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14. ABSTRACT Combat-related burns have largely been caused by explosions in recent conflicts and frequently overlap with traumatic brain injury (TBI) diagnosis. The purpose of this study is to produce a preclinical model of concomitant burn and blast induced TBI to understand the impact of the combined injury on the anemia of critical illness (ACI) and neuroinflammation and to validate IL-1R1 targeted therapies as an intervention. We have found no evidence that concomitant injury exacerbates systemic inflammation or ACI compared to burn alone. Given ACI is primarily a consequence of burn in the combined injury model, and we recently reported IL-1 α / β blockade markedly attenuates post burn ACI, it is highly likely the therapy will be effective in the combined injury model. We utilized RNAseq to determine if concomitant injury amplifies neuroinflammation and identified transcriptional changes that reflect those induced by individual injuries as well as several changes that are unique to concomitant injury. Lipocalin-2 (Lcn2), a common inflammatory biomarker, demonstrated the largest fold change following concomitant injury so we used Lcn2 expression as an endpoint for initial therapeutic studies. IL-1 α / β blockade markedly attenuated systemic inflammation and Lcn2 expression in the brain following either prophylactic or therapeutic administration of the neutralizing antibodies.					
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1. INTRODUCTION

Explosions are the leading cause of burn injuries in recent conflicts, have contributed to approximately 14% of the 352,612 traumatic brain injury (TBI) diagnosis in the US military between 2000 and 2016, and in many cases lead to complex injuries in which burn injury and TBI overlap. Anemia is a nearly universal pathophysiological consequence of burn injury and could lead to serious long-term neurological complications when overlapping with TBI, as low hemoglobin levels have been linked to poor neurologic outcomes in TBI patients and long-term nervous system-related morbidity may already be anticipated in victims of severe burn. At some point anemia must be corrected in TBI patients to guard against secondary brain injury but the appropriate hemoglobin target for correction has not been established due to concern transfusion could also cause harm. Similar concerns have led to conservative transfusion protocols in burn patients, yet to this day patients with 20-60% total body surface area burns typically receive an amount of blood that is roughly equivalent to vascular volume. There are currently no therapeutic alternatives to reduce transfusion requirements in burn or trauma victims because erythropoietin (EPO) and iron supplements fail to effectively promote erythropoiesis in these patients. Resistance to EPO and iron supplements is characteristic of the anemia of critical illness (ACI), an inflammation driven anemia that limits iron availability, reduces red blood cell lifespan, and impairs erythropoiesis. The purpose of this study is to determine if concomitant burn/TBI amplifies the severity of ACI and/or neuroinflammation and to validate IL-1R1 targeted therapies as an approach for intervention.

2. KEYWORDS

Anemia of Critical Illness (ACI), Traumatic Brain Injury (TBI), erythropoietin (EPO)

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim 1: Determine if concomitant burn/TBI amplifies the severity of ACI.

Task 1: IACUC Approval (1-2 months) **Completed.**

Task 2: Post Injury Day One Endpoints (2-8 months) **75% Complete, See Changes/Problems**

Task 3: Post injury Day Seven Endpoints (2-9 months) **Completed.**

Milestone: Model produced and characterized/report pathophysiological impact of concomitant injury on ACI and neuroinflammation (8-9 months) **75% Complete, See Changes/Problems**

Specific Aim 2: Determine if targeting of IL-1R1 ligands can attenuate ACI following concomitant Burn/TBI.

Task 1: Dose Confirmation for Antibodies (8-9 months) **Completed**

Task 2: Therapeutic Day One Endpoints (9-16 months) **50% Complete**

Task 3: Therapeutic Day Seven Endpoints (9-16 months) **0% Complete**

Milestone: IL-1R1 ligand blockade studies complete/ report efficacy for ACI and neuroinflammation following concomitant injury (16-17 months) **0% Complete**

Specific Aim 3: Determine if small molecule IRAK4 inhibitors can attenuate ACI following concomitant Burn/TBI.

Task 1: IRAK4 inhibitor (AS2444697) dose determination (16-17 months) 0% Complete

Task 2: IRAK4 inhibitor (PF06650833) dose determination (16-17 months) 0% Complete

Task 3: Most Effective Therapeutic Day One Endpoints (17-24 months) 0% Complete

Task 4: Most Effective Therapeutic Day Seven Endpoints (17-24 months) 0% Complete

Milestone: IRAK4 inhibitor studies complete/ report efficacy for ACI and neuroinflammation following concomitant injury (24-25 months) 0% Complete

- What was accomplished under these goals?

For aim 1 we have completed post injury day 7 blood count studies. The results show red blood cells, hemoglobin and hematocrit are all reduced by burn injury but not TBI and no further reduction occurred in the Burn+TBI group (**Fig.1**). Hypochromic reticulocytes, a measure of iron restricted erythropoiesis, were increased by burn alone and no further increase occurred in the Burn+TBI group. Overall, these data indicate burn injury is the primary driver of ACI in the combined injury model. Other parameters suggest combined injury leads to a small but statistically significant increase in circulating neutrophils, platelets, and reticulocytes compared to single injury models.

