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T cells from burn-injured mice demonstrate a loss of sensitivity to glucocorticoids

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D'Elia M, Patenaude J, Dupras C, Bernier J. T cells from burn-injured mice demonstrate a loss of sensitivity to glucocorticoids. *Am J Physiol Endocrinol Metab* 299: E299–E307, 2010. First published June 1, 2010; doi:10.1152/ajpendo.00084.2010.—Glucocorticoids (GC) are steroid hormones that modulate T cell functions and restrain their hyperresponsiveness following stimulation. Naive T lymphocytes are sensitive to GC but become more resistant when they are activated. A balance between activation and inhibition signals is important for a targeted and effective T cell response. Thermal injury is characterized by an immune dysfunction and hyperactive T cells visible at day 10 postburn. In this study, our objective was to evaluate T cell sensitivity to GC following thermal injury and to identify mechanisms that could modulate their sensitivity. One mechanism that we hypothesized was increased p38 mitogen-activated protein kinase (MAPK) activity that could lead to GC resistance. Male C57BL/6 mice underwent a full-thickness 20% total body surface area. At 10 days postinjury, splenic T cells were isolated. Glucocorticoid receptor (GR) expression was higher in T cells from burn-injured mice. Interestingly, these cells were also less sensitive to GC-induced apoptosis prior to and poststimulation. Furthermore, anti-CD3-activated T cells from burn-injured mice showed increased proliferation and CD25 expression, which resisted corticosterone's (CORT) suppressive effect. Anti-CD3-activated $CD4^+CD44^+$ memory cells from burn-injured mice expressed the highest level of CD25 and were resistant to CORT. Increased phosphorylation of p38 MAPK was also noted in activated T cells from burn-injured mice. Pharmacological inhibition of p38 MAPK decreased cell proliferation and normalized interferon-γ (IFNγ) production. In conclusion, we demonstrate that a unique event like burn injury induces a loss of sensitivity to GC in splenic T cells and have identified p38 MAPK as a key modulator for this resistance.

T lymphocyte; resistance; mitogen-activated protein kinase

ENDOGENOUS GLUCOCORTICOID (GC) hormones are highly produced following thermal injury. A small number of studies focusing on the early events postburn have reported the effects of GC released in response to this trauma on the immune system. Most of them suggested a proapoptotic role for GC on thymocytes and mature T cells, while Faunce et al. have shown that GC protected burned mice from severe immune dysfunction that occurred on days 1 and 2 postburn by regulating interleukin-6 (IL-6) production (17, 20, 21, 32). GC exerts their action by suppressing both innate and acquired immunity and by inhibiting the production of proinflammatory mediators (19). T lymphocytes are a major sensitive target for GC action on acquired immunity. GC counteract T cell activity by promoting their apoptosis, reducing proliferation, decreasing high-affinity IL-2 receptor-α chain (CD25) expression, and inhibiting IL-2 and interferon-γ (IFNγ) production (35, 38). Overall, these events cause a shift in T cell-mediated response from T helper-1 (Th1) to Th2 phenotype, which in burn-injured patients was related to impaired immunity (15). T cell dysfunction occurring past day 10 postinjury has been linked to increased morbidity and mortality of burn-injured patients (2). Also, previous reports from our group and others' have noted hyperresponsive T lymphocytes on day 7 and later postburn injury (23, 27, 34). Furthermore, we have shown in a time course study that T cell functions were modified at day 10 postburn injury. Splenic T lymphocytes demonstrated an activated phenotype, spontaneous apoptosis, and an overall unresponsiveness to antigen-induced stimulation (34).

Normal T cell activation leads to increased intracellular signaling mediated by mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase (JNK), and p38 (14). In T lymphocytes, p38 MAPK is activated by T cell receptor (TCR)/CD3, triggering alone or in synergy with CD28 cross-linking (8, 40). Moreover, p38 MAPK can be activated in response to IL-2 and IL-7 (9). p38 MAPK is involved in the expression of several cytokines (IFNγ, IL-2, and IL-10), cell proliferation, and induction of apoptosis (14). Regarding their phenotype, p38 MAPK activation is closely related to Th1 response in committed $CD4^+$ T cells and controls IFNγ expression (4, 39).

Few studies have evaluated MAPK expression or its activity following burn injury, and all of them have been interested in the early immune response going up to day 3 postburn. Choudhry et al. (7) have reported decreased p38 MAPK activity in anti-CD3-stimulated T cells isolated form rat mesenteric lymph node at day 2 postburn. They noted decreased T cell proliferation associated with reduced MAPK activity. Other studies showed a perturbation of Ca^{2+} mobilization in T cells isolated from burn-injured animals resulting in attenuated MAPK activation (18). Li et al. have associated high corticosterone (CORT) levels present in burn-injured rats at days 1 and 2 postinjury to decreased p38 MAPK activity resulting in lower IL-2 production and cell proliferation (30). At a cellular level, GC repress p38 MAPK by promoting the dual MAPK phosphatase-1 (MKP-1) expression, which dephosphorylates p38 and attenuates cell activation (29).

In chronic inflammatory diseases, resistance to GC has been related to several mechanisms: reduced number of glucocorticoid receptors (GR), altered affinity of the GR for the ligand, and reduced ability of the GR to bind to DNA by posttranscriptional modification (1, 6). Among candidates to mediate the latter event, MAPKs were identified. Recently, Irusen et al. (24) have associated GR phosphorylation mediated by p38 MAPK with reduced responsiveness to dexamethasone in peripheral blood mononucleated cells (PBMCs) from patients with steroid-resistant asthma. Data from in vitro studies showed that phosphorylation sites in the amino-terminal region

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(at Ser^{203}, Ser^{211}, and Ser^{226}) are critical for GR function (47). Recently, Szatmary et al. (44) have confirmed that suppression of GR function by activated p38 MAPK is a physiologically important mechanism for GC resistance in patients with chronic inflammatory disease.

Resistance to GC is an acquired situation where cells that are exposed during a long period of time to high levels of GC develop a loss of sensitivity to these hormones. Endogenous GC production is maximal following thermal injury, and the effect of this single event has already been associated with increased apoptosis seen in the thymus and spleen at days 1 and 2 postburn followed by a general recovery at day 5 postburn (12, 21). GC responsiveness in T cells that either were selected or survived during these crucial first 5 days postburn has never been addressed. Since T cell dysfunction has been demonstrated at day 10 postburn injury by our group and others (23, 27, 34) and was linked to their hyperactive status, we hypothesize that this situation could be the result of a loss of sensitivity to GC.

