Chronic stress induces persistent low-grade inflammation

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ABSTRACT

Introduction: This study sought to determine if the systemic cytokine profile of rodents subjected to chronic restraint stress leads to persistent low-grade inflammation.
Methods: Male Sprague-Dawley rats were subjected to restraint stress for a total of seven or fourteen days. Urine norepinephrine (NE), plasma interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), C-reactive protein (CRP) were assessed with ELISA. Liver expression of IL-6 and TNF-α were assessed with real time PCR.
Results: Chronic stress at 7 and 14 days sequentially increased plasma acute phase reactants (NE, IL-6, TNF-α, and CRP), liver IL-6 expression, hematopoietic progenitor cell mobilization, and decreased erythroid progenitor colony growth. Weight gain was reduced by chronic stress compared to each models' naïve counterpart.
Conclusions: Combining this model with trauma and sepsis models will allow evaluation of the contribution of persistent inflammation in disease progression and outcomes.

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Introduction

Acute inflammation is a short-term defense response to injury and is required for healing and tissue repair.[1,2] During acute inflammation, pro-inflammatory cytokines, such tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are released. In addition, there is vasodilation, increased vascular permeability, activation of complement, and leukocyte migration. The acute inflammatory response should be terminated when it is no longer necessary, to prevent unnecessary damage to tissue. Resolution of acute inflammation occurs with the release of transforming growth factor-beta (TGF-beta), interleukin-10 (IL-10), upregulation of anti-inflammatory molecules such as interleukin-1 receptor antagonists and prostaglandin E2.[2,3] The pro-inflammatory response is balanced by an anti-inflammatory response to resume a state of homeostasis.

If there is repeated stress, neuroendocrine dysregulation is prolonged, chronic inflammation occurs which alters organ function. Chronic stress is associated with persistent activation of the hypothalamic-pituitary-adrenal axis, which leads to continuously elevated levels of stress hormones, such as glucocorticoid and catecholamines.[4,5] Several authors have previously noted that the transition from neutrophil to mononuclear cell infiltrate is a hallmark trait of acute to chronic inflammation, with IL-6 and its soluble receptor, sIL-6Ra, playing an important role.[3,6–8] During chronic stress, activated polymorphonuclear cells (PMNs) release sIL-6Rα, which combines with IL-6 to activate endothelial cells to produce monocyte chemotactic protein-1 (MCP-1), stimulating monocyte recruitment.[6,8]

In-hospital mortality following infection and trauma have improved, but long-term morbidity and mortality remain high.[9–12] The mechanisms linking stress, the immune system, and disease progression are not well understood. Patients with long-term critical illness frequently progress to a state of persistent inflammation, immunosuppression, and catabolism (PICS).[7,13] PICS is characterized by increased plasma concentrations of IL-6 as well as anemia.[13] In addition, Gentile et al. proposed that during PICS, myeloid-derived suppressor cells (MDSCs) appear in the bone marrow and secondary lymphoid organs following the initial insult,

resulting from a process known as emergency myelopoiesis.[13] These cells produce TNF-α, MCP-1, stromal-derived factor 1 (SDF-1), nitric oxide and reactive oxygen species, eliciting a prolonged pro-inflammatory environment.[13–15]

Chronic critical illness has not been well-studied in animal models. Restraint stress to induce acute stress has been more thoroughly studied in rodents, but not in the context of chronic critical illness.[16,17] Majority of animal models focus on the acute phase of sepsis and trauma. The current study examines a rodent model of chronic stress that leads to persistent inflammation. Rodents were evaluated at seven and fourteen days following chronic stress to determine a time course for inflammation and organ dysfunction.

Methods

Animals

Male Sprague-Dawley rats age 8–9 weeks (Charles River, Wilmington, MA) were housed in pairs under barrier-sustained conditions with a 12-h light/dark cycle, and with free access to chow and water. Female rodents were excluded from this study due to possible estrous cycle effect on systemic stress state as well as evidence of sex-related differences in leukocyte genomic response during severe injury and hormonal differences accounting for improved survival in sepsis.[18] Animals were given at least a 5-day acclimation period before chronic restraint stress was initiated. Animals were randomly assigned to one of four groups: naïve weighed for 7 days (n = 6), naïve weighed for 14 days (n = 3), chronic stress (CS) for 7 days (n = 14), or chronic stress for 14 days (n = 4). After seven or fourteen days of handling or restraint stress, the rats were anesthetized using a rodent cocktail consisting of ketamine (80–100 mg/kg) and (xylazine 5–10 mg/kg), then underwent sacrifice through thoracotomy and exsanguination via cardiac puncture. Blood, bone marrow from the left femur, lung, spleen and liver tissue were collected at the time of sacrifice. The average percent weight change was calculated for each rodent by subtracting the starting weight from the sacrifice day weight and dividing the difference by the starting weight.