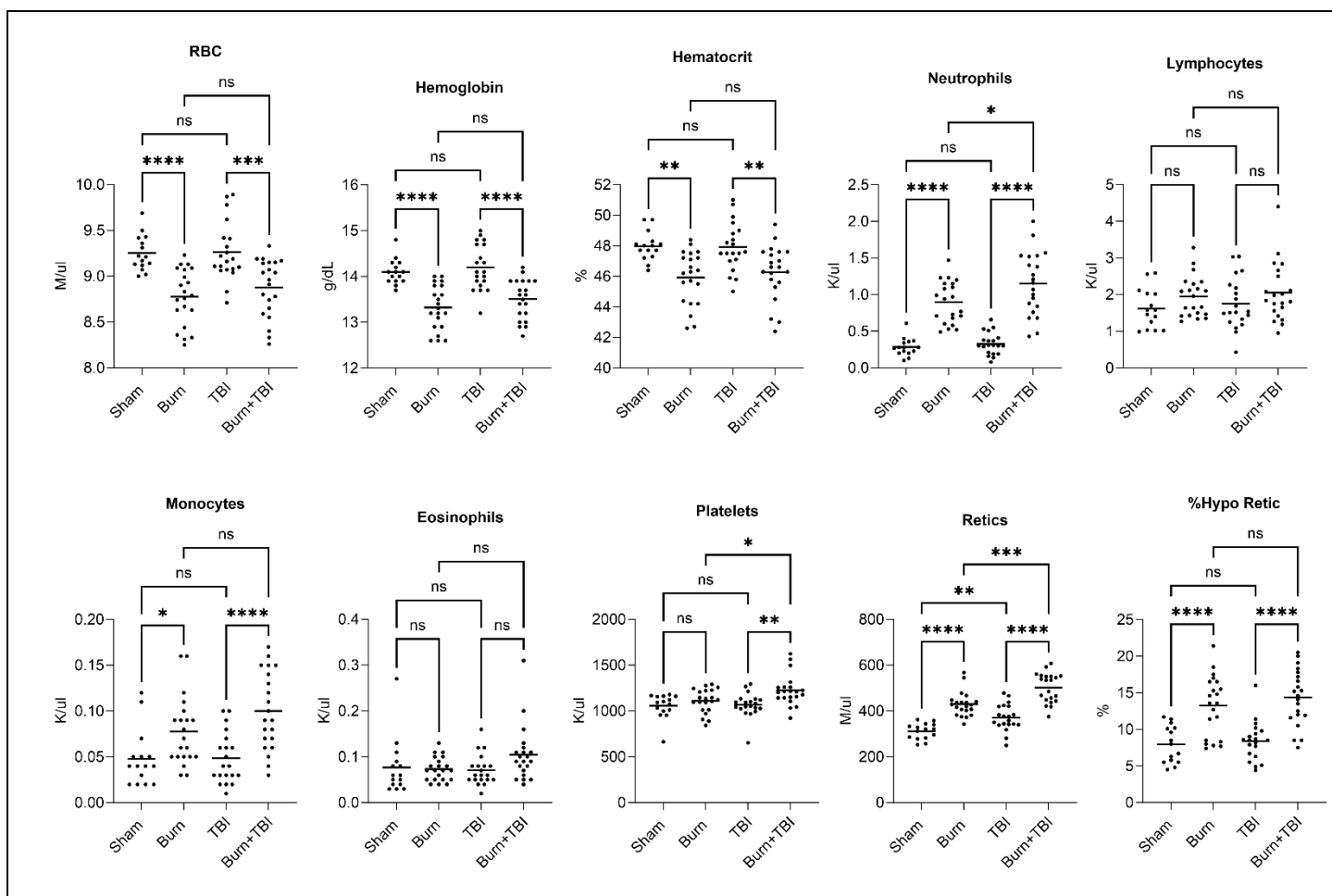
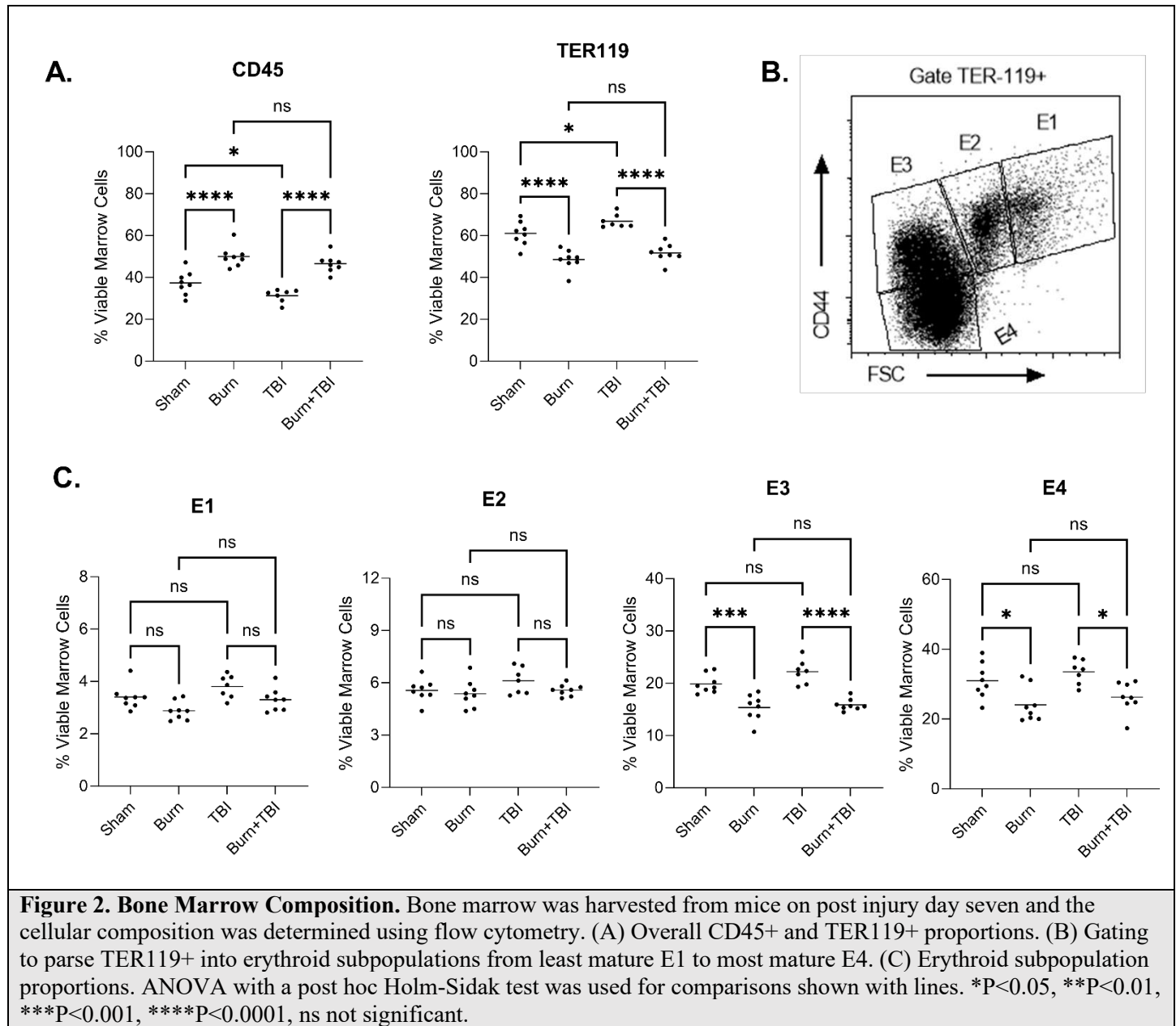


Figure 1. Complete Blood Counts. Blood was collected from mice on post injury day seven and run on an ADVIA 120 Hematology System. ANOVA with a post hoc Holm-Sidak test was used for comparisons shown with lines. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns not significant.

