functio laesa; loss of function **[67]**.



[68]

Figure 1.3: Inflammatory stages of wound healing. *Representation of a non-burn wound between 24-48hr following initial injury where platelet aggregation has formed a fibrin clot and infiltrating innate immune cells such a neutrophils are present to aid in clearance of debris and bacteria. (Adapted from [68]).*

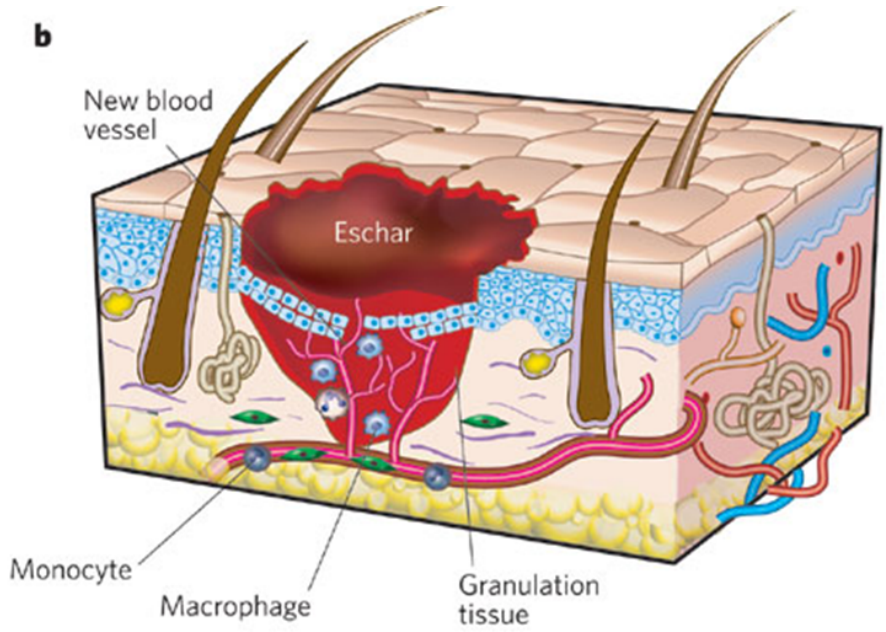
1.3.2: Proliferation phase of wound healing

The proliferation phase occurs within days of the injury, there is formation of granulation tissue and subsequently development of the extracellular matrix (ECM) which enables re-epithelialisation to occur (Figure 1.4) [69]. By day 4 the surrounding fibroblasts invade the wound and start to produce the extracellular matrix required to support growth and migration of new cells and micro-vessels at the wound base, known as granulation tissue [70]. The provisional matrix is comprised of fibrin and fibronectin, these act as a scaffold for the migration of epithelial cells in addition to providing a template for the formation of the granulation tissue [64]. If at this stage the balance of the

cytokine milieu is not favourable, proliferation can be interrupted and a chronic wound can ensue [71-74].

At the time of initial injury, the tissue destruction and hypoxia stimulate activated macrophages to release fibroblast growth factor and angiogenesis factors. In addition, epidermal cells are stimulated to release vascular endothelial-cell growth factor (VEGF). In response to these growth factors Endothelial cells are stimulated and migrate into the wound site forming new vessels [64]. As epidermal cells migrate the ECM degrades, they dissect the wound and separate healthy tissue from the eschar [64, 75].

Wound contraction is initiated early following injury and peaks at 14 days post. This process is secondary to modified contractile fibroblasts that contain the actin filament (similar to filaments found in smooth muscle) known as myofibroblasts [76]. This process occurs greatest in full thickness injuries and can account for a 40% reduction in the wound size [76]. This process is greater in non-human mammalian skin such as mice due to the presence of the panniculous carnosus. The process of wound contraction is likely a protective evolutionary mechanism that follows wounding to limit haemorrhage.

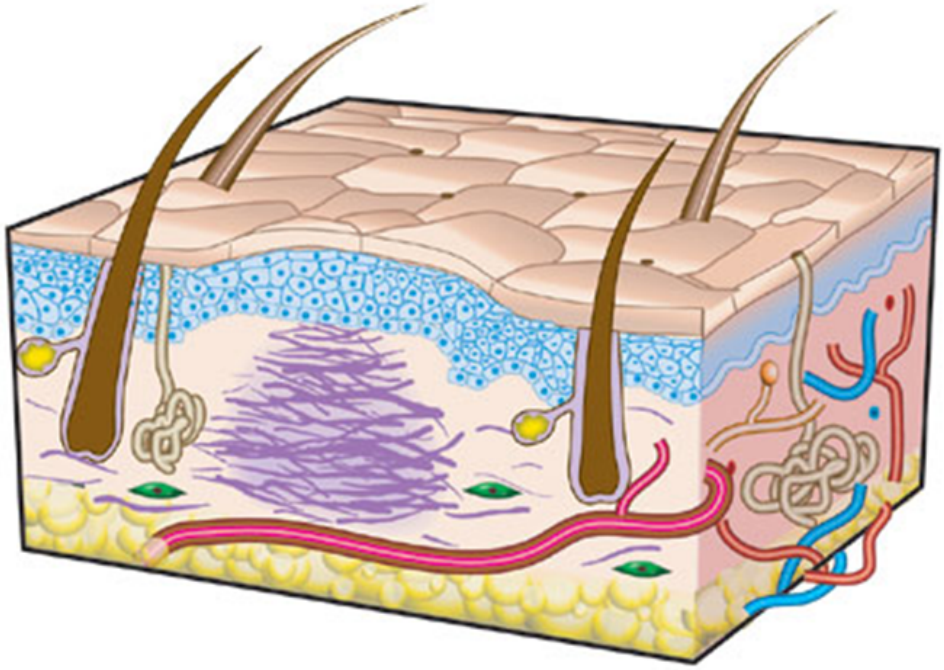


[68]

Figure 1.4: Proliferative stage of wound healing. *Represents a wound between 5-10 days post injury. An eschar has formed, the innate cells present have reduced and neovascularisation and epithelial migration occur under the eschar. (Adapted from [68]).*

1.3.3: Remodelling phase of wound healing

A relatively acellular scar replaces granulation tissue when there is an adequate amount of collagen within the wound space (Figure 1.5). The few remaining fibroblasts within the scar assume the myofibroblast phenotype approximately 2 weeks following injury. They become more contractile and are able to secrete collagen proteins into the extracellular matrix [60].This allows for connective tissue compaction and further wound contraction. As the scar matures, collagen turnover occurs and the type III collagen is gradually replaced by type I. This is a more fibrous collagen forming longer stands, resulting in the rigid structure that we recognise as scar [64, 77].



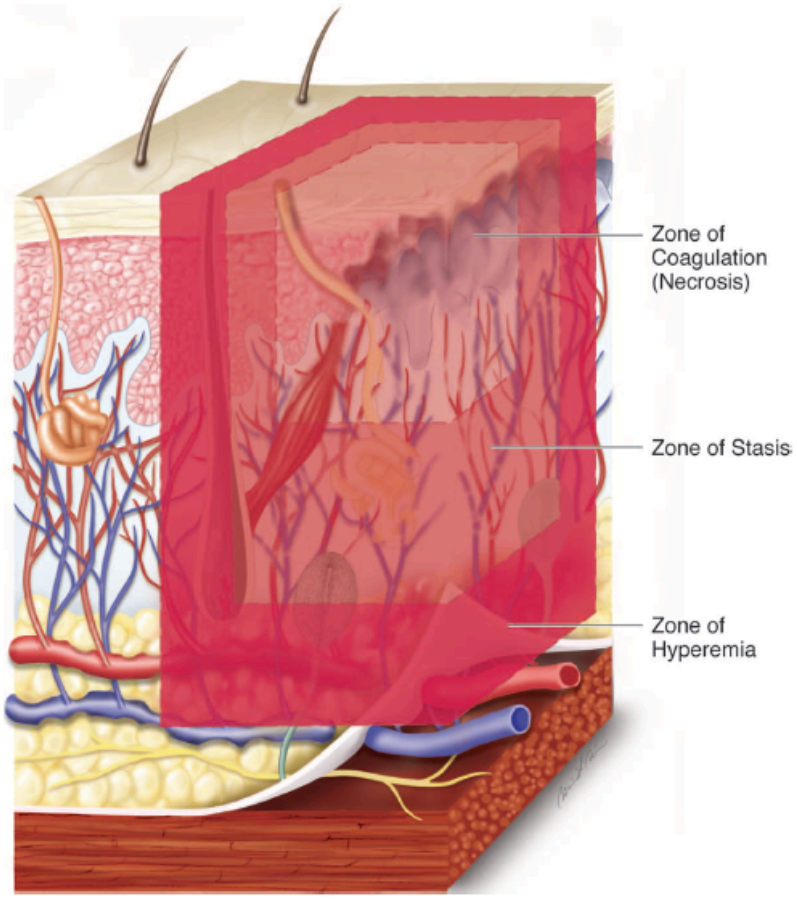
[68]

Figure 1.5: Remodelling Stage of Wound Healing. *Represents a scar approximately 1 month post injury. Collagen has been laid down by fibroblasts; the wound has contracted and does not contain normal skin appendages. As a result restoration of function is not complete in scarred skin as it is unable to perform all the same functions as seen in un-scarred skin (Adapted from [68]).*

1.3.4: Thermal injury

A burn wound can be created by thermal, chemical, electrical, friction or ultra violet light injury which induce protein denaturation and cell death [78]. A burn wound differs to an excised wound and is described by Jackson's model where 3 zones of injury are recognised; the central zone of coagulation, the zone of stasis and the outer zone of erythema (Figure 1.6) [79]. The

pathophysiology of burn injury is different to other injury mechanisms in several ways. Firstly the damage or heat effect causes increased capillary permeability that allows for plasma leak and as such patients are susceptible to volume depletion and shock. Secondly, anaemia and blood loss does not solely occur from haemorrhage as seen with other traumatic wounds. It is a cumulative process that includes losses from the burn wound itself, red blood cell sequestration and erythrocyte damage. Further to this, there are often losses following the surgical management of the burn wound in addition to myelosupression and anaemia of critical illness [80].



[56]

Figure 1.6: Jacksons' model of burn injury. *Outlining the 3 zones of injury seen following a burn injury. At the peripheral edge of the injury is the zone of hyperaemia, within this zone lays the zone of stasis, and finally the central zone of coagulation.*

In the zone of coagulation there is protein denaturation resulting in alteration of tissue structures and properties as well as complete destruction of the sub-papillary vasculature [56, 81]. In the zone of stasis there is oedema and inflammation secondary to vasodilation and increased microvascular permeability. This zone has the potential for dynamic changes, specifically wound progression or wound salvage. Where there is a reduction in perfusion further ischemia and wound progression ensue increasing the area of necrosis [82]. In contrast, appropriate resuscitation and increase perfusion may result in tissue salvage [81]. The zone of hyperaemia is the outermost zone where the perfusion is increased secondary to the inflammatory response. In large TBSA burns this area can cause large fluid shifts and haemodynamic changes in the patient, including shock. This area will recover without intervention unless complicated by sepsis or profound hypotension [56, 81].

Burn wounds are complex, often they are not uniform with a variety of different depths, making clinical assessment of wounds challenging. Although the mechanism and pathophysiology of injury differs from that of other cutaneous injury, it is believed that burns heal with the same overlapping phases as previously discussed. The depth of a burn impacts the rate of healing as healing is facilitated by the presence of remaining or surrounding viable cells from the dermis and epidermis that secrete the necessary growth factors and cytokines to drive healing [78].

Erythema describes a superficial burn that involves the epidermis: It heals rapidly and seldom leaves a scar. A partial thickness burn can be superficial or

deep depending on the depth of involvement of the dermal layers. A superficial partial thickness burn extends into the papillary dermis, resulting in blistering (separation of the dermo-epidermal junction), erythema and oedema. Whereas a deep partial thickness burn extends into the reticular dermis, and is accompanied by slow capillary refill and therefore appears pale in association with reduced sensation and oedema [56]. A full thickness burn extends into the subcutaneous tissues, burn injuries beyond the fascia and into the muscle or bone are considered a sub-dermal burn, the skin will be charred or pale in appearance and is insensate due to the destruction of cutaneous innervation [79].

As previously discussed, burn wounds are capable of dynamic change, including progression [83]. Burn wound progression is the process whereby the damage in the zone of stasis can progress during the acute inflammatory phase secondary to microvascular damage compromising vessel patency resulting further ischaemia and a deeper burn wound [82, 83]. There are other processes that may also contribute to the progression of a burn wound, such as prolonged survival of peripheral neutrophils which has previously been observed in burn patients [56]. This prolonged neutrophil survival may contribute to burn wound progression as there is extended exposure to release of reactive oxygen species (ROS) & proteases, in addition neutrophils can also aggregate in the venules surrounding the burn wound resulting in stasis and subsequent damage via a pressure gradient [56]. Both apoptosis and necrosis occur during burn wound progression, where apoptosis involves shrinkage and condensation of cell contents and the DNA fragments; necrosis involves cellular swelling with depletion of intracellular stores [56]. This unique

feature of burn wound progression is important clinically as there is greater morbidity and mortality associated with increased depth, therefore prevention of progression with appropriate resuscitation, oedema control and nutrition are required to limit the adverse outcomes associated with depth [84]. At present the exact mechanisms of the pathophysiology of the burn that alters the wound that results in progression remains unclear.

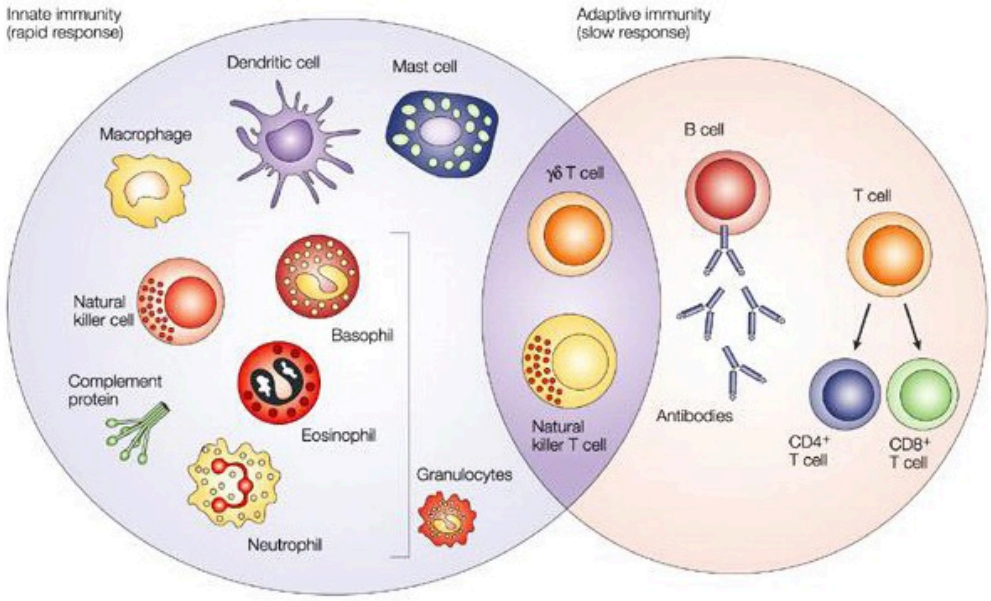
1.4: The Immune System

The immune system functions to provide recognition and defense against injury and disease as well as promote tissue repair. In humans and other mammals the immune system is classified into the innate and adaptive systems (previously known as humoral and cell-mediated responses).

1.4.1: Innate cells

The innate system is a non-specific first response to injury or a disease process. It rapidly responds to danger signals (section 1.4.1.1) and generates a response that either extinguishes the threat or engages the more specific antigen dependent adaptive T cell response via antigen presenting cells. The threshold for this activation is complex and multi-factorial (section 1.4.3). The cells of the innate system are categorised as granulocytes and agranulocytes (Figure 1.7) [85]. Lymphocytes, which are agranulocytes are part of the adaptive system and are engaged by antigen presenting cells (APCs) such as

dendritic cells (DCs) which bridge the innate and adaptive responses. In addition to the cellular components, the innate system it is also comprised of the complement system, which will not be discussed in this thesis.



[86]

Figure 1.7: The Innate and adaptive systems. *The innate and adaptive systems have differing cell types that complement the role of each system. The innate cells are comprised of the granulocytes; which release their contents to facilitate cleansing of cellular debris and microbes. The agranulocytes include the monocyte/macrophages and dendritic cells. These cells in addition to specialised T cells bridge the gap between the innate and adaptive systems. The adaptive system primarily includes the B and T lymphocytes that are antigen specific and have 'memory' function.*

Granulocytes (aka polynuclear cells) are a collection of innate cells that have granules within their cytoplasms. The granulocytes encompass several cells types, including the neutrophils, eosinophils, basophils and mast cells [85].

Neutrophils arise from haemopoetic stem cells and are the dominant white cell in the systemic circulation of humans, making up to 50-70%, whereas in mice the populations are between 10-25% [51]. Their half-life is approximately 6-8 hours, with the marrow producing 5-10x10^{10} cells/day [69, 85]. Following injury and the initiation of the inflammatory cascade, there is an intravascular chemokine gradient generated which aids the neutrophils in locating the necrotic foci [69]. These cells contain granules that when released facilitate lysis of microbes (particularly pyogenic bacteria) and cellular debris aiming to cleanse the wound and reduce bacterial load [85].

Eosinophils are derived from the bone marrow, following release from the marrow they circulate and traffic to peripheral sites (predominately mucosa) where they are involved in immune surveillance. They play a significant role in allergy and have the capacity for extracellular killing (proficient for parasites) via release of pre-formed granules. When activated, these granules generate a respiratory burst (active oxygen metabolites) in addition to pro-inflammatory cytokine production or release (IL-13, IFN-γ and TNFα) [87, 88]. Eosinophils contain/produce over 35 chemokines, cytokines or growth factors and as such play a large role in inflammation, both injury and allergy [88].

Basophils account for less than 1 % of the white cell population and are associated with allergic immune responses. Upon activation they degranulate and release pre-formed granules of histamine, which promotes vasodilation at the site of injury in addition to erythema and oedema classically associated with inflammation [85].

Agranulocytes (aka mononuclear cells), unlike the granulocytes contain no granules within their cytoplasm and populations include monocytes, macrophages and lymphocytes [85].

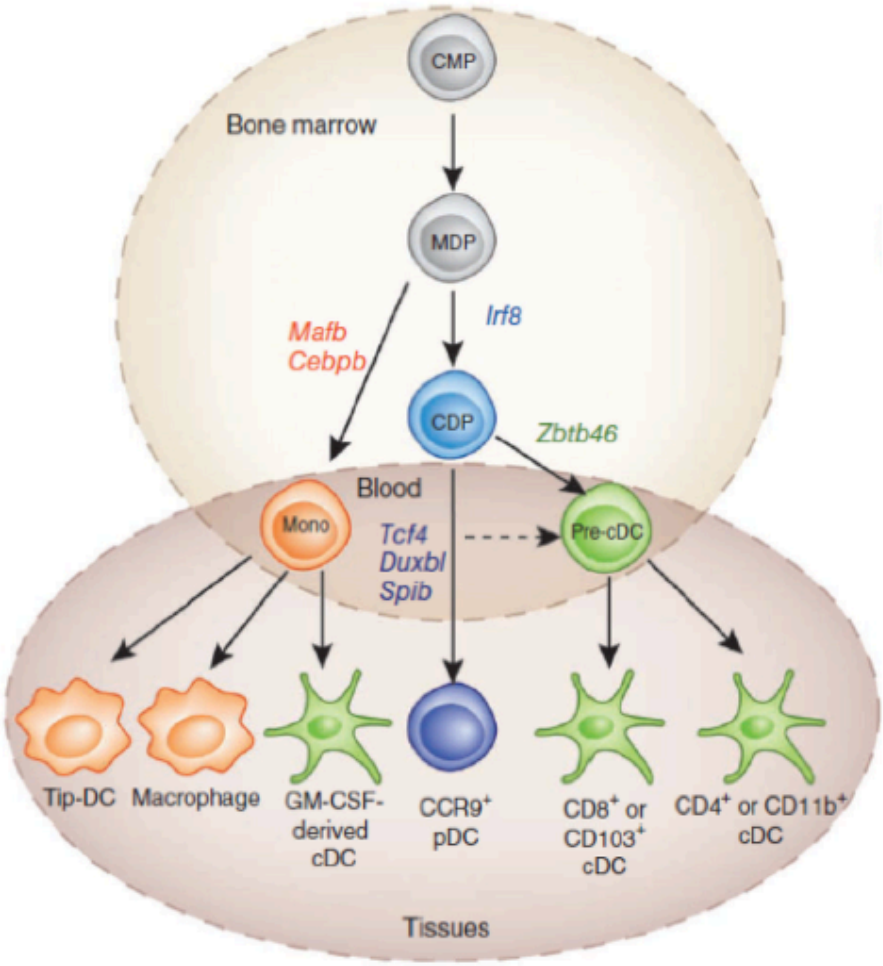
Monocytes are short-lived circulating antigen presenting cells derived from the bone marrow. In steady state conditions monocytes have a short cell cycle and undergo spontaneous apoptosis, however in the presence of a stimulus apoptosis is delayed [89, 90]. The recruitment of monocytes from the bone marrow occurs secondary to high levels of chemokines including MCP-1 and GM-CSF [91]. Following extravasation into the tissues, monocytes differentiate into macrophages or dendritic cells, under the influence of GM-CSF and IL-4, this process is inhibited by IFN-γ and TGF-β [92].

Macrophages are derived from monocytes and following their differentiation they settle in the tissues and mature. In contrast to neutrophils, these cells are long-lived and are found in abundance in connective tissues, blood vessels and lymphoid tissue where they commonly encounter foreign material. They are able to phagocytose and are capable of combating microbes that reside within host cells. In addition they can also present antigen to T cells and therefore engage the adaptive response in a similar way to dendritic cells [87].

1.4.2: Dendritic cells (DCs)

Dendritic cells are specialised antigen presenting cells (APC) that bridge the gap between the innate and the adaptive systems. Antigens captured by DCs are processed and presented to $CD4^+$ T cells via MHC class II presentation or to $CD8^+$ T cell via MHC class I, a process known as cross-presentation. When presentation occurs in an inflammatory setting, the threshold for T cell activation is reached and the adaptive immune response is engaged.

Dendritic cells have several lineages and can be characterised as conventional (cDCs) which are mostly circulatory or plasmacytoid (pDCs) which reside in the tissues. During DC differentiation and homeostasis, the lymphoid tissue resident cDCs, pDCs and monocytes share a common granulocyte macrophage precursor (GMP) that differentiates to a macrophage-dendritic cells progenitor (MDP) (Figure 1.8) [93, 94]. From this stems the common dendritic cell precursor (CDP) which gives rise to the aforementioned pre-DC and pDC. The pre-DCs circulate and either resides in the lymphoid or non-lymphoid tissue where they differentiate to their terminal sub-types. Depletion of a subset of T cells known as T regulatory cells appears to augment pre-cDC and cDC division, particularly in the lymphoid organs [93].

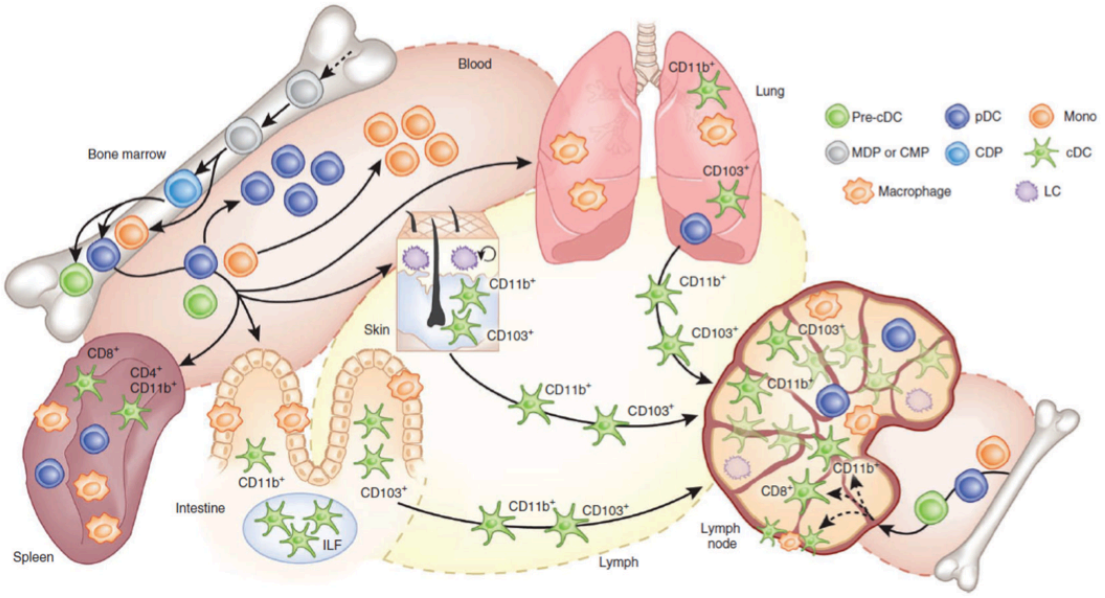


[95]

Figure 1.8: Dendritic cell lineage. *Illustrating the common dendritic cell precursor (CDP) from which stems the pre-DCs that differentiate further and terminate as either lymphoid resident subpopulations (CD8+) or non-lymphoid tissue resident subpopulations (CD11b+).*

Dendritic cells are capable of presentation of antigen to T cells in situ, in addition, a proportion of a skin subtype, $CD8a^-$ DCs are capable of migration to local skin draining lymph nodes where they signal the adaptive immune response (Figure 1.9). During normal immune surveillance (tolerance) no T cell response will be initiated, however during infection or inflammation these DC will mature during migration to the draining lymph node and their

phenotype, maturation status and cytokine production profile (IL-12) leads to the onset of an adaptive immune response.



[95]

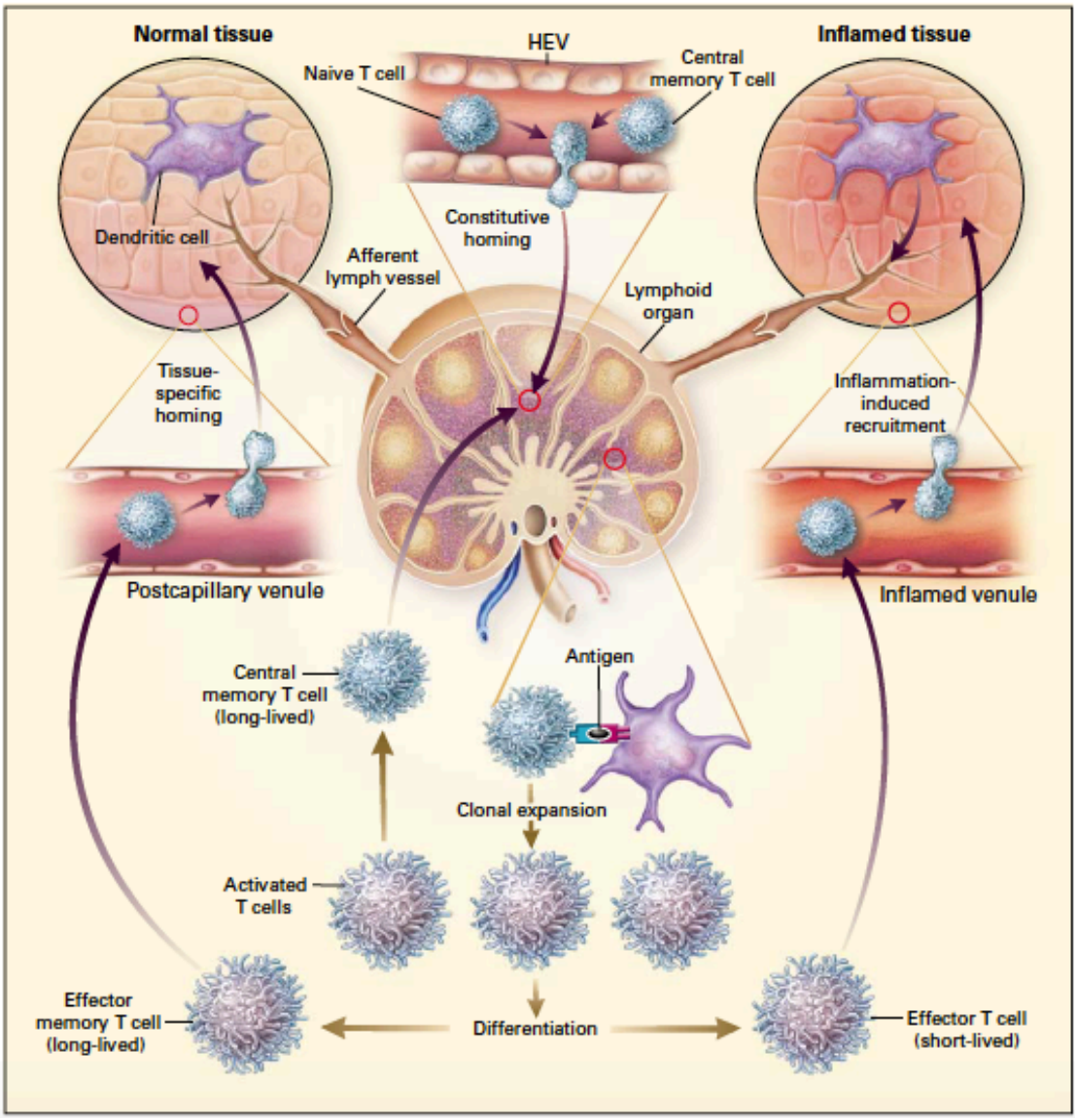
Figure 1.9: Tissue resident dendritic cell subpopulations. *$CD8a^{-}CD103^{+}$; $CD8a^{-}CD11b^{hi}$ dendritic cells shown here migrating to the draining LN following antigen presentation. Here they engage with $CD4^{+}$ T cells or $CD8^{+}$ T cells depending on the DC subpopulation. CD11b+ DCs are of myeloid origin.*

1.4.3: Adaptive cells

The adaptive immune system is primarily antigen dependent and aims to provide an appropriate response to the injury to allow for restoration of normal function [85]. This response is initiated by the inflammatory cells of the innate system in addition to antigen presenting cells, such as DCs.

T cells comprise 50-60% of the total blood lymphocytes, the remainder are made up from B-cell populations [96].

Naïve T cells have a unique T cell antigen receptor (TCR), which has an affinity for its antigen in addition to major histocompatibility complex (MHC). These cells sample their environment in the secondary lymphoid tissue via their interaction with APC such as DCs [97]. During the process of immune tolerance (i.e. in the steady-state) a small number of DCs mature and migrate to the node where they present self-antigen. Should a T cell have escaped central tolerance and engages with the DC carrying self-antigen apoptosis should occur [98]. These lymphocytes mature and differentiate following engagement with APCs in the presence of co-stimulatory signals and the required cytokine profile. It has been observed that the threshold for activation, i.e. the amount of antigen dose required to activate T cells is variable with some authors postulating that T cells make an assessment of antigen dose that ultimately determine their activation [97]. This is often observed during inflammation, but can arise in its absence, resulting in autoimmune disease. Following activation and differentiation to their terminal effector functions, they are able to release from the lymphoid tissue to migrate to the periphery to exert their function (Figure 1.10) [97].

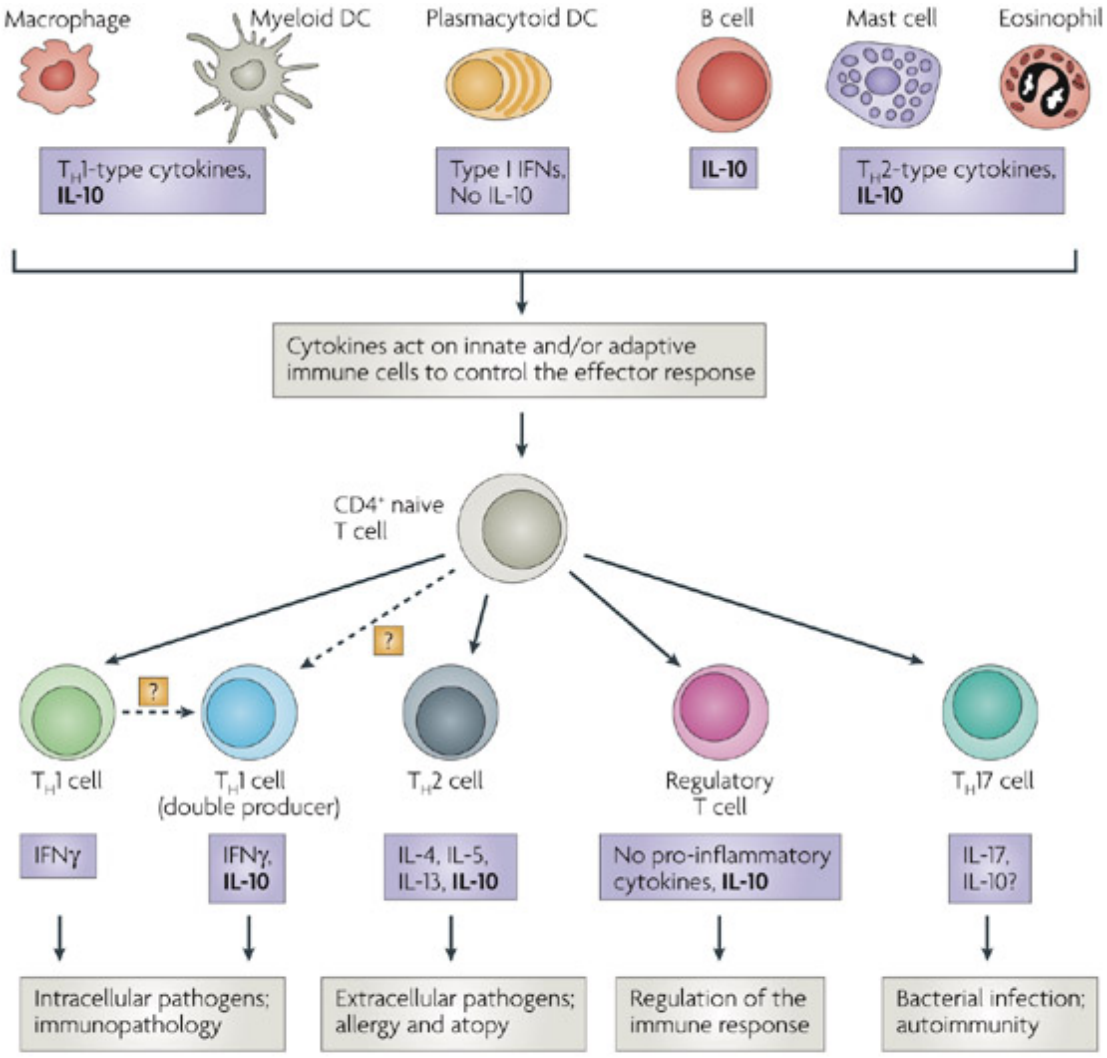


[99]

Figure 1.10: T Cell tolerance and activation: *Dendritic cells migrate to the draining lymph nodes and lymphoid tissues and engage with naïve T cells via antigen recognition. In the stead-state, when the threshold is not reached, apoptosis should occur, a process known as 'tolerance'. During inflammatory conditions, the threshold is reached and T cell clonal expansion occurs with activation and differentiation.*

T lymphocytes are classified according to their surface markers as well as their cytokine production profile, at present numerous different $CD4^+$ sub-populations have been described. In this thesis, T-helper 1 (Th1) T cells, T-

helper 2 (Th2) T cells, T-helper 17 (Th17) and T regulatory (Treg) cells will be discussed (Figure 1.11).



[100]

Figure 1.11: T cell differentiation. *During the inflammatory phase innate cells secret cytokines that influence the T cell response generated. In addition to antigen presentation, the co-stimulatory cytokine present drives naïve CD4⁺ T cell differentiation to either Th1, Th2, Th17 or T regulatory cells.*

1.4.3.1: T cell sub-populations

$CD4^+$ T-helper 1 (Th1) cells secrete cytokine such as IFN-γ and IL-12 which drive inflammation via recruitment of more T-helper and Natural Killer (NK) cells, which is necessary during the acute phase of healing to facilitate the clearance of necrotic debris and reduced microbial load in order to prepare the wound for re-epithelialisation [101-103].

$CD4^+$ T helper 2 (Th2) cells secrete IL-4, IL-5 and IL-13, which are modulatory cytokines that control and cease the pro-inflammatory phase [101]. Induction of Th2 cells is facilitated by the presence of IL-4 and the down regulation of IL-12 (that is associated with a Th1 type response). Further to this, IL-5 production increases which induces terminal differentiation, proliferation and increases survival of eosinophils', which also play a role in the Th2 response via their secretion of Th2 chemokines and cytokines [85, 88].

A balance between the $CD4^+$ Th1 and Th2 response is required to provide the optimum basis for appropriate clearance of debris/microbes (during the pro-inflammatory response) and wound healing that is accomplished following induction of the anti-inflammatory response. Should this balance between Th1 and Th2 be disrupted, the patient may be susceptible to potential morbidity as a consequence of either a prolonged Th1 pro-inflammatory phase that can result in additional host injury secondary to over exposure to inflammatory cells such as neutrophils resulting in SIRS and chronic wound formation. Alternatively, an early or prolonged Th2 anti-inflammatory phase pushes the immune response to a state of suppression where the patient becomes more

vulnerable to wound infection or sepsis. This state of immune suppression known as CARS is preceded by SIRS suggesting that this would not occur in isolation and requires an initial abnormal Th1 inflammatory response.

The differentiation of naïve T cells to Th17 T cells occurs under the control of both TGF-β and IL-6. In the absence of IL-6, TGF-β alone aids in the differentiation of Tregs from naïve $CD4^+$ T cells [104]. Th17 T cells are a type of CD4+ T cell that produce effector cytokines, including IL-17, IL-6, IL-21 and IL-22, which are pro-inflammatory and lead to the recruitment of neutrophils. These primed neutrophils secrete chemokines such as MCP-1 that recruits further Th17 T cells, thus forming a positive feedback loop [105]. These Th17 T cells have been identified as protective against a variety of microbes, including klebsiella and candida albicans, both are uncommon but problematic wound infections in the immunocompromised patient [101].

T regulatory cells (Tregs) make up to 5-10% of $CD4^+$ T cells [106]. They are classified into 2 groups, natural (nTregs) and induced (iTregs). Natural Tregs are produced in the thymus and are present in the circulation where they are capable of expansion following exposure to danger signals and APCs. These cells appear to exert their suppressive/regulatory effect via a cell-to-cell-contact manner without the need for cytokine secretion or antigen, the process of which is not wholly understood [106]. Induced Tregs are converted in the periphery from naïve $CD4^+$ T cells that acquire a regulatory function following exposure to a pathogen or damaged autologous cells which is antigen specific in relation to the nature of the insult [107]. The iTregs exert their regulatory effect via the secretion of cytokines such as IL-10 and TGF-β [106]. Tregs are commonly identified by the markers $CD4^+CD25^+FoxP3^+$,

where $CD25^+$ enables the regulatory function via the IL-2R and $FoxP3^+$ represent the functional marker Forkhead/winged helix family of transcriptional regulators, which at present is specific to Treg cells [108].

$CD8^+$ T cells are referred to as cytotoxic T lymphocytes and make up 20-25% of T cells in the peripheral blood pool [109]. These cells play an important role during viral infection and cancer cell eradication [96]. Once activated $CD8^+$ T cells ($CD8^+CD25^+$) act via a perforin-dependent pathway on virally infected and foreign cells. They are activated by an APC binding via MHC class I to their T cell receptors (TCR) [96]. The cytotoxic $CD8^+$ T cells are able to apoptose the APC via cell lysis following antigen recognition, in addition they also act by secreting IFN-γ [85].

The skin itself contains T cell populations, including gamma-delta (γδ) T cells, natural killer (NK) T cells, and alpha-beta T cells which make up a third of the skins T cells [47]. Gamma-delta T cells are the predominant type of T cells in murine skin. When activated they express Macrophage inflammatory protein 1α (MIP1-α), Macrophage inflammatory protein 1a (MIP1-β), RANTES (Regulated on Activation, Normal T Expressed and Secreted), KC, IFN-γ, IL-2 and IL-13 which contribute to the recruitment of innate and adaptive cells during the inflammatory phase following cutaneous injury [49].

1.5: The Immune Response to Injury

In response to cutaneous injury, an immune response is triggered. The immune response orchestrates a complex cascade of events that aims to reduce microbial infection, clear necrotic cellular debris and coordinate repair, ultimately striving to restore function. It is broadly divided into the innate and the adaptive responses that have been discussed (section 1.4.1 – 1.4.3). The innate cells are rapidly recruited, which provides a generic and board response to injury. If required the innate system engages cells of the more specific adaptive system via APCs [85].

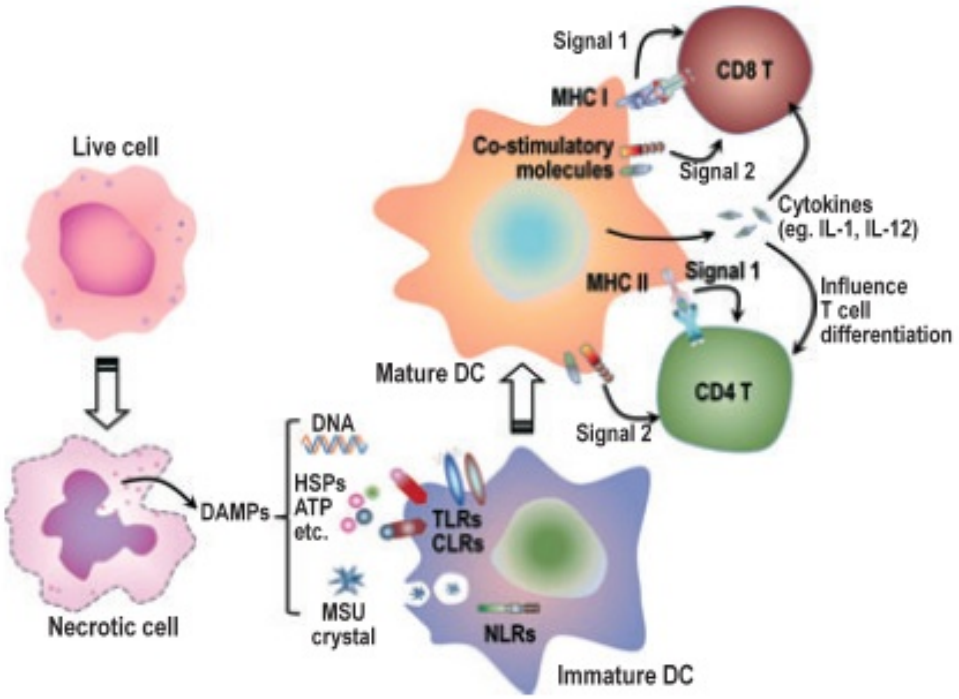
1.5.1: DAMPS, chemokines and inflammatory cytokine induction

Cells of the innate immune system are recruited by the release of cytokines and chemokines at the injury site. Damaged keratinocytes in the epidermis release a number of cytokines from pre-formed pools including IL-1α, MCP-1, GM-CSF and TNF-α [91, 110, 111]. Interleukin-1α attracts circulating neutrophils and monocytes to the wound, it facilitates in the induction of T cells via increasing IL-2 production and induces the production of other pro-inflammatory cytokines such as IL-6, TNF-α and GM-CSF [73, 85, 112].

In consequence to tissue injury and cell damage there is release of mitochondrial DNA fragments and adenosine triphosphate (ATP) from the nucleus that can activate the Toll Like Receptor (TLR) pathways and thus

recruit the innate system in the form of neutrophils. These signals are known as Danger Associated Molecular Patterns (DAMPs) and also include the alarmins and the Pathogen Associated Molecular Patterns (PAMPs) (Figure 1.12) [113-115]. The alarmins are danger signals released secondary to tissue injury, they include the heat shock proteins (HSPs), annexins, defensins S100 and high mobility group box 1 protein (HMGB-1) [114]. PAMPs are microbial molecules that form recognisable profiles, which can be commensal or pathogenic microbes that have traversed the skin barrier [114]. These molecular patterns are recognised by pattern recognition receptors (PRRs) which are expressed by innate cells including neutrophils, macrophages and dendritic cells to initiate the inflammatory immune response [116]. The Pattern recognition receptors (PRRs) include the Toll Like Receptors (TLR), mannose receptor (MR), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) [113, 116]. TLRs are the dominant PRR for microbes; with 10 TLR having being described in the literature with each recognising various patterns and associations. Some are expressed on the cell surface and recognise bacteria, fungi and protozoa, and others lie within endocytic compartments and recognise viral nucleic acid self-damaged DNA fragments [116].

In addition to activation of various PRRs the inflammasome is simultaneously activated. The inflammasome is a large multi-protein complex that detects aggregated PAMPs and DAMPs on cell surfaces or within endosomes [115, 117]. Following activation of the inflammasome, pro-IL-1β, -IL-18 and -IL-3 are converted to their active cytokine profiles and are released to further stimulate an inflammatory response [118].



[119]

Figure 1.12: DAMPs engaging with immature DCs. *Engagement of the DCs PRRs with co-stimulatory signals allows maturation of the DCs and subsequent recruitment of appropriate $CD4^+$ or $CD8^+$ T cells via antigen presentation.*

Chemokines are a subset of cytokines that aid the recruitment of cells by stimulating chemotaxis as well as the extravasation of leukocytes from the peripheral blood. Chemokines are produced in response to inflammatory cytokines such as IL-1 and TNF-α [85]. There are 4 described subtypes of chemokines, the CXC, CC, CX3C and C-chemokines. Of these 4 subtypes, the CXC and CC chemokines have a prominent role in wound healing, specifically the recruitment of neutrophils, re-epithelialisation and angiogenesis [69, 91]. The CXC cytokines are more specific for neutrophils and lymphocytes, whereas the CC chemokines for monocytes, basophils, eosinophils and NK cells [85].

Macrophage Chemo-Attractant Protein (MCP-1), also known as CCL2, is released by damaged keratinocytes following injury and serves to attract macrophages, T cells, natural killer cells (NK) and dendritic cells (DCs) to the site of injury [73, 91, 120].

Macrophage inflammatory protein 1a (MIP1-α) is also known as CCL3 is released from damaged endothelial cells and aids in recruitment and differentiation of monocytes to macrophages [121]. Levels of MIP1-α peak in wounds around day 1 post injury, correlating with maximal macrophage infiltration [91].

Macrophage inflammatory protein 1b (MIP1-β) also known as CCL4 attracts NK cells and monocytes to the wound site and increases following extensive cutaneous injury [122].

Granulocyte-macrophage colony stimulating factor (GM-CSF) is secreted predominately by eosinophils [73]. GM-CSF plays an important role in the inflammatory phase following injury as it increases the number of neutrophils recruited to the wound site and eosinophil survival. In addition it increase keratinocyte proliferation via direct action & indirectly via up-regulating IL-6 and therefore aids in the process of re-epithelialisation [73].

Granulocyte colony-stimulating factor (G-CSF) is a modulatory cytokine, which is able to increase leukocyte count (predominately neutrophils) via increasing proliferation, cell survival and mobilisation [123]. In monocytes it has been reported to decrease the rate of deactivation following surgical injury, thus further encouraging a pro-inflammatory Th1 type (stimulatory)

response [42]. In contrast to these pro-inflammatory effects, G-CSF is associated with antagonising some pro-inflammatory cytokines such as IL-1 and TNF-α.

RANTES (Regulated on Activation, Normal T Expressed and Secreted) is also know as CCL5, its release (from eosinophils) is stimulated by INFγ and its acts to attracts T cells, eosinophils and basophils to the site of tissue damage thus levels increase following significant cutaneous injury [88, 124].

EOTAXIN, also known as CCL11 is a chemokine highly specific for eosinophil chemotaxis to the peripheral tissues, its release is stimulated by eosinophils as well as epithelial cells. Elevated levels are associated with allergy and chronic inflammation [88, 125].

KC is also known as CXCL1 is secreted by damaged epithelial cells and attracts neutrophils to the area of tissue injury, it is raised acutely in injury, both cutaneous and visceral [125].

1.5.2: Innate cell response to injury

Neutrophils develop in the bone marrow and following differentiation they are released into the circulation where they are able to extravasate into the tissues [123]. They are the first nucleated cell to arrive at the wound site following skin injury which can be achieved within minutes following injury, peaks at 24hrs post and levels can be sustained for several days [126].

Neutrophils are recruited to the area of injury following the actions of IL-1β and TNF-α that are released on keratinocyte damage. These cytokines play a role in increasing in the permeability of endothelial cells and expression of adhesion molecules such as β2-Integrins. These changes to the endothelium allows trafficking of innate cells from the circulation to the area of injury, a process that can be observed clinically as tissue oedema and erythema [111]. In addition, the release of IL-1β and TNF-α from damaged keratinocytes facilitates in the recruitment of neutrophils from the bone marrow, aids in neutrophil migration and activation, enhances phagocytosis and reduces neutrophil apoptosis. Further to this, these cytokines play a role in macrophage activation & production of other inflammatory cytokines such as IL-1 and IL-6 [110, 127, 128].

Priming of neutrophils following injury is a two-step process, which includes the presence of pro-inflammatory cytokines (TNFα, IL-1β and GM-CSF) and engagement of their TLR (DAMPs/PAMPs) [105]. The primed neutrophils release reactive oxygen species and tissue toxic enzymes, which are able to debride the necrotic tissue and microbes that is found in wounded tissue. However, if neutrophils are excessively primed, these mechanism that aim to cleanse the wound can result in host tissue injury via cleavage of the extracellular matrix, elastin and collagen. This leads to further damage resulting in a self-perpetuating cycle and ultimately an altered inflammatory process and non-healing wounds [66, 129].

Neutrophil apoptosis is regulated by macrophages via reduction of IL-23, IL-17 and G-CSF [130]. Where neutrophil apoptosis is delayed post injury there is accumulation of primed neutrophils in the tissues, which if substantial

can lead to Systemic Inflammatory Reponses Syndrome (SIRS) and eventually Multi-Organ Dysfunction Syndrome (MODS) secondary to vascular occlusion, ischaemia and subsequent necrosis [105]. In the absence of injury, impaired neutrophil apoptosis is associated with autoimmune disease [105].

Monocytes are present in two forms, circulating ($CD1c^{+}CD16^{-}$) and tissue resident ($CD14^{-}CD16^{+}$) [130]. Monocytes are recruited from the circulation to the wound under the influence of IL-1α, TNF-α and IFN-γ. Here they differentiate into macrophages or dendritic cells. Despite the differing lineage, they retain many similar features including; phagocytosis; secretion of various cytokines/chemokines; and antigen presentation to T cells [24].

Following differentiation, tissue macrophages are present in wounds from 12 hours, peaking between day 2 to 4 and remain in the wound until approximately day 6 [126]. Here they phagocytose and cleanse the wound of debris and bacteria in preparation for wound healing. The adherence of monocytes and macrophages to the extracellular matrix in the wound bed induces the expression of TNF-α, TGF-β and growth factors such as colony stimulating factor 1 (CSF1) and platelet derived growth factor (PDGF) [64]. These growth factors and cytokines aid in promoting angiogenesis, keratinocyte activity stimulators (re-epithelialisation) and the process of collagen synthesis [64, 131]. These cells therefore facilitate in the transition from the inflammatory phase through to wound closure.

Three populations of tissue macrophages have been described related to their response to cytokines; classic, wound healing, and regulatory macrophages.

Classic macrophages are activated in response to INFγ and TNFα secreted from Th1 T cells resulting in production of IL-12 and IL-10 by the macrophage. Wound healing macrophages are activated in response to IL-4 from Th2 T cells resulting in reduced secretion of IL-12 and IL-10. Regulatory macrophages are activated in response to prostaglandin 2 (PGE2) and IL-10 resulting in increased secretion of IL-10 and reduced secretion of IL-12, and TGFβ. In combination, this cytokine milieu suppresses the immune response [105].

Tissue macrophages sample their surrounding by phagocytosis, engulfing antigens. After engagement with a pattern recognition receptor (PRR), activation occurs amplifying their phagocytic ability and enabling them to secrete cytokines and chemokines [85]. These are both pro-inflammatory (IL-1, IL-6 and TNF-α) and modulatory cytokines (IL-10, IL-12 and TGF-β) enabling macrophages to influence the immune response in both a pro-inflammatory or suppressive manner [102, 111, 125]. In contrast to DCs, tissue macrophages in the skin do not drain to the lymph nodes and have weak antigen presenting capacity, thus do not play as significant role in T cell activation compared to DCs [98].

