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Impaired CD4$^+$ T-cell proliferation and effector function correlates with repressive histone methylation events in a mouse model of severe sepsis

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Immunosuppression following severe sepsis remains a significant human health concern, as long-term morbidity and mortality rates of patients who have recovered from life-threatening septic shock remain poor. Mouse models of severe sepsis indicate this immunosuppression may be partly due to alterations in myeloid cell function; however, the effect of severe sepsis on subsequent CD4$^+$ T-cell responses remains unclear. In the present study, CD4$^+$ T cells from mice subjected to an experimental model of severe sepsis (cecal ligation and puncture (CLP)) were analyzed *in vitro*. CD4$^+$CD62L$^+$ T cells from CLP mice exhibited reduced proliferative capacity and altered gene expression. Additionally, CD4$^+$CD62L$^+$ T cells from CLP mice exhibit dysregulated cytokine production after *in vitro* skewing with exogenous cytokines, indicating a decreased capability of these cells to commit to either the T_H1 or T_H2 lineage. Repressive histone methylation marks were also evident at promoter regions for the T_H1 cytokine IFN-γ and the T_H2 transcription factor GATA-3 in naïve CD4$^+$ T cells from CLP mice. These results provide evidence that CD4$^+$ T-cell subsets from post-septic mice exhibit defects in activation and effector function, possibly due to chromatin remodeling proximal to genes involved in cytokine production or gene transcription.

Key words: CD4$^+$ T cell · Epigenetics · Inflammation · Mouse · Sepsis

Introduction

Recent clinical and experimental studies have indicated that the long-term effects of severe inflammatory events often include suppression of immune system functions. For example, the long-term survival of patients after recovery from severe septic shock is significantly reduced as compared to the unaffected age-matched population, and this increased morbidity correlates with both decreased overall health quality and increased prevalence of infection with opportunistic pathogens [1, 2]. In addition to clinical studies, mouse models provide additional evidence for immunosuppression following severe sepsis. For example, DC from post-septic mice have been shown to be deficient in their ability to produce IL-12, a cytokine important for the promotion of T_H1 immune responses and the clearance of bacterial and viral pathogens [3, 4]. Additionally, post-septic mice are susceptible to infection with the opportunistic fungi *Aspergillus fumigatus*, succumbing to airway challenge at conidia doses that are well tolerated by sham surgery mice [5, 6]. Understanding the cellular and molecular mechanisms underlying the long-term immunosuppression following severe sepsis is critical for the development of treatments and therapies for patients in the years following recovery from a severe inflammatory episode.

Previously published results by our laboratory and others have indicated that the innate immune system suffers from multiple deficiencies following severe sepsis. However, little is understood

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about the long-term effects of severe sepsis on the adaptive immune system. During the acute phase of severe sepsis, lymphocytes (including T and B cells) undergo significant apoptosis in lymphoid tissues, including the spleen and thymus [7–10]. As previous studies have indicated that innate immune cells suffer from long-term deficiencies in proinflammatory cytokine production, it is possible that adaptive immune cells may suffer similar deficiencies following severe sepsis.

Suppression of IL-12 production by DC following severe sepsis may partially be due to epigenetic regulation of the *Il12* gene locus, specifically through modification of histone tails with suppressive marks resulting in transcriptional inaccessibility. Of particular interest is the increase in repressive histone modifications at the *Il12* locus, including methylation of histone 3 at lysine 27 (H3K27) and loss of the activating methylation event at histone 3 lysine 4 (H3K4) [3]. These epigenetic events are of particular interest because they are often thought to be heritable from parent to daughter cell [11]; in this way, epigenetic regulation of proinflammatory genes following severe sepsis may be passed on to daughter cells, perpetuating the immunosuppressed phenotype for an extended period of time, long after recovery from severe sepsis [12]. Epigenetic gene regulation is essential for the maturation and activation of numerous immune cells, and it plays a central role in lineage commitment in $CD4^+$ T cells [13, 14].

The purpose of this study was to investigate the effects of severe sepsis on the phenotype and function of $CD4^+$ T cells in a mouse model. As previous studies have indicated that the immune environment following sepsis is biased away from T_H1 toward T_H2 responses [15], our initial hypothesis was that post-septic T cells would show a bias toward T_H2 cytokine production. To investigate this, $CD4^+$ T cells from post-septic mice were isolated and assayed for *ex vivo* proliferation and cytokine production, along with their ability to skew toward T_H1 or T_H2 cytokine production in response to *in vitro* stimuli. Results indicate that $CD4^+$ T cells from post-septic mice have deficiencies in proliferative capacity and proinflammatory cytokine production; specifically, they appear to have difficulty in T_H lineage commitment, as assayed by cytokine production *in vitro*. These deficiencies were associated with epigenetic modifications in histone methylation at gene loci important for lineage commitment in T_H1 and T_H2 T cells.

Results

$CD4^+$T cells are reduced in spleens of mice at 14 days following cecal ligation and puncture

Previous studies have indicated that severe sepsis results in a significant apoptotic event, resulting in a significant loss of leukocytes in lymphoid and peripheral tissue during the acute phase of inflammation. To investigate the effect of severe sepsis on T-cell populations at time points post-sepsis, spleens of sham surgery (sham) and cecal ligation and puncture (CLP) mice were harvested at 14 days post-surgery and analyzed *via* flow cytometry for the presence of $CD4^+$ T cells and various $CD4^+$ T-cell subsets. During the acute phase of this sepsis model, mice subjected to CLP experience a high mortality rate, with an average mortality of 40–60% *per* cohort by day 4 post-surgery, with no mortality apparent in sham surgery mice. At the time of analysis (day 14 post-surgery), surviving CLP mice no longer display overt indications of inflammation.

Total percentages of lymphocytes are unchanged between sham and CLP spleens at day 14 post-sepsis (Fig. 1A). However, total numbers of lymphocytes are significantly reduced in CLP spleens, largely due to a reduction in total viable cell counts (Fig. 1B). The percentage of $CD4^+$ T cells in the spleens is significantly reduced in CLP mice (Fig. 1C), and this reduction is reflected in their total number (Fig. 1D). To analyze the relative

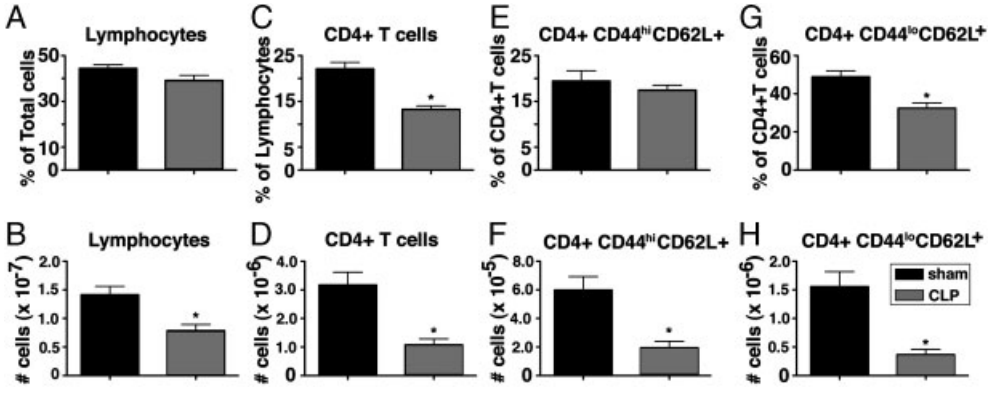

Figure 1. Percentages and total numbers of $CD4^+$ T cells and T-cell subsets in the spleen of post-septic mice. Spleens from sham surgery ("sham") or cecal ligation and puncture ("CLP") mice were analyzed *via* flow cytometry for the presence of $CD4^+$ T cells and various $CD4^+$ T-cell subsets. Total numbers of cells were obtained using a hemocytometer and trypan blue staining for the enumeration of viable cells. (A) Percentage and (B) total numbers of viable lymphocytes were calculated using forward and size scatter profiles for gating on viable lymphocytes. (C) Percentage and (D) total numbers of $CD4^+$ T cells were obtained from the viable cell gate using antibodies to CD3ε and CD4. (E) Percentages and (F) total numbers of $CD44^{hi}$ $CD62L^+$ T cells, and (G) percentages and (H) total numbers of $CD44^{lo}$ $CD62L^+$ T cells were obtained from the $CD4^+$ T-cell gate. Data presented represent the mean ± SEM, representative of two separate experiments, $n = 5–6$ *per* group. *$p<0.05$ *versus* sham.