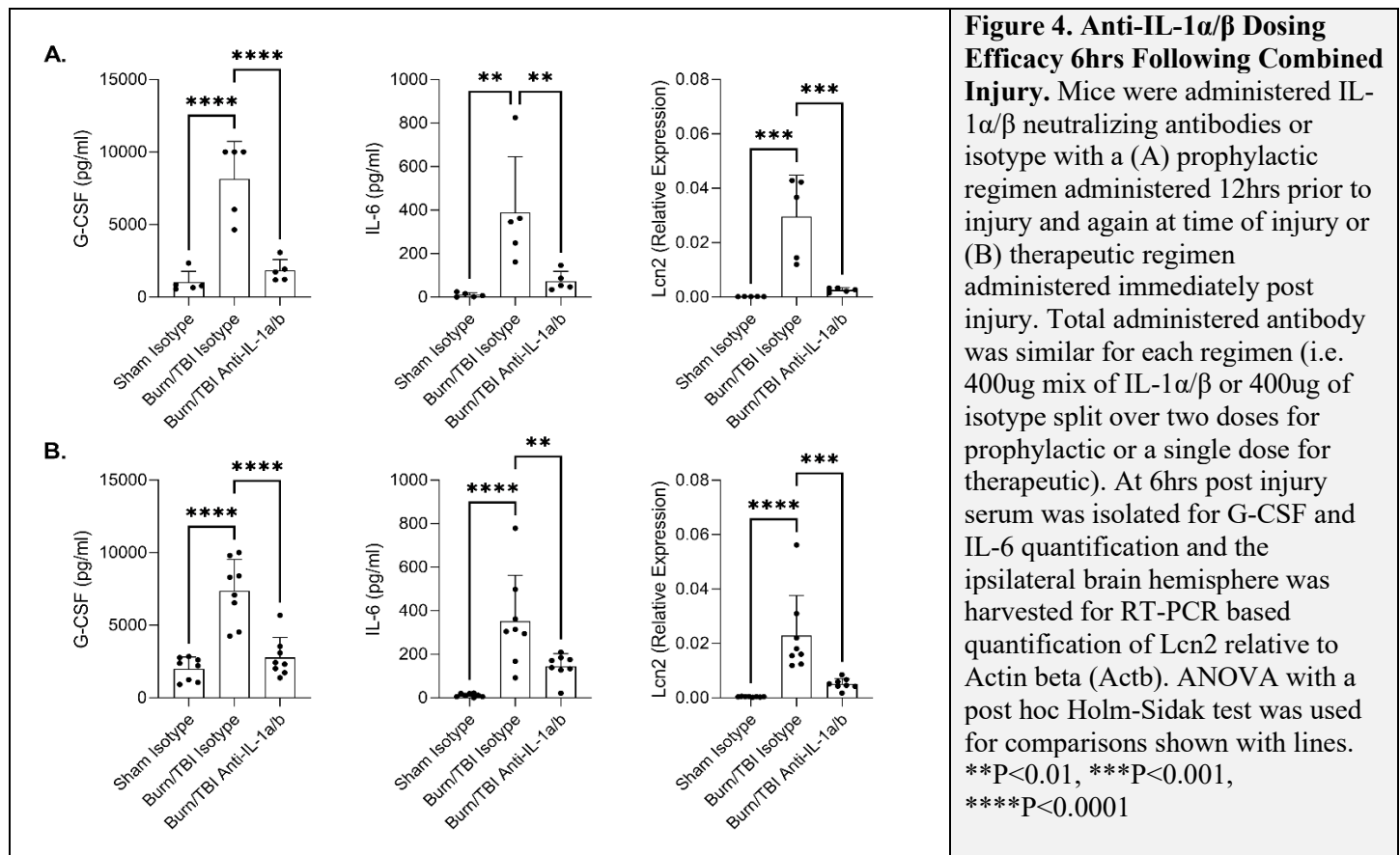
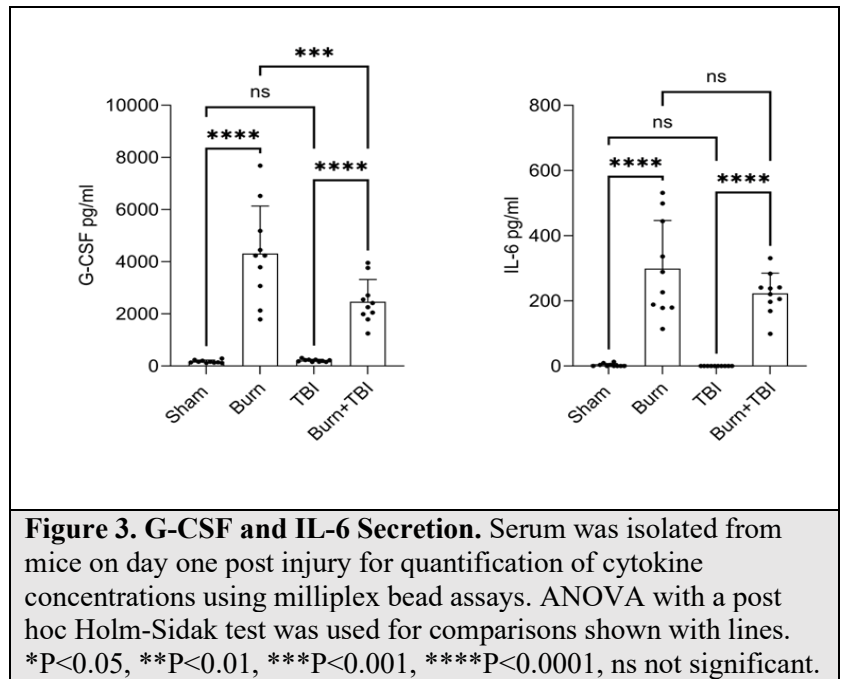
Aim 1 results from day 7 bone marrow composition studies are consistent with burn injury being the primary driver of ACI in the combined injury model. Burn injury increases CD45+ cells and decreases TER119+ cells in the bone marrow and the changes in the Burn+TBI group are no different (**Fig.2A**). Further, parsing the TER119+ cells into various stages of increasing maturity from E1 through E4 indicates the composition of erythroid cells is similar in the Burn and Burn+TBI groups with reductions primarily seen in E3 and E4 populations (**Fig.2B&C**).



Aim 1 results for day 1 post injury G-CSF and IL-6 quantification indicates concomitant Burn+TBI does not enhance the systemic secretion of these factors which we have found are key mediators of ACI (**Fig.3**). Therefore, all data collected to assess worsening of ACI are congruent and indicate burn injury is the primary driver of ACI in the combined model of Burn+TBI.

To assess neuroinflammation in aim 1 we harvested the ipsilateral hemisphere (side exposed to blast tube) for RT-PCR and could not detect significant upregulation of Gfap at 24hrs following Burn+TBI. Given the Robson groups prior data from TBI alone were limited to the ipsilateral cortex we suspected the expression could be

regional and moved to RNAseq to interrogate changes that can be readily detected in the entire ipsilateral hemisphere. We performed RNAseq at two time points, 6hr and 24hrs post injury. The dataset is still being analyzed but we readily identified Lipocalin-2 (Lcn2) as a highly upregulated gene in all injury models at 6hrs post injury and found that Lcn2 remained highly elevated in each group involving burn at 24hrs post injury. Given Lcn2 expression is common marker of an inflammatory response in tissues and has been suggested as a therapeutic target in models of brain injury, we used this gene along with G-CSF and IL-6 secretion as an initial assessment of anti-IL α/β blockade dosing efficacy. Data generated from this set of experiments confirmed our prophylactic dosing was sufficient to markedly attenuate G-CSF and IL-6 secretion as well as the induction of Lcn2 expression within the brain following concomitant Burn+TBI (Fig.4A). We also found a therapeutic dosing regimen was effective which is promising given the therapy would only be useful if it is effective when delivered at some point post injury (Fig.4B).