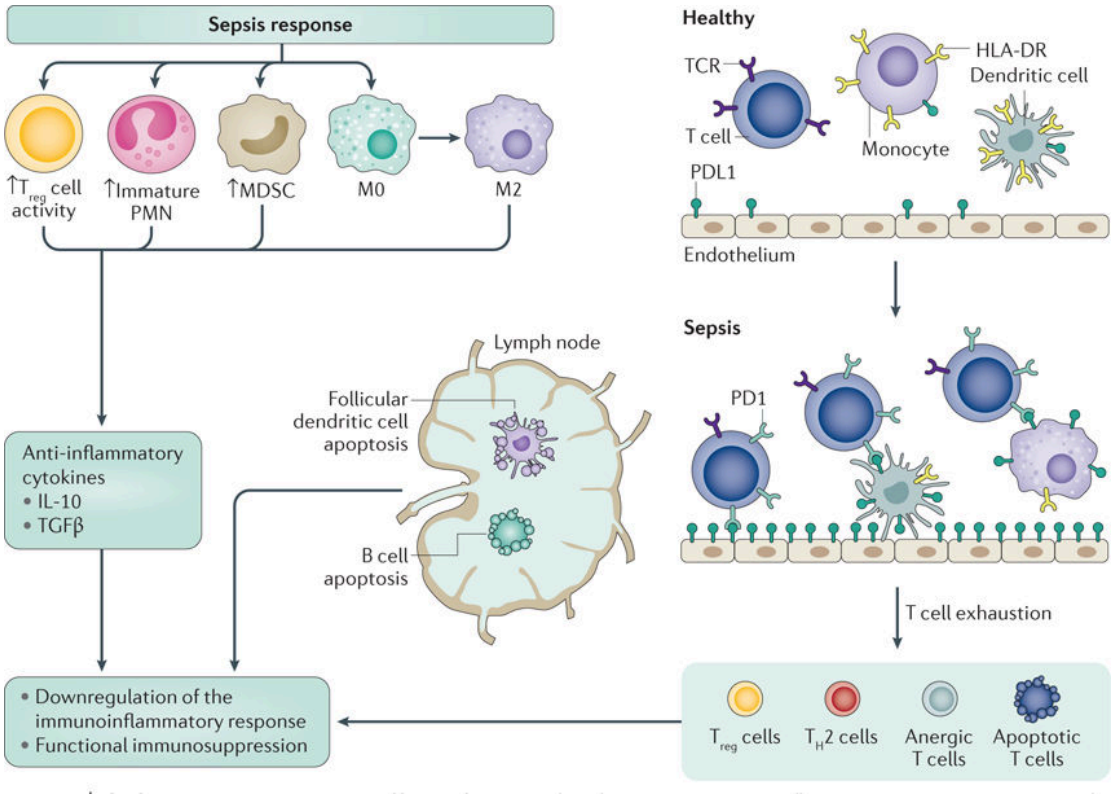
1.5.3: Dendritic cells and injury

When cutaneous injury occurs, Langerhans cells (LC) and dendritic cells (DCs) capture antigens and migrate via the lymphatic channels to the draining lymph node where they encounter naïve T cells. This occurs within the first 24

hours and peaks at 72 hours post injury [132]. Dendritic cells are primed via the engagement of a damage associate molecular pattern (DAMP) that binds to its toll like receptor (TLR) in the presence of inflammatory cytokines such as IFN-γ, GM-CSF and IL-4 (Figure 1.11) [121, 133].

Dendritic cells acquire a mobile state that allows their translocation from the site of antigen uptake (i.e. the skin) to the site of antigen presentation (i.e. the draining lymph node) where they engage with T cells. Both IL-1 and TNF-α produced by DCs, keratinocyte and fibroblast are involved in this process of antigen presentation and translocation [47]. Following injury to the skin, the dermal DCs are responsible for the initial T cell recruitment, thus mobilisation occurs swiftly with a substantial number of DCs found in the draining lymph node by 24 hours post injury which peaks at day 2 [134]. The T cell response generated following engagement with the DC is dependent on the cytokine milieu at the time of presentation. In the presence of IL-12, a Th1 (pro-inflammatory) response ensues, whereas when IL-10 is secreted a Th2 type response is generated [121] [135] [102].

With extensive injury a SIRS state is generated which can alter the normal differentiation of DC precursors due to the level of the pro-inflammatory cytokine present. As a result, they do not acquire their full antigen presentation functions and are unable to mature appropriately resulting in low or no antigen capture and thus altered presentation to T cells. In this environment a state of immunosuppression ensues (CARS) as a T cell response cannot be generated thus compromising the adaptive response (Figure 1.13) [127].



[136]

Figure 1.13: DC response and SIRS. *During the steady state, DC traverses through their cell cycle from pre DCs maturing to DCs under the influence of steady state cytokines and then apoptosis. When a systemic inflammatory response occurs secondary to injury cells of the innate and adaptive systems are exhausted. Further recruitment of myeloid precursors occurs. However the cells, such as the pre DCs are unable to mature and upregulate their MHC Class II expression. Consequently, antigen presentation is limited to T cells. Concurrently, Treg and M2 macrophages are activated and produce an anti-inflammatory milieu. In this environment immunosuppression ensues, as an effector T cell response cannot be generated.*

The conventional dendritic cells (cDCs) are predominately circulatory and are identified by their surface marker $CD11c^+$ in conjunction with MHC class II high expression [49, 94, 98]. To classify DCs further, those that are resident to the lymph nodes express $CD8a^+$, whereas the migratory dermal DCs express $CD8a^-$.

These migratory dermal CD8a$^-$ DCs traffic from the skin to the draining lymph node during inflammation and surveillance. Here they present antigen specifically to the CD4$^+$ or CD8$^+$ T cells and can either engage the adaptive response or contribute to immune tolerance (Section 1.4.3).

The dermal DCs can be further subdivided based on their surface markers and function. Three subsets of interest have been identified which include; CD103$^+$CD11b^{lo} (CD103$^+$ DCs), CD11b^{hi}CD103$^-$ (CD11b^{hi} DCs) and CD11b^{lo}CD103$^-$ (CD11b^{lo} DCs).

The CD103$^+$ DCs play a role in CD8$^+$ T cell activation during viral immunity as these cells capture necrotic material and cross present to MHC class I thus engaging with CD8$^+$ T cells [49, 94, 137, 138]. They behave in a similar manner to the CD8α$^+$ DCs that are found in the lymph nodes [98].

During infectious challenges, both viral and fungal, these CD103$^+$DCs are the dominant migratory DCs that present to CD8$^+$ T cells which is associated with concurrent increased production of IL-12, IFN-γ, IL-2 and TNF-α thus promoting both Th1 and Th17 T cell responses which is required to manage the microbial challenge [139-141]. In addition to recruiting T helper cells, the CD103$^+$ DCs have been implicated in the production of Treg cells in the lymph nodes via a retinoic acid dependent mechanism [141].

The CD11b^{hi} (the most abundant dermal DC) and CD11b^{lo} populations can stimulate a Th1, Th2 or Th17 immune response on presentation of antigen to CD4$^+$ T cells which is dependent on both the DC maturation status and simultaneous cytokine production [98]. In similarity to the CD103$^+$ DCs, these CD11b^{hi} dermal DCs also produce retinoic acid (RA), which is necessary for

the conversion of naïve $CD4^+$ T cells to Treg cells in the presence of TGF-β. Implying that these $CD11b^{hi}$ dermal DCs also induce the generation of Tregs [49, 110].

The lymphoid tissue resident $CD8a^+$ DCs captures circulating antigen that leech into the systemic circulation following injury. In the setting of injury and the presence of cytokines such as IFN-γ, GM-CSF and IL-4, a priming signal is received via their TLR following exposure to antigen [121, 133]. In response to this these cells are able to produce high levels of IL-12p70 which amplifies their ability to present to naïve lymphoid $CD4^+$ T cells [142]. In addition, these lymphoid resident DCs are also able to accept antigen from migratory dermal $CD8a^-$ DCs via antigen cross-dressing, as well as cross-presenting to $CD8^+$ T cells [143, 144]. These cells therefore play a vital role generating a Th1-type response during viral exposure/infection [49, 138]. In the steady state, the $CD8a^+$ resident DCs are necessary in the induction of tolerance to cell associated antigens present during apoptosis as the $CD8a^+$ DCs internalise dead cells [144, 145].

1.5.4: T Lymphocytes and injury

As previously discussed (section 1.4.3), Th1 T cells are pro-inflammatory thus enhance the clearance of intracellular pathogens via production of IFN-γ and support the inflammatory response via the production of IL-2, IL-12 and IFN-γ [42, 146]. The Th2 response is not suppressive, but modulatory via the

secretion of cytokines IL-3, IL-4 and IL-5. These allow for cell proliferation and remodeling which does not occur in the presence of inflammation.

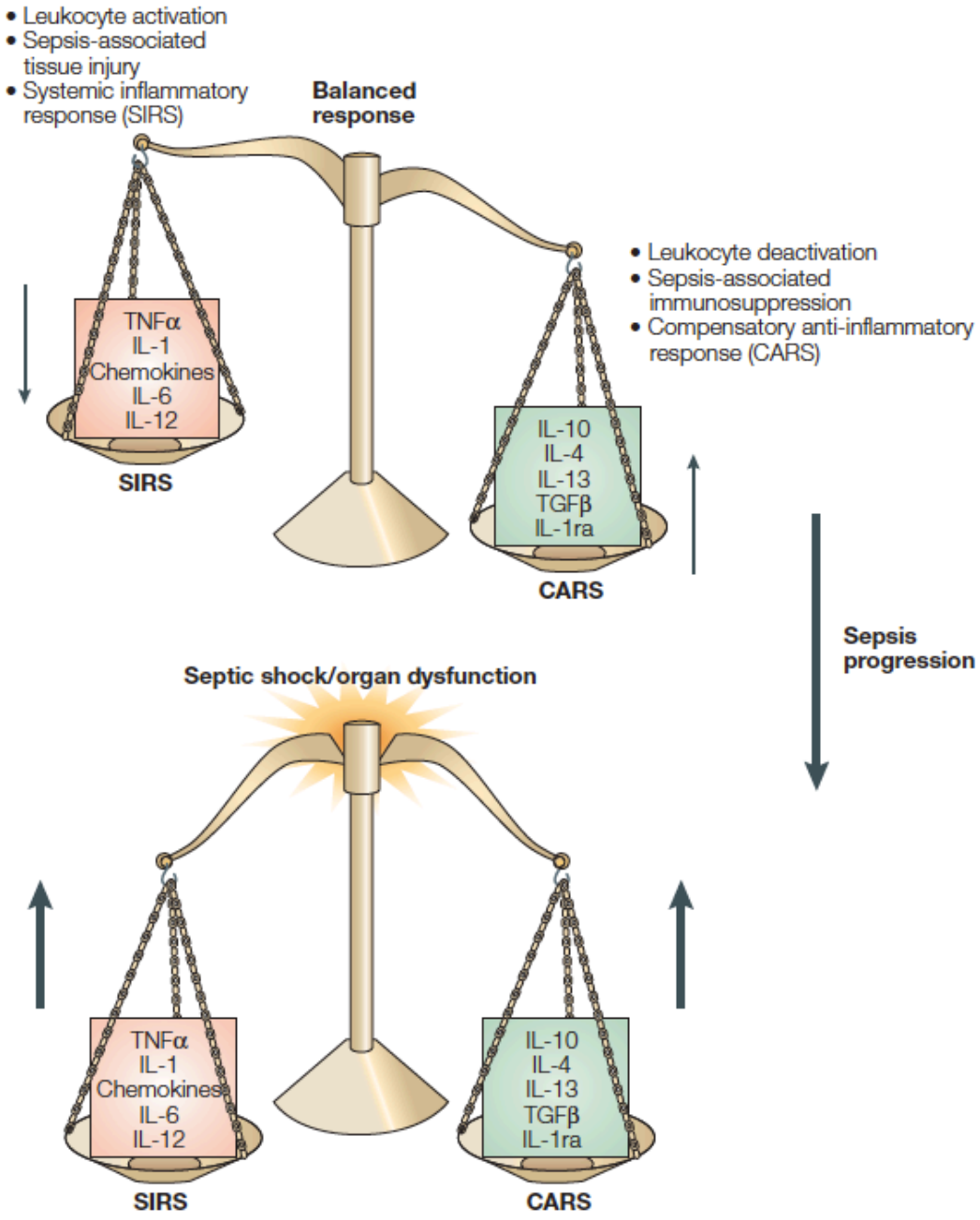
In the injured state T cells are recruited once a their threshold for activation is reached via their interaction with APCs whilst in the context of inflammatory co-stimulators & cytokines in order to initiate the adaptive response [47]. The response of the T cells appears to be specific to the injury itself with significant alterations in gene expression (both up and down-regulation) between two murine injury models has been observed [22, 28]. However the details of the mechanisms leading to this have not been confirmed.

On activation, $CD4^+$ T cells up-regulate their CD25 expression. The marker CD25 is the IL-2 receptor that allows for IL-2 stimulation of T cells to induce further proliferation and increase T cell survival [147, 148].

T cells can be recruited from the skin itself, the draining lymph nodes and from the circulation. The T cells which are resident to the skin can interact locally with skin resident DCs to induce a local T cell response [98]. Whilst those activated and released from the draining lymph node migrate to the site of injury via the actions of cytokine and chemokines. In brief, IFN-γ induces a cytokine cascade that recruits Th1 cells, whereas Th2 cells are recruited via IL-4 and Il-13 promoting chemokines such as MCP-1 and EOTAXIN [149]

The literature suggests that the T cell response pivots on the innate inflammatory response i.e. should this be inappropriate it will be reflected in the adaptive response mounted. For example, in cases of major injury where there is excessive innate inflammation the major lymphocyte populations including the $CD4^+$, $CD8^+$ and NK T cells can be altered resulting in a state of

subsequent immunocompromise [150]. Using cytokine profiles as a measure of activity, it appears that the Th1 CD4+ T cell populations are reduced following major trauma (ISS >20) [41, 101, 102, 151]. It has been proposed that this is secondary to an increase in Th1 T cell apoptosis whilst the Th2-type T cells are preserved (Figure 1.14) [41]. As a result there is a relative increase towards the Th2-type cell response and subsequent dampening of the inflammatory phase, evident by the paucity of Th1 inflammatory cytokine IFN-γ and the increase in modulatory cytokines IL-3, IL-4 and IL-5 [42, 101, 151]. As a consequence pro-inflammatory cytokines and innate cells are reduced in the wound site, thus reducing the microbial defensives. Clinically, in both surgical and trauma patients, this can allow the development of sepsis secondary to CARS where T cells fail to proliferate, produce IL-2 and IFN-γ, thus levels of IL-10 and IL-4 rise (Figure 1.12) [41, 152].



[153]

Figure 1.14: The Th1 to Th2 shift. *Following trauma there is an initial Th1-type response, which generates copious pro-inflammatory cytokines, is clinically apparent as SIRS. Subsequently, a shift towards the Th2 type response occurs. A Th2 type response is less efficient with regards to microbial challenges that can clinically manifests as immunosuppression with infection and subsequent multi-organ dysfunction.*

In combination with the shift towards a Th2-type response, the $CD8^+$ T cells have been shown to increase their proliferation in response to apoptosis of $CD4^+$ T cells following significant cutaneous injury [154]. This may be a protective mechanism to allow for some active defence against pathogens.

Following injury Tregs exert their regulatory effect by secreting IL-10. This results in reduced antigen presentation by DCs secondary to trapping of the MHC class II molecules and reduction of co-stimulatory molecules that DCs express and/or secrete. Antigen presentation to T cells is therefore disrupted thus ceasing engagement of T helper cells via the reduced production of IL-2 [107, 127]. Additionally, the Treg secreted IL-10 conditions $CD4^+$ T cells to become unresponsive to antigens and thus lose capacity to produce cytokines, resulting in further suppression [107]. Aside from their manipulation of the cytokine milieu, Tregs can also act via a direct T cell to T cell interaction, resulting in the suppression of the proliferation of T cells via a reduction in the production of IFN-γ by both CD4+ and $CD8^+$ T cells [20].

To prevent systemic immunosuppression, a negative feedback loop for Tregs occurs which is IL-2 dependant. During the inflammatory phase post wounding IL-2 is released and stimulates the activated Tregs transiently. As a consequence to the Treg proliferation the inflammatory response is dampened and the drive for Treg proliferation is subsequently terminated due to reduced IL-2 levels [155]. This aims to prevent negative sequelae of Treg cells such as autoimmunity.

1.6: Summary

Following cutaneous injury there is a cascade of events, involving the release of cytokines, chemokines and recruitment of the innate cells that activates the adaptive immune response. Where there is excessive inflammation SIRS ensues followed by CARS that can lead to sepsis and MODS. In addition, excessive inflammation at the wound site can perpetuate delayed healing and a chronic wound which increases the risk of hypertrophic or keloid scarring [156]. It is well documented that this imbalance in the immune response occurs in severe burns, which can have devastating consequence to the patient with regards to infectious complications, however other cutaneous injury models have been poorly explored.

The literature suggests that injury aetiologies have a similar immunological trend initially, however these later diverge and result in differences in the inflammatory response between the different injury models [22, 28]. These immunological differences are observed clinically following burn and non-burn injury. Specifically a gender dimorphism exists which is highly suggestive that there is recognition of injury type rather than injury result [5, 23, 29, 30, 34, 35]. To date there has been little focus on comparing two cutaneous models of injury to establish if there is a difference in the immune response to different injuries in addition to establishing what this difference may be.

The majority of work has focused on outcome in severe injury, however recent research has shown that even non-severe burn injuries of less than 10% TBSA are associated with long term sequelae including increased risk of

malignancy and cardiovascular disease [5, 38], suggesting that there are sustained impacts of burn trauma. However it is not clear what underpins this association. There is some indication that a measurable long-term impact can be seen in non-burn cutaneous injury though the extent of the long-term consequences remains to be explored.

Thus, understanding the immune response to specific cutaneous injury is essential in order to predict response and manage patients appropriately to limit a potentially avoidable negative outcome. This difference may underlie the clinically apparent compensatory anti-inflammatory response syndrome and associated long-term sequelae of burn injury, which is not seen to the same extent in other trauma.

1.7: Hypothesis

The immune response to burn injury differs from the immune response to non-burn injury (excisional injury).

1.7.1: Aims

Aim 1: To test the hypothesis that aetiology of injury (burn and non-burn, non-severe cutaneous models) impacts the immune response.

Aim 2: To compare immune status long after non-severe burn and non-burn (excisional) injury.

1.8: Experimental Design

There are several models used to reproduce consistent burn injuries with respect to TBSA and depth [124, 157, 158]. In this laboratory a standard model of burn injury is used to produce a moderate size burn injury, comprising of 8% TBSA [157]. Where TBSA is calculated using the Meeh equation (S=kW(2/3)), where k = 9.82 (for C57BL/6J mice [87]), and W = the average weight at the time of injury (19.3g) [87].

This injury model is used as it creates a full thickness contact burn injury that extends to the fascia (including the panniculus carnosus). It is reproducible, consistent in its size and depth, and represents the extent of injury found in the vast majority of burn patients presenting to hospital in developed countries and in Western Australia [10, 12].

In this study a comparable cutaneous injury model was used where a full thickness skin excision was performed using a described method to the same depth as the burn injury model and was matched in location and TBSA [159].

1.9: Significance

It is well known that the immune response to skin injury is a critical regulator of the subsequent healing response. Not only is it crucial for preventing infection subsequent to trauma but it also plays an important role in scar formation and repair. This study will compare the impact of aetiologies of cutaneous injury on the immune response. This information will help guide further research aiming to optimise treatment with respect to modulating the immune response to injury to improve clinical outcome with regards to limiting infectious complications, optimising scarring and improving function in both burn and trauma patients. Furthermore this study may partially elucidate some of the immune changes relevant to the evolving association with long-term consequences that occurs in burn injury survivors. This may have significant clinical impact as it may represent a large patient population group at risk of potentially preventable sequelae.

Chapter 2

Materials and Methods

2.1: Injury Procedure

All experiments were approved by the University of Western Australia Animal Ethics Committee (Perth, Western Australia; Animal Ethics Approval Number RA/3/100/1155) (Appendix A) and all experiments conducted in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.1.1: Animals

Nine week old, female C57BL/6J mice (Animal Resource Centre, Perth, Western Australia) were used for all studies. All mice were received aged 8 weeks and kept for one week prior to the experiment to acclimatise. The mice were housed in the Pre-Clinical Facility at the University of Western Australia in standard housing with food (dry), water (acidified) and enrichment

2.1.2: Time points, groups and experiment overview

Experiments were divided into 6 time points with respect to the day of sample collection; Day 1, 3, 7, 14, 28 and 84 (Table 2.1). Within each of these time

points were three groups; burn, excision and control. The mice were randomly selected for both time point and group and each group housed in group specific cages of 4 to provide companionship.

Time from Injury	Burn	Excision	Control	Total	Haematology n =			Cytokines n =			DC n =			T Cell n =		
					B	E	C	B	E	C	B	E	C	B	E	C
Day 1	9	11	6	26	8	8	3	5	5	3	6	8	5	7	11	6
Day 3	8	9	6	23	8	8	6	5	5	4	9	9	6	8	9	6
Day 7	7	7	5	19	7	7	5	5	5	3	7	7	5	7	7	5
Day 14	13	13	6	32	12	13	6	5	5	3	8	8	6	7	8	6
Day 28	7	8	5	20	7	7	5	5	5	2	8	7	4	8	7	5
Day 84	7	8	3	18	6	8	3	5	5	3	7	8	3	7	8	3
				138												

Table 2.1: Experimental groups, time points and corresponding numbers for each experiment; *Total numbers within each injury and time point group is shown in addition to the subsequent number in each of the specific experiments. Numbers differ from the total number in the group due to inadequate sample/experimental error. Note, day 14 required greater numbers as data was lost during the FACS analysis due to non-viable cells, as such the experiment required repeating.*

Figure 2.1: Schematic overview of methods; *Mice were allocated into burn, excision or control groups. Under anaesthetic burn, excisional or sham injury was performed. All injury groups received post operative IM burprenorphine at time 0 and 12hrs post. All groups received paracetamol in their drinking water for 5 days post operatively. On day 1,3,7,14,28 or 84 cardiac puncture was performed and blood collected was separated into whole blood and sera with subsequent haematology or cytokine profiling. Euthanasia followed cardiac puncture & lymph nodes were harvested and processed for FACS analysis of dendritic or T cell profiles. Skin samples were taken and stained with H&E.*

2.1.3: Anaesthetic and analgesia

Mice were anaesthetised in a closed chamber with continuous flow of Isoflurane 2.5%. Following induction, anaesthetic was maintained via a facemask. In burn and excision groups Temgesic ® (Buprenorphine Hydrochloride 0.3mg/ml) was used for post-operative analgesia. A dose equivalent of 0.1mg/kg (0.001mg) was administered intramuscularly (I.M) following anaesthesia and prior to the injury, in addition a further injection was administered at 12 hours post injury. Soluble paracetamol was dissolved in drinking water for the administration of oral analgesia at a dose of 1mg/ml for a maximum of five days in all groups with the expected amount of consumption of 20ml/day/mouse based on their required fluid intake. The Buprenorphine was not administered to the sham group to prevent to unnecessary respiratory depression or apnoea that may occur in the absence of hyper-stimulation that occurs during pain/noxious stimulation. The implications of this are considered in the discussion (Section 4.7.1)

2.1.4: Burn injury procedure

A full thickness burn injury was performed using a previously described method which produces a full thickness burn to the level of the fascia (including the panniculus carnosus layer (PC)) [157]. For each time point the mice were anaesthetised as described above. The dorsal surface was shaved using clippers and prepped with Inadine® solution. A cylindrical brass rod weighing 65g with a circular diameter of 19mm was used to generate a full

thickness burn to the mid-lower dorsal area. The brass rod was heated to 95^0C in a thermometer controlled water bath and applied with equal pressure for 10 seconds. Using the Meeh formula (S = kW(2/3)) to calculate to surface area, with K = 9.82 (for C57BL/6J mice [87]), and the average weight (W) at the time of injury as 19.3g therefore the sum equates to approximately 8% (7.7%) of the total body surface area. The wounds were left undressed to prevent agitation to the mice in attempts to remove while grooming. There were no cases of wound infection. One mouse died in the day 28 burn group at day 8-post injury. A post mortem was conducted to investigate for infection and concluded autolysis as the cause of death (see Appendix B).

2.1.5: Excisional injury procedure

A full thickness skin excision was performed using a described method [159]. For each time point the mice were anaesthetised as described above. The dorsal surface was shaved and prepped as per the burn group. The area to be excised was marked with ink from a circular stencil with a matching diameter to the brass rod (19mm) generating an equivalent TBSA of 8% (7.7%) as the injury group average weight prior to injury was also 19.3g. Under loupe magnification, the skin was incised using tenotomy scissors along the inked marking to the mid-lower dorsal. The incision was dissected down to the level deep to the panniculus carnosus and superficial to the fascia thus matching the depth of the burn model. The area marked was excised en bloc. There was minimal or no bleeding on excision of the tissue. The wounds

were left undressed to prevent agitation to the mice in attempts to remove while grooming. There were no cases of wound infection. One mouse from the day 84 excision group was culled at day 36 post injury due to an elevated animal monitoring score in excess of 2 (see section 2.1.7). Following examination by the approved personnel they suspected ulcerative dermatitis as the cause for the distress that was thought to be secondary to excessive grooming (see Appendix C).

2.1.6: Control procedure

Control mice were anaesthetised as previously described and anaesthetic was maintained for the approximate duration of the injury groups (5 minutes). Soluble paracetamol (1mg/ml) was provided in their drinking water for 5 days.

2.1.7: Recovery and monitoring

All mice were observed during recovery from anaesthetic and/or injury in housing with foam bedding. All mice were monitored and scored twice daily (B.D) for five days post injury and once daily thereafter up to day 14. Mice monitoring was inclusive of signs of dehydration, distress, pain and infection (See). As per the conditions of the Animal Ethics Committee, a score of 1 resulted in increased monitoring to three times daily (T.D.S) until score returns to normal (0). Should the animal score ≥ 2 the Chief Investigator and Acting Technician in Charge would consider euthanasia (see below procedure).

2.2: Whole Blood and Serum Collection

Blood was collected from each group for all time points. The sample was obtained via cardiac puncture as this maximised amount of blood collection, approximately 500-800μL each mouse that was required for both whole blood and serum collection. A previously described protocol for cardiac puncture was used [160], in brief, the mouse was anaesthetised as previously described and positioned supine. Percutaneous cardiac puncture was performed; Maximal blood volume obtainable was aspirated using a 1ml syringe and dispensed between two tubes; Microvette® 500 EDTA and Microvette® 500 Serum tubes for whole blood and serum samples respectively.

2.2.1: Euthanasia

The anaesthetic was continued following blood collection (see above method) and animals euthanised via a Lethabarb© (Pentobarbitone Sodium) cardiac injection of a dose equivalent of 150mg/kg (2.7mg). Following injection, anaesthesia was ceased and the mice were observed until the toe pinch reflex was absent.

2.2.2: Sample preparation

Whole blood samples (collected in Microvette® 500 EDTA tubes) were analysed for haematology using ADVIA® 2120i Haematology System at Murdoch University Veterinary Hospital Clinical Pathology Department. All samples were kept at 4^0C until processed.

Serum samples (collected in Microvette® 500 Serum tubes) were processed as per manufacturer's instructions. In brief, the samples were allowed to clot at room temperature for 30 minutes, followed by centrifugation at 13,000rpm to separate the clot. Serum was aspirated immediately and stored at -20^0C.

2.2.3: Whole blood data analysis

Descriptive analysis completed for whole blood results using Excel. Statistical analysis was performed using Excel Program and GraphPad Prism 7.0e®

2.3: Cytokine Analysis

Cytokine profiling assays were performed on a selection of the sera collected using a Bio-Plex Pro™ Mouse Cytokine 23-plex assay kits as per the manufacturer's instructions. The plate holds 78 wells for sampling, thus just over half of the data was analysed. The well allocation was evenly distributed across the groups and time points (see table 2.1).

2.3.1: Bio-Plex assay preparation

	1	2	3	5	5	6	7	8	9	10	11	12
A	S	S	B	B	B503	E903	B407	C207	E1014	B528	E728	E184
B	S	S	B501	E901	B603	C103	B507	C307	E1114	B628	C128	E284
C	S	S	B601	E1001	B703	C203	E107	B814	E1214	B728	C128	E384
D	S	S	B701	E1101	B803	C303	E207	B914	E1314	B828	B184	E484
E	S	S	B801	C101	E503	C403	E307	B1014	C114	E328	B284	E584
F	S	S	B901	C201	E603	B107	E407	B1114	C214	E428	B384	C184
G	S	S	E701	C301	E703	B207	E507	B1214	C314	E528	B484	C284
H	S	S	E801	B403	E803	B307	C107	E914	B428	E628	B584	C384

Table 2.2: Cytokine assay 96 well plate layout. *A 96 well plate was used. This encompassed 16 wells for standards (S) and blanks (B); 78 wells were used for the burn (B), excision (E) and controls (C). Numeration represents the animal number and time point, i.e. B501 is burn specimen 5 day 1.*

The cytokine plate was organised including 16 wells with predetermined locations for the standards and blank wells (Table 2.2). The assay reagents were equilibrated to room temperature. Bio-plex standard diluent was reconstituted and diluted to form the standards. The 8-point standard dilution series and blanks were prepared (Figure 2.1). Briefly, 72ul of diluent was added to tube S1, 150ul was added to tubes S2 through S8 and the blank. 128ul of reconstituted diluent was transferred to S1; serial dilution of 50ul between tubes S1 to S8 was performed, with vortexing.

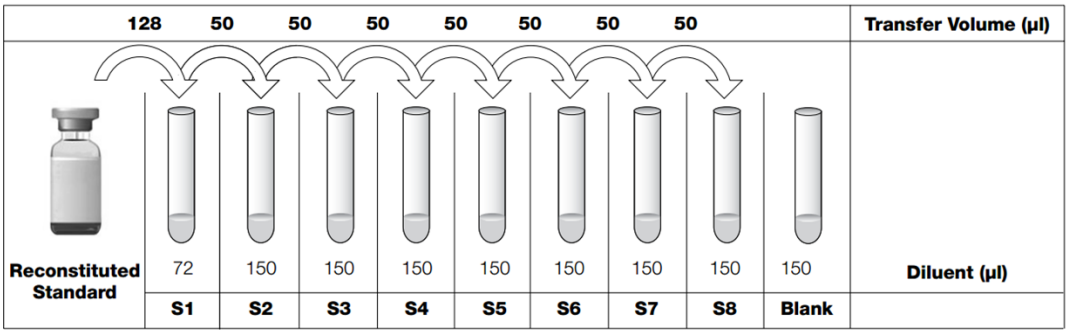


Figure 2.2: Standards and blanks preparation. *The standards and blanks were reconstituted to 8 standards (S1-S8) and a blank (B).*

The serum samples were thawed from -20°C at room temperature and kept at 4°C. Once the serum samples were thawed, they were diluted in the Bio-Plex sample diluent using a 1:4 ratio (1 volume of sample for 3 volumes of sample diluent) in this case 50ul of serum sample to 150ul diluent sample.

The coupled beads were prepared in assay buffer; this was performed protected from light due to fluorescence sensitivity. The beads were allowed to equilibrate at room temperature for 20 minutes prior to use. For this assay, x1 couple beads were required. Prior to dilution, the beads were vortexed for 30 seconds. Each well requires 50µl of coupled beads (x1), therefore 575ul of x10 couple beads were added to 5175ul of buffer solution, total volume of 5750ul (this calculation includes a 20% excess to account for transfer loss).

The detection antibodies were prepared during the primary incubation, as they require preparation 15 minutes prior to use. The detection antibodies were vortexed for 20 seconds, followed by a further 30 second vortex, 300µl was then transferred into 2700µl of detection antibody diluent was added to a 15ml tube.

The Streptavidin-PE is prepared during the detection antibody secondary incubation. In brief, 5940µl of assay buffer is added to a 15ml tube, the

Streptavidin-PE is vortexed for 20 seconds then spun for 30 seconds, 60µl of Streptavidin-PE is pipetted in to the 15ml tube containing the assay buffer, diluting the Streptavidin to a x1 concentration. Calculations used include a 25% excess to compensate for transfer losses.
The Bio-Plex® MAGPIX™ Multiplex Reader system (Bio Rad®) was warmed up for 30 minutes prior to use.

2.3.2: Running the assay

The filter plate was pre-wet with 100µl of assay buffer and the liquid was removed using vacuum filtration. The diluted coupled beads were vortexed for 30 seconds and 50µl of x1 beads were added to each well of the assay plate. The wells were washed twice by adding 100µl of wash buffer that was then removed via vacuum filtration.
The diluted standards, blanks and samples were vortexed gently for 3 seconds, 50µl added to each applicable well. The plate was incubated at room temperature on a shaker for 30 minutes.
Following the primary incubation of the samples, wells were washed three times with 100µl of assay buffer and liquid removal by vacuum manifold.
The detection antibody mix was then gently vortexed for 3 seconds and 25µl added to each well. The plate was then incubated with shaking at room temperature for 30 minutes.
Following the secondary incubation with detection antibody, the wells were washed three times, using the previously described method. The Streptavidin-

PE was vortexed for 5 seconds, and then 50µl added to each well, and the plate incubated at room temperature with shaking for 10 minutes.
Following the tertiary incubation with streptavidin-PE the plates are washed three times, using the previously described method.
Finally, Assay buffer, 125ul was added to each well, and the plate was shaken at 1100rpm for 30 seconds prior to being placed on the Bio-plex analyser (Perkin Elmer CS 1000 Autoplex Analyzer with Sheath Delivery System301-3913-BSIINV with Luminex xMAP® Technology).

2.4: Lymph Node Preparations and Analysis

To assess migration of dendritic cells and nature of T cell responses to the injury, the wound skin draining lymph nodes were harvested from all groups at all time points. Both inguinal nodes were harvested as the draining lymph nodes due to the size and location of the injury.
Following blood collection and euthanasia as described above, the lymph nodes were stored at 4^{0}C in PBS until processed. Processing commenced within an hour of collection of all samples in order to capture live cells for analysis.

2.4.1: Preparation of lymph node single cell suspension

Lymph node digest was performed cold at 4 degrees using a described protocol [161], in brief the lymph nodes were chopped with a scalpel for 1 minute then digested with a solution of Collagenase D (1.5mg/ml;

Worthington® Biochemical, Lakewood, NJ) and Pancreatic type I DNase (0.1mg/ml; Sigmal-Aldrich® St. Louis, MO) in 10% FBS-GKN (Fetal Bovine Serum- Glucose Potassium Sodium solution D-glucose, 5.5mM KCl, 137mM NaCl, 25mM Na_2HPO_4, 5.5mM $NaH_2PO_4.2H_2O$ solution) using 5ml per lymph node pair. The solution was incubated at 37^0C in an agitated incubator for 30 minutes. Digested samples were further aggravated (using manual pipetting) before filtering through a 100 µm yellow cell strainer and washed with 10% FBS-GKN to reach a total volume of 25ml. The suspension was centrifuged at 1700rpm for 5 minutes, after which all samples were placed on ice.
The supernatant was aspirated and cells resuspended in 200µl of 0.5% BSA/ GKN solution and incubated with 1.0µl anti-Fc block (anti-mouse CD16/CD32 purified eBioscience®) for 15 min on ice. Cells were centrifuged as previously before the addition of phenotyping mABs to prevent any nonspecific binding prior to the addition of the anti-mouse antibodies.
Cells from the suspension were counted using Trypan Blue at a 1:9 ratio (10µl: 90µl).

2.4.2: Lymph node cell staining

2-5 x 10^6 cells were added to each FACS tube (BD Pharmigen®) for both the dendritic cell stain and the T cell stain. Cell suspensions were centrifuged at 1800rpm for 5 minutes, and the supernatant was discarded. Cells were re-suspended in a 200µl GKN/BSA with primary anti-mouse antibody conjugates for determination of DC and T cell populations.

All antibodies were used as direct conjugates to FITC, Phycoerythrin (PE), PE-Cy7, allophycocyanin (APC), APC-Cy7, or biotin and Streptavidin conjugated PE-Cy5 (BD Biosciences, San Jose, CA) as required. Appropriately matched IgG isotype controls (BD Pharmingen, Western Australia) and cytometer compensation settings adjusted using single-stained controls were used for each experiment

2.4.3: Dendritic cell antibody staining

DC populations were identified using combinations of fluorochrome-labeled mAbs including; anti-CD4 APC (BioLegend®), anti-CD11cPE (N418), anti-CD11bAPC Cy7 (M1/70), anti-CD8a FE Cy7 (53-6.7), anti-CD103bio (M290 BD Pharmigen®), anti-I-A/I-E FITC (2G9 eBioscience®) primary antibody stain. All labeling was performed in glucose sodium potassium buffer containing 0.2% BSA for 30 minutes on ice. Following incubation 2mls of GKN/BSA 0.1% was added and re-centrifuged at 1800rpm for 5 minutes. The supernatant was aspirated and resuspended in 200µl of PE-Cy5 Streptavidin conjugated antibodies and incubated for 15minutes at 4^0C. After which 2mls GKN/BSA 0.1% solution was added to each and repeat centrifuge at 1800rpm for 5 minutes. The supernatant was aspirated and resuspended in 250µl GKN/BSA 0.1% solution. Samples were stored at 4^0C over night then processed on the Flow cytometer.

2.4.4: T Cell antibody staining

T-cell populations were identified in ILN digests using the fluorochromes anti-CD4 APC Cy7, anti-CD25 APC, anti-CD8 PECy7 to identify the T cell subtypes (BD Pharmigen®). Anti-Ki67 (BD Pharmigen®) was used to identify proliferating T cells and a FOXP3 intracellular staining kit (eBiosciences, San Diego, CA) was use to determine FOXP3 intracellular staining. All Abs were used as direct conjugates to FITC, Phycoerythrin (PE), PE-Cy7, allophycocyanin (APC), APC-Cy7, or biotin and Streptavidin conjugated PE-Cy5 (BD Biosciences) as required.

The cells were incubated at 4^0C for 30 minutes. Following incubation 2mls of GKN/BSA 0.1% was added and re-centrifuged at 1800rpm for 5 minutes. Following the primary staining, cells were washed with 2mls of GKN/BSA 0.1% and centrifuged at 1800rpm for 5 minutes. The supernatant was discarded and cells were resuspended in 250µl GKN/BSA 0.1% solution. Samples were stored at 4^0C over night. The following day cells were processed using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience®), as per manufacturer's instructions. In brief, cells were fixed using 700 µl of Fix/Perm Solution and incubated in the dark for 30 minutes at 4^0C. Cells were washed with 1ml x1 Fix/Perm Buffer Solution and centrifuged at 1800rpm for 5 minutes. The supernatant was discarded and cells were resuspended in 100µl fix/perm buffer solution with 1.5 µl of Fc block added. Cells were incubated at 4^0C for 15 minutes. Without washing, 1.5µl of Foxp3-PE and Ki67-FITC antibodies (eBioscience®) were added. Cells were

incubated at 4^0C for 30 minutes. Following incubation cells were washed in 2ml of x1 fix/perm buffer solution, and then centrifuged at 1800rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 250µl GKN/BSA 0.1% solution.

Both the DC and T cell samples were stored at 4^0C and transferred to the Telethon Institute of Child Health Research for processing.
Samples were run using LSRII flow cytometer (BD Biosciences) and analysed using FlowJo software (TreeStar, Ashland, OR).
Staining for surface molecules was reported as the frequency of cells within a population expressing the marker of interest.

2.4.5: Splenic cytokine assays

At the indicated time points, spleens were collected and digested to single-cell suspensions as described previously. Cells were prepared in RPMI 1,640 with glutamine (Invitrogen Life Technologies, Australia) supplemented with 10% fetal calf serum, 20 µgml− 1 gentamycin, and 20 µM 2-ME and plated at 3 Å~ 105 cells per well in 96-well plates (Nunclon, Nunc, Denmark) with Concavalin A at 10 ngml−1 in triplicate. At 48 hours of culture, triplicate supernatants were pooled and stored at −20 °C prior to cytokine analysis.

2.5: Statistical Analysis

Microsoft Excel Program was used for data collection and GraphPad Prism7.0e® was used for statistical analysis. As our sample size was small, normal distribution was not assumed and a non-parametric ANOVA test (Kruskal-Wallis) with Dunns multiple comparison test was used. A p-value of <0.05 was considered statistically significant.

Chapter 3

Results

3.1: Summary of Results

	Day 1				Day 3			
	C	H	DCs	TC	C	H	DCs	TC
B	↑↑IL-6 ↑TNFα ↑IL-10 ↑MCP1 ↑MIP1α ↑MIP1β ↑KC ↑↑GCSF ↑EOTAXIN ↑IL-12p70 ↑IL-5 ↑IL-13		↓CD103+ MFI	↑CD8+ # ↑↑CD8+CD25+ #	↑GCSF	↑↑Mono	↑DC# ↑CD8α+ # ↑CD8α- # ↑CD11b+ # ↑CD11b- # ↓DC% ↓CD103+ % ↓DC MFI ↓↓CD8α+ MFI ↓↓CD8α- MFI ↓↓CD103+ MFI	↑CD4+ # ↑CD8+ # ↑CD8+CD25+ # ↓CD8+ Ki67+ %
E	↑IL-6 ↑↑KC ↑↑GCSF			↑CD4+ # ↑CD8+ # ↑↑CD8+CD25+ #	↑↑IL-6 ↑GCSF ↑IL-13		↑DC# ↑CD8α+ # ↑CD8α- # ↑CD11b- #	↑CD4+ # ↑CD4+ CD25+ # ↑CD8+ #
	Day 7				Day 14			
	C	H	DCs	TC	C	H	DCs	TC
B	↑IL-6 ↑↑GCSF	↑Neuts	↑CD8α- # ↑CD11b+ #			↑Neuts	↑DC # ↑CD8α # ↑CD11b+ # ↓CD11b- MFI	↑CD4+Ki67+ % ↑↑Tregs # ↑↑Tregs %
E	↑IL-10 ↑IL-12p70	↑↑Neuts		↑↑CD4+ Ki67+ % ↑CD8+ CD25+ %				
	Day 28				Day 84			
	C	H	DCs	TC	C	H	DCs	TC
B			↓CD11b- # ↓CD11b+ # ↓CD11b+ MFI ↓CD11b- MFI	↓CD4+CD25+ # ↑CD4+Ki67+ % ↓CD4+CD25+ %	↑IL-10	↓WCC # ↓Lymph ↓Eosin	↓CD11b+ MFI	
E							↑CD4+ #	

Table 3.1; Summary of significant results throughout all time points. *Where (B) represents the burn group and (E) represents excision group. Results summarised included: C - cytokines; H – haematology; DCs – Dendritic cells; TC – T cells; # - Cell number; % - Cell Frequency; ↑ - Increased significantly; ↓ Decreased significantly*

3.2: Injury Impact on Growth

The weight of the mouse was used to assess clinical dehydration and general recovery post operatively. There were no significant changes observed in weight between the burn & excision groups within the specified days. There was increased weight observed in both groups throughout the time points (day 1 -84) as the mice increased in maturity and reached adult size (Figure 3.1).

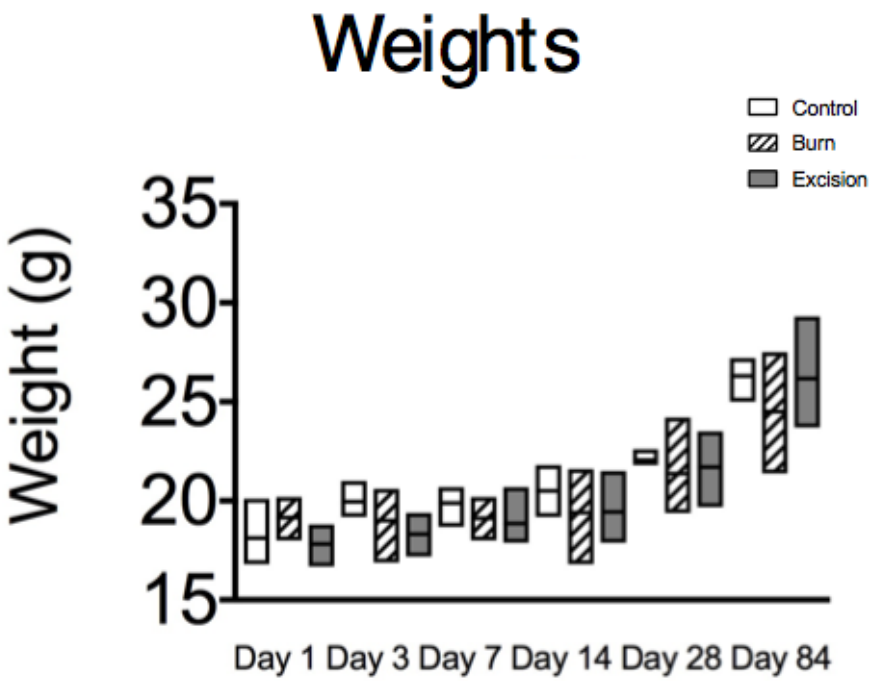


Figure 3.1 Weight changes in mice post burn and excision injury. *Weights were measured twice daily in each group from day 1 – 7 post injury/sham. Weights were once daily in each group from day 14 – 28, then once weekly from day 28-84 post injury/sham. Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. N=3-14 mice/group*

3.3: Burn Injury Induces a Rapid Systemic Cytokine and Chemokine Response

Significant trauma induces an early inflammatory cytokine responses including IL-1α, IL-1β, tumour necrosis factor-α (TNF-α), and IL-6 [162]. This response facilitates the coordination of removal of cellular debris, defends against pathogen exposure and initiates tissue repair.

Sera was isolated from all groups (burn, excision and control) on day 1, 3, 7, 14, 28 & 84 post injury/control. The serum was examined for inflammatory cytokines (Figure 3.2), chemokines (Figure 3.3) and T cell cytokines (Figure 3.4).

At day 1 post-burn injury, IL-6 levels were significantly increased (Figure 3.2). Excision injury also induced a significant increase in IL-6 production at day 1, however its peak level was not until day 3, compared with both control and burn injury (Figure 3.2). TNF-α sera concentrations were significantly elevated at day 1 post burn compared with the excisional injury. However, in the excision group TNF-α levels did not significantly increase.

Interleukin 10 was also significantly elevated in the burn group at day 1 compared to the control, in addition there was a later peak at day 84 compared to the excision. Whereas in the excision group IL-10 trended towards an increase at day 3 with significant elevation at day 7 compared to the control (Figure 3.2).

There was no significant difference in sera concentrations of IFN-ɣ in either injury group at any time. Due to data loss in some of the IL-α groups resulting in an n=1 statistical significance could not be drawn (data not shown).

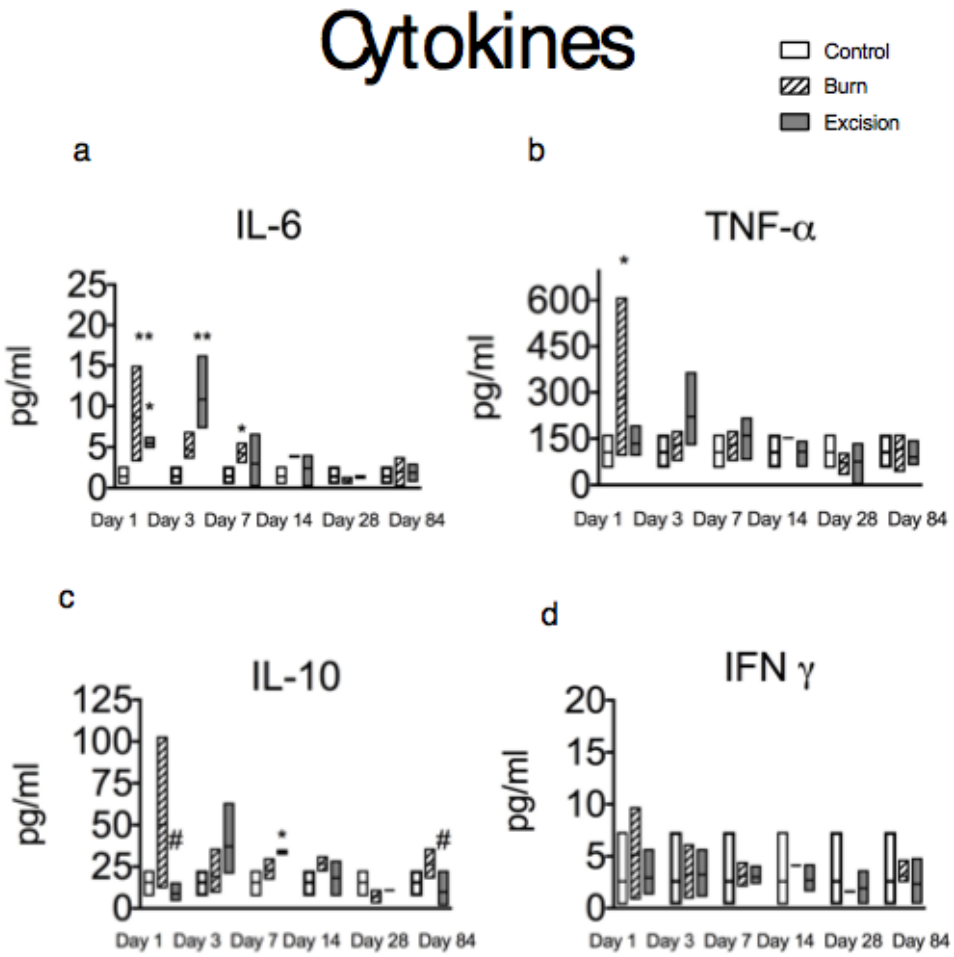


Figure 3.2 Inflammatory cytokines following burn and excision injury:
*Inflammatory cytokines were assessed from the sera of each group; control, burn and excision on day 1 - 84 post injury/sham. Graphs show prominent cytokines involved in the inflammatory phase, including: IL-6 (a), TNF-a (b), IL-10 (c) and IFN-ɣ (d).Graphs represent mean + range. Significance is indicated by *(p=0.05) and ** (p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=2-5 mice/group, for at least two independent experiments.*

Chemokines are responsible for the recruitment of innate inflammatory cells that are required during the inflammatory response. Levels were assessed in each of the groups and all time points (Figure 3.3).

monocyte chemoattractant protein 1 (MCP1) is a monocyte, immature DC, and memory T-cell attractant. At day 1, burn injury induced elevated levels in the sera when compared with the excision injury and control (Figure 3.3a). Macrophage inflammatory protein 1α (MIP1α/CCL3) and macrophage inflammatory protein 1β (MIP1β/CCL4) attract monocytes, T cells, and polymorphonuclear leukocytes. Their sera levels were also significantly elevated at day 1 post burn compared with excision (Figure 3.3b & c). No significant change in MCP1, MIP1α, and MIP1β was observed after excision. KC (CXCL1) is a neutrophil attractant that also induces angiogenesis. Elevated levels of KC were detected at day 1 both post excision and burn compared with control (Figure 3.3d).

Serum granulocyte colony stimulating factor (G-CSF) was next examined as its secretion stimulates the release of granulocytes from bone marrow in addition to increasing neutrophil proliferation and mobilisation [42]**.** A significant increase in G-CSF levels after burn injury was detected at day 1 through to 7 when compared to control levels (Figure 3.3e). Following excisional injury a 7-fold increase in G-CSF at day 1 occurred compared to both the burn and control groups. This significant elevation persisted to day 3. EOTAXIN (CCL11) is an eosinophil, basophil, mast cell, T-helper 2 cell, and platelet attractant [125]. This chemokine was significantly elevated at day 1 above the control in burn injury alone (Figure 3.3f). GM-CSF showed a trend towards elevation in the burn injury at day 1 and excision at day 3, however neither were found to be significant. RANTES showed no significant change in either burn of injury group at any time point, thus data not shown.

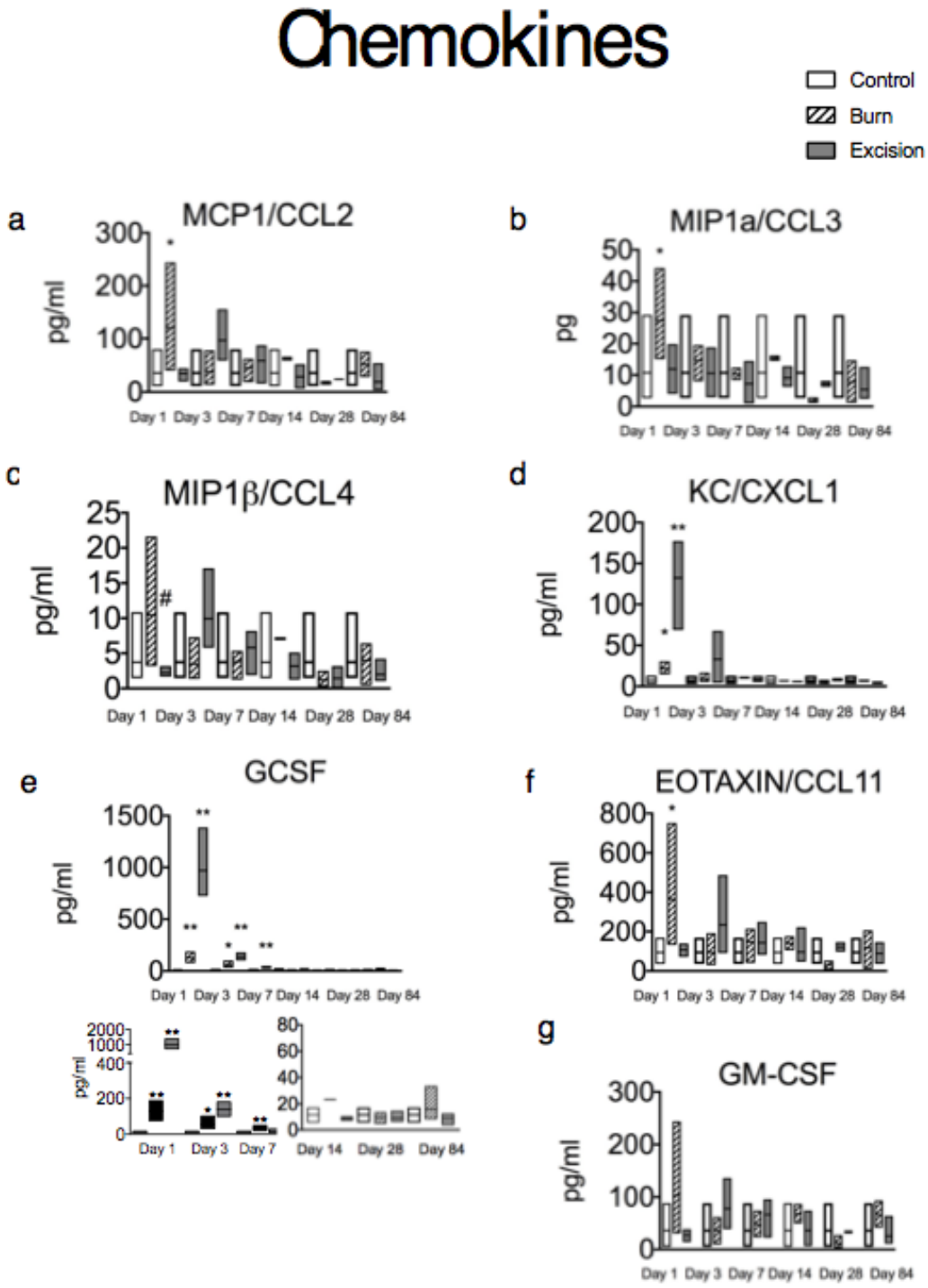


Figure 3.3 Chemokine changes in burn and excision injury. *Chemokines were assessed from the sera of each group; control, burn and excision on day 1 - 84 post injury/ sham. Graphs show prominent chemokines involved in the innate and adaptive systems, including: MCP-1/CCL2(a), MIP1-a/CCL3 (b), MIP1-b/CCL4 (c) KC/CXCL1 (d) GCSF as an overview from day 1-88 and expanded views day 1-7 and day 14-84 (e), EOTAXIN (f) and GM-CSF (g). Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=2-5 mice/ group, for at least two independent experiments.*

T cell–modulating cytokines were next examined (Figure 3.4). Both IL-3 and IL-4 showed a trend towards elevation in both injury groups at day 1 in the burn and day 3 in the excision, however results here were not found to be significant (Figure 3.4a & 3.4c).

