Burn-related morbidity and mortality can in part be attributed to immune dysfunction and wound-healing complications.1 These conditions enhance the risk of infections, leading to the development of sepsis and multiple organ failure (MOF).2,3 It is also well established that inflammation plays a major role in wound healing involving a wide range of immune cells.4,5

In this regard, T-cells regulate the activity of other immune cells by releasing T-cell cytokines and thereby shaping the immune response.6 T helper 1 (Th1) cells release interleukin (IL)-2, interferon (IFN)γ, and tumor necrosis factor (TNF)β and initiate a cell-mediated immune response, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 and support humoral immune responses.6 Therefore, the induction of an appropriate Th1/Th2 response is crucial to control infections. In this regard, major burns in experimental models and in patients have been shown to decrease the Th1 (IFNγ) response and increase a Th2 (IL-10) response. This altered immune response was correlated with increased septic events in patients and decreased resistance to infection in the experimental systems.7–9 A recently defined Th cell population, termed as Th17 cells, which secrete IL-17, are described as key players in chronic inflammation and autoimmunity.10,11 Th17 cells also play a critical role in maintaining barrier integrity of the skin, lung, and gut.12

Our recent studies have demonstrated the induction of a Th17 response after burn.13,14 Gamma-δ T-cells are major producers of the IL-17 cytokines and play a major role during inflammation and wound healing.15,16 Our studies have also clearly demonstrated that γδ T-cells, are central in the response to burn injury.17,18 Nonetheless, the relationship of γδ T-cells to T-cell cytokine responses at the injury site after burn remains unidentified.
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METHODS

Mice
C57BL/6 male mice weighing 18 to 25 g (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Mice were allowed to acclimatize for at least 1 week before experimentation and maintained in ventilated cages under specific pathogen-free conditions. Animals were randomly assigned into either sham or burn group. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the experiments were performed in accordance with the National Institutes of Health guidelines for the care and handling of laboratory animals.

Burn Procedure
Mice received a scald burn as described previously. In brief, the mice were anesthetized by intraperitoneal injection of ketamine/xylazine, and the dorsal surface was shaved. The anesthetized mouse was placed in a custom-insulated mold exposing 12.5% of its TBSA along the right dorsum. The mold was immersed in 70°C water for 10 seconds to produce a third-degree burn. The burn procedure was repeated on the left dorsal side yielding a total burn size to be 25% TBSA. Previous studies have verified this injury to be a full-thickness burn with damage to the epidermal, dermal, and subdermal layers. The mice were then resuscitated with 1 ml of Ringer’s lactate solution administered by intraperitoneal injection and returned to their cages. The cages were placed on a heating pad until the mice were fully awake, at which time they were returned to the animal facility. Sham treatment consisted of anesthesia and resuscitation only.

Skin Tissue Collection and Processing
At 3 hours, 3 days, or 7 days after burn or sham procedure, skin samples were collected and wet weight was measured. Normal noninjured skin was collected from sham and injured skin, including the wound margin, was collected from burn mice. The burn injured skin was excised, down to the level of the musculofascia, including the submucosal layer by sharp dissection and processed to isolate single cells for flow cytometry.

Skin Tissue Digestion and Single-Cell Isolation
Full-thickness skin tissues were collected and washed in phosphate-buffered saline with 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO, Grand Island, NY). Skin tissues were collected in a 60-mm petri dish (Corning, Tewksbury, MA) and minced with scissors into small pieces that were approximately 2–3 mm in size and put into dispase II (0.05%; Roche, Indianapolis, IN) medium for overnight digestion at 4°C on orbital rocker. The next day, skin samples were further minced into smaller pieces and then digested by agitating in trypsin-GNK (0.3%; glucose/dextrose, NaCl and KCl buffer; Sigma, St Louis, MO) for 30 minutes at 37°C in water bath shaker. Heat inactivated fetal bovine serum (GIBCO), 50 μM of 2-Mercaptoethanol (Sigma-Aldrich), 2 mM of L-glutamine (GIBCO), 1 mM of sodium pyruvate (GIBCO), 100 μM nonessential amino acids (GIBCO), 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO) supplemented with 10 U/ml murine recombinant IL-1β (BD Biosciences, San Jose, CA). Cells were counted and cultured at a density of 1 × 10⁶/ml in a 12-well plate overnight. The next day, the cells were collected after passing them through a 70-μm mesh and used for flow cytometry.

Flow Cytometry
The isolated skin cells were washed in staining buffer (PBS with 0.2 % bovine serum albumin and 0.09% NaN₃) and treated with Fc-blocking antibody (anti-cluster of differentiation (CD)16/CD32, BD Biosciences) for 15 minutes. The cells were then stained with the following directly conjugated antibodies: anti-CD3 (phycoerythrin or allophycocyanin-cyanin 7) in combination with anti-β T-cell receptor (PerCP-Cy5.5) and anti-δ T-cell receptor (fluorescein isothiocyanate). After 30 minutes of incubation on ice, the cells were washed and resuspended in staining buffer.