Initial analysis of the RNAseq data suggest a majority of the differentially expressed genes in the Burn+TBI group can be attributed to either TBI or Burn alone (i.e. change in the Burn+TBI group but also similarly changed in one of the single injury models) or change in a similar direction in all injury groups. However, we have identified several genes in which differential expression is augmented or uniquely altered by the combined injury. As an example, we filtered genes based on Burn+TBI vs Sham using a false discovery rate (FDR) ≤ 0.05 and a fold change of ± 1.5 to identify a large set of genes that are altered by combined injury, then we used a more stringent FDR of ≤ 0.01 and a smaller fold change of ± 1.25 to capture genes where changes are either augmented or uniquely altered by combined injury. These criteria identified 137 genes as augmented or unique to combined injury at the 6hr time point in which the majority were downregulated (Fig.5). There were fewer genes that met this criterion at the 24hr time point (Fig.6). Importantly, several genes identified at the 24hr post injury time point are specifically expressed in microglia such as downregulation of Siglech, P2ry12, and upregulation of Gpr84. This pattern of distinct changes in direction of microglia specific genes may indicate combined injury alters microglial phenotypes.

In summary, data to this point show combined injury does not amplify the systemic secretion of the ACI driver cytokines, G-CSF and IL-6, so it is not surprising that burn injury alone is the major factor leading to the reduction of circulating red blood cells and impairment of medullary erythropoiesis. However, the combined injury leads to complex changes in the brain that reflect both burn and/or TBI induced changes as well as amplification. Further, our initial studies focused on Lcn2 expression in the brain because this gene demonstrated the greatest fold change in all injury models at 6hrs post injury and remained highly elevated in both Burn and Burn+TBI groups 24hrs after injury. The initial studies

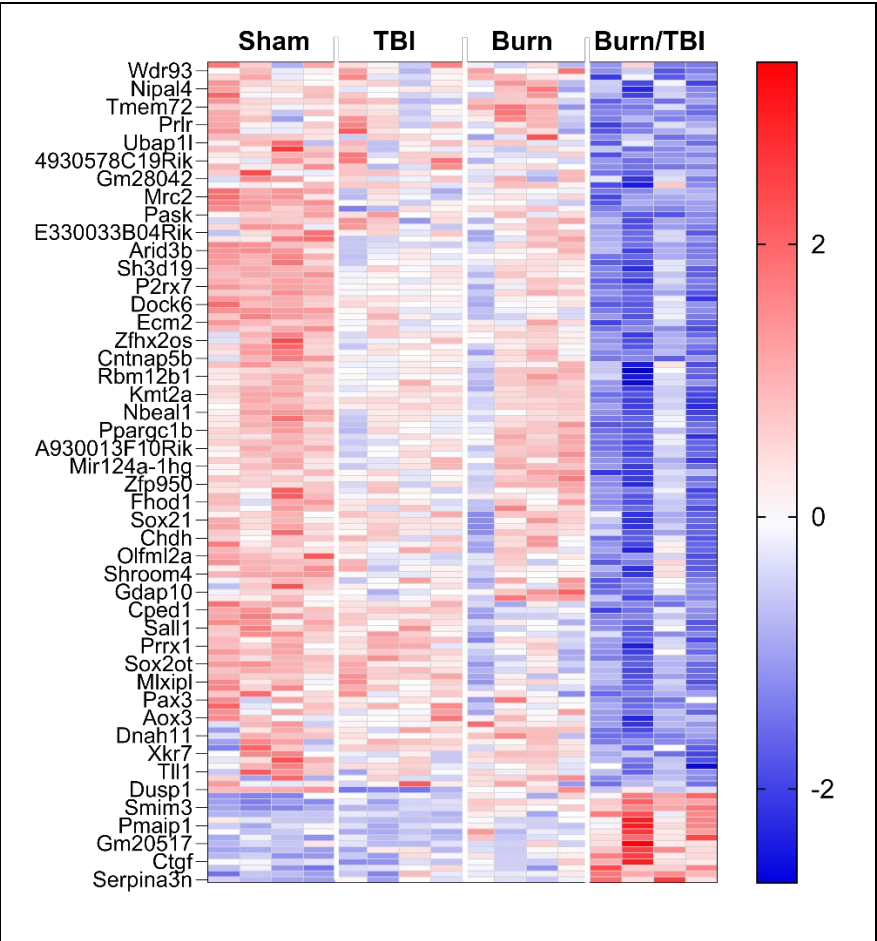


Figure 5. Augmented Gene Expression Patterns 6hrs Post Combined Injury. Ipsilateral hemisphere bulk RNAseq data colored by Z-Score.

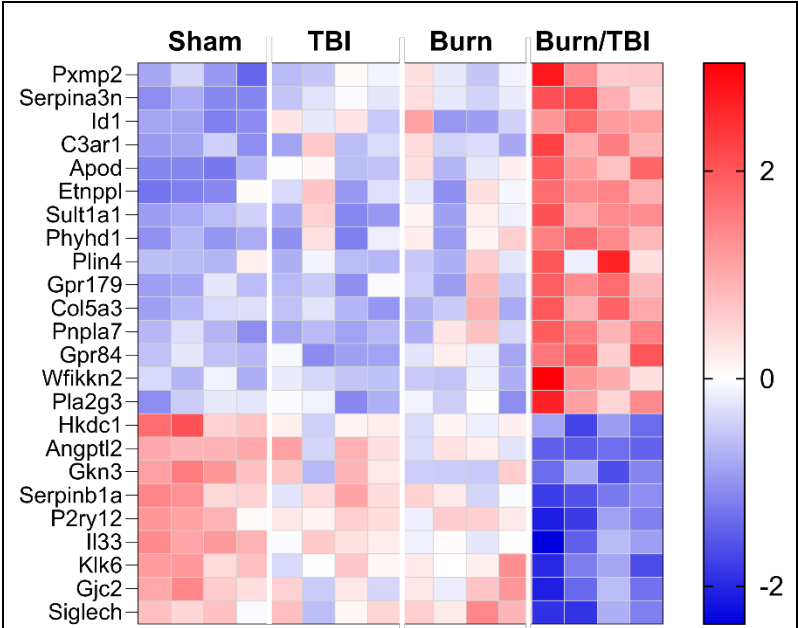


Figure 6. Augmented Gene Expression Patterns 24hrs Post Combined Injury. Ipsilateral hemisphere bulk RNAseq data colored by Z-Score.

showed effective IL-1 ligand blockade markedly reduced G-CSF and IL-6 secretion and Lcn2 expression in the brain. We found IL-1 ligand blockade effectively attenuates ACI following burn injury alone and burn is the primary driver of ACI in the combined model, it is likely IL-1 blockade will also attenuate ACI in the combined model which could have beneficial effects as anemia is associated with poor neurological outcomes. While we have only assessed a single gene to this point, the marked attenuation of Lcn2 is promising and suggests there is at least some IL-1R1 dependence of the brain's response to combined injury.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report

- **How were the results disseminated to communities of interest?**

Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**

We expect to follow the experiments outlined in the SOW with minor modifications explained further in the Changes/Problems section.

