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.

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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).

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

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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

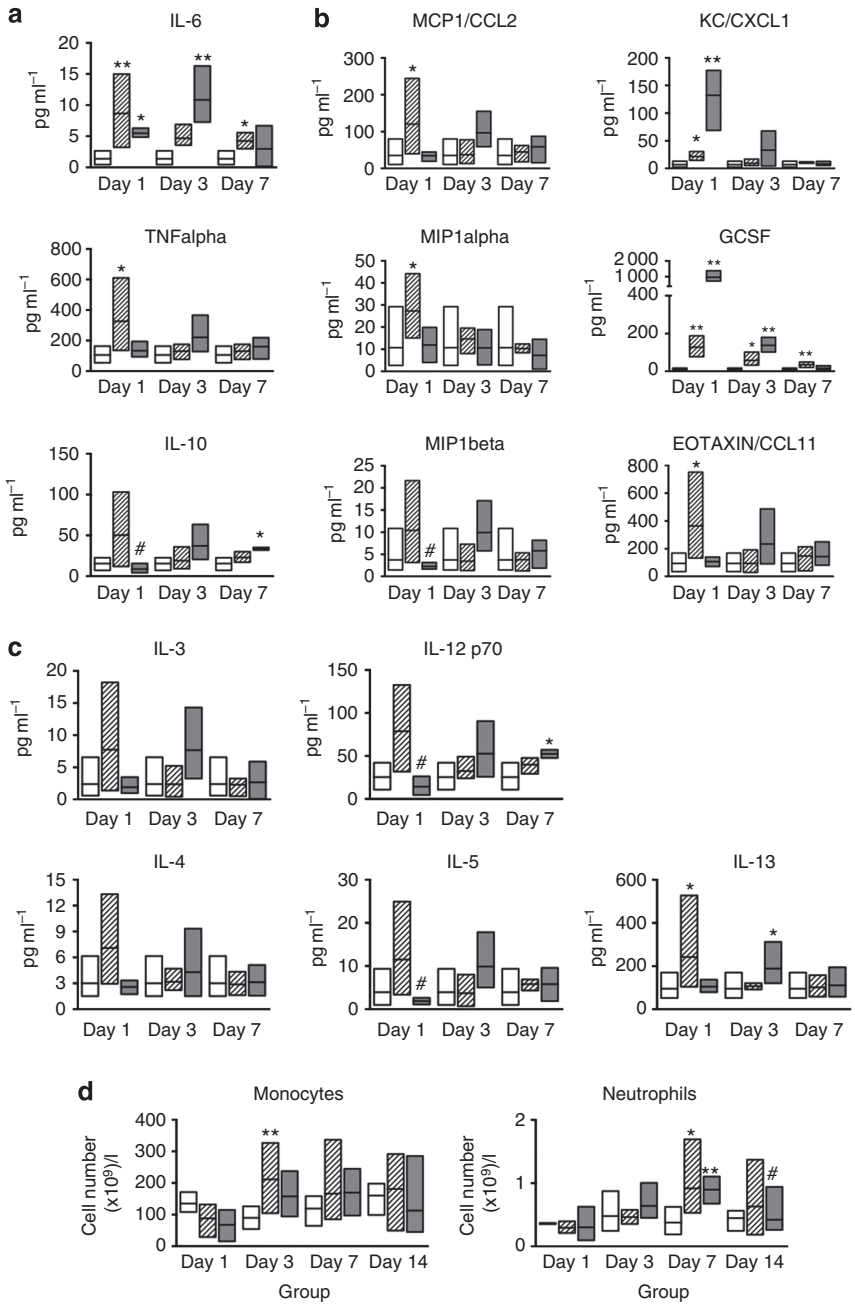

Figure 1. Temporal cytokine, chemokine, and hematology changes in burn and excision injury. Mice received sham (control), burn, or excision injury, and sera or whole blood were collected at the indicated time points for immune assay or hematology, respectively. Inflammatory cytokines IL-6, TNFα, and 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

