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Up-Regulation of Cell Surface Toll-Like Receptors on Circulating γδ T-Cells Following Burn Injury

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Abstract

Burn injury is associated with profound inflammation and activation of the innate immune system involving γδ T-cells. Similarly, toll-like receptors (TLR) are associated with activation of the innate immune response; however, it is unclear whether TLR expression is altered in γδ T-cells after major burn injury. To study this, male C57BL/6 mice were subjected to burn injury (25% TBSA) and 1 or 7 days thereafter, blood and spleen cells were isolated and subjected to FACs analysis for TLRs and other phenotypic markers (γδ TCR, αβ TCR, CD69, CD120b). A marked increase in the number of circulating γδ T-cells was observed at 24 hr. post-burn (14% vs. 4%) and a higher percentage of these cells expressed TLR-2. TLR-4 expression was also increased post-burn, but to a lesser degree. These changes in TLR expression were not associated with altered CD69 or CD120b expression in γδ T-cells. The mobilization of, and increased TLR expression in, γδ T-cells was transient, as phenotypic changes were not evident at 7 days post-burn or in γδ T-cells from the circulation or spleen. The increases in TLR expression were not observed in αβ T-cells after burn injury. In conclusion, 24 hr. after burn injury mobilization of γδ T-cells with increased TLR expression was observed. This finding suggests that this unique T-cell population is critical in the innate immune response to injury, possibly through the recognition of danger signals by TLRs.

Keywords

trauma; SIRS; T-cell receptors; cytokines; chemokines

INTRODUCTION

Thermal injury remains a significant health problem. Despite advances in patient care, immunosuppression, increased susceptibility to sepsis, wound healing complications, and multiple organ failure remain major concerns in this severely compromised and unique patient population. Major burn injury is associated with an activation of the innate immune system and inflammation [1;2]. It is believed that the activation of the innate immune system contributes to the development of a systemic inflammatory response (SIRS) and subsequent multiple organ failure [1;3;4].

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The involvement of γδ T-cells in a wide variety of disease processes is indicative of an important role for this T-cell subset in both innate and acquired immunity [5–7]. In this regard, studies from our laboratory suggest an important role for γδ T-cells in the immune response to thermal injury [8–11]. In these studies, increased activation of the circulating γδ T-cells was observed and the findings also indicated that γδ T-cells have a role in post-burn immune dysfunction, neutrophil-mediated tissue damage and wound healing. Normally, γδ T-cells are present in only small numbers in peripheral lymphoid tissues (i.e., spleen), but relatively abundant in the skin epithelia, intestine, uterus and tongue [5]. However, γδ T-cells have a phenotype of spontaneous activation and under pathological conditions they can quickly expand and infiltrate lymphoid compartments and other tissues [12;13].

The identification of a family of mammalian receptors related to Drosophila Toll has demonstrated how cells of the innate immune system are capable of recognizing and reacting to a wide variety of microbial antigens [14]. This family of receptors are known as Toll-like receptors (TLRs) and consists of 10 members that have shared structural homology and signaling components [15–17]. TLRs are primary sensors of microbial products and activate signaling pathways that lead to the induction of inflammatory genes. They belong to a family of IL-1R-related molecules that recognize conserved pathogen-associated molecules such as LPS (TLR-4) bacterial lipopeptide (TLR-2) [18]. Recent studies have shown that γδ T-cells can, upon activation express TLRs [19–21]. While studies have shown that TLRs and TLR responsiveness are involved in the post-burn inflammatory response by macrophages [1;18], it remains unknown whether TLRs are involved in the activation of γδ T-cells after major burn injury.

MATERIALS AND METHODS

Animals

C57BL/6 male mice (18–22 g; 8–10 weeks of age, Charles River Laboratories, Wilmington, MA) were used for all experiments. The mice were allowed to acclimatize in the animal facility for at least one week prior to experimentation. Animals were randomly assigned into either a thermal injury group or a sham treatment group. The experiments in this study were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham where the animal studies were performed. In addition, the animal experiments were performed in accordance with the National Institutes of Health guidelines for the care and handling of laboratory animals.

Thermal injury procedure

Mice received a scald burn as described previously [22]. Briefly, the mice were anesthetized by intra-peritoneal injection of ketamine/xylazine and the dorsal surface was shaved. The anesthetized mouse was placed in a custom insulated mold exposing 12.5% of their total body surface area along the right dorsum. The mold was immersed in 70°C water for 10 sec, producing a burn injury. The mouse was repositioned in the mold exposing the left dorsum, and the mold was re-immersed in 70°C water for 10 sec. The resulting injury covered 25% total body surface area, and previous studies have verified this injury to be a full-thickness burn, as defined by injury to the epidermal, dermal, and subdermal layers [23]. 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 for 2 hr until the mice were fully awake, at which time they were returned to the animal facility. Sham treatment consisted of anesthesia and resuscitation with Ringer's lactate solution only. The mice were not given analgesics post-procedure due to the profound immunomodulatory properties of NSAIDs and opiates [24–26].