Here, we report that isolated T cells from burn-injured mice are less sensitive to GC at day 10 postburn. Decreased GC sensitivity was mediated by T cell activation and high p38 MAPK activity. Furthermore, we demonstrate that a single event like a thermal injury can induce in splenic T cells a loss of sensitivity to GC and that inhibition of p38 MAPK can normalize their functions.

MATERIALS AND METHODS

Animals. All experiments were performed on 6-wk-old male mice: C57BL/6 (Charles River Laboratories, St-Constant, QC, Canada). The mice were acclimatized for a period of 2 wk prior to the initiation of any procedures and were housed in a central animal facility under strictly controlled temperature, relative humidity, and a 12:12-h light-dark cycle. They were kept in cages, each containing five mice. Standard chow (Richmond Standard Lab Diet; Lab Diet, Richmond, IN) and water were provided ad libitum. The Institutional Animal Care Committee reviewed and approved all procedures performed in accordance with the Canadian Council on Animal Care guidelines.

Burn injury. Mice were subjected to a 20% total body surface area burn (TBSA) as described before (12). Briefly, mice were randomly assigned to a control or burn group, each containing eight animals. After anesthesia and shaving of the dorsum, animals were placed in a mold and immersed for 7 s in water at 90°C to produce a full thickness to cover 20%. The sham group was immersed in water at room temperature (22°C). Both sham- and burn-injured mice were resuscitated after the procedure with 2 ml of saline given intraperitoneally containing buprenorphine. Flamazine (Smith & Nephew, Montreal, QC, Canada), a topical antiseptic cream, was directly applied on the wound to reduce the risk of infection.

T lymphocyte purification. On day 10 after treatment, the animals were euthanized. Spleens were removed and prepared individually as single-cell suspensions by digestion with collagenase D (1 mg/ml)/Dnase (20 μg/ml; Roche Diagnostics, Laval, QC, Canada) solution in RPMI 1640 (Sigma-Aldrich, Oakville, ON, Canada). The suspensions were washed three times in HBSS and depleted of red blood cells by osmotic shock. Cells were resuspended in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and antibiotic solution (100 IU/ml penicillin + 100 μg/ml streptomycin, Sigma-Aldrich). Cell viability was determined by trypan blue exclusion and was consistently greater than 90%. T cells were purified from mouse spleen cell suspensions by use of a MACS separation column system with a Pan T cell isolation kit (Miltenyi biotec, Auburn, CA). T lymphocyte purity was determined by flow cytometry on a FACScan flow cytometer (Becton-Dickinson) using anti-mouse CD3 FITC-conjugated antibody (eBioscience, San Diego, CA) and was reproducibly higher than 95%.

Apoptosis determination. T cell apoptosis was evaluated by quantifying DNA sub-G0/G1 events in cell cycle. Briefly, 1×10^6 isolated T cells from sham- and burn-injured mice were cultured in 24-well plates in complete RPMI supplemented with 10% FCS (Sigma-Aldrich) for 24 h in the presence of increasing concentrations of CORT (1 to 0.01 μM). Control samples and cells were treated or not with 100 nM RU-486 (Sigma-Aldrich) a GR inhibitor, 30 min prior to CORT exposition. Following culture, cells were treated as previously described (10), and apoptosis was quantified by flow cytometry on a FACScan flow cytometer (Becton-Dickinson) by measuring the precentage of cells in phase sub-G0/G1.

T lymphocyte phenotyping. Cells were analyzed by three-color flow cytometry. Splenic T lymphocytes (1×10^5 cells/100 μl) were incubated for 30 min on ice in the presence of fluorochrome-conjugated anti-mouse mAb: rat anti-CD4 FITC-labeled molecule (eBioscience) or rat anti-CD8a FITC-labeled molecule (eBioscience) with anti-CD69 phycoerythrin(PE)-Cy5-conjugated molecule (eBioscience) or anti-CD25 phycoerythrin (PE)-conjugated molecule (eBioscience) for activation detection, biotinylated anti-CD62L L-selectine molecule (Cedarlane Laboratories, Hornby, ON, Canada) for naive T cell detection, or biotinylated anti-CD44 molecule (eBioscience) for effector/memory T cell detection. Cells were washed twice with cold HBSS, 0.1% BSA, and 0.01% sodium azide (Sigma-Aldrich) and analyzed on a FACScan flow cytometer (Becton-Dickinson). WinMDI software was used for the analysis.

In vitro proliferation assay. For lymphocyte proliferation studies, Anti-CD3 (clone 17A2, eBioscience) coated 96-well flat-bottom tissue culture plates were prepared by adding 100 μl per well of the diluted antibody (10 or 1 μg/ml) in PBS and incubating plates overnight at 4°C. Plates were washed three times with PBS prior to addition of T lymphocytes. Isolated T cells (2×10^5 per well) were cultured in complete RPMI supplemented with 10% FCS. In another experiment, purified T cells were exposed for 30 min with either 10 μM MAPK p38 inhibitor SB-203580 (Sigma-Aldrich) or DMSO vehicle (Fisher Scientific) and subsequently transferred to tissue culture plates. Cells were stimulated in the presence or absence of 0.1 to 0.001 μM CORT (Sigma-Aldrich). All cultures were performed in triplicates, including the control groups without mitogen. Cells were cultured for 60 h at 37°C with 5% CO_2 in a humidified incubator, and all cultures were pulsed with 1 μCi/well of [^{3}H]TdR activity specific (GE Healthcare Bio-Sciences, Baie-d'Urfee, QC, Canada). Cells were harvested 12 h later onto a glass fiber filter using a Skatron cell harvester (Skatron Instruments, Sterling, VA). Radioactivity was determined with a liquid scintillation counter (LKB Wallac).

Carboxyfluorescein succinimidyl ester labeling. Cells were diluted (1×10^7 cells/ml) in a solution of PBS supplemented with 0.5% BSA and incubated for 10 min at 37°C with carboxyfluorescein succinimidyl ester (CFSE) at a final working concentration of 5 μM (Molecular Probes, Eugene, OR). CFSE was subsequently neutralized by adding complete ice-cold RPMI to the cells followed by 5 min of incubation on ice and washed twice with ice-cold RPMI. CFSE-labeled T cells were cultured for 72 h in 24-well plates with the same conditions as described previously. CFSE labeling efficiency was measured before culture by flow cytometry. Cells were analyzed on a FACScan flow cytometer. Histogram analyses were performed using WinMdi software by gating only live cells.