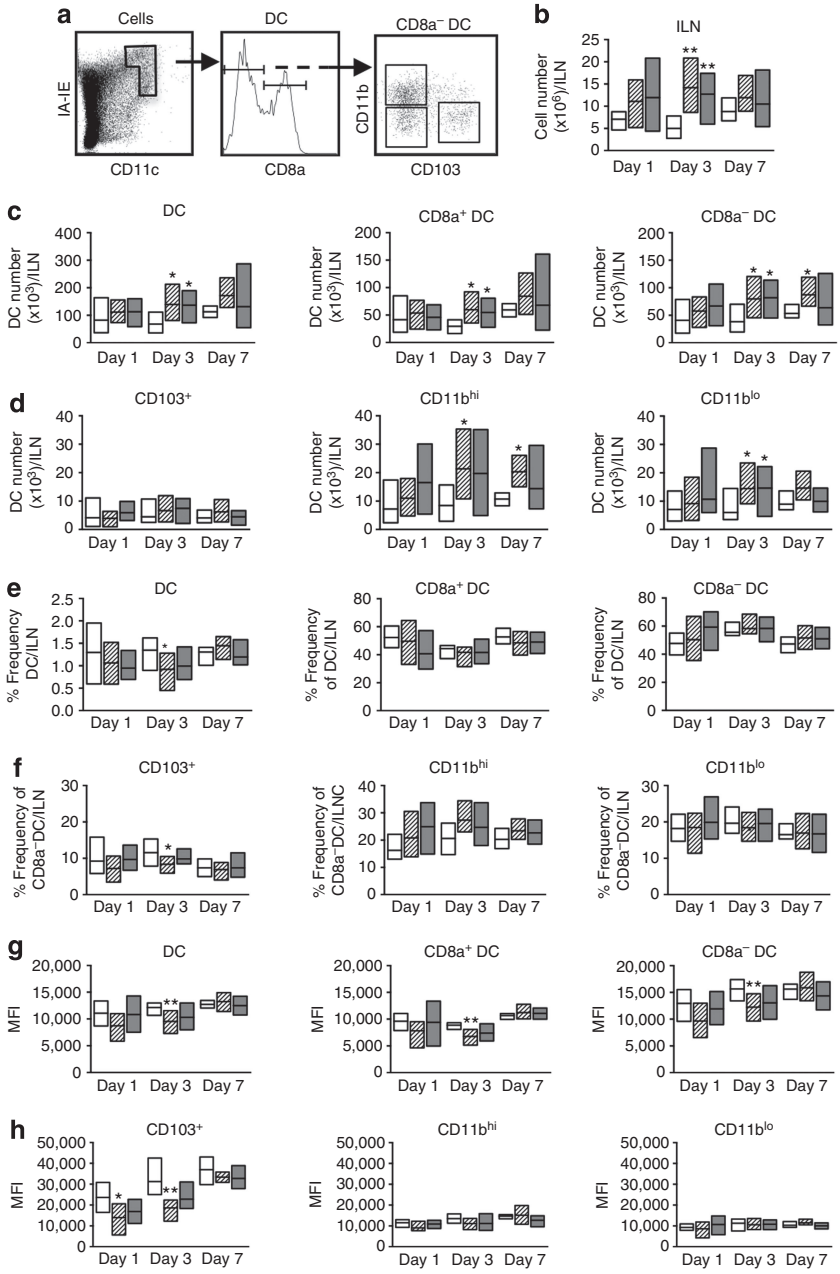

Figure 2. Dendritic cell responses in local draining lymph nodes after burn injury and excision injury. Mice received sham (control), burn, or excision injury, and ILNs were harvested to single suspensions at the indicated time points. Dendritic cells were gated CD11c$^+$ MHC II$^+$ and further gated 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).