Collection of cell populations

Anti-coagulated whole blood was obtained at 1 or 7 days after thermal injury or sham procedure by cardiac puncture. Splenocyte and MLN cells were collected at 7 days post-injury and cell suspensions prepared in PBS as described elsewhere [27;28].

Determination of cellular phenotype

Blood, Spleen or Mesenteric Lymph Node (MLN) cells were stained with a combination of antibodies (γδ TCR, αβ TCR, TLR-2, TLR-4, CD69, CD120b) conjugated to either FITC or PE to assess cellular phenotype. The manufacturer's recommended methodology for each cell population was employed (BD Pharmingen). Appropriate isotype controls were included. FITC and PE were analyzed with a Becton-Dickinson LSR II flow cytometer (BD Biosciences, Mountain View, CA). Unless noted otherwise, the entire lymphocyte and monocyte populations (as determined for forward and side scatter) were gated in the analysis. A minimum of 20,000 events was collected and WinMIDI 2.8 software was used to analyze the results.

Analysis of cytokine levels

Plasma was collected and stored at −80° C until being assayed for TNF-α, IL-6, IL-10, KC and MCP-1. The Cytometric Bead Array (CBA; BD Pharmingen) and CBA Mouse Flex Sets were used for all analyses according to the manufacturer's instructions with some modification. In brief, 25 μl of mixed capture beads were incubated with 25 μl of plasma for 1 hr at room temperature in the dark. Twenty-five μl of PE detection reagent was then added and incubated for 1 hr. The immunocomplexes were then washed twice with wash buffer and centrifuged at 200 × g for 5 min. Analysis was then carried out using the LSR II flow cytometer (BD Biosciences) and the data was processed with the accompanying FACSDiva and FCAPArray software.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons were analyzed using ANOVA. A P value of < 0.05 was considered to be statistically significant for all analyses.

RESULTS

Effect of burn injury on circulating immune cells

At 24 hrs after burn injury a profound change in the phenotype of the circulating immune cells was observed (Fig. 1). Based on the analysis of forward and side scatter the granulocyte and lymphocyte/monocyte populations could be identified. Approximately a 2-fold increase in the percentage of granulocytes was observed in the blood on burn injured mice as compared with shams ($p<0.05$). In parallel, an approximate 50% drop in the percentage of circulating lymphocytes and monocytes was observed; however, due to high variability between animals statistical significance was not observed. Further analysis of the cells in the lymphocyte and monocyte population revealed that the percentage of the cells positive for γδ TCR was significantly greater ($p<0.05$) in the burn group (9.9±1.5% vs. 18.9±3.6% for sham and burn, respectively; mean ± SEM; n=6/group. Fig. 2).

TLR expression by circulating T-cells early post-injury

At 24 hrs after burn injury the percentage of the γδ T-cells expressing TLR-2 or TLR-4 significantly increased as compared to sham animals ($p<0.05$; Fig. 3). A significantly greater proportion of the γδ T-cells in the burn group where TLR-2^+ as compared with γδ T-cells that were not expressing TLR-2 ($p<0.05$; Fig 3A). Gamma-delta T-cells in the burn group had similar percentages for the TLR-4^+ and TLR-4^- populations (Fig. 3B). No such

differences were observed in γδ T-cells from sham animals. In contrast to γδ T-cells, the expression of TLR-2 or TLR-4 on αβ T-cells at 24 hrs after burn injury was not different from that of sham animals (Fig. 4). Moreover, the majority of the αβ T-cells were negative for TLR expression.

The circulating γδ T-cells were further analyzed for the expression of the T-cell activation markers CD69 and CD120b (p75 TNFR2). In contrast to TLR-2 and -4 expressions, expression of CD69 and CD120b was not different between the sham and burn groups (Table 1).

Burn injury induction of a systemic inflammatory response

Burn injury induced a systemic inflammatory response at 24 hrs post-injury as evidenced by the increased plasma levels of IL-6 and KC as compared to sham animals ($p<0.05$; Fig. 5). Plasma levels of these inflammatory mediators was increased approximately 10–12-fold. In contrast, TNF-α, IL-10, and MCP-1 plasma levels were not different between sham and burn animals at 24 hrs post-injury.

TLR expression by circulating tissue-fixed T-cells late post-injury

The increased expression of TLRs on circulating γδ T-cells appeared to be transient, as analysis of cells at 7 days post-injury revealed no differences between the sham and burn groups (Fig. 6A, 6B). Similarly, no differences in TLR expression were observed in circulating αβ T-cells at 7 days post-injury (Fig. 6C, 6D).