Chronic stress

Chronic stress consisted of 2 h of restraint stress daily for seven or fourteen days based on a validated model shown to be associated with bone marrow dysfunction worsened by restraint stress after traumatic injury compared to traumatic injury alone.[19] Also, signs of sepsis and multiple organ failure occur in days-weeks in rodents as compared to weeks-months in humans.[18] The rodents were placed in a nose cone animal cylinder (Kent Scientific Corporation, Torrington, CT) which was adjusted to restrict its movements. During the 2 h period, the restraint cylinder was rotated one hundred eighty degrees every 30 min, and alarms were sounded for 2 min directly adjacent to the cylinders at the time of each rotation. At the end of 2 h, the rats were placed back in their housing where they had free access to food and water.

Urine catecholamines

Urine norepinephrine was measured on sacrifice day as a surrogate for catecholamine levels in the blood. Norepinephrine (NE) was analyzed rather than epinephrine because previous work has shown that persistent elevation of NE is associated with persistent injury-associated anemia.[19,20] Spontaneous urine samples were collected daily during chronic stress and stored at −20 °C. Urine norepinephrine was measured by enzyme linked immunosorbent assay (LDN Immunoassay and Diagnostics, Nordhorn, Germany) according to manufacturer instructions.

Plasma cytokines

Plasma interleukin 6 (IL-6), C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), and stromal cell-derived factor 1 (SDF-1) were measured on day 7 and 14 by enzyme linked immunosorbent assay (R&D Systems, Thermo Fisher, Invitrogen, MyBioSource Inc, MyBioSource Inc, respectively) (Minneapolis, MN; Waltham, MA; Carlsbad, CA; San Diego, CA; San Diego, CA, respectively). All samples were run following the manufacturer's protocol.

Liver, spleen and bone marrow cytokines

Liver and spleen expression of IL-6 and TNF-α was measured using real-time polymerase chain reaction (RT PCR). RT PCR was performed using SYBR Green Real-Time PCR Master Mix (Applied Biosystems, Foster City, CA). Primers were designed from specific mRNA sequences using the OligoPerfec Designer (ThermoFisher), ideally with 80–140 base pairs and a melting temperature of 60 °C (Table 1). RNA isolation was performed using PureLink RNA Mini Kit (Invitrogen). RNA concentrations and purity were determined by measuring the ratio of the ultraviolet absorbance at 260 nm and 280 nm. RNA was reverse transcribed to cDNA using the Applied Biosystems protocol. The Mx3005P QPCR System was used for amplification (Agilent Technologies, Santa Clara, CA).

Hematopoietic progenitor cell mobilization

Peripheral whole blood from cardiac puncture on the day of sacrifice was used to determine HPC mobilization by flow cytometry. Markers CD-71 and CD-117 (c-kit) were used to identify hematopoietic progenitor cells as they are found on early erythroid progenitors. CD-71 is a marker for BFU-E, one of the earliest erythroid progenitors.[21] CD-117 is an early erythroid receptor which is expressed on progenitor cells such as CFU-GEMM, BFU-E, and CFU-E.[22] 100 μl of whole blood was incubated in the dark with mouse anti-rat CD-71 antibody conjugated with fluorescein isothiocyanate (BD Pharmingen, San Jose, CA) and rat anti-mouse CD117 antibody conjugated with allophycocyanin (Southern BioTech, Birmingham, AL). 2 ml of BD Phosflow Lyse/Fix Buffer was added then the solution was spun at 400 g for 6 min. The fluid was decanted and 2 ml 1x PBS was added to the tube. This procedure of adding phosphate-buffered saline (PBS), spinning the solution and decanting the fluid was repeated until it was clear. Once it was clear, the final fluid was drawn off and 500 ml of Stain Buffer fetal bovine serum (FBS) was added. The blood sample was then run on BD LSR II flow cytometer equipped with FACSDiva software (BD Biosciences, San Jose, CA) to enumerate CD CD71+117+ cells.