4. IMPACT

- **What is the impact on the development of the principal disciplines of the project?**

Nothing to Report

- **What is the impact on other disciplines?**

Nothing to Report

- **What is the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change.**

We anticipated running a limited set of RT-PCR to assess neuroinflammation with Gfap being a key gene based on prior data from the Robson Lab which assessed Gfap gene expression and Gfap staining in the ipsilateral cortex. We found a minor but statistically significant increase at 6hrs following combined injury that had normalized by 24hrs when we assessed the entire ipsilateral hemisphere. We suspected the Gfap could be regional and performed RNAseq on the ipsilateral hemisphere to identify genes that were altered in the larger brain specimen given the model is new and we have not established if specific regions are more impacted than others. We have now identified hundreds of genes that are impacted by individual injuries and/or combined

injuries. Further we found there are changes that persist across the 24-hour period and others that are dependent on timing. Given this is a new model and a major goal is to understand how IL-1 targeting therapies can modify neuroinflammation, the best approach going forward would be to assess therapies using RNAseq to understand how it all works. This would increase our expenditures but many of the additional expenses can be supported with some internal funds Dr. Gardner received. While these changes are within the scope of the project, we plan to reach out to our Grants Officer about any expenditures that could limit our ability to fully complete any tasks in our SOW. As an example, we were not able to complete serum iron measures because we could not isolate enough serum from trunk blood captured following decapitation to quantify both cytokines and iron. Trunk blood was required because other euthanasia methods are likely to artifact gene signatures in the brain. Given the hypochromic reticulocyte data shown in (**Fig.1**) indicates iron available for erythropoiesis is similarly reduced following burn or combined injury it is likely serum iron levels are similarly reduced. If we did not repeat experiments to capture the serum iron data, we could transition those mouse and reagent expenses to RNAseq studies that would provide a comprehensive assessment of IL-1 therapies on the brain following combined injury.

- **Actual or anticipated problems or delays and actions or plans to resolve them.**

Other than what is mentioned above, our Hemavet stopped operating very early after we started the study. Internal funding was used to purchase a new Hemavet but there was an extended period before we had an operating unit. To prevent any delays, we used fee for service ADVIA 120Hematology System at a core facility to capture the complete blood counts for aim 1.

- **Changes that had a significant impact on expenditures.**

See above.

6. PRODUCTS

- **Journal publications**

Noel, J. G., S. W. Ramser, L. Pitstick, H. S. Goetzman, E. L. Dale, A. Potter, M. Adam, S. S. Potter, and J. C. Gardner. 2023. IL-1/MyD88-Dependent G-CSF and IL-6 Secretion Mediates Postburn Anemia. *Journal of immunology* (Baltimore, Md. : 1950) 210: 972-980. (Funding was acknowledged, See Appendix).

*Note this manuscript identifies IL-1R1 signaling as a key mechanism driving post burn ACI and was honored as a Top Read in the *Journal of Immunology* (See Appendix)

- **Other publications**

C. O'Connell, E. Reeder, R. Brown, M. Robson, and J. Gardner. Concomitant Mild Traumatic Brain and Burn Injury Elicits a Unique Gene Expression Profile within the Brain. *Journal of Pharmacology and Experimental Therapeutics* June 2023, 385 (S3) 165. (Funding was acknowledged, See Appendix).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

There was no change in the effort for Dr. Gardner (PI) or Dr. Robson (Co-I) from the original proposal.

Our Research Associate position was filled at the beginning of the project by Satarupa Sengupta, PhD and Dr. Sengupta's effort is exactly as specified for the Research Associate in the original proposal.

Christopher O'Connell is a graduate student in University of Cincinnati James L Winkle College of Pharmacy that is fully supported by a graduate stipend in the Robson Lab. Chris has a strong interest in gene expression analysis and pharmacogenomics, so he has been participating in the RNAseq analysis and was first author on the abstract above. Chris has also been working along with Dr. Gardner and Robson when the injury models are performed and some other aspects of the project with Dr. Sengupta.

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable

9. APPENDICES

IL-1/MyD88–Dependent G-CSF and IL-6 Secretion Mediates Postburn Anemia

John G. Noel,* Seth W. Ramser,* Lori Pitstick,* Holly S. Goetzman,* Elizabeth L. Dale,[†] Andrew Potter,[‡] Mike Adam,[‡] S. Steven Potter,[‡] and Jason C. Gardner*

The anemia of critical illness (ACI) is a nearly universal pathophysiological consequence of burn injury and a primary reason burn patients require massive quantities of transfused blood. Inflammatory processes are expected to drive postburn ACI and prevent meaningful erythropoietic stimulation through iron or erythropoietin supplementation, but to this day no specific inflammatory pathways have been identified as a critical mechanism. In this study, we examined whether secretion of G-CSF and IL-6 mediates distinct features of postburn ACI and interrogated inflammatory mechanisms that could be responsible for their secretion. Our analysis of mouse and human skin samples identified the burn wound as a primary source of G-CSF and IL-6 secretion. We show that G-CSF and IL-6 are secreted independently through an IL-1/MyD88–dependent mechanism, and we ruled out TLR2 and TLR4 as critical receptors. Our results indicate that IL-1/MyD88–dependent G-CSF secretion plays a key role in impairing medullary erythropoiesis and IL-6 secretion plays a key role in limiting the access of erythroid cells to iron. Importantly, we found that IL-1 α / β neutralizing Abs broadly attenuated features of postburn ACI that could be attributed to G-CSF or IL-6 secretion and rescued deficits of circulating RBC counts, hemoglobin, and hematocrit caused by burn injury. We conclude that wound-based IL-1/MyD88 signaling mediates postburn ACI through induction of G-CSF and IL-6 secretion. *The Journal of Immunology*, 2023, 210: 972–980.

The anemia of critical illness (ACI) is a sudden reduction in red cell mass associated with inflammation that occurs in nearly all patients requiring a week or more of intensive care (1, 2) and a primary reason burn patients require transfusions (3). Conservative protocols for postburn transfusion have been adopted based on an association between poor medical outcomes and the frequency of transfusion (4, 5). A protocol designed to maintain hemoglobin in a range of 7–8 μ g/dl is safe and significantly reduces blood use, yet patients with 20–60% total body surface area burns still require an amount of blood that is roughly equivalent to vascular volume or four times this amount for larger burns (5). There are no therapeutic alternatives to transfusion because erythropoietin (EPO) (6–9) and iron (10) supplements fail to promote effective erythropoiesis following severe burn or trauma. The impaired response to EPO and iron supplements has largely been ascribed to inflammatory processes but the precise mechanisms remain elusive. Thus, a more complete understanding of the inflammation-based pathogenesis of ACI could facilitate strategies for intervention.