T-cells isolated from the spleen and MLN were assessed for and TLR-2 or TLR-4 expression. The data presented in Table 2 indicates that the vast majority of the tissue-fixed T-cells were of the αβ TCR phenotype. The expression TLR-2 or TLR-4 on either T-cell population was negligible, irrespective of sham treatment or burn injury

DISCUSSION

Major injuries, such as burn injury, induce a profound immunoinflammatory response that ultimately can predispose the host to various types of opportunistic infections. Toll-like receptors (TLRs) can recognize conserved microbial antigens and/or host stress or danger signals induced by injury. Thus, injury-induced alterations in TLR expression or responsiveness by the injured host may contribute to the development of infectious complications and the potential for the development of multiple system organ failure and death. The studies presented here were conducted to determine whether changes in TLR expression by γδ T-cells after burn injury were associated with the inflammatory response. We have made a number of important observations concerning the post-burn immunoinflammatory response. First, a profound change in the circulating immune cell populations was observed relatively early post-burn (i.e., 24 hr) that was associated with an increased circulating γδ T-cells, granulocytes and a systemic inflammatory response. Secondly, these γδ T-cells were primed for TLR reactivity, as evidenced by increased expression of TLR-2 and TLR-4. Third, these changes in TLR expression were specific for the γδ TCR T-cell subset in the circulation and transient, since it was not observed in the spleen, MLN or blood at 7 days post-injury.

TLRs are cell surface molecules that participate in innate immunity by recognizing conserved motifs on pathogens These pathogen-associated molecular patterns (PAMP) are displayed in a variety of microorganisms [17;29], as well as endogenous danger/damage associated molecular pattern molecules (DAMP's) [30]. Increased TLR reactivity by macrophages has been implicated in a number of immunopathological aspects of burn injury [1;15;18]. These studies have primarily focused on responses to the TLR-4 ligand LPS. This

enhanced TLR reactivity appears to be in part related to increased expression of intracellular TLR-4-MD2, rather than cell surface expression of the receptor complex [18]. Our laboratory and others [18;31;32] have also shown TLR-4 reactivity is associated with an increase in p38 MAPK activity. Nonetheless, TLR signaling pathways converge in the activation of the transcription factor NF-κB, which controls the expression of an array of inflammatory associated genes [30]. In contrast to macrophages, the analysis of TLR expression by T-cells post-injury has been very limited. A recent study by Cairns et al. [33] have shown that late post-burn injury (i.e., 14 days) increased expression of TLR-4 was evident on splenic T-cells. The current findings are consistent with the work Cairns et al. with regard to T-cell expression of TLRs; however, TLR expression was seen earlier, only in the circulation and specific for the γδ T-cell subset. This apparent discrepancy is most likely related to the time post-injury used for analysis (i.e., 1–7 days vs. 14 days). Moreover, while patient studies indicate mobilization and activation of γδ T-cells in SIRS patients at 24 hr (i.e., 0–1 day) [34], it remains to be determined whether changes in γδ T-cell TLR expression occur earlier than 24 hrs. post-injury, as mobilization of this cell population has been observed at 3–4 hr after burn injury or sepsis in mouse injury models [9;35].

Gamma-delta T cells responses have observed in disease states of by an infectious and non-infectious nature, suggesting that this unique T-cell subset has a primary role in immunoregulation and the protection of host tissues against the damaging side-effects of immune responses [36]. There are a number of features which distinguish γδ T-cells from "traditional" αβ T-cells. Most γδ T-cells have a phenotype of spontaneous activation with a rapid turnover rate [37] and quickly expand upon activation by pathogens or inflammatory stimuli [13]. A critical role for γδ T-cells has been demonstrated with regard to inflammation, pro-inflammatory cytokine (i.e., TNF-α) production [38] and tissue repair [39]. They also can regulate Th1-type and Th2-type T-cell phenotypes and establishment of protective $CD8^+$ responses [40]. A clinical study has demonstrated activated γδ T-cells are present in the circulation of SIRS patients [34]. We have also shown that early post-burn (3 hr) in our murine model there is a mobilization of γδ T-cells to the circulation [9]. In that study the circulating γδ T-cells were in an primed state as evidenced by increased CD120b expression [41]. In contrast, our findings at 24 hr post-burn indicate that, while increased number of γδ T-cells are present in the circulation, their phenotype has changed and the cells, in a classical sense, are not activated, since CD120b and CD69 expression was unaltered. While the increased expression of TLR-2 and TLR-4 suggests that these γδ T-cells may be more reactive towards PAMPs and DAMPs [29;30], it remains to be determined whether this phenotypic change significantly alters the response to TLR ligands post-burn. Chemokine receptors are expressed on both circulating and tissue γδ T-cells that allow them to extravasate and migrate to sites of tissue damage or infectious foci. Gamma-delta T-cells can also produce chemokines and thereby contribute to the recruitment of additional γδ T-cells [9;42;43]. It can be suggested that injury induces an early activation and migration of γδ T-cells from epithelial rich tissue to the lymph/circulation and subsequently to burn injured tissue to help control inflammation and/or initiate the healing process. Previous studies have support such a concept for γδ T-cells under other pathological conditions [44;45]. Moreover, this process appears to be, in part, TLR-dependent [46]. Nonetheless, it remains to be yet determined whether or not such trafficking of γδ T-cells occurs post-burn.