Bone marrow cellularity and erythroid progenitor colonies

Bone marrow was harvested from the left femur and BM erythroid progenitor colony growth assays were performed, including colony-forming unit granulocyte, erythrocyte, monocyte megakaryocyte (CFU-GEMM), burst-forming unit erythroid (BFU-E), and colony forming unit erythroid (CFU-E). The femur is flushed with Iscove's Modified Dulbecco's Medium (IMDM) + 2% Fetal Bovine Serum (FBS) (Stem Cell, Vancouver, Canada) until all bone marrow had been flushed out. A 1:100 dilution of this cell suspension was made in IMDM+2%FBS and the cells are counted in a TC-20 Automated Cell Counter (Bio Rad). 10 μl of the diluted cells were mixed with 10 μl of trypan blue dye and placed in the cell

Table 1
Polymerase chain reaction primers and conditions.

Gene	Forward Primer	Reverse Primer	Base Pair Position in mRNA sequence	qPCR Product Size (bp)	Annealing Temp (°C)
IL-6	5′-TGATGGATGCTTCCAAACTG -3′	5′-GAGCATTGGAAGTTGGGGTA -3′	352–581	229	56
TNF-α	5′- GAAACACACGAGACGCTGAA -3′	5′- GAAAGCCCATTGGAATCCTT -3′	1034–1115	82	56
Beta-actin	5′-AGCCATGTACGTAGCCATCC -3′	5′-ACCCTCATAGATGGGCACAG -3′	468–582	115	60

counter to determine the total, live and percentage of live cells. 50,000 bone marrow cells in 1 ml methocult (SF M3436 for CFU-E and BFU-E from Stem Cell, and HSC012 from R&D Systems for CFU-GEMM) were added to 35 mm Petri dish. The cells were then incubated in a CO_2 incubator maintained at 36C, 5% CO_2 in air and $\geq$95% humidity. The colonies are enumerated after 7–14 days by a blinded observer.

Statistical analysis

Statistical analysis and figure production were performed using GraphPad Prism Version 6.05 (GraphPad Software, La Jolla, CA) to calculate one-way analysis of variance with Bonferroni's or Tukey's multiple comparisons test. Significance was set at $\alpha = 0.05$ and data were reported as mean ± standard deviation.

Results

Urine catecholamines

Urine norepinephrine concentration in the CS 7-day rodents was 504% higher than that of the naïve rodents, reaching statistical significance ($p = 0.03$) (Fig. 1). Urine norepinephrine concentration in the CS 14-day rodents decreased significantly compared to the 7-day model, with a 92% decrease ($p = 0.04$) (Fig. 1).

Plasma acute phase reactants

Plasma IL-6 concentration increased significantly with respect to naïve rodents in both the CS 7 and 14-day group with a 137% and 177% increase respectively ($p < 0.01$ and $p < 0.01$ respectively) (Fig. 2a). Plasma TNF-α concentration in the CS 7 day rodents did not significantly change compared to naïve, however the CS 14 day TNF-α concentration increased 250% compared to naïve ($p < 0.01$). The plasma concentration was also significantly increased from the CS 7 to CS 14-day rodents with a 227% increase ($p < 0.01$) (Fig. 2b). Plasma CRP concentration increased significantly from naïve to the
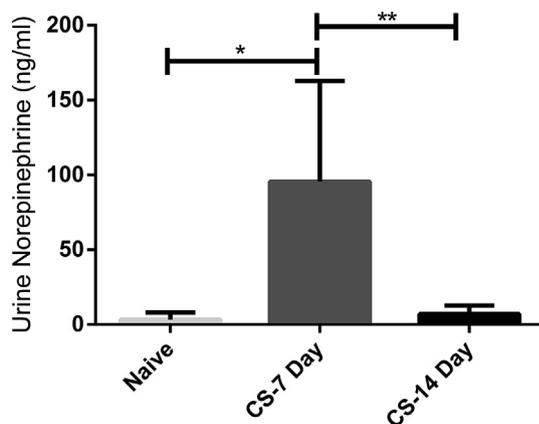
CS 7-day rodents with a 100% increase ($p < 0.01$), then increased again to the CS 14-day rodents with a 118% increase from naïve ($p < 0.01$) (Fig. 2c). Plasma SDF-1 concentration was not significantly altered at 7 or 14 days after chronic stress (Fig. 2d).