proportion of naïve and activated T cells in the $CD4^+$ T-cell pool, cells were further analyzed for the expression of CD44 and CD62L, cell surface markers that can be used to delineate subpopulations of $CD4^+$ T cells. No significant difference was observed in the percentage of $CD4^+$ T cells that were both $CD44^{hi}$ and $CD62L^+$ in the spleens of CLP mice (Fig. 1E); however, total numbers of these cells were reduced in CLP mice due to the overall reduction in $CD4^+$ T-cell numbers in these mice (Fig. 1F). In contrast, the percentage of $CD4^+$ T cells that were both $CD44^{lo}$ and $CD62L^+$ was significantly reduced in CLP spleens as compared to sham mice (Fig. 1G). This reduction in $CD4^+CD44^{lo}$ $CD62L^+$ T cells was reflected in total numbers as well (Fig. 1H).

Expression of CD44 and CD62L can delineate naïve *versus* antigen-experienced T cells; however, these marks are not sufficient to delineate recently activated T cells from memory T-cell lineages. To determine whether the modulations in $CD4^+CD62L^+$ T-cell populations was due to modulations in memory T cells, $CD62L^+$ T cells from sham and CLP spleens were analyzed for the expression of the chemokine receptor CCR7 [16]. In both sham (Fig. 2A) and CLP (Fig. 2B) mice, less than 1% of the $CD62L^+$ T cells in the spleen were CCR7+, indicating that the vast majority of the $CD62L^+$ T cells were not memory T cells. Repeated analysis of multiple sham and CLP spleens at 14 days post-sepsis shows no significant differences in percentages of $CD4^+$ $CD62L^+CD44^{hi}$ CCR7 cells between sham and CLP mice (Fig. 1E; $27.18 \pm 3.3\%$ for sham *versus* $33.18 \pm 2.7\%$ for CLP, $p > 0.05$).

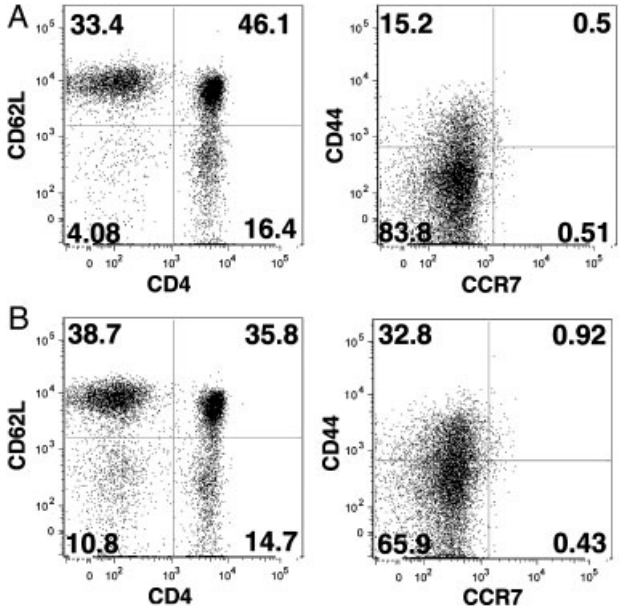

Figure 2. Surface marker profiles of splenic $CD4^+CD62L^+$ T cells from sham and CLP mice at 14 days following surgery. Spleens from (A) sham and (B) CLP mice 14 days following surgery were analyzed *via* flow cytometry for the presence of $CD4^+CD62L^+$ T cells, and these cells were analyzed for the surface expression of CD44 and CCR7. Flow diagrams are representative of spleens from individual animals, $n = 5$–6 mice *per* group. Data are representative of two separate experiments.

$CD4^+CD62L^+$ T cells from CLP mice exhibit decreased proliferative capacity *in vitro*

To analyze the proliferative capacity of T-cell subsets post-sepsis, $CD4^+CD62L^-$ and $CD62L^+$ T cells were purified from spleens of sham and CLP mice at day 14 post-sepsis and were stimulated *in vitro* with αCD3/αCD28. $CD4^+CD62L^-$ T cells from CLP mice exhibited a slight decrease in proliferative capacity as compared to sham mice; however, this difference was not significant ($p > 0.05$) (Fig. 3A). In contrast, $CD4^+CD62L^+$ T cells from CLP mice showed a significant decrease in proliferative capacity as compared to sham $CD4^+CD62L^+$ T cells (Fig. 3B). Addition of exogenous IL-2 did not affect the proliferation of $CD4^+CD62L^-$ T cells from either sham or CLP mice, with no significant differences observed between surgery groups or between αCD3/αCD28 alone and αCD3/αCD28/IL-2 culture conditions (Fig. 3A). In a similar fashion, addition of exogenous IL-2 did not affect the proliferation of $CD4^+CD62L^+$ T cells from either sham or CLP mice, with CLP T cells exhibiting a significant decrease in proliferation (Fig. 3B).

To determine whether the decrease in proliferation observed in $CD4^+CD62L^+$ T cells from CLP was due to activation-induced cell death, sorted $CD4^+CD62L^+$ T cells were stimulated *in vitro* with αCD3/αCD28 for 24 h, and viability was assessed using vital dye inclusion and flow cytometry. After 24 h of *in vitro* stimulation, there was an apparent increase in the number of dead/dying $CD4^+$ T cells in CLP cultures as compared to sham (Fig. 3C). Analysis of multiple repeated cultures indicated a significant decrease in the percentage of viable $CD4^+CD62L^+$ T cells in CLP cultures as compared to sham, following 24 h of *in vitro* polyclonal stimulus (Fig. 3D).

$CD4^+CD62L^+$ T cells from CLP mice exhibit decreased JNK and ERK1/2 phosphorylation

While the loss of viable lymphocytes following activation may provide one explanation for the reduction in proliferative capacity exhibited by $CD4^+CD62L^+$ T cells from CLP mice, it does not provide a mechanism for the decreased proliferative capacity of the cells that remain viable. One possible alternative mechanism for the decrease in proliferation may be decreased intracellular signaling in $CD4^+CD62L^+$ T cells from CLP mice. To test this possibility, purified $CD4^+CD62L^+$ T cells from sham and CLP mice were stimulated *in vitro* with αCD3/αCD28, and total cellular protein was harvested at varying time points between 0 and 20 h for analysis of signal transduction protein phosphorylation.