Western blot. Cells (5×10^5) were diluted in sample buffer, subjected to SDS-PAGE, and electroblotted onto nitrocellulose membrane (Bio-Rad, Hercules, Ca) for anti-GR (sc-1004; Santa Cruz biotechnology, Santa Cruz, CA), for anti-MKP-1 (sc-370; Santa Cruz Biotechnology) and anti-heat shock protein 70 (HSP70) and HSP90 (Stressgen Bioreagents, Ann Arbor, MI) blots or polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for anti-phospho-p38 MAPK (Biosource) and anti-p38 MAPK (sc-535, Santa Cruz Biotechnology) blots. The membranes were washed in Tris-buffered saline (TBS; 50

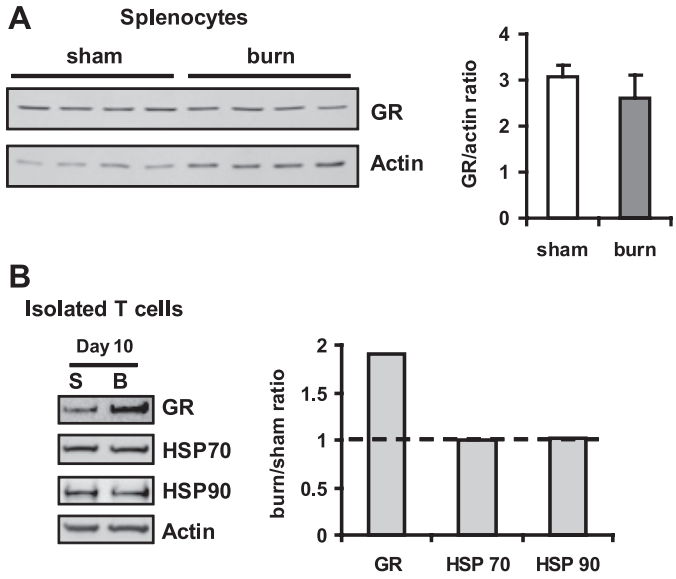

Fig. 1. Burn injury selectively increases glucocorticoid (GC) receptor (GR) expression in isolated T cells at day 10 postburn. Cells (5×10^6; *A*, splenocytes or *B*, T cells) were isolated from sham and burn-injured mice at day 10 postburn injury, protein were extracted in sample buffer, and GR and heat shock protein (HSP) protein levels were analysed by Western blot. For GR analysis in splenocytes, each lane represents cells extracted from one mouse, and results are presented as grouped values. For isolated T lymphocytes, each lane represents grouped individuals, and results are presented graphically as a burn vs. sham ratio. One representative of 4 independent experiments is shown.

mM Tris, pH 8.0, and 150 mM NaCl) and probed according to the manufacturer's indication. Specific antibody-antigen complexes were identified using a horseradish peroxidase-labeled anti-rabbit antibody (sc-2004, Santa Cruz biotechnology) or anti-mouse antibody (GE Healthcare Biosciences) and ECL Western blotting detection reagents (GE Healthcare Biosciences). Membranes were stripped and reprobed with antibody against mouse actin (A4700, Sigma-Aldrich) to evaluate protein loading in each sample.

Cytokine assays. Cells were cultured for 72 h as described previously (In vitro proliferation assay). IL-2 and IFNγ levels in culture supernatants were assayed by sandwich ELISA using paired antibodies. Briefly, Maxisorp 96-well-flat-bottom Nunc immunoplates (Fisher) were coated with purified rat anti-mouse IL-2 (BD Biosciences, Mississauga, ON, Canada) or purified rat anti-mouse IFNγ (BD Biosciences), and the assays were performed according to the manufacturer's indications using, respectively, biotin rat anti-mouse IL-2 or biotin rat anti-mouse IFNγ (both BD Biosciences). The final step of the assays was performed using 2,2′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Sigma), and for each well the optical density at 405 nm was determined on a Powerwave X microplate reader (Bio-Tek Instruments). The concentrations of IL-2 and IFNγ in the samples were determined by interpolation against a standard curve produced with murine recombinant IL-2 or IFNγ (both eBioscience), respectively.

Statistical analysis. All data are expressed as means ± SE. Data were analyzed by one-way ANOVA comparison test using the Tukey posttest with the GraphPad InStat 3.05 software program (GraphPad Software, San Diego, Ca). Differences between groups were considered statistically significant when the probability by chance was less than 5% ($P < 0.05$). All experiments were performed in quadruplicates to confirm our results.

RESULTS

GR is specifically elevated in T cells from burn-injured mice. We demonstrated on day 10 postburn injury, increased cell activation in purified T lymphocytes in the absence of accessory cells or reducing reagent (11). In healthy mice, T cell activation was associated with increased GR expression (28). We first investigated GR expression in total spleen cells and in purified T lymphocytes from sham- and burn-injured mice at day 10 postburn. Whereas splenocytes from both sham- and burn-injured mice showed similar GR levels (Fig. 1*A*), GR was highly expressed in purified splenic T cells from burn-injured mice (Fig. 1*B*). To verify whether other GR complex components were modulated as well by burn injury, we also measured heat shock protein (HSP)70 and HSP90 expression in isolated T cells. These proteins act as chaperones and are essential for inactive GR conformation. Interestingly, although GR levels were strongly increased following burn injury, HSPs levels were similar to control values. This results shows that GR is specifically upregulated in isolated T cells at day 10 postburn injury and suggests a possible role for GC in T cell-mediated responses following thermal injury.

T lymphocytes from burn-injured mice are less sensitive to GC-induced apoptosis. Increased GR expression in T cells after burn injury can modify their sensitivity to GC. Like other nuclear hormone receptors, GR function may be affected by an oxidizing environment, as documented after a major burn injury (25). For this reason, we studied GR functionality in a complete medium without any reducing reagent such as β-mercaptoethanol (ME). The presence of a reducing agent has been demonstrated in numerous in vitro or in vivo studies to affect ligand binding on nuclear hormone receptors (16, 45). To verify whether GR expression in purified T cells correlated with GC-induced apoptosis, lymphocytes were exposed to CORT concentrations ranging from 1 to 0.01 μM for 24 h. Apoptosis was subsequently determined as described above. Compared with control cells, T lymphocytes from burn-injured mice were significantly less sensitive to GC-induced apoptosis when exposed to 1 μM CORT (Fig. 2). To confirm this, we used the pharmacological inhibitor RU-486, which is known to block GR agonist activity by competitive binding to the ligand-binding domain (26). As expected, RU-486 prevented GC-induced apoptosis in purified T cells from both sham- and