Organ dysfunction

Liver IL-6 expression increased from naïve to CS 7 day, then again from CS 7 day to CS 14 day. Expression at 14 days was significantly elevated compared to both naïve and 7-day rodents with a 180% ($p = 0.01$) and 40% increase ($p = 0.03$) (Fig. 3a). Spleen IL-6 expression was significantly increased at 7 days compared to naïve (350% increase, $p = 0.01$), then significantly decreased at 14 days compared to 7days (87% decrease, $p = 0.01$).

Liver and spleen TNF-α expression significantly increased from naïve to CS 7 day with a 1567% ($p = 0.02$) and 285% ($p = 0.03$) increase respectively (Fig. 3b). Expression in both liver and spleen then significantly decreased from 7 days to 14 days with a 97% ($p = 0.03$) and 92% ($p = 0.01$) (Fig. 3b).

Bone marrow cellularity and progenitor growth

In the CS 7-day model, bone marrow cellularity was significantly decreased compared to the naïve rodents with a 24% decrease in cellularity ($p = 0.01$). In the CS 14-day model, bone marrow cellularity increased from the naïve group and was significantly increased compared to the CS 7-day group with a 56% increase ($p < 0.01$).

Erythroid progenitor CFU-GEMM was not significantly affected by CS in either the 7 or 14-day group (data not shown). However, both progenitor groups BFU-E and CFU-E were significantly suppressed by restraint stress. In the CS 7-day model, BFU-E was suppressed from the naïve group significantly by 19% (52 vs 42 colonies/plate, ($p < 0.01$). After 14 days of chronic stress, BFU-E growth decreased by 27% compared to naïve (52 vs 37 colonies/plate, $p < 0.01$) (Fig. 4a). CFU-E growth decreased 24% in the 7 day chronic stress group compared to naïve ($p < 0.01$) (Fig. 4b). CFU-E growth was also significantly suppressed following 14 days of chronic stress compared to both naïve and 7 day chronic stress with a 37% ($p < 0.01$) and 17% ($p < 0.01$) decrease, respectively (Fig. 4b).

Hematopoietic progenitor cell mobilization

Hematopoietic progenitor cell mobilization to the peripheral blood was measured identifying CD71$^+$CD117$^+$ cells in whole blood. Hematopoietic progenitor cell mobilization to the peripheral blood was increased 511% following seven days of chronic stress when rodents compared to naïve (Fig. 5). Following 14 days of chronic stress, there was a significant increase in hematopoietic progenitor cells in the peripheral blood compared to both naïve and CS 7 day with a 2780% ($p < 0.01$) and 287% increase ($p < 0.01$) (Fig. 5).