Following stimulation, $CD4^+CD62L^+$ T cells from sham mice exhibited a rapid increase in intracellular p-JNK, with the peak observed concentration of p-JNK to total JNK observed after 15 min of *in vitro* stimulation (Fig. 4A). In contrast, $CD4^+$ $CD62L^+$ T cells from CLP mice exhibited a decreased concentration of p-JNK to total JNK, with the maximum observed difference between sham and CLP T cells at 15 min of *in vitro*

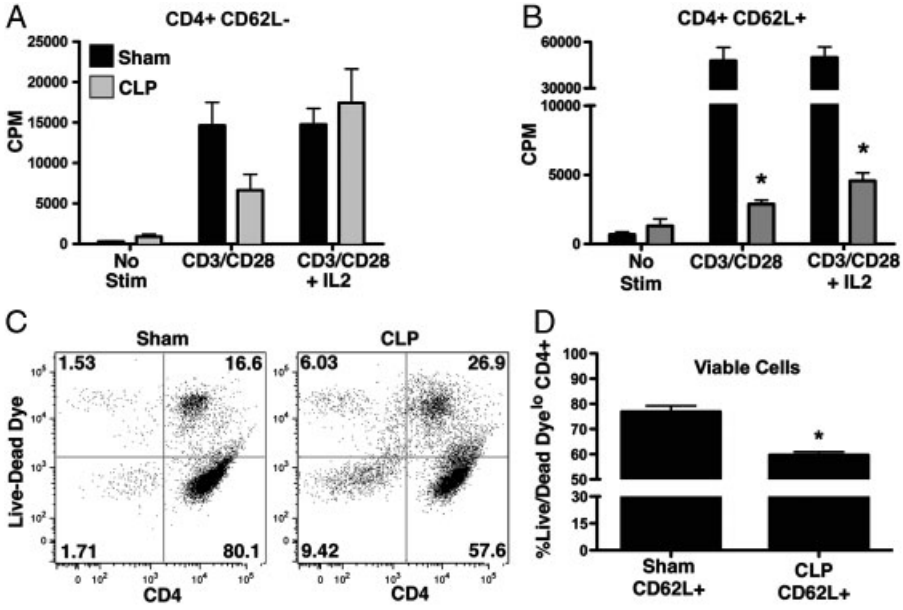

Figure 3. $CD4^+CD62L^+$ T cells from CLP mice exhibit decreased proliferative capacity *in vitro* in response to polyclonal stimulus. (A) $CD4^+CD62L^-$ and (B) $CD4^+CD62L^+$ T cells from sham and CLP mice were isolated from spleens 14 days following surgery utilizing bead antibodies and magnetic columns (MACS), and were stimulated for 72 h *in vitro* with 1 μg/mL plate-bound αCD3 and 3 μg/mL soluble αCD28 in 96-well flat-bottom plates. Where indicated, cell culture media was supplemented with 10 U/mL recombinant IL-2. Data presented represent the mean ± SEM, representative of three separate experiments utilizing pooled spleens from 5 to 6 mice *per* group. (C) Representative flow diagrams of *in vitro* restimulated $CD4^+CD62L^+$ T cells from sham and CLP mice. Following stimulation with αCD3/αCD28, cells were analyzed for viability by flow cytometry using antibodies to CD3ε and CD4 (for gating), and LIVE/DEAD dye exclusion (Invitrogen). Non-viable cells are identified by bright staining with the LIVE/DEAD dye. (D) Percentage of viable $CD4^+$ cells ($LIVE/DEAD^{lo}$) in cultures of restimulated $CD4^+CD62L^+$ T cells from sham and CLP mice. Data represent the mean ± SEM of triplicate cultures using pooled spleens from 5 to 6 mice *per* group. *$p<0.05$ *versus* sham.

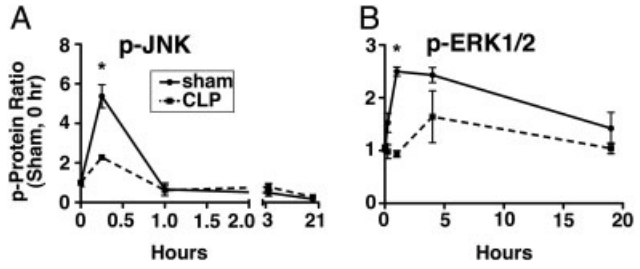

Figure 4. Phosphorylation of signal transduction proteins in sham and CLP $CD4^+CD62L^+$ T cells following *in vitro* polyclonal stimulus. $CD4^+CD62L^+$ T cells from sham and CLP mice were isolated from spleens 14 days following surgery utilizing bead antibodies and magnetic columns (MACS) and were cultured for the indicated time points (0–20 h) in the presence of αCD3/αCD28. At the indicated time points, total cellular protein was isolated using cell lysis reagents (Bio-Rad), the protein lysate was clarified *via* centrifugation and analyzed for the relative abundance of both total and phosphorylated (A) JNK and (B) ERK1/2 using a multiplex bead assay technique (Luminex). *y*-axis values represent the relative ratio of phosphoprotein to total protein in each sample. Values represent the mean ± SEM of triplicate cell cultures for each time point, with cells isolated from the spleens of 3–6 mice *per* group. (*) = $p<0.05$ *versus* sham at each individual time point indicated.

stimulation (Fig. 4A). Observed concentrations of p-JNK rapidly declined in both sham and CLP T cells after 1 h, and remained similar at all time points observed, up to 20 h after the start of the culture (Fig. 4A). Additionally, concentrations of p-ERK1/2 were significantly decreased in CLP $CD4^+CD62L^+$ T cells as compared with sham, with the maximum observed difference between sham and CLP T cells observed after 1 h of *in vitro* stimulation (Fig. 4B). Unlike p-JNK, this deficiency in ERK1/2 phosphorylation was observed at all time points, with concentrations of p-ERK1/2 between sham and CLP $CD4^+CD62L^+$ T cells only becoming equivalent after 20 h of stimulation (Fig. 4B). In addition, relative levels of p-Akt, p-IκB-α and p-p38 MAPK were analyzed; however, no significant differences in the concentrations of these phosphoproteins were observed in sham *versus* CLP $CD4^+CD62L^+$ T cells at all time points analyzed (data not shown).

Dysregulated mRNA expression in $CD4^+CD62L^+$ T cells from CLP mice

The apparent conflicting phenomena of poor proliferation/inhibited T_H skewing potential and increased pan-cytokine expression directly *ex vivo* suggested that numerous disparate gene pathways were affected in post-septic $CD4^+CD62L^+$ T cells. To investigate this possibility, mRNA from resting and activated $CD4^+CD62L^+$ T cells from sham and CLP mice was harvested and analyzed *via* quantitative real-time PCR using superarray analysis for multiple target genes involved in T-cell activation, signal transduction, gene expression and effector function. A total of 84 mRNA targets were analyzed for each assay, and significance of fold increase/decrease was calculated by comparing the values

for each gene product with the mean and SD of gene expression across the entire superarray.

Analysis of gene expression between sham and CLP $CD4^+CD62L^+$ T cells after 6 h of *ex vivo* rest in minimal media identified numerous genes that were both up- and downregulated in post-septic T cells (Fig. 5A). Of particular interest was the extreme downregulation of *Cd4* and *Cd28*, which encode surface receptors critical for $CD4^+$ T-cell activation (Fig. 5A). Additional downregulated genes involved with T-cell receptor interactions include *Icos* and *Tnfrsf4*, which encode the costimulatory receptors ICOS and OX40, respectively (Fig. 5A). Overall, a majority of the surface receptor genes that showed significant modulation were downregulated, with only four of the total 12 showing upregulation in CLP $CD4^+CD62L^+$ T cells (*Cd40*, *Igsf6*, *Tlr4* and *Tlr6*) (Fig. 5A). In contrast, numerous genes involved with cytokine and chemokine expression were upregulated in CLP $CD4^+CD62L^+$ T cells (Fig. 5A). These include genes encoding both secreted proteins (*Il15*, *Il18*, *Il27*, *Spp1*) and receptors (*Ccr2*, *Il4ra*). Of particular interest was the apparent downregulation of *Il2* and *Ifng* mRNA, which encode cytokines important for T_H1 responses characteristic of sepsis (Fig. 5A). Genes downregulated in CLP $CD4^+CD62L^+$ T cells also include *Ccr4*, *Il4ra* and *Il27ra*, which all encode cell surface receptors. (Fig. 5A).