Levels of IL-12p-70 were significantly increased in the burn injury at day 1 compared to the excision which showed increasing levels from day 3 which reached significant elevation above the control at day 7 (Figure 3.4b).

The burn injury group had elevated IL-5 levels compared to the excision at day 1 alone. The excision group showed an apparent increase at day 3, however this was not found to be significant.

When looking at IL-13, the burn group had significantly raised levels at day 1 against the control and the excision group rose later at day 3 (Figure 3.4e).

IL-2 and IL-17 are both Th2 associated cytokines. These were also examined in the sera with IL-2 found to be increased significantly in the burn injury alone at day 14 only.

Levels of sera IL-17 could not be assessed due to an n=2 in some of the control sample time points, thus significance could not be accurately established and the data is not shown.

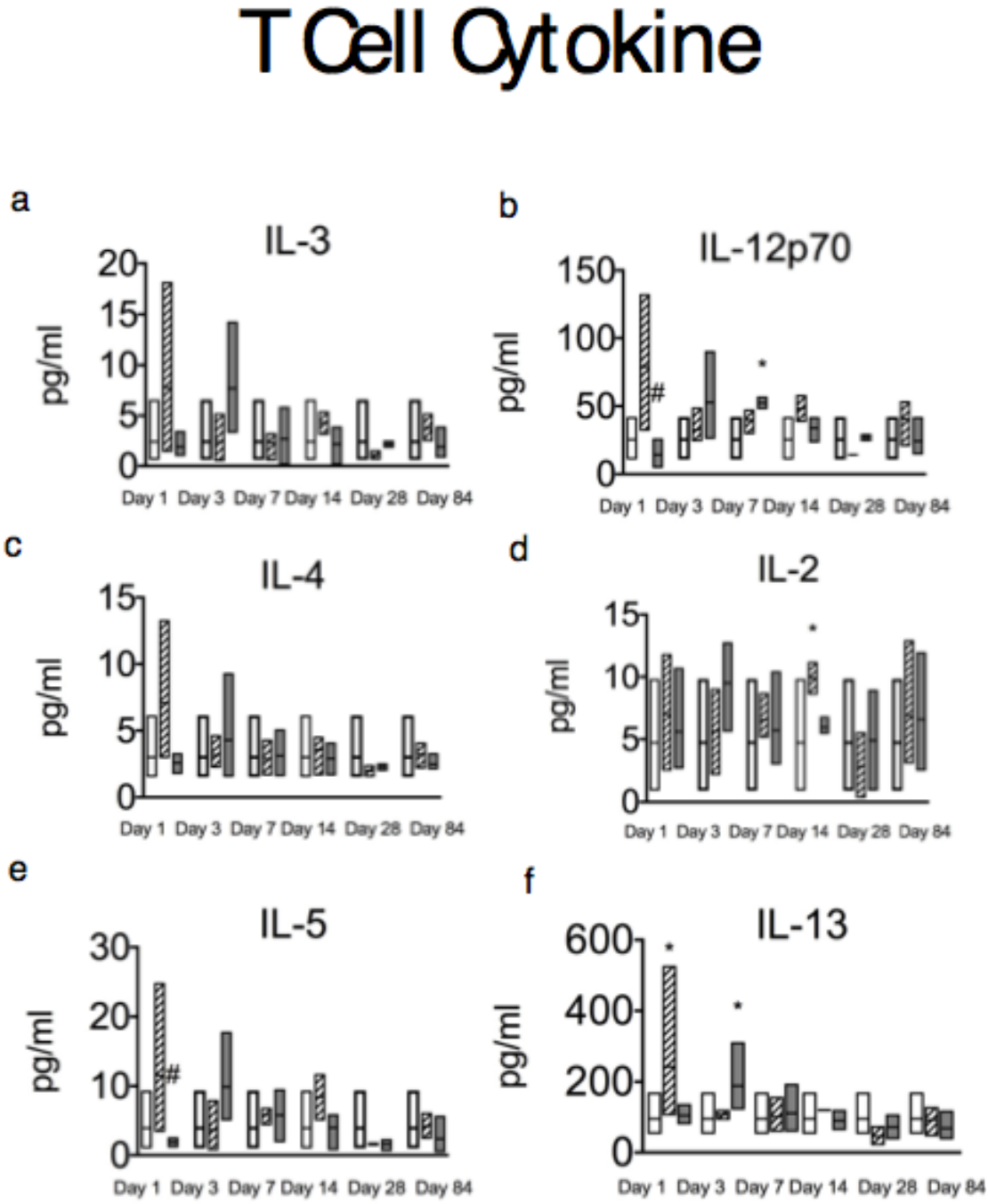


Figure 3.4: T cell modulating cytokine changes in burn and excision injury;
Cytokines that modulate the T cell response were assessed from the sera of each group: control, burn and excision on day 1 - 84 post injury/sham. Graphs represent the cytokines IL-3 (a), IL-12p70 (b), IL-4 (c), IL-2 (d), IL-5 (e) and IL-13 (f).
*Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=2-5 mice/group, for at least two independent experiments.*

The data thus far indicates a difference in both the timing and profile of the cytokine and chemokine production between burn and excision injury. The burn induced an earlier release of inflammatory cytokines and a different chemokine response consisting of those that promote monocyte/macrophage recruitment & differentiation (MCP1, MIP1α, MIP1β, and EOTAXIN). Whereas, the excision group showed a later and less amplified inflammatory cytokine levels with a chemokine response that predominantly recruits neutrophils (KC and GCSF).

3.4: Burn Injury Induces Changes in Systemic Monocyte and Neutrophil Levels

Whole-blood counts were obtained at days 1, 3, 7, 14, 28 and 84 post injury. There were no significant acute alterations to the total white cell count (WCC) in either injury group (Figure 3.5a). The total WCC was divided to examine the cell numbers for neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The lymphocyte cell count reflects circulating T and B cells and specific sub-types were not examined in the systemic compartment. Neutrophil cell counts were elevated post burn (<0.05) and excision (<0.01) at day 7 compared to the control. In the burn injury group this significant elevation was sustained until day 14 when compared with the excision group (Figure 3.5b). A significantly increased monocyte count was observed at day 3 post burn when compared with control with no changes observed in the excision group (Figure 3.5c). However, later at day 84 the burn group showed reduced total WCC levels compared to the control, which appears to be secondary to a significant reduction in lymphocyte and eosinophil populations in the burn injury (Figure 3.5a,d & e).

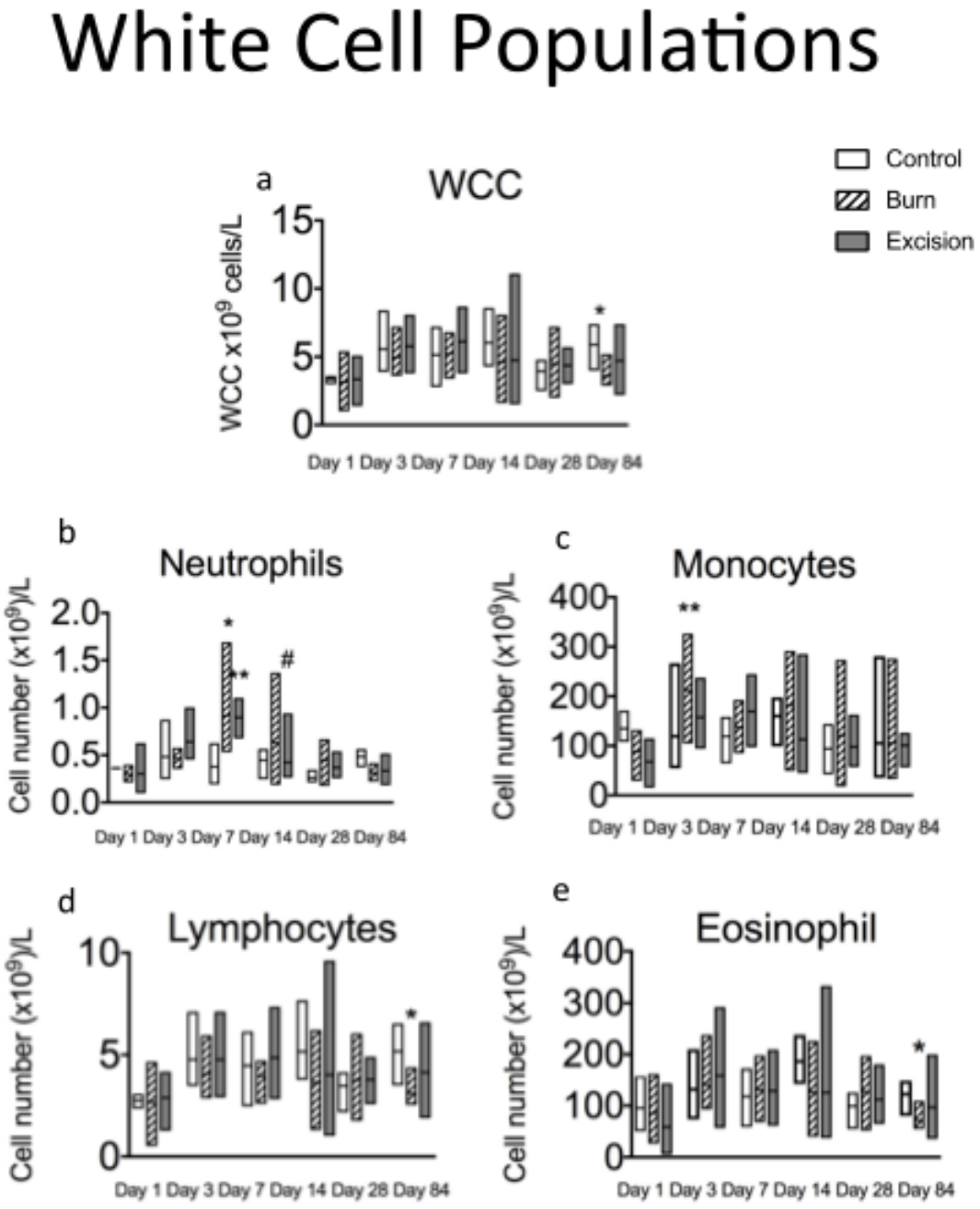


Figure 3.5: Innate cell numbers in burn and excision injury. *Whole blood was collected from control, burn and excisional injury groups on days 1 - 84 and analysed for basic haematology and innate cell profiles. Graphs represent white cell populations including; total white cell count (a) neutrophils, (b) monocytes (c) lymphocytes (d) and eosinophils (e). Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-13 mice/group, for at least two independent experiments.*

3.5: Changes in Dendritic Cell Population and Maturation are Different Following Burn and Excision Injury

The draining inguinal lymph nodes (ILNs) were harvested at all time points post injury and assessed using flow cytometry. Dendritic cells (DCs) and their subsets were examined for cell number, frequency and maturation status. Dendritic cell populations were identified as major histocompatibility complex **(**MHC) class II high and $CD11c^{+}$ [49, 94] . This cell population was subsequently gated on $CD8a^{+}$ and $CD8a^{-}$ representing DCs resident to the lymph node and those that have migrated from the skin, respectively. The latter $CD8a^{-}$ DC that circulate from the dermis during surveillance and inflammation were further gated into $CD103^{+}CD11b^{lo}$ ($CD103^{+}$ DC), $CD11b^{hi}CD103^{-}$ ($CD11b^{hi}$ DC), and $CD11b^{lo}CD103^{-}$ ($CD11b^{lo}$ DC) subsets (Figure 3.6a). This gating strategy provides information on resident DC filtering blood antigens and migratory DC presenting antigen from the periphery, which modulate adaptive T-cell responses via induction of $CD4^{+}$ and/or $CD8^{+}$ T–cell populations.

Inguinal lymph node (ILN) total cell number was significantly increased after burn and excision injury at day 3 compared with controls (Figure 3.6b). Total DC cell number, resident $CD8a^{+}$ DC number, and $CD8a^{-}$ DC number were all significantly increased at day 3 in both injury groups when compared with the control. In the burn injury total DC number had a trend towards elevation at day 7 and was significantly elevated above the control at day 14 (Figure 3.6c). In the $CD8a^{-}$ population, the burn also had significant elevation at day 7 and 14 which was not seen in the excisional model (Figure 3.6e).

The $CD8a^-$ DC subpopulation cell numbers were further analyzed. There was no increase in the $CD103^+$ DCs in either the burn or excision groups. In the $CD11b^{hi}$ DC populations numbers were significantly increased compared with controls at day 3,7 and 14 in the burn injury group only (Figure 3.6f). In the $CD11b^{lo}$ DC populations, both burn and excisional injuries had significantly elevated levels above the control at day 3 (Figure 3.6g).

In the later time points when healing was complete a significantly reduced number of $CD11b^{lo}$ DCs were seen after burn injury compared with the control. In addition to this, the excision group also showed some persistent changes in the DC populations with significantly elevated total DCs at day 84 compared with control (Figure 3.6c).

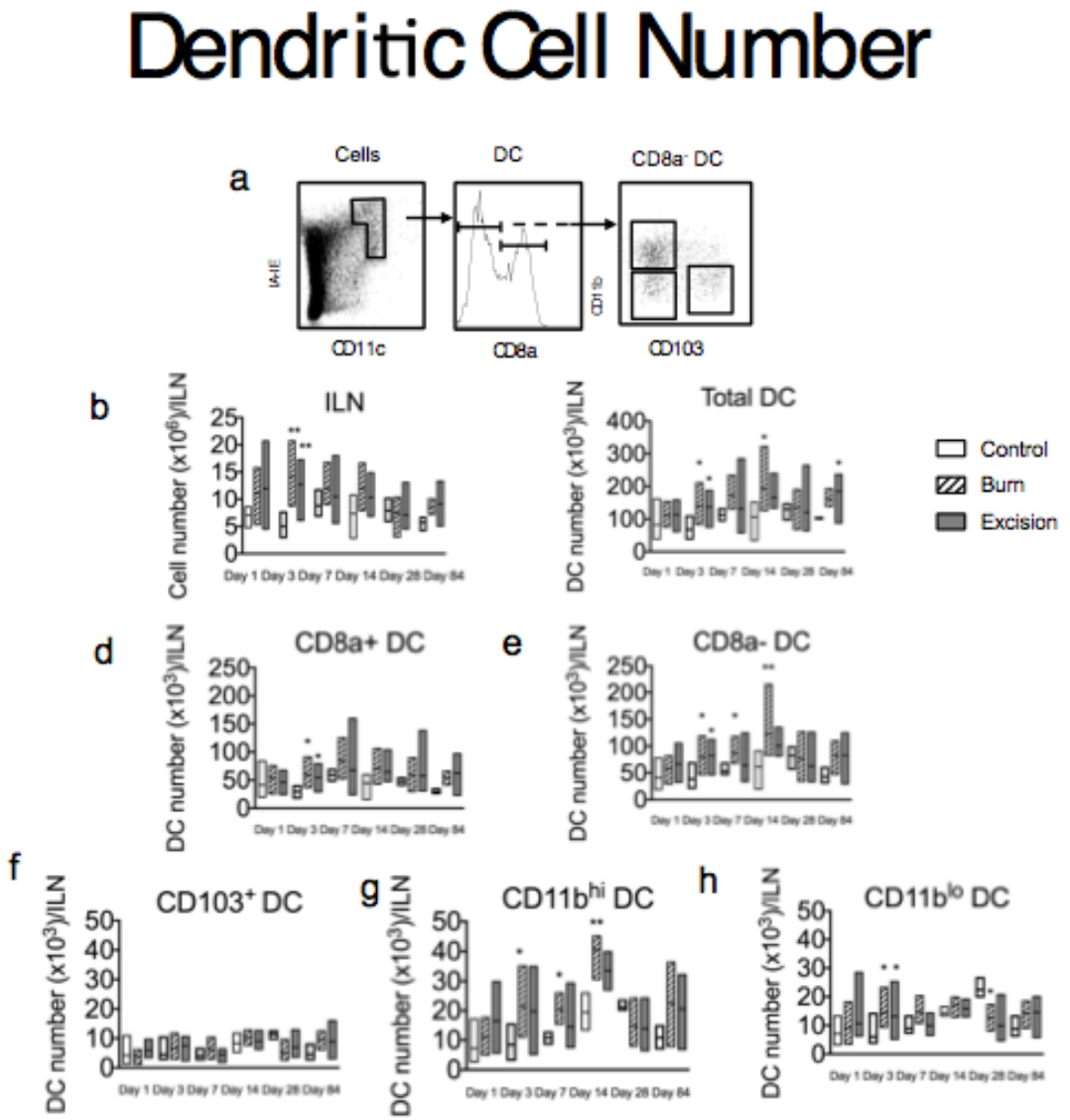


Figure 3.6: Dendritic cell following burn and excision injury. *Inguinal lymph nodes were harvested from each group day 1 - 84 post injury/sham. Inguinal lymph nodes were digested to a single cell suspension. Cells were gated at CD11c⁺ MHC class II^{hi} & further gated using CD8a, then CD11b and CD103 to establish resident lymph node and migratory skin populations (a). The CD8α⁻ DC were subsequently delineated according to CD11b and CD103 expression, as indicated. The graphs represent the total cell numbers in the paired inguinal lymph nodes (ILN) (b), total DC number (c), CD8α⁺ DC (d) and CD8α⁻ DC (e). The CD8α⁻ DC subpopulations numbers are represented next; CD103⁺ DC (e), CD11b⁻ DC (f) and CD11b⁺ DC (g). Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-11 mice/ group, for at least two independent experiments.*

In addition to the dendritic cell number data, the burn injury group had a significant decrease in the percentage of DC cells in the draining ILN that (DC % frequency) at day compared with control (Figure 3.7a). No changes were observed in the excision group.

There were no significant changes in either of the injury groups when looking at the different populations of DCs ($CD8a^+$ or the $CD8a^-$). However, within the $CD8a^-$ DC subpopulations, there was a significant decrease in the $CD103^+$ DC percentage frequency post burn when compared with control at day 3 (Figure 3.7d). There were no other significant changes in the $CD103^+$, $CD11b^{hi}$, or $CD11b^{lo}$ percentage frequencies in the burn or excision groups (Figure 3.7e & f).

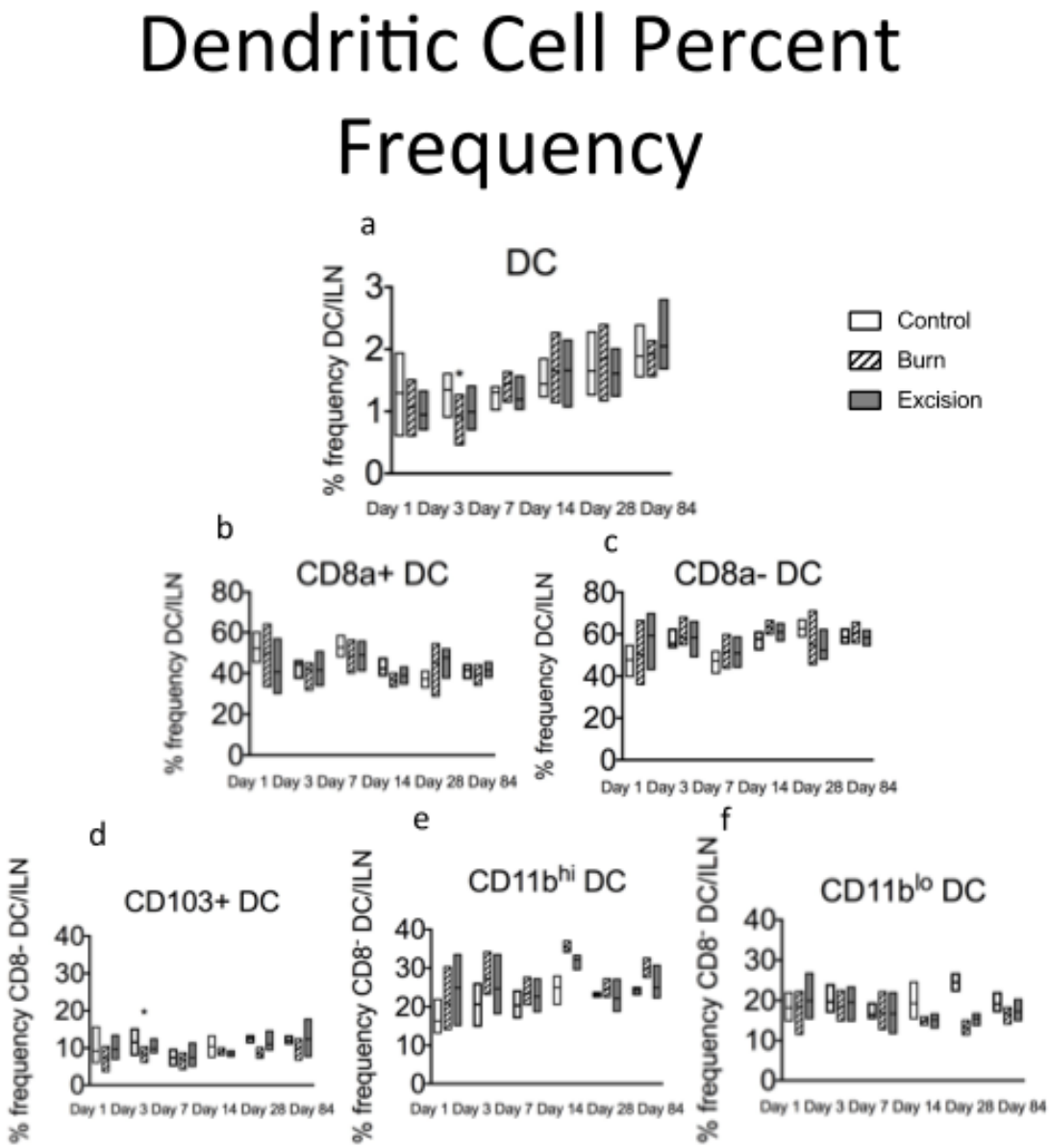


Figure 3.7: Dendritic cell frequency following burn and excision injury. *Inguinal lymph nodes were harvested from each group day 1 - 84 post injury/sham. Inguinal lymph nodes were digested to a single cell suspension. Cells were gated at CD11c^{+} MHC class II^{hi} to establish DC populations were gated using CD8a, then CD11b and CD103 to establish resident lymph node and migratory skin populations (Figure 3.6a). The CD8α^{-} DCs were subsequently delineated according to CD11b and CD103 expression, as indicated. The graphs represent the total DC frequency (a), CD8α^{+} DC frequency (b) and CD8α^{-} DC frequency (c). The CD8α^{-} DC subpopulations frequencies are represented next; CD103^{+} DC (d), CD11b^{-} DC (e) and CD11b^{+} DC (f). Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-11 mice/group, for at least two independent experiments.*

The activation status of the DC populations was also determined by analysing the up regulation of Mean Fluorescence Intensity (MFI) of MHC II expression (Figures 3.8). Reduced MHC II expression on DCs compromises their ability to prime T-cell responses and may potentiate a tolerant response.

The total DC MFI was significantly decreased at day 3 in the burn injury alone when compared with control. Furthermore, there were significant reductions in the MHC II MFI in the $CD8\alpha^{+}$ DC and $CD8\alpha^{-}$ DC subsets after burn injury compared with the control (Figure 3.8b & c).

Within the migratory $CD8\alpha^{-}$ DC the subsets also showed significant reductions the MCH II MFI in the burn injury group, specifically the $CD103^{+}$ DC subset at day 1 and day 3 compared with control (Figure 3.8d).

Interestingly these changes in activation of the migratory $CD8\alpha^{-}$ DCs was found to have significantly reduced MHC II MFI at the later time points of day 28 and 84. The $CD11b^{hi}$ were found to be significantly reduced compared to the control post burn on day 28 and 84, whereas the $CD11b^{lo}$ DCs at day 14 and 28 (Figure 3.8e & f).

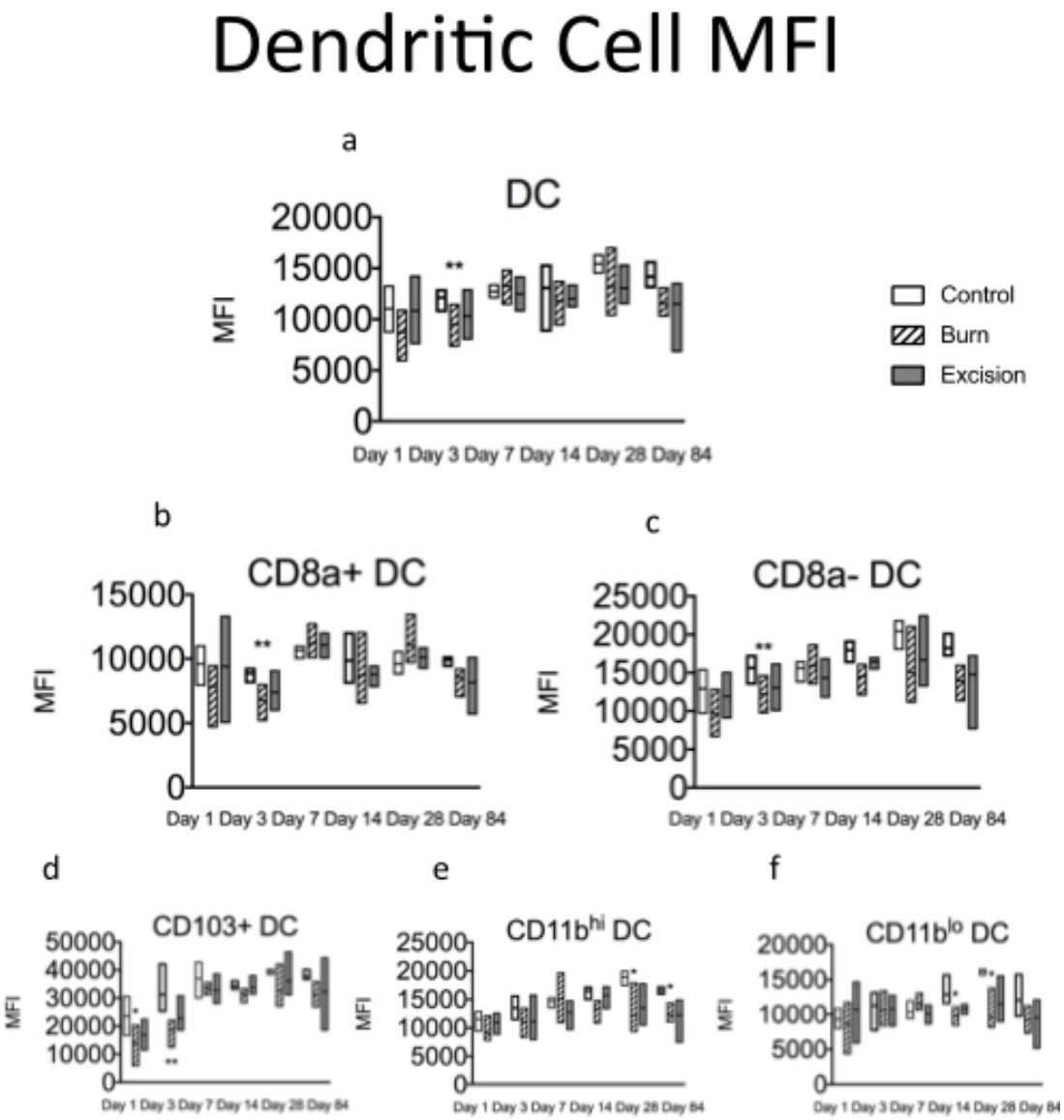


Figure 3.8: Dendritic cell MHC class II surface expression following burn and excision injury. *Inguinal lymph nodes were harvested from each group day 1 - 84 post injury/sham. Inguinal lymph nodes were digested to a single cell suspension and gated at CD11c^{+} MHC class II^{hi} to establish DC populations. This was further gated using CD8a, CD11b and CD103 to establish resident lymph node and migratory skin populations (Figure 3.6a). The CD8α^{-} DCs were subsequently delineated according to CD11b and CD103 expression, as indicated. Cell surface expression of MHC II as determined by MFI is presented in the graphs for each of the cell populations. The graphs represent total DC MFI (a), CD8α^{+} DC MFI (b) and CD8α^{-} DC MFI (c). The CD8α^{-} DC subpopulations CD103^{+} DC (d), CD11b^{-} DC (e), and CD11b^{+} DC (f).Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-11 mice/group, for at least two independent experiments.l*

3.6: $CD4^+$ and $CD4^+$ T Regulatory Cell Responses Differ Between Burn and Excision Injury

Inguinal lymph node (ILN) cells were examined for T populations including $CD4^+$ T cells, $CD8^+$ T cells, and T regulatory (Treg) cells by flow cytometry. Subsequently $CD4^+$ T-cell number, proliferation, activation, and Treg cell induction was determined (Figures 3.9a).

The $CD4^+$ T cell number was significantly increased at day 1 in the excision compared to the control and both the burn and excision at day 3 compared to the control (Figure 3.9b). In the dividing $CD4^+$ cells ($Ki67^{hi}$) there was an earlier increase in the excision group at day 7 post injury compared to the control. Whereas in the burn group, there was significant increase compared to the control from day 14 which continued and was elevated above the excision group at day 28 (Figure 3.9e).

In the $CD4^+CD25^+$ (activated) T cells there was a significant elevation at day 3 in the excision group compared to the control. In contrast the burn group showed depressed levels in both cell number and frequency later at day 28 (Figure 3.9c & f).

No significant acute changes were observed in $CD4^+$ Treg cell numbers in either injury group, however there was an observed upward trend in both until day 14 where the burn group reached significant elevation above the control group in both number and compared to the excision group in frequency (Figure 3.9d & g).

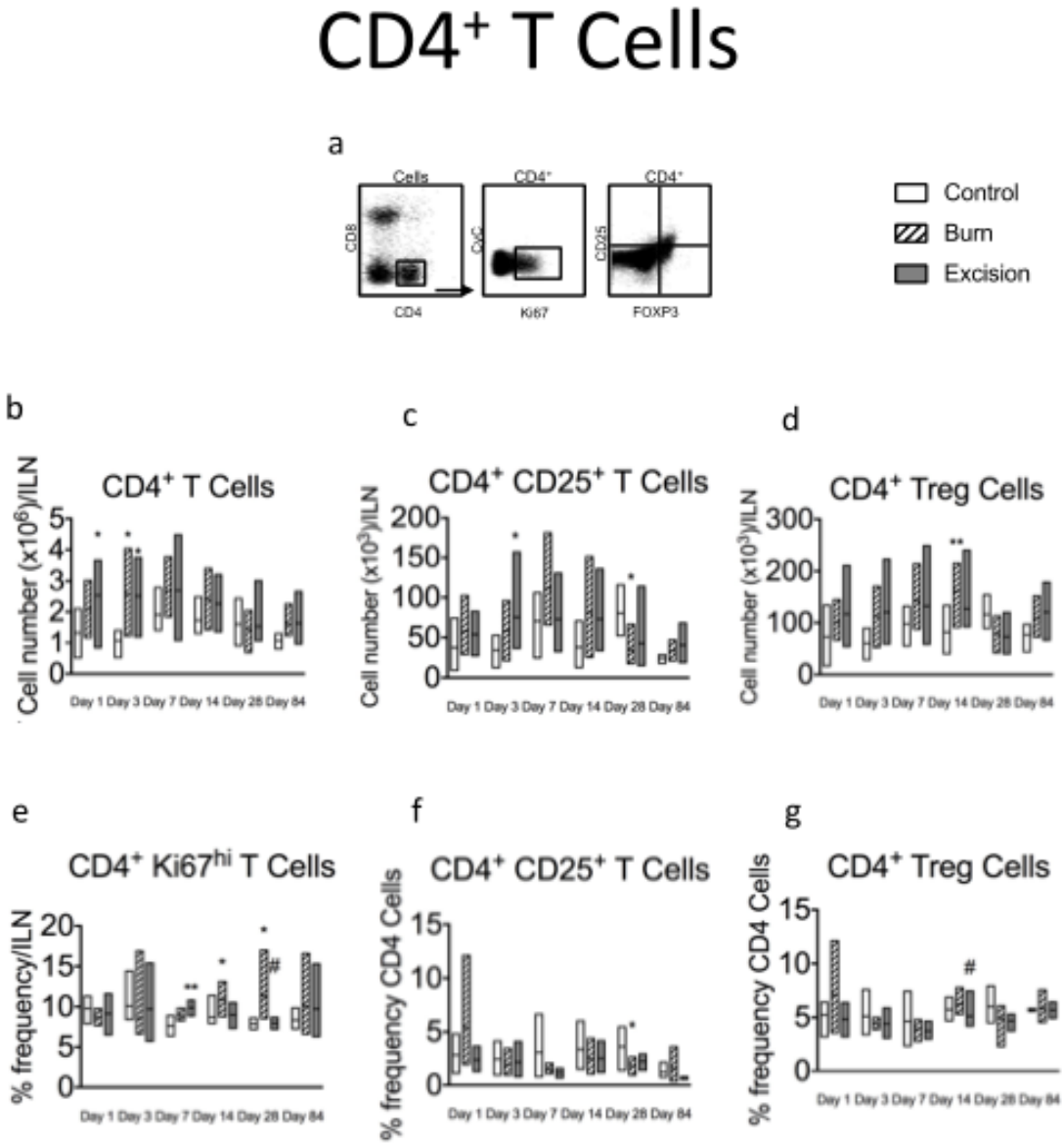


Figure 3.9: CD4+ T cell number changes following burn and excision injury.
*Inguinal lymph nodes were harvested from each of the groups at day 1 - 84 post injury/sham, and digested to a single cell suspension. Cells were gated as CD4+, the CD4+ population was further gated for expression of CD25, Ki67 and FoxP3 (a). The graphs represent the number of the total CD4+ T cells (b). The subsequent graphs represent the cell count of the subpopulations of CD4+cells including CD4+CD25+ T Cells (c), CD4+ Tregs cells (CD4+CD25+FoxP3+) (d), in addition to the percentage frequency of CD4+Ki67hi (e), CD4+CD25+ (f) and CD4+ Tregs cells (CD4+CD25+FoxP3+) (g). Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-11 mice/group, for at least two independent experiments.*

3.7: $CD8^+$ T Cell Response Differ Between Burn and Excision Injury

The $CD8^+$ T cell number, proliferation, and activation were also determined by flow cytometry (Figures 3.10a). The $CD8^+$ T cell number was significantly increased at day 1 post burn compared to the control and day 3 in both the burn and excision groups compared to the control (Figure 3.10b). when looking at the dividing status ($Ki67^{hi}$) of the $CD8^+$ there was a significant decrease in the frequency in the burn group compared to the control at 3 (figure 3.10d).

In the $CD8^+CD25^+$ (activated) T cells, there was a significant elevation in the number in both the burn and excision groups at day 1 compared to control which persisted in the burn group until day 3 post injury (Figure 3.10c). There was no significance found in the percentage frequency in these cells (Figure 3.10e)

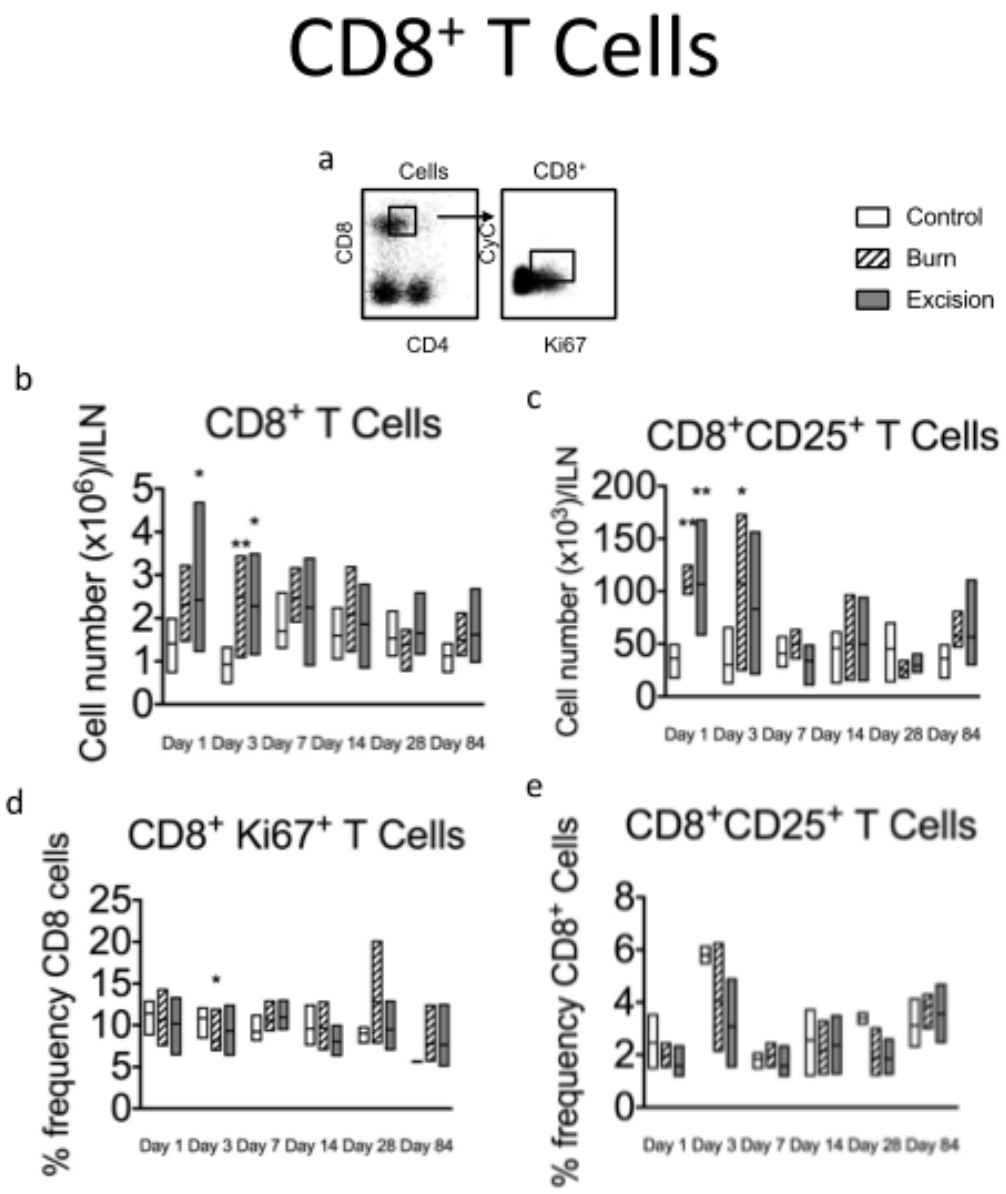


Figure 3.10: CD8⁺ T cell changes following burn and excision injury. *Inguinal lymph nodes were harvested from each of the groups at day 1 - 84 post injury/sham, and digested to a single cell suspension. Cells were gated as CD8⁺, the CD8⁺ population was further gated for expression of CD25 and Ki67 (a). The graphs represent the number of the total CD8⁺ T cells (b). The subsequent graphs represent the cell count of the subpopulations of CD8⁺cells including, CD8⁺CD25⁺ T cells (c), in addition to the percentage frequency of CD8⁺Ki67^{hi} (d) and CD8⁺CD25⁺ (e)*

*Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-11 mice/group, for at least two independent experiments.*

3.8: Burn Injury Leads to Suppressed Acute Splenic T-cell Cytokine Responses

Stimulation of T cells isolated from the spleen was conducted in cell culture using Concavalin A to assess T-cell function after burn injury. At day 7 post burn, the T-cell response was reduced compared with control animals (Figure 3.11). There was significantly reduced production of IL-1β in the splenic T cells at day 7 that was not reflected in the sera (Figure 3.11a & b). IL-17 was also significantly reduced in splenic T cells following burn injury at day 7, (Figure 3.11i). The systemic reflection of this cytokine could not be assessed due to an n=2 in some of the control sample time points, thus significance could not be accurately established. Both IL-6 and IL-2 showed a trend to reduced production (P=0.06) at day 7 in the splenic T cells (Figure 3.11c & e). In the systemic system the IL-6 (which has previously be discussed) was significantly elevated the burn group from day 1-7(Figure 3.11e). In the sera, systemic IL-2 levels were found to be significantly elevated in the burn group at day 14 only (Figure 3.11f). There were no significant changes to the IL-12p40 levels either in the splenic T cells or sera (Figure 3.11g & h).
By Day 14, T-cell cytokine production in response to Concavalin A had returned to normal levels (data not shown).

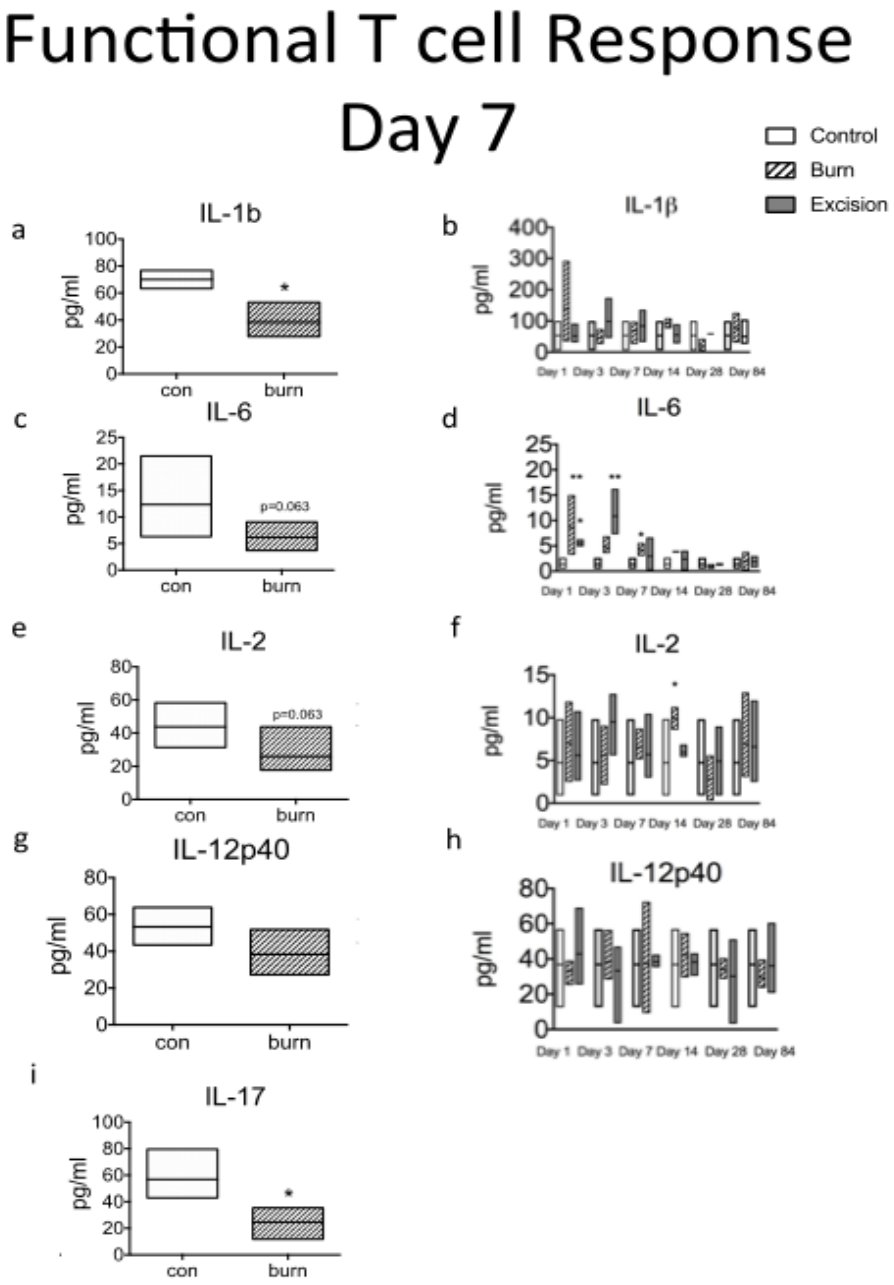


Figure 3.11: Burn injury leads to suppressed acute splenic T cell cytokine responses. *Spleen cells (3 x 10^{5}cells/mL) were stimulated with 10μM concavalin A and cytokine levels determined in supernatant at 48 h. At Day 14 there was no significant difference between burn injury and control spleen cultures stimulated with concavalin A (data not shown). The subsequent grafts represent the functional T cell response in splenic T cells with the comparable serum value, including; IL-1β (a & b); IL-6 (c & d); IL-2 (e & f); IL-12p40 (g & h); IL-17 (i).*

*Graphs represent mean + range. Significance is indicated by *(p=0.05) and **(p=<0.01) compared to control; # (p<0.05) compared to burn injury group. n=3-11 mice/group, for at least two independent experiments.*

Chapter 4

Discussion

4.1: Injury and the Impact of Immune Dysfunction

The word ‘injury’ is defined as a stress that exceeds physiological tolerance resulting in physical harm or damage [163]. Worldwide, injury is a leading cause of morbidity and as such has huge implications medically and economically. To date there has been little focus on non-severe burn injury despite this accounting for almost 90% of admissions in burn units in the developed countries [10]. As such, non-severe models of injury have been used in this thesis to facilitate translation of this work to what we frequently see clinically.

Immunological dysfunction is apparent in several clinical forms following injury. In the acute setting this is observed clinically as Systemic Inflammatory Response Syndrome (SIRS), characterised by hypo/hyperthermia, tachycardia, tachypnoea and leucocytosis. This can progress to Multi Organ Dysfunction Syndrome (MODS) where there is a failure to maintain homeostasis and subsequent circulatory collapse. In trauma, this process can be primary occurring in consequence to the initial insult or secondary to an abnormal host response [24]. In the intermediate phase, Compensation Anti-Inflammatory Response Syndrome (CARS) can occur in consequence to SIRS and is characterised by leukopenia, infection susceptibility and failure to clear infection [24-27]. This clinical status observed is a reflection of the Th1/

Th2 paradigm where there is an imbalance in T helper 1 (Th1) and T-Helper 2 (Th2) cells [164]. In burn injury this can result in infectious challenges, poor wound healing and chronic wound formation resulting in increased scar-associated morbidity with poor functional outcome [164]. In addition to these early and intermediate sequelae recent studies have demonstrated a long-term impact following burn injury that includes an increased risk of malignancy, infectious susceptibility as well as cardiac dysfunction [3, 7, 38-43]. It has been proposed the latter is a sequalae of the documented prolonged and complex metabolic changes that occur post burn injury and work in this area is on-going [7, 39]. With respect to the increased risk of malignant change and infectious susceptibility, the mechanism is at present unknown but it is believed it is related to immunological changes. The role of stress at the cellular level or indeed changes in the immune system and its subsequent function require further investigation.

Burn injury in isolation is already a global burden due to scarring and loss of function [165]. With the developing association of long term consequences post burn injury, the impact these injuries have clinically and economically is set to rise. The knowledge of the mechanisms involved provides potential future therapeutic opportunities to reduce the acute consequences and limit the global burden of burn injury.

4.2: Neither Non-severe Burn nor Excision Injury Disrupted Growth Potential in Mice

Adolescent mice were used for the experimental model and therefore reached maturity and adult size during the course of the experiment. The weight of the mice was measured throughout the duration of the experiments and was documented in all groups at all time points. The data showed no significant disruption to the terminal growth in either the burn or the excision injury as demonstrated by the final time point weight on day 84 (Figure 3.1). In contrast to this data other published work looking at the long term outcome in paediatric burns growth has been reported to be impacted in consequence to the metabolic and hormonal disturbances [7, 166]. Metabolic and hormonal data was not collected in this experimental model, however in other murine non-severe burn injury models the consequence of metabolic dysfunction has shown significant impact to both distant skeletal and cardiac muscle dysfunction [38, 167]. To better establish the details of the relationship between metabolic dysfunction and immunological changes in burns of this size warrants further investigation.

4.3: Non-severe Burn Injury Generates a Different Cytokine Response Compared to Excisional Injury

Cutaneous injury results in damage to multiple structures and cell types; denaturation of proteins occurs, there is disruption of plasma membrane integrity and subsequent secretion of mediators such as nitric oxide (NO) and reactive oxygen species (ROS) [125]. In response to this soup-pot of cells and cellular debris, pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10 in addition to chemokines are released to recruit inflammatory cells to the area of injury and initiate the innate immune response [149, 168, 169].

Despite the non-severe model used in this thesis an inflammatory response in both models was observed, however the nature and temporal release of these cytokines and chemokines were different.

In this thesis, Interleukin 6 (IL-6) was elevated early at day 1 in the burn injury with a secondary peak occurring later at day 7. Whereas, in the excisional injury a modest elevation occurred at day 1 reaching significance at day 3.
In the context of injury and in support of the data observed in this study, IL-6 has been shown to be released early following both burn and non-burn injury and positively correlates with the size of the burn or extent of the trauma as assessed by the injury severity score (ISS) [170-174]. In similarity, different IL-6 profiles have previously been observed where levels of IL-6 were found to be higher in burn injury comparison to injury secondary to elective surgery [170].

Production of IL-6 is induced by the presence of pathogen associated molecular patterns (PAMPs) as well as other inflammatory cytokines such as IL-1α and TNF-α [111]. IL-6 is produced by a number of cell types, including neutrophils, monocytes and lymphocytes [111]. In the context of the model used in this thesis, the IL-6 data suggests the burn injury resulted in an earlier and more prolonged exposure to PAMPs and other inflammatory cytokines. The source of the IL-6 release may be from recruited neutrophils, monocytes and lymphocytes in the wound as systemic levels of these cells showed a trend towards depression signifying egress to the wound bed.

High levels of IL-6 are associated with increased morbidity and mortality following both burn and non-burn injury and as such, it is regarded as a marker of severity [158, 171]. The data presented here demonstrates that similar changes occur in a non-severe injury model and as such may have clinical implications. The IL-6 data demonstrates differing profiles and temporal release of IL-6 in the two injury models showing support towards an aetiology specific immune response.

A significant elevation in sera TNF-α levels was observed at day 1 following burn injury compared to control levels. In similarity to the IL-6 data, the excisional injury trended towards a peak at day 3, however was not found to be significant.

TNF-α is produced by monocytes, macrophages and T cells in response to high levels of other inflammatory cytokines such as IL-1, IL-2, IL-12 and IFN-γ following injury [111, 152, 175, 176]. Together with IL-1β, TNF-α promotes further pro-inflammatory cytokine release specifically IL-6 in addition to

increasing the local concentration of neutrophils [85, 110, 127], both of which were apparent in the burn injury group. Should this become uncontrolled a state of SIRS will arise which may result in concurrent tissue damage [177]. In relation to this sustained or abnormally elevated levels of TNF-α are associated with poor clinical outcome [164]. The production of TNF-α requires modulation via the influence of other cytokines to limit adverse outcome. Modulatory cytokines such as IL-10 and IL-13 as well as Th2 cytokines IL-4 and IL-13 act to reduced levels of TNF-α [111, 152, 175, 176]. In this data, both IL-10 and IL-13 were found to be significantly elevated in the burn group at day 1. The Th2 cytokines IL-4 and IL-3 also demonstrated a trend towards elevation at this same time point in the burn group, however it was not found to be significant. These concurrent levels of modulatory Th2 cytokines may represent measures to control the elevated TNF-α following the burn injury and minimise host tissue damage.