Weight change

All study rodents gained weight over the course of the study, but

Fig. 1. Chronic stress increased urine epinephrine. Seven days of restraint stress significantly increased urine norepinephrine, while it decreased from 7 to 14 days of stress. *$p < 0.05$ vs. naïve; **$p < 0.05$ CS 7 Day vs. CS 14 day.
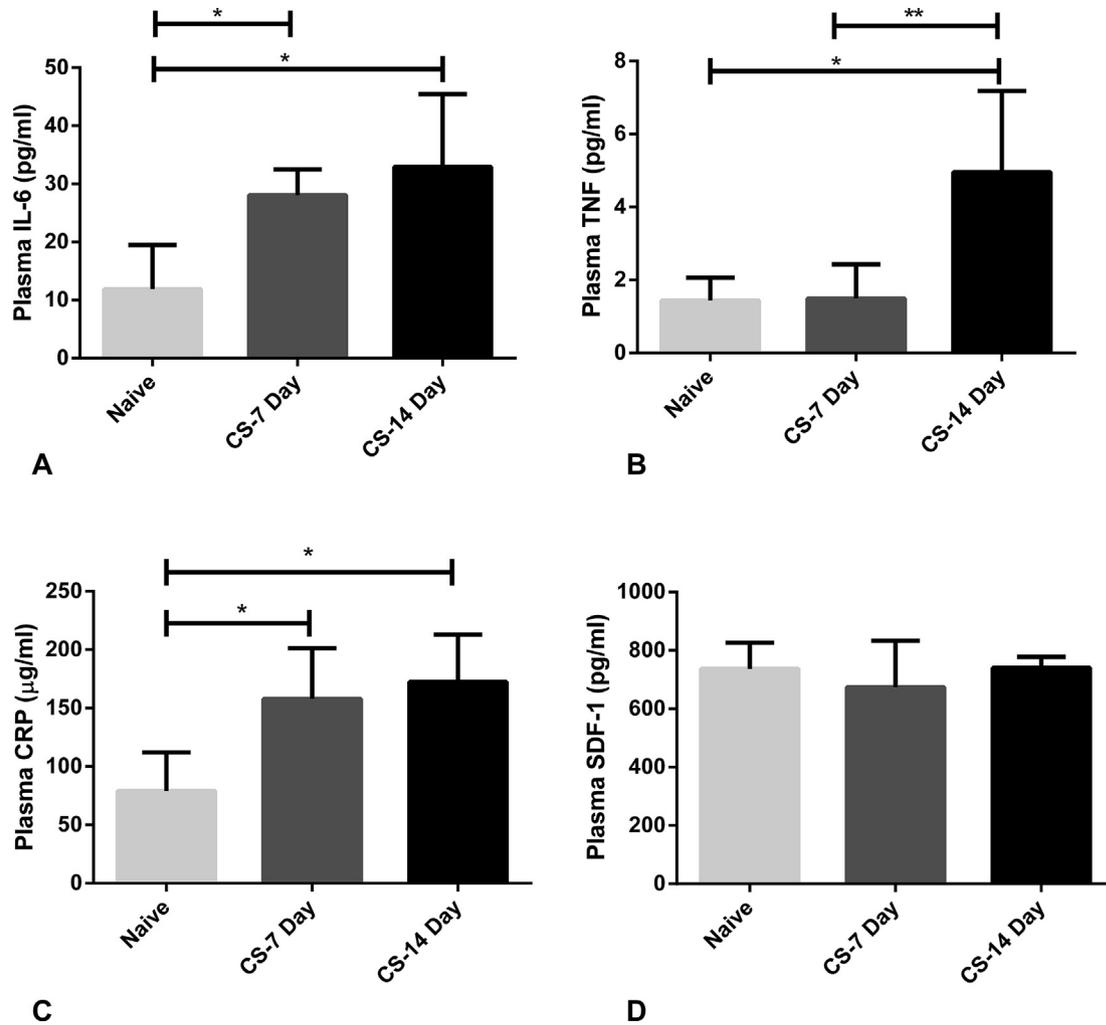
Fig. 2. A-D. Chronic stress increased plasma acute phase reactants. 2A. Plasma IL-6 increased significantly from naïve to 7 days of restraint stress, then again from 7 to 14 days of stress. **2B.** Fourteen days of restraint stress significantly increased plasma TNF-α. **2C.** Plasma CRP increased significantly from naïve to 7 days of restraint stress, then again from 7 to 14 days of stress. **2D.** Plasma SDF-1 was not significantly affected by restraint stress. *p < 0.05 vs. naïve; **p < 0.05 CS 7 Day vs. CS 14 day.

the CS 7-day rodents gained significantly less weight than their naïve counterparts by 50% (p < 0.01). The CS 14-day CS rodents gained less weight than their naïve counterparts (33%), but gained significantly more weight than the 7-day CS rodents (171%) (p < 0.01).

Discussion

These findings indicate that this rodent model of chronic restraint stress led to a systemic cytokine milieu associated with persistent low-grade inflammation, hypercatecholaminemia, increased acute phase reactants and evidence of organ dysfunction, similar to the persistent inflammation, immunosuppression and catabolism syndrome (PICS) described by Mira at el.[23] Mira describes that trauma and/or infection leads to systemic inflammatory response syndrome (SIRS); those that do not progress to death from multiple organ failure (MOF), will either recover and reach homeostasis, or enter a stage of chronic critical illness (defined as > 14 days in intensive care unit with organ dysfunction) with ongoing immunosuppression, inflammation, and protein catabolism. Our study shows that daily restraint stress without physical trauma can also lead to elements of CCI and PICS and is an acceptable model of persistent inflammation.

