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

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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.


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; Henschen 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).
RESULTS

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

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

Changes in dendritic cell population and maturation are different following burn and excision injury
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, CD8α-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 CD8a, 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+, CD11bhi, or CD11blo (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, CD11bhi DC, and CD11blo 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, CD11bhi DC, or CD11blo 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 < 0.01 compared with control; ***P < 0.001 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, CD11bhi DC and CD11blo DC numbers were significantly increased compared with controls at day 3 (Figure 2d) with CD11bhi number remaining elevated after burn injury at day 7 (Figure 2d).
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 CD8α− 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+, CD11bhi, or CD11blow 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 CD8α− DC and CD8α−γ DC after burn injury compared with control (Figure 2g). Within the CD8α− 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 (Ki67hi) 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 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 Ki67hi 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).
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 CD11bhi DC in the ILN after burn compared with the control (Figure 4b). Moreover, there was reduced MHC II MFI on CD11bhi and CD11blo DC post burn compared with the control (Figure 4b). 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 Concanavalin 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 Concanavalin 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
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 CD8α+ 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 CD8α+ 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/maturaiton 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 proliferation 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.

**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$_2$HPO$_4$, 5.5 mM NaH$_4$PO$_4$·2H$_2$O).

**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/E (2G9), CD11c (N418), CD11b (M1/70), CD6a (53-6.7), or CD103/3 bio (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 cytokometer 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% isoflurane, 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 000g 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

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**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.
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 μg ml⁻¹ gentamycin, and 20 μg 2-ME and plated at 3 x 10⁵ cells per well in 96-well plates (Nunclon, Nunc, Denmark) with Concanavalin 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.

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**REFERENCES**


Beatty SR, Rose CE Jr, Sung SS. (2007) Diverse and potent chemokine production in RPMI 1,640 with glutamine (Invitrogen Life Technologies, Australia) supplemented with 10% fetal calf serum, 20 μg ml⁻¹ gentamycin, and 20 μg 2-ME and plated at 3 x 10⁵ cells per well in 96-well plates (Nunclon, Nunc, Denmark) with Concanavalin 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.


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


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


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