Significantly raised IL-12p70 levels were also observed at day 1 post burn injury in comparison to the excision model. The excision group had increasing levels from day 3, which reached significance later at day 7 when compared with the control. IL-12 is a Th1 cytokine and is associated with the development of an inflammatory $CD4^+$ Th1 type response in addition to the induction and proliferation of cytotoxic CD8+ T cells in burn injury [96, 154]. This early rise of IL-12p70 in the burn injury group correlates with the presence of other inflammatory $CD4^+$ Th1 cytokines which include IL-6 and TNF-α [178]. The elevated levels of IL-12p70 may be responsible for the

significant elevation seen in the following days in the $CD8^+$ T cell populations in the lymph nodes.

The IL-10 data in this thesis showed an earlier rise after burn injury at day 1 when compared to the excision model which showed a trend towards elevation at day 3 and significantly elevated at day 7 when compared to the control. In addition, IL-10 was the only cytokine significantly elevated at the latest time point of day 84 in the burn group compared to the excision group.

IL-10 is a modulating cytokine that is synthesized by a number of cells including monocytes, dendritic cells and $CD4^+$ Th2 T cells [168]. As a modulatory cytokine, IL-10 acts to inhibit IFN-γ, TNF-α and IL-1β therefore inhibiting Th1 T cell proliferation thus dampening the inflammatory response [85, 125, 178, 179].

Early elevation in Th2 cytokines occurs in consequence to a hyper-acute elevation of inflammatory Th1 type cytokines following burn injury [122]. Given the previous literature findings, the elevation of IL-10 seen in the burn injury at day 1 may have been released by local or recruited Th2 T cells in consequence to the elevated Th1 cytokines IL-6 and TNF-α. Unlike the burn, the excision model had significantly elevated IL-10 at day 7 only. This may be due to the later and less amplified inflammatory response found in this group at day 3 and as such the requirement to reduce the Th1 cytokine as promptly was not warranted.

The T cell data from this thesis was from cells harvested from the wound draining lymph node that are activated in response to dendritic cell (DC)

interaction and presentation of antigen. As such, this may explain why elevation of the $CD4^+$ T cells was not evident until day 3 in the burn group. Considering this, it is possible that the T cells responsible for this cytokine profile are local cutaneous T cells such as the gamma-delta (γδ) T cells which when activated secrete IL-13 in addition to a host of chemokines which were also found to be significantly elevated in the burn group at day 1, including Macrophage inflammatory protein 1α (MIP1-α) and Macrophage inflammatory protein 1a (MIP1-β) [47, 49]. It was out of the scope of this work to investigate the functional cells in the wound bed and surrounding tissues, however further work in this area could clarify the results with respect to the source of cytokine release. Despite the ambiguity, the IL-10 profile in context of the cytokine profile gives further evidence that in this burn injury model there was an earlier drive towards a Th2 cell type response when compared to the excision.

EOTAXIN attracts eosinophils, basophils, mast cells and Th2 cells that are all involved in the acute phase following burn injury [125]. In compliment to the IL-10 data, EOTAXIN levels were also significantly elevated at day 1 post burn that was not seen in the excision injury. Although not significant, the haematology data observed relatively low circulating eosinophils at day one in comparison to the later time points which would correlate with the EOTAXIN data and indicate migration of these cells from the systemic circulation and to the wound site.

These findings suggest that this burn injury model has a similar picture to that which occurs in clinical studies where there is a correlation between early increased levels of IL-10 in association with reduced production of pro-inflammatory cytokines and down-regulation of MHC class II expression on

antigen presenting cells [91, 125, 152, 176]. These actions aim to reduce the inflammatory phase to make way for wound healing & resolution of inflammation, however if the Th2 response is inappropriately high immunosuppression occurs and recognised clinically as CARS [146, 158, 164]. Furthermore, the IL-10 profile seen here in the burn injury may also play a role in the differences observed in the dendritic cell data as IL-10 influences dendritic cell differentiation, maturation and function when presenting to T cells [102, 121, 135]. The details of the dendritic cell data will be discussed later in this chapter (Section 4.4).

The data from the cytokine profiles show that the non-severe burn injury model in this thesis caused early elevation of both pro-inflammatory Th1 and modulatory Th2 cytokine in the acute phase post injury. When compared to the excision injury of the same size and depth the same response was not observed. It is possible in the burn injury group the Th1 cytokines are released earlier than the Th2 cytokines rather than concurrently. However this may not have been captured by the cytokine assessment at day 1. The Th2 cytokine levels appear to represent a counter response to dampen an unfavorably high pro-inflammatory response. As such, the data shows concurrent elevation of both Th1 and Th2 cytokines at day 1. This process has been observed before in other studies where raised Th2 cytokines increase in response to elevated Th1 cytokines following burn injury [7, 39, 122, 180]. Interestingly, this cytokine profile was not observed in the excision injury. The data strongly suggests that there is an acute ‘inflammatory storm’ response to burn injury that is absent after an excisional injury of equivalent extent.

It is possible that the excision model represents an appropriate inflammatory response where there is first a modest increase in inflammatory cytokines followed by later resolution as represented by th2 cytokines.

The cytokine data in the burn injury appears to fit with the cytokine profiling in previous murine and clinical studies [7, 21, 39, 118, 122, 124]. The magnitude of this initial innate inflammatory response directly corresponds to the deregulation of the adaptive response and therefore may explain the persistent changes seen in the later time points in the burn injury group [20, 158]. The known clinical consequences of this cytokine profile in the early phase post burn injury correlate positively with the extent of tissue damage, development of post injury complications (such as sepsis and ICU admissions) and a higher mortality rate [172, 181-184]. The relationship of the initial immune response and the subsequent immunocompromise offer supporting evidence to the evolving association with long term consequences (such as malignancy and infection vulnerability) seen following burn injury [5, 14, 36].

To gage a more accurate timing of release and the specifics of the profile further studies could be done looking at interval time points within the first 24hrs to assess which cytokine type (Th1 or Th2) rose first in the burn injury.

Cytokines analysed but not shown/discussed include; IL-9, RANTES, IL-1α. This is due to an n=1 in some of the groups/time points due to inaccurate plate readings. As such, values had to be omitted and the n value was too low to draw significance.

4.4: Injury Aetiology Induces Different Chemokine Profiles

Chemokines are a subset of cytokines that aid the recruitment of cells by stimulating chemotaxis, including the extravasation of leukocytes from the peripheral blood [85]. Neutrophils are the first nucleated cell to arrive at the wound site following skin injury [126]. Monocytes are next to be recruited, where they promptly differentiate into macrophages or dendritic cells in the wound which phagocytose and secrete cytokines and chemokines to manipulate the immune response to the injury via their engagement with the adaptive system [24].

4.4.1: Excision injury promotes chemokine expression that drives neutrophil recruitment

KC (CXCL1) levels were significantly elevated in both the burn and excision models at day 1 against the control, however the excision group had a more pronounced elevation with a p value of <0.01. KC is secreted by damaged epithelial cells and attracts neutrophils and lymphocytes to the area of tissue injury [85, 125].

The Granulocyte Colony-Stimulating Factor (G-CSF) data demonstrates a different profile between the two injury models with the excision group inducing an earlier and greater (10-fold) increase at day 1 which remained elevated until day 3 against the control. Whereas, in the burn injury levels

were elevated moderately from day 1 to 7 compared to the control. G-CSF is a modulatory cytokine that promotes neutrophil function, antagonises pro-inflammatory cytokines such as IL-1, INF-γ and TNF-α, as well as reducing monocyte deactivation thus promoting a Th2 type response [42, 185].

The circulating neutrophil levels trended towards low levels in the early time points of day 1 and 3, followed by a significant elevation in both the burn and excision models at day 7 which persisted in the burn to day 14. In the burn group the neutrophilia and raised G-CSF were prolonged in comparison to the excision group (day 14 and 7 respectively). Further to the chemokine data, IL-6 was also elevated at day 7 in the burn group. IL-6 modulates the growth and differentiation of neutrophil progenitors in the bone marrow and also attracts neutrophils to the site of injury [42, 73, 85, 169].

Both injury models had elevated KC and G-CSF; however the profile of these chemokines differed in the two injury groups. The excision model appeared to induce an earlier and greater response in these chemokines compared to the burn which is suggestive of a greater requirement of neutrophils in the excisional wound. The burn injury model demonstrated elevation in both KC and G-CSF which has previously been observed in other burns literature [85, 118, 124, 125]. However, when compared to the excision profile and taken in context with the neutrophil data, this profile observed in the burn injury group may represent neutrophil dysfunction. This has previously been reported in burn injury and G-CSF has been used to facilitate restoration of neutrophil balance [185, 186]. Clinically, high activation or prolonged peripheral neutrophils are associated with burn wound progression and the development of SIRS, whereas low activation is related to sepsis [56] [187] [130]. Implying

that neutrophils are required during the inflammatory phase but in a balanced manner. In burn injury neutrophilia has been observed to occur in association with dysfunctional chemotaxis to the wound site [187]. This may explain the relationship in burns with the development of SIRS due to the increased levels of circulating neutrophils and delayed infiltration into the wound site. Alternatively, the data presented may suggest that burn injury generates a prolonged neutrophil requirement at the wound site compared to the excisional model.

4.4.2: Burn injury promotes chemokine expression that drives monocyte to macrophage differentiation

Monocyte chemotactic protein (MCP-1), Macrophage inflammatory protein 1a (MIP1-α) and Macrophage inflammatory protein 1b (MIP1-β) are chemokines that are all involved during pro-inflammatory response to promote monocyte recruitment to the wound bed, release of progenitors from the bone marrow and facilitate in monocyte to macrophage differentiation (Section 1.5.1) [49, 121] [91, 188]. In addition to this, MIP-1α is released by damaged epithelial cells and activated γδ T cells [49, 121]. MIP-1β attracts natural killer (NK) T cells and monocytes to the wound site [122].

The data showed MCP1, MIP-1α and MIP-1β were all elevated at day 1 in burn injury when compared to the control with no significant elevation being observed in the excision model. This suggests a high influx of monocytes to the wound tissues. The haematology data shows a trend towards monocyte

depletion at day 1 in the burn. Although this was not found to be significant it is suggestive of an egress of these cells to the wound site in response to the chemotractive nature of these chemokines. There was significant elevation of systemic monocytes in the burn group at day 3 which indicated the response to the haematological roles of these chemokines in repopulating the peripheral pools. In keeping with this, similar chemokine findings have been reported in other burns literature [71, 122, 125, 189]. Alternatively, the monocyte elevation at day 3 in the burn injury may indicate a loss of monocyte egress from the circulatory system secondary to immaturity which has previously been reported following trauma [190].

Other cytokines including IL-4, IL-10 and GM-CSF play a role in promoting monocyte to macrophage differentiation [92]. IL-10 it is considered modulatory as it is not only capable of suppressing the inflammatory response but also complements IL-6 and its actions when released during the inflammatory phase by facilitating the drive of monocyte towards macrophage differentiation [42, 73, 85, 168, 169]. As previously discussed, both IL-10 and IL-6 were significantly elevated at day 1 in the burn group with IL-6 levels remaining elevated until day 7. Although not significant, both IL-4 and GM-CSF showed a trend towards elevation in the burn group at day 1. Collectively, the chemokine and cytokine data is suggestive that the burn injury demonstrates a promotion towards monocyte to macrophage differentiation.

The chemokine data in the context of the haematology profiles indicate that the burn and excision injury behave differently with regards to the innate cells they require. The excision injury appeared to recruit neutrophils, whereas the

burn injury showed preference to monocytes. Furthermore, the burn injury demonstrated a drive towards monocyte to macrophage differentiation.

Analysis of differences in wound infiltrates from both injury types was out of the scope of this research. However, this work would be beneficial to correlate these chemokine findings and compare the systemic innate cells to those that have infiltrated into the wound or scar and have a greater understanding of the immune response on the pathophysiological process.

4.5: Cutaneous Injury Alters Dendritic Cell Profiles

Dendritic cells are specialised antigen presenting cells (APC) that bridge the gap between the innate and the adaptive systems. During homeostasis a tolerogenic T cell response is induced. In the presence of inflammation and/or infection DCs up-regulate their MHC class II expression and become capable of engaging with the adaptive system and inducing a T cell response. The DC activation/maturation status of the DCs impacts the ensuing T cell response. Skin migratory DC subsets are able to differentially stimulate $CD4^+$ and $CD8^+$ T cell responses in the lymph node in order to tailor the response required [191].

The lymph node populations were examined via FACS. The total inguinal lymph node (ILN) count data show a trend towards elevation in both injury models by day 1 post injury, which reached significance when compared to the control at day 3. Despite the injury model being non-severe this data

confirms engagement of the adaptive system as there has been an increase in the cellular populations in the regional draining lymph node basins.

Expanding on the lymph node populations, cells were stained and gated for migratory and lymph node resident dendritic cell (DC) populations.

A significant increase in the total number of DCs in the draining inguinal lymph node (ILN) after both burn and excision occurred when compared with the control at day 3. In addition, the burn had a mid-point significant elevation above the control at day 14 and the excision had a later elevation at day 84.

Considering the analysis of the DC sub-populations the early elevation of total DCs in the two injury models was secondary to an increase in both the resident lymph node $CD8a^+$ and migratory $CD8a^-$ populations above the control. The burn group appeared to generate a greater response compared to the excision, however this was not found to be significant. This data implies expansion of the resident DC populations as well as adequate migration of the DCs from the skin to the draining lymph node in response to the non-severe cutaneous injury. This data appears to correlate with other authors who reporting similar findings with an increased number of DCs in the draining lymph nodes during the acute phase post burn injury (day 1 – 3) [192].

Despite the resident $CD8a^+$ DCs being elevated at day 3 in both injury models, further study of these DCs demonstrated a reduced activation status as indicated by MFI in the burn group when compared to the control, no changes to the activation status were observed in the excision group. The lymph node resident $CD8a^+$ cells play an essential role in the development of tolerance to cell-associated antigens presented during apoptosis [145, 193].

Therefore following burn injury and subsequent reduction in $CD8a^+$ DC activation may result in reduced tolerance to self and pave the way towards the development of long-term immune consequences.

The migratory $CD8a^-$ DCs were also elevated in both injury models at day 3, which persisted in the burn injury until day 14. Similarly to the resident $CD8a^+$ DCs there was an associated reduced activation status in the burn injury alone at day 3. Activation of dendritic cells is a complex multi-staged process that involves the engagement of Toll Like Receptors (TLRs) in the presence of inflammatory cytokines such as interferon type 1, GM-CSF and IL-4 [121, 133, 194, 195]. Neither TLRs nor IFN-1 were examined in this thesis thus the influence of these in the immune response following injury cannot be establish. However, both GM-CSF and IL-4 were examined, both showing a trend towards depression at day 3 in the burn group only, however neither were found to be significant. Given the context of literature surrounding DC maturation, these cytokine/chemokine changes may be responsible or the reduced activation status seen in the burn injury.

In further support, high circulating IL-10 leves are also known to suppress DC maturation and antigen presentation [127]. As discussed previously (section 4.3), IL-10 was significantly elevated at day 1 compared to the excision that showed a later and less pronounced elevation at day 7. This early elevation in IL-10 may have further contributed to the suppression and reduced maturation status in this DC population that we see only in the burn.

The migratory $CD8a^+$ cells were further sub-categorised into $CD103^+$, $CD11b^{hi}$, and $CD11b^{lo}$ as they differentially stimulate $CD4^+$ and $CD8^+$ T cells in

the lymph node, thus altering the adaptive response generated [98, 191, 196, 197]. Presentation to $CD4^+$ T cells is preference in the presence of IL-12 at the time of antigen presentation, and as such a Th1 (pro-inflammatory) response ensues [121] [135]. Whereas in the presence of IL-10 a Th2 type response is generated which ultimately results in dampening of the Th1 response [102]. With regards to cross presentation to $CD8^+$ T cells concurrent high levels of IL-12 and TNF-α have been found to promote a Th1 T cell response [139-141].

The $CD103^+$ DCs showed no significant change to their number following either injury; however a reduced maturation status at day 1 and 3 in association with depressed frequency at day 3 occurred after burn injury alone. Altered maturation status would result in poor T cell activation in burn injury. As these migratory DCs are able to stimulate both $CD4^+$ and $CD8^+$ T cells, a process known as cross presentation they play a prominent role during infectious challenge, particularly in viral and fungal immunity [98, 138, 139, 196, 198, 199] [139-141]. The consequence of immaturity in these DC populations may explain the high infectious rates observed in burn patients [15, 16]. In addition to maturation disruption that was observed here, these cutaneous $CD103^+$ DCs have previously been found to be dysfunctional following burn injury where unregulated IL-10 and reduced IL-12 levels resulted in reduced co-stimulatory signals and subsequent poor stimulation of T cells [200].

The most pronounced changes post burn injury were in the migratory $CD11b^{hi}$ DCs subsets which showed significantly elevated levels above the control from day 3 to 14 with normal activation status. There were no significant

changes seen in the excision injury model. In the later time points (day 28 and 84) despite no significant alteration to their numbers in the lymph nodes these cells were found to have suppressed activation, again this was not observed in the excision group. These migratory DCs are the most abundant subtype in murine skin and predominantly present to $CD4^+$ T cells to preferentially stimulate a Th2 response. However, a Th1 or Th17 response can be generated depending on the DCs maturation status and chemokine milieu present [98, 199]. In this data there was no significant corresponding cytokine changes that support the changes seen in this DC population from day 3 - 14.

The $CD11b^{hi}$ and the $CD103^+$ DCs behave similarly and are implicated in the development of tolerance to antigen via retinoic acid that enables these DCs to induce T regulatory cells [198, 201]. Interestingly, the Tregs were significantly elevated (number and frequency) in the burn group alone at day 14 echoing the $CD11b^{hi}$ data. Tregs are known to release IL-10 as part of their modulatory function [107, 127]. The raised IL-10 at day 84 may represent a remaining reflection of the earlier Treg activation. Additionally, the late changes to the $CD11b^{hi}$ DC activation may be in response to this later IL-10 elevation as it is known to suppress DC maturation and antigen presentation [127]. This data indicates that the burn injury model in this thesis showed changes associated with tolerance induction at day 14 as well as long term disrupted activation of these cell types. This DC data shows further supportive evidence that the immunological changes post burn injury are long term. Furthermore, these changes are not successfully demonstrated in the size matched excision model indicating a difference between the two aetiologies.

In the migratory CD11b^{lo} DCs both injury groups had significantly elevated levels with normal activation status at day 3 when compared with the control. Later at day 28, the burn injury developed significantly suppressed levels that corresponded with a preceding reduced activation status at day 14 that continued day 28. The CD11b^{lo} act in a similar manner to the CD11b^{hi} and activate CD4^{+} T cells [98]. The early change in this DC population appears to correspond with the CD4^{+} T cell data where both injury groups had elevated levels above the control at day 3. This data may demonstrate the expected course following a cutaneous injury (both burn and excision) where there has been migration of cutaneous DCs to the draining lymph node with an associated elevation in T cell numbers to generate the required adaptive response. In the later data, there is suppressed CD11b^{lo} DCs in the burn injury demonstrating further differences between the injury models as there were no long-term changes seen in the excision injury. These migratory CD11b^{lo} DCs are found in the draining lymph node in both the steady state as well as during inflammatory conditions [98]. As such, suppression of these DCs indicates persistent immune compromise following burn injury that may explain the long-term morbidity observed following burn injury [3, 5, 7, 37].

The DC changes in migration and activation of the skin resident DCs to the draining lymph nodes post injury may be sufficient enough to impact antigen presentation to T cells and induce a different adaptive immune response that is aetiology dependent. The cause of late depletion in DCs may be related to the significant elevation of IL-10, however the source of this cytokine remains uncertain and warrants further investigation. Interestingly, recent evidence in

burn injury has indicated the reduction in DCs may be secondary to bone marrow monocyte progenitors lacking DC differential potential secondary to a high MafB expression which exerts an inhibitory role in monocyte-derived DC differentiation with a preference towards monocyte to macrophage differentiation [202, 203]. The data presented in this thesis shows support of this literature, with evidence of monocyte to macrophage differentiation (Section 4.3.2) in the context of reduced DC populations.

4.6: Injury aetiology induces different T cells responses

T cells are recruited to the site of injury following antigen presentation in the presence of co-stimulatory molecules in the draining lymph node [47]. There are various types of T cells depending on the required action and the concurrent cytokine secretion including $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and $CD4^+$ T regulatory cells [150]. T cells engage with antigen presenting cells such as dendritic cells in the draining lymph node as well as in the primary lymphoid tissues (the spleen). During the steady state, this is a tolerogenic process to maintain homeostasis. However during inflammatory states, the T cells migrate to the site of injury and the response is determined by the activation and maturation status of the DCs [200, 204-206]. In both burn and non-burn trauma, findings have suggested that the Treg cells can induce an anti-inflammatory state observed clinically as delayed immunosuppression thus rendering the patient more susceptible to infection and sepsis [20, 40, 158, 180, 207]. To date, there has been little work that

demonstrates the long term impact this early immune-compromise has post injury.

4.6.1: Injury aetiology impacts activated $CD4^+$ T cell function

The excision injury showed a significant increase in $CD4^+$ T cell number from day 1 to 3 when compared to the control, whereas the burn group showed a later response at day 3. As discussed previously (section 4.4), these elevations appear to been in keeping with the expected response to injury where a T cell response is induced [49, 208]. Based on the DC data, it may be in consequence to elevated migratory $CD11b^{lo}$ DCs at day 3.

$CD4^+$ activation was determined via $CD25^+$ expression and was increased in the excision injury at day 3. The data from the activated T cells corresponds appropriately when considering time matched cytokine profiles (section 4.2) where the excision injury had elevated levels of IL-6 suggesting activation of the Th1 type response. This was followed by significant elevation of modulatory and Th2 cytokines (IL-10 and IL-12p70 respectively) at day 7 indicating resolution of the inflammatory phase. This data indicates the expected post injury response with demonstrated increased frequency of activated $CD4^+$ T cells following trauma [180]. In complement to this data, proliferation as determined by Ki64 showed significant elevation at later time points of day 7 in excisional injury which may indicate repopulation of these cells following earlier elevation.

In the burn injury, there was a trend towards elevation later than in the excision at day 7, however this was not found to be significant. The delayed elevation in the activated $CD4^+$ T cells in the burn injury group may be secondary to altered DC maturity (section 4.4) preventing successful presentation to T cells and thus stifling T cell activation. These changes in the burn demonstrate how an early immune challenge creates a domino effect with subsequent poor engagement of the adaptive system. This would be in keeping with the evolving picture of immune alteration following burn injury. Furthermore, the burn injury had depressed number of activated $CD4^+$ T cells after healing at day 28. In the proliferating data (Ki64) there were increased levels at day 14 and 28 implying the CD4+ T cells are present and proliferating but not activated. Additionally, sera IL-2 shows elevation at day 14 in the burn group suggesting attempts to increase population as IL-2 is known to promote induction of T cells, stimulate further proliferation and increase T cell survival [73, 85, 112] [147, 148]. This rebound effect has previously been observed following burn injury but in the cytotoxic $CD8^+$ T cells population [209]. Other studies have similarly reported reduced activated $CD4^+$ T cells during the acute phase post burn injury, however there is little data on the long term changes [210]. The alterations in T cell populations previously reported have occurred in substantial burn injury, however here it has been demonstrated that changes also occur in a non-severe model [158]. Without appropriate T cell response one would expect poor response to infective challenge (bacterial and viral), which in the intermediate term post injury would results in infectious susceptibility and poor clearance of sepsis. In relation to this, burn injury patients are known to have a higher rate of wound

infections and sepsis in comparison to other trauma and as such the T cell data presented here is highly suggestive of the underpinning process [15, 16]. In the long term, if T cell anergy or deletion tolerance persist the development of autoimmune disorders or malignant change can occur [148, 211]. Should this immune response observed in this murine burn model translate to patients following burn injury, both severe and non-severe injury, it may explain the findings relating to the increased incidence of malignancy and infectious sequalae in the clinical and population studies.

4.6.2: T regulatory cells were activated following burn injury

In this study, elevated T regulatory (Treg) numbers were observed in the draining lymph nodes of the burn group at day 14 post injury, with no changes seen in the excision group. This may be due to the DC response observed in the burn injury where the migratory $CD11b^{hi}$ DCs (which stimulate production of Tregs) was elevated. Following activation of the adaptive immune system the Treg cells orchestrate the T cell response in order to dampen the inflammatory state and make way for healing and remodelling to occur [151, 212]. As such, this increase of Tregs in the burn injury may be responsible for the reduction in activated $CD4^+$ T cells seen in the burn injury at day 28, as neither Treg elevation nor reduced $CD4^+$ activation was seen in the excision injury. Tregs are induced or activated following engagement with DCs in consequence to an exaggerated inflammatory response. The activation of the Tregs in the burn injury and not in the excision implied that the burn induced a

greater but poorly controlled inflammatory response that required regulation. The Treg data consolidates the findings that burn and excision injury induce different immune responses.

Other studies have also reported later increases in Tregs in association with low levels of IFN-γ and higher levels of IL-10 following burn injury [20]. These changes to the cytokine profile can inhibit the Th1 type response, thus making way for increased action of a Th2 type response and promoting the immunosuppressive state seen clinically in burn patients. Treg secretion of IL-10 renders $CD4^+$ T cells unresponsive to antigens and therefore cease to produce cytokines, thus perpetuating the immunosuppressive state [107]. In further support of the data, the burn injury demonstrated increased IL-10 in the burn group at day 84 in association with activated $CD4^+$ T cells suppression, implying that these changes may be secondary to Treg influence.

These late differences in the $CD4^+$ T cell and Treg populations profiles implicates the development of T cell anergy, exhaustion or deletion tolerance to self-antigens in this burn injury model in consequence to the innate cytokine profile, innate cell recruitment and altered DC maturation.

4.6.3: Injury aetiology impacts the cytotoxic CD8+ T cell response

With regards to the cytotoxic $CD8^+$ T cells in the draining lymph nodes elevated levels compared to control were seen in the excision injury at day 1

in both the excision and burn injury models on day 3. Cytotoxic T cells are vital to the control of viral infection and malignant cell eradication and predominantly secrete Th2 type cytokines following injury [96, 109]. The markers of $CD8^+$ T cell activation ($CD25^+$) were also assessed and both burn and excisional injuries had significantly elevated levels compared to the control at day 1 which persisted to day 3 in the burn injury group. In the burn injury group these elevations in the $CD8^+$ T cells were associated with a significant reduction in the proliferation of these cells as indicated by low Ki67 which was not seen in the excisional injury. Demonstrating a different cytotoxic T cell response in the two aetiologies. In burn injury, altered $CD8^+$ T cell function has similarly been observed with functional studies in the acute phase demonstrating that these cells take on more of a Th1 cytokine profile in addition to reduced activity [109]. This is further supportive data showing evidence of immune suppression following burn injury.

4.7: Burn Injury Leads to Suppressed Acute Splenic T-cell Cytokine Responses

Analysis of T-cell activation using cells isolated from the spleen after a burn injury showed that T cell cytokine production in response to stimulation was suppressed at day 7. Levels of IL-1b and IL-17 were significantly reduced compared to the control, whereas IL-6, IL-12p40 and IL-2 were all reduced but significance was not reached. By day 14, levels had recovered. The functional T cell data from the splenic cells show transient suppression, which in time

returns to normal. T cell lymphoid tissue changes have been found to behave in a compartmentalised manner with the response primarily related to the zone of injury, i.e. within the draining lymphatic tissue [158]. This suggests that the effect on the function of the cells within the ‘compartment/lymph node’ would be greater than observed in the less affect the primary lymphoid tissue of the spleen. The lymph node cell population changes more likely reflect alterations in dendritic cell presentation and maturity. This has previously been reported after UV exposure of the skin and may also occur after burn injury [213]. Therefore, characterisation of long-term changes in dendritic cell function after a non-severe burn, in particular the ability to prime T cells, is an important next step. In addition, comparison data from the secondary lymphoid tissues (inguinal lymph nodes) would be valuable to assess the functional impact to these cells.

4.8: The Impact of Injury Aetiology on the Immune Response

Altered immune response following injury is not a novel concept, with other literature similarly concluding that different injuries induced a different immune response [22, 28]. The collective work following the ‘Host Response to Inflammation’ collaborative produced some insightful work in this area and concluded that injuries of differing aetiology induced different gene up/down-regulation which results in a tailored immune response to the injury [22, 28]. Other studies have similarly published findings that suggest injury aetiology influences the immune response generated and impacts clinical outcome [21].

Specifically, burn patients had higher IL-6 and IL-8 compared to non-burn trauma which correlated with poorer outcome despite the same injury severity score (ISS) [21]. In addition to the acute cytokine/genetic differences early post injury, differences between injury aetiology and infectious risk has been acknowledged. The literature reports that the rate of infection is known to be higher in burn injury when compared to other trauma [15, 16]. This is seen in both this data as well as the wider literature in burn injury and is suspected to be related to adaptive dysfunction and early Th2 elevation [214].

Furthermore, there has been a body of literature looking into the gender dimorphism following injury. Although the model used in this thesis was gender matched, the consensus from these works provides supporting evidence towards the development of different immune responses specific to the aetiology of injury. In murine burn injury studies, male mice have higher pro-inflammatory cytokines levels such as IL-6, in association with decreased IL-2 and INF-γ during the acute phase post injury where consequentially reduced CD4+ T cell proliferation is observed [34, 35]. In the female mice the same process occurred but in a delayed manner (day 7) [34, 35]. Clinically, male burn injury patients have a lower morbidity and mortality in both the acute and long-term post injury, with female patients having greater morbidity and mortality, specifically an increased risk of malignancy has been observed [5, 29, 30]. In contrast, the opposite is seen in non-burn injury with females having a lower morbidity and mortality in both trauma and surgical injury [31, 32]. These gender difference have also been observed in a large epidemiological study that combined burn survivor data from Western Australia and Scotland [5]. Although there were differences between the

cohorts with regards to gender and socioeconomic risk factors etc. the authors used well accepted methods to adjust for and correct the data. Their findings showed that female burn injury patients had an increased incidence of malignancy compared to expected (for age/gender match) which was not observed in the male burn population, suggesting a change post burn to the function of the immune response in females [5]. These findings collectively indicated that gender influences the immune response, and as such alters its function following exposure to different injury with subsequent long-term changes. The mechanisms of this have not been fully established, but it has been suggested it is secondary to the overlap that exist between the endocrine and immune systems as the differences reduced following menopause [29, 30, 34].

There are some published reports that have not established a difference between injury responses. Of particular interest, a genomic study comparing the response of injury in patients and murine models reported that the aetiology of injury had minimal effect on the clinical response in humans, in addition to reporting a low correlation of murine models to human data [215]. Since publication the limitations of this study have been discussed openly as it presents a one-dimensional view of the genomic response to injury and the findings do not appear to correlate with many previously well described correlations between murine and human immune responses [21, 48]. Specifically, the analysis of the peripheral blood alone, the limited diversity in murine strain and inconsiderate statistical analysis of data [216]. It is also worth considering that despite minimal genomic difference observed by the authors there may still be a considerable variation in the immune response

generated by that different gene expression between different injury and aetiology.

To date there is paucity in the literature with regards to the comparison of different aetiology on the same tissue type. The injury models used in the previously discussed literature are limited as they do not involve the same tissues (i.e. cutaneous, visceral, long bone, sepsis etc.) and as such it makes it difficult to draw conclusions on whether it is the aetiology of injury or the type of tissue injured that lead to their interesting findings. In consequence of this, it makes the findings of these studies difficult to translate into clinical practice. The data presented in this thesis compares injury to the same tissue type and demonstrates clearly a divergence in the immune response in the two cutaneous injury models. Specifically there was a greater inflammatory response with both Th1 and Th2 cytokines released early in addition to chemokine promotion of monocyte recruitment with macrophage (rather than DC) differentiation. There was engagement of Treg cells and subsequently reduced white cell populations and lymphocytes, in addition to reduced DC maturation and poor CD4 activation. Further to this, there is indication that in the burn injury the immunological changes are evident long after healing which may go some way to explaining the long-term morbidity that occurs post burn injury [5-7, 13, 36, 37, 124]

4.8.1: Injury model and eschar impact the immune response

When interpreting the data from this thesis, consideration of the limitations of the injury models used (thermal verses sharp) must be acknowledged. The pathophysiology process of the tissue injury in each model differs. Specifically, in incised wounds the damage does not grossly extend beyond the boundary of the incision, which is not the case in thermal burn wounds where there are radiating zones of injury around the point of initial contact (a process originally described by Jackson) [79].

Opiate analgesics in the form of buprenorphine were used at time 0 and 12 hours post injury in keeping with accordance of the animal ethics issued (see Appendix A). Opiates were not issued in the control mice post sham procedure due to ethics committee guidance with regards to risk of respiratory depression in the absence of painful stimulation. The literature around the use of opiate analgesia and its immune consequences is well established and thus must be discussed. What appears to be consistent within the literature is the variance in the impact to the immune response based on the specific opioid used with the focus of work looking into fentanyl, morphine and buprenorphine [217-221]. From this work, buprenorphine appears to have the least impact on the immune response with minimal effect reported in animal studies [221]. As such, it is difficult to determine the impact the absence of buprenorphine administration in the control model made in the results in this thesis.

In addition the presence or absence of an eschar must also be explored. The excision model created a size and depth matched cutaneous wound with a parameter of damaged cells around the circumference and base, however it did not create a formal eschar that resulted following the burn injury (Figure 4.1).

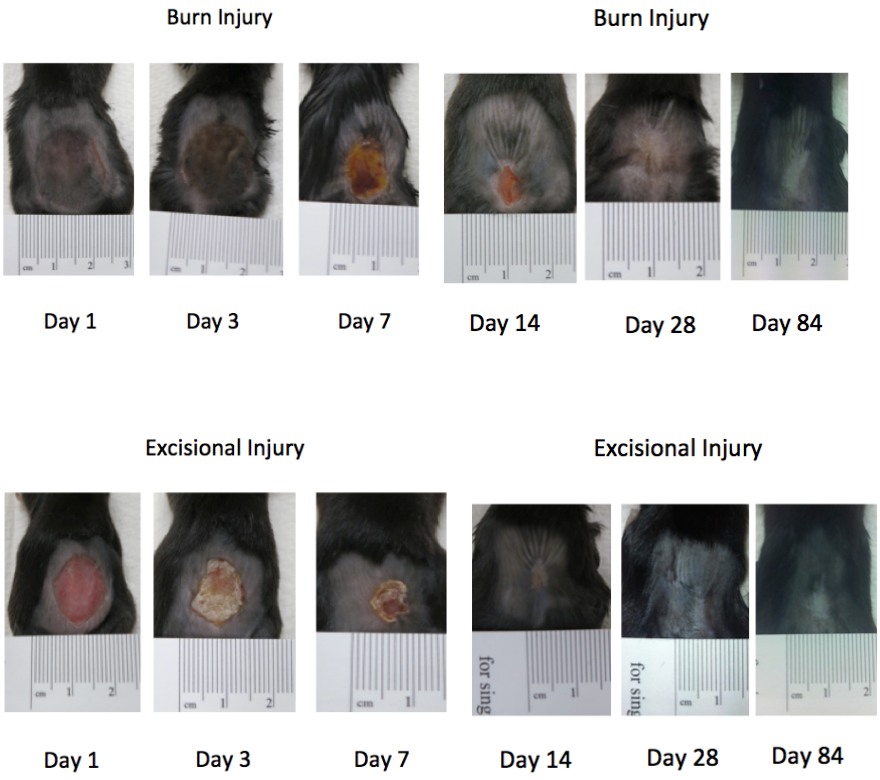


Figure 4.1: A demonstration of wound size and phases of wound healing observed in this study. *Note the presence of the burn eschar formation and progression and the eschar-like wound in the excision model on days 1, 3 and 7 post injury. All wounds were healed between day 14 and day 28.*

The eschar is a dry cellular mass that forms in full thickness wounds over the area of necrosis [79]. The presence of this eschar and its role in burn wound healing has been well investigated with regards to the management and timing of excision [2, 19, 222-227]. Despite this, there appears to be variability in both the literature and in clinical practice. The disparity in practice may be

influenced by the complex nature of burn wound progression that is not yet wholly understood and as such requires clinical judgment to determine between adequate excision and skin salvage [84, 228]. In several older clinical studies, they concluded that early excision of the burn eschar and coverage with skin graft (between 48-72hrs) proved beneficial in reducing the hyperinflammatory (as determined by reduced IL-6, IL-1β, IL-10 and TNF-α) and hypermetabolic process (as determined by reduced growth factor levels and hormone production) as well as reducing mortality in severe burn injury [19, 223-226]. Whereas other studies suggested a later excision (4-10 days) was optimal to improve patient prognosis [2, 227].

Subsequent work completed from this laboratory following the findings in this thesis concluded that early excision was preferable to late in a murine model, as early (day 1) excision of the burn wound was found to preserve a more appropriate innate and adaptive immune response to cutaneous injury when compared to late excision (day 8) [229].

Collectively, the data from this thesis, the subsequent work from this laboratory and clinical studies where removal of the eschar has been shown improve outcome are highly suggestive of the burn eschar contributing further to the inflammatory response and subsequent influence on the direction of the immune response [222, 226]. It is possible the eschar act as a nidus of damaged self-antigen (DAMPS and PAMPS) that drive the initial hyper-inflammatory response observed in the burn injury model. The findings from the gender studies shows supportive evidence that the immune response to injury is related to immune recognition of the aetiology of injury rather than the tissue damaged or the subsequent development of an eschar [34, 35] [31,

32]. However, there appears to be some role and contribution from the eschar presence [229]. To establish this and determine its level of contribution, an experimental model where two injury aetiologies, both of which result in the formation of an eschar would need to be developed and examined in both genders. Furthermore, it would be neglectful to not consider and investigate in future studies other complexities and compounding factors when assessing and analysing the immune response including; the mechanical force to the cells i.e. thermal vs sharp; the impact of the peripheral/surrounding zones of injury and the microbial exposure.

4.9: The Immune Response to Burn Injury Has Long-term Implications

To date, there have been many studies that have demonstrated long-term morbidity in burn patients [5-7, 13, 36, 37, 124]. With much of the focus having been on the metabolic changes that occur in consequence to the hypermetabolic state that is observe during the acute phase post burn [7, 39]. In compliment to this work, other burns studies have reported in non-severe models that burn patients have greater risk of developing both skeletal and cardiac complications, both in the intermediate and long-term [38, 167]. In parallel to the metabolic work, others have observed long-term changes in the cytokine profile post burn injury, which echoes the data in this thesis [7, 124]. The authors report changes in the cytokine profile as long as three years post major burn. From these studies it is clear there is change in the immune function that remains late post burn and that these changes correlate with

clinically observed hypermetabolic morbidity. However, what is not evident from these studies is how these changes link with other aspects of the immune system. The data from this thesis goes some way to indicating these associations and paves the way for further and more focused study.

The data in this thesis was examined until day 84 post injury and was found to show long-term immune changes. All wounds were healed between day 14 and 28 however, changes were still observed in the burn injury day 28 and 84 that was not seen to the same extent in the excision model. At day 28 the migrating skin dendritic cells had depressed maturation and number in the $CD11b^{lo}$ population. In the $CD11b^{hi}$ maturation was reduced from day 28 and continued until day 84. The DC changes were reflected in the T cell lymph node data at day 28 where the activated $CD4^+$ T cells were reduced in the burn that was associated with a rebound elevation in the proliferating $CD4^+$ T cells. In addition, the burn injury had associated elevated IL-10 levels and depressed white cell populations, including neutrophils, eosinophils and lymphocytes at day 84. This immunological picture seen in the burn injury may represent a process of T cell anergy or deletion tolerance to self-antigens that is secondary to abnormal DC antigen presentation. In consideration of these findings, recent clinical and population based studies elude to altered immune function following burn by way of increased risk of subsequent infection (unrelated to the burn scars/wounds); increased incidence of gastrointestinal disease as well as increased malignant development [5, 6, 13, 36, 37]. These later changes to the DC and subsequently the T cells observed in this thesis may contribute to a state of immune vulnerability which is observe clinically in the findings of these previous studies as increased

incidence of infection and cancers that is observed in burn patients**.** Further to this, this burn injury sustained was a non-severe injury model yet still demonstrated an immunological impact. This has great clinical and economic relevance as these non-severe burns represent a large proportion of clinical workload in burn units [1] [2] [10]. As such, the net is cast wider with regards to those patients that may be affected by the sequelae of their burn injury.

4.10: Study Limitations

The use of a murine model for human pathology and in particular immunology has long been established and well investigated with regards to its comparability to human biology [48, 51]. Although some literature may dispute its relevance, many others show support [48, 215]. The murine model has many advantages for immunological study, including immunological similarity to humans; age and gender consistency, in additional to practical advantages with regards to size and care of the animals. Despite this, it is necessary to acknowledge its limitations and differences. In the skin, structural differences are apparent with the presence of the panniculus carnosus, which is a contractile layer of tissue that facilitates greatly in wound contraction. In humans, this layer is mostly absent, with remnants remaining in layers such as the platysma [50]. In addition, mice have greater density of hair, which has implications in wound healing due to the presence of epithelial cells lining the follicles [50]. With regards to the immune system there are some differences in the type of DCs and T cells present in mice and the equivalent in humans

needs to be considered during translation to human pathology or in planning of clinical studies [51]. In this work, the murine model shows that there is a difference between aetiologies of injury in mice and as such it is necessary to verify these findings in patient based studies.

The time points of data collection were from day 1 to day 84 post injury to establish a broad range of both acute and long-term immune profiles following burn and excisional injury. However, assessment of earlier and later time points would display a more complete comprehension of the changes following injury. In particular establishing whether the changes observed at 3 months persist to 6 or 12 months will be important to determine the likely long-term impact of the changes observed.

Analysis of the cellular influx into the wound bed would be helpful to establish the dispersion of innate cells. Furthermore, functional studies to assess cytokine production of the innate cells at the wound site would also facilitate clarification source of the systemic cytokine profile observed. However this work was out of the scope of this research project.

Functional studies of the dendritic cells were not performed in this study. In the context of microbial or malignant challenge this data would give a better insight as to how aetiology of injury alters cellular function of the immune response rather than cellular and cytokine profiling alone.

Wound size at the different time points post injury was not measured. This information would have been helpful with the analysis as well as determine time to healing and the impact the immune response has on this. From our observation, all wounds healed between day 14 – 28. The wound sizes at day 1 were equal

and all wounds were healed by day 84, thus the data from these time points are reflective of the same wound/scar and differences in the immune responses are still observed. In order to quantify the impact of wound healing in the two models and correlate this with the immune system, growth factor analysis would also need to be considered.

4.11: Future Work

Future work would include the addition of extended time points for data collection, including prior to day 1 and post day 84 at up to 12 months post injury. Previous literature has observed earlier cytokine changes following injury which may have been missed in this study [124]. Furthermore, the data collection could be extended to beyond day 84 to assess the chronic implications following burn injury, which may facilitate explanation of the increased incidence of malignancy following burn injury [5].

To assess current clinical practice in full thickness burn injury, the addition of a further group to assess the impact of excision of the burn eschar. Since the completion of this thesis this work has been completed and published (Appendix E [229]). This data demonstrated that changes following burn injury can be reduced or eliminated with early excision of the burn, thus potentially reducing some of the clinically recognised morbidity associate with burn injury [5, 7].

Increased malignancy is seen in ageing, where changes to the functioning of the immune system also occur, known as immune-senescence [230, 231]. Ageing is

associated with reduced DC antigen uptake and migration, and a reduced level of circulating DC that leads to diminished naïve T cell activation. $CD4^+$ T cell function also declines with age, where Tregs increase, thus contributing to $CD4^+$ and $CD8^+$ T cell immunosuppression. Aged mice also have elevated levels of IL-10 which had been associated with cancer development as this modulatory cytokine may prime Treg cells and/or dampen immune responses [231]. It is therefore possible that burn injury prematurely ages the immune system, as in similarity to some of the aging studies this data showed a down-regulation of DC and T cell responses in addition to elevated IL-10 at the latest time point [5]. To assess this, functional studies would be beneficial to establish changes in the dendritic and T cells in response to the different aetiologies of injury.

Finally, to establish the similarity between murine and human pathology, a clinical study will need to be conducted to assess immune system function in burn injury survivors to determine the proportion of T cells, B cells and DC subsets and their activation levels in peripheral blood mononuclear cells.

4.12: Conclusion

Severe burns have a profound hyper-inflammatory response followed by immunocompromise. Recent research has shown that even in non-severe burn injuries there are long term sequelae including increased risk of malignancy, infection, gastrointestinal and cardiovascular disease supporting that immunological changes observed in this study occur in humans and are sustained [5].

The literature suggests that burn and non-burn injury have similar immunological trends; however burn injury shows some key differences. Here, it has been shown that the immune response to different cutaneous injury (burn and excision) differs in the temporal cytokine response resulting in promotion of monocyte to macrophage differentiation in burn injury with subsequent alteration to the dendritic and T cell response. Despite the size of the injury model used, changes were still observed long after healing, suggesting alteration to the immune system post injury.

Understanding the immune response to cutaneous injury is essential to understand the drivers of clinical outcome and assess consequence to surgical procedures in addition to injury. At present there is still need for further work in this area to generate the evidence necessary to tailor the clinical management, reduce patient morbidity and improve patient outcomes.

References

1. Murray, C.J.L., et al., *Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: A systematic analysis for the Global Burden of Disease Study 2010.* The Lancet, 2013. 380 (9859): p. 2197-2223.
2. Pavoni, V., et al., *Outcome predictors and quality of life of severe burn patients admitted to intensive care unit.* Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine, 2010. 18(1): p. 24.
3. Duke, J.M., et al., *Long term cardiovascular impacts after burn and non-burn trauma: A comparative population-based study.* Burns, 2017.
4. Ryan, C.M., et al., *Recovery trajectories after burn injury in young adults: does burn size matter?* Journal of Burn Care & Research, 2015. 36(1): p. 118-129.
5. Duke, J.M., Bauer, J., Fear, M. W., Rea, S., Wood, F. M., & Boyd, J., *Burn injury, gender and cancer risk: population-based cohort study using data from Scotland and Western Australia.* BMJ Open, 2014. 4(1): p. e003845.
6. Stevenson, A.W., et al., *Burn leads to long-term elevated admissions to hospital for gastrointestinal disease in a West Australian population based study.* Burns, 2017. 43(3): p. 665-673.
7. Jeschke, M.G., et al., *Long-term persistance of the pathophysiologic response to severe burn injury.* PloS one, 2011. 6(7): p. e21245.
8. Committee, N.B.C.R., *NBCR Committee Report: Standards and Strategy for Burn Care–A Review of Burn Care in the British Isles*. 2001, NBCRC.
9. Dunn, K., *The epidemiology and continued monitoring of burn injury in England and Wales*, in *Injury Prevention*. 2010, Group.bmj.com.
10. Duke, J., et al., *Urban compared with rural and remote burn hospitalisations in Western Australia.* Burns, 2012. 38(4): p. 591-8.
11. Rea, S. and F. Wood, *Minor burn injuries in adults presenting to the regional burns unit in Western Australia: A prospective descriptive study.* Burns, 2005. 31 (8): p. 1035-1040.
12. (NNBC), N.N.f.B.C., *National burn care referral guidance*, N.H.S.N.S. Services, Editor. 2012. p. 10.
13. Fear, V.S., et al., *Burn Injury Leads to Increased Long-Term Susceptibility to Respiratory Infection in both Mouse Models and Population Studies.* 2011.
14. Fear, V., et al., *Burn injury leads to increased long-term susceptibility to respiratory infection in both mouse models and population studies.* 2017.
15. Sharma, B.R., *Infection in patients with severe burns: causes and prevention thereof. .* Infectious disease clinics of North America, 2007. 21(3): p. 745-759.

16. Tran, N.K., Wisner, D. H., Albertson, T. E., Cohen, S., Greenhalgh, D., Palmieri, T. L., ... & Kost, G. J. , *Multiplex polymerase chain reaction pathogen detection in patients with suspected septicemia after trauma, emergency, and burn surgery. .* Surgery, 2012. 151(3): p. 456-463.
17. Wysocki, A.B., *Evaluating and managing open skin wounds: colonization versus infection.* AACN Advanced Critical Care, 2002. 13(3): p. 382-397.
18. Erol, S., Altoparlak, U., Akcay, M. N., Celebi, F., & Parlak, M. , *Changes of microbial flora and wound colonization in burned patients.* Burns, 2004. 30(4): p. 357-361.
19. Barret, J.P., & Herndon, D. N. , *Effects of burn wound excision on bacterial colonization and invasion.* Plastic and reconstruction surgery, 2003. 111(2): p. 744-750.
20. Choileain, N.N., et al., *Enhanced regulatory T cell activity is an element of the host response to injury.* Journal of Immunology, 2006. 176 (1): p. 225-236.
21. Mace, J.E., et al., *Differential expression of the immunoinflammatory response in trauma patients: Burn vs. non-burn.* Burns, 2012. 38 (4): p. 599-606.
22. Lederer, J.A., et al., *Comparison of longitudinal leukocyte gene expression after burn injury or trauma-hemorrhage in mice.* 2008.
23. Cassidy, J.T., Phillips, M., Fatovich, D., Duke, J., Edgar, D., & Wood, F., *Developing a burn injury severity score (BISS): Adding age and total body surface area burned to the injury severity score (ISS) improves mortality concordance.* Burns, 2013.
24. Davies, M. and P.O. Hagen, *Systemic inflammatory response syndrome.* British Journal of Surgery, 1997. 84(7): p. 920-935.
25. Efron, P. and L.L. Moldawer, *Sepsis and the dendritic cell.* Shock, 2003. 20(5): p. 386-401.
26. Bone, R.C., *Sir isaac newton, sepsis, SIRS, and CARS.* Critical Care Medicine, 1996. 27(7): p. 1125-1128.
27. Ward, N.S., Casserly, B., & Ayala, A, *he compensatory anti-inflammatory response syndrome (CARS) in critically ill patients.* Clinics in Chest Medicine, 2008. 29(4): p. 617-625.
28. Brownstein, B.H., et al., *Commonality and differences in leukocyte gene expression patterns among three models of inflammation and injury.* Physiol Genomics, 2006. 24(3): p. 298-309.
29. McGwin Jr, G., et al., *Gender differences in mortality following burn injury.* Shock, 2002. 18(4): p. 311-315.
30. O'Keefe, G.E., J.L. Hunt, and G.F. Purdue, *An evaluation of risk factors for mortality after burn trauma and the identification of gender-dependent differences in outcomes.* Journal of the American College of Surgeons, 2001. 192(2): p. 153-160.
31. Wichmann, M., et al., *Incidence and mortality of severe sepsis in surgical intensive care patients: the influence of patient gender on disease process and outcome.* Intensive care medicine, 2000. 26(2): p. 167-172.
32. George, R.L., et al., *Age-related gender differential in outcome after blunt or penetrating trauma.* Shock, 2003. 19(1): p. 28-32.

33. Mostafa, G., Huynh, T., Sing, R. F., Miles, W. S., Norton, H. J., & Thomason, M. H., *Gender-related outcomes in trauma.* Journal of Trauma-Injury, Infection, and Critical Care, 2002. 53(3): p. 430-435.
34. Gregory, M.S., et al., *Gender difference in cell-mediated immunity after thermal injury is mediated, in part, by elevated levels of interleukin-6.* Journal of leukocyte biology, 2000. 67(3): p. 319-326.
35. Plackett, T.P., R.L. Gamelli, and E.J. Kovacs, *Gender-Based Differences in Cytokine Production after Burn Injury: A Role of Interleukin-6.* Journal of the American College of Surgeons, 2010. 210(1): p. 73-78.
36. Duke, J.M., et al., *Burns and long-term infectious disease morbidity: A population-based study.* Burns, 2017. 43(2): p. 273-281.
37. Duke, J.M., et al., *Mortality After Burn Injury in Children: A 33-year Population-Based Study.* 2015.
38. O'Halloran, E., et al., *The impact of non-severe burn injury on cardiac function and long-term cardiovascular pathology*, in *Sci Rep*. 2016.
39. Jeschke, M.G., et al., *Burn size determines the inflammatory and hypermetabolic response.* Critical care (London, England), 2007. 11 (4): p. R90.
40. O'Sullivan, S.T. and T.P.F. O'Connor, *Immunosuppression following thermal injury: The pathogenesis of immunodysfunction.* British Journal of Plastic Surgery, 1997. 50 (8): p. 615-623.
41. Mannick, J.A., M.L. Rodrick, and J.A. Lederer, *The immunologic response to injury.* Journal of the American College of Surgeons, 2001. 193 (3): p. 237-244.
42. Menger, M.D. and B. Vollmar, *Surgical trauma: hyperinflammation versus immunosuppression?* Langenbeck's Archives of Surgery, 2004. 389(6): p. 475-484.
43. Menges, P., et al., *Surgical trauma and postoperative immune dysfunction.* European Surgical Research, 2012. 48 (4): p. 180-186.
44. Zaidi, Z. and S. Lanigan, *Skin: Structure and Function*, in *Dermatology in Clinical Practice*. 2010, Springer London. p. 1-15.
45. Cartotto, R., et al., *The Acute Respiratory Distress Syndrome (ARDS) in mechanically ventilated burn patients: An analysis of risk factors, clinical features, and outcomes using the Berlin ARDS definition.* Burns, 2016. 42(7): p. 1423-1432.
46. Di Meglio, P., G.K. Perera, and F.O. Nestle, *The Multitasking Organ: Recent Insights into Skin Immune Function.* Immunity, 2011. 35(6): p. 857-869.
47. Förster, R., Braun, A., & Worbs, T., *Lymph node homing of T cells and dendritic cells via afferent lymphatics.* Trends in Immunology, 2012. 33(6271-280).
48. Mestas, J., & Hughes, C. C. , *Of mice and not men: differences between mouse and human immunology. .* Journal of Immunology, 2002. 172(5): p. 2731-2738.
49. Heath, W.R., & Carbone, F. R., *The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells.* Nature Immunology, 2013. 14(10): p. 978-985.