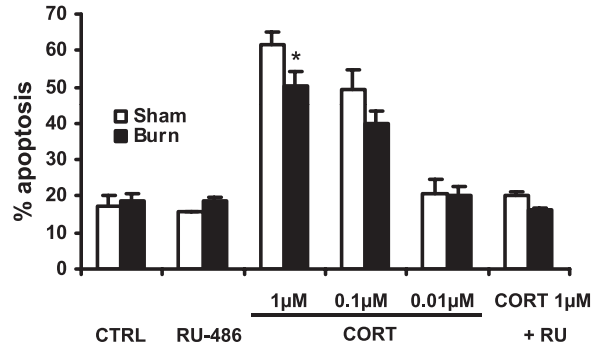

Fig. 2. Splenic isolated T cells from burn-injured mice are less sensitive to CORT-induced apoptosis at day 10 postburn injury. Isolated T cells (1×10^6) from sham- and burn-injured mice were exposed to CORT (1 to 0.01 μM) in cell medium culture for 24 h. Cells were treated with 1 μM RU-486 or left untreated 30 min prior to CORT exposition to specifically inhibit GC-induced apoptosis. Cell apoptosis was evaluated by flow cytometry as the percent DNA content sub-G_0. Data represent mean values of pooled T cells ± SE ($n = 8$ mice per group; $^*P < 0.05$). Data are representative of 4 independent experiments.

burn-injured mice. This result suggests that T cells from burn-injured mice are more resistant to GC-induced apoptosis.

T cells from burn-injured mice are highly proliferative and demonstrate resistance to the antiproliferative effect of GC. We (11) recently showed that anti-CD3 mAb-stimulated T cells from burn-injured mice were highly proliferative in the absence of ME. We next investigated in similar conditions their sensitivity to a GC-mediated antiproliferative effect. T cells from sham- and burn-injured mice were stimulated with plate-bound anti-CD3 in the presence of CORT with concentrations ranging from 0.1 to 0.001 μM (Fig. 3). T cells from burn-injured mice were more responsive to plate-bound anti-CD3 than control cells. Lower concentration of CORT had no effect on T cell response from both groups. In contrast, addition of 0.1 μM CORT significantly reduced T cell proliferation from sham mice as previously reported (41). Interestingly, the proliferative response of T lymphocytes from burned mice remained unchanged in the presence of 0.1 μM CORT, pointing out a resistance of these cells to CORT.

GC-resistant T cells from burn-injured mice display greater cell division and lower activation-induced cell death. On the basis of in vitro proliferation results, T cells from burn-injured mice proliferate more and are resistant to CORT's antiproliferative effect. To determine whether GC action was associated either with an increase in cell division or in the number of stimulated cells, T lymphocytes from both groups were labeled with CFSE, and their division profile was evaluated by flow cytometry (Fig. 4*A*). CFSE is partitioned between daughter cells during each cell division. Because daughter cell fluorescence intensities are approximately halved after each division, the intensity of a cell compared with its intensity at the time of staining provides information about the number of cell divisions it has undergone. As expected, nonstimulated cells were positive for CFSE staining, whereas no cell division peaks were noticeable. Purified T cells from both sham- and burn-injured mice responded to anti-CD3 stimulation (M1 marker on the histograms; Fig. 4*A*). However, burn-injured animals exhibited a greater number of highly proliferative cells, represented on the histograms by M4 and M5 markers (19% for

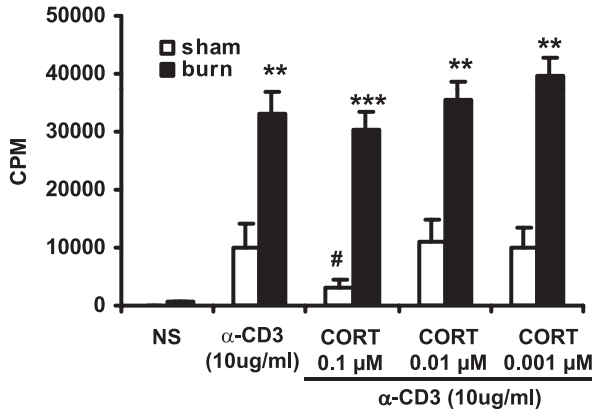

Fig. 3. Stimulated T cells from burn-injured mice are resistant to CORT antiproliferative effect. T cells from sham- and burn-injured mice were isolated at day 10 postburn injury. Cells were cultured (2×10^5 cells/well in 96-well plates) and stimulated with plate-bound anti-CD3 (10 μg/ml) for 60 h. [^{3}H]thymidine was added for the last 12 h of cell culture. Data represent mean values of cpm ± SE ($n = 8$ mice per group; #$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data are representative of 4 independent experiments.

burn-injured mice vs. 8% for control animals). The presence of 0.1 μM CORT greatly inhibited cell division for sham-injured animals, as only 2% of these cells reached the M4 and M5 markers. Nevertheless, 50% of them underwent one cell division cycle. The presence of 0.1 μM CORT had no effect on T cell division from burned mice, as 21% of these cells were found in markers M4 and M5. As seen in T cells from control animals, ~50% of T cells from burned mice responded to anti-CD3 mAb. However, the number of cells with three cycles of division or more was two times greater in cells from burn-injured mice. Overall, stimulated T cells from burned mice showed a reduced number of cells that were dividing but a general increase in the number of division cycles.

T cells from burn-injured mice are less sensitive to apoptosis. We next wished to analyze whether increased cell division correlated with either decreased activation-induced cell death (AICD) or GC-induced apoptosis. T lymphocytes from burn-injured mice were significantly less sensitive to AICD after anti-CD3 stimulation (Fig. 4*B*). In fact, whereas 50% of stimulated T cells from sham-injured mice exhibited fragmented DNA (sub-G0) only 35% of the cells from burn-injured animals were in the same situation (apoptosis index = 0.7). When stimulated T lymphocytes were exposed to CORT, cell death was increased for both groups, but the difference that existed between stimulated sham- and burn-injured T cells was still present. Together, these results suggest that T cells from burn-injured mice are highly proliferative and less sensitive than control cells to CORT-induced apoptosis or the CORT antiproliferative effect.