For intracellular cytokine staining, cells were stimulated first for 4 hours with phorbol-12-myristate-13-acetate (1 μg/ml; Calbiochem) and ionomycin (1 μg/ml, Calbiochem) and then for 2 hours with, 1 μl/ml Brefeldin A (GolgiPlug, Billerica, MA; BD Biosciences). After surface staining cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) for 20 minutes in the dark. After washing twice with permeabilization buffer (BD Biosciences), intracellular cytokines were stained using anti-IFNγ (Pacific blue), anti-IL-10 (phycoerythrin), or anti-IL-17 (phycoerythrin) antibodies. After 30 minutes on ice, the cells were washed and resuspended in staining buffer.

Appropriate isotype controls were used for all staining. All data were acquired using an LSRII (BD Biosciences) and analyzed using FlowJo (Tree Star, Rani, Zhang, and Schwacha 47
A profound influx of total CD3⁺ T-cells was observed at the wound site at 3 hours, 3 days, and 7 days after burn (Table 1). The absolute numbers of T-cells were significantly increased as early as 3 hours postburn (1.7-fold) and remained increased up to 7 days (3.9-fold) in comparison with the T-cell numbers in skin from sham mice (Table 1).

The majority of T-cells in the uninjured skin of sham mice were of the γδ T-cell receptor lineage (80–90%; Table 1). At 3 hours and 7 days postburn, γδ T-cell numbers were significantly reduced as compared with sham skin (Table 1). The absolute number of γδ T-cells at the wound site, however, did not significantly change between 3 hours and 7 days.

In contrast, the overall percentage of γδ T-cells of the CD3⁺ T-cell population was reduced by 70 to 95% at the wound site as compared with sham skin because of the influx of large numbers of αβ T-cells. Both, the percentages (data not shown) and the absolute numbers of γδ T-cells at the wound site were significantly increased at all time points after burn, contributing to approximately 95% of the total T-cells (Table 1). Alpha-β T-cell numbers increased by 19-fold as early as 3 hours postburn and remained increased at 3 and 7 days (20-fold–25-fold) postinjury (Table 1).

Profiles After the Burn Injury

Skin cells were stained with T-cell and cytokine markers to investigate their cytokine profile. IFNγ, IL-10, and IL-17 were used as the representative cytokines for the Th1, Th2, and Th17 profiles, respectively. The gating strategy is shown in Figure 1A. Representative dot plots from 3 hours, 3 days, and 7 days postburn are shown in Figure 1B. The percentages of the γδ T-cells positive for different cytokines after sham procedure remained comparable at all time points (data not shown). Representative dot plots for sham group at the 3-hour time point are shown (Figure 1B).

In the sham group, the majority of the γδ T-cells did not produce cytokines. In the cytokine-producing γδ T-cells the majority were IFNγ⁺ (~26%) as compared with IL-10⁺ (~6%) and IL-17⁺ (~3%; Figure 1B). After burn, there was a progressive increase in the percentage of cytokine-producing cells with time, and by 7 days postinjury ~90% of the cells produced IFNγ, and ~60% of the cells produced either IL-10 or IL-17 (Figure 1B and C). While this represents a significant 3-fold increase in IFNγ⁺ γδ T-cells after burn, the increase in IL-10⁺ γδ T-cells was 9-fold, and IL-17⁺ γδ T-cells were increased by more than 20-fold, representing a shift from a Th1 profile in sham γδ T-cells to a mixed IL-10/IL-17 profile in burn γδ T-cells (Figure 1B and C).

In addition to changes in the percentage of γδ T-cells positive for a given cytokine, median fluorescence intensity (MFI) was also measured (Figure 1C). MFI measures the shift in the fluorescence intensity of a population of cells. In this case, MFI is the measurement of the expression levels of different cytokines associated with the T-cells, whereas an increase in MFI would be indicative of that cell producing more of a specific cytokine.

The IFNγ MFI value remained comparable at 3 hours postburn, but was enhanced significantly at 3 and 7 days postburn (1.8-fold and 2.7-fold, respectively) when compared with the sham group. While γδ T-cells from sham skin produced very little IL-10 and IL-17, the MFI values increased significantly after burn. IL-10 MFI values remained unchanged at 3 hours postburn but were significantly enhanced by approximately 2.7-fold at 3 days postburn and remained increased at 7 days (2.4-fold) after burn as compared with the shams (Figure 1C). IL-17
MFI values were increased significantly at 3 hours (1.5-fold), 3 days (2.3-fold), and 7 days (2-fold) (Figure 1C).