50. Rittié, L., *Cellular mechanisms of skin repair in humans and other mammals*, in *J Cell Commun Signal*. 2016. p. 103-20.
51. Mestas, J., & Hughes, C. C. , *Of mice and not men: differences between mouse and human immunology.* Journal of Immunology, 2002. 172(5): p. 2731-2738.
52. Merad,M., Ginhoux,F., and Collin, M., *Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells.* Nature Reviews Immunology, 2008. 8: p. 13.
53. Menon, G.K., *New insights into skin structure: scratching the surface.* Advanced Drug Delivery Reviews, 2002. 54, Supplement(0): p. S3-S17.
54. Rodemann, H.P. and H.-O. Rennekampff, *Functional Diversity of Fibroblasts*, in *Tumor-Associated Fibroblasts and their Matrix*. 2011, Springer. p. 23-36.
55. Valladeau, J., et al., *Langerin, a Novel C-Type Lectin Specific to Langerhans Cells, Is an Endocytic Receptor that Induces the Formation of Birbeck Granules.* Immunity, 2000. 12(1): p. 71-81.
56. Shupp, J.W., et al., *A review of the local pathophysiologic bases of burn wound progression.* Journal of Burn Care and Research, 2010. 31 (6): p. 849-873.
57. Herndon, D.N., *Total burn care*. 1996: Saunders.
58. Guo, S., & DiPietro, L. A, *Factors affecting wound healing. .* Journal of Dental Research, 2010. 89(3): p. 219-229.
59. Gosain, A., & DiPietro, L. A, *Aging and wound healing.* World Journal of Surgery, 2004. 28(3): p. 321-326.
60. Clark, R.A., *Basics of cutaneous wound repair.* J Dermatol Surg Oncol, 1993. 19(8): p. 693-706.
61. Scott, J.R., Muangman, P., & Gibran, N. S., *Making sense of hypertrophic scar: a role for nerves. .* Wound Repair and Regeneration, 2007. 15(1): p. S27-31.
62. Murray, P.J., & Wynn, T. A., *Protective and pathogenic functions of macrophage subsets.* Nature Reviews Immunology, 2011. 11(11): p. 723-737.
63. Engelmann, B. and S. Massberg, *Thrombosis as an intravascular effector of innate immunity.* Nature Reviews Immunology, 2012.
64. Singer, A.J. and R. Clark, *Cutaneous wound healing.* N Engl J Med, 1999. 341(10): p. 738-746.
65. Witte, M.B. and A. Barbul, *General principles of wound healing.* Surgical Clinics of North America, 1997. 77(3): p. 509-528.
66. Neher, M.D., et al., *Molecular mechanisms of inflammation and tissue injury after major trauma-is complement the "bad guy"?* Journal of Biomedical Science, 2011. 18 (1)(90).
67. Medzhitov, R., *Inflammation 2010: new adventures of an old flame.* Cell 2010. 140(6): p. 771-776.
68. Gurtner, G.C., et al., *Wound repair and regeneration.* Nature, 2008. 453(7193): p. 314-321.
69. Raman, D., Sobolik-Delmaire, T., & Richmond, A, *Chemokines in health and disease. .* Experimental Cell Research, 2011. 317(5): p. 575-589.

70. Clark, R.A., *The molecular and cellular biology of wound repair*. 1996: Springer.
71. Low, Q.E.H., et al., *Wound Healing in MIP-1α−/− and MCP-1−/− Mice*, in *Am J Pathol*. 2001. p. 457-63.
72. Stadelmann, W.K., et al., *Physiology and healing dynamics of chronic cutaneous wounds.* The American Journal of Surgery, 1998. 176(2): p. 26S-38S.
73. Barrientos, S., et al., *Growth factors and cytokines in wound healing.* Wound Repair and Regeneration, 2008. 16(5): p. 585-601.
74. Mirza, R.E. and T.J. Koh, *Contributions of Cell Subsets to Cytokine Production during Normal and Impaired Wound Healing.* Cytokine, 2015. 71(2): p. 409-12.
75. Paladini, R.D., et al., *Onset of re-epithelialization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16.* J Cell Biol, 1996. 132(3): p. 381-97.
76. Li, J., J. Chen, and R. Kirsner, *Pathophysiology of acute wound healing.* Clinics in dermatology, 2007. 25(1): p. 9-18.
77. Welch, M.P., G.F. Odland, and R.A. Clark, *Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction.* J Cell Biol, 1990. 110(1): p. 133-45.
78. Singh, V., et al., *The pathogenesis of burn wound conversion.* Annals of plastic surgery, 2007. 59(1): p. 109-115.
79. Jackson, D.M., *The diagnosis of the depth of burning.* British journal of surgery, 1953. 40(164): p. 588-596.
80. Posluszny, J.A. and R.L. Gamelli, *Anemia of Thermal Injury: Combined Acute Blood Loss Anemia and Anemia of Critical Illness.* J Burn Care Res, 2010. 31(2): p. 229-42.
81. Orgill, D.P., Porter, S.A.,Taylor,H.O., *Heat injury to cells in perfused systems.* Annals of the New York Academy of Sciences, 2006. 1066(1): p. 106-118.
82. Tan, J.Q., et al., *The roles of autophagy and apoptosis in burn wound progression in rats.* Burns, 2013. 39(8): p. 1551-6.
83. Zawacki, B.E., *Reversal of capillary stasis and prevention of necrosis in burns.* Annals of surgery, 1974. 180(1): p. 98.
84. Johnson, R.M. and R. Richard, *Partial-thickness burns: identification and management.* Advances in skin & wound care, 2003. 16(4): p. 178-187.
85. Delves, P.J., Martin, S. J., Burton, D. R., & Roitt, I. M., *Roitt's essential immunology*. 12th ed. Vol. 20. 2011: John Wiley & Sons.
86. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nat Rev Cancer, 2004. 4(1): p. 11-22.
87. Cheung, M.C., et al., *Body surface area prediction in normal, hypermuscular, and obese mice.* J Surg Res, 2009. 153(2): p. 326-31.
88. Davoine, F. and P. Lacy, *Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity.* Front Immunol, 2014. 5: p. 570.

89. Parihar, A., Eubank, T. D., & Doseff, A. I., *Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death.* Journal of Innate Immunity, 2010. 2(3): p. 204-215.
90. Fahy, R.J., Doseff, A. I., & Wewers, M. D., *Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pathway that is blocked by endotoxin and is independent of caspase-1.* Journal of Immunology, 1999. 163(4): p. 1755-1762.
91. Werner, S. and R. Grose, *Regulation of wound healing by growth factors and cytokines.* Physiological reviews, 2003. 83(3): p. 835-870.
92. Relloso, M., et al., *DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents.* J Immunol, 2002. 168(6): p. 2634-43.
93. Liu K, V.G., Schwickert TA, Guermonprez P, Meredith MM, Yao K, Chu FF, and R.A. Randolph GJ, Nussenzweig M, *In vivo analysis of dendritic cell development and homeostasis.* Science, 2009. 324(5925): p. 6.
94. Hashimoto, D., Miller, J., & Merad, M. , *Dendritic cell and macrophage heterogeneity in vivo.* Immunity, 2011. 35(3): p. 323-335.
95. Satpathy, A.T., et al., *Re(de)fining the dendritic cell lineage.* Nature Immunology, 2012. 13(12): p. 1145.
96. Cairns, B.A., Maile, R., Buchanan, I., Pilati, D., deSerres, S., Collins, E. J., ... & Meyer, A. A. , *CD8+ T cells express a T-helper 1–like phenotype after burn injury. .* Surgery. 130(2): p. 210-216.
97. Henrickson, S.E., et al., *T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation.* Nature Immunology, 2008. 9(3).
98. Malissen, B., Tamoutounour, S., & Henri, S., *The origins and functions of dendritic cells and macrophages in the skin.* Nature Reviews Immunology, 2014. 14(6): p. 417-428.
99. von Andrian, U.H. and C.R. Mackay, *T-cell function and migration. Two sides of the same coin.* N Engl J Med, 2000. 343(14): p. 1020-34.
100. O'Garra, A., & Vieira, P. , *TH1 cells control themselves by producing interleukin-10. .* Nature Review Immunology. 7(6): p. 425-428.
101. Sasaki, J.R., Q. Zhang, and M.G. Schwacha, *Burn induces a Th-17 inflammatory response at the injury site.* Burns, 2011. 37(4): p. 646-51.
102. Schwacha, M.G., *Macrophages and post-burn immune dysfunction.* Burns, 2003. 29 (1): p. 1-14.
103. Langrish, C.L., et al., *IL−12 and IL−23: master regulators of innate and adaptive immunity.* Immunological reviews, 2004. 202(1): p. 96-105.
104. Rendon, J.L., & Choudhry, M. A., *Th17 cells: critical mediators of host responses to burn injury and sepsis. .* Journal of Leucocyte Biology, 2012. 92(3): p. 529-538.
105. Kimura, F., et al., *Immunosuppression following surgical and traumatic injury.* Surg Today, 2010. 40(9): p. 793-808.
106. Ramsdell, F., *Foxp3 and natural regulatory T cells: key to a cell lineage?* Immunity, 2003. 19(2): p. 165-168.
107. Adalid-Peralta, L., et al., *Mechanisms Underlying the Induction of Regulatory T cells and Its Relevance in the Adaptive Immune*

Response in Parasitic Infections. International journal of biological sciences, 2011. 7(9): p. 1412.
108. Hori S, N.T., Sakaguchi S, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. 299: p. 5.
109. Cairns, B.A., Maile, R., Buchanan, I., Pilati, D., deSerres, S., Collins, E. J., ... & Meyer, A. A. , *CD8+ T cells express a T-helper 1–like phenotype after burn injury.* Surgery. 130(2): p. 210-216.
110. Guilliams, M., Crozat, K., Henri, S., Tamoutounour, S., Grenot, P., Devilard, E., ... & Malissen, B. , *Skin-draining lymph nodes contain dermis-derived CD103− dendritic cells that constitutively produce retinoic acid and induce Foxp3+ regulatory T cells.* Blood, 2010. 115(10): p. 1958-1968.
111. Giannoudis, P.V., Hildebrand, F., & Pape, H. C., *Inflammatory serum markers in patients with multiple trauma CAN THEY PREDICT OUTCOME?* Journal of Bone & Joint Surgery, British Volume, 2004. 86(3): p. 313-323.
112. Wood, L.C., et al., *Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis.* J Invest Dermatol, 1996. 106(3): p. 397-403.
113. Matzinger, P., *The danger model: a renewed sense of self.* Science Signaling, 2002. 296(5566): p. 301.
114. Bianchi, M.E., *DAMPs, PAMPs and alarmins: all we need to know about danger.* Journal of Leucocyte Biology, 2007. 81(1): p. 1-5.
115. Gentile, L.F. and L.L. Moldawer, *What Is the Role for the Inflammasome in Burn Injury and Sepsis?* Shock, 2012. 37(1): p. 124-125 10.1097/SHK.0b013e31823b7643.
116. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* International reviews of immunology, 2011. 30(1): p. 16-34.
117. Walle, L.V., & Lamkanfi, M. , *Inflammasomes: caspase-1-activating platforms with critical roles in host defense. .* Frontiers in Microbiology, 2011. 2(3): p. 4-10.
118. Osuka, A., et al., *A protective role for inflammasome activation following injury.* Shock, 2012. 37 (1): p. 47-55.
119. Rock, K.L., J.-J. Lai, and H. Kono, *Innate and adaptive immune responses to cell death.* Immunological Reviews, 2011. 243(1): p. 191-205.
120. Yoshimura, T., Galligan, C., Takahashi, M., Chen, K., Liu, M., Tessarollo, L., & Wang, J. M., *Non-myeloid cells are major contributors to innate immune responses via production of monocyte chemoattractant protein-1/CCL2.* Frontiers in Immunolgy, 2013. 4.
121. Hochrein, H., et al., *Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells.* The Journal of experimental medicine, 2000. 192(6): p. 823-834.
122. Jeschke, M.G., Chinkes, D. L., Finnerty, C. C., Kulp, G., Suman, O. E., Norbury, W. B., ... & Herndon, D. N, *The pathophysiologic response to severe burn injury.* Annals of Surgery, 2008. 248(3): p. 387.
123. *Neutrophils: Between Host Defence, Immune Modulation, and Tissue Injury.* 2017.

124. Finnerty, C.C., et al., *Cytokine expression profile over time in burned mice.* Cytokine, 2009. 45(1): p. 20-25.
125. Orman, M.A., et al., *Comparison of the cytokine and chemokine dynamics of the early inflammatory response in models of burn injury and infection.* Cytokine, 2011. 55(3): p. 362-71.
126. Martin, P., D'Souza, D., Martin, J., Grose, R., Cooper, L., Maki, R., & McKercher, S. R., *Wound healing in the PU. 1 null mouse—tissue repair is not dependent on inflammatory cells. .* Current Biology, 2003. 13(13): p. 1122-1128.
127. Vega-Ramos, J., Roquilly, A., Asehnoune, K., & Villadangos, J. A. , *Modulation of dendritic cell antigen presentation by pathogens, tissue damage and secondary inflammatory signals.* Current Opinion in Pharmacology, 2014. 17: p. 64-70.
128. Youn, Y.K., C. LaLonde, and R. Demling, *The role of mediators in the response to thermal injury.* World Journal of Surgery, 1992. 16 (1): p. 30-36.
129. Dovi, J.V., A.M. Szpaderska, and L.A. DiPietro, *Neutrophil function in the healing wound: adding insult to injury?* THROMBOSIS AND HAEMOSTASIS-STUTTGART-, 2004. 92: p. 275-280.
130. Kimura, F., et al., *Immunosuppression following surgical and traumatic injury.*
131. Linares, H.A., *From wound to scar. .* Burns, 1996. 22(5): p. 339-253.
132. Belz GT, S.C., Kleinert L, Reading P, Brooks A, Shortman K, Carbone FR, and H. WR, *Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus.* Proceedings of the National Academy of Sciences of the United States of America, 2004. 101(23): p. 6.
133. Schulz, O., Edwards, A. D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., & Reis e Sousa, C, *CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal.* Immunity, 2000. 13(4): p. 10.
134. Henri, S., Guilliams, M., Poulin, L. F., Tamoutounour, S., Ardouin, L., Dalod, M., & Malissen, B., *Disentangling the complexity of the skin dendritic cell network. .* Immunology and Cell Biology, 2010. 88(4): p. 366-375.
135. Wu J, X.A., Chen W, *Cytokine regulation of immune tolerance.* Burns & Trauma, 2014. 2(1): p. 7.
136. Hotchkiss, R.S., et al., *Sepsis and septic shock.* Nature Reviews Disease Primers, 2016. 2: p. 16045.
137. Edelson, B.T., Wumesh, K. C., Juang, R., Kohyama, M., Benoit, L. A., Klekotka, P. A., ... & Murphy, K. M. , *Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8α+ conventional dendritic cells.* The Journal of Experimental Medicine, 2010. 207(4): p. 823-836.
138. Maldonado-López, R., De Smedt, T., Pajak, B., Heirman, C., Thielemans, K., Leo, O., ... & Moser, M, *Role of CD8alpha+ and CD8alpha-dendritic cells in the induction of primary immune responses in vivo.* Journal of Leucocyte Biology, 1999. 66(2): p. 5.

139. Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., ... & Heath, W. R., *Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells.* Nature Immunology, 2009. 10(5): p. 488-495.
140. Igyártó, B.Z., Haley, K., Ortner, D., Bobr, A., Gerami-Nejad, M., Edelson, B. T., ... & Kaplan, D. H. , *Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses.* Immunity, 2011. 35(2): p. 260-272.
141. doyaga, J., Fiorese, C., Zbytnuik, L., Lubkin, A., Miller, J., Malissen, B., ... & Steinman, R. M., *Specialized role of migratory dendritic cells in peripheral tolerance induction. .* Journal of Clinical Investigation, 2013. 123(2): p. 844-854.
142. Hochrein H, S.K., Vremec D, Scott B, Hertzog P, O'Keeffe M, *Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets.* Journal of Immunology, 2001. 166(9): p. 8.
143. Allan, R.S., Smith, C. M., Belz, G. T., van Lint, A. L., Wakim, L. M., Heath, W. R., & Carbone, F. R, *Epidermal viral immunity induced by CD8α+ dendritic cells but not by Langerhans cells.* Science, 2003. 301(5641): p. 4.
144. Schulz, O., & Reis e Sousa, C, *Cross−presentation of cell−associated antigens by CD8α+ dendritic cells is attributable to their ability to internalize dead cells.* Immunology, 2002. 107(2): p. 7.
145. Qiu, C.H., Miyake, Y., Kaise, H., Kitamura, H., Ohara, O., & Tanaka, M. , *Novel subset of CD8α+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. .* Journal of Immunology 2009. 182(7): p. 4127-4136.
146. Mosmann T, C.R., *Th1 and Th2 Cells: Different patterns of lymphokine secretions leads to different functional properties.* Annual Review Immunology, 1989. 7: p. 29.
147. Thornton, A.M. and E.M. Shevach, *Suppressor effector function of CD4+ CD25+ immunoregulatory T cells is antigen nonspecific.* The Journal of Immunology, 2000. 164(1): p. 183-190.
148. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., & Toda, M. , *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* The Journal of Immunology, 1995. 155(3): p. 1151-1164.
149. Schön, M.P., T.M. Zollner, and W.H. Boehncke, *The Molecular Basis of Lymphocyte Recruitment to the Skin: Clues for Pathogenesis and Selective Therapies of Inflammatory Disorders.* Journal of Investigative Dermatology, 2003. 121(5): p. 951-962.
150. Lennard, T., et al., *The influence of surgical operations on components of the human immune system.* British journal of surgery, 1985. 72(10): p. 771-776.
151. MacConmara, M.P., Tajima, G., O'Leary, F., Delisle, A. J., McKenna, A. M., Stallwood, C. G., ... & Lederer, J. A, *Regulatory T cells suppress antigen-driven CD4 T cell reactivity following injury.* Journal of Leucocyte Biology, 2011. 89(1): p. 137-147.

152. Hensler, T., Hecker, H., Heeg, K., Heidecke, C. D., Bartels, H., Barthlen, W., ... & Holzmann, B. , *Distinct mechanisms of immunosuppression as a consequence of major surgery.* Infection and Immunity, 1997. 65(6): p. 2283-2291.
153. Buras, J.A., B. Holzmann, and M. Sitkovsky, *Model organisms: Animal Models of sepsis: setting the stage.* Nature Reviews Drug Discovery, 2005. 4(10): p. 854.
154. Maile, R., Barnes, C. M., Nielsen, A. I., Meyer, A. A., Frelinger, J. A., & Cairns, B. A. , *Lymphopenia-induced homeostatic proliferation of CD8+ T cells is a mechanism for effective allogeneic skin graft rejection following burn injury.* The Journal of Immunology, 2006. 176(11): p. 6717-6726.
155. Toda, A. and C.A. Piccirillo, *Development and function of naturally occurring CD4+ CD25+ regulatory T cells.* Journal of leukocyte biology, 2006. 80(3): p. 458-470.
156. van den Berg, L.M., de Jong, M. A., de Witte, L., Ulrich, M. M., & Geijtenbeek, T. B. , *Burn injury suppresses human dermal dendritic cell and Langerhans cell function.* Cellular Immunology, 2011. 268(1): p. 29-36.
157. Bruen, K.J., et al., *Real-time monitoring of keratin 5 expression during burn re-epithelialization.* Journal of Surgical Research, 2004. 120 (1): p. 12-20.
158. Guo, Z., et al., *Burn injury promotes antigen-driven Th2-type responses in vivo.* Journal of Immunology, 2003. 171 (8): p. 3983-3990.
159. Komi-Kuramochi, A., et al., *Expression of fibroblast growth factors and their receptors during full-thickness skin wound healing in young and aged mice.* Journal of Endocrinology, 2005. 186 (2): p. 273-289.
160. Hoff, J., *Methods of Blood Collection in the Mouse.* Technique, 2000. 29 (10): p. 7.
161. Wikstrom, M.E., et al., *Influence of mucosal adjuvants on antigen passage and CD4+ T cell activation during the primary response to airborne allergen.* Journal of Immunology, 2006. 177 (2): p. 913-924.
162. Sheridan, R.L., *A great constitutional disturbance.* New England Journal of Medicine, 2002. 345(17): p. 1271-1272.
163. *The Oxford Dictionary of English*, in *Oxford Dictionary of English*, A. Stevenson, Editor. 2010, Oxford University Press.
164. Wynn, T.A., *Fibrotic disease and the Th1/Th2 paradigm.* Nature Reviews Immunology, 2004. 4(8): p. 583.
165. Lim, S.S., et al., *A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010.* The Lancet, 2013. 380(9859): p. 2224-2260.
166. Finnerty, C.C., et al., *Cytokine expression profile over time in severely burned pediatric patients.* Shock (Augusta, Ga.), 2006. 26 (1): p. 13-19.
167. O'Neill, T.B., et al., *The effect of localised burn injury on skeletal muscle in a mouse model.* Journal of Burn Care and Research, 2011. Conference: 43rd Annual Meeting of the American Burn Association Chicago, IL United States. Conference Start: 20110329 Conference End: 20110401. Conference Publication: (var.pagings). 32: p. S74.

168. Vicari, A.P., Chiodoni, C., Vaure, C., Aït-Yahia, S., Dercamp, C., Matsos, F., ... & Caux, C., *Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti–interleukin 10 receptor antibody.* The Journal of Experimental Medicine, 2002. 196(4): p. 541-549.
169. De, A.K., K. Laudanski, and C.L. Miller-Graziano, *Failure of monocytes of trauma patients to convert to immature dendritic cells is related to preferential macrophage-colony-stimulating factor-driven macrophage differentiation.* The Journal of Immunology, 2003. 170(12): p. 6355-6362.
170. Biffl, W.L., et al., *Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation?* Annals of surgery, 1996. 224(5): p. 647.
171. Biffl W L, M.E.E., Moore F A, Peterson V M, *Interleukin-6 in the injured patient: Marker of injury or mediator of inflammation?* Annals of Surgery, 1996. 224(5).
172. Baigrie, R., et al., *Systemic cytokine response after major surgery.* British Journal of Surgery, 1992. 79(8): p. 757-760.
173. Pape, H.C., Grimme, K., van Griensven, M., Sott, A. H., Giannoudis, P., Morley, J., ... & Krettek, C. , *Impact of intramedullary instrumentation versus damage control for femoral fractures on immunoinflammatory parameters: prospective randomized analysis by the EPOFF Study Group. .* Journal of Trauma-Injury, Infection, and Critical Care, 2003. 55(1): p. 7-13.
174. Hietbrink, F., Koenderman, L., Rijkers, G. T., & Leenen, L. P. H., *Trauma: the role of the innate immune system. .* World Journal of Emergency Surgery, 2006. 1(1): p. 15.
175. Skrzeczyńska−Moncznik, J., Bzowska, M., Grage−Griebenow, E., Zembala, M., & Pryjma, J., *Peripheral Blood CD14high CD16+ Monocytes are Main Producers of IL−10. .* Scandinavian Journal of Immunology, 2008. 67(2): p. 152-159.
176. Strong, V.E.M., Mackrell, P. J., Concannon, E. M., Naama, H. A., Schaefer, P. A., Shaftan, G. W., ... & Daly, J. M. , *Blocking prostaglandin E2 after trauma attenuates pro-inflammatory cytokines and improves survival. .* Shock, 2000. 14(3): p. 374-379.
177. Baue, A.E., R. Durham, and E. Faist, *Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle?* Shock, 1998. 10(2): p. 79-89.
178. O'Garra, A., & Vieira, P. , *TH1 cells control themselves by producing interleukin-10.* Nature Review Immunology. 7(6): p. 425-428.
179. Neidhartdt R, K.M., Steckholzer U, Safret A, Ungethue, U, Trentz O, Ertel W, *Relationship of interleukin-10 plasma levels to severity of injurry and clinical outcome in injured patients.* The Journal of Trauma: Injury, Infection and Critical Care, 1997. 5(42): p. 9.
180. MacConmara, M.P., et al., *Increased CD4+ CD25+ T regulatory cell activity in trauma patients depresses protective Th1 immunity.* Ann Surg, 2006. 244(4): p. 514-23.
181. Mokart, D., Capo, C., Blache, J. L., Delpero, J. R., Houvenaeghel, G., Martin, C., & Mege, J. L. , *Early postoperative compensatory anti−*

inflammatory response syndrome is associated with septic complications after major surgical trauma in patients with cancer. . British Journal of Surgery, 2002. 89(11): p. 1450-1456.
182. Shenkin, A., Fraser, W. D., Series, J., Winstanley, F. P., McCartney, A. C., Burns, H. J., & Van Damme, J. , *The serum interleukin 6 response to elective surgery. .* Lymphokine research, 1988. 8(2): p. 123-127.
183. Cruickshank, A.M., Fraser, W. D., Burns, H., Van Damme, J., & Shenkin, A. , *Response of serum interleukin-6 in patients undergoing elective surgery of varying severity.* Clinical Science, 1990. 79(2): p. 161-165.
184. Drost, A.C., Burleson, D. G., Cioffi Jr, W. G., Jordan, B. S., Mason Jr, A. D., & Pruitt Jr, B. A., *Plasma cytokines following thermal injury and their relationship with patient mortality, burn size, and time postburn. .* Journal of Trauma-Injury, Infection, and Critical Care, 1993. 35(3): p. 335-339.
185. Sartorelli, K.H., Silver, G. M., & Gamelli, R. L. , *The effect of granulocyte colony-stimulating factor (G-CSF) upon burn-induced defective neutrophil chemotaxis. .* Journal of Trauma and Acute Care Surgery, 1991. 31(4): p. 523-530.
186. Peter, F.W., et al., *The effect of severe burn injury on proinflammatory cytokines and leukocyte behavior: Its modulation with granulocyte colony-stimulating factor.* Burns, 1999. 25 (6): p. 477-486.
187. Moore, F.A., Sauaia, A., Moore, E. E., Haenel, J. B., Burch, J. M., & Lezotte, D. C., *Postinjury multiple organ failure: a bimodal phenomenon. .* Journal of Trauma-Injury, Infection, and Critical Care, 1996. 40(4): p. 501-512.
188. Charo, I.F., & Ransohoff, R. M. , *The many roles of chemokines and chemokine receptors in inflammation. .* New England Journal of Medicine, 2006. 354(6): p. 610-621.
189. Heinrich, S.A., et al., *Elevated monocyte chemoattractant protein-1 levels following thermal injury precede monocyte recruitment to the wound site and are controlled, in part, by tumor necrosis factor-alpha.* Wound Repair Regen, 2003. 11(2): p. 110-9.
190. Decker D, T.R., Springer W, Lauschke H, Hirner A, von Ruecker A, *Abdominal surgical interventions: local and systemic consequences for the immune system--a prospective study on elective gastrointestinal surgery.* Journal Surgical Research, 2005. 126(1): p. 6.
191. Malissen, B., Tamoutounour, S., & Henri, S., *The origins and functions of dendritic cells and macrophages in the skin. .* Nature Reviews Immunology, 2014. 14(6): p. 417-428.
192. Kataranovski, M., et al., *Increased activity of lymph node cells in experimental thermal injury: Changes in accessory cells in injured area-draining lymph nodes.* Burns, 2000. 26 (6): p. 525-534.
193. Qiu, C.H., Miyake, Y., Kaise, H., Kitamura, H., Ohara, O., & Tanaka, M. , *Novel subset of CD8α+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens.* Journal of Immunology, 2009. 182(7): p. 4127-4136.
194. Alloatti, A., et al., *Dendritic cell maturation and cross−presentation: timing matters!* Immunological Reviews, 2018. 272(1): p. 97-108.

195. Pantel, A., et al., *Direct Type I IFN but Not MDA5/TLR3 Activation of Dendritic Cells Is Required for Maturation and Metabolic Shift to Glycolysis after Poly IC Stimulation*, in *PLoS Biol*. 2014.
196. Sung, S.S.J., Fu, S. M., Rose, C. E., Gaskin, F., Ju, S. T., & Beaty, S. R. , *A major lung CD103 (αE)-β7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins.* The Journal of Immunology, 2006. 176(4): p. 2161-2172.
197. Sung, S.S.J., Fu, S. M., Rose, C. E., Gaskin, F., Ju, S. T., & Beaty, S. R. , *A major lung CD103 (αE)-β7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. .* The Journal of Immunology, 2006. 176(4): p. 2161-2172.
198. Semmrich, M., Plantinga, M., Svensson-Frej, M., Uronen-Hansson, H., Gustafsson, T., Mowat, A. M., ... & Agace, W. W. , *Directed antigen targeting in vivo identifies a role for CD103+ dendritic cells in both tolerogenic and immunogenic T-cell responses.* Mucosal Immunology, 2012. 5(2): p. 150-160.
199. Beaty, S.R., C.E. Rose, and S.-s.J. Sung, *Diverse and Potent Chemokine Production by Lung CD11bhigh Dendritic Cells in Homeostasis and in Allergic Lung Inflammation.* 2007.
200. Fazal, N., *OX62+ OX6+ OX35+ rat dendritic cells are unable to prime CD4+ T cells for an effective immune response following acute burn injury.* Results in Immunology, 2013(3): p. 64-72.
201. Heath, W.R., & Carbone, F. R., *Dendritic cell subsets in primary and secondary T cell responses at body surfaces.* Nature Immunology, 2009. 10(12): p. 1237-1244.
202. Howell, K., et al., *High MafB expression following burn augments monocyte commitment and inhibits DC differentiation in hemopoietic progenitors.* Journal of Leukocyte Biology, 2012. 91(1): p. 69-81.
203. Williams, K.N., et al., *Dendritic Cell Depletion in Burn Patients Is Regulated by MafB Expression.* Journal of Burn Care & Research, 2012. 33(6): p. 747-758.
204. Shen, H., et al., *Burn Injury Triggered Dysfunction in Dendritic Cell Response to TLR9 Activation and Resulted in Skewed T Cell Functions.* PLoS ONE, 2012. 7(11): p. e50238.
205. Patenaude, J., D'Elia, M., Hamelin, C., & Bernier, J. , *Selective effect of burn injury on splenic CD11c+ dendritic cells and CD8α+ CD4− CD11c+ dendritic cell subsets.* Cellular and Molecular Life Sciences, 2010. 67(8): p. 1315-1329.
206. Hubert, F.X., Voisine, C., Louvet, C., Heslan, M., & Josien, R., *Rat plasmacytoid dendritic cells are an abundant subset of MHC class II+ CD4+ CD11b− OX62− and type I IFN-producing cells that exhibit selective expression of Toll-like receptors 7 and 9 and strong responsiveness to CpG. .* The Journal of Immunology, 2004. 172(12): p. 7485-7494.
207. Schneider, D.F., Glenn, C. H., & Faunce, D. E., *Innate lymphocyte subsets and their immunoregulatory roles in burn injury and sepsis.* Journal of Burn Care & Research, 2007. 28(3): p. 356-379.
208. Wendland, M., Willenzon, S., Kocks, J., Davalos-Misslitz, A. C., Hammerschmidt, S. I., Schumann, K., ... & Förster, R., *Lymph node T*

cell homeostasis relies on steady state homing of dendritic cells. Immunity, 2011. 35(6): p. 945-957.
209. Hunt, J.P., et al., *The Effector Component of the Cytotoxic T-Lymphocyte Response Has a Biphasic Pattern after Burn Injury.* Journal of Surgical Research, 1998. 80(2): p. 243-251.
210. Sayed, S., Bakry, R., El-Shazly, M., El-Oteify, M., Terzaki, S., & Fekry, M. , *Effect of major burns on early and late activating markers of peripheral blood T lymphocytes. .* Annals of Burns and Fire Disasters, 2012. 25(1): p. 17.
211. Sakaguchi, S., *Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses.* Annu. Rev. Immunol., 2004. 22: p. 531-562.
212. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work.* Nature Reviews Immunology, 2008. 8(7): p. 523-532.
213. Ng, R.L.X., et al., *Altered Immunity and Dendritic Cell Activity in the Periphery of Mice after Long-Term Engraftment with Bone Marrow from Ultraviolet-Irradiated Mice.* 2013.
214. Patil NK, L.L., Bohannon JK, Guo Y,Hernandez A, Fensterheim B,, *IL-15 Superagonist Expands mCD8+ T, NK and NKT Cells after Burn Injury but Fails to Improve Outcome during Burn Wound Infection.* 2017.
215. Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., ... & Schoenfeld, D. A. , *Genomic responses in mouse models poorly mimic human inflammatory diseases. .* Proceedings of the National Academy of Sciences, 2013. 110(9): p. 3507-3512.
216. Osterburg, A.R., Hexley, P., Supp, D. M., Robinson, C. T., Noel, G., Ogle, C., ... & Babcock, G. F. , *Concerns over interspecies transcriptional comparisons in mice and humans after trauma. .* Proceedings of the National Academy of Sciences, 2013. 110(36): p. E3370-E3370.
217. Molina-Martínez, L.M., C. González-Espinosa, and S.L. Cruz, *Dissociation of immunosuppressive and nociceptive effects of fentanyl, but not morphine, after repeated administration in mice: Fentanyl-induced sensitization to LPS.* Brain, Behavior, and Immunity, 2014. 42(Supplement C): p. 60-64.
218. Alexander, M., et al., *Opiate Analgesics Contribute to the Development of Post-Injury Immunosuppression1.* Journal of Surgical Research, 2005. 129(1): p. 161-168.
219. Martucci, C., A.E. Panerai, and P. Sacerdote, *Chronic fentanyl or buprenorphine infusion in the mouse: similar analgesic profile but different effects on immune responses.* Pain, 2004. 110(1): p. 385-392.
220. Sacerdote, P., *Opioids and the immune system.* Palliat Med, 2006. 20 Suppl 1: p. s9-15.
221. Pergolizzi, J., et al., *Opioids and the Management of Chronic Severe Pain in the Elderly: Consensus Statement of an International Expert Panel with Focus on the Six Clinically Most Often Used World Health Organization step III Opioids (Buprenorphine, Fentanyl, Hydromorphone, Methadone, Morphine, Oxycodone).* Pain Practice, 2018. 8(4): p. 287-313.

222. C., W., et al., *Full-Thickness Thermal Injury Delays Wound Closure in a Murine Model.* http://www.liebertpub.com/wound, 2015.
223. BURKE, J.F., BONDOC, C. C., & QUINBY, W. C., *Primary burn excision and immediate grafting: a method shortening illness.* Journal of Trauma and Acute Care Surgery, 1974. **14**(5): p. 389-395.
224. Barret, J.P., & Herndon, D. N., *Principles and practice of burn surgery.* . 2005: Springer Science & Business.
225. Barret, J.P., & Herndon, D. N., *Modulation of inflammatory and catabolic responses in severely burned children by early burn wound excision in the first 24 hours.* Archives of Surgery, 2003. **138**(2): p. 127-132.
226. Herndon, D.N., et al., *A comparison of conservative versus early excision. Therapies in severely burned patients.* Ann Surg, 1989. **209**(5): p. 547-53.
227. Saaiq, M., S. Zaib, and S. Ahmad, *Early excision and grafting versus delayed excision and grafting of deep thermal burns up to 40% total body surface area: a comparison of outcome.* Ann Burns Fire Disasters, 2012. **25**(3): p. 143-7.
228. Papp, A., et al., *The progression of burn depth in experimental burns: a histological and methodological study.* Burns, 2004. **30**(7): p. 684-690.
229. Fear, V.S., et al., *Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model.* Burns, 2016. **42**(4): p. 815-824.
230. Goronzy, J.J., & Weyand, C. M. , *Understanding immunosenescence to improve responses to vaccines.* Nature Immunology, 2013. **14**(5): p. 428-436.
231. Jackaman, C., Dye, D. E., & Nelson, D. J., *IL-2/CD40-activated macrophages rescue age and tumor-induced T cell dysfunction in elderly mice.* Age, 2014. **36**(3): p. 1315-1328.

Appendix

RESEARCH APPLICATION

OFFICE USE ONLY This proposal is approved for the period: **From:**	**FILE REF: RA/3/100/** **To:**
Signature AEC Chair:	Date:

PROTOCOL DETAILS	
Protocol Title: *(full title)*	Comparison of the effects of burn and non-burn skin trauma on the innate and adaptive immune response using a mouse model
Chief Investigator: (full name)	Mark Fear
School: (Centre/Dept)	Burn Injury Research Unit, School of Surgery
Please indicate if this is an	**INITIAL APPLICATION** ☒ or **RENEWAL** ☐
*If this is a **Renewal** of a previous protocol, please provide a short summary (less than 300 words) which outlines all changes.*	
Application can only be approved for the period of funding and may not exceed 5 years. At the conclusion of the funding period a Renewal application will be required.	
Expected commencement date: 01-10-2012 (dd/mm/yy)	**Completion date**: 31-09-2015 (dd/mm/yy)
Commercial in confidence:	**Yes** ☐ **No** ☒
NHMRC Classification Category (Compulsory): ***http://www.research.uwa.edu.au/staff/forms***	3a, 4a

All research involving the use of animals must comply with the *Animal Welfare Act (2002)* and the requirements of the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th Edition 2004) *'The Code'. Please see*

www.nhmrc.gov.au/health_ethics/animal/issues.htm ***Code of Practice***
www.austlii.edu.au/au/legis/wa/consol_act/awa2002128/ ***Animal Welfare Act* (2002)**

Applications received after 4pm on the submission closing date will be held over for consideration at the next available meeting. For Submission deadlines and AEC meeting dates - *Please refer* www.research.uwa.edu.au/staff/animals/ethics-committee

The Animal Welfare Veterinary Advisors (AWVAs) and the Animal Welfare Officer (AWO) are available for advice on specific aspects of your application.

Animal Welfare Veterinary Advisors - 6488 4549 / 4700 - vets-research@uwa.edu.au *or*
Animal Ethics Office – 6488 7887 / 2516 / 3033 - aeo@uwa.edu.au

This application to be discussed with the Manager Animal Care and Veterinary Services, or Curator for other facilities	**Signature of MACVS/Curator**
Dr Malcolm Lawson, 6488 6616, malcolm.lawson@uwa.edu.au or **facility curator for non ACVS or non UWA sites**	

Please indicate below all documents attached to support this document. Attachments should be clearly named and labelled ie Attachment 1,2, etc.		✓
Permission to Use Animals (PUAs) form for each investigator, *if required.*	**Section 2 & 3**	
Notification Form – Department of Agriculture and Food, Government Western Australia	**Section 10**	
Permits eg Transport / AQIS / Department of Environment and Conservation (DEC) etc (NB: Provide a copy of the application to DEC for permit)	**Section 10C**	
Risk Assessment	**Section 11**	
Genetically Modified Organisms: OGTR assessment / Phenotype Reports	**Section 12B**	
Flow Diagram/s	**Section 13A**	✓
Monitoring Sheets		✓
Glossary of Terms		

1. SPONSORS - SOURCE OF FUND AND STATUS OF OUTCOME

Source of Funding: Fiona Wood Foundation **Approved? Pending** [ ] **Yes** [**X**] **No** [ ]

ID Number (if approved): N/A

OTHER APPROVAL (if applicable)

Does this project involve more than one AEC? **Yes** [] **No** [**X**]

*If **Yes**, an Inter-Institutional Agreement is required. Please arrange this with the Animal Ethics Office, complete the following details and attach a copy of the approved application.*

Name of the Institution and approval number and the Outcome:

Approved [ ] **Not Approved** [ ] **Under consideration** [ ]

PREVIOUS APPROVAL (if applicable)

Protocol title:

AEC Approval no:

Unexpected deaths or adverse events:

Please attach list of publications arising:

2. CHIEF INVESTIGATOR

Title, first name, middle, last name A/Prof Mark Fear		**Staff number (UWA)** 00058472
Qualifications: PhD		
Work mailing address /school (include UWA MBDP): M318, Burn Injury Research Unit		
Email: mark@mccomb.org.au	**Phone**:6488 8133	**Mobile**: 0411 355944

Please detail the relevant experience you have (including the number of years) in the procedures/techniques to be used in this project.

I have previously conducted burn procedures in mice, rat and pigs. I have also previously conducted rodent studies on dermal templates using the excision model that will be the basis of the experiments proposed in this application.

Over the past six years I have been chief investigator on several projects involving burn injury and wound repair, and have experience of monitoring wounded animals, with both burn injury and excisional skin injury. I have been responsible for anesthesia, analgesia, euthanasia and monitoring of animals in these previous projects, which have resulted in published papers (selection of papers involving the rodent model are listed below)

I am currently Chief Investigator on ongoing projects related to burn injury using rodent models (RA3/100/1032, RA3/100/899, RA3/100/951).

1. Morellini NM, **Fear MW**, **Rea S**, West AK, Wood FM, Dunlop SA. Burn injury has a systemic effect on reinnervation of skin and restoration of nociceptive function. Wound Repair Regen. 2012 May-Jun;20(3):367-77.
2. Anderson JR, Zorbas JS, Phillips JK, Harrison JL, Dawson LF, Bolt SE, **Rea SM**, Klatte JE, Paus R, Zhu B, Giles NL, Drummond PD, Wood FM, **Fear MW**. Systemic decreases in cutaneous innervation after burn injury. J Invest Dermatol. 2010 Jul;130(7):1948-51.
3. Morellini NM, Giles NL, **Rea S**, Adcroft KF, Falder S, King CE, Dunlop SA, Beazley LD, West AK, Wood FM, **Fear MW**. Exogenous metallothionein-IIA promotes accelerated healing after a burn wound. Wound Repair Regen. 2008 Sep-Oct;16(5):682-90.

Animal Competency and Experience *AEO checked* ❑

Have you obtained UWA Permission to Use Animals (PUA) within the last 5 years? Yes [X] **No** [ ]

Please note: All personnel named on the protocol must apply for a PUA.

*If **No**,* Please download a PUA form available from our website and attach to this application.
http://www.research.uwa.edu.au/staff/forms

PUA application attached [ ]

3. CO-INVESTIGATOR/S **(copy and paste additional tables as required for each co- investigator)**

Title, first name, middle, last name Professor Suzanne Rea		**Staff /Student number (UWA)**
Qualifications: MBBS, FRACS		
Work mailing address /school(include UWA MBDP): M318, Burn Injury Research Unit		
Email: Suzanne.rea@health.wa.gov.au	**Phone**: 9202 1145	**Mobile**: 0413 827186

What is your Role in this project?

Prof Rea is a Burns consultant with the Burns Service,WA. Prof Rea will conduct and/or monitor the surgical procedures (excisional and burn injury) and also assist with monitoring post-injury.

Please detail the relevant experience you have (including the number of years) in the procedures/techniques to be used in this project.

Prof Rea has worked together with the CI for the last 5 years on burn injury related projects on mouse, rat and pig models of injury. She has previously conducted the excisional injury model together with the CI on this proposal to investigate a novel dermal template for stability in vivo. Prof Rea has also been responsible, together with the CI, for anesthesia and euthanasia using the established protocols described here in the previous research. Prof Rea is a co-author on all papers listed above from recent rodent injury model research projects.

Animal Competency and Experience *AEO checked* ❑

Have you obtained UWA Permission to Use Animals (PUA) within the last 5 years? Yes [X] **No** [ ]

Please note: All personnel named on the protocol must apply for a PUA.

*If **No**,* Please download a PUA form available from our website and attach to this application.
http://www.research.uwa.edu.au/staff/forms

PUA application attached [ ]

3. CO-INVESTIGATOR/S(copy and paste additional tables as required for each co- investigator)

Title, first name, middle, last name Emily O Halloran	**Staff /Student number (UWA)**
Qualifications: MBBS	
Work mailing address /school(include UWA MBDP): M318, Burn Injury Research Unit	

Email: ohalloranemily@gmail.com	**Phone**: 6488 8587	**Mobile**: 0424433839

What is your Role in this project?

Dr O Halloran is currently studying for her Masters of Surgery within our group and has been a key person working on the project RA3/100/1032 (impact of non-severe burn injury on skeletal muscle and role of the inflammatory response). Dr O Halloran is now very competent with mouse handling and monitoring techniques. Dr O Halloran will contribute to supervising Samantha Valvis with monitoring and euthanasia of the animals together with the CI Fear.

Please detail the relevant experience you have (including the number of years) in the procedures/techniques to be used in this project.

Dr O Halloran has completed the PAWES course and spent the past 8 months working on a project involving non-severe burn injury and the impact on skeletal muscle using the mouse animal model that will be used for this project. During this time she has gained considerable experience with the mouse model of skin injury and with monitoring the animals under the supervision of A/Prof Fear and Prof Rea, and she is now very competent with handling and monitoring animals with the types of protocols involved here.

Animal Competency and Experience ***AEO checked*** ❑

Have you obtained UWA Permission to Use Animals (PUA) within the last 5 years? Yes [X] **No** [ ]

Please note: All personnel named on the protocol must apply for a PUA.

If ***No****,* Please download a PUA form available from our website and attach to this application.
http://www.research.uwa.edu.au/staff/forms

NB- PUA will be applied for on arrival and completion of PAWES course PUA application attached [ ]

3. CO-INVESTIGATOR/S (copy and paste additional tables as required for each co-investigator)

Title, first name, middle, last name Samantha Valvis	**Staff /Student number (UWA)**

Qualifications: MBBS

Work mailing address /school(include UWA MBDP): M318, Burn Injury Research Unit

Email: samantha_valvis@hotmail.com	**Phone**: 6488 8597	**Mobile**:

What is your Role in this project?

Dr Valvis is currently a core surgical trainee who will be undertaking a Masters in Surgery as a research project within our team. Dr Valvis will be involved in assisting with the surgical procedures (together with Prof Rea) and in monitoring animals. Dr Valvis will join the group in August 2012 and is registered for the PAWES course. After this she will apply for permission to use animals (PUA). Her role will also be in the analysis of samples collected after euthanasia of the animals.

Please detail the relevant experience you have (including the number of years) in the procedures/techniques to be used in this project.

Dr Valvis has no prior animal experience. However, she will complete the PAWES course and subsequently be supervised closely by CI Fear and CO-I Rea until she is demonstrably competent with monitoring and relevant procedures. The AEC will be notified once the CI deems that Dr Valvis is competent to conduct the experiments with limited supervision. No burn injury or excisional procedures will be carried out by Dr Valvis alone without either Prof Rea or A/Prof Fear assisting and supervising.

Animal Competency and Experience ***AEO checked*** ❑

Have you obtained UWA Permission to Use Animals (PUA) within the last 5 years? **Yes** [] **No** [**X**]

Please note: All personnel named on the protocol must apply for a PUA.

If ***No****,* Please download a PUA form available from our website and attach to this application.
http://www.research.uwa.edu.au/staff/forms

NB- PUA will be applied for on arrival and completion of PAWES course PUA application attached [ ]

4. EMERGENCY CONTACT PERSONNEL - DURING STUDY

Name: Mark Fear, Emily O Halloran, Samantha Valvis (all details on monitoring sheets)

What is their role in this project? Chief Investigator

Email: mark@mccomb.org.au **Phone**: 6488 8133 **Mobile**: 0411 355944

After hours/emergency contact number:0411 355944

5. ANIMAL MONITOR - DURING STUDY - ANAESTHESIA, SURGERY & POST- OP RECOVERY PERIOD

Name: Mark Fear, Emily O Halloran and Samantha Valvis

Details of their relevant experience: A/Prof Fear has 6 years of experience with animal monitoring after skin injury. Dr Valvis will be a new student working on this project and will be closely supervised and assisted with monitoring by A/Prof Fear until deemed competent.

Email: mark@mccomb.org.au **Phone**: 6488 8133 **Mobile**: 0411 355944

After hours/emergency contact number: 0411 355 944

6. PERSONNEL RESPONSIBLE FOR EUTHANASIA

Name: Mark Fear, Emily O Halloran and Samantha Valvis

Details of their relevant experience: As described above, A/Prof Fear has 6 years of experience with skin injury experiments in rodent models and the subsequent euthanasia and tissue collection procedures. Dr Valvis will be a new student working on this project and will be closely supervised and assisted with monitoring by A/Prof Fear and Emily until deemed competent. The animals are euthanased by lethal injection of pentobarbitone (IP) and this has been done for many animals previously (and is current procedure for approved related protocols). A/Prof Fear will be the person responsible for ensuring Samantha is adequately supervised until competent to carry out any procedures.

Email: mark@mccomb.org.au **Phone**: 6488 8133 **Mobile**: 0411 355944

After hours/emergency contact number:0411 355 944

7. REVIEWER – INDEPENDENT EXTERNAL (non UWA staff)
Please note this person may be asked to review aspects of this application

Title and name: Zee Upton

Position /Business address / contact details: Assistant Dean (Research), Head of Tissue Repair and Regeneration Facility, Queensland University of Technology

Email: z.upton@qut.edu.au **Phone**: 07 3138 9639 **Mobile**:

8. OVERVIEW AND GENERAL AIM OF THE PROJECT
Must be written in lay language (i.e. as if it was a press release) and must not exceed one A4 page.

Burn injury causes a large immune response from the body. In particular, there is a lot of inflammation associated with burn injury. However, it is also thought that burn injury can cause suppression of the later immune response (the adaptive immune response), leading to susceptibility to infection.
One key question is whether burn injury causes the same response as any other type of trauma, either to the skin or to other organs (such as in a road accident), or whether burn injury, because of

the type of damage to the skin, actually causes different changes in the body's response to other injuries.
From a clinical perspective, the currently used Injury Severity Score (ISS), which is used for trauma as a tool to help predict outcome, is thought to underestimate the impact of a burn injury. But whether this is because burn has unique impacts with the immune system, or is just due to the scoring system used, is not clear.
It would be very useful for the accurate assessment of burn injury (and therefore to guide treatment options, which is critical to outcome for the patients) to understand whether burn injury causes different responses in the immune system than other injury types, or it causes the same response which is dependent on solely on the extent of injury. It is also necessary to further clarify whether burn injury really does suppress the longer term immune response, and if so how this occurs, as burn patients can often be susceptible to infection and this has significant consequences both for treatment and scar outcomes.
This project will use the mouse model of burn injury, and also excisional injury (surgical skin removal) to look at whether injuries of the same extent BUT DIFFERENT ETIOLOGY (Surgical trauma or burn) to the skin cause the same or different immune responses. We will monitor the short term inflammatory response and also the longer term response (looking at cells at the injury site and in lymph nodes). We will also look at the response to the injury site as well as systemic (serum) changes. This data will be important in our design of studies in patients, as we can tightly control the injury size and monitor the immune response much more closely (for example using lymph node tissue samples as well as injury site tissue samples) using the mouse model than we can in patients.
The outcome of this project will be that we can clearly understand the impact on the immune system of a burn injury compared to other types of trauma. This will be important in guiding clinicians' assessment of burn injury severity and therefore how best to treat them. Also - currently, the use of the ISS score to assess burn injury severity and guide treatment does not appear to work well. This project will help to explain why.
Finally, a clear understanding of the interaction between the immune system and a burn injury will be important in developing new treatments that limit systemic problems associated with burn injury.

9. ANIMAL SUMMARY (add additional lines as required)

	Species scientific & Common name ONE SPECIES PER LINE	Strain	NHMRC Animal Species Code	Dietary Modifications Yes/No	Total number required (over the life of the project)	Vendor / Source (ARC/Jackson labs/ Wandalup/Murdoch)
1.	Mouse	C57BL/6		No	140	ARC
2.						
3.						
4.						

Justification of Number of Animals Requested (ie number in each experimental group)

This project will require 140 animals in total. This will provide n=10 for each group at each time point - excision injury at day 1, day3, day7, day14, day28 and day84 (n=60 total) and burn injury at day 1, day3, day7, day14, day28 and day84 (n=60 total). There will also be a group of 10 baseline controls. Finally, there is an additional 10 animals requested in the ethics to provide for any potential post-euthanasia sample or data loss. The analysis post-euthanasia will involve Flow cytometry, lymph node isolation and cell culture, wound tissue removal and serum collection. This provides a number of steps at which experimental errors may be encountered, so the additional 10 animals will be used if required due to problems with any individual samples.

The numbers requested are based on our own experience of analysing the inflammatory response after skin injury. We have substantial preliminary data that demonstrates with the group size requested differences between the inflammatory response (as assayed by cytokine levels and cell counting) and the adaptive immune response (through lymph node cell isolation and flow cytometry) of clinical significance can be detected. These numbers will provide the ability to detect a difference (at $p<0.05$) in the extent of inflammation (level of response) of 10% between excisional

and burn injury. This is similar for other aspects of the immune response. Overall profiles of cytokines and cellular responses will be detected at much greater levels of sensitivity. **NOTE** – this is a greater level of sensitivity than for some previous experiments as this is to better understand the normal progression of two injury types, and it is expected the differences observed may be smaller than with our studies looking at wound healing intervention.
If any experimental group size is less than 10, have you provided statistical justification or an example of an informative study of this type that used a similarly small sample size?
Not applicable
If your experimental group size varies across groups, have you provided justification for this variation?
Not applicable
Has a biostatistician been consulted? **Yes** [] **No** [X] If ***Yes***, please provide contact details below.
Research Assistant Professor Hilary Wallace provides statistical support within the Burn Injury Research Unit. Dr Wallace has extensive statistical experience and knowledge in experimental design.