Restraint stress led to a significant increase in urine norepinephrine concentration after 7 days. Urine was used as a surrogate for plasma as urine was more readily available, could be easily collected daily, and reflects systemic metabolism. Supraphysiologic norepinephrine has been shown to be associated with persistent anemia and systemic inflammation following trauma.[19,20,24–31] Using a rodent model of lung contusion, hemorrhagic shock and chronic restraint stress, Alamo and Fonseca et al. demonstrated that increased plasma and urine norepinephrine concentrations were significantly increased on post-injury day 7, which was also associated with increased systemic acute phase reactants like IL-6.[24,26,29]

Chronic restraint stress increased the plasma acute phase reactants IL-6, TNF-α, and CRP from naïve to 7 days–14 days of restraint stress. In a normal model of *acute* human healing, TNF-α reaches its peak plasma concentration within 82 min after traumatic injury then significantly decreases in the late trauma group.[32] TNF-α from monocytes and endothelium induce IL-6 production which is first detected in the plasma of trauma patients within an hour of trauma.[2] In the early hours following trauma, IL-6 induces the secretion of C-reaction protein (CRP). While our model time points were more extended than minutes to hours, the concentrations of IL-6, TNF-α, and CRP not only remained elevated at 14
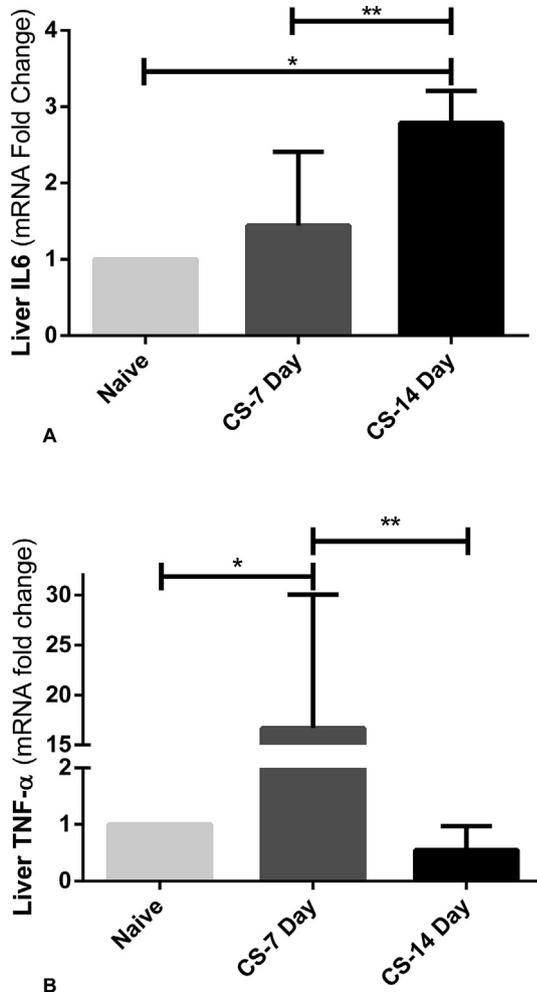
A