While the regulation of TLR expression is not clearly understood [47], cell surface TLR expression is likely to be limited to avoid excessive activation. Thus, the transient nature of the post-burn increase in γδ T-cell TLR expression is likely to be protective. Studies have shown that TLR expression is transiently upregulated on monocytes in response to LPS or TNF-α [48;49]. Moreover, the activation of TLRs can induce the expression of genes that regulate cell migration [50]. Thus, the post-burn changes in γδ T-cell TLR expression may

be related to the early inflammatory response (i.e., TNF-α) and important in their mobilization and migration.

The findings presented here further support a role for γδ T-cell in post-burn immunopathology. Previous studies demonstrated high mortality in mice lacking γδ T-cells during the initial 48 h post-burn period [8], a role for γδ T-cells in post-burn end organ injury [9], and an important role in post-burn wound healing [10;11]. We have extended these observations by demonstrating a potential role for TLRs in the γδ T-cells response to burn injury. It is likely that the activation and migration of γδ T-cell during the initial post-injury period (i.e., 24 hr.) is overall protective to the host, most likely via regulation of inflammation and initiation of healing processes. Nonetheless, additional studies are needed to develop a more comprehensive understanding of this unique T-cell subset and their role in post-injury immunopathology.

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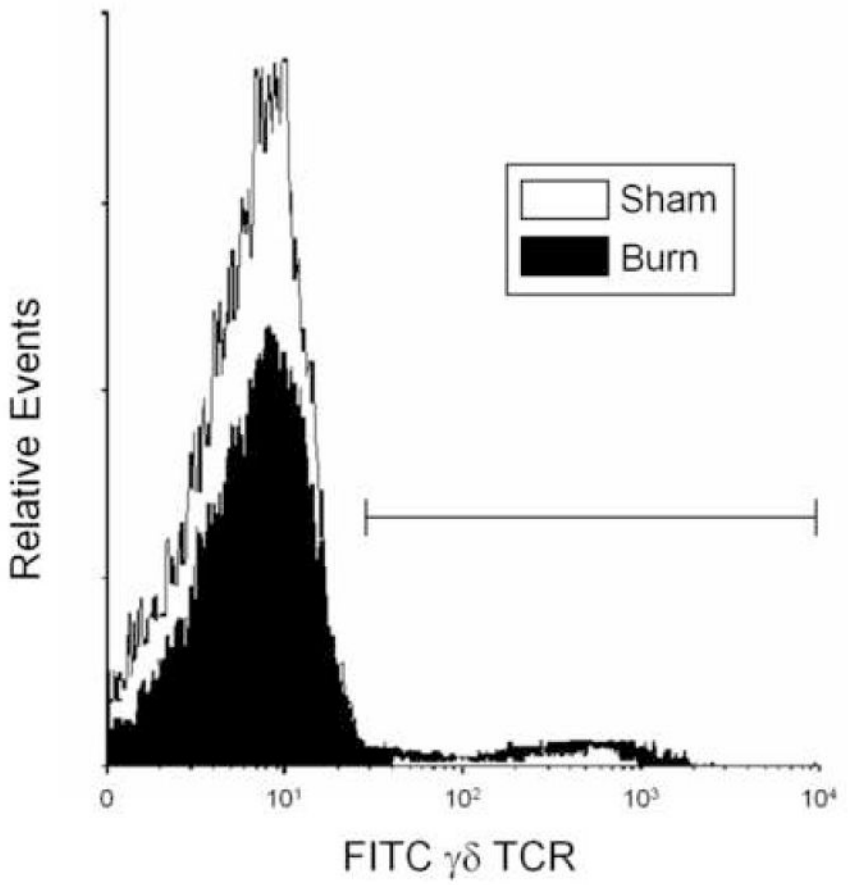

Figure 1. Effect of burn injury on circulating immune cell populations

Blood was collected from mice at 24 hr after sham or burn injury. The lymphocyte/monocyte and granulocyte populations were identified by forward (FSC) and side scatter (SSC) and analyzed as a percentage of the total cell population. Data are mean ± SEM for n=6/group. * $p<0.05$ vs. respective sham group.