Similar to γδ T-cells, the majority of the αβ T-cells did not produce cytokines in the sham group. In the cytokine-producing αβ T-cells, the majority were
IFNγ+ (~40%) at 3-hour time point as compared with IL-10+ (~6%) and IL-17+ (~6%) (Table 2) in both sham and burn groups. These values remained comparable at 3 days postburn. A significant increase in the percentage of αβ T-cells positive for IFNγ, IL-10, and IL-17 was observed at 7 days postburn. However, unlike skin αβ T-cells, no preference for Th1 (IFNγ), Th2 (IL-10), or Th17 (IL-17) profile was evident (Table 2). MFI values for IFNγ, IL-10, or IL-17 did not change after burn as compared with that of shams (Table 3).

**DISCUSSION**

Considering the implication of Th profile in the wound-healing process, the current study was performed to assess the cytokine phenotype of epidermal γδ T-cells at the burn wound site. The findings demonstrate the development of a mixed Th2 and Th17 response at the burn wound site, which likely plays an important role in subsequent inflammation and wound-healing complications. Specifically, our findings show an increase in the Th2 cytokine, IL-10+ and Th17 cytokine, IL-17+ γδ T-cells after burn. While a significant increase in Th1 cytokine, IFNγ+ γδ T-cells was also evident after the injury, the increase in Th2 and Th17 positive γδ T-cells was far greater (by more than 10-fold–20-fold for Th2 and Th17, respectively) than Th1 positive γδ T-cells. These changes represent a shift from a Th1 profile in sham γδ T-cells to a mixed IL-10/IL-17 profile in burn γδ T-cells. In contrast, wound αβ T-cells cytokine profile did not change.

T-cells are the critical component of the immune system and Th1 and Th2 subtypes are the most recognized T-cell population as first described in 1980s. While IL-2 and IFNγ are the signature cytokines produced by Th1 cells, IL-4 and IL-10 are the hallmark cytokines produced by Th2 cells. Development of a distinct Th1 or Th2 profile influences susceptibility and outcomes from various diseases. Studies by different groups have shown that in allergic diseases, such as atopic dermatitis and allergic asthma, allergen-specific T-cells acquired the Th2 phenotype and produced IL-4, IL-5, IL-6, IL-10, and IL-13. These findings suggest a Th2-bias in the development of allergic diseases. In contrast, Th1-directed responses have been shown to be involved in the pathogenesis of organ-specific autoimmune disorder (rheumatoid arthritis), psoriasis, and graft vs host disease.

Gamma-δ T-cells are functionally specialized and are involved in different disease processes. With regard to trauma, studies have shown the presence of activated γδ T-cells in the circulation of patients with severe systemic inflammatory response syndrome. Studies from our laboratory have established an important role of γδ T-cells in burn-induced immunopathology influencing macrophage function, distal organ injury, and wound repair. Importantly, we observed that γδ T-cells are important in the recruitment of inflammatory cells to the injury site after burn, as γδ T-cell deficient mice displayed a significant reduction in the cellular filtrate. While in the study herein we observed that the total number

**Table 2. Alpha-β T-cell cytokine phenotypes (% positive)**

<table>
<thead>
<tr>
<th>Time Postburn</th>
<th>Sham</th>
<th>Burn</th>
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<tbody>
<tr>
<td>IFNγ</td>
<td>38.7±2.9*</td>
<td>22.2±6.8</td>
</tr>
<tr>
<td>IL-10</td>
<td>6.2±1.8</td>
<td>10.9±2.2</td>
</tr>
<tr>
<td>IL-17</td>
<td>6.2±0.4</td>
<td>8.6±2.7</td>
</tr>
</tbody>
</table>

IFN, interferon; IL, interleukin.

*Data are expressed as mean ± SEM (n = 3–8 mice/group).

<table>
<thead>
<tr>
<th>Time Postburn</th>
<th>Sham</th>
<th>Burn</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>874.4±48.1*</td>
<td>1054.2±104.7</td>
</tr>
<tr>
<td>IL-10</td>
<td>766.9±164.7</td>
<td>749.3±93.0</td>
</tr>
<tr>
<td>IL-17</td>
<td>652.2±20.4</td>
<td>725.8±7.8</td>
</tr>
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IFN, interferon; IL, interleukin.

*Data are expressed as mean ± SEM (n = 3–8 mice/group).
of T-cells at the burn wound site increases and are predominantly γδ T-cells, concurrent findings have shown that the γδ T-cell influx was dependent upon the presence of γδ T-cells, as this T-cell infiltration of the wound site was not evident in mice deficient in γδ T-cells. Moreover, the absolute numbers of γδ T-cells in the burn wound were comparable with that of uninjured skin, but were activated with increased expression of TLR2, TLR4, and CD69. Thus, while γδ T-cells are not the predominant T-cell at the wound site they are essential in the initiation and propagation of the inflammatory infiltration.