DOES THE PROTOCOL INVOLVE ANY OF THE FOLLOWING? **Yes** [ ] **No** [**X**]
If ***Yes***, please complete the details below

	Species	**Strain**	**Total number required**
Creation of hybridoma			
In vivo (ascites) production of monoclonal antibodies			
Genetically modified mice			
Other genetically modified animals			
Transplantation			
• autograft			
• allograft			
• xenograft			
- cells			
- tissue			
- organs			

10. HOUSING AND LOCATION OF ANIMALS (SIGNATURES REQUIRED)

UWA: (ACVS)
- **QEII**: M Block
- **Shenton Park**: BRF, NAF, SRF
- **Crawley Campus:** PCF, LAF

OTHER UWA Facilities:
- **Fremantle Hospital** – Z Block
- **Allandale Farm / Ridgefield Farm**
- **Animal Biology**
- **Royal Perth Hospital**

Facility room number/ zone	**Species** (and/or strain if applicable)	**Gender**	**Age or initial weight**	**Reproductive status**	**Manager UWA (ACVS) Facilities / Curator Other UWA Facilities** **Signature and Date**
PCF	Mouse C57BL/6J	F	9 weeks	Non-pregnant	

* Locations not covered by a scientific licence require a **Notification to the Department of Agriculture and Food**, Government Western Australia. The Proforma to be attached to this application is available from the website at http://www.research.uwa.edu.au/staff/forms/animals

A. WHAT IS THE MAXIMUM LENGTH OF HOLDING IN WEEKS?

13 weeks

B. WHAT ADDITIONAL ENRICHMENT WILL BE PROVIDED FOR ANIMALS WHICH WILL BE HELD LONGER THAN 3 MONTHS?

No animals will be held for LONGER than 3 months. However, 20 animals will be held for 13 weeks (3 months duration). All animals, even those only held for a short duration, will be provided with additional enrichment. Enrichment materials will include soft paper tissue, coarse paper tissue, cotton nestlets, a wood gnawing block and a handful of rodent pellets in the cage for foraging. In addition, cardboard rolls (tunnels) will be provided. These types of enrichment will be alternated and/or mixed over time (changed weekly) to provide a more stimulating environment, which reduces anxiety.

C. PLEASE DETAIL THE METHOD OF TRANSPORT AND ANY ANIMAL WELFARE IMPLICATIONS:

Animals will not be transported after delivery to PCF by ARC. Animals will undergo surgery and euthanasia in PCF.

[ ] **I have attached copies of any necessary transport and acquisition permits/approvals**.

11. SAFETY AND HEALTH RISKS

Does project involve use of:	**Y/N**	**Approval Y/N/ Pending**	**If *YES*, explain risks involved**	**Precautions to protect staff and/or animals**
Teratogens or carcinogens	N			
S7, 8 ,9 drugs *(a copy of the permit will be required)*	Y		S8 drug used is buprenorphine which is used as an analgesic post-injury. The concentrations and quantities required for rodent work provide minimal risk since it is S8 drug due to potential for abuse.	S8 drugs are kept in a safe within PCF surgery and use of burprenorphine monitored and logged in accordance with requirements.
Radioisotopes or x-rays	N			
Other potentially infectious or hazardous (chemical/physical / biological) agents which may pose a health risk to staff or animals	N			

IDENTIFY POTENTIAL RISKS TO STAFF AND HOW THEY WILL BE MANAGED AND MINIMISED

We believe there are minimal risks for staff other than the possibility of bites and/or scratches from animal handling and needle stick injuries that could possibly occur during the administration of analgesic. All staff and students are trained in proper handling of hypodermic needles and animal restraint, and all investigators have extensive experience and have completed the PAWES course (or potential students will undergo PAWES course, training and monitoring as required). If injury occurs, individuals will be directed to obtain a tetanus injection.

[ ] **I have attached a Risk Assessment**

12. GENE TECHNOLOGY / BIOLOGICAL SAFETY

A. PLEASE PROVIDE DETAILS OF ANY BIOLOGICAL SAFETY CONCERNS TO STAFF, AND HOW THEY WILL BE MANAGED

N/A

B. ARE YOU DEALING WITH GENETICALLY MODIFIED ORGANISMS? **Yes** [ ] **No** [N]

If *Yes*, please provide details of phenotypic expression.

[ ] Established Phenotype report attached?

[ ] **I have attached copies of required permits/approvals**.

Dealing Type: **EXEMPT DEALING**[ ] **NLRD** [ ] **DNIR** [ ] **DIR** [ ]

Exempt Dealings - Requires IBC assessment
Notifiable Low Risk Dealings (NLRD) - requires IBC assessment and notification to OGTR
Dealings Not involving an Intentional Release of a GMO into the Environment (DNIR) - Requires IBC Assessment & application to OGTR for a licence.
Dealings involving an Intentional Release of a GMO into the Environment (DIR) - Requires IBC Assessment and application to OGTR for a licence.

IBC Approval Number: **Approval Status:** **Pending** [ ] **Yes** [ ] **No** [ ]
***(if approved*)**

IF USING GMOS, HAVE ALL INVESTIGATORS UNDERTAKEN THE (COMPULSORY) GENE TECHNOLOGY REGULATION TRAINING COURSE? **Enrolled [ ] Yes [ ] NO [ ]**

If *NO,* or Enrolled, please specify which investigator/s have not completed the course:

[ ]**AQIS Permit is attached (please supply 1 photocopy)**
[ ] **I have attached copies of all required permit/approvals**.

13. EXPERIMENTAL PROPOSAL – Cost Benefit Analysis

A. FLOW DIAGRAM OUTLINING THE PROJECT AND THE PROCEDURES – Can be attached as separate sheet/s. Must include a time line and list of procedures to clearly illustrate what will happen to each group of animals from acquisition to disposal.

Day -7 – animals arrive at PCF (n=140)
7 days acclimatization

Day 0 – Control animals are euthanized (n=10) – normal skin, serum and inguinal lymph nodes collected for analysis

Day 0 – 60 animals receive 19 mm diameter full-thickness skin excision under anesthesia. Wound is dressed using 3M Tegaderm absorbent clear acrylic dressing, a transparent sterile dressing commonly used for burn and surgical wound covering in patients. Animals are allowed to recover with buprenorphine analgesia. Paracetamol added to drinking water for 5 days post-injury

Day 0 - 60 animals receive 19 mm diameter full-thickness burn injury under anesthesia. Wound is dressed using 3M Tegaderm absorbent clear acrylic dressing, a transparent sterile dressing commonly used for burn and surgical wound covering in patients. Animals are allowed to recover with buprenorphine analgesia. Paracetamol added to drinking water for 5 days post-injury

Full thickness excisional injury is created using a skin punch biopsy to mark the skin for excision and then the skin is removed using surgical scissors by a plastic surgeon (Prof Rea). This creates an accurate reproducible injury size.

The full-thickness burn injury is created using a brass rod of 19mm diameter heated to 95 C and applied to the skin for 10 seconds (as per the current approved protocol for RA3/100/899, RA3/100/1032 and RA3/100/951).

Day 1 – 10 excision and 10 burn injured animals euthanized (n=20 total). Serum, wound and normal skin samples and inguinal lymph nodes collected for analysis

Day 3 – 10 excision and 10 burn injured animals euthanized (n=20 total). Serum, wound and normal skin samples and inguinal lymph nodes collected for analysis

Day 7 – 10 excision and 10 burn injured animals euthanized (n=20 total). Serum, wound and normal skin samples and inguinal lymph nodes collected for analysis

Day 14 – 10 excision and 10 burn injured animals euthanized (n=20 total). Serum, wound and normal skin samples and inguinal lymph nodes collected for analysis

Day 28 - 10 excision and 10 burn injured animals euthanized (n=20 total). Serum, wound and normal skin samples and inguinal lymph nodes collected for analysis

Day 84 - 10 excision and 10 burn injured animals euthanized (n=20 total). Serum, wound and normal skin samples and inguinal lymph nodes collected for analysis

[ ] **Attached is a separate flow diagram**

B. THE "COST" OF THE RESEARCH TO THE ANIMALS – Please describe any potential harms, pain, distress, that may arise from the procedures being performed.

NOTE: IF YOU STATE THAT THERE IS NO LIKELY WLEFARE IMPACT, THIS MUST BE FULLY SUPPORTED BY APPROPRIATE EVIDENCE.

If using genetically modified animals or mutant, please attach phenotype reports.

As the animal is under general anaesthetic, it will not experience any pain during the surgical excisional or burn injury procedure. Whilst under anesthesia, the animals receive buprenorphine analgesic, which will minimise any pain on recovery. The 19mm diameter wound is full-thickness, and has the potential to cause pain to the animals. However, full-thickness injuries are known to be much less painful than partial thickness injuries in patients (a key sign of a full-thickness injury is the absence of reported pain). The use of buprenorphine analgesia at time of injury and 12 hours post-injury, together with paracetamol in the water, has been adequate to prevent any observable signs of pain in the animals. The excision procedure (of similar size) in humans is conducted under local anesthetic and no additional pain relief is required. The use of full thickness excision, as with the use of a full-thickness burn model, is to minimize pain and distress to the animals by creating a wound that has minimal active innervation and nociception. The wound surface area is 3 cm^2 in total area, which equates in the age and strain of mice being used (9 week old female C57BL6/J weighing 20-22g) to approximately 7% of the total body surface area.

All animals will have a dressing placed over the wound and this will be attached using tissue glue. The tissue glue will be used to adhere the dressing more permanently, so the dressing will cover the wound and overlap normal tissue, which is where glue will be applied and dressing adhered to the animal. The wounds will be on the back of the mouse away from the limbs, and should not impede movement or cause discomfort as animals move.

After the biopsy is taken, animals are provided with paracetamol in drinking water as an additional precaution against pain. This is unlikely to be required in the case of excisional injury, but will be provided for all animals.

At time of analysis, animals will be anesthetised and euthanized, and therefore the ultimate cost to the animals is death. No additional pain will be experienced by the animals as all analysis will be done on euthanized animals.

Animals will be extensively monitored for any sign of stress indicated by lack of grooming, eating and drinking, general alertness, responsiveness and abnormal behaviour. Signs of skin infection will also be monitored.

C. POTENTIAL "BENEFITS" OF THE RESEARCH – to humans, animals, or the environment.

The potential benefits of this research are primarily for people that suffer skin injury, either through burn or other injury, and in particular those patients that do not heal well and therefore often need surgical procedures to try to improve the scar outcome.

This work will add to our understanding of how the body's immune system responds to burn injury and other skin trauma and how this differs between the two injury types. This will provide information on how best to change or moderate the immune response in these different injury types, and improve our understanding of the interaction between skin trauma and the individual. This information will be useful to design patient centred studies in the short term, and also in the design of

new tools to predict the severity of a burn injury similar to the widely used Injury Severity Score (ISS), which does not seem to work well for burn injury.

14. ADDRESSING THE THREE R'S – ALL 3 SECTIONS MUST BE COMPLETED.
If Genetically modified animals are to be used also provide justification for the strain

A. REPLACEMENT – describe the alternatives to animal use that you have considered and/or adopted. **See the Australian Code of Practice Section 1.8**

The healing of injuries involves a cascade of complex processes leading to the formation of new tissue. *In vitro* models are useful for isolating specific events or mechanisms of the process of wound healing, but fall short in modeling the complex in vivo situation during wound healing, where inflammation, nerve regrowth, revascularization and many other processes are all intertwined to repair the damage. Since this project is aimed at understanding the effect of skin trauma on the immune response, the animal model is necessary so that all the different factors which underlie changes associated with wound healing are active. In particular, to examine systemic and localized inflammation to the injury site requires the use of an animal model.

B. REDUCTION – describe the ways that you propose to minimise the use of animals.
See the Australian Code of Practice Section 1.9 – 1.13

This study will analyse changes in the lymph nodes (inguinal, as the most proximal to the injury site), the wound site, normal uninjured skin and in the circulation (serum sampling). By conducting all these different analyses on each animal we can minimize the numbers required and also increase the power of the experiment by having matched data of all aspects of the immune response from each individual animal. Because this increases the complexity of the post-euthanasia experimental procedures, an additional 10 mice are requested to provide for potential sample loss or experimental problems. However, the intensive analysis means we do not need separate groups of animals to assess the cellular response, or the serum cytokines, or the wound changes, and so by analysing all aspects in each animal we substantially reduce the animal numbers required.

The group numbers have been chosen to provide significance at a level that we believe is clinically relevant, rather than large group numbers that will identify even very small changes in the immune response which would not be important in terms of clinical and translational impact.

Therefore the numbers have been reduced to the minimum required to progress this research and answer the proposed questions.

C. REFINEMENT – **Also complete the tables at Section 15 & 16.**
Step-by-step description of procedures. What will happen to animals and their tissues identifying the actions taken at each step to minimise suffering and distress
See the Australia Code of Practice Section 1.14 – 1.28.

Animal Welfare and Veterinary Advisors will be invited to observe initial surgeries and recovery of animals. The Animal Welfare Officer (AWO) and veterinary advisors have attended previous experiments involving both excisional injury (RA3/100/1067) and the burn injury (RA3/100/1032). As part of this process, the analgesia was changed to include a second burprenorphine injection at 12 hours post-injury, as some animals exhibited signs of distress within this time period. Subsequently, with the addition of the 12 hour buprenorphine injection, recovery seems better and continued monitoring suggests this is a good analgesic regime for the injury types included here.

As part of our refinements and in consultation with the AWO we have also investigated the use of carprofen, but this did not provide good analgesia and the optimal protocol is as included here. We also cannot use carprofen in the types of experiment detailed here as we are investigating the immune response which will be altered by the use of NSAID type analgesia.

The technician in charge will also be requested to provide observations on animal behaviour and responses to treatment. Continual monitoring will allow refinements for this type of injury to be proposed and developed as part of a continual process to improve the research outcomes and the animal welfare in our experiments.

The use of a transparent wound dressing allows close monitoring of the wound and potential infection status without interference in the wound healing process or unnecessary dressing changes. This reduces the impact on the animals of unnecessary handling.

15. NON SURGICAL PROCEDURES

FULL DESCRIPTION OF ALL NON SURGICAL PROCEDURES. ***Please also include details in the table below.***
If substances are being administered to animals please include details of route, volumes, frequency, intervals and duration.

Two non-surgical procedures are used – intramuscular injection of buprenorphine (for analgesia) and intraperitoneal injection of pentobarbitone for euthanasia.

1. Buprenorphine injection-intramuscular injection using insulin syringe in the shoulder. Initial injection is given while animal is under anesthesia and therefore has no impact on the animal at the time.
2. Second injection of burprenorphine at 12 hours post-injury animal is not under anesthesia and therefore animal is restrained briefly for im injection.
3. IP injection of pentobarbitone requires restraint.

All investigators have experience of animal handling and restraint for these injections (Samantha Valvis will be supervised and trained).

Note: Only the title of the procedure should be listed in column one of table. Full details of the procedures must be completed in the relevant space below each table.

Type of non surgical procedure to be carried out	**Expected impacts of the procedure**	**Expected frequency of adverse impacts**	**Refinement taken to minimise impacts**
e.g. gavage	*Minor discomfort rarely substance enters airway or oesophagus is damaged*	*Some discomfort on each occasion. Substance in airway or oesophageal damage in less than 1 in 1000 administrations.*	*Good handling to minimise discomfort and observation after dosing with humane killing of any animal showing signs of mis-dosing or damage.*
Analgesia – injection of buprenorphine im at time of injury	None – animals under anesthesia at time of injection		
Analgesia – injection of buprenorphine im 12 hours post-injury	Minor discomfort from	always	
Euthanasia – ip injection of pentobarbitone	Minor discomfort from handling of animals and injection (and ultimately death of animal)	always	

Which investigator as detailed in Section 3 or 4 will perform these procedures?

A/Prof Mark Fear, Prof Suzanne Rea, Emily O Halloran and Samantha Valvis will conduct analgesia injections. Only A/Prof Fear, Emily and Samantha will conduct IP injections for euthanasia.

16. SURGICAL PROCEDURES

A. FULL DESCRIPTION OF ALL SURGICAL PROCEDURES. ***Please also include details in the tables below.***

Methods

Full thickness surgical excisional wound injury

Mice aged 9 weeks will be obtained and given 7 days to acclimatise to handling and the PCF environment. On the day of surgery, they will be given a general anaesthesia using isoflurane administered by anaesthetic chamber and then maintained by face-mask. The surgery will be conducted in the small animal surgery room in PCF zone B. Prior to surgery, the surgeon will be wearing sterile gloves for the procedure duration. The equipment used is all single use sterile equipment except for clippers used to shave the animal which are washed in 80% Ethanol prior to use.

Whilst under anaesthesia, the mouse will have the area to be wounded shaved and cleaned with Betadine. A sterile single use 19mm punch biopsy will be used to create an incision on the back of the mouse. The outline will then be removed by the surgeon using surgical scissors. The wound will

be on the back, away from all limbs, and on one side of the animals away from the spine. The wound will be covered with Tegaderm using tissue adhesive to maintain in place. All animals will receive analgesia (buprenorphine 0.1 mg/kg intramuscular) immediately prior to surgery whilst under anesthesia with an additional injection of buprenorphine (0.1 mg/kg intramuscular) at 12 hours post-surgery, with oral paracetamol for the 5-day post-surgery period (in drinking water 1mg/ml).

Full thickness burn injury

Mice aged 9 weeks will be obtained and given 3 days to acclimatise to handling and the PCF environment. On the day of surgery, they will be given a general anaesthesia using isoflurane administered by anaesthetic chamber and then maintained by face-mask.
Whilst under anesthetic, the mouse will have the area to be wounded shaved and cleaned with betadine. A 19mm diameter full-thickness burn injury will be created by an experienced burns surgeon (Prof Rea), using a brass rod heated to 95C in contact for 10 seconds. The wound will be on the back, away from all limbs, and on one side of the animals away from the spine. The wound will be covered with Tegaderm using tissue adhesive to maintain in place. Animals will receive analgesia (buprenorphine 0.1 mg/kg intramuscular) immediately prior to burn injury whilst under anesthesia, with an additional injection of buprenorphine (0.1 mg/kg intramuscular) at 12 hours post-injury, with oral paracetamol for the 5-day post-surgery period (in drinking water 1mg/ml).

ALL PROCEDURES

Animals are recovered in the surgery in individual cages and are monitored until they are fully recovered from the anaesthesia. The procedure and duration of anaesthesia is less than 10 minutes in total, and the animals do not require a heatpad for surgery or warm recovery box, as recovery is very quick and normal ambient temperature is sufficient.
Once recovered animals will be allowed feed and water ad libitum. At days 1, 3, 7, 14, 28 and 84 post-surgery, animals will be euthanased and serum and tissue samples will be obtained.

Analgesia protocol

As stated above, we intend to use buprenorphine initially followed by oral paracetamol. Because the inflammatory response is critical to this investigation, we cannot use an anti-inflammatory type analgesic such as carprofen without substantially influencing the experimental results (we have also previously investigated the use of carprofen and this was not beneficial). From our experience, and currently approved protocols, we are confident the current analgesia provides good pain relief and minimizes discomfort for the animals.

Monitoring

Animals will be closely monitored (see attached sheets), and any animals that show signs of distress or infection will be euthanized. It should be noted that TEGADERM (the dressing to be used) is a transparent dressing that allows observation of the wound, and therefore monitoring for changes in colour or wound appearance to assess presence of localised infection is manageable.
No prophylactic antibiotics will be used (as is standard clinical practice for burn patients), and they will not be used in the case of infection (which is an unlikely event not previously seen in our animal work) as the infection would have too large an impact on healing and inflammation and therefore impact on the data. However, as infection is an adverse event, the AWO and ACVS staff will be notified if infection does occur and the animal welfare implications and continuation of the procedures will be discussed.

Type of <u>surgical</u> procedure to be carried out	**Expected impacts of the procedure**	**Expected frequency of adverse impacts**	**Refinement taken to minimise impacts**
e.g. insertion of catheter	*Pain*	*Always*	*Analgesia*
Induction of anesthesia	Minor discomfort	Always	Optimised protocol
Excisional injury	Post-procedural pain	always	Appropriate analgesia
Burn Injury	Post-procedural pain	always	Appropriate analgesia
Serum and tissue analysis	Euthanasia	always	Careful handling and optimised protocol
Which investigator as detailed in Section 3 or 4 will perform these procedures?			

Prof Rea will perform surgical procedures listed above except for euthanasia will be A/Prof Mark Fear and Samantha Valvis

B. ANAESTHETICS AND NEUROMUSCULAR BLOCKADE

Species	Agent (s)	Dose	Route	Frequency	Duration
e.g. Rat		*80 mg/kg*	*Ip/ im*	*Once only*	*Single injection is sufficient for the 10 minute procedure*
Mouse	Isoflurane	By induction	Anesthetic chamber and mask	Once only	Maintained with face mask for duration of procedure

Which investigator as detailed in Section 3 or 4 will perform these procedures?

Prof Rea and A/Prof Fear will induce anesthesia.

C. ANALGESICS - Analgesics to be used intra- and post-operatively

Species	Agent	Dose	Route	Frequency & Duration
e.g. Mouse	*buprenorphine*	*0.05-0.1mg/kg*	*Sub cut*	*Initial dose given at induction of anaesthesia then continued every 8 hours for 3 days post-op*
Mouse	Burprenorphine	0.1mg/kg	intramuscular	Initial dose at induction of anesthesia. Additional dose at 12 hours post anesthesia.
Mouse	Paracetamol	1mg/ml	Drinking water	Drinking water contains 1mg/ml for 5 days post-surgery. Consumption per mouse approx. 20ml/day.

Which investigator as detailed in Section 3 or 4 will perform these procedures?

Prof Rea will administer buprenorphine at time of surgery. At 12 hours post-surgery, administration will be by A/Prof Fear and/or Samantha Valvis (once trained and competent).

17. ANIMAL WELL-BEING

A. POST-PROCEDURAL PAIN AND DISTRESS – How will pain and distress be monitored, scored and treated.

All mice will be monitored continuously during anaesthesia and until fully recovered from anaesthesia. To date, no mice have shown signs of distress in any experiments. Mice will be monitored for their behaviour, grooming and lack of activity. In previous experiments with the burn injury and excisional injury (eg RA3/100/899, or RA3/100/1067), mice have recovered and exhibit normal behaviour within minutes of recovery from the anaesthesia. No adverse events or unplanned euthanasia has been recorded as a result of the burn injury or excisional procedure to date.

B. MONITORING SCHEDULE – provide a "Post-Procedural Monitoring Sheet" and /or "Long Term Monitoring Sheet"

Monitoring sheets are attached.

For the acute post-injury phase (5 days), animals are monitored twice daily, including weight of the animals and close monitoring of the wound site for signs of infection as well as hydration. After 5 days post-injury, the wound healing is progressing and animals are monitored once per day until 14 days post-injury, when wound will be completed healed and animal recovered from the surgery/injury procedure. For the period 5-14 days, wound site is still monitored as well as animal behaviour, but weight and hydration are no longer required as the animals are substantially recovered at this point.

C. CRITERIA FOR EUTHANASIA - How will animals be assessed for euthanasia.

If animals display a lack of activity or abnormal behaviour and signs of distress after the injury they will be euthanased.

D. WHAT % OF ANIMALS DO YOU EXPECT TO DIE OR REQUIRE INTERVENTION EUTHANASIA DURING THIS PROJECT? PLEASE EXPLAIN LIKELY REASONS FOR THE ANTICIPATED LOSS RATE.

We expect that there will be less than 1% of animals requiring intervention euthanasia for this project. The anesthetic and surgical procedure does carry a level of risk, and this cannot be completely eliminated, but the protocols are well established and optimized and the team

involved very experienced, and we expect this to minimize any potential interventional euthanasia.

Potential cause of death or euthanasia	Impact on welfare	Steps taken to minimise impact	Percentage of animals affected
e.g. vessel rupture	*irreversible haemorrhage*	*haemorrhage apparent to surgeon, animal would be euthanased whilst still under general anaesthesia*	*<1%*
Anesthesia	Death of animal under anesthesia – no additional impact	Anesthesia protocol is optimised to minimize possibility of adverse reaction.	<1%

PLEASE NOTE THAT ALL UNEXPECTED /UNPLANNED DEATH/S MUST BE REPORTED PROMPTLY TO THE AEC.

18. COMPLETION OF EXPERIMENT – Fate of the animals at the end of the experiment?

Researchers are expected to share animal tissue where possible via Ethitex: **www.ethitex.com.au**
To register please contact the Animal Ethics Office at aeo@admin.uwa.edu.au

A. WHAT ARRANGEMENTS HAVE YOU USED TO SHARE TISSUE?

Collaborators are made aware of any ongoing animal projects that may provide material for use after euthanasia for other projects, for example as a source of primary cells for culture.

B. ARE ALL THE ANIMALS EUTHANASED AT THE END OF THE EXPERIMENT? **Yes [X] No [ ]**

If *No*, What is the fate of non-euthanased animals?

If *Yes*, please complete the table below.

METHOD OF EUTHANASIA – provide details of the generic constituents (not the trade name), the dose rate as mg/kg, and the route of administration.

Species	Agent	Dose	Route
e.g. Rat	*Pentobarbitone*	*>160mg/kg*	*Intraperitoneal injection*
Mouse	Pentobarbitone	>160mg/kg	Intraperitoneal injection

C. DETAIL HOW DEATH WILL BE CONFIRMED:

Lack of respiration for 2 minutes, permanent cessation of heartbeat, lack of reflexes

D. METHOD OF DECONTAMINATION /DISPOSAL OF GMOS.

N/A

E. METHOD AND DETAILS OF CARCASS DISPOSAL

Incineration

19. DECLARATION

I/we, the undersigned:

(i) have read and agree to abide by the conditions and constraints of the WA Animal Welfare Act 2002 and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004);

(ii) understand any non-compliance with the Code of Practice must be reported immediately to the AEC and may result in the withdrawal of project approval and possible disciplinary action;

(iii) understand that in keeping with AEC and Animal Facility policy, all animals are to be monitored as detailed in the application. The Animal Welfare Officer (AWO) has the authority to euthanase distressed animals. Every attempt will be made to inform the CI before any action is taken;

(iv) understand It is the responsibility of the CI to maintain animal records annually to the AEC on animal usage;

(v) understand that in the event of an animal death, or an unplanned euthanasia, we will immediately report the death to the AEO, complete a Notification of UNEXPECTED DEATH FORM and email to AEO within 48 hours, and arrange for an autopsy to be carried out and the results of the autopsy report to be sent to the AEO;

(vi) will ensure that the qualifications and/or experience of all listed personnel are appropriate to the procedures to be performed;

(vii) certify that the resources in the school or department, including housing and personnel, are appropriate for the welfare of the animals and the satisfactory completion of the project;

(viii) will maintain in strict confidence all information contained in this application and acknowledge that the information is both true and accurate.

[X] **I agree to all of the above**

CHIEF INVESTIGATOR - It is the responsibility of the CI to obtain all required signature/s on the application form

PRINT NAME MARK FEAR SIGNATURE DATE 20-07-2012

CO-INVESTIGATOR

PRINT NAME SUZANNE REA SIGNATURE DATE 20-07-2012

CO-INVESTIGATOR

PRINT NAME EMILY O HALLORAN SIGNATURE E O'Halloran DATE 20-07-2012

CO-INVESTIGATOR

PRINT NAME SAMANTHA VALVIS SIGNATURE DATE 20-07-2012

As Head of the School (or Acting), I acknowledge that I have reviewed this application and I confirm that sufficient financial and other resources are available to enable the research to occur in compliance with the Regulations above.

I understand that when I am named on an application as an investigator, the Dean of the Faculty will be required to sign below.

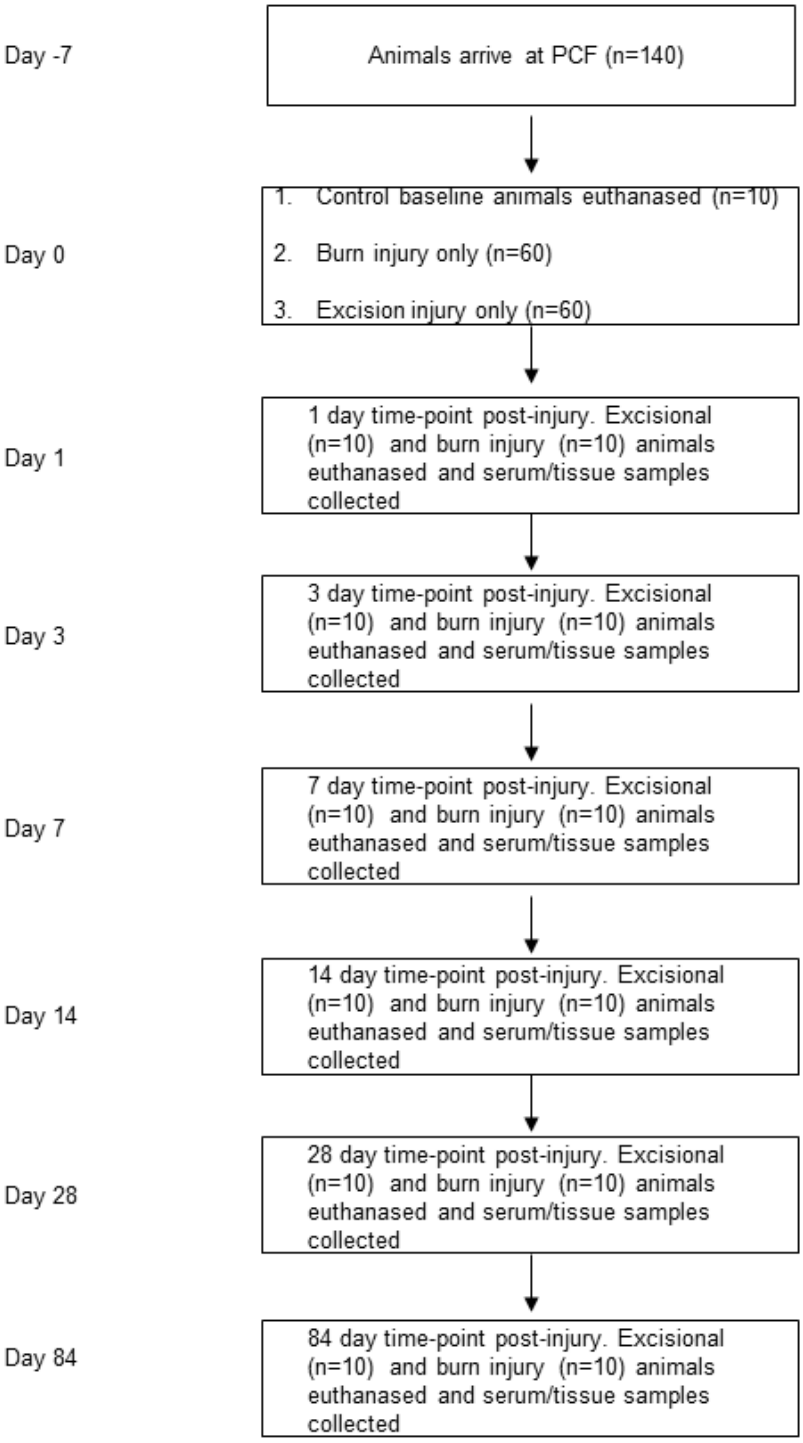
[ ] A review of this application has taken place within the school / faculty.

HEAD OF SCHOOL/(*DEAN OF FACULTY) **Acting [ ]**

PRINT NAME JEFF HAMDORF SIGNATURE DATE 2/8/12

CRICOS Provider Code: 00126G Updated May 2012

Comparison of the effects of burn and non-burn skin trauma on the innate and adaptive immune response



MONITORING SCHEDULE FOR MICE POST BURN AND/OR EXCISIONAL INJURY (up to 5 days)

PROTOCOL NUMBER:

CHIEF INVESTIGATOR: **MARK FEAR**

EMERGENCY CONTACTS:

Mark Fear	**0411 355 944**
Emily O Halloran	**0424 433 839**
Samantha Valvis	**TBA**
AWO	**0428 021 529**

Objective: *To monitor for signs of discomfort or infection post-injury*

Species Mouse (C57BL/6)
Procedure Burn Injury

DATE										
TIME	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
Observations (Yes or No)	YES	YES	YES	YES	YES	YES	YES	YES	YES	YES
Weight (actual, % loss)										
Active?										
Drinking/hydrated										
Normal gait/posture?										
Vocalization?										
Wound edges red?										
Swelling around injury site										
Exudate from injury site										
Pain score (1-4)										
Grooming										
Facial grimace										
Social behaviour										
OTHER (detail)										
Observers initials										

A score of 1 will result in additional monitoring of the affected animal to 3 times per day until score returns to 0 or increases (for euthanasia)

A score of ≥ 2 will result in euthanasia

PAIN ASSESSMENT SCORING

0 = normal behaviour and physiology
1 = mild behaviour and physiological changes (decreased food/water consumption, slightly depressed, minor guarding of incision site)
2 = moderate pain (includes observation in group 1 plus swelling/redness/discharge at surgical site, reluctance to move, guarding with vocalization or aggression

WEIGHT ASSESSMENT SCORING

0 = Less than 5% weight loss
1 = Between 5%-10% weight loss
2 = >10% weight loss

HYDRATION ASSESSMENT SCORING

0= Skin instantly returns to normal after being pinched
1= Skin slightly delayed in returning to normal
2= Skin remains bunched after pinching and does not return to normal

Water will be changed to normal water if score of 1.

Signs of Infection - Exudate/swelling

0 = no exudate/swelling
1 = small amount of exudate/swelling infection unlikely
2 = moderate exudate/swelling infection possible

MONITORING SCHEDULE FOR MICE POST BURN AND/OR EXCISIONAL INJURY (6 to 14 days post-intervention)

PROTOCOL NUMBER:

CHIEF INVESTIGATOR: **MARK FEAR**

EMERGENCY CONTACTS:

Mark Fear	**0411 355 944**
Emily O Halloran	**0424 433 839**
Samantha Valvis	**TBA**
AWO	**0428 021 529**

Objective: *To monitor for signs of discomfort or infection post-injury*

Species Mouse (C57BL/6)
Procedure Burn Injury

DATE									
TIME	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Observations (Yes or No)	YES	YES	YES	YES	YES	YES	YES	YES	YES
Active?									
Normal gait/posture?									
Vocalization?									
Wound edges red?									
Swelling around injury site									
Exudate from injury site									
Pain score (1-4)									
Grooming									
Facial grimace									
Social behaviour									
OTHER (detail)									
Observers initials									

A score of 1 will result in additional monitoring of the affected animal to 3 times per day until score returns to 0 or increases (for euthanasia)

A score of ≥ 2 will result in euthanasia

THE UNIVERSITY OF WESTERN AUSTRALIA
Achieving International Excellence

Research Ethics and Biosafety Office
Research Services
Phone: +61 8 6488 2516 / 3033 / 7887
Fax: +61 8 6488 8775
email: aeo@uwa.edu.au
MBDP:M459

Our Ref: RA/3/100/1155

25 September 2012

Dr Mark Fear
Surgery (School of)
MBDP: M318

Dear Doctor Fear

ANIMAL ETHICS RESEARCH APPLICATION - APPROVED

Comparison of the effects of burn and non-burn skin trauma on the innate and adaptive immune response using a mouse model

On behalf of the Animal Ethics Committee (AEC), I am pleased to advise you that your modified application for the above research project has been approved from **01 October 2012** to **30 September 2015**.

In approving this protocol, the AEC would like you to note the following items and conditions of approval:

- Your adherence to all provisions contained within the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th edition, 2004) '*the Code*'.
- Submission of a satisfactory Annual Report and a State Government Animal Use Statistical Return to the AEC for each calender year (due each January).
- Compliance with the protocol including adhering to the specified procedural techniques and monitoring regimes.
- All unexpected deaths and unplanned euthanasia **must be** reported immediately to the Animal Ethics Office with a completed 'Notification of Animal Death' form, available from http://www.research.uwa.edu.au/staff/forms. Please note that this also refers to all animal deaths in field research projects and wildlife projects.

The AEC would like to remind all investigators using animals that while animal technicians are responsible for the daily care of animals, and play a major role in alerting investigators should an emergency arise, the ultimate responsibility for animal welfare (including monitoring requirements) lies with the investigator. Investigators must maintain their own animal records in order to comply with the *Animal Welfare Act 2002 (WA)* and the *Code of Practice*, to enable accurate completion of the animal usage to be reported to the AEC.

It is a requirement of *the Code* § 3.3.24 that when an animal dies unexpectedly, or is euthanased due to unforseen complications, an autopsy should be performed by a person with appropriate qualifications and/or experience.

If you have any concerns with regard to animal wellbeing or animal welfare, one of the following persons must be notified immediately:

Animal Welfare Officer - awo@uwa.edu.au; T: (08) 6488 7882 M: 042 802 1529
Animal Welfare and Veterinary Advisor - Vets-research@uwa.edu.au; T: (08) 6488 4549 / 4700

Page 2.../

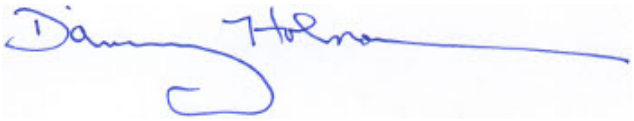
Doctor Mark Fear

25 September 2012

RA/3/100/1155: Comparison of the effects of burn and non-burn skin trauma on the innate and adaptive immune response using a mouse model

If you have any queries, please do not hesitate to contact the Animal Ethics Office as listed above.

Yours sincerely



Professor D'Arcy Holman
Chair, Animal Ethics Committee

Animal species and quantities approved for the above protocol from 01 October 2012 to 30 September 2015

.

Species Name	Approved
C57BL/6 {220}	140

Gross Anatomical Report

Date	**20/2/2013**	**Facility**	PCF	**Zone**	B
Animal found dead or culled (method of euthanasia if applicable)			Found Dead		
Species and strain			C57bl/6j		
AEC#	100/1155	**CI**	**Fear**		
Gender	**Female**	**DOB**	**10wks**	**Genotype (e.g. +ve)**	
ID/Pen #	Tail Markings (V small white tip on end of tail)	**Parent ID # or Animal Facility Source**	**ARC**		
Biological Risk (compulsory)	None	**OGTR status** and **IBC #**	**N/A**		

History – phenotype – treatments
Arrived in PCF on the 5/2/13 housed with 4 other mice. All had burns surgery 7% to dorsum on 12/2/2013 all recovered well gaining weight were bright and alert, wounds healing well. Cage was changed on the 19/2/2013 and all were fine. On 20/2/2013 one of the mice was found dead in cage, there was quite a bit of blood in cage so initially thought fighting was the cause but there are no obvious injuries on mouse other than the burns site. All other mice are fine.

External examination:
Weight: 16.49 grams
2cm x 1cm full thickness wound on the dorsal lumbosacral region. See image below.

Internal Examination: Subcutis had a green discolouration and organs appeared to be liquefying,
Final Comment: Inconclusive. Autolysis.

Lauren Callahan
ACS Veterinary Officer
Animal Care Services
Biomedical Researcher Facility

School of Veterinary & Biomedical Sciences
South St, Murdoch
Western Australia 6150
Telephone: (61-8) 9360 2356
Facsimile: (61-8) 9310 4144
Duty Pathologist: 04202 77743

MURDOCH UNIVERSITY
PERTH, WESTERN AUSTRALIA

ANATOMIC PATHOLOGY NECROPSY REPORT
FINAL

Pathology No: 13/223
Date In: 17/4/2013
Pathologist: Dr Ziyuan Lim and Dr Nahiid Stephens

Owner's Details:	***Consulting Veterinarian:*** *Dr Marilyn Davies*
Marilyn Davies **University Of Western Australia** **Animal Care Unit** **35 Stirling Highway** **Nedlands WA 6009** **Ph: 6488 6661** **Fax: 6488 6677** **marilyn.davies@uwa.edu.au**	***Date of Consult: 17/4/2013***

Patient's Details:	
Clinic Number:	***Name: Anatomic Pathology***
Species Breed: Mouse	***Strain: C57BL/6J***
Gender: Female	***D.O.B: 8 weeks on 8-03-13***
Current Age: 12 – 13 weeks	

History:
AEC # RA/3/100/1155

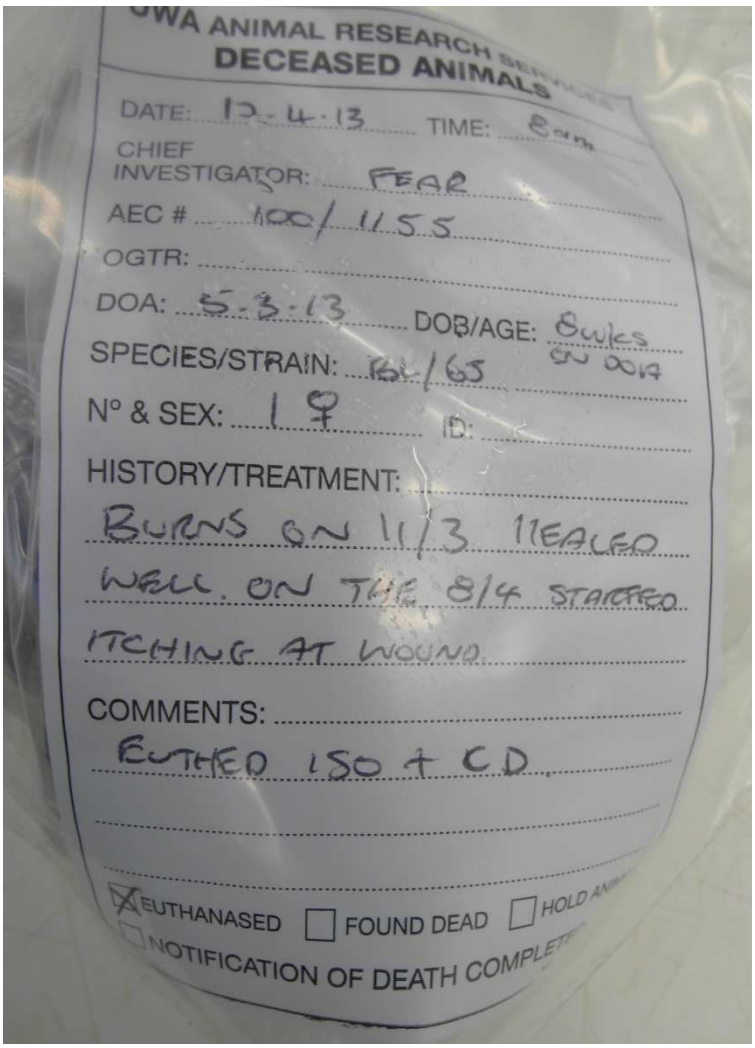
Underwent burn surgery on 11-03-13 and mouse recovered well. Burn injury appeared to be healing well up until the final 3 x 3mm area observed on 8-04-13 when barbering appeared on the mouse. She was singularly housed at this time. Further observations over the next week still showed excessive grooming and irritation of the hind area.

Suspect ulcerative dermatitis.
Request culture of the wound.

Note: This is an OGTR Facility. Dispose of carcass and tissue by incineration

Submission: 20.3g black mouse submitted in a transparent plastic bag contained within another transparent ziplock bag [Fig. 1 & 2].
Status: Post euthanasia.
Post mortem interval: Unknown; examined at 16.30pm on the 17/4/13.
Post mortem decomposition: Minimal.
Identifying features: Inner plastic bag had a white adhesive label [Fig. 1 & 3].

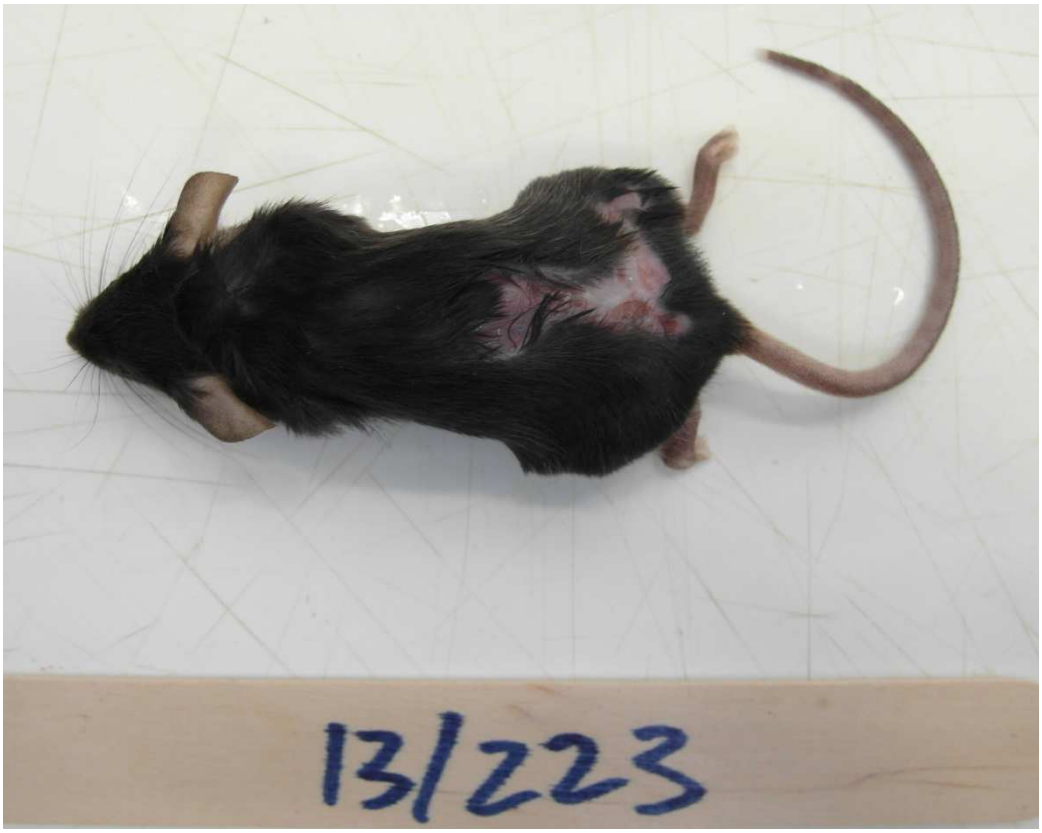
[Figure 1 & 2: Submission; showing the front (left) and back (right) of the bag]



[Figure 3: Close up of tag]

□ **External examination**
Not examined/ No visible lesions/ **See Below**
□ **Skin and subcutis**
Not examined/ No visible lesions/ **See below**
□ **Body cavities**
Not examined/ No visible lesions/ **See below**
□ **Respiratory system**
Not examined/ No visible lesions/ **See below**
□ **Cardiovascular system**
Not examined/ **No visible lesions**/ See below
□ **Alimentary system**
Not examined/ **No visible lesions**/ See below
□ **Lymphoreticular system**
Not examined/ **No visible lesions**/ See below
□ **Urogenital system**
Not examined/ **No visible lesions**/ See below
□ **Endocrine system**
Not examined/ **No visible lesions**/ See below
□ **Musculoskeletal system**
Not examined/ **No visible lesions**/ See below
□ **Nervous system**
Not examined/ **No visible lesions**/ See below

Visible lesions:
Significant External Findings
External examination, skin and Subcutis:
An irregularly-shaped roughly 30 X 20 mm alopecic area was present on the dorsal caudal back; within this there were five to six 3-5mm ovoid, red, slightly depressed lesions (ulcers), the surfaces of which were covered with mild amounts of dried translucent yellow material (exudative crusts) [Fig. 4].



[Figure 4: Dorsum of mouse; showing alopecic and ulcerative areas]

Significant Internal Findings
Body Cavities:
There was a mild amount (about 0.1-0.2mls) of red fluid noted in the peritoneal cavity (sanguineous fluid ddx: post mortem changes, haemorrhage) and 2 irregularly-shaped 5mm long clumps of dark red gelatinous material located in the caudal pleural cavity (blood clots i.e. haemorrhage).

Gross Summary:
1. *Skin and subcutis:*
   Moderate, subacute, multifocal to coalescing, fibrinosuppurative, ulcerative and alopecic dermatitis.

2. *Body cavities:*
   Pleural cavity: Moderate, acute haemothorax.

Gross Comment:
The loss of hair and presence of ulcerative and crusty wounds on the dorsal back is consistent with barbering with possible secondary bacterial infection. No obvious associated ectoparasites were noted on gross examination, however microscopic ectoparasites cannot be ruled out. Culture is underway and it is hoped that histology will further characterise the cause of the presumed barbering.

The haemothorax is likely unrelated to the barbering and, as the mode of euthanasia is unknown, may be attributable to iatrogenic causes.

Ancillary Tests:

1. Anaerobic and aerobic culture was performed on an aseptically collected sample of affected skin:

64

VETPATH
LABORATORY SERVICES

Specialist Diagnostic Services
T/A Western Diagnostic Pathology
(Vetpath Laboratory Services) ACN: 007 190 043
84 007 190 043

39 Epsom Avenue, Ascot WA 6104
PO Box 18, Belmont WA 6984
Tel: (08) 9259 3666
Fax: (08) 9259 3627
A/Hrs: 0418 916 436

Lab No : 220019
Client : DAVIES/13/223 1354.13

Date: 18/04/2013
Collection Date: Date not supplied
Species: Other 6700

Murdoch University/Clin Path Lab
South Street
MURDOCH WA 6150

Tests :	**Specimens :**	
Aer+Anaer C&S	Skin Lesion - H	26/18

MICROBIOLOGY 1 REPORT

WET MICRO

GRAM

NO POLYMORPHS SEEN
EPITH V.OCC
NO BACTERIA SEEN
NO FUNGAL ELEMENTS SEEN

CULTURE

Aerobic	No Growth
Anaerobic	No growth
Broth	No growth

Comments FINAL REPORT: 20/04/2013

Senior Scientist Vincent Wycoco

2. Gram Twort histochemistry was carried out on the skin section (H13-338A) and no bacteria were identified.

Histopathological findings:
H13-338 A Skin:
There is focal epithelial loss (ulceration) and the adjacent epithelium displays decreased layers of cells with several of the cells appearing flattened (erosion). The rest of the epithelium is slightly thickened caused by increased layers within the stratum spinosum (hyperplasia; acanthosis). The dermis is diffusely infiltrated (more densely infiltrated superficially directly subjacent to the ulcer) by moderate numbers of neutrophils and macrophages, some mast cells as well as rare lymphocytes and plasma cells. A Gram Twort stain fails to identify any bacteria; likewise no ectoparasites or fungal organisms are seen.

H13-338 A, B, C Lung, Heart, Liver, Kidney, Adrenal, Spleen, Brain, Stomach, Small intestines, Large intestines:
There are no abnormalities detected.

Final Diagnosis:
1. *Skin and subcutis:*
   Moderate, chronic, multifocal to coalescing, neutrophilic and histiocytic, ulcerative and alopecic dermatitis with mild acanthosis.

2. *Body cavities:*
   Pleural cavity: Moderate, acute haemothorax.

Final Comment:
Histopathological examination confirms the gross diagnosis of an ulcerative dermatitis however the inciting cause could not be identified. Although the changes (acanthosis, increased numbers of macrophages and mast cells) are consistent with ulcerative dermatitis of ectoparasitic origin (i.e. ascariasis) it is aetiologically non-specific and hence ectoparasitism cannot be definitively diagnosed on the basis of the section examined. There were no aetiological agents (e.g. ectoparasites, bacteria) identified either in culture or on histological examination of the tissue. Despite this, external parasites cannot be definitively ruled out based on the skin section examined; indeed in many cases of ectoparasitism there is a paucity of parasites, however they can nevertheless be clinically significant due to the secondary hypersensitivity-related dermal inflammation that ensues.

The haemothorax is likely unrelated to the skin lesions and, as the mode of euthanasia is unknown, may be attributable to iatrogenic causes.

Yours Sincerely,

Dr Ziyuan Lim, BSc BVMS (Murdoch)
Resident in Anatomic Pathology

Nahiid Stephens BSc BVMS (Hons) MANZCVSc (Vet Path)
Lecturer in Veterinary Pathology

Checklist: (**√** = no gross lesions; **H** = sample for histology; **C** = culture; **P** = photographed)									
	Eyes	√H	Lungs	√H	Stomach	√H	Adrenals		Other organs
HC	Skin		Bronch LN	√H	S. intestine		Testes		(list)
	Head LN	√H	Heart		Caecum	√H	Ovaries		
√H	Tongue	√H	Liver	√H	Colon		Meninges		
√H	Oesophagus		Gall bladder		Mesent LN	√H	Brain		
	Thyroid	√H	Spleen	√H	Kidneys	√H	Bone Marrow		
	Parathyroid	√H	Pancreas	√H	Bladder				
	Thymus		Forestomachs						
Frozen samples:□ Liver □ Fat □ Kidney □ Brain □ Other (list)									

Disease Process 1	**INC**	**Disease Process 2**	
System 1	**SKI**	**System 2**	
General Cause 1	**UNK**	**General Cause 2**	
Aetiology 1		**Aetiology 2**	
Common Name 1	**Ulcerative dermatitis**	**Common Name 2**	

The Immune Response to Skin Trauma Is Dependent on the Etiology of Injury in a Mouse Model of Burn and Excision

Samantha M. Valvis[1], Jason Waithman[2], Fiona M. Wood[1,3,4], Mark W. Fear[1,3,5] and Vanessa S. Fear[2,5]

Skin trauma has many different causes including incision, blunt force, and burn. All of these traumas trigger an immune response. However, it is currently unclear whether the immune response is specific to the etiology of the injury. This study was established to determine whether the immune response to excision and burn injury of equivalent extent was the same. Using a mouse model of a full-thickness 19 mm diameter excision or 19 mm diameter full-thickness burn injury, we examined the innate immune response at the level of serum cytokine induction, whole-blood lymphocyte populations, dendritic cell function/phenotype, and the ensuing adaptive immune responses of CD4 and CD8 T-cell populations. Strikingly, both the innate and adaptive immune system responses differed between the burn and excision injuries. Acute cytokine induction was faster and different in profile to that of excision injury, leading to changes in systemic monocyte and neutrophil levels. Differences in the immune profile between burn and excision were also noted up to day 84 post injury, suggesting that the etiology of injury leads to sustained changes in the response. This may in part underlie clinical observations of differences in patient morbidity and mortality in response to different skin injury types.