Analysis of mRNA expression of transcription factor and signal transduction proteins indicated numerous genes that were downregulated in post-septic $CD4^+CD62L^+$ T cells prior to activation. These include genes involved in both T_H1 (*Tbx21*) and T_H2 (*Gata3*) responses, as well as transcription factors associated with T-cell activation (*Nfatc2*, *Nfatc2ip*, *Nfatc3*) (Fig. 5A). Additionally, mRNA encoding signal transduction proteins (*Jak1*, *Mapk8*) as well as negative regulators of cytokine signaling (*Socs5*) were downregulated in CLP $CD4^+CD62L^+$ T cells (Fig. 5A). Of the gene transcription and signal transduction mRNA analyzed, only *Cebpb* was found to be upregulated in CLP $CD4^+CD62L^+$ T cells (Fig. 5A).

Analysis of gene expression between sham and CLP $CD4^+CD62L^+$ T cells after 6 h of *ex vivo* stimulation (αCD3/αCD28) identified numerous genes that were upregulated in post-septic T cells (Fig. 5B). The vast majority of these upregulated genes were involved with chemokine (*Ccr2*, *Ccr3*, *Ccr5*, *Ccl5*) and cytokine (*Il4*, *Il6*, *Ifng*, *Il12rb2*, *Il13ra1*, *Il15*, *Il17a*, *Il18*, *Il18bp*, *Il27)* responses (Fig. 5B). The increase in *Il18bp* mRNA was

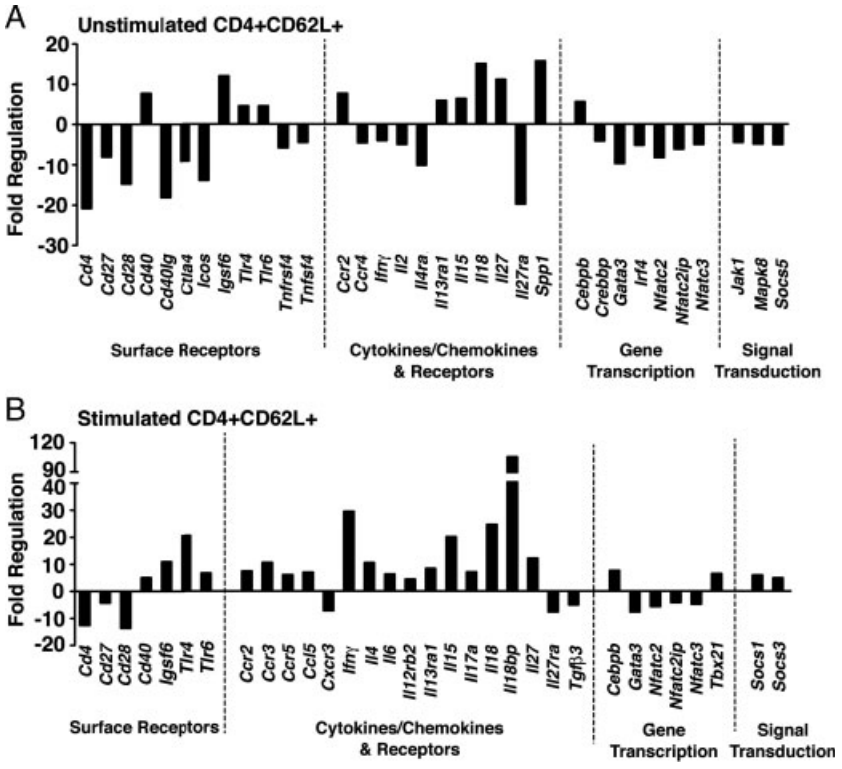

Figure 5. mRNA expression in splenic sham and CLP $CD4^+CD62L^+$ T cells during *ex vivo* rest and restimulation. $CD4^+CD62L^+$ T cells from sham and CLP mice were isolated from spleens 14 days following surgery utilizing bead antibodies and magnetic columns (MACS), and were either (A) rested in minimal media or (B) restimulated with 1 μg/mL plate-bound αCD3 and 3 μg/mL soluble αCD28 for 6 h directly *ex vivo*. Following either rest or restimulation, total RNA from cell cultures was isolated utilizing a spin column method (Qiagen) and converted to cDNA following the manufacturer's protocol (SA Biosciences). Gene expression was then analyzed using a 96-well superarray containing primers for genes involved with immune cell activation and effector function (T_H1-T_H2-T_H3 superarray, SA Biosciences). Plates were analyzed using a ABI standard 7500 real-time PCR system, and data were analyzed using the manufacturer's web-based software suite. Data reported represent genes that were up- or downregulated above the average amount for all genes, and *p*-values below 0.05 were considered statistically significant. mRNA that were not significantly up- or downregulated are not shown. Values represent the mean of two separate experiments, $n = 3$ replicate plates *per* experimental condition.

the most striking, with an over 100-fold increase in CLP $CD4^+CD62L^+$ T cells as compared to sham (Fig. 5B). Levels of *Cxcr3* and *Tgfb3* mRNA were decreased in CLP $CD4^+CD62L^+$ T cells as compared to sham, indicating certain chemokine and cytokine genes that were negatively regulated in post-septic T cells (Fig. 5B). Additionally, increases in *Tbx21*, *Socs1* and *Socs3* were observed in CLP $CD4^+CD62L^+$ T cells after stimulation (Fig. 5B). In a similar fashion as unstimulated cells, CLP $CD4^+CD62L^+$ T cells exhibited lower levels of *Cd4* and *Cd28* as compared to sham T cells (Fig. 5B). Additionally, levels of *Gata3*, *Nfatc2*, *Nfatc2ip* and *Nfatc3* remained decreased in CLP T cells as compared to sham after stimulation, indicating no effect of stimulus on the relative expression of these transcription factor mRNA (Fig. 5B).

$CD4^+CD62L^+$ T cells from CLP mice exhibit dysregulated cytokine expression after *in vitro* skewing

To determine the effects of septic shock on the ability of surviving $CD4^+CD62L^+$ T cells to commit to either the T_H1 or T_H2 lineage, cells were purified from spleens of sham and CLP mice at 14 days post-surgery and skewed *in vitro* utilizing polyclonal stimulus (αCD3/αCD28) and exogenous cytokine stimulus. After four days of stimulus and three days of rest, cells were restimulated with αCD3/αCD28 for 48 h, and resulting cytokine expression was analyzed *via* multiplex bead assay. In response to T_H1 skewing stimulus (IL-12+αIL-4), sham $CD4^+CD62L^+$ T cells expressed high levels of IL-2 and the T_H1 cytokine IFN-γ after restimulation, characteristic of T_H1 cells (Fig. 6A). In contrast, while CLP $CD4^+CD62L^+$ T cells made comparable levels of IL-2 in response to restimulation, levels of IFN-γ were significantly reduced as compared to sham skewed cells (Fig. 6A). Neither sham nor CLP $CD4^+CD62L^+$ T cells produced the T_H2 cytokine IL-4 in response to restimulation in the T_H1 skewing culture condition.