CD4$^+$ T cells are less sensitive to CORT, as demonstrated by sustained CD25 expression postactivation. IL-2 receptor-α chain (CD25) is one of the first markers increased following T cell activation. Interestingly, GC decreases its expression at a transcriptionnal level (3). Thus, flow cytometry evaluation of CD25 expression on anti-CD3-stimulated T cells exposed to CORT is an indirect method to visualize their responsiveness to GC. Flow cytometry analysis was performed on CD4$^+$, CD8$^+$, CD62$^+$ (naive), and CD44$^+$ (memory) splenic T cells to determine CORT's effect on CD25 expression. As expected, anti-CD3 stimulation increased CD25 expression in all subpopulations, with close to 80% expression in gated viable cells from burn-injured mice, but only between 20 and 60% in cells from sham-injured animals. However, although CORT decreased CD25 expression in all sham subgroups, CD4$^+$ and CD44$^+$ burn T cells were totally resistant to CORT effect (Fig. 5*A*). We next confirmed which subpopulation was less sensitive to CORT by looking at their individual expressions poststimulation and when exposed to CORT. Consistant with CD25 expression, both naive (CD62$^+$) and memory (CD44$^+$) CD4$^+$ subtypes demonstrated the greatest resistance to the CORT effect in burn-injured mice (Fig. 5*B*), wherease CD8$^+$ T cell subtypes demonstrated the same sensitivity as per CD25 expression (data not shown).

Anti-CD3-stimulated T cells from burn-injured mice show sustained p38 MAPK activation. T cell activation leads to signal transduction through MAPK proteins. It was previously demonstrated that anti-CD3 mAb stimulation could activate p38 MAPK in T cells (13). Moreover, p38 MAPK was also shown to induce GC resistance through GR phosphorylation (24). Conversely, GC are able to induce expression of MKP-1, a phosphatase that inactivates p38 MAPK (29). To study p38

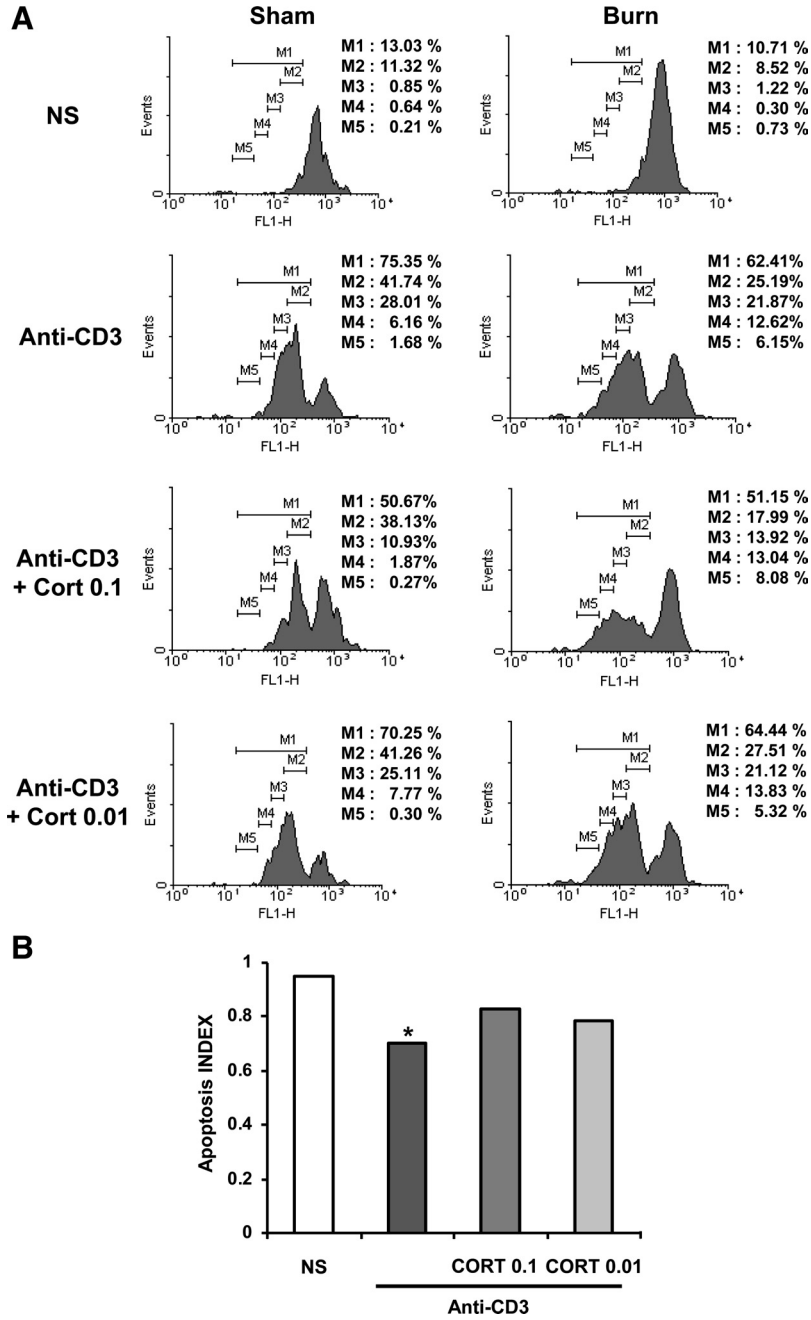

Fig. 4. Anti-CD3-stimulated T cells from burn-injured mice are less sensitive to CORT suppressive effect as shown by increased cell division cycle and lower apoptotic cell death. Splenic T cells from sham- and burn-injured mice were isolated at day 10 postburn. Cells were labeled with 5 μM CFSE, plated (2×10^6 cells/well in 24-well plates), and stimulated with plate-bound anti-CD3 (10 μg/ml) for 72 h. *A*: cells were exposed to CORT (0.1 and 0.01 μM) or left untreated. Cells were analyzed by flow cytometry, and histograms were generated. One representative of 4 independent experiments is shown. *B*: M1 marker represents total cell division; M2-M5 regions represent corresponding cell division peak. Apoptosis was evaluated by flow cytometry as percent DNA content sub-G_0 and presented as an index ratio between burn- and sham-injured T cells. Data represent mean values of pooled T cells ± SE ($n = 8$ mice per group; $^*P < 0.05$) and are representative of 4 independent experiments.