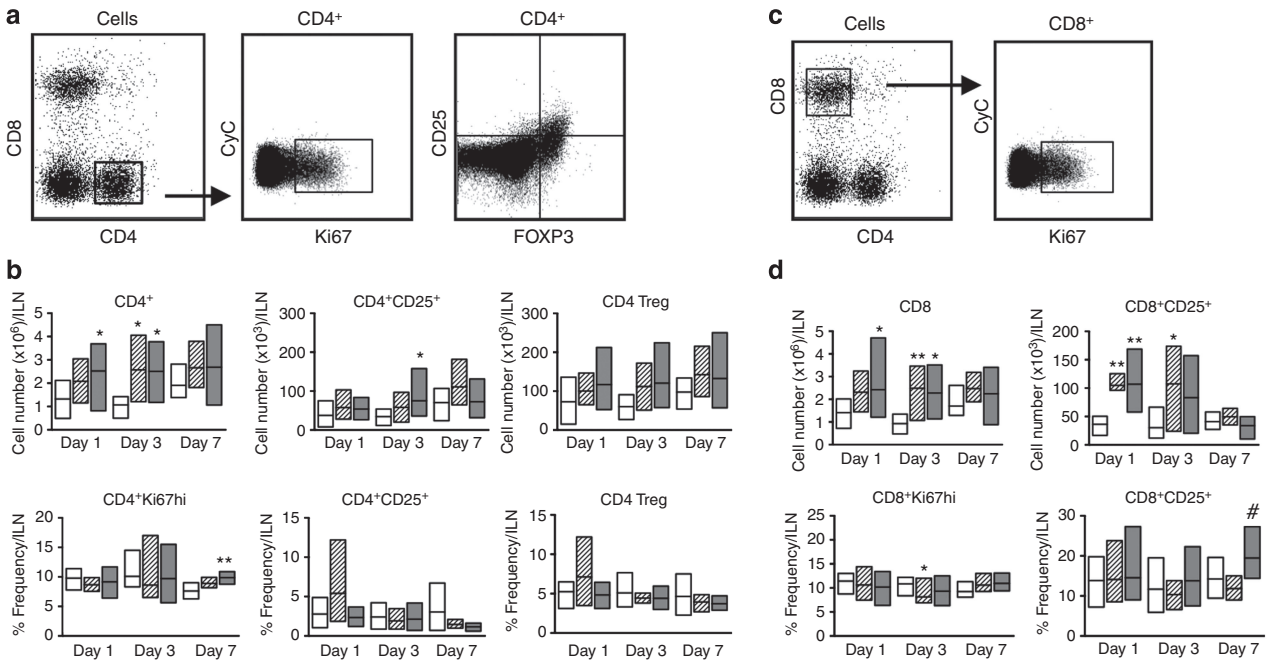

Figure 3. CD4 and CD8 cellular response to burn injury and excision injury. Mice received sham (control) burn or excision injury, and ILNs were harvested to single suspensions at the indicated time points. Dot plots indicate flow cytometric gating strategy for $CD4^+$ cells that were further gated on $Ki67^{hi}$, $CD25^+$, 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).

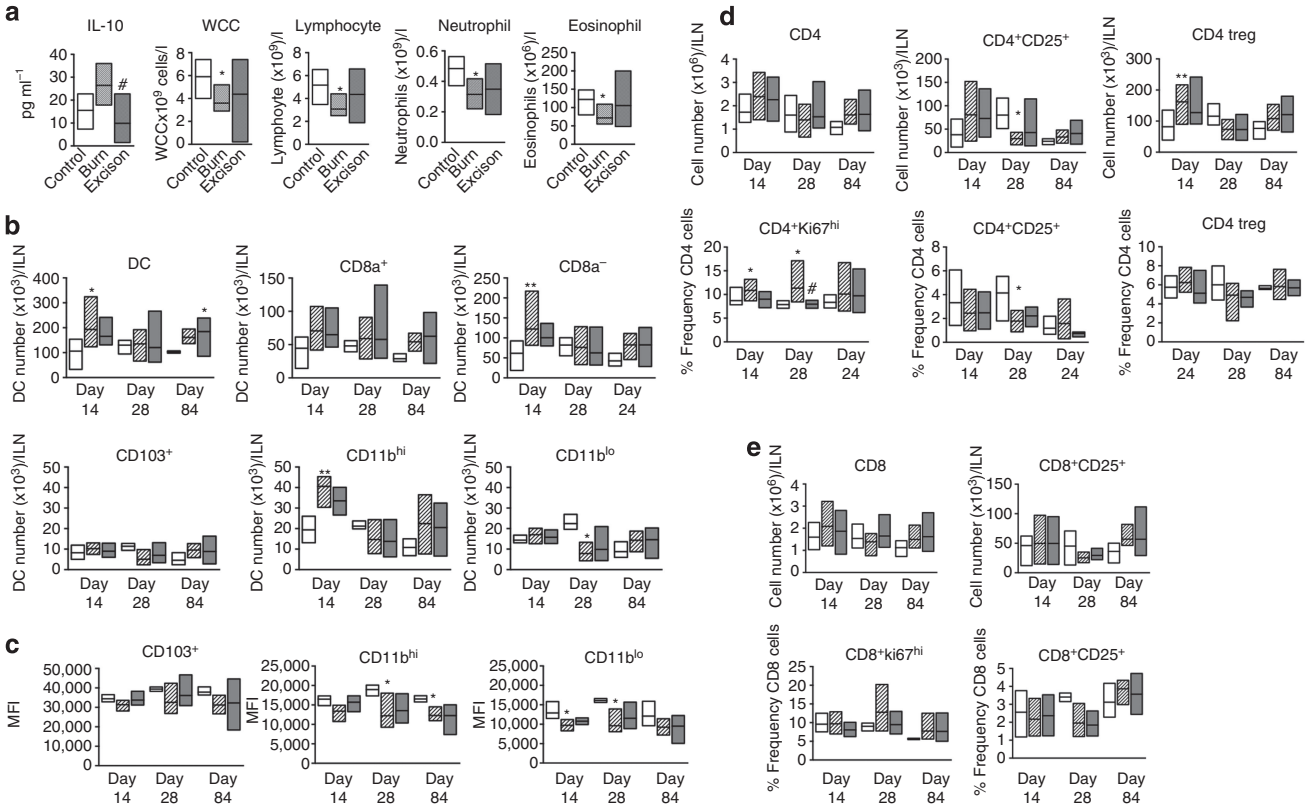

Figure 4. Innate and adaptive immune responses in the remodeling phase of burn injury and excision injury. Mice received sham (control), burn, or excision injury. Sera or whole blood were collected at day 84, and IL-10, WCC, lymphocytes, neutrophils and eosinophils were measured (**a**). ILNs were harvested 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

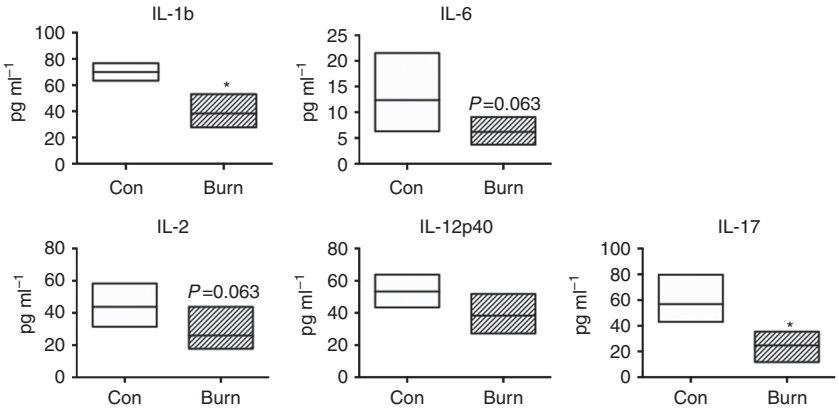

Figure 5. Burn injury leads to suppressed acute splenic T-cell cytokine responses. Spleen cells (3×10^5cells per ml) were isolated and stimulated with 10 ng ml^{-1} concavalin A and cytokine levels determined in supernatant at 48 hours. Bars indicate control (white bars) and burn injury day 7 post injury (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.

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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