In response to T_H2 skewing stimulus (IL-4+αIL-12+αIFN-γ), $CD4^+CD62L^+$ T cells from sham mice produced high levels of IL-2 and the T_H2 cytokine IL-4 after restimulation, characteristic of T_H2 cells (Fig. 6B). In contrast to the T_H1 cultures, $CD4^+CD62L^+$ T cells from CLP mice produced similar levels of IL-4 as compared to sham T_H2 cultures (Fig. 6B). However, these cells also produced high levels of the T_H1 cytokine IFN-γ in response to restimulation ($p<0.05$ as compared to sham T_H2), which is uncharacteristic of T_H2 cultures (Fig. 6B). No significant differences were observed in levels of IL-2 produced between sham and CLP T_H2 cultures (Fig. 6B).

$CD4^+CD62L^+$ T cells from CLP mice exhibit increased H3K27 methylation at *Ifng* and *Gata3* genes

Previous studies indicate that post-septic innate immune cells exhibit increased repressive histone methylation marks at promoter regions of proinflammatory cytokines, suggesting a possible epigenetic mechanism for immunosuppression following sepsis. To determine whether similar epigenetic modifications may be playing a role in the dysregulated T_H responses of post-septic T cells, sham and CLP $CD4^+CD62L^+$ T cells were analyzed *via* ChIP assay for the relative amount of activating (histone 3 lysine 4 dimethylation, H3K4) and repressing (histone 3 lysine 27 dimethylation, H3K27) histone modifications at the promoter regions of genes essential for T_H lineage commitment. Cells were analyzed directly *ex vivo* to ascertain their epigenetic status prior to activating stimulus *in vivo* or *in vitro*.

Analysis of H3K4 dimethylation showed no significant differences between $CD4^+CD62L^+$ T cells from sham and CLP mice at either T_H1 or T_H2 gene promoter regions (Fig. 7A). Relative levels of H3K4

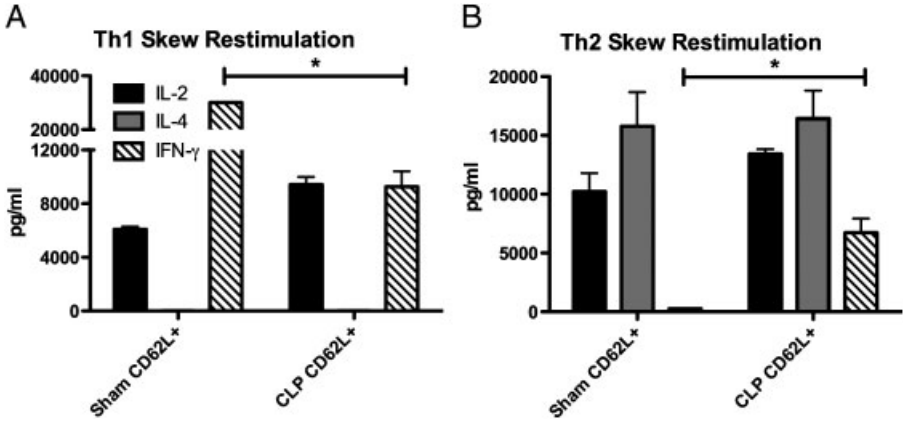

Figure 6. Cytokine expression of splenic $CD4^+CD62L^+$ T cells following *in vitro* skewing in the presence of exogenous cytokines. $CD4^+CD62L^+$ T cells from sham and CLP mice were isolated from spleens 14 days following surgery utilizing bead antibodies and magnetic columns (MACS) and were cultured for 4 days in the presence of polyclonal stimulus (αCD3/αCD28) and exogenous cytokines. (A) For T_H1 skewing, cells were cultured in the presence of rIL-12 (10 ng/mL) and αIL-4 (10 μg/mL). (B) For T_H2 skewing, cells were cultured in the presence of rIL-4 (10 ng/mL) and αIL-12 and α IFN-γ (both at 10 μg/mL). Following skewing, cells were enumerated using a hemocytometer and vital dye, re-plated in equivalent numbers and rested for 3 days in minimal media and were then restimulated with αCD3/αCD28 for an additional 48 h. Cell culture supernatants were harvested and analyzed utilizing a multiplex cytometric bead assay (Luminex). Data presented represent the mean ± SEM, representative of two separate experiments, $n = 3$ replicate wells for each cell type. *$p<0.05$ *versus* sham.

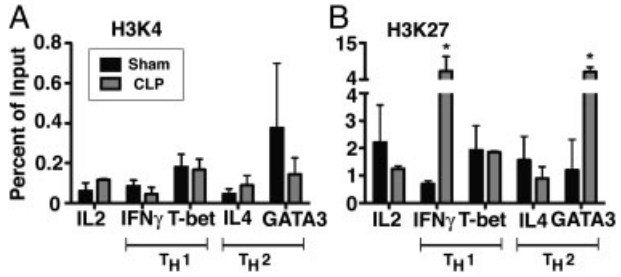

Figure 7. Methylation of histones associated with promoter regions of genes involved in T_H lineage commitment. $CD4^+CD62L^+$ T cells from sham and CLP mice were isolated from spleens 14 days following surgery utilizing bead antibodies and magnetic columns (MACS), and were analyzed *via* ChIP assay using antibodies directed to (A) methylation of lysine 4 on histone 3 (H3K4) or (B) methylation of lysine 27 on histone 3 (H3K27) ChIP-enriched genomic DNA was analyzed *via* quantitative real-time PCR for the promoter regions of indicated genes and was compared with a pre-enriched sample ("input") to generate relative values. Data presented represent the mean ± SEM, representative of three separate experiments, $n = 3$ replicates. $^*p<0.05$ *versus* sham.

methylation at the IL-2 promoter were low in both sham and CLP T cells, indicative of their non-activated state (Fig. 7A). No significant differences were observed in H3K4 methylation at T_H1 gene loci *Ifng* (T_H1-specific proinflammatory cytokine) or *Tbet* (T_H1-specific transcription factor). Additionally, no significant differences were observed in H3K4 methylation at T_H2 gene loci *Il4* (T_H2-specific proinflammatory cytokine) or *Gata3* (T_H2-specific transcription factor) (Fig. 7A). While there appears to be a decrease in H3K4 methylation at the *Gata3* promoter in CLP $CD4^+CD62L^+$ T cells, this result is not statistically significant, due to the increased variation in methylation values for sham $CD4^+CD62L^+$ T cells (Fig. 7B).

In contrast to H3K4, analysis of H3K27 dimethylation showed significant differences between $CD4^+CD62L^+$ T cells from sham and CLP mice. In a similar fashion to H3K4 methylation at the *Gata3* promoter region, H3K27 methylation was decreased at the *Il2* promoter in $CD4^+CD62L^+$ T cells from CLP mice as compared to sham; however, these results were not statistically significant (Fig. 7B). A significant increase in H3K27 methylation was observed at the *Ifng* promoter region of $CD4^+CD62L^+$ T cells from CLP mice as compared to sham, indicating repressive epigenetic modification of this T_H1 proinflammatory cytokine; however, no significant differences were observed between sham and CLP T cells at the *Tbet* promoter region (Fig. 7B). In a similar fashion to *Tbet*, no significant differences were observed in H3K27 methylation levels at the *Il4* promoter region; however, a significant increase in H3K27 methylation was observed at the *Gata3* promoter region in CLP T cells as compared to sham, approaching levels comparable to H3K27 methylation at the *Ifng* promoter (Fig. 7B).