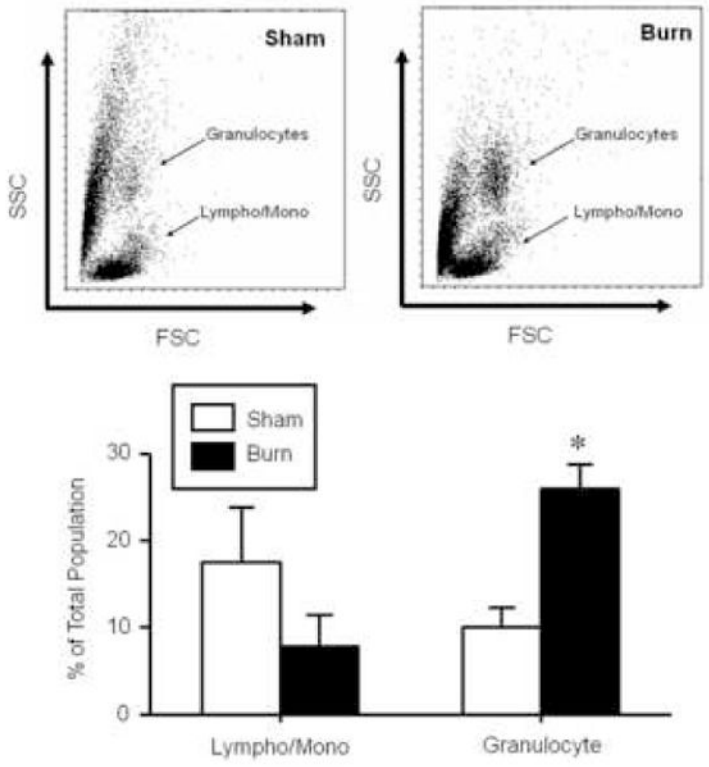

Figure 2. Effect of burn injury on circulating γδ T-cells

Blood was collected from mice at 24 hr after sham or burn injury and stained for expression of γδ TCR as described in the materials and methods. The lymphocyte/monocyte population was identified by forward and side scatter and analyzed FITC γδ TCR. Bar indicates γδ TCR+ population. Data are representative of 6 experiments.

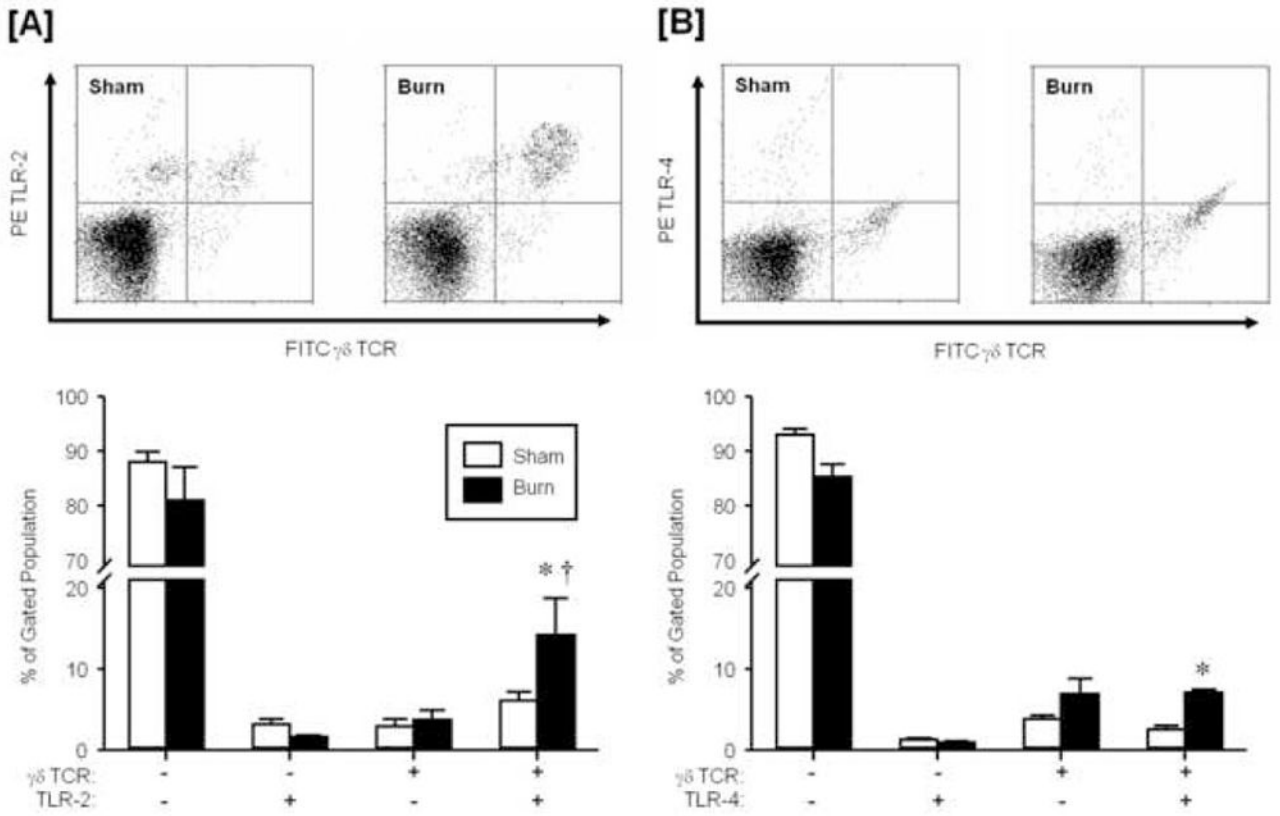

Figure 3. TLR expression on circulating γδ T-cells

Blood was collected from mice at 24 hr after sham or burn injury and stained for expression of γδ TCR and TLR-2 [**A**] or TLR-4 [**B**] as described in the materials and methods. The lymphocyte/monocyte population was identified by forward and side scatter and analyzed FITC γδ TCR and PE TLR-2 or TLR-4. Data are mean ± SEM for n=4/group. * $p<0.05$ vs. respective sham group. † vs. respective gd TCR^+ $TLR\text{-}2^-$ group.