Postburn ACI involves all of the pathological features of inflammatory anemia, including iron restriction (11, 12), hemolysis (13–15), a reduced RBC lifespan (16), and an attenuated response to EPO (7–9). Although the overall pathology is complex, studies have linked features of ACI associated with erythropoietic impairment to myeloid-biased lineage commitment (17–20) and hematopoietic

progenitor mobilization (21–25), mechanisms expected to diminish the pool of EPO-responsive cells in the marrow and in turn reduce the number of circulating erythrocytes. Catecholamine and β -adrenergic stimulation play a role in both mechanisms (20, 22, 23, 25), and benefits of β -adrenergic receptor antagonism on erythropoiesis following trauma have been associated with attenuated G-CSF secretion (23, 25). Our studies confirmed that G-CSF secretion plays a key role in the postburn impairment of medullary erythropoiesis (26, 27), and we recognized that G-CSF secretion leads to attenuation of EPO signaling in bone marrow erythroid cells (26). G-CSF blockade improved EPO signaling and largely rescued erythroid cellularity in the marrow; however, G-CSF blockade was not sufficient to rescue a peripheral anemic response in ACI mice (26). We also assessed a role for M-CSF in postburn ACI based on studies that proposed that β -adrenergic stimulation could contribute to postburn myeloid bias through augmenting M-CSF receptor expression in LSK cells (19, 20). Instead, postburn M-CSF blockade led to an exacerbation of ACI associated with impaired iron recycling and increased inflammation (28). The exacerbation of iron-restricted erythropoiesis caused by postburn M-CSF blockade was at least in part dependent on enhanced IL-6 secretion. Notably, IL-6 is an established mediator of inflammation-induced iron restriction (29), and there is no known mechanism in which G-CSF restricts iron availability. Because burn injury initiates concomitant and durable secretion of G-CSF and IL-6

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The raw fastqs and count matrix presented in this article have been submitted to the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214717>) under accession number GSE214717.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ACI, anemia of critical illness; DPBS, Dulbecco's PBS; EBI, erythroblastic island; EPO, erythropoietin; KO, knockout; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA-seq; UMAP, uniform manifold approximation and projection; UMI, unique molecular identifier.

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in both mice (26, 27) and humans (30, 31), our prior studies suggested that G-CSF and IL-6 secretion could mediate different features of ACI.

The purpose of this study was to determine whether secretion of G-CSF and IL-6 plays a nonoverlapping role in postburn ACI and to understand how the secretion of each mediator is induced. Our results indicate that wound-based IL-1/MyD88 signaling is a key mechanism that leads to independent secretion of G-CSF and IL-6 following burn injury and identify distinct features of postburn ACI that are associated with the secretion of each mediator.

Materials and Methods

Mice and injury model

Female C57BL/6J, B6.129P2(SJL)-Myd88^{tm1.1Defr/J}, B6(Cg)-Tlr4^{tm1.2Karp/J}, and B6.129-Tlr2^{tm1Kir/J} mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and used in studies at 8–12 wk of age. A flame burn injury model was used to produce a full-thickness insensate lesion encompassing 15% of total body surface area, as previously described (27). In brief, mice were anesthetized with isoflurane and covered with a flame-resistant template exposing shaved dorsal skin. The target area was saturated with 0.5 ml of absolute ethanol and ignited for 10 s in the burn group or allowed to evaporate without ignition in the sham group. Immediately after the procedure, all mice received 0.5 ml of saline for volume resuscitation via the i.p. route. Mice were sacrificed by i.p. injection of Euthasol (Virbac, Fort Worth, TX). All animal procedures were approved by the University of Cincinnati Institutional Care and Use Committee.

Administration of neutralizing Abs

Abs were administered i.p. in a 100- μ l vol of PBS 12 h prior to injury, at the time of injury, and then daily until the day prior to harvest for all studies. Anti-G-CSF clone 67604 (R&D Systems, catalog no. MAB414), anti-IL-6 clone MP5-20F3 (Bio X Cell, catalog no. BE0046), and isotype clone HRPN (Bio X Cell, catalog no. BE0088) were used in G-CSF and IL-6 neutralization studies as cocktails adjusted to control for different dose requirements. Briefly, mice were administered 100 μ g of isotype, 90 μ g of isotype + 10 μ g of anti-G-CSF, or 10 μ g of isotype + 90 μ g of anti-IL-6 Abs such that all groups received 100 μ g of Ab with each injection. Combined IL-1 α and IL-1 β blockade was performed by administering 200 μ g of anti-IL-1 α clone ALF-161 (Bio X Cell, catalog no. BE0243) and 200 μ g of anti-IL-1 β clone B122 (Bio X Cell, catalog no. BE0246) together as a mixture, and groups of sham or burn injured mice received 400 μ g of Armenian hamster IgG (Bio X Cell, catalog no. BE0091) as the Ab control.

Serum cytokine and iron assays

Blood from cardiac puncture was collected in serum separator tubes (Microvette 500 Z-Gel, Sarstedt), and serum was stored at -80°C until analysis of cytokines using Milliplex MAP kits (Millipore, Billerica, MA) or determination of serum iron levels using a QuantiChrom iron assay kit (BioAssay Systems, Hayward, CA).

Flow cytometry

Bone marrow cells were flushed from each femur with 1.5 ml of ice-cold HBSS and passed through a 70- μ m filter. Isolated cells were spun at $400 \times g$ in a 4°C centrifuge, resuspended in Dulbecco's PBS (DPBS) containing a 1:1000 dilution of the viability dye eFluor 780 (eBioscience) and incubated at 4°C for 30 min according to the manufacturer's instructions. After washing twice in ice-cold FACS buffer (DPBS containing 0.5% BSA and 0.1% sodium azide), cells were blocked with rat serum and mouse Fc Block (BD Biosciences) for 10 min on ice and stained with fluorescent-labeled Abs for 30 min. The Ab panel for analysis of erythroid cells included TER-119 (clone TER-119, FITC labeled), CD71 (clone R17217, PE labeled), CD44 (clone IM7, allophycocyanin labeled), and CD45 (clone 30-F11, PE-Cy7 labeled), all from eBioscience. Cells were washed three times with ice-cold FACS buffer prior to performing flow cytometry on an LSR II (BD Biosciences) or an Attune (Thermo Fisher Scientific). Data were analyzed using FCS Express 7 (De Novo Software).