Th1/Th2 polarization has also been shown in the area of trauma research. Major injury has been documented to induce T-cell specific changes in the immune system. The disruptions of the natural and adaptive immunity after traumatic and burn injuries are related to the increased susceptibility to systemic inflammatory response syndrome, sepsis, and MOF. Furthermore, there is evidence that suggest that trauma induces a polarized Th2 lymphocyte response and the suppression of the Th1 response that may further predispose patients to systemic inflammatory response syndrome, sepsis, and MOF. Different studies have been conducted to elucidate those cytokines and Th profiles and inflammatory markers that occur in traumatic and burn injury through animal models and patient populations. In this regard, Lyons et al showed an increased Th2 response (IL-10) in peripheral blood mononuclear cells (PBMC) of burn and trauma patients as compared with PBMC from healthy volunteers at 7 to 14 days postinjury. A significant correlation between increased IL-10 and subsequent septic events was found in the first 10 days after injury. In a year-long longitudinal study, Tredget et al have also demonstrated a polarized Th2 cytokine response in patients with hypertrophic scarring after burn. The study demonstrates that circulating lymphocytes shift their cytokine response away from an IFNγ-mediated Th1 response to a predominantly Th2 response, which persisted for more than 12 months. The present study herein is consistent with these findings showing a shift toward a Th2 response. We demonstrated the presence of Th2 polarization by the wound γδ T-cells at the injury site early postinjury (ie, 7 days), when the immune system is still in the inflammatory phase rather than in remodeling phase. In another study, Neidhardt et al found a correlation between IL-10 levels in plasma and injury severity score. Increased levels of IL-10 have been associated with multiple organ dysfunction syndrome and sepsis in trauma patients. Yeh et al demonstrated a significant difference between the survival of burn patients and the levels of serum IL-10, which were further correlated to patients with TBSA of greater or less than 50%. Guo et al showed that burn injury favors antigen-driven Th2 response in vivo in the mouse burn model. Taking all these studies together, our present study further confirms the presence of a Th2 bias after burn injury.

A recently defined Th17 response involving T-cells that produce IL-17 has been shown to play a pivotal role in chronic inflammation and autoimmune disorders. IL-17 acts on different cell types such as neutrophils, fibroblasts, epithelial cells, and endothelial cells. Different sources of IL-17 have been identified, including CD8 T-cells, natural killer cells, γδ T-cells, and neutrophils. On the basis of these observations, an important and unidentified role of Th17 response in the development of immune complications after injury may exist. Gamma-δ T-cells, which are important in postburn inflammation and wound healing, have also been shown to be a major source of IL-17. These studies have demonstrated a causative relationship between IL-17, γδ T-cells and survival after sepsis.

Studies by Finnerty et al have shown the increased levels of circulating IL-17 in pediatric burn patients, as well as in mouse burn model. Other studies have also documented increased levels of IL-17 during pulmonary complications and in sepsis models. These studies have demonstrated a role of IL-17 in recruiting immune cells, such as neutrophils and propagating inflammation. Recenly, we have demonstrated the increased expression of IL-17 in different tissue beds, such as cardiac and skin tissue as early as 3 hours after burn injury. The early activation in cardiac and skin IL-17 levels is consistent with our present findings. We have shown in the present study herein an association between IL-17 and wound gamma-delta T-cells, irrespective of the time post-burn evaluated. This is in agreement with the other studies that have shown that γδ T-cells are an important source of IL-17. Therefore, we speculate that the increase in the skin tissue levels of IL-17 after burn is because of the activation of resident γδ T-cells.

An important question is whether this mixed IL-10 and IL-17 response after burn injury is beneficial or detrimental to the healing response. Recent findings by Rutz et al have reviewed the coexistence of IL-10 and IL-22 (cytokine produced by Th17 cells) during inflammation. Another study by Lemaitre et al have shown the coexistence of Th2 and Th17 in a posttransplant oblitative airway disease. They showed in a mouse trachea transplant model, cyclosporin A treatment favored Th2 and Th17 responses as coexisting pathways mediating chronic rejection of the tracheal allograft. Therefore, the Th2 or Th17 response appears to be detrimental.
In conclusion, T-cells of the γδ T-cell receptor lineage have a significant role in the burn wound-healing process through the development of a mixed Th2 and Th17 response at the injury site. Specific increase of these cytokines suggests that they may serve as unique targets for therapeutic manipulation to improve wound healing.

The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. The authors declare that they have no conflict of interest.

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M.R. was responsible for experimental design, animal and flow-cytometry experiments, data analysis, scientific interpretation, and draft of the article. Q.Z. was responsible for the animal experiments. M.G.S. was responsible for scientific conception, design and helped to draft the article.

REFERENCES