Journal of Investigative Dermatology (2015) **135,** 2119–2128; doi:10.1038/jid.2015.123; published online 7 May 2015

INTRODUCTION

Injury to the skin initiates an immune response, which is the key driver of repair (Gurtner *et al.*, 2008). However, it is not clear whether the immune response is etiology dependent.

Patients with significant skin injuries do appear to differ in their responses dependent on etiology. This is evident in the sexual dimorphism observed where female patients have a lower incidence of sepsis and mortality after surgical or blunt force trauma, whereas after burn injury male patients fare better, with a 2-fold increase in mortality observed in females with equivalent total body surface area burn injury (O'Keefe *et al.*, 2001; McGwin *et al.*, 2002). Following burn injury, all patients have a poorer outcome than predicted by the injury severity scoring system (Cassidy *et al.*, 2013), whereas recent evidence shows an increased risk of cancer in burn injury patients, in particular females (Duke *et al.*, 2011, 2014). This may be indicative of a unique impact of burn injury on the immune system, leading to significant acute and long-term consequences.

Post injury there is increased prevalence of blood stream infections and mortality (Sharma, 2007; Tran *et al.*, 2012). This is attributed to disturbances in the skin, including changes in microflora (Wysocki, 2002; Barret and Herndon, 2003; Erol *et al.*, 2004) and persistent innate pro-inflammatory or the systemic inflammatory response syndrome (Ni Choileain and Redmond, 2006). Finally, this increase in morbidity and mortality is compounded by compensatory anti-inflammatory responses and suppressed T-cell responses (Ni Choileain and Redmond, 2006).

In burn patients, early investigations suggested that increased susceptibility to infection is due to hyperactivity of the monocyte/macrophage system on a backdrop of insufficient CD4 and C8 T cell responses (Murphy *et al.*, 2004; Samonte *et al.*, 2004). The adaptive immune system following burn injury adopts a suppressive phenotype with reported reduced T-helper 1 and cytotoxic T-cell responses (Hunt *et al.*, 1998) and increased T-regulatory (Treg) cell activity (Teodorczyk-Injeyan *et al.*, 1988; Hultman *et al.*, 1995; Kelly *et al.*, 1997, 1999; Lederer *et al.*, 1999; Guo *et al.*, 2003; Hanschen *et al.*, 2011; MacConmara *et al.*, 2011). Similarly, trauma injury immune responses include increased Treg activity (Ni Choileain and Redmond, 2006) and a reduced T-helper 1 response (Beilin *et al.*, 2006).

[1]*Burn Injury Research Unit, University of Western Australia, Perth, Western Australia, Australia;* [2]*Telethon Kids Institute, University of Western Australia, Perth, Western Australia, Australia;* [3]*Fiona Wood Foundation, Perth, Western Australia, Australia and* [4]*Burns Service of Western Australia, WA Department of Health, Perth, Western Australia, Australia*

Correspondence: Mark W. Fear, Burn Injury Research Unit, M318, University of Western Australia, 35 Stirling Highway, Perth, Western Australia 6009, Australia. E-mail: Mark.fear@uwa.edu.au

[5]*These authors contributed equally to this work.*

Abbreviations: GCSF, granulocyte colony–stimulating factor; ILN, inguinal lymph node; MFI, Mean Fluorescence Intensity; MHC, major histocompatibility complex; Treg, T regulatory

Received 17 November 2014; revised 16 March 2015; accepted 18 March 2015; accepted article preview online 31 March 2015; published online 7 May 2015

Research in burn patients with sepsis has revealed a significant decrease in circulating conventional dendritic cells (DCs) and plasmacytoid DCs (D'Arpa *et al.*, 2009). Burn injury has been shown to decrease conventional DC (Patenaude *et al.*, 2010; Shen *et al.*, 2012) and plasmacytoid DC (Shen *et al.*, 2012) number in both local draining lymph tissue and the spleen, with DCs possessing an anti-inflammatory phenotype and dysfunctional T-cell-priming ability in the acute post-injury phase (Patenaude *et al.*, 2010; Van den Berg *et al.*, 2011).

Here, we have investigated the impact of burn and excisional injury on the immune system. We examined the innate immune response at the level of induction of cytokine, chemokine, and DC function/phenotype, as well as the ensuing adaptive immune responses in CD4 and CD8 T cell–populations. Strikingly, we observed different profiles of innate and adaptive immune system changes, both acute and in the longer term in response to the burn and excision injury. This work suggests that etiology of injury is an important determinant of the immune response.

RESULTS

Burn injury induces a rapid systemic cytokine and chemokine responses, which differs from that of excision injury

Serum was isolated from control, burn, and excision groups on days 1, 3, and 7 post injury and examined for inflammatory cytokine profiles (Figure 1). Severe trauma induces inflammatory cytokine responses including IL-1α, IL-1β, tumor necrosis factor-α, and IL-6 (Sheridan, 2001). Post-burn injury at day 1, IL-6 was significantly increased (Figure 1a). Excision injury induced significantly increased IL-6 production at day 1, with a peak level at day 3, compared with control and burn injury (Figure 1a). At day 1 post burn tumor necrosis factor-α and IL-10 sera concentrations were significantly elevated compared with the excision injury. Following excision, tumor necrosis factor-α does not significantly increase, whereas IL-10 appears to be increasing at day 3 with a significant increase at day 7 (Figure 1a). There was no significant difference in production of IL-1α or IL-1β (data not shown).

Chemokine levels were also assessed (Figure 1b). Burn injury induced elevated levels of serum monocyte chemoattractant protein 1 (MCP1), a monocyte, immature DC, and memory T-cell attractant at day 1 compared with the excision injury and control (Figure 1b). macrophage inflammatory protein 1α (MIP1α) (CCL3) and macrophage inflammatory protein 1β (MIP1β) (CCL4) attract monocytes, T cells, and potentially polymorphonuclear leukocytes. Their expression was also significantly elevated at day 1 post burn compared with excision (Figure 1b). No significant change in MCP1, MIP1α, and MIP1β was observed after excision.

KC (CXCL1) is a neutrophil attractant that also induces angiogenesis. Elevated levels of KC were detected at day 1 both post excision and burn compared with control. Granulocyte colony–stimulating factor (GCSF) stimulates granulocyte release from the bone marrow and increases neutrophil proliferation and mobilization. A significant increase in GCSF levels after burn injury was detected at day 1 through to 7 (compared with control (Figure 1b)). Excision injury induced a 7-fold increase in GCSF at day 1, with significant increases in GCSF compared with the burn and control groups persisting to day 3 (Figure 1b). The response appears greater but for a shorter duration after excision injury. Eotaxin (CCL11) an eosinophil, basophil, mast cell, T-helper 2 cell, and platelet attractant was significantly elevated above control at day 1 following burn injury only (Figure 1b).

T cell–modulating cytokines were examined (Figure 1c), with burn injury showing a trend for the highest levels of all five cytokines tested and significantly increased IL-13 at day 1 (Figure 1c). This cytokine profile is indicative of a pro-T-helper 2 cell environment (Pulendran *et al.*, 2010).

These data indicate significant differences both in the timing and profile of inflammatory cytokine and chemokine production between burn and excision. Burn induced an acute inflammatory response consisting of MCP1, MIP1α, MIP1β, and eotaxin on day 1. Alternately, the day 1 response following excision injury consisted predominantly of elevated KC and GCSF.

Burn injury induces changes in systemic monocyte and neutrophil levels that are significantly different to those induced by excision

Whole-blood counts were obtained at days 1, 3, 7, and 14 post injury (Figure 1). A significantly increased monocyte count was observed at day 3 post burn compared with control (Figure 1d). Neutrophil cell counts were elevated post burn and excision at day 7, and this was sustained in the burn injured group compared with the excision group at day 14 (Figure 1d). The differences in hematology in part mirror the differences in cytokine and chemokine production observed. Increases in MCP1, MIP1α, and MIP1β after burn injury appear to lead to increases in monocyte production for example. These data also support previous findings, indicating an important role for these chemokines in burn injury but not excision (Heinrich *et al.*, 2003; Low *et al.*, 2001). In contrast, the acute increase in GCSF is not mirrored by a significant increase in neutrophil number until day 7. This may be due to egress of neutrophils from the blood to the excision site. Analysis of differences in wound infiltrate from both injury types will be important to assess this further. No changes in whole-blood white cell count, eosinophil, or lymphocyte levels were detected (data not shown).

Changes in dendritic cell population and maturation are different following burn and excision injury

To compare DC responses in the lymph node, the inguinal draining lymph nodes (ILNs) were harvested at days 1, 3, and 7 post injury. DC subset number, frequency, and maturation status were assessed using FACS. Dendritic cells were identified as the major histocompatibility complex (MHC) II^{hi} and $CD11c^{+}$ cell population, with subsequent gating on $CD8a^{+}$ (resident lymph node DC population) and $CD8a^{-}$ expression (migratory DC population from the skin). The latter $CD8a^{-}$ DC that circulate from the dermis during surveillance and inflammation were further gated into $CD103^{+}CD11b^{lo}$ ($CD103^{+}$ DC), $CD11b^{hi}CD103^{-}$ ($CD11b^{hi}$ DC), and $CD11b^{lo}CD103^{-}$ ($CD11b^{lo}$ DC; Figure 2a) as these subtypes present differentially to CD4 and CD8 T cells. This gating

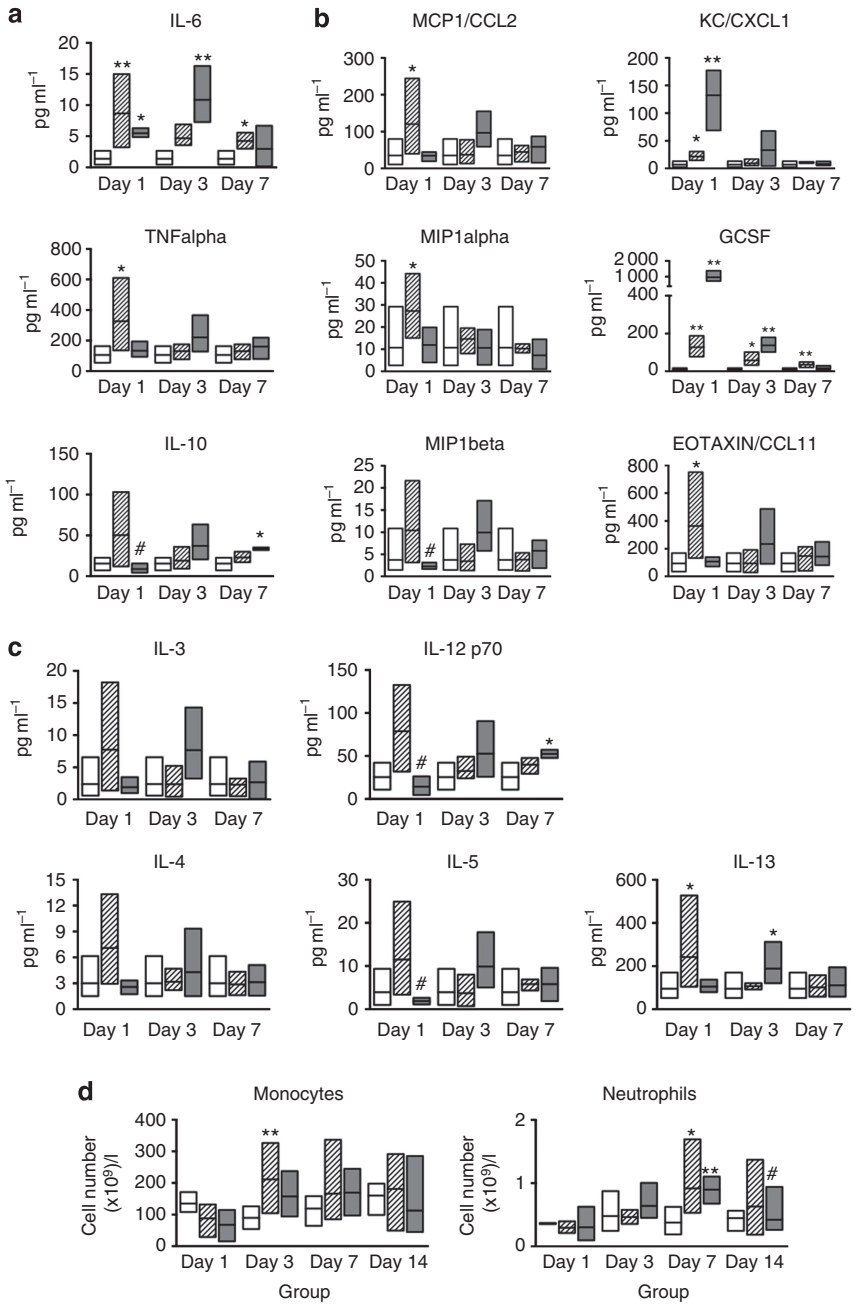


Figure 1. Temporal cytokine, chemokine, and hematology changes in burn and excision injury. Mice received sham (control), burn, or excision injury, and sera or whole blood were collected at the indicated time points for immune assay or hematology, respectively. Inflammatory cytokines IL-6, TNFα, and IL-10 (**a**), Chemokines monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1α (MIP1α), macrophage inflammatory protein 1β (MIP1β), KC/CXCL1, GCSF, and Eotaxin (**b**), and T-cell-modulating cytokines IL-3, IL-12p70, IL-4, IL-5, and IL-13 (**c**). Bar graphs indicate control (white bars), burn injury (hatched bars), and excision-injury (gray bars). Results are shown as mean+SEM. $n = 5$–8 mice per group for at least two independent experiments. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with burn injury group; $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with burn injury. Hematology profile for monocytes (**d**) and neutrophils (**d**). Bars indicate control (white bars), burn injury (hatched bars), and excision injury (gray bars). Results are shown as median and range (0–100th percentile). $n = 6$–8 mice per group and at least two independent experiments. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with burn injury group; $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with burn injury. GCSF, granulocyte colony–stimulating factor; TNF-α, tumor necrosis factor-α.

strategy provides information on resident DC filtering blood antigens and migratory DC from the periphery, which modulate adaptive T-cell responses via induction of CD4 and/or CD8 T–cell populations. In particular, CD8a-CD103+ DC are implicated in stimulation of viral immunity via cross-presentation of antigen to CD8 T cells (Sung *et al.*, 2006; Malissen *et al.*, 2014). Alternately, under steady state conditions both CD11b and CD103 have been implicated in the induction of Treg cells. However, this has been found to be tissue dependent (Semmrich *et al.*, 2012).

ILN total cell number was significantly increased after burn and excision injury at day 3 compared with controls

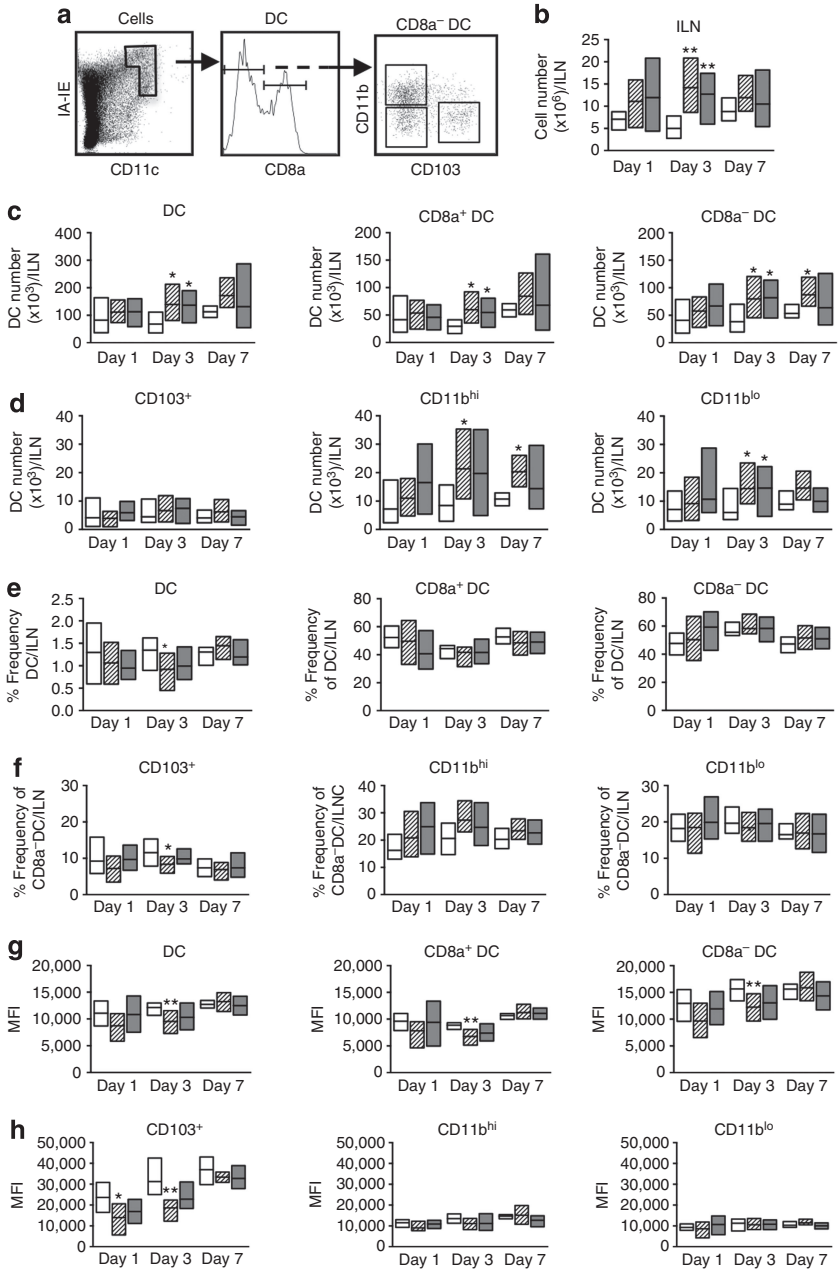


Figure 2. Dendritic cell responses in local draining lymph nodes after burn injury and excision injury. Mice received sham (control), burn, or excision injury, and ILNs were harvested to single suspensions at the indicated time points. Dendritic cells were gated CD11c$^+$ MHC II$^+$ and further gated for subpopulations using CD8α, then CD11b and CD103 (**a**). Graphs show ILN total cell number (**b**), total DCs, CD8a$^+$ DC, and CD8a$^-$ DC number (**c**), and the number of CD8a$^-$ DC that was CD103$^+$, CD11b^{hi}, or CD11b^{lo} (**d**). Lymph node percentage frequency of total DCs, the percentage frequency of CD8a$^+$ DC and CD8a$^-$ DC in total DCs (**e**), and the percentage frequency of CD103$^+$ DC, CD11b^{hi} DC, and CD11b^{lo} DC in the CD8a$^-$ DC compartment (**f**) are as indicated. Expression of MHC II as determined by MFI is presented for total DCs and DC gated CD8a$^+$ and CD8a$^-$ (**g**). Data indicate MHC II MFI on CD8a$^-$ DC population further gated for CD103$^+$ DC, CD11b^{hi} DC, or CD11b^{lo} DC sub-populations (**h**). Bars indicate control (white bars), burn injury (hatched bars), and excision injury (gray bars). Results are shown as median and range (0–100th percentile). $n = 5$–8 mice per group, for at least two independent experiments. $^*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with burn injury group; $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with burn injury. ILN, inguinal lymph node; MFI, Mean Fluorescence Intensity; MHC, major histocompatibility complex.

(Figure 2b). Total DC cell number, resident CD8a$^+$ DC number, and CD8a$^-$ DC number were all significantly increased at day 3 post injury compared with the control, whereas after burn injury the increase in CD8a$^-$ cells was sustained to day 7 (Figure 2c).

The CD8a$^-$ DC subpopulation cell numbers were further analyzed. There was no increase in CD103$^+$ DC. However, CD11b^{hi} DC and CD11b^{lo} DC numbers were significantly increased compared with controls at day 3 (Figure 2d) with CD11b^{hi} number remaining elevated after burn injury at day 7 (Figure 2d).

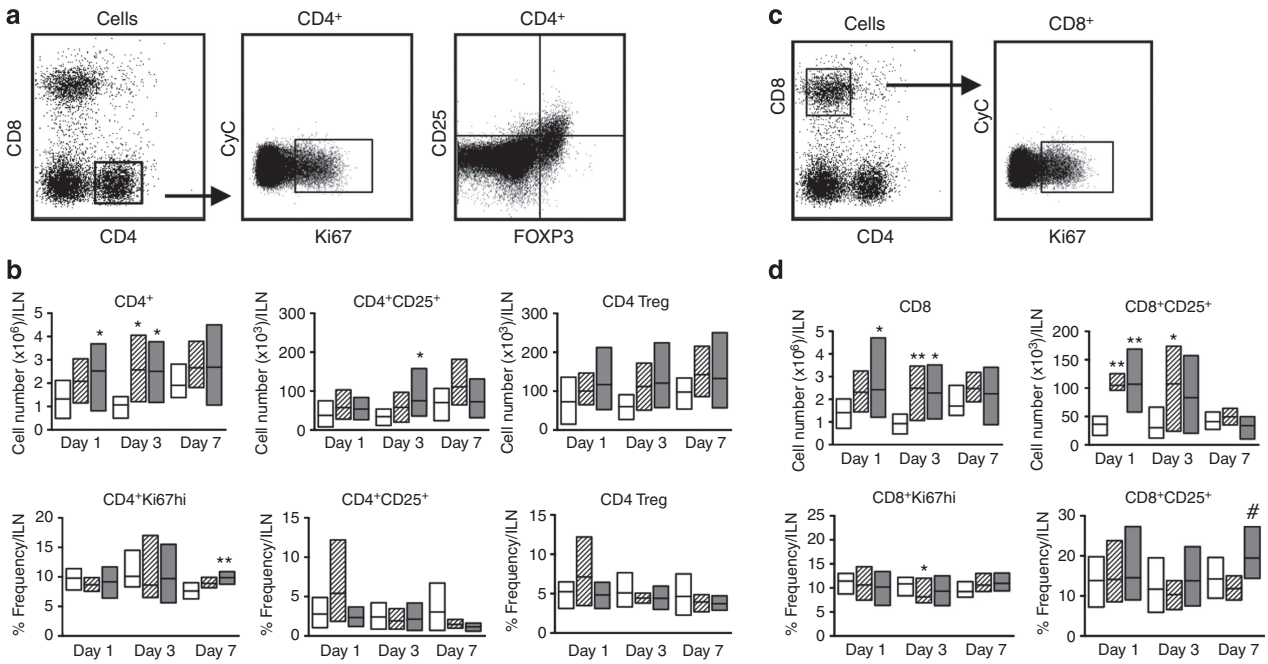


Figure 3. CD4 and CD8 cellular response to burn injury and excision injury. Mice received sham (control) burn or excision injury, and ILNs were harvested to single suspensions at the indicated time points. Dot plots indicate flow cytometric gating strategy for $CD4^+$ cells that were further gated on $Ki67^{hi}$, $CD25^+$, or $CD25^+FoxP3^+$ cells (**a**). ILN $CD4^+$, $CD4^+CD25^+$, and CD4 Treg ($CD25^+FoxP3^+$) cell number and the percentage frequency of $Ki67^{hi}$, $CD25^+$, and Treg ($CD25^+FoxP3^+$) in CD4+ cell population are indicated (**b**). $CD8^+$ cells gating strategy is indicated in dot plots showing further gating on $Ki67^{hi}$ cells (**c**). ILN $CD8^+$ and $CD8^+CD25^+$ cell number and the percentage frequency of $Ki67^{hi}$ and $CD25^+$ cells in CD8 cell population are shown (**d**). Bars indicate control (white bars), burn injury (hatched bars), and excision injury (gray bars). Results are shown as median and range (0–100th percentile)., $n = 5–8$ mice per group, for at least two independent experiments. $^*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with burn injury group; $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with burn injury. ILN, inguinal lymph node; Treg, T regulatory.

There was a significant decrease in the percentage of cells that were DCs (DC % frequency) at day 3 post burn compared with control (Figure 2e). Within the $CD8a^-$ DC populations, we observed a significant decrease in the $CD103^+$ DC subset percentage frequency compared with control at day 3 after burn injury (Figure 3f), with no other significant changes in the $CD103^+$, $CD11b^{hi}$, or $CD11b^{lo}$ percentage frequencies.

To determine the activation status of the DC populations, we determined Mean Fluorescence Intensity (MFI) of MHC II expression (Figures 2g and h). DC MHC II MFI was significantly decreased at day 3 in the burn injury only compared with control. Furthermore, MHC II MFI was significantly reduced for $CD8a^+$ DC and $CD8a^-$y DC after burn injury compared with control (Figure 2g). Within the $CD8a^-$ migratory CD103+ DC subset, there was a significant reduction in MHC II MFI at day 1 and day 3 post burn compared with control (Figure 2h).

CD4 and CD8 T–cell activation and Treg cell responses differ between burn and excision injury

CD4 T-cell number, proliferation, activation, and Treg cell induction in the ILN were determined by flow cytometry (Figures 3a and b). CD4 cell number was significantly increased at day 3 after burn injury and more acutely at days 1 and 3 after excision injury compared with controls. There was a further significantly increased frequency of dividing CD4 cells ($Ki67^{hi}$) in the excision injury group at day 7 compared with control. There was a significant change in the excision $CD4^+CD25^+$ cell number at day 3 compared with the control; however, there was no change in the $CD4^+CD25^+$ percentage frequency in either injury group (Figure 3b). No changes were observed in CD4 Treg cell numbers or the percentage frequency (Figure 3b).

CD8 T-cell number, proliferation, and activation were also determined by flow cytometry (Figures 3c and d). CD8 cell number was significantly increased at day 3 post burn and days 1 and 3 post excision compared with control. There was a decrease in the frequency of $Ki67^{hi}$ dividing cells in this population at day 3 post burn compared with control. An increase in $CD8^+CD25^+$ cell numbers, indicative of cell activation, was observed at days 1 and 3 for burn injury and day 1 alone for excision compared with control (Figure 3d).

Long-term alterations in immune profiles following burn and excision injury

Sera and whole blood were examined for cytokine and hematology at 84 days post injury. At Day 84, there was a significant increase in IL-10 in the burn group compared with the excision group (Figure 4a). In addition, there was a significantly decreased white cell count and lymphocyte cell number for burn injury compared with controls (Figure 4a).

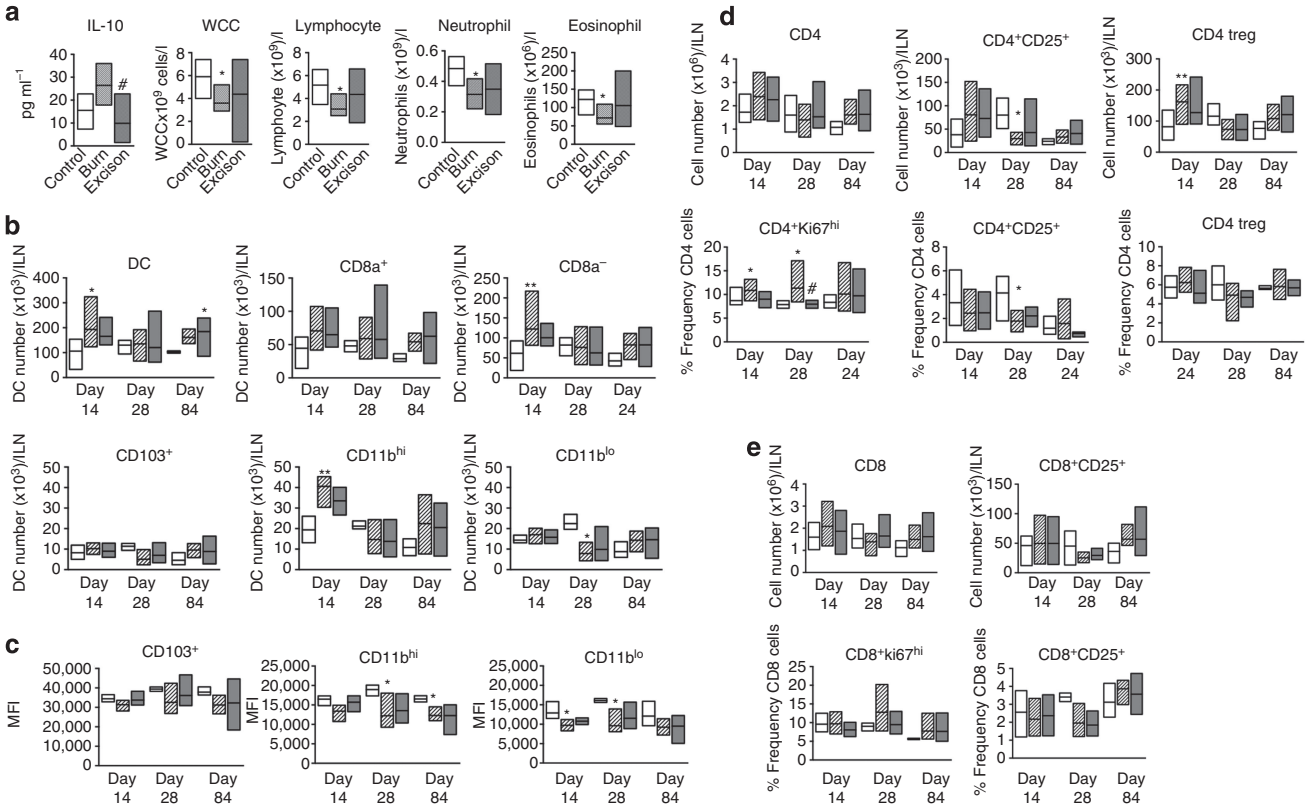


Figure 4. Innate and adaptive immune responses in the remodeling phase of burn injury and excision injury. Mice received sham (control), burn, or excision injury. Sera or whole blood were collected at day 84, and IL-10, WCC, lymphocytes, neutrophils and eosinophils were measured (**a**). ILNs were harvested to single suspensions at the indicated time points and total DCs, $CD8a^+$ DC, $CD8a^-$ DC, and $CD8a^-$ DC subpopulations $CD103^+$, $CD11b^{hi}$, and $CD11b^{lo}$ numbers determined (**b**) as per previous gating strategy. Expression of MHC II as determined by MFI is presented for total DCs, and DC gated $CD8a^+$ and $CD8a^-$, and for $CD8a^-$ DC population further gated for $CD103^+$ DC, $CD11b^{hi}$ DC, or $CD11b^{lo}$ DC sub-populations (**c**). ILN $CD4^+$, $CD4^+CD25^+$, and CD4 Treg ($CD25^+FoxP3^+$) cell number and the percentage frequency of $Ki67^{hi}$, $CD25^+$, and Treg ($CD25^+FoxP3^+$) in CD4+ cell population are indicated (**d**). ILN $CD8^+$ and $CD8^+CD25^+$ cell number and the percentage frequency of $Ki67^{hi}$ and $CD25^+$ cells in CD8 cell population are shown (**e**). Bars indicate control (white bars), burn injury (hatched bars), and excision injury (gray bars). Results are shown as median and range (0–100th percentile). $n = 5$–8 mice per group, for at least two independent experiments. $^*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with burn injury group; $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with burn injury. ILN, inguinal lymph node; MFI, Mean Fluorescence Intensity; MHC, major histocompatibility complex; Treg, T regulatory; WCC, white cell count.

There was also a significantly reduced cell number for eosinophils and neutrophils in the burn injury group only (Figure 4a).

DC cell number was significantly elevated at day 84 post excision compared with control. At day 28, we observed a significantly reduced number of $CD11b^{lo}$ DC in the ILN after burn compared with the control (Figure 4b). Moreover, there was reduced MHC II MFI on $CD11b^{hi}$ and $CD11b^{lo}$ DC post injury, and this extended to day 84 for the $CD11b^{hi}$ DC subset (Figure 4c).

Reduced MHC II expression on DC compromises their ability to prime T-cell responses and may potentiate a tolerant response. Accordingly, although we observed an increase in proliferating CD4 T-cell frequency and number, there was a concomitant reduction in CD25 expression at day 28 after burn injury (Figure 4d). This may indicate a downregulation of the CD4 T-cell response. Indeed, CD4 Treg cell numbers were significantly increased at day 14 after burn compared with the control, but there was no increase in the percentage frequency of CD4 cells (Figure 4d).

CD8 T-cell number and proliferation were not changed at day 14 through day 84 after burn or excision (Figure 4e). However, a reduction in CD8 T-cell CD25 expression was observed at day 28 for excision injury, which may indicate less responsive CD8 cells at this time.

Burn injury leads to suppressed acute splenic T-cell cytokine responses

Stimulation of T cells isolated from the spleen was conducted in cell culture using Concavalin A to assess T-cell function after burn injury. At day 7 post burn, the T-cell response was reduced compared with control animals (Figure 5) with significantly reduced production of IL-1β and IL-17 and a trend to reduced production of IL-2 and IL-6 ($P = 0.06$). By Day 14, T-cell cytokine production in response to Concavalin A had returned to normal levels (data not shown).

DISCUSSION

Early following injury the innate immune response includes a cytokine cascade with release of tumor necrosis factor-α and

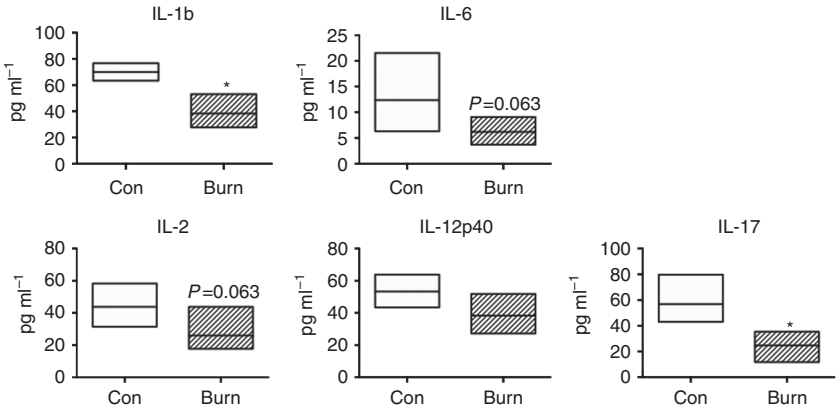


Figure 5. Burn injury leads to suppressed acute splenic T-cell cytokine responses. Spleen cells (3×10^5cells per ml) were isolated and stimulated with 10 ng ml^{-1} concavalin A and cytokine levels determined in supernatant at 48 hours. Bars indicate control (white bars) and burn injury day 7 post injury (hatched bars). Results are shown as median and range (0–100th percentile, $n = 5$ mice per group). At Day 14, there was no significant difference between burn injury and control spleen cultures stimulated with concavalin A (data not shown).

IL-1β to stimulate IL-6 production. IL-6 increases proliferation of polymorphonuclear leukocytes and inhibits monocyte to inflammatory DC differentiation, instead of promoting monocyte to macrophage differentiation following injury (Chomarat *et al.*, 2000; De *et al.*, 2003). In addition, IL-10 released after trauma is monocyte to macrophage promoting (Vicari *et al.*, 2002). Indeed, high levels of innate cytokines have been correlated with increased severity of tissue trauma (Shenkin *et al.*, 1989; Cruickshank *et al.*, 1990; Mokart *et al.*, 2002) and development of post-operative complications (Baigrie *et al.*, 1992). Research indicates that the magnitude of the innate inflammatory response directly corresponds to the subsequent downregulation of ensuing immune response following trauma (Tschoeke and Moldawer, 2005; Ni Choileain and Redmond, 2006). Burn and excision injury, in this study, differ in their temporal release and pattern of innate cytokine production, with an acute innate cytokine response in burn injury that could potentially mediate monocyte to macrophage development. Notably, excision induced an early neutrophil chemoattractant response with no significant increase in macrophage inducing cytokines MCP/MIP1α/β, delayed onset of IL-6 production, and lower IL-10 induction when compared with the burn. These differences in acute cytokine profile after wounds of equivalent extent suggest that the impact and subsequent changes in immune response are related to the injury etiology.

Dendritic cells link the innate and adaptive arms of the immune system. Resident ILN CD8a$^+$ DCs are important for tolerance induction to cell-associated antigens present during apoptosis (Qiu *et al.*, 2009). Here, we show a significant increase in the ILN resident CD8a$^+$ DC population after burn and excision with reduced activation status. This may assist with dampening of an escalating immune response to tissue necrosis/apoptosis antigen presentation during injury.

Skin migratory DC subsets are able to differentially stimulate CD4 and CD8 T-cell responses (Sung *et al.*, 2006; Malissen *et al.*, 2014) in the lymph node. The CD103$^+$ DC present antigen to both CD4 (Beaty *et al.*, 2007; Jakubzik *et al.*, 2008; Semmrich *et al.*, 2012) and CD8 T cells (Jakubzik *et al.*, 2008; Beauchamp *et al.*, 2010; Semmrich *et al.*, 2012), whereas the CD11b$^+$ DC predominantly present antigen to CD4 T cells (Beaty *et al.*, 2007). During homeostasis, a tolerogenic T-cell response is induced, whereas during inflammation and/or infection these DC subsets upregulate MHC II expression and are capable of inducing a T-cell response. The activation/maturation status determines the development of a T-cell response. Our data indicate temporal changes in DC populations following burn and excision injury with notable differences in CD103$^+$ frequency and maturity, and CD11b DC cell number following burn compared with excision. These temporal changes in resident and skin migratory DC populations in the draining lymph node may be sufficient to induce differential trauma-dependent CD4 T-cell responses.

A CD4 T-cell increase was observed after both burn and excision. However, only the excision group displaying an increase in CD4 cell proliferation (day 7). Interestingly, there was an increased CD4 T-cell proliferative response late (day 28) following initial burn-injury suppression in this population. This rebound effect in burn injury has previously been observed for a cytotoxic CD8 population (Hunt *et al.*, 1998).

The effect of burn injury has been reported to stimulate a more robust CD8 T–cell proliferative response compared with their CD4 T-cell counterpart (Buchanan *et al.*, 2006). In this study, total CD8 T-cell number increased in the skin-draining lymph node after burn and excision. However, in the burn injury, there was a notable decrease in CD8 T cell–proliferative response.

The changes in systemic cytokine and hematology profiles as well as lymph node cell populations indicate that the impact of the burn was substantially different to that of excision. Additional work assessing wound infiltrate will be important to shed light on localized changes as well. Of particular note in this data, profound changes to the dendritic cell populations were observed, indicating a loss of activation and maturity after the burn injury that was not observed after

excision. Importantly, these changes appeared to be sustained until the final time point analyzed. Analysis of T-cell activation using cells isolated from the spleen after a burn injury showed that T-cell cytokine production in response to stimulation was suppressed acutely but had recovered to normal levels by day 14 post injury. Together, this suggests that functionally the T cells are normal after transient suppression and that therefore the effects observed in cell populations more likely reflect changes in dendritic cell presentation and maturity. This has previously been reported after UV exposure of the skin (Ng *et al.*, 2013) and may also occur after burn injury. Therefore, characterization of long-term changes in dendritic cell function after a non-severe burn, in particular the ability to prime T cells, is an important next step.

In summary, these data show significant differences between the immune response to burn and excisional injury. However, the injury model used is a non-severe injury (which represents close to 90% of presentations in developed countries), and it is possible responses converge as injury severity increases. Here we observed an early acute inflammatory response to burn injury that is delayed following excision. Interestingly, cytokine and chemokine profiles indicate an early macrophage-promoting environment after burn injury, whereas excisional injury has an acute neutrophil attractant response with a delayed macrophage-promoting environment. Furthermore, number and activation status in resident and migratory DC subsets in the skin-draining lymph nodes displayed temporal changes following burn or excision injury. These changes may underpin the absence of a CD4 T cell–proliferative response and decreased CD8 T-cell proliferation following burn injury (day 7 and day 3, respectively). Excision injury presented with a CD4 T cell–proliferative response at day 7.

The immediate lack of adaptive immune responses after burn injury likely contributes to increased infectious complications observed in patients including frequent *Pseudomonas aeruginosa* infections, which hamper recovery (Guggenheim *et al.*, 2009; Singer and McClain, 2002) with reactivation of latent viral infection potentially a further complication (Haik *et al.*, 2011). Although this period of suppression was followed by a rebound effect in CD4 T–cell proliferation, this was concomitant with a reduced CD25 expression profile, which may be potentiating a process of anergy or deletion tolerance to self-antigens in this remodeling phase. This may contribute to the increased incidence of cancer observed in burn patients and requires further investigation.

MATERIALS AND METHODS

Mice

Adult 9-week-old female C57BL/6 mice were housed under pathogen-free conditions with food and water provided *ad libitum*. Approval was obtained by the University of Western Australia Animal Ethics Committee, and all experiments performed in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care and Use of Animals for Scientific Purposes.

Full-thickness burn and excision trauma procedure

Nine-week-old C57BL6/J female mice ($n = 140$, $n = 10$ per group per time-point) received either a full-thickness 19-mm diameter burn wound following a previously described protocol (Giles *et al.*, 2008) or a full-thickness 19 mm diameter excision by outlining the area with a 19 mm template and surgically removing the tissue under anesthesia. This equates to ~8% total body surface area (a non-severe injury model). Sham injury mice received no surgical treatment but underwent anesthesia. Animals were administered analgesic (buprenorphine, 0.1 mg kg^{-1}) intramuscularly immediately post injury and at 12 hours. Water was instilled with paracetamol (1 mg ml^{-1}) for 5 days following surgery.

ILN tissue preparation

ILN preparations were performed as described previously (Fear *et al.*, 2011). In brief, ILN from individual mice were subjected to type IV collagenase digestion (1.5 mg ml^{-1}; Worthington Biochemical, Lakewood, NJ) with type I DNase (0.1 mg ml^{-1}; Sigma-Aldrich, St. Louis, MO) to prepare single-cell suspensions. All digestions and washes were performed in glucose sodium potassium buffer (11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na_2HPO_4, 5.5 mM $NaH_2PO_4.2H_2O$).

FACS analysis and antibodies

Single-cell suspensions were FcR blocked (2.4G2; BD Biosciences, San Jose, CA) prior to the addition of phenotyping antibodies. Airway and draining lymph node DC populations were identified using combinations of fluorochrome-labeled mAbs (BD Pharmingen, Sydney, NSW, Australia) to mouse I-A/I-E (2G9), CD11c (N418), CD11b (M1/70), CD8a (53-6.7), or CD103bio (M290). All labeling was performed in glucose sodium potassium buffer containing 0.2% BSA for 30 minutes on ice. T-cell populations were identified in ILN digests using the fluorochromes CD4, CD8, CD25, and Ki67 (BD Biosciences). A FOXP3 intracellular staining kit (eBiosciences, San Diego, CA) was used to determine intracellular FOXP3 staining. All Abs were used as direct conjugates to FITC, Phycoerythrin (PE), PE-Cy7, allophycocyanin (APC), APC-Cy7, or biotin and Streptavidin conjugated PE-Cy5 (BD Biosciences) as required. Appropriately matched IgG isotype controls (BD Pharmingen) and cytometer compensation settings adjusted using single-stained controls were used for each experiment. Samples were collected using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Blood and sera collection

Mice were anaesthetized in a closed chamber with 2.5% isofluorane, placed under 2.5% isoflurane nose-cone and 1 ml blood collected by cardiac heart puncture. Bloods (0.5 ml) were placed in heparin collection tubes for hematology whole-blood analysis at Clinical Pathology, Murdoch University (Murdoch, WA, Australia). In addition, blood (0.5 ml) was collected in serum tubes, stored at 4 °C for 30 minutes, centrifuged at 13 000*g* for 30 minutes, and sera stored at −20 °C.

Cytokine analysis

Cytokine analysis on sera was performed using the Bioplex Pro mouse Cytokine, 23-Plex Assay (Bio-Rad, Gladesville, NSW, Australia) according to the manufacturer's protocol. In brief, premixed standards were reconstituted to 50 ng/ml and serially

diluted for an 8-point standard curve. Premixed beads (50 µl) were washed twice in a 96-well plate and 50 µl standard or sample added to each well. The plate was incubated at room temperature for 30 min with shaking. After washing, beads were suspended in 125 µl of Bio-Plex assay buffer and read on a Bioplex System (Bio-Rad, NSW, Australia).

Spleen cytokine assays

At the indicated time points, spleens were collected and digested to single-cell suspensions as described previously. Cells were prepared in RPMI 1,640 with glutamine (Invitrogen Life Technologies, Australia) supplemented with 10% fetal calf serum, 20 $\mu g\,ml^{-1}$ gentamycin, and 20 µM 2-ME and plated at 3×10^5 cells per well in 96-well plates (Nunclon, Nunc, Denmark) with Concavalin A at 10 $ng\,ml^{-1}$ in triplicate. At 48 hours of culture, triplicate supernatants were pooled and stored at −20 °C prior to cytokine analysis.

Statistical analyses

All results were analyzed using Prism 5 (Graphpad software, San Diego, CA). Differences between groups were compared using the Kruskal–Wallis test with Dunn's test for multiple comparisons. Correction for multiple testing was done using Bonferroni correction. Values are presented as median and range (0–100th percentile).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments. MWF is supported by Chevron Australia. The authors also acknowledge the support of the Fiona Wood Foundation for funding of this work.

REFERENCES

Baigrie RJ, Lamont PM, Kwiarkowski D *et al.* (1992) Systemic cytokine response after major injury. *Br J Surg* 79:757–60

Barret JP, Herndon DN (2003) Effects of burn wound excision on bacterial colonization and invasion. *Plast Reconstr Surg* 111:751–2

Beilin B, Greenfeld K, Abiri N *et al.* (2006) Anesthiologists at work: an increase in pro-inflammatory and Th2 cytokine production, and alterations inproliferative immune responses. *Acta Anaesthesiol Scand* 50:1223–8

Beauchamp NM, Busick RY, Alexander-Miller MA (2010) Functional divergence among CD103+ dendritic cell subpopulations following pulmonary poxvirus infection. *J Virol* 84:10191–9

Beaty SR, Rose CE Jr, Sung SS. (2007) Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation. *J Immunol* 178:1882–95

Buchanan IB, Maile R, Frelinger JA *et al.* (2006) The effecto of burn injury on CD8+ and CD4+ T cells in an irradiation model of homeostatic proliferation. *J Trauma* 61:1062–8

Cassidy JT, Phillips M, Fatovich D *et al.* (2013) Developing a burn injury severity score (BISS): adding age and total body surface area burned to the injury severity score (ISS) improves mortality concordance. *Burns* 40:805–13

Chomarat P, Banchereau J, Davoust J *et al.* (2000) IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 1:510–4

Cruickshank AM, Fraser WD, Burns HJ *et al.* (1990) Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. *Clin Sci (Lond)* 79:161–5

D'Arpa N, Accardo-Palumbo A, Amato G *et al.* (2009) Circulating dendritic cells following burn. *Burns* 35:513–8

De AK, Laudanski K, Miller-Graziano CL *et al.* (2003) Failure of monocytes af trauma patients to convert to immature dendritic cells is related to preferential macrophage-colony-stimulating factor-driven differentiation. *J Immunol* 170:6355–62

Duke JM, Bauer J, Fear MW *et al.* (2014) Burn injury, gender and cancer risk: population-based cohort study using data from Scotland and Western Australia. *Br Med J Open* 4:e003845

Duke J, Rea S, Semmens J *et al.* (2011) Burn injury and cancer risk: a state-wide longitudinal study. *Burns* 38:340–7

Erol S, Altoparlak U, Akcay MN *et al.* (2004) Changes of microbial flora and wound colonization in burned patients. *Burns* 30:357–61

Fear VS, Burchell JT, Lai SP *et al.* (2011) Restricted aeroallergen access to airway mucosal dendritic cells in vivo limits allergen-specific CD4+ T cell proliferation during the induction of inhalation tolerance. *J Immunol* 187:4561–70

Giles N, Rea S, Beer T *et al.* (2008) A peptide inhibitor of c-Jun promotes wound healing in a mouse full-thickness burn model. *Wound Repair Regen* 16:58–64

Guggenheim M, Zbinden R, Handschin AE *et al.* (2009) Changes in bacterial isolates from burn wounds and their antibiograms: a 20-year study (1986-2005). *Burns* 35:553–60

Guo Z, Kavanagh E, Zang Y *et al.* (2003) Burn injury promotes antigen-driven Th2-type responses *in vivo*. *J Immunol* 171:3983–90

Gurtner G, Werner S, Barrandon Y *et al.* (2008) Wound repair and regeneration. *Nature* 453:314–32

Haik J, Weissman O, Stavrou D *et al.* (2011) Is prophylactic acyclovir treatment warranted for prevention of herpes simplex virus infections in facial burns? A review of the literature. *J Burn Care Res.* 32:358–62

Hanschen M, Tajima G, O'Leary F *et al.* (2011) Injury induces early activation of T-cell receptor signaling pathways in CD4+ regulatory T cells. *Schock* 35: 252–7

Heinrich SA, Messingham N, Meredith GS *et al.* (2003) Elevated monocyte chemoattractant protein-1 levels following thermal injury precede monocyte recruitment to the wound site and are controlled, in part, by tumor-necrosis factor-a. *Wound Rep Regen* 11:110–9

Hultman CS, Cairns BA, deSerres S *et al.* (1995) Early complete burn wound excision partially restores CTL function. *Surgery* 118:421–9

Hunt JP, Hunter CT, Brownstein MR *et al.* (1998) The effector component of the cytotoxic T-lymphocyte response has a biphasic pattern after burn injury. *J Surg Res* 80:243–51

Jakubzik C, Helft J, Kaplan TJ *et al.* (2008) Optimization of methods to study pulmonary dendritic cell migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen. *J Immunol Methods* 337:121–31

Kelly JL, Lyons A, Soberg CC *et al.* (1997) Anti-interleukin-10 restores burn-induced defects in T cell function. *Surgery* 122:146–52

Kelly JL, O'Suilleabhain CB, Soberg CC *et al.* (1999) Severe injury triggers antigen-specific T-helper cell dysfunction. *Shock* 12:39–45

Lederer JA, Rodrick ML, Mannick JA. (1999) The effects of injury on the adaptive immune response. *Shock* 11:153–9

Low QE, Drugea IA, Duffner LA *et al.* (2001) Wound healing in MIP-1alpha(-/-) and MCP1(−/−) mice. *Am J Pathol* 159:457–63

MacConmara MP, Tajima G, O'Leary F *et al.* (2011) Regulatory T cells suppress antigen-driven CD4 T cell reactivity following injury. *J Leukoc Biol* 89:137–47

Malissen B, Tamoutounour S, Henri S. (2014) The origins and functions of dendritic cells and macrophages in the skin. *Nat Rev Immunol* 14:417–28

McGwin G Jr, George RL, Cross JM *et al.* (2002) Gender differences in mortality following burn injury. *Shock* 18:311–5

Mokart D, Capo C, Blache JL *et al.* (2002) Early postoperative compensatory anti-inflammatory response syndrome is associated with septic complications after major surgical trauma in patients with cancer. *Br J Surg* 89:1450–6

Murphy TJ, Paterson HM, Mannick JA *et al.* (2004) Injury, sepsis, and the regulation of Toll-like receptor responses. Review. *Crit Care Med* 75:400–7

Ng RL, Scott NM, Strickland DH *et al.* (2013) Altered immunity and dendritic cell activity in the periphery of mice after long-term engraftment with bone marrow from ultraviolet-irradiated mice. *J Immunol* 190:5471–84

Ni Choileain NN, Redmond PH. (2006) Cell response to surgery. Review. *Arch Surg* 141:1132–40

O'Keefe GE, Hunt JL, Purdue GF (2001) An evaluation of risk factors for mortality after burn trauma and the identification of gender-dependent differences in outcomes. *J Am Coll Surg* 192:153–60

Patenaude J, D'Elia M, Hamelin C *et al.* (2010) Selective effect of burn injury on splenic CD11c(+) dendritic cells and CD8alpha(+)CD4(-)CD11c(+) dendritic cell subsets. *Cell Mol Life Sci* 67:1315–29

Pulendran B, Hua Tang H, Manicassamy S. (2010) Programming dendritic cells to induce T_H2 and tolerogenic responses. *Nat Immunol* 11:647–55

Qiu CH, Miyake Y, Kaise H *et al.* (2009) Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. *J Immunol* 182:4127–36

Samonte VA, Goto M, Ravindranath TM *et al.* (2004) Exacerbation of intestinal permeability in rats after a two-hit injury: burn and enterococcus faecalis infection. *Crit Care Med* 32:2267–73

Semmrich M, Plantinga M, Svensson-Frej M *et al.* (2012) Directed antigen targeting *in vivo* identifies a role for CD103+ dendritic cells in both tolerogenic and immunogenic responses. *Mucosal Immunol* 5: 150–60

Sharma BR. (2007) Infection inpatients with severe burns: causes and prevention thereof. Review. *Infect Dis Clin North Am* 21:745–59

Shen H, de Almeida PE, Kany KH *et al.* (2012) Burn injury triggered dysfunction in dendritic cell response to TLR9 activation and resulted in skewed T cell functions. *PLoS One* 7:e50238

Shenkin A, Fraser WD, Series J *et al.* (1989) The serum interleukin 6 response to elective surgery. *Lymphokine Res* 8:123–7

Sheridan RL (2001) A great constitutional disturbance. *N Engl J Med* 345:1271–2

Singer AJ, McClain SA (2002) Persistent wound infection delays epidermal maturation and increases scarring in thermal burns. *Wound Repair Regen* 10:372–7

Sung SS, Fu SM, Rose CE Jr *et al.* (2006) A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing langerin and tight junction proteins. *J Immunol* 176:2161–72 Erratum in: J Immunol 176(9): 5683

Teodorczyk-Injeyan JA, Sparkes BG, Falk RE *et al.* (1988) IL-2 secretion and transmembrane signaling in burned patients. *J Trauma* 28:152–7

Tran NK, Wisner DH, Albertson TE *et al.* (2012) Multiplex polymerase chain reaction pathogen detection inpatients with suspected septicemia after trauma, emergency, and burn. *Surgery* 151:456–63

Tschoeke SK, Moldawer LL. (2005) Human leukocyte antigen expression in sepsis: what have we learned? *Crit Care Med* 33:236–7

Van den Berg LM, de Jong MA, Witte LD *et al.* (2011) Burn injury suppresses human dermal dendritic cell and langherhans cell function. *Cell Immunol* 268:29–36

Vicari AP, Chiodoni C, Vaure C *et al.* (2002) Reversal of tumour induced cell paralysis by CpG immunostimulatory oligoneucleotide and interleukin 10 receptor anitibody. *J Exp Med* 196:541–9

Wysocki AB (2002) Evaluating and managing open skin wounds: colonization versus infection. Review. *AACN Clin issues* 13:382–97

Available online at www.sciencedirect.com
ScienceDirect
journal homepage: www.elsevier.com/locate/burns

Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model

Vanessa S. Fear [a,b], Wee-Peng Poh [b], Samantha Valvis [a], Jason C. Waithman [b], Bree Foley [b], Fiona M. Wood [a,c,d], Mark W. Fear [a,d,*]

[a] Burn Injury Research Unit, School of Surgery, University of Western Australia, Perth, Western Australia, Australia
[b] Telethon Kids Institute, University of Western Australia, Perth, Western Australia, Australia
[c] Fiona Wood Foundation, Perth, Western Australia, Australia
[d] Burns Service of Western Australia, WA Dept. of Health, Perth, Western Australia, Australia

ARTICLE INFO

Article history:
Accepted 5 January 2016

Keywords:
Burn
Excision
Immune response
Timing

ABSTRACT

Background: Burn excision has emerged as the dominant clinical paradigm in treatment of deep burns. Surgical intervention is common but the timing of wound excision is a balance between wound depth assessment, avoidance of infection and unnecessary intervention. However the physiological impact of timing of excision and consequences for the immune response are not well understood.