Skin RT-PCR

Skin was excised from the burn wound margin or dorsal skin of sham mice and processed in RNAlater stabilization solution (Ambion, catalog no. AM7021). Total RNA was isolated with the mirVana microRNA isolation kit (Ambion, catalog no. AM1561), and the DNA-free kit (Ambion, catalog no. AM1906) was used to eliminate DNA contamination. A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used to convert

1 μ g of RNA to cDNA prior to performing TaqMan gene expression assays for Csf3 (assay ID: Mm00438334_m1), Il6 (assay ID: Mm00446190_m1), and hypoxanthine guanine phosphoribosyl transferase (Hprt; assay ID: Mm03024075_m1) from Thermo Fisher Scientific. Results are reported as relative expression compared with the housekeeping Hprt.

Isolation of skin cells and single-cell RNA sequencing

Single-cell suspensions were obtained by mincing skin collected from the burn wound margin with a razor and dissociation in digestion buffer (HBSS with metals, 10 mM HEPES, 0.2% BSA, 500 U/ml type 4 collagenase, 100 U/ml DNase I, and 2.5 U/ml dispase) for 30 min at 37°C . Isolated cells were passed through a 70- μ m filter, washed with buffer (0.2% BSA in DPBS), and RBCs were lysed. After washing, cells suspended in buffer were mixed with OptiPrep density gradient medium (Sigma-Aldrich, catalog no. D1556) to achieve a density of 1.15 g/ml and then overlaid with 1.12 g/ml OptiPrep followed by buffer alone. Viable cells were collected from the interface between the 1.12 g/ml OptiPrep and buffer at the top of the gradient following centrifugation at $800 \times g$ for 20 min. The isolated cells were washed twice in ice-cold buffer and examined using trypan blue dye exclusion to determine concentration and viability. Concentration was adjusted to 700–1200 cells/ μ l and 9600 cells were loaded into the 10x Genomics chromium controller for 10x Genomics single-cell sequencing to target recovery of 6000 cells, and a Gel Bead-in-Emulsion (GEMs) was generated. 10x Genomics 3' v3.1 chemistry was used. Libraries were prepared according to the 10x Genomics protocol, and 14 cycles were used for cDNA amplification. Single-cell libraries were sequenced using the Illumina NovaSeq 6000.

Data analysis methods for single-cell RNA sequencing

After sequencing, the raw fastqs were processed through Cell Ranger v3.1.0 to obtain a gene-barcode unique molecular identifier (UMI) count matrix. Raw fastqs and the count matrix are available under GEO accession number GSE214717 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214717>). Cell-type clustering and marker genes were identified using Seurat 4.2.0 (32). Data were prefiltered at both the cell and gene level with the removal of cells with low library complexity (<500 expressed genes) as well as those with a high percentage (>30%) of UMIs mapping to mitochondrial genes. Genes were selected that had >0 UMIs in more than three cells. Genes were log normalized and the data were scaled around the mean regressing out the nCount_RNA feature. The most variable genes were used for the principal component analysis. Significant principal components were determined using the JackStraw function. Data were visually represented after clustering using Seurat's uniform manifold approximation and projection (UMAP) function using the umap-learn and correlation Python implementation of UMAP using the first 15 significant dimensions. The FindAllMarkers function was used to determine the top marker genes for each cluster with a minimum of 25% of cells expressing the gene within the cluster and a minimum log fold change threshold of 0.25.

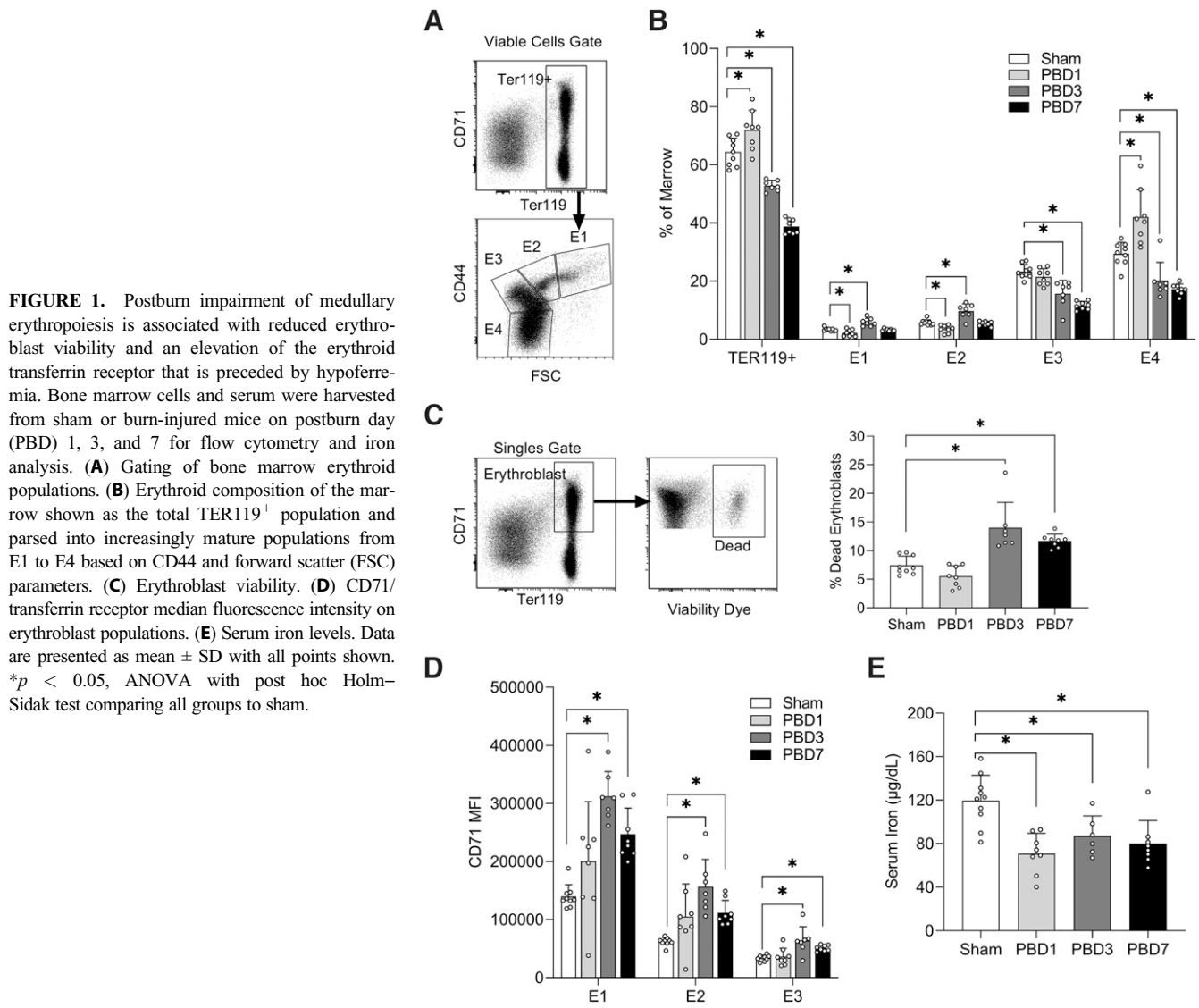
Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. The Student *t* test was used for two-group comparisons, and ANOVA with a posthoc Holm–Sidak test was used for comparisons involving three or more groups as specified in the figure legends. Significant differences were determined at *p* values of <0.05.