B

Fig. 3. A-B. Chronic stress increased expression of liver IL-6 and TNF-α. 3A. Fourteen days of restraint stress significantly increased liver expression of IL-6 compared to naïve and 7 days of stress. **3B.** Seven days of restraint stress significantly increased spleen expression of TNF-α. *p < 0.05 vs. naïve; **p < 0.05 CS 7 Day vs. CS 14 day.
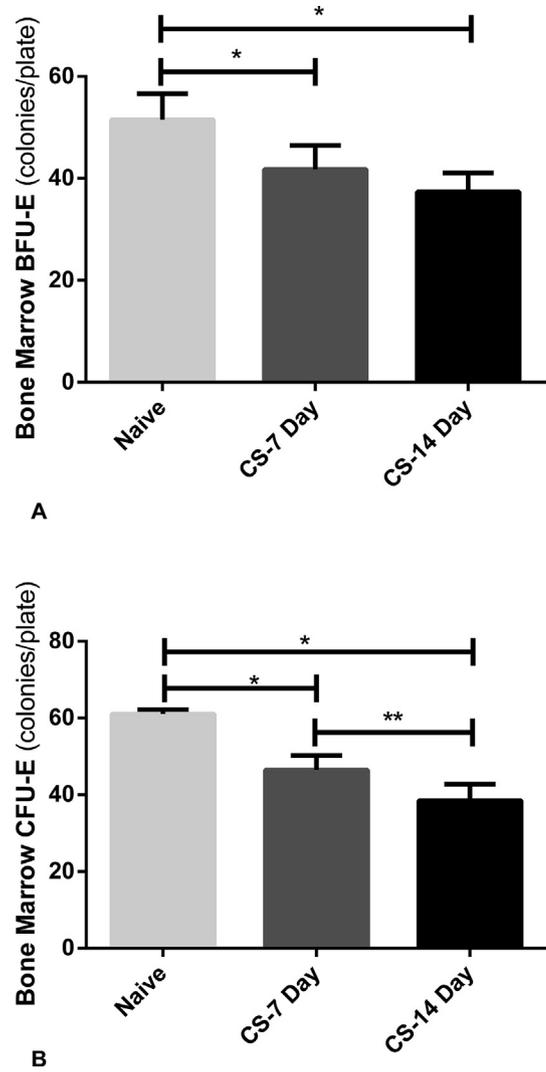

A

B

Fig. 4. A-B. Chronic stress suppressed bone marrow erythroid progenitor colony growth. 4A. BFU-E was significantly suppressed after seven days of restraint stress, then further suppressed after 14 days of stress. **4B.** CFU-E was significantly suppressed after seven days of restraint stress, then further suppressed after 14 days of stress. *p < 0.05 vs. naïve; **p < 0.05 CS 7 Day vs. CS 14 day.

days, but were increased at that time point demonstrating that chronic restraint stress was associated with a plasma cytokine milieu of chronic inflammation. These findings are in accordance with previous reports measuring acute phase reactants in rodents undergoing acute (forced swimming) and chronic stress (restraint stress).[33] IL-6 and TNF-α were found to be greater in both the acute and chronic stressed groups.

As previously stated, increased levels of systemic IL-6 are seen in inflammation and stress. This lab has previously proposed that the hypercatecholamine state seen after trauma and/or stress, leads to increased IL-6 production by the liver and downstream iron metabolism dysfunction.[24] Briest et al. has also explained that systemic IL-6 is an indicator to defense mechanisms like the liver, to produce acute phase reactants.[34] Our study demonstrated that liver IL-6 expression was significantly increased after 7 days of CS then even further increased after 14 days of CS. Liver TNF-α expression significantly increased after 7 days of CS then decreased from 7 to 14 days. Spleen IL-6 and TNF-α expression increased after 7 days of CS, then decreased after 14 days. The spleen is an immune organ innervated by sympathetic nerves.[35] Laukova et al. used Sprague-Dawley rats in a stress protocol using immobilization boards for 2 h daily for 7 days. They found that repeated stress was associated with elevated expression of IL-6 and TNF-α in the spleen on the day of sacrifice.[35] These findings reflect the increased liver and spleen
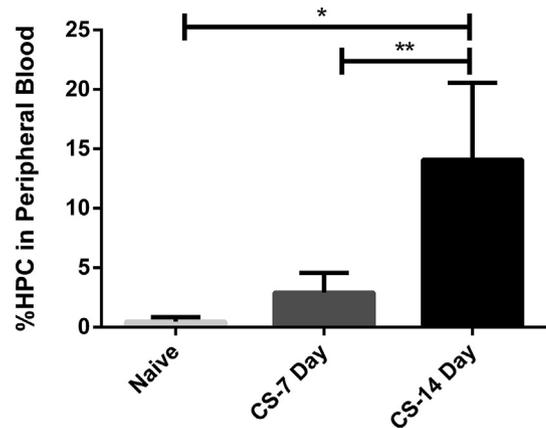

Fig. 5. Chronic stress increased HPC mobilization. HPC mobilization to the peripheral blood was significantly increased after 7 days of restraint stress, then further increased after 14 days of stress. HPC: hematopoietic progenitor cell; *p < 0.05 vs. naïve; **p < 0.05 CS 7 Day vs. CS 14 day.

expression of IL-6 and TNF-α in our model of chronic restraint stress and how this increased expression is associated with our additional findings.