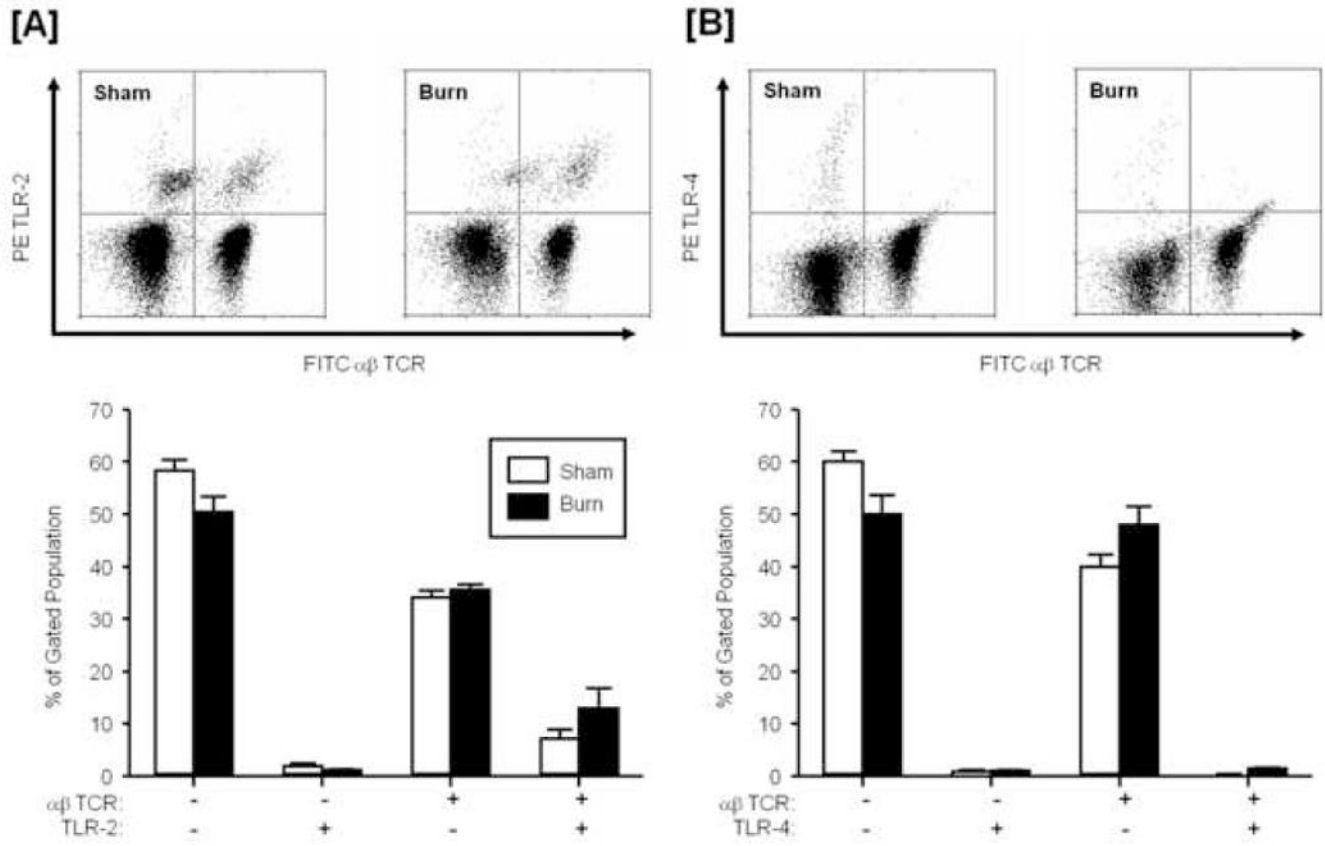

Figure 4. TLR expression on circulating αβ T-cells

Blood was collected from mice at 24 hr after sham or burn injury and stained for expression of αβ TCR and TLR-2 [**A**] or TLR-4 [**B**] as described in the materials and methods. The lymphocyte/monocyte population was identified by forward and side scatter and analyzed FITC αβ TCR and PE TLR-2 or TLR-4. Data are mean ± SEM for n=4/group.

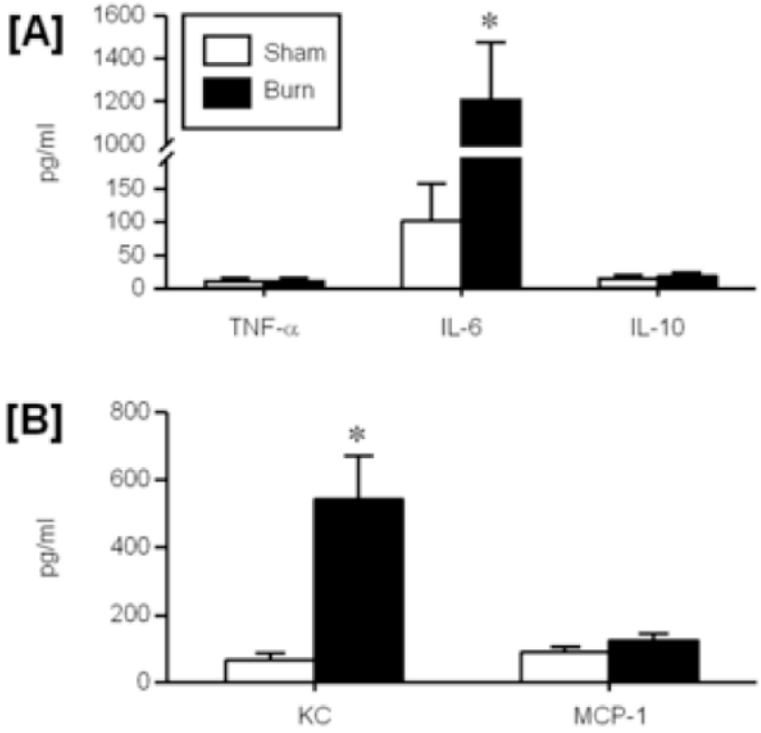

Figure 5. The impact of burn injury on systemic cytokine and chemokine levels

Plasma was collected from mice at 24 hr after sham or burn injury and assayed for the cytokines TNF-α, IL-6 and IL-10 [**A**] and the chemokines KC and MCP-1 [**B**] as described in the materials and methods. Data are mean ± SEM for n=5/group. * $p<0.05$ vs. respective sham group.