Discussion

There are numerous mechanisms that underlie the immune suppression observed following polymicrobial sepsis, including impaired activation of macrophages and DC, and suppressed cytokine and chemokine production by numerous immune cell types. These immune deficiencies manifest themselves in decreased survival of both human patients in follow-up studies and in secondary infections of mice following experimental sepsis. Previous studies have investigated $CD4^+$ T-cell functions following severe sepsis, often in the context of acute inflammation [17–19] and/or in concert with other post-septic accessory cells, such as DC [20]. In this study, $CD4^+$ T cells from post-septic mice were analyzed for their proliferative capability and effector cytokine function following acute inflammation, and these cells were studied *in vitro* in the absence of accessory cells so as to asses defects in $CD4^+$ T-cell function that were due to cell-intrinsic factors. These studies indicate that $CD4^+$ T cells from post-septic mice exhibit variations in proliferative capability and gene regulation as compared to T cells from sham surgery mice. These variations include decreased proliferation *in vitro*, correlating with decreased signal transduction *via* protein phosphorylation and decreased expression of cell surface receptor and gene transcription mRNA in the $CD4^+CD62L^+$ T-cell subset. However, $CD4^+$ T cells from post-septic mice also exhibited increased non-specific effector cytokine production *in vitro*, along with impaired ability to produce T_H-lineage specific cytokines following *in vitro* skewing and reactivation. Finally, analysis of histone modifications in CLP $CD4^+CD62L^+$ T cells indicates increases in H3K27 proximal to genes important for $CD4^+$ T-cell polarization, suggesting an epigenetic-based mechanism for the non-specific *in vitro* cytokine production by these cells.

Previous reports in both mouse and human studies indicate that severe sepsis results in the loss of peripheral T cells, due in part to both cell-contact-dependent [9, 21, 22] and cell-contact-independent [23] mechanisms. Consistent with these previously published results, our data indicate a significant reduction in total lymphocytes, including $CD4^+$ T cells and T-cell subsets, in the spleens of CLP mice 14 days post-surgery. While this reduction was significant for both percentages and total numbers of $CD4^+$ T-cell subsets, the effects were largely due to global reductions in total numbers of splenic lymphocytes. However, variations in percentages of $CD44^{lo}CD62L^+$ T cells, which are thought to be largely antigen-inexperienced or "naïve" T cells, appear to be independent of the reduction in percentage of total $CD4^+$ T cells in the spleen. This is indicated by the lack of reduction in the percentage of $CD44^{hi}CD62L^+$ T cells, which share a basic surface phenotype with antigen-experienced or "memory" T cells. The reduction in percentages of $CD44^{lo}CD62L^+$ T cells in the spleen of CLP mice may be a result of activation during sepsis, either through antigen stimulation by bacterial components or as a result of cytokine stimulus. However, it does not appear that this activation results in an increase in $CD4^+$ T cells with a memory phenotype, as both percentages and total numbers of $CD44^{hi}CD62L^+$ T cells were not increased in CLP spleens. In addition, $CD4^+CD62L^+$ T cells from CLP mice did not show any increase in surface CCR7 expression, providing further evidence that severe sepsis did not result in an expansion of the $CD4^+$ memory T-cell pool 14 days following sepsis.

Previous reports utilizing CLP models in mice indicate that post-septic lymphocytes exhibit a reduction in proliferative capability *in vitro* [24]; however, proliferative responses in $CD4^+$ T-cell subsets can differ, as shown in studies analyzing $CD4^+$ $CD25^-$ and $CD4^+CD25^+$ subsets in WT and cytokine knockout mice following CLP [25]. In this study, comparison of $CD4^+$ T-cell subsets based on the surface expression of CD62L indicates a reduction in proliferative capacity in post-septic T cells and that this deficiency is confined to the $CD62L^+$ T-cell subset. As IL-2 is a potent proliferative signal for effector cells, it was hypothesized that the reduction in proliferation by post-septic $CD4^+CD62L^+$ T cells was due to a reduction in IL-2 production. Previous studies have indicated that splenocytes from post-septic mice show decreases in IL-2 production [15], providing evidence for this hypothesis. However, addition of exogenous IL-2 to the culture media did not rescue the proliferation of $CD4^+CD62L^+$ T cells from CLP mice, indicating that IL-2 is not involved with the reduction in proliferative capacity in these cells.

The reduction in proliferation observed in $CD4^+CD62L^+$ T cells following *in vitro* stimulation may be affected by the viability of these cells after stimulation, as well as their ability to transmit signals to the nucleus in response to TCR stimulation *via* protein phosphorylation. In the former instance, activation-induced cell death in previously activated T cells [26] may explain the reduced proliferative capacity of CLP T cells. Analysis of viability of *in vitro* restimulated CD4+$CD62L^+$ T cells indicated a significant increase in cell death in CLP T-cell cultures as compared to sham, suggesting that *in vitro* restimulation may be initiating activation-induced cell death in these cells. Additionally, intracellular signaling in CLP $CD4^+CD62L^+$ T cells appears impaired, as evidenced by decreased levels of phosphorylated JNK and ERK1/2 following *in vitro* stimulation. As JNK signaling proceeds directly downstream of TCR and CD28 [27], this may suggest an early signal transduction defect governing the proliferation defect observed. ERK1/2 has also been shown to be involved with transmitting TCR signals to the nucleus though interactions with upstream adaptor proteins such as Bam32, and that inhibition of ERK signaling can inhibit T-cell proliferation [28, 29]. These deficiencies in protein phosphorylation may provide an explanation for the apparent inability of exogenous IL-2 to rescule the proliferation of CLP T cells, as these adaptor molecules work through TCR/CD28 mediated signaling.

Analysis of global gene regulation in $CD4^+CD62L^+$ T cells from sham and CLP mice indicates numerous genes with differing expression patterns in T cells following sepsis. For example, reductions in mRNA coding for the costimulatory ligands CD4, CD28, CD40L, CTLA4 and ICOS suggests that post-septic $CD4^+$ $CD62L^+$ T cells may have a reduced capacity for activation by accessory cells, providing one possible mechanism for immunosuppression following severe sepsis. In addition, downregulation of mRNA for numerous gene transcription and signal transduction proteins may also result in $CD4^+$ T-cell dysfunction post-sepsis. For example, NFATc2 has been shown to positively regulate ICOS expression in murine T cells [30], which may explain the link between decreased expression of both *Nfatc2* and *Icos* in post-septic $CD4^+CD62L^+$ T cells. Additionally, studies with $Irf4^{-/-}$ mice in experimental models of parasite infection indicate a role for this gene in protecting $CD4^+$ T cells from apoptosis [31]; therefore, reduced expression of *Irf4* in post-septic $CD4^+$ $CD62L^+$ T cells may play a role in the loss of these cells in the spleen due to increased apoptosis. Of particular interest is the upregulation of *Cebpb* in post-septic $CD4^+CD62L^+$ T cells, as C/EBP has been shown to promote IL-4 production in T cells [32]. Increased C/EBP expression in post-septic T cells may promote T_H2 responses in these cells, which is a hallmark of immune responses post-sepsis [33].