MAPK's role in T cells postburn injury, cells from sham- and burn-injured mice were stimulated with plate-bound anti-CD3 mAb for 3 days of cell culture. To determine GC influence on p38 activity, stimulated cells were also exposed to 0.1 or 0.01 μM CORT or left untreated. p38 MAPK expression and its activated form, shown by its phosphorylation status, were analyzed. Anti-CD3 stimulation induced p38 MAPK activation in cells from both groups. However, when cells were exposed to 0.1 μM CORT, only T lymphocytes from burn-injured mice demonstrated a sustained p38 MAPK phophorylation (Fig. 6*A*). On the other hand, cells from sham-injured animals were sensitive to GC, as p38 MAPK phosphorylation decreased significantly ($P < 0.05$) following their exposure to 0.1 μM CORT (Fig. 6*B*). As expected, MKP-1 expression was induced when T cells were stimulated (Fig. *6C*). However, T cells from burn-injured mice failed to further upregulate MKP-1 expression when exposed to CORT. These results suggest that p38 MAPK is activated in stimulated T cells and that p38 MAPK sustained phosphorylation in CORT-treated cells from burn-injured mice contributes to GC resistance.

p38 MAPK pharmacological inhibition normalizes T cell proliferation and IFNγ production by burn-injured mice. To confirm a relationship between p38 MAPK activation, T cell proliferation, and CORT sensitivity, p38 MAPK activity was inhibited with 10 μM SB-203580, a specific p38 MAPK inhibitor. Purified T cells from sham- and burn-injured mice were exposed to the inhibitor or left untreated for 30 min and then stimulated with anti-CD3 mAbs. Cell proliferation was

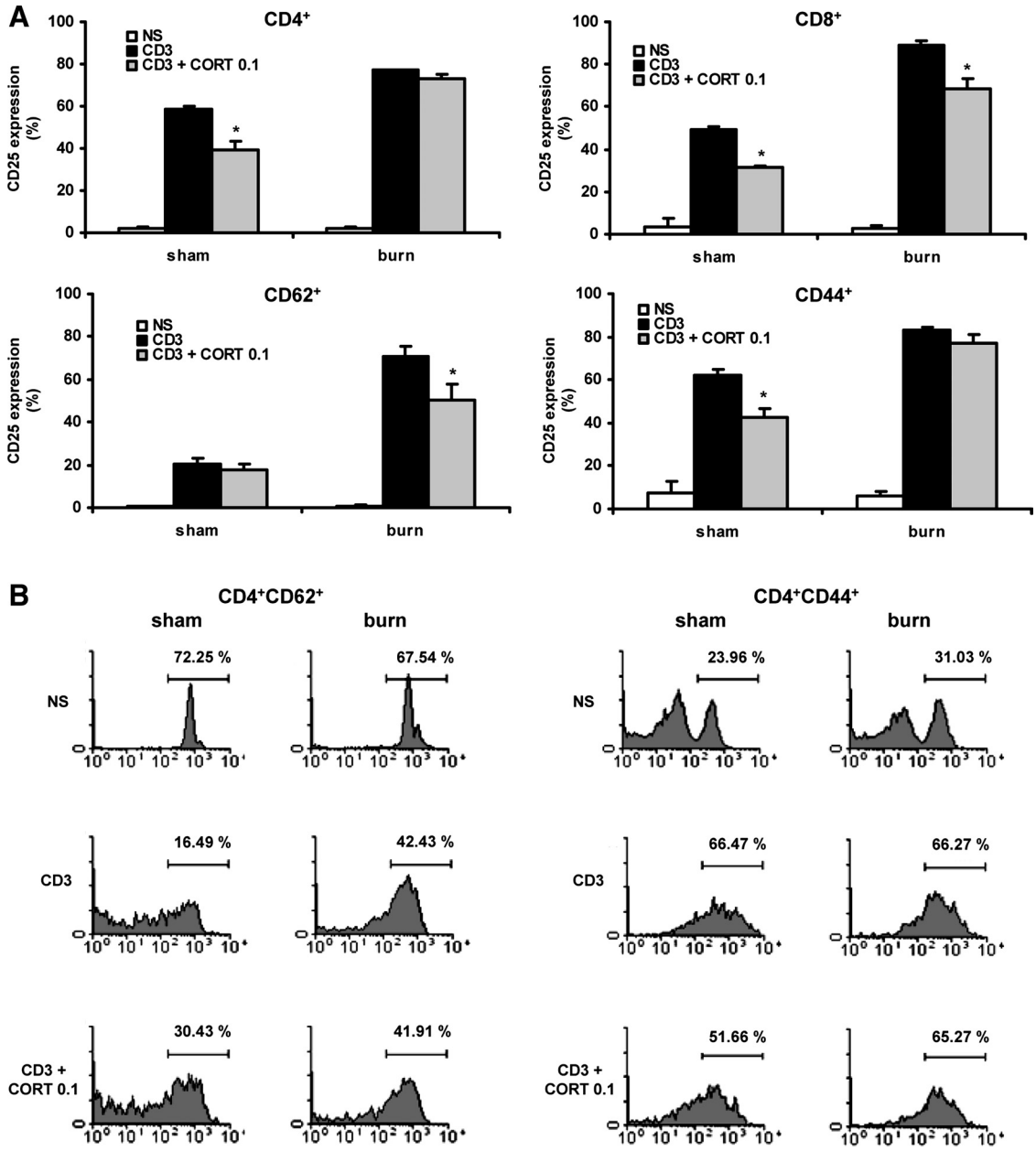

Fig. 5. Anti-CD3-stimulated T cells from burn-injured mice demonstrate high CD25 expression. Splenic T cells from sham- and burn-injured mice were isolated at day 10 postburn injury. Cells were plated (2×10^6 cells/well in 24-well plates) and stimulated with plate-bound anti-CD3 (10 μg/ml) for 72 h. Cells were exposed to CORT (0.1 μM) or left untreated. CD25 expression was evaluated by flow cytometry on gated $CD4^+$, $CD8^+$, $CD44^+$, and $CD62^+$ T cells, and histograms were generated. Data in *A* represent mean values of 2 independent experiments ± SE (*$P < 0.05$). Gated $CD4^+$ T cells were also characterized by flow cytometry and identified as either $CD62^+$ or $CD44^+$ cells. Their respective percentages were calculated on each histogram (*B*).

next determined. T cells from burn-injured mice treated with the inhibitor showed a significantly reduced proliferation ($P < 0.001$). p38 MAPK inhibition did not render these cells sensitive to GC, as their proliferation was not further reduced when exposed to 0.1 μM CORT. This result demonstrates that p38 MAPK-specific inhibition normalized the proliferation of T cells from burn-injured mice. As cell proliferation was not further reduced by CORT, p38 MAPK may indirectly account for GC resistance of these cells (Fig. 7*A*).