Results

Postburn impairment of medullary erythropoiesis is associated with reduced erythroblast viability and an elevation of the erythroid transferrin receptor that is preceded by hypoferrremia

In initial studies we examined the temporal pattern of ACI features that could be linked to postburn G-CSF and/or IL-6 secretion. We previously found that the reduction of erythroid composition of the marrow and erythroblast viability were features of G-CSF-dependent ACI on postburn day 7 (26). Time-course experiments assessed erythroid composition of the bone marrow during 1 wk postburn by gating on TER119⁺ cells and then CD44 versus forward light scatter to resolve distinct stages of erythroid differentiation as previously described (33) (Fig. 1A). TER119⁺ cell composition of the marrow was reduced by postburn day 3 and continued to decrease through day 7, with the major reduction occurring in later stage E3 and E4 erythroid cells (Fig. 1B). Erythroblast viability was quantified over time by gating on CD71⁺TER119⁺ cells without prior exclusion of



viability dye–positive cells and then quantifying the percentage of dead erythroblasts as the proportion of erythroblasts that are viability dye positive. The percentage of dead erythroblasts increased by postburn day 3 and remained increased on postburn day 7 (Fig. 1C). In our prior study involving M-CSF blockade we identified an elevation of transferrin receptor (CD71) on erythroid populations at postburn day 7 as a marker of iron-restricted erythropoiesis that was exacerbated by M-CSF blockade in an IL-6–dependent manner (28). Time-course experiments assessed erythroid transferrin receptor levels by gating as in Fig. 1A and then quantifying CD71 median fluorescence intensity on erythroid populations E1–E3. Transferrin receptor levels were significantly increased on erythroid subpopulations by postburn day 3 and remained elevated on day 7 (Fig. 1D). Circulating iron levels were significantly decreased on postburn day 1 and remained low throughout the study (Fig. 1E). These results established the time frame in which distinct features of ACI can be assessed and indicated that a reduction in circulating iron precedes the postburn elevation of the erythroid transferrin receptor.

G-CSF and IL-6 are secreted independently following burn injury and have distinct effects on serum iron levels

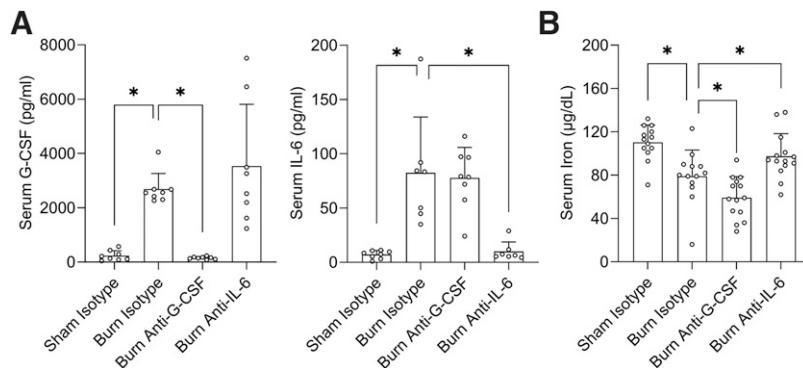
Pleiotropic effects of IL-6 on diverse cell types and inflammatory responses could modulate postburn G-CSF secretion either positively or negatively, and prior reports indicate that mobilizing doses

of G-CSF can increase systemic levels of IL-6 (34, 35). We isolated serum from mice subjected to G-CSF or IL-6 blockade on postburn day 7 to determine whether blocking one cytokine modified secretion of the other and found that the neutralization protocols specifically reduced the cytokine that was targeted (Fig. 2A). We also assessed serum iron levels in mice subjected to G-CSF or IL-6 blockade and found that postburn IL-6 blockade improved circulating iron levels whereas G-CSF blockade led to a further decrease (Fig. 2B). These results demonstrated that G-CSF and IL-6 are secreted independently following burn injury and that IL-6 plays a role in mediating the postburn hypoferrremic response.

Postburn G-CSF blockade rescues bone marrow erythroid cellularity whereas IL-6 blockade attenuates elevation of the erythroid transferrin receptor

Because G-CSF and IL-6 were independently secreted and had distinct effects on serum iron levels following burn injury, we assessed the impact of each cytokine on medullary erythropoiesis as in Fig. 1. Postburn G-CSF blockade improved the erythroid composition of the marrow whereas IL-6 blockade did not (Fig. 3A). IL-6 blockade uniquely attenuated elevation of the erythroblast transferrin receptor that is induced following burn injury (Fig. 3B), and both neutralization protocols improved erythroblast viability, although G-CSF blockade was more apparent (Fig. 3C). Furthermore, a combined G-CSF

FIGURE 2. G-CSF and IL-6 are secreted independently following burn injury and have distinct effects on serum iron levels. Mice were administered 100 μ g of isotype, 90 μ g of isotype + 10 μ g of anti-G-CSF, or 10 μ g of isotype + 90 μ g of anti-IL-6 Abs 12 h prior to injury, at the time of injury, and then daily until collection of serum for cytokine and iron analysis on day 7 after injury. **(A)** Serum G-CSF and IL-6 levels. **(B)** Serum iron levels. Data are presented as mean $\pm$ SD with all points shown. * p < 0.05, ANOVA with post hoc Holm–Sidak test comparing all groups to burn isotype.



and IL-6 neutralization strategy rescued each of the features in the marrow of burn-injured mice that could be attributed to the individual cytokines: erythroid composition of the marrow was rescued, elevation of the erythroblast transferrin receptor was attenuated, and erythroblast viability was normalized (Supplemental Fig. 1). Overall, these results were consistent with distinct effects of the cytokines in postburn ACI; that is, G-CSF plays a key role in reducing erythroid cellularity of the marrow, and IL-6 limits the access of erythroid cells to iron.

Postburn secretion of G-CSF and IL-6 is MyD88-dependent but does not require TLR4 or TLR2

Because G-CSF and IL-6 were independently secreted and mediated distinct features of postburn ACI, we investigated mechanisms that

might be responsible for the secretion of both mediators. Damage-associated molecular patterns, endogenous molecules secreted by immune cells or released from injured cells, are expected to trigger the postburn inflammatory response through engagement of pathogen-recognition receptors (36). Among pathogen-recognition receptors, TLRs have received the most attention, in particular TLR2 and TLR4, which are primed to respond to ligands following burn injury (37). MyD88 is an essential adapter protein for signaling to occur through most TLRs (38) and members of the IL-1 receptor family (39). Therