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

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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.

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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.