IFNγ production in T cells is controlled mainly by signal transduction through p38 MAPK, while IL-2 is partially regulated by this kinase (39). As T cell proliferation depends on cytokine production and cells from burn-injured mice are highly proliferative, we wanted to investigate whether first it was related to increased IL-2 and/or IFNγ production, and second whether p38 MAPK inhibition had any influence on their levels. To address this, cell culture supernatants were collected and cytokine levels measured. As expected, both INFγ and IL-2 levels were significantly elevated in cells from burn-injured mice, consistent with their increased proliferation (Fig. 7, *B* and *C*). Interestingly, production of both cytokines was significantly reduced ($P < 0.05$) when cells from both groups were exposed to 0.1 μM CORT. Furthermore, p38 inhibition normalized IFNγ production by cells from thermally

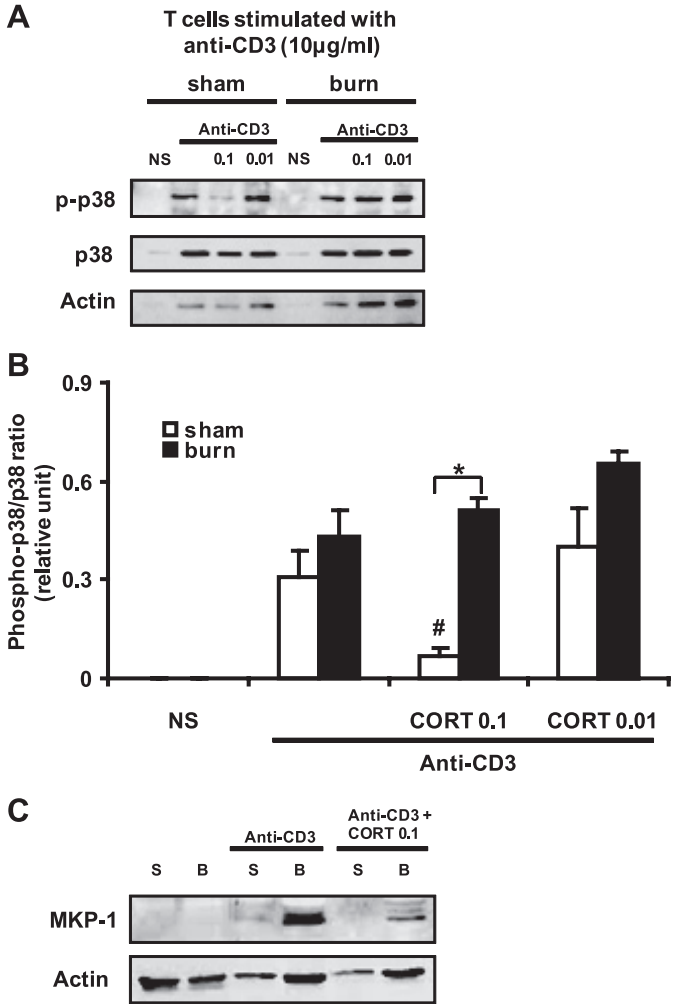

Fig. 6. GC-resistant T cells from burn-injured mice show sustained expression of p38 MAPK following anti-CD3 stimulation. Isolated T cells from sham- and burn-injured mice were plated (2×10^6 cells/well in 24-well plates) and stimulated with 10 μg/ml plate-bound anti-CD3 for 72 h. Cells were left untreated or exposed to CORT (0.1 and 0.01 μM). Phosphorylated and nonphosphorylated p38 MAPK forms were evaluated by Western blotting (*A*). Each lane represents grouped individuals from sham- and burn-injured mice ($n = 8$ mice/group) and 1 of 4 independent experiment is shown. Results are also expressed as a phospho-p38 vs. p38 ratio (*B*), and data represent mean values of 4 independent experiments ± SE (#$P < 0.05$, *$P < 0.05$). One representative blot for MAPK phosphatase-1 (MKP-1) expression in T cells is shown (*C*).