Methods: Mice were subject to full-thickness burn (<8% TBSA) followed by early (day 1) or late (day 8) surgical excision. Draining lymph nodes, wound tissue and sera were collected longitudinally at day 2 and day 6 after excision and analyzed for cytokine, dendritic cell and T cell profiles using FACS and multiplex ELISA assays.

Results: Delayed excision after injury initiated acute and severe inflammatory responses, with high levels of inflammatory cytokines, increased chemokine responses, and elevated Th2 promoting cytokines compared to early excision. Cellular inflammation in the wound was exacerbated with elevated neutrophils, eosinophil and monocytes. Wound cellular innate immune response decreased after late excision with a loss of inflammatory dendritic cells (DC), decreased NKT cells, and inhibition of NK cell activation. Systemically late excision increased trafficking conventional CD8α^- DC to the lymph node, but there was no apparent DC activation. This was reflected in the induction of CD4T regulatory (Treg) cells and suppression of CD8T cell proliferation after late excision. No suppression was observed with early excision.

Conclusion: This data suggests early excision of the wound, during the phase of immune down-regulation initiated by the burn, maintains an innate and adaptive immune cell response. In contrast, late wound excision induced a severe inflammatory response, with subsequent down-regulation of innate and adaptive immune cell responses. Therefore timing of excision is critical in affecting the immune response to burn.

* *Corresponding author at*: Burn Injury Research Unit, M318, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia. Tel.: +61 864888133.
E-mail addresses: vanessa.fear@uwa.edu.au (V.S. Fear), matthew.poh@uwa.edu.au (W.-P. Poh), Samantha.valvis@health.wa.gov.au (S. Valvis), Jason.Waithman@telethonkids.org.au (J.C. Waithman), Bree.foley@telethonkids.org.au (B. Foley), Fiona.Wood@health.wa.gov.au (F.M. Wood), mark.fear@uwa.edu.au (M.W. Fear).
http://dx.doi.org/10.1016/j.burns.2016.01.013

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

1. Introduction

Early excision of burn in patients has in part decreased mortality and reduced length of stay [1]. However, the optimal timing of burn excision remains unknown. In clinical practice, timing of excision has varied from less than 24 h [2], to 4 days [3], to 4–8 weeks [4]. The nature of burn and wound progression makes the decision to excise complex [5]. Clinicians must determine injury severity, forecasting the degree of thermal injury progression including heat transfer, tissue edema and decreased wound perfusion. The general health of the patient must also be assessed with respect to the systemic impact of the burn with malfunction of essential metabolic processes, risk of infection and exacerbation of inflammation [6].

In burn in murine models early excision and late excision do not differ in wound closure rate [7]. However patient studies show significant effects of the timing of excision, with reports of beneficial effects of burn excision within 48 h for severe burns [8]. In addition, prospective studies indicate early excision (within 72 h) reduced mortality in patients aged 17–30 with no inhalation injury [9]. Other studies have shown a better prognosis for severe burn patients who underwent early excision surgery within 10 days of injury [10] whilst more recent studies indicate optimal excision of burn to be 4–7 days [11].

The magnitude of the innate inflammatory response to injury has been shown to directly correspond to the subsequent down-regulation of ensuing immune responses [12,13]. In addition, high levels of innate cytokines have been correlated with increased severity of tissue trauma [14–16] and development of post-operative complications [17]. However there is a paucity of information that directly relates inflammatory, innate and adaptive immune responses to early and late excision after burn.

This study directly investigates the immune response induced by late and early excision of the burn in a murine model of non-severe burn. The systemic inflammatory response, wound inflammatory and innate cell responses and cell-mediated immunity were all assessed.

2. Methods

2.1. Mice

Adult 9 week old female C57BL/6 mice were housed under pathogen free conditions with food and water provided *ad libitum*. Approval was obtained by the Telethon Kids Institute, Animal Ethics Committee (AEC#272), all experiments performed in accordance with the National Health and Medical Research Council Australia Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Full thickness burn and excision trauma procedure

9 week old C57BL6/J female mice ($n = 80$, $n = 5$/group/time-point) received a full thickness 19-mm diameter burn wound following a previously described protocol [18]. This equates to approximately 8% total body surface area injury (TBSA (a non-severe injury model)). Sham injury mice received no surgical treatment but underwent anesthesia and received the same analgesic protocol [18]. Mice received sham or burn on day 0 followed by sham or excision surgery at day 1 or day 8. Excision surgery removed the entire 19 mm diameter burned area as previously described [18].

2.3. ILN tissue preparation

Inguinal lymph node preparations as described in detail previously [19]. In brief, ILN from individual mice were subjected to type IV collagenase digestion (1.5 mg/ml; Worthington Biochemical, Lakewood, NJ) with type I DNase (0.1 mg/ml; Sigma–Aldrich) to prepare single cell suspensions. All digestions and washes were performed in glucose sodium potassium buffer (GKN; 11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na_2HPO_4, 5.5 mM $NaH_2PO_4{\cdot}2H_2O$).

2.4. Skin tissue preparation

Skin wound sections were chopped and subjected to type IV collagenase digestion, with Dispase (2 mg/ml; Roche) for 2 h at 37 °C with shaking. Digested tissue was filtered through 100 μM mesh filters to prepare single cell suspensions. All digestions and washes were performed in GKN buffer.

2.5. FACS analysis and antibodies

Single-cell suspensions were FcR blocked (2.4G2; BD Biosciences) prior to the addition of phenotyping antibodies. ILN DC populations were identified using combinations of fluorochrome labeled mAbs (BD Pharmingen) to mouse I-A/I-E (2G9); CD11c (N418), CD11b (M1/70), CD8a (53–6.7), CD103 (M290), B220 (RA3-6B2), CD19. T cell and innate cell populations were identified in ILN digests using the fluorochromes CD3, CD4, CD8, CD25, CD44, TCR γ/δ, and NK1.1 (PK136). Labeling was performed in GKN buffer containing 0.2% BSA for 30 min on ice. A FOXP3 intracellular staining kit (eBiosciences, San Diego, CA) was used to determine intracellular Granzyme B, Ki67 (BD Biosciences), and FOXP3 staining. All Abs were used as direct conjugates to FITC, Phycoerythrin (PE), PE-Cy7, allophycocyanin (APC), APC-Cy7, Alexa Fluor 700, Brilliant Violet 421, Brilliant Violet 650, Brilliant Violet 711, Brilliant Violet 786, PE-CF594, or biotin and Streptavidin conjugated Brilliant Violet 421 (BD Biosciences, San Jose, CA) as required. Appropriately matched IgG isotype controls (BD Pharmingen, WA) and cytometer compensation settings adjusted using single-stained controls were used for each experiment. Samples were collected using an LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

2.6. Sera collection

Mice were warmed for 10 min in a heating box and 0.2 ml blood collected by tail bleed, collected in serum tubes, stored at 4 °C for 30 min, centrifuged at 13,000 × *g* for 30 min and sera collected and stored at −20 °C.

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

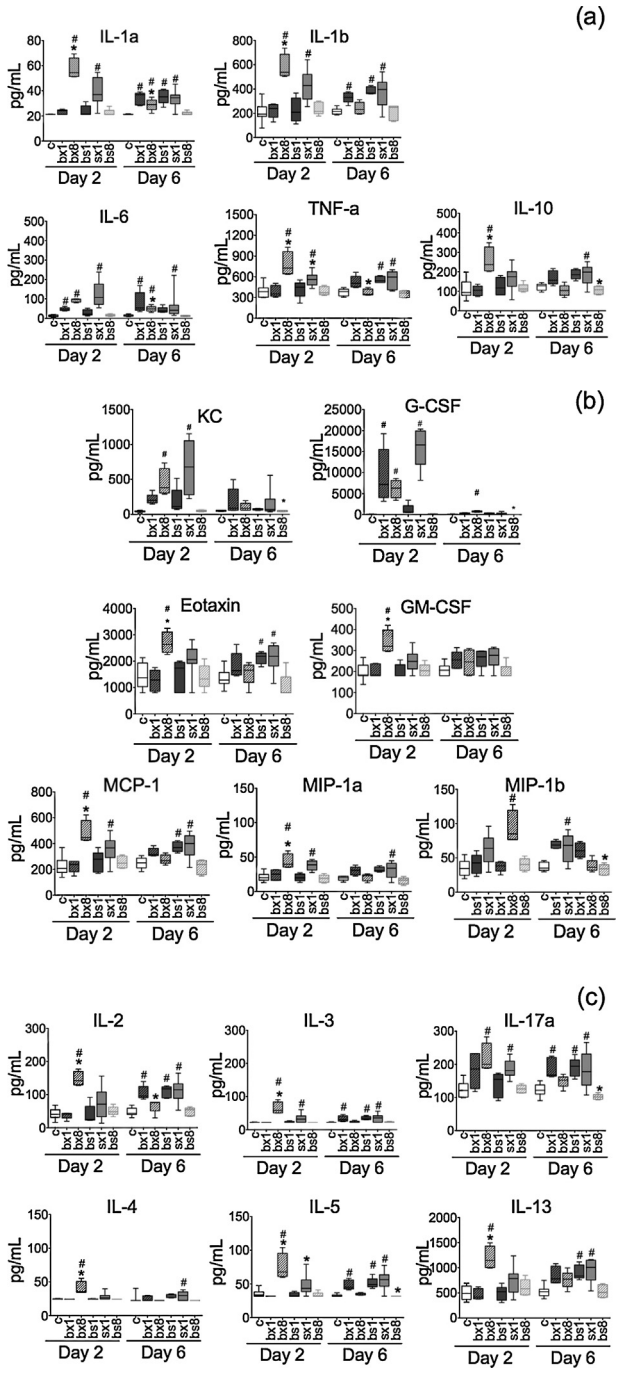


Fig. 1 – Late excision of burn exacerbates cytokine and chemokine responses. Mice received a combination of sham operation, burn and/or excision surgery and sera were collected at the indicated time-points for immune assay. (a) Inflammatory cytokines IL-1α, IL-1β, IL-6, TNFα and IL-10. (b) Chemokines KC, GCSF, Eotaxin, GM-CSF, MCP1, MIP1α, MIP1β, and (c) T cell affected cytokines IL-2, IL-3, IL-17α, IL-4, IL-5 and IL-13. Bar graphs indicate control (c) – sham injury day 0/sham surgery day 1; bs1 – burn day 0/sham surgery day 1; sx1 – sham injury day 0/excision surgery day 1; bx1 – burn day 0/excision surgery day 1; bx8 – burn day 0/excision surgery day 8; and, bs8 – burn day 0/sham surgery day 8, as indicated. Results are shown as mean ± SEM, $n = 5–10$ mice/group, for at least two independent experiments, $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to bx1.

2.7. *Cytokine analysis*

Sera cytokine analysis was performed using the Bioplex Pro mouse Cytokine, 23-Plex Assay (Bio-Rad, NSW, Australia) according to manufacturer's protocol. In brief, premixed standards were reconstituted to 50 ng/ml and serially diluted for an 8-point standard curve. Premixed beads (50 μl) were washed twice in a 96-well plate and 50 μl standard or sample added to each well. The plate was incubated at RT for 30 min with shaking. After washing beads were suspended in 125 μl of Bio-Plex assay buffer and read on a Bioplex System (Bio-Rad, NSW, Australia).

2.8. *Statistical analyses*

All results were analyzed using Prism 6 (GraphPad software). Differences between groups were compared using Kruskal–Wallis test with Dunn's test for multiple comparisons. All test groups were compared to control and all groups compared to burn excision at day 1 to identify significant changes from baseline and early excision. p-Value of <0.05 was considered statistically significant.

3. Results

3.1. *Late excision of burn leads to an exacerbated cytokine response*

Experimental groups received either burn or sham injury on day 0, followed by surgical excision or sham operation on day 1 or day 8 after injury. Experimental groups consist of control (con; sham injury day 0/sham surgery day 1), burn/sham day 1 (bs1; burn day 0/sham surgery day 1), sham/excision day 1 (sx1; sham injury day 0/excision surgery day 1), burn/excision day 1 (bx1; burn day 0/excision surgery day 1), burn/excision day 8 (bx8; burn day 0/excision surgery day 8) and burn/sham day 8 (bs8; burn day 0/sham surgery day 8). Sera was isolated from the six treatment groups at day 2 or day 6 after the excision or sham surgery and cytokine profiles obtained (Fig. 1). All levels were compared to control levels and to the levels after burn and excision at day 1. At day 2 after surgery sham/excision day 1 and burn/excision day 8 serum cytokines IL-1α, IL-1β were significantly elevated compared to control and burn/excision day 1 whilst IL-6 was significantly elevated compared to control (Fig. 1a). At day 6 levels remained elevated in the burn excision day 8 group whilst significantly elevated levels of IL-1α and IL-1β were observed in the burn/sham day 1, with significantly increased IL-6 also observed in the burn/excision day 1. TNFα and IL-10 were significantly elevated at day 2 compared to the control and burn/excision day 1. On day 6, TNFα was significantly elevated for the sham/excision day 1. These data indicate an early pro-inflammatory response after late excision which appears to be sustained.

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

Chemokine levels were also assessed (Fig. 1b). KC (CXCL1), a neutrophil attractant increased significantly at day 2 after sham/excision day 1 and burn/excision day 8 compared to control. Granulocyte colony stimulating factor (G-CSF) increases neutrophil mobilization and proliferation. A significant increase in G-CSF occurred at day 2 after sham/excision day 1 and burn/excision day 8, and this persisted at day 6 in the latter group compared to control. Eotaxin, an eosinophil, basophil, mast cell, Th2 cell and platelet attractant was significantly increased in the burn/excision day 8 group at day 2 compared to control and burn/excision day 1 group. At day 2 GM-CSF, a cytokine that increases DC maturation and function as well as macrophage activity [20], had significantly elevated levels after burn/excision day 8 compared to both control and burn/excision day 1 groups. MCP1, a monocyte, immature DC and memory T cell attractant was significantly increased at day 2 in the burn/excision day 8 compared to both control and burn/excision day 1. At day 6 there was significantly increased MCP-1 in the burn/sham day 1 and sham/excision day 1 groups. Levels in the burn/excision day 8 were not significantly different from control. MIP1α (CCL3) and MIP1β (CCL4) attract monocytes, T cells and polymorphonuclear cells (PMNs). MIP1α levels were significantly elevated at day 2 for the burn/excision day 8 compared to control and burn/excision day 1 groups. MIP1β levels were significantly increased at day 2 for burn excision/day 8 group compared to control. This indicates significant induction of KC, G-CSF, GM-CSF, MCP-1, MIP1α and MIP1β chemokine responses specific to late excision after the burn.

T cell affected cytokines were also assessed (Fig. 1c). IL-2 and IL-3 were significantly elevated on day 2 after burn/excision day 8 and this was down-regulated by day 6. Importantly, the increase in IL-2 and IL3 was also significant when compared to the burn excision at day 1. There was significantly elevated IL-2 at day 6 for the burn/sham day 1, sham/excision day 1 and burn/excision day 1 groups, and this was not observed in the burn/sham day 8. IL-17 at day 2 significantly increased for the sham/excision day 1 and burn/excision day 8, and at day 6 for the burn/sham day 1. IFN-γ showed a significant increase at day 6 after sham/excision day 1 only, with no other significant changes between groups for IFNγ, IL-12p40 or IL-12p70 (data not shown). Strikingly, IL-4, IL-5 and IL-13 were strongly up-regulated at day 2 in the burn/excision day 8 compared to control and burn/excision day 1 groups with significant increases at day 6 for IL-5 and IL-13 in burn/sham day 1 and sham/excision day 1. These data indicate that in the late burn excision group there was an early release of IL-2 and pro-Th2 cytokines.

3.2. *Cellular inflammation is increased after late surgical excision of burn*

Cellular inflammation in the wound was also investigated ([21], gating strategy, Fig. 2A).

Wound cell numbers were significantly increased at day 2 after burn/sham day 1 and burn/excision day 8. At day 6 wound cell numbers were significantly increased in the sham/excision day 1, burn/excision day 1, and burn/excision day 8 groups compared to control (Fig. 2b). PMNs were next examined in the wound (Fig. 2c). There was significantly increased early neutrophil number in the wound (day 2) for the burn/excision day and burn/sham day 8 groups. At day 6 neutrophil cell numbers were significantly increased for the sham/excision day 1, burn/excision day 1 and burn/excision day 8 groups. Notably the burn/sham day 8 neutrophil cell numbers were similar to control. Eosinophil cell numbers in the wound were significantly increased in the burn/excision day 8 compared to the burn/sham day 1 and burn/excision day 1 at day 2. Macrophage cell numbers were not significantly altered between treatment groups. Monocyte cell numbers were significantly increased at day 2 for the burn/excision day 8 compared to the burn/excision day 1; and at day 6 the burn/sham day 8 monocyte numbers were significantly increased compared to the control. These data indicate an early wound cellular infiltrate of eosinophil and monocytes specific to late excision of the burn.

LC cell numbers were significantly increased at day 2 and day 6 for burn/sham day 8 compared to the control and burn/excision day 8, respectively (Fig. 2d). The cDC cell numbers were not significantly altered at either day 2 or 6 between all treatment groups (Fig. 2d). Interestingly, the Ly6C^{hi} DC percentage frequency of cDC in the burn/excision day 1 was significantly increased at day 2 and 6 compared to the control and significantly increased at day 2 compared to the burn/excision day 8. These data indicate reduced frequency of LC and DC, in particular monocyte-derived DC, in the wound site after late excision of the burn.

NKT and NK were next examined in the wound at day 6 (Fig. 2e). NKT cell numbers were significantly decreased in burn/excision day 8 compared to the control and burn/excision day 1 groups. Conversely, the NK cell numbers in the wound were significantly elevated for the burn/excision day 1 and burn/excision day 8 compared to the control. NK cell activation, as determined by CD11b up-regulation [22] was significantly increased in the burn/sham day 1, sham/excision day 1 and burn/excision day 1. Notably, the burn/excision day 8 did not show NK cell activation on CD11b compared to the control. These data indicate a loss of NK cell activation in the wound after late excision of the burn, whereas early excision induced NK cell activation.

3.3. *Innate immune cell responses in the wound draining lymph node*

Activation of innate immune cell, NKT, NK and γδT, populations in response to burn and excision surgery were next examined in the wound draining lymph node (inguinal lymph node, ILN (gating strategy, Fig. 3a)). Total ILN cell numbers (Fig. 3b) were significantly increased at day 2 in the burn/sham day 1, sham/excision day 1 and burn/excision day 8 groups. Notably, there were significantly increased cell numbers at day 6 in the ILN for burn/excision day 8 compared to the burn/excision day 1 group.

NKT, NK and γδT cell numbers were determined in the ILN. There were significantly elevated cell numbers of NKT, NK and γδT cells at day 2 in burn sham and sham excision groups compared to the control (Fig. 3c). Strikingly, at day 6 NKT, NK and γδT cell numbers were significantly elevated in the burn/excision day 8 compared to burn/excision day 1 and control.

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

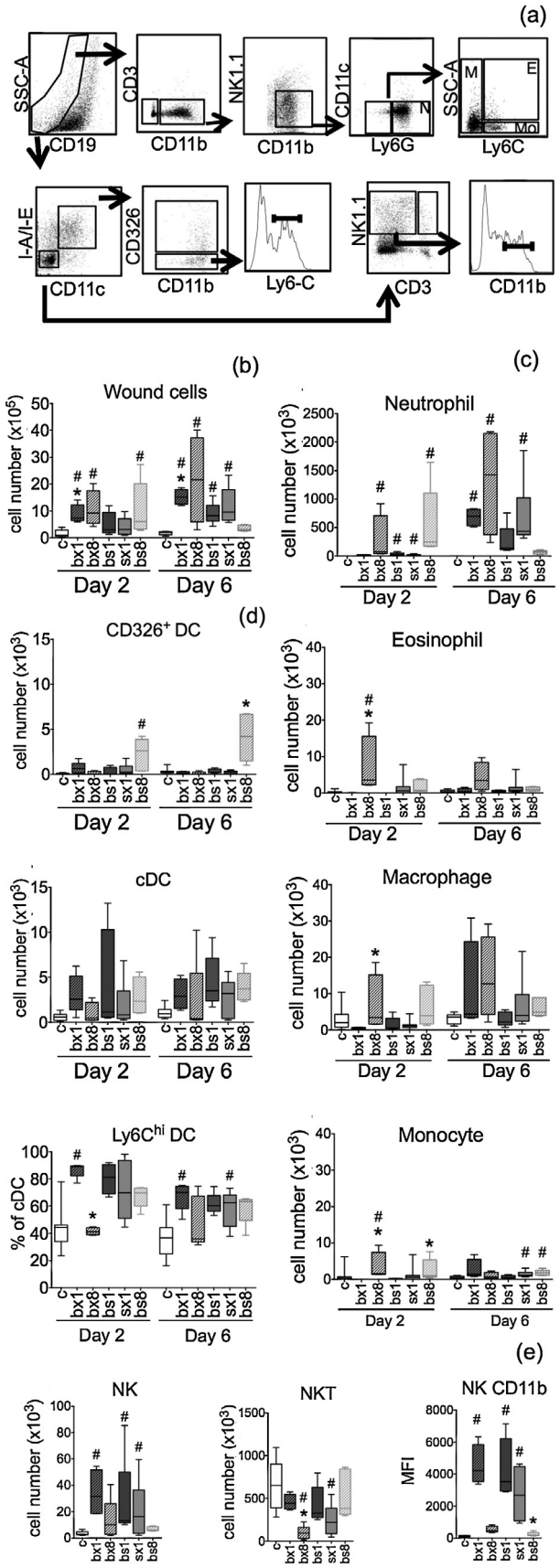


Fig. 2 – Wound increased inflammatory cell response and decreased innate cell response after late excision of the burn. Mice were subject to a combination of sham operation, burn and/or excision surgery and wound cells harvested to single cell suspension. (a) After live cell gating on forward and side scatter, CD19-cells were further gated $CD3^-$, $CD11b^+$, and $NK1.1^+$ cells were gated out. Remaining cells were then gated $Ly6G^{hi}$ (neutrophils), or $Ly6G^{lo}$ with further gating on SSC-$A^{hi}Ly6C^{lo}$ (macrophages) and $Ly6C^{hi}$SSC-A^{lo} (monocytes), and SSC-$A^{hi}Ly6G^{hi}$ (eosinophils). Dendritic cell were gated $CD19^-$, I-A/I-$E^{hi}CD11c^{hi}$ with subsequent gating on $CD326^+$ (LCs), $CD326^-$ (DC), and the DC were further stratified for $Ly6C^{hi}$ (monocyte derived DC). Innate lymphoid cells were gated $CD19^-$I-A/I-E^-CD11c^-, and subsequently $CD3^+NK1.1^+$ (NKT cells) or $CD3^-NK1.1^+$ (NK cells), NK cells were further gated on $CD11b^{hi}$. (b) Total cell number in wound. (c) Neutrophil, eosinophil, macrophage and monocyte cell number. (d) LC and DC cell numbers, and $Ly6C^{hi}$ percentage frequency in DC population. (e) Innate immune cells NKT and NK cell number at day 7, and $CD11b^{hi}$ percentage frequency of NK cells. Bar graphs indicate control (c) – sham injury day 0/sham surgery day 1; bs1 – burn day 0/sham surgery day 1; sx1 – sham injury day 0/excision surgery day 1; bx1 – burn day 0/excision surgery day 1; bx8 – burn day 0/excision surgery day 8; and, bs8 – burn day 0/sham surgery day 8, as indicated. Results are shown as mean ± SEM, $n = 5–10$ mice/group, for at least two independent experiments, $^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to bx1.

Within the NKT cell population, at day 2, there were significantly decreased frequency of NKT cells harboring granzyme B in the sham/excision day 1, with a similar trend in the burn/excision day 1 group compared to control (Fig. 3d). Up-regulation of CD25 on NKT cells was significantly increased at day 2 in the sham/excision day 1 and burn/excision day 8 compared to both control and burn/excision day 1 groups (Fig. 3d). This difference was sustained to day 6. Similarly NKT Ki67 expression, a marker of proliferation, was significantly decreased in the burn/excision day 1 compared to the burn/sham day 1 and burn/excision day 8 at day 2. The sham/excision day 1 had significantly increased Ki67 expression in NKT cells at day 6 compared to the control. Therefore in late excision of burn there appears to be activation/proliferation of NKT cells in the ILN that is not observed after early excision of the burn.

NK cell activation was next examined in the ILN after injury. We observed a significant decrease in granzyme B expression in the ILN for the sham/excision day 1 and trend for a decrease in the burn/excision day 8 group compared to the burn/excision day 1 group at day 2. At day 6, the burn/excision day 8 group showed significantly less granzyme B expression in NK cells than the burn/excision day 1 group. Conversely, there was a significant increase in CD25 expression at day 2 for the sham/excision day 1 and burn/excision day 8 groups compared to control which persisted to day 6 (Fig. 3e). Interestingly, at day 6 burn/sham day 1, sham/excision day 1 and burn/excision day 1 all had significantly increased NK cell proliferation as determined by Ki67 expression. Interestingly at day 2 burn/excision day 8 had significantly suppressed Ki67 expression compared to the control and this was

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

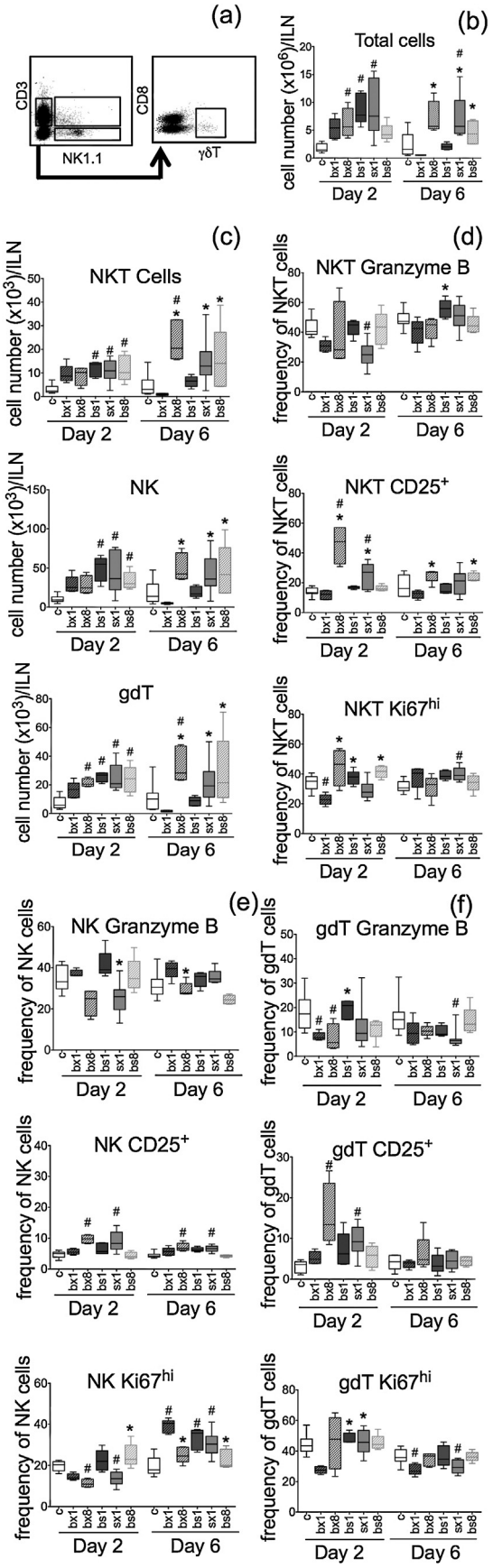


Fig. 3 – Different innate immune cell response in the ILN after early and late excision of burn. Mice were subject to a combination of sham operation, burn and/or excision surgery and ILN cells harvested to single cell suspension at times indicated. (a) Gating strategy, live cells were gated on forward and side scatter, and NKT cells gated on $CD3^+NK1.1^+$, NK cells gated on $CD3^-NK1.1^+$, and γδT cells gated $CD3^+$ then γδ TCR^+. (b) ILN total cell numbers. (c) NKT, NK and γδT cell numbers in ILN. (d) NKT percentage frequency of cells with expression of granzyme B, CD25 and $Ki67^{hi}$. (e) NK percentage frequency of cells with expression of granzyme B, CD25 and $Ki67^{hi}$. (f) γδT percentage frequency of cells with expressing granzyme B, CD25 and $Ki67^{hi}$. Results are shown as mean ± SEM, $n = 5–10$ mice/group, for at least two independent experiments, $^*p < 0.05$ compared to control; $^\#p < 0.05$ compared to bx1.

suppressed at day 6 compared to the burn/excision day 1 group. Therefore ILN NK cell numbers increased for burn/sham day 1 and sham/excision day 1, however with a combination of burn and excision no increase was observed. Notably there was a loss of cell proliferation for the NK cell population after late excision of the burn.

γδT frequency of granzyme B cells were significantly reduced at day 2 in both burn excision groups compared to control and at day 6 in the sham/excision day 1 compared to the control (Fig. 3f). There was increased CD25 expression at day 2 for the sham/excision day 1 and burn excision/day 8, compared to control (Fig. 3f). These data indicate a γδT response in the late excision after burn not observed in the early surgical excision of the burn.

3.4. *Lymph node dendritic cell response after early and late burn excision*

Total lymph node DC populations LCs, plasmacytoid DC (pDC), cDC with delineation of $CD8\alpha^+$ and $CD8\alpha^-$ to determine resident and trafficking population, respectively, and CD11b and CD103 to determine $CD8\alpha^-$ DC subsets were determined (Fig. 4a).

At day 2 after burn/sham day 1 there was a significant increase in the cell number of LC, pDC and cDC compared to the control (Fig. 4b). On day 6 there was a significant change in the LC, pDC and cDC populations which were reduced in the burn/excision day 1, compared to the burn/excision day 8 group. Further examination of cDC, resident and trafficking, revealed a significant increase in $CD8a^+$ DC cell frequency for burn/excision day 1 compared to burn/excision day 8 and control groups; and the converse was true for the $CD8a^-$ trafficking DC population (Fig. 1c). Examination of $CD8a^-$ trafficking DC subsets revealed significant cell frequency changes in the burn/sham day 1, sham/excision day 1 and burn/excision day 1 for $CD11b^{lo}$ DC that was not apparent in the burn/excision day 8 (Fig. 4d). These data indicate increased DC in the lymph node after late burn excision, in particular $CD8a^-$ cDC, however no changes in trafficking cDC profiles indicative of activation were observed.

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

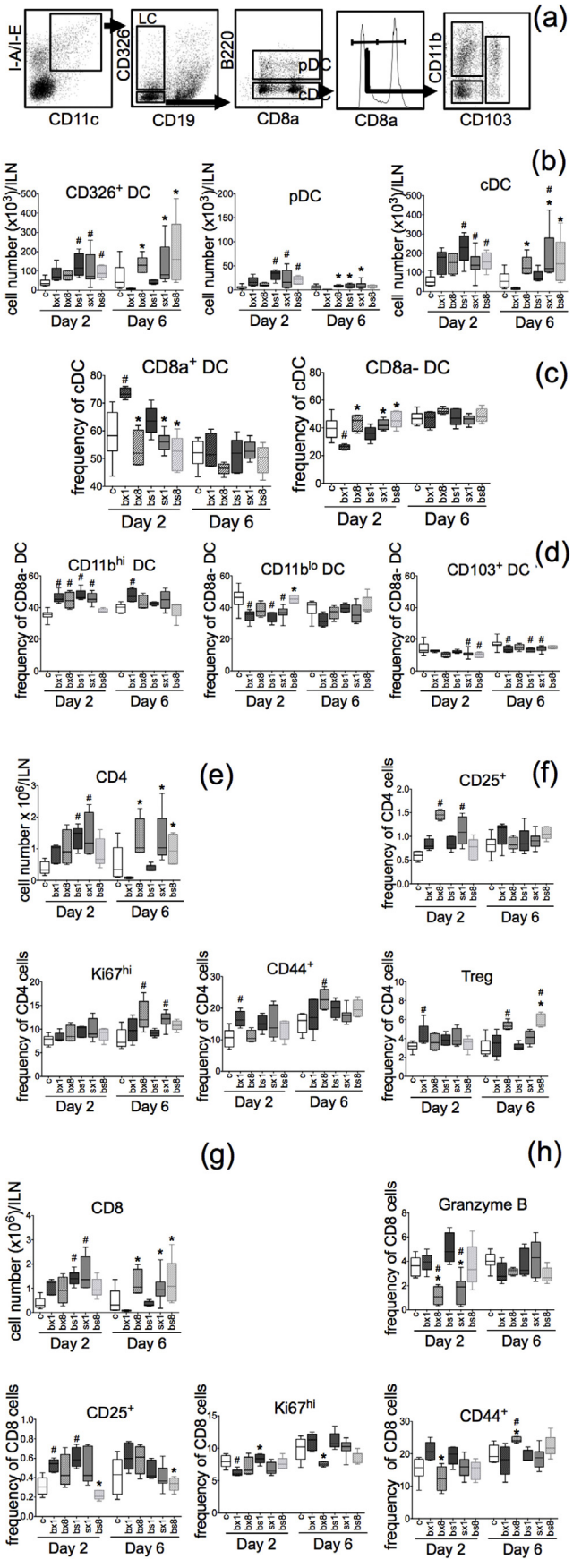


Fig. 4 – Altered immune response to early and late excision of burn. Mice were subject to a combination of sham operation, burn and/or excision surgery and ILN cells harvested to single cell suspension at times indicated. (a) DC gating strategy, after forward and side scatter gating, DCS were gated I-A/I-E^{hi} CD11c^{hi}. DC subpopulations were determined as CD19-CD326^{+} for LCs, CD19^{-}CD326^{-} then B220^{+} for pDC, and CD19^{-}CD326^{-} and B220^{-} for cDC. The cDC were further gated CD8a^{+} and CD8a^{-}, the latter DC subpopulation were then gated CD103^{+}, CD11b^{hi} and CD11b^{lo}. (b) ILN cell number for CD326+ (LCs), pDC and cDC. (c) cDC percentage frequency of resident CD8α^{+} and trafficking CD8α^{-} DC. (d) CD8a^{-} DC percentage frequency of CD11b^{hi}, CD11b^{lo} and CD103^{+} subsets. (e) ILN total cell number of CD4^{+} T cells. (f) CD4T cell percentage frequency CD25^{+}, Ki67^{hi}, CD44^{+} and Treg. (g) ILN cell number of CD8^{+} T cells. (h) CD8T cell percentage frequency of granzyme B^{+}, CD25^{+}, Ki67^{hi} and CD44^{+} cells. Results are shown as mean ± SEM, n = 5–10 mice/group, for at least two independent experiments, $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to bx1.

3.5. *T cell response after early and late burn excision*

Lymph node CD4 and CD8T cells at day 2 were increased significantly following burn/sham day 1 and sham/excision day 1 (Fig. 4e, g). Significantly reduced CD4 and CD8T cell numbers were observed by day 6 for burn/excision day 1 compared to burn excision/day 8. Further analysis of CD4T cells indicated increased frequency of CD25 expression at day 2, with increased proliferation at day 6 for the sham/excision day 1 and burn/excision day 8 compared to control. At day 6 in the burn/excision day 8 and burn/sham day 8 the frequency of Treg cells significantly increased compared to control (Fig. 4f).

Within the CD8T cell compartment (Fig. 4h) there was a reduction in Granzyme B percentage frequency of cells at day 2 in the sham/excision day 1 and burn/excision day 8 compared to control and burn/excision day 8 groups. CD25 expression, at day 2, increased in the burn/sham day 1 and burn/excision day 1 compared to control. Proliferation, as determined by Ki67^{hi} expression, was significantly reduced at day 2 in the burn/excision day 1 compared to the control. Additionally, there was significantly reduced Ki67^{hi} expression in the day 6 burn/excision day 8 compared to the burn/excision day 1 group. Only the burn/excision day 8 showed increased CD44 expression at day 6 compared to control and burn/excision day 1 group, although CD44 expression was significantly less at day 2 in burn/excision day 8 compared to the burn/excision day 1 group.

These data indicate differential CD4 and CD8T cell responses to early and late excision after burn. A CD4T cell response was observed after late excision, with development of Treg cells that was not observed after early excision. CD8T cells were less activated after late excision and developed CD44 expression indicative of a T effector or memory cell response.

4. Discussion

Here we demonstrate a differential immune response between early and late excision of a burn. The data suggests early

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

excision is less disruptive and damaging to the subsequent immune responses and is therefore likely to be beneficial to patient outcomes.

We [18], and others [23–26], have previously shown that burn and excision injury induces an inflammatory response including IL-1α, IL-1β, TNFα, and IL-6. The stimulation of the inflammatory response is a suggested cause of decreased vascular tone and cardiac insufficiency after thermal injury. IL-1 increases adhesion molecule expression on endothelial cells to allow transmigration of immune cells and induces hyperalgesia, vasodilation and hypertension [27]. Further IL-1α stimulates monocyte production of IL-1β, amplifying inflammation and recruiting macrophages [28]. TNFα, which promotes fever and tachycardia, was similarly exacerbated after late excision of burn.

IL-1β and TNFα stimulate IL-6 production leading to hepatic release of C-reactive proteins and other acute phase proteins [12]. IL-6 influences inflammation *via* stimulation of bone marrow release of PMN cells [29], and is associated with increased severity of tissue trauma [16], and multiple organ failure [29]. Further IL-6 induces macrophage prostaglandin E2 (PGE2) release, known to decrease DC function [30] and inhibit T cell mitogenesis and IL-2 production [31,32].

Interestingly IL-1, IL-6 and TNFα are all potent inducers of GM-CSF [20], and overexpression of GM-CSF leads to macrophage accumulation and severe damage to tissue [33]. Surprisingly, GM-CSF treatment increases the frequency of Treg cells in autoimmune disease [34]. We show significantly increased GM-CSF specific to late excision of burn where a Treg response developed. This may exacerbate increased infection rates in patients in intensive care with delayed burn surgery [35].

Late burn excision induced a prominent chemokine response with early release of the chemoattractants KC (CXCL1), Eotaxin (CCL11), MCP-1 (CCL2), MIP-1a (CCL3), MIP-1b (CCL4) and GM-CSF. This increased chemokine response correlated with an acute inflammatory cell response of neutrophils, eosinophils and monocytes in the wound and was specific to late excision. Notably there was also a loss of innate cells in the wound including LCs and inflammatory DCs. The changes observed in cytokine profiles broadly suggest early excision can ameliorate the inflammatory storm after injury and these effects are not observed with late excision. This is supported by a previous study that showed successive delays in burn excision decreased any positive effects on measured cytokine levels [36].

NKT cell number and NK cell maturation/activation were down-regulated in the wound after late excision. In the ILN after late excision there was also a decrease in NK cell proliferation that may reflect the observed loss of DC activation signal at this site. Interestingly high expression of IL-4 has been linked with repression of some key effector functions of NK cells favoring tolerogenic or Th2 responses [37]. Other research has similarly observed innate immune cell paralysis [38] with normal NK cell number and reduced NK cell function in both thermal and trauma injury [39]. NKT and NK cell are early producers of IFNγ during inflammation [38], and dysfunction of these cellular responses at the wound site may lead to the observed lack of an IFNγ response.

Adaptive cell cytokine responses specific to late burn excision included IL-2, IL-3, IL-17, IL-4, IL-5 and IL-13, and these were not elevated after early excision. The adaptive immune response after late burn excision included the development of CD4 Treg cells and increased CD8T effector or memory cells in the skin draining lymph node. This was not observed after early excision. Our study identifies dysfunction of adaptive immunity after burn treatment with late excision. Potentially this will manifest later as a tolerance to self-antigen or inactivity toward microbial pathogens. Similarly after UVR exposure, the adaptive immune response is suppressed with enhanced suppression by Treg cells [40], in combination with DC dysfunction [41] that is suggested to lead to reduced immune responses [42]. Accordingly, bacterial infection rates are higher after late excision compared to early excision of burn wounds [43].

In other research a reversal of innate NK cell dysfunction occurs with pre-operative administration of IFNα [44]. Notably, late burn excision IL-6 potentiation of PGE_2 levels would lead to suppression of type I IFN production. Decreased type I IFN, an important signaling cytokine in protection against microbial pathogens, would render patients more susceptible to infection. Therefore, PGE_2 inhibitor [32] or type I IFN administration may limit immune dysfunction and increased infection.

Whilst many of these observed changes are indicative of increased susceptibility to infection, in this study functional studies were not completed. It will be critical to supplement these observational studies with further functional work. Indeed, others have assessed functional changes and observed changes in T cell proliferation and cytotoxic T cell function after burn, in part changed by excision and/or grafting [45,46]. Similar assays, as well as investigation into responses to infection and or cancer in these models will provide important insight into the physiological consequences of the burn on immune system function, in the acute phase and in the long-term. In addition, the use of grafting to more closely mimic clinical practice could also provide more insight into the relevance of different changes after burn [45,46].

This study directly compared the immune responses after early and late wound excision following burn. The data highlights the extreme inflammation and subsequent immune dysfunction specific to late excision (day 8) that will directly impact on organ dysfunction and susceptibility to infection. In addition, recent work demonstrating the importance of timing and type of inflammatory response to scar formation and patient outcome suggests excision timing is likely to also impact on scar [47]. Early surgical intervention is supported to reduce the systemic impact of burn. Alternatively, treatment with immune-modulatory agents may be beneficial in cases where early excision is not feasible after burns.

Conflict of interest

The authors state there are no conflicts of interest.

Funding source

This project was supported by the Fiona Wood Foundation. MF is supported by Chevron Australia.

Acknowledgements

Mark Fear is supported by Chevron Australia. The authors also acknowledge the support of the Fiona Wood Foundation for funding of this work.

REFERENCES

[1] Ong YS, Samuel M, Song C. Meta-analysis of early excision of burns. Burns 2006;32:145–50.
[2] Engrav LH, Heimbach DM, Reus JL, Harner TJ, Marvin JA. Early excision and grafting vs. non-operative treatment of burns of indeterminant depth: a randomized prospective study. J Trauma 1983;23:1001–4.
[3] Sorenson B. Acute excision or exposure treatment? Results of a randomized controlled clinical trial. Scand J Plast Reconstr Surg 1979;13:115–8.
[4] Mohammadi AA, Bakhshaeekia AR, Marzban S, Abbasi S, Ashraf AR, Mohammadi MK, et al. Early excision and skin grafting versus delayed skin grafting in deep hand burns (a randomized clinical controlled trial). Burns 2011;37:36–41.
[5] Rousseau A-F, Massion PB, Laungani A, Nizet J-L, Damas P, Ledoux D, et al. Toward targeted early burn care: lessons from a European survey. J Burn Care Res 2014;35:e234–9.
[6] Singh V, Devgan L, Bhat S, Milner SM. The pathogenesis of burn wound conversion. Ann Plast Surg 2007;59:109–15.
[7] Wu JC, Rose LF, Christy RJ, Leung KP, Chan RK. Full-thickness thermal injury delays wound closure in a murine model. Adv Wound Care 2014;4:83–91.
[8] Barret JP, Herndon DN. Effects of burn wound excision on bacterial colonization and invasion. Plast Reconstr Surg 2003;111:744–50.
[9] Herndon DN, Barrow RE, Rutan R, Rutan TC, Desai MH, Abston S. A comparison of conservative versus early excision therapies in severely burned patients. Am J Surg 1989;209:547–53.
[10] Pavoni V, Gianesello L, Paparella L, Buoninsegni LT, Barboni E. Outcome predictors and quality of lifer of severe burn patients admitted to intensive care unit. Scan J Trauma Resusc Emerg Med 2010;18:24.
[11] Saaiq M, Zaib S, Ahmad S. Early excision and grafting versus delayed excision and grafting of deep thermal burns up to 40% total body surface area: a comparison of outcome. Ann Burns Fire Disasters 2012;25:143–7.
[12] Ni Choileain NN, Redmond PH. Cell response to surgery. Rev Arch Surg 2006;141:1132–40.
[13] Tschoeke SK, Moldawer LL. Human leukocyte antigen expression in sepsis: what have we learned. Crit Care Med 2005;33:236–7.
[14] Shenkin A, Fraser WD, Series J, Winstanley FP, McCartney AC, Burns HJ, et al. The serum interleukin 6 response to elective surgery. Lymphokine Res 1989;8:123–7.
[15] Mokart D, Capo C, Blache JL, Delpero JR, Houvenaeghel G, Martin C, et al. Early postoperative compensatory anti-inflammatory response syndrome is associated with septic complications after major surgical trauma in patients with cancer. Br J Surg 2002;89:1450–6.
[16] Cruickshank AM, Fraser WD, Burns HJ, Van Damme J, Shenkin A. Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. Clin Sci 1990;79:161–5.
[17] Baigrie RJ, Lamont PM, Kwiarkowski D, Dallman MJ, Morris PJ. Systemic cytokine response after major surgery. Br J Surg 1992;79:757–60.
[18] Valvis SM, Waithman H, Wood FM, Fear MW, Fear VS. The immune response to skin trauma is dependent on the etiology of injury in a mouse model of burn and excision. J Invest Dermatol 2015;35:2119–28.
[19] Fear VS, Burchell JT, Lai SP, Wikstrom ME, Blank F, von Garnier C.. et al. Restricted aeroallergen access to airway mucosal dendritic cells in vivo limits allergen-specific CD4+ T cell proliferation during the induction of inhalation tolerance. J Immunol 2011;187:4561–70.
[20] Shi Y, Lu CL, Roberts AI, Das J, Xu G, Ren G, et al. Granulocyte-macrophage colony stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. Rev Cell Res 2006;16:126–33.
[21] Rose S, Misharin A, Perlman H. A novel Ly6C/Ly6G based strategy to analyze the mouse splenic myeloid compartment. Cytometry A 2013;81A:343–50.
[22] Walzer T, Vivier E. G-protein-coupled receptors in control of natural killer cell migration. Rev Trends Immunol 2011;32:486–92.
[23] De AK, Laudanski K, Miller-Graziano CL. Failure of monocytes of trauma patients to convert to immature dendritic cells is related to preferential macrophage-colony-stimulating factor-driven differentiation. J Immunol 2003;170:6355–62.
[24] Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat Immunol 2000;1:510–4.
[25] Vicari AP, Chiodoni C, Vaure C, Aït-Yahia S, Dercamp C, Matsos F, et al. Reversal of tumour induced cell paralysis by CpG immunostimulatory oligoneucleotide and interleukin-10 receptor antibody. J Exp Med 2002;196:541–9.
[26] Sheridan RL. A great constitutional disturbance. N Engl J Med 2002;345:1271–2.
[27] Hersiger S, Simmen H-P, Werner CML, Wanner GA, Rittirsch D. Danger signals activating the immune response after trauma. Mediators Inflamm 2012;2012:1–10.
[28] Rider P, Carmi Y, Guttman O, Braiman A, Cohen I, Voronov E, et al. IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol 2011;18:4835–43.
[29] Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. J Trauma 1995;39:411–7.
[30] Scott NM, Ng RLX, Gorman S, Norval M, Waithman J, Hart PH. Prostaglandin E_2 imprints a long-lasting effect on DC progenitors in the bone marrow. J Leukoc Biol 2014;95:225–32.
[31] Phipps RP, Stein SH, Roper RL. A new view of prostaglandin E regulation of the immune response. Immunol Today 1991;12:349–52.
[32] Coulomb F, Jaworska J, Verway M, Tzelepis F, Massoud A, Gillard J, et al. Targeted prostaglandin E_2 inhibition enhances antiviral immunity through induction of type I interferon and apoptosis in macrophages. Immunity 2014;40:554–68.
[33] Lang RA, Metcalf D, Cuthbertson RA, Lyons I, Stanley E, Kelso A. Transgenic mice expressing a hematopoietic macrophage-colony stimulating factor (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. Cell 1987;51:675–86.
[34] Gangi E, Vasu C, Cheatem D, Prabhakar BS. IL-10-producing CD4+ CD25+ regulatory T cells play a critical role in granulocyte-macrophage colony-stimulating factor-induced suppression of experimental autoimmune thyroiditis. J Immunol 2005;174:7006–13.
[35] Fagan SP, Bilodeau M-L, Goverman J. Burn intensive care. Surg Clin N Am 2014;94:765–79.

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013

[36] Chang KC, Ma H, Liao WC, Lee CK, Lin CY, Chen CC. The optimal time for early burn wound excision to reduce pro-inflammatory cytokine production in a murine burn injury model. Burns 2010;36:1059–66.
[37] Marcais A, Viel S, Grau M, Henry T, Marvel J, Walzer T. Regulation of mouse NK cell development and function by cytokines. Frontiers Immunol 2013;4:450.
[38] Schneider DR, Glenn CH, Faunce DE. Innate lymphocyte subsets and their immunoregulatory roles in burn injury and sepsis. J Burn Care Res 2007;28:365–79.
[39] Blazar BA, Rodrick ML, O'Mahony JB, Wood JJ, Bessey PQ, Wilmore DW, et al. Suppression of natural killer-cell function in humans following thermal and trauma injury. J Clin Immunol 1986;6:26–36.
[40] Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, et al. Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+ CD25+ cells in the draining lymph nodes. J Immunol 2007;179:6273–83.
[41] Ng RX, Scott NM, Strickland DH, Gorman S, Grimbaldeston MA, Norval M, et al. Altered immunity and dendritic cell activity in the periphery of mice after long-term engraftment with bone marrow form ultraviolet-irradiated mice. J Immunol 2013;2:5471–84.
[42] Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by UVR: more that just the effects of vitamin D? Rev Nat Rev Immunol 2011;11:548–96.
[43] Saaiq M, Ahmad S, Zaib MS. Burn wound infections and antibiotic susceptibility patterns at Pakistan Institute of Medical Sciences, Islamabad, Pakistan. World J Plast Surg 2015;4:9–15.
[44] Tai L-H, Zhang J, Scott KJ, de Souza CT, Alkayyal AA, Ananth AA, et al. Perioperative influenza vaccination reduces postoperative metastatic disease by reversing surgery-induced dysfunction in natural killer cells. Clin Cancer Res 2013;19:5104–15.
[45] Hultman CS, Cairns BA, deSerres S, Frelinger JA, Meyer AA. Early, complete burn wound excision partially restores cytotoxic T lymphocyte function. Surgery 1995;118:421–9.
[46] Schwacha MG, Knöferl MW, Chaudry IH. Does burn wound excision after thermal injury attenuate subsequent macrophage hyperactivity and immunosuppression? Shock 2000;14:623–8.
[47] van den Broek LJ, van der Veer WM, de Jong EH, Gibbs S, Niessen FB. Suppressed inflammatory gene expression during human hypertrophic scar compared to normotrophic scar formation. Exp Dermatol 2015;24:623–9.

Please cite this article in press as: Fear VS, et al. Timing of excision after a non-severe burn has a significant impact on the subsequent immune response in a murine model. Burns (2016), http://dx.doi.org/10.1016/j.burns.2016.01.013