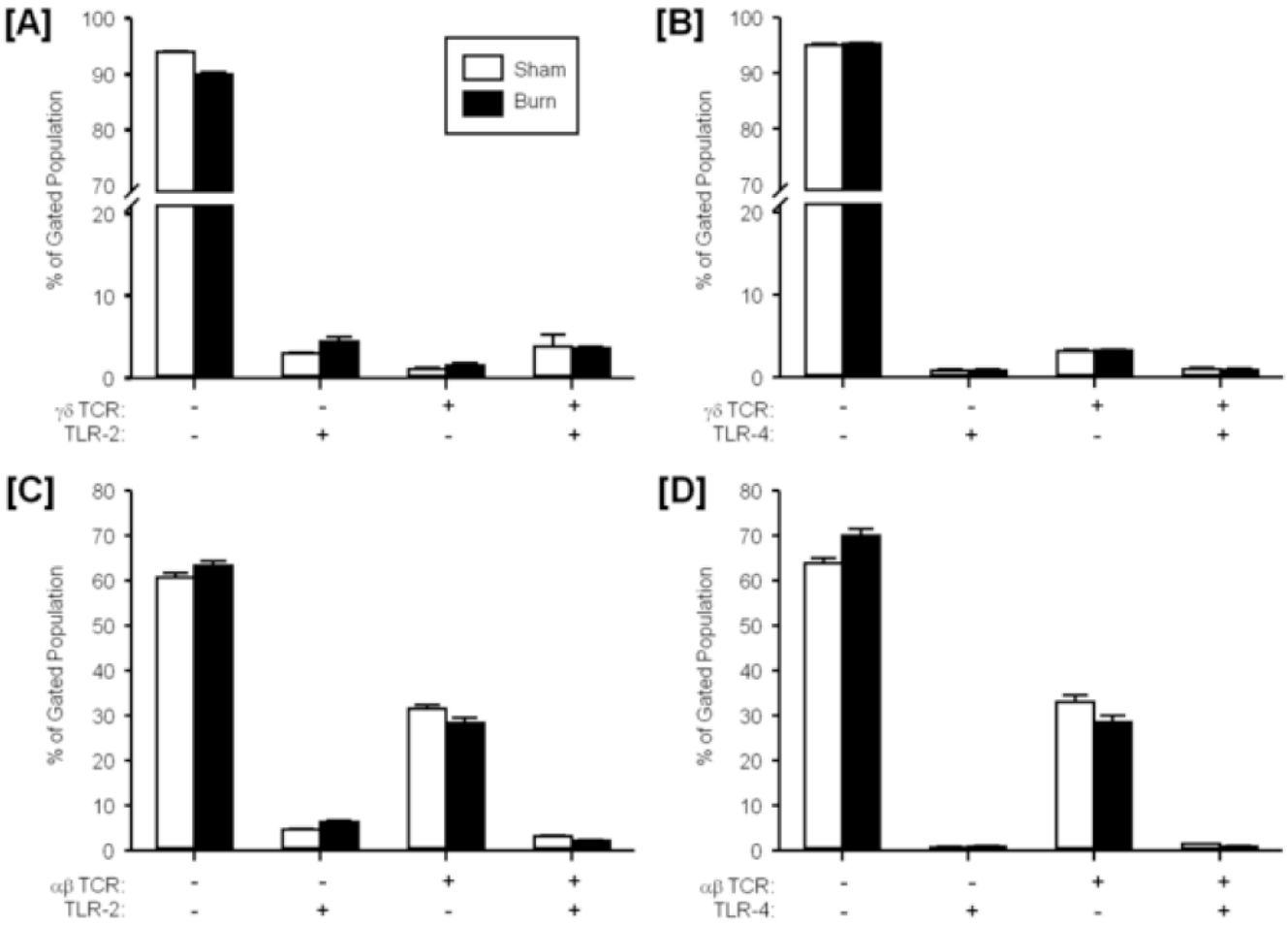

Figure 6. TLR expression on circulating T-cells late post-injury

Blood was collected from mice at 7 days after sham or burn injury and stained for expression of γδ TCR and TLR-2 [**A**], γδ TCR and TLR-4 [**B**], αβ TCR and TLR-2 [**C**] and γδ TCR and TLR-4 [**D**] as described in the materials and methods. The lymphocyte/monocyte population was identified by forward and side scatter and analyzed FITC TCR and PE TLR-2 or TLR-4. Data are mean ± SEM for n=4/group.

Table 1

Expression of activation markers on circulating γδ T-cell at 24 hr post-injury

	γδ TCR$^+$	γδ TCR$^+$ CD69$^+$	γδ TCR$^+$ CD120b$^+$
Sham	14.6±0.9[a]	4.8±1.6	7.2±0.9
Burn	20.6±3.6[b]	3.8±1.1	7.6±1.8

[a] Data are expressed mean ± SEM of % of the gated lymphocyte population (n = 5–6/group).

[b] $p<0.05$ as compared with sham.

Table 2

Expression of TLRs on splenic and MLN T-cells at 7 days post-injury

	γδ TCR$^+$	γδ TCR$^+$ TLR-2$^+$	γδ TCR$^+$ TLR-4$^+$	αβ TCR$^+$	αβ TCR$^+$ TLR-2$^+$	αβ TCR$^+$ TLR-4$^+$
Spleen (n=4)						
Sham	2.5±0.9[a]	1.1±0.4	0.3±0.1	30.9±2.1	1.2±0.1	0.4±0.1
Burn	1.8±0.5	0.8±0.2	0.2±0.1	28.4±0.6	1.0±0.2	0.4±0.1
MLN (n=3)						
Sham`	1.7±0.1	0.4±0.1	0.8±0.1	49.2±0.3	0.4±0.1	1.6±0.3
Burn	1.7±0.3	0.3±0.1	0.3±0.1	54.3±0.6	0.8±0.1	3.7±0.3

[a]Data are expressed mean ± SEM of % of the gated lymphocyte population.

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