Surprisingly, $CD4^+CD62L^+$ T cells from post-septic mice exhibited increases in specific cytokine mRNA directly *ex vivo*, including *Il15*, *Il18*, *Il27* and *Spp1*, as lymphocytes from post-septic mice and humans are not often considered to have increased propensity for cytokine production. However, one previous report indicates that splenocytes from CLP mice exhibit increased production of both IL-15 and IL-18 in a secondary infection model [34], indicating that these specific cytokine responses may not be adversely affected by severe sepsis. Recent studies have implicated IL-27 as an immunosuppressive cytokine in certain conditions [35–37]; increased production of IL-27 prior to activation may be one mechanism behind the decreased proliferative capacity of $CD4^+CD62L^+$ T cells. Additionally, IL-27 production has been shown to be increased in CLP mice, indicating a role of IL-27 in the acute phase of sepsis [38]. The role of the protein product of *Spp1* (osteopontin) in T-cell responses is less clear; certain studies indicate that it supports T_H1 responses [39], while others indicate that it is dispensable for immune protection against viral infection [40]. Increased expression of *Spp1* directly *ex vivo* may be a consequence of the T_H1-dominated immune response during sepsis, or a compensatory response based on an as-yet-unknown mechanism.

As $CD4^+CD62L^+$ T cells exhibited decreased proliferative capacity *in vitro*, it was hypothesized that these cells would also show a trend toward decreased gene expression following *in vitro* stimulus. Surprisingly, the opposite appears true, especially in regards to chemokine/cytokine mRNA. Of the chemokine and cytokine mRNA analyzed, 14 of 17 genes were upregulated. Included in these were numerous secreted proteins (*Ccl5*, *Ifng*, *Il4*, *Il6*, *Il15*, *Il17a*, *Il18*, *Il27*) as well as mRNA for chemokine receptors (*Ccr2*, *Ccr3*, *Ccr5*) and cytokine receptors (*Il12rb1*, *Il13ra1*). As mentioned previously, increases of T_H2 cytokines such as IL-4 are expected as a consequence of severe sepsis; however, the increase in both T_H1 and T_H17 cytokines is largely unexpected. Increases in systemic IFN-γ and IL-17 have been noted in both humans and mice following significant burn trauma [41, 42], and IFN-γ is an important protective cytokine during acute inflammation in sepsis [43]; however, the conventional understanding of immune responses post-sepsis involves a shift away from T_H1 responses toward T_H2 responses. In this case, an increase in chemokines and cytokines from three major effector T-cell lineages were observed, indicating a non-specific increase in gene expression in post-septic $CD4^+CD62L^+$ T cells.

Interestingly, while certain patterns of expression for gene transcription factors remained similar in stimulated *versus* unstimulated $CD4^+CD62L^+$ T cells from CLP mice (*Cebpb*, *Gata3*, *Nfatc2*, *Nfatc2ip*, *Nfatc3*), others were lost (*Crebpb*, *Irf4*) or upregulated (*Tbx21*). As with the cytokine and chemokine data, upregulation of the T_H1 transcription factor T-bet [44] was unexpected, as post-septic immunity in both mice and humans is thought to be dominated by T_H2 responses. One possible mechanism for the observed T_H2 bias in previous studies is the concurrent upregulation of *Socs1* and *Socs3* in activated CLP $CD4^+CD62L^+$ T cells, which encode the suppressor-of-cytokine-signaling proteins 1 and 3, respectively. SOCS1 has been shown to inhibit T_H1 polarization through interruption of the IFN-γ signaling pathway [45], and SOCS3 can inhibit T_H1 polarization by inhibiting IL-12 signals in concert with STAT5a [46]. These results suggest a possible feedback loop whereby $CD4^+CD62L^+$ T cells from post-septic mice may produce increased T_H1 cytokines while lacking the ability to properly respond to them in an autocrine fashion.

The unexpected observation that $CD4^+CD62L^+$ T cells from CLP mice exhibit increased production of both T_H1 and T_H2 cytokines (by mRNA) suggested that these cells had an impaired ability to commit to either T_H lineage. To test this hypothesis, $CD4^+CD62L^+$ T cells from sham and CLP mice were skewed *in vitro* using recombinant cytokines and polyclonal stimulus, and their ability to produce T_H1 or T_H2 cytokines upon restimulation was analyzed. CLP $CD4^+CD62L^+$ T cells produced less IFN-γ in T_H1 culture conditions as compared to sham, indicating a deficiency that was not overcome with exogenous cytokine stimulus. In addition, CLP $CD4^+CD62L^+$ T cells produced both IFN-γ and IL-4 following T_H2 culture conditions, indicating an impaired ability to commit to the T_H2 effector lineage in post-septic cells. Unlike the T_H1 cultures, the $CD4^+CD62L^+$ T_H2 cultures from CLP mice produced similar levels of IL-4 as sham T cells; however, the concomitant production of IFN-γ may be detrimental when these mice attempt to mount a T_H2 immune response *in vivo*.

As CLP $CD4^+CD62L^+$ T cells exhibit dysfunctional gene regulation *in vitro*, we next sought to investigate the epigenetic regulation of select cytokines and transcription factors important for T_H1 and T_H2 lineage commitment. Modifications of histone tails proximal to gene promoters can have either a positive or negative effect on gene expression and subsequent cell phenotype and function, and epigenetic mechanisms tightly regulate the lineage commitment program of $CD4^+$ T cells [14]. In this study, methylation of histone 3 at lysine 4 (H3K4, activating [47]) and methylation of histone 3 at lysine 27 (H3K27, repressing [48]) was analyzed at the promoters of IL-2, IFN-γ and T-bet (T_H1), and IL-4 and GATA-3 (T_H2) using a standard ChIP assay. Previous reports indicate that sepsis can affect methylation patterns of cytokine genes in immune cells, specifically in the promoter region of the IL-12 gene in DC [3]; based on these results, we hypothesized that similar mechanisms may underlie $CD4^+$ T-cell dysfunction following CLP. No significant differences were observed in H3K4 levels at gene promoters between sham or CLP $CD4^+CD62L^+$ T cells; in contrast, increased levels of H3K27 were observed at the promoters of the T_H1 cytokine IFN-γ and the T_H2 transcription factor GATA-3 in CLP $CD4^+CD62L^+$ T cells.

This reciprocal silencing of genes important for both T_H1 and T_H2 lineages may provide one mechanism for the impaired lineage commitment observed in CLP $CD4^+CD62L^+$ T cells *in vitro*. Recent studies of histone methylation in $CD4^+$ T cells have indicated that certain genes (such as *Gata3*) responsible for T_H lineage commitment are "plastic" and can contain both H3K4 and H3K27 methylation marks prior to cell differentiation [49]. Therefore, increased repressive H3K27 methylation in post-septic $CD4^+CD62L^+$ T cells may negatively regulate T_H lineage commitment through modulation of basal methylation levels. In this model, the increase in repressive histone methylation would interfere with the ability of post-septic $CD4^+$ T cells to stabilize the T_H1 or T_H2 locus in a transcriptionally activated state, resulting in impaired lineage commitment in these cells. This predicted phenotype is similar to the impaired lineage commitment observed herein (*via* cytokine expression). Further studies are planned to address these issues, including kinetic analysis of histone methylation in sham and CLP $CD4^+CD62L^+$ T cells following activation, as well as analysis of the expression patterns of proteins involved in histone modifications (such as methyltransferases and demethylases) in sham and CLP T cells.

Previously published reports that studied $CD4^+$ T-cell responses during sepsis have focused primarily on either the acute phase of septic shock [17, 18, 43] or on dysfunction proximal in time to the initial insult [9, 50–52]. Little is understood about $CD4^+$ T-cell dysfunction following severe sepsis and its impact on sepsis-induced immunosuppression in both mice and humans. In this study, $CD4^+$ T cells from CLP mice were shown to have both functional and transcriptional defects that were maintained up to 14 days following surgery, indicating that sepsis-induced defects in gene regulation may be maintained in peripheral T cells well after inflammation has subsided. $CD4^+$ T-cell subsets from CLP mice exhibited decreased proliferative capacity, along with decreased survival and protein phosphorylation after stimulation *in vitro*. In addition, these cells exhibited modulations in the expression of surface receptor, cytokine and gene transcription mRNA, an impaired ability to commit to the T_H1 or T_H2 lineage, and increases in repressive histone modifications at gene promoters essential for both lineages. Our current hypothesis is that these defects in gene expression and regulation result in an impaired ability of post-septic $CD4^+$ T cells to mount a directed T_H response to subsequent inflammatory stimuli, which, in parallel with deficiencies in APC function, results in immunosuppression following severe sepsis.