Bone marrow erythroid progenitor colonies BFU-E and CFU-E were significantly suppressed following 7 days of CS and ever further suppressed following 14 days CS. Previous studies have demonstrated that the addition of chronic stress to a rodent model of trauma led to increasingly suppressed bone marrow erythroid progenitor colonies than with trauma alone.[19,24] The sympathetic nervous system terminates peripherally in organs like the bone marrow, secreting norepinephrine at times of stress.[36] This model of chronic stress was associated with significantly elevated concentrations of urine norepinephrine. Fonseca et al. demonstrated that under physiologic conditions, norepinephrine accentuates BFU-E and CFU-E growth, but at severe stress levels, norepinephrine inhibits BFU-E and CFU-E growth.[29] Additionally, studies using chemical sympathectomy show that erythroid progenitor colony (BFU-E and CFU-E) growth is inhibited in rodents with supraphysiologic concentration of norepinephrine.[31] This relationship between norepinephrine and suppression of bone marrow erythroid progenitor growth reflect our study results.

Bone marrow cellularity was significantly decreased after 7 days of chronic stress, and HPC mobilization to the peripheral blood was significantly increased after 7 days of CS, then even further increased after 14 days. Previous studies using a rodent model of traumatic injury have shown that adding chronic restraint stress to the injury model significantly increased HPC mobilization to the peripheral blood and led to persistently increased concentration of plasma G-CSF.[37] Previous work has shown that mobilization of hematopoietic stem cells (HSCs) into the peripheral blood is mediated by sympathetic innervation of the HSC bone marrow niche.[38] Katayama et al. demonstrated in a mouse study that adrenergic bone marrow norepinephrine mediates G-CSF osteoblast suppression (to suppress bone formation), bone CXCL12 downregulation, and HPC mobilization.[39] These findings correlate with this current study's findings of increased norepinephrine concentration associated with significantly increased HPC mobilization to the peripheral blood following chronic restraint stress.

Chronic restraint stress was associated with decreased daily weight gain. Both 7 and 14 days of CS led to significantly less weight gain compared to their naïve counterparts. When PICS follows the SIRS response protein catabolism is associated with weight loss and decreased functional status.[13,23] Additionally, a characteristic response to a stressor is inhibition of food intake, increased heat production and increased activity with changes in body weight and hypothalamic-pituitary-adrenal (HPA) axis.[16] Harris notes that chronic psychological stress is more likely to cause weight loss than weight gain in rodent models.[16] In a separate study, immune activation by chronic immune challenges induced a depressive-like syndrome in rodents characterized by anhedonia, body weight loss and anorexia mediated by splenic TNF-α production.[40] Additionally, TNF-α has been shown to upregulate major inflammatory mediators like nitric oxide and matrix metalloproteinases.[34] When treatment attenuated adrenocortical activation and splenic TNF-α, many of the associated behavioral effects were mitigated.[40] These results are similar to our current study as the naïve counterpart rodents gained more weight over the study period, while the rodents who underwent daily psychological stress gained significantly less weight.

Limitations

The major limitation of this model is that there are only two time points for sample collection and analysis when stress-induced systemic cytokine changes are known to vary throughout minutes/ hours/days/weeks. A more thorough investigation would include time points starting at day 1 and extending out to day 14. Time points beyond 14 days raise concern for habituation which is more prominent in murine models.

Conclusion

Chronic restraint stress induced an overall state similar to chronic critical illness and persistent inflammation, immunosuppression, and catabolism syndrome. Stress increased urine norepinephrine, plasma IL-6, TNF-α, and CRP concentration, liver and spleen IL-6 and TNF-α expression, decreased bone marrow cellularity and erythroid progenitor growth, increased HPC mobilization, and decreased daily weight gain. Combining this model with trauma and sepsis models will allow evaluation of the contribution of persistent inflammation in disease progression and outcomes.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amjsurg.2019.07.006.

Conflicts of interest

The authors have no conflicts of interest to disclose but do acknowledge the following grant funding.

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