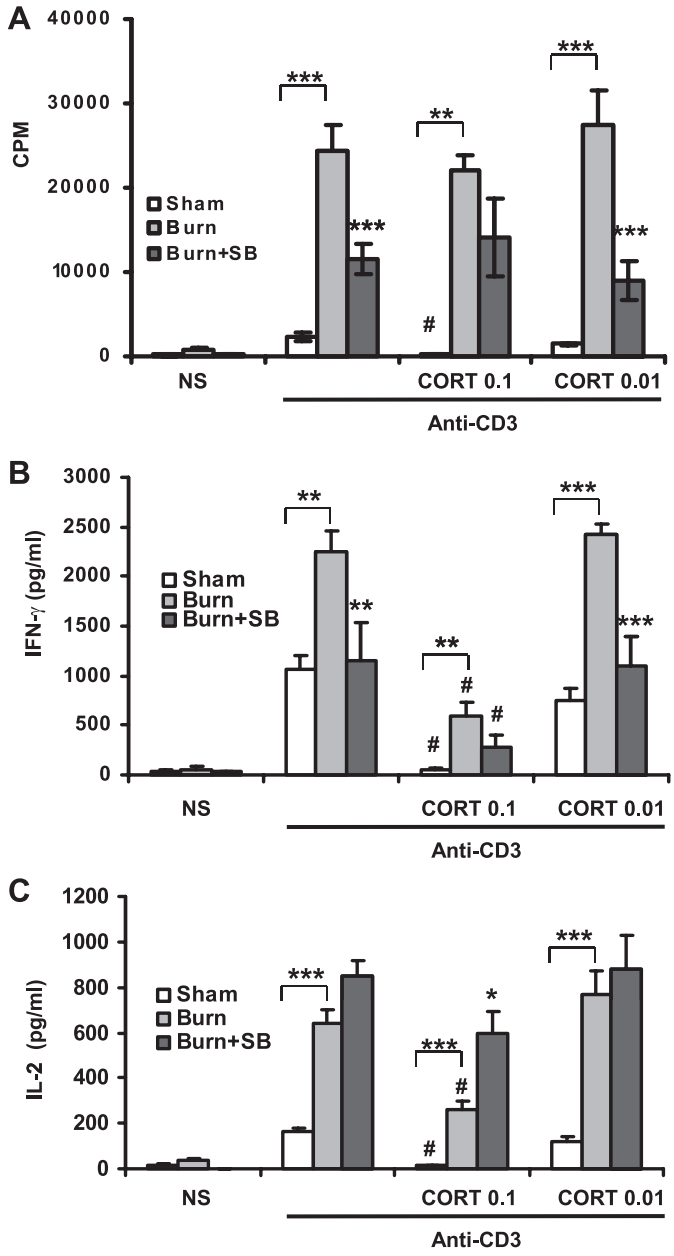

Fig. 7. p38 MAPK inhibition decreases cell proliferation and IFNγ production without affecting IL-2 production in isolated T cells from burn-injured mice. Splenic T cells from sham- and burn-injured mice were purified at day 10 postburn injury. Cells were cultured (2×10^5 cells/well in 96-well plates) and left untreated or exposed to p38-specific inhibitor SB-203580 (0.1 μM) for 30 min before stimulation with 10 μg/ml plate-bound anti-CD3 for 60 h and left untreated or exposed to CORT (0.1 and 0.01 μM). [^{3}H]thymidine was added for the last 12 h of cell culture. Proliferative responses are shown as cpm (*A*). Data represent mean values of cpm ± SE ($n = 8$ mice per group; **$P < 0.01$, ***$P < 0.001$). Data are representative of 4 independent experiments. Anti-CD3-stimulated T cells from burn-injured mice produce more IFNγ (*B*) and IL-2 (*C*) and are less sensitive to GC inhibitory effect than control mice. Treatment of burn-injured T cells p38 MAPK inhibitor decreased IFNγ but not IL-2 production to control levels. Cell culture supernatants were collected and cytokines were quantified. Data represent mean values ($n = 8$ mice per group; **$P < 0.01$, ***$P < 0.001$, and #$P < 0.05$, different from anti-CD3-stimulated cells for the same group). Data are representative of 4 independent experiments with three replicates each.

injured mice while significantly increasing IL-2 production. These results suggests that T cells from burn-injured mice are mainly Th1 committed, as they produce high levels of IFNγ, and that p38 MAPK inhibition normalizes IFNγ production without inhibiting IL-2 production.

DISCUSSION

Immunosuppression is defined as an inability to respond adequately to mitogenic or antigenic challenge either in vivo or in vitro (48). The stress response that follows thermal injury has an impact on mature peripheral T lymphocytes (17, 21, 30). Moreover, it was reported that the p38 MAPK pathway contributes to the immune dysfunction seen in sepsis (18, 42). This study has focused on evaluating in vitro sensitivity to CORT on

purified T lymphocytes from sham- and burn-injured mice. We hypothesized that increased in vivo splenic T cell activation reported on day 10 postburn injury might correlate with GC resistance and that p38 MAPK could be responsible for this situation.

First, we were able to show a specific increase in GR expression in T cells from burn-injured mice. Elevated GR levels were also observed in splenic T lymphocytes from mice exposed to stress-inducible concentrations of CORT in vivo (37). We can hypothesize that the stress response associated with thermal injury triggers the same change in our model. GC sensitivity in T cells does not necessarily correlate with GR levels, as many other mechanisms exist in these cells that may interfere with GC signaling. Transrepression has been described as an interaction between transcription factors (activator protein-1, nuclear factor of activated T cells, signal transducer and activator of transcription-5), which bind the GR and inhibit the transcription of genes under its control (5). As T cells from burn mice show an activated phenotype at day 10 postburn injury, we cannot rule out the possibility that transcription factors may interact with the GR and inhibit its binding to the DNA.

Our next step was to verify whether T cell proliferation was sensitive to CORT's inhibition effect. As previously shown, anti-CD3 mAb-stimulated T cells from burn-injured mice are highly proliferative (11). Moreover, in this report we have demonstrated that their proliferation was completely resistant to the CORT inhibition effect. Flow cytometry analysis revealed that some specific T cell subpopulations were responsible for their overall increased proliferation. These highly cycling cells demonstrated high CD25 expression following anti-CD3 stimulation, which was completely resistant to the CORT inhibition effect. These cells were identified as $CD4^+$ T cells with both naive ($CD62^+$) and memory ($CD44^+$) showing the same pattern when exposed to CORT. This result confirms our previous report, which showed a specific effect on $CD4^+$ T cells at day 10 postburn (34).

Splenic T cells from burn-injured mice also showed sustained p38 MAPK activation, which was resistant to CORT inhibition and correlated with high proliferation. This result was related to a failure in MKP-1 upregulation, a mechanism also seen in alveolar macrophages isolated from patients with severe asthma insensitive to corticosteroid treatments (43). In our model, pharmacological p38 MAPK inhibition reduced significantly T cell proliferation and IFNγ production in the burn-injured group. Interestingly, GC resistance was not related directly to increased p38 MAPK activation, since its pharmacological inhibition did not completely restore GC sensitivity. Therefore, it appears that alterations in the p38 MAPK pathway may account for only a portion of the changes that contribute to increased proliferation and indirectly to GC resistance observed in T cells from burn-injured mice.

Another possible mechanism to explain a loss of sensitivity to GC in activated T cells from burn-injured mice is high IL-2 production following anti-CD3 stimulation. We have demonstrated that T cell proliferation correlates with IL-2 levels but also p38 MAPK activity. Interestingly, another study has noted that IL-2-dependent T cell proliferation partially relied on the p38 MAPK signaling pathway (9). Indeed, it was defined that IL-2 inhibited GR nuclear translocation and transcriptional activity through a mechanism involving a direct interaction with STAT5, which led to GC resistance (5, 22). In our model, p38 MAPK inhibition, while decreasing IFNγ production, increased IL-2 secretion in activated T cells from burn-injured mice. Increased IL-2 production by T cells in which p38 MAPK was inhibited is documented in several studies (42, 43, 46).

Although cell proliferation, CD25 expression, and p38 MAPK phosphorylation were completely resistant to CORT-induced suppression, IFNγ and IL-2 production remained CORT sensitive. p38 MAPK inhibition led to decreased cell proliferation and normalized IFNγ production while not restoring complete GC sensitivity. This study is the first to demonstrate that a single event like thermal injury can lead to GC resistance in T cells and identifies p38 MAPK as a potential target for pharmacological intervention. Interestingly, in a different model, p38 MAPK inhibition improved survival in endotoxin shock and in a cecal ligation and puncture model of sepsis (33).

GC resistance in T cells following thermal injury may be part of a complex syndrome called critical illness-related corticosteroid insufficiency (CIRCI), which is defined as an inadequate corticosteroid activity for the severity of the illness of a patient (31). This would explain why the use of high-dose corticosteroids in patients with severe sepsis failed to improve outcome and was associated with increased complications. This finding prompted renewed interest in a replacement therapy with low doses of corticosteroids during longer periods (36). Taking this into account, a potential therapy using specific MAPK pharmacological inhibitors could enhance the therapeutic benefits of low-dose GC administered to patients with septic shock.

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DISCLOSURES

No conflicts of interest are reported by the authors.

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