Materials and methods

Mice

Female C57BL/6 mice (6–8 wk of age; Taconic Farms, Germantown, NY, USA) were housed under specific pathogen-free

conditions at the Unit for Laboratory Animal Medicine of the University of Michigan and treated in accordance with the guidelines of the animal ethical committee.

CLP

CLP surgery was performed on mice as described previously [4]. For CLP, the cecum was punctured seven times with a 21-gauge needle. The average mortality rate for mice subjected to CLP in this study was 40–60% by day 4 after surgery.

Flow cytometry

At day 14 post-surgery, mice were sacrificed and spleens were harvested. Single-cell suspensions were obtained by processing the spleens through sterile 40-μm filters, and ammonium chloride lysis buffer was used to eliminate erythrocytes. Cells were stained with the following fluorescent antibodies and secondary reagents (when applicable) in flow cytometry buffer (phosphate buffered saline, 1% w/v bovine serum albumin, 0.05% w/v sodium azide): FITC-CD3e (145-2C11, BD Biosciences, Franklin Lakes, NJ, USA), PerCP-Cy5.5-CCR7 (4B12, Biolegend, San Diego, CA, USA), APC-CD62L (MEL-14, BD Biosciences), Biotin-CD44 (IM7, Biolegend), Pacific Blue-CD4 (RM4-5, Biolegend), Streptavidin-Pacific Orange (Invitrogen, Carlsbad, CA, USA), and LIVE/DEAD violet dye (Invitrogen). Cells were fixed in 4% paraformaldehyde and analyzed on a LSR II (BD Biosciences). Flow cytometry data were analyzed using FlowJo 8.8.6 (Tree Star, Ashland, OR, USA).

$CD4^+$ T-cell isolation and cell culture

At day 14 post-surgery, mice were sacrificed and spleens were harvested as mentioned previously. For purification of $CD4^+$ CD62L and $CD4^+CD62L^+$ T cells, ferromagnetic beads were utilized (Naïve $CD4^+$ T-cell isolation kit, Miltenyi Biotech, Auburn, CA, USA) according to the manufacturer's instructions. For cell culture and restimulation assays, cells were cultured in RPMI 1640 (Mediatech, Herndon, VA, USA) supplemented with 10% FBS (Atlas Biologicals, Ft. Collina, CO, USA), penicillin/streptomycin, L-glutamine, MEM-non-essential amino acids, Na-pyruvate (Lonza, Basel, Switzerland) and 2-ME (Sigma-Aldrich, St. Louis, MO, USA). Flat-bottom 96-well plates were coated previously with 1 μg/mL αCD3 (BD Biosciences, San Jose, CA, USA). For *in vitro* skewing, culture medium was supplanted with the following recombinant cytokines when indicated: 10 U/mL IL-2 (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL IL-4 or IL-12 (R&D Systems, Minneapolis, MN, USA). Additionally, the following blocking antibodies were used when indicated: α-IL-4, α-IL-12 and α-IFN-γ, all at 10 μg/mL (eBioscience, San Diego, CA, USA).

Thymidine proliferation assay

For analysis of *in vitro* proliferation, freshly isolated $CD4^+$CD62L and $CD4^+CD62L^+$ T cells from sham and CLP mice 14 days post-surgery were stimulated with plate-bound α-CD3 and soluble α-CD28 for 72 h. During the final 6 h, cells were labeled with 1 μCi/well of ^{3}H-thymidine. After 6 h of incubation with radiolabeled thymidine, cells were harvested onto glass filters and analyzed using a beta scintillation counter (Becton-Dickinson, Franklin Lakes, NJ, USA).

Multiplex cytokine/phosphoprotein analyisis

Concentrations of indicated cytokines in culture supernatants and phosphoproteins in cell lysates were analyzed using a Luminex Bio-Plex 200 system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol, as previously described [3]. For phosphoprotein analysis, cell cultures were first lysed using the manufacturer's cell lysis solution (Bio-Rad), and clarified lysates were analyzed in a similar fashion to cytokine analysis using beads directed to both total and phosphorylated JNK, ERK1/2, Akt, I-κBα and p38 MAPK. Plates were washed and read using a Luminex Bio-Plex 200 system plate reader. For cytokine analysis, murine stock cytokines of known concentrations (provided with the kit) were used to generate standard curves. The threshold of each cytokine was routinely <5 pg/mL. For phosphoprotein analysis, ratios of the relative levels of phospho- to total protein were used to generate values.

Quantitative real-time PCR

$CD4^+CD62L^+$ T cells from sham and CLP mice were isolated from spleens 14 days post-surgery, and were either rested (cell culture media) or stimulated (αCD3/αCD28) for 6 h in 96-well plates. Following incubation, total RNA was extracted from these cells utilizing RNeasy Mini spin columns (Qiagen, Valencia, CA, USA) and cleaned using the RNeasy MinElute spin column kit (Qiagen). Following isolation, mRNA was converted to cDNA and analyzed on a T-cell gene superarray following the manufacturer's protocol (SA Biosciences, Fredrick, MD, USA) using the supplier's kits for genomic DNA cleanup and RT-PCR. Superarray plates (T_H1-T_H2-T_H3 superarray, PAMM-034, SA Biosciences) were analyzed in an ABI 7500 standard qPCR light cycler (Applied Biosystems, Foster City, CA, USA). Resulting data were analyzed using the manufacturer's web-based analysis suite (RT^2 Profiler PCR Array Data Analysis, SA Biosciences), which identified statistical significance of variations in gene expression between experimental groups. The full list of genes included in the superarray analysis can be found at the following web address: http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-034A.html.

ChIP

$CD4^+CD62L^+$ T cells from sham and CLP mice were analyzed directly *ex vivo* for histone modifications as previously described [3]. Sonication was performed using a Branson Sonifier 450 (VWR, West Chester, PA, USA) under the following conditions: four times for periods of 30 s each. Immunoprecipitation was performed with the following antibodies: anti-H3K4me3 (ab8580; Abcam, Cambridge, MA, USA) and anti-H3K27me2 (07-452; Upstate Biotechnology), overnight at 4°C with gentle rotation. DNA was subjected to real-time PCR utilizing primers for the promoter regions of the indicated cytokine or transcription factor genes. Primers for promoter regions of IL-2 [53], IFN-γ [54], IL-4 [55] and GATA3 [56] were as previously described. Primers for T-bet were as follows: 5′-ACCAGGCTGGCCTCGAA-3′ and 5′-TGGCGCACGCCTTTAATC-3′.

Statistical analysis

Significance was calculated utilizing repeated measures ANOVA when necessary, followed by *post hoc* Bonferroni tests for significance between experimental groups. For single-group analysis, two-tailed Student's *t*-tests were used to determine significance. Analysis of significance for mRNA expression was performed by the manufacturer's web-based analysis suite, as previously mentioned. In all cases, $p<0.05$ were considered statistically significant. Data analysis was performed with GraphPad Prism v5.0a for Macintosh (GraphPad software, San Diego, CA, USA).

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Abbreviation: **CLP:** cecal ligation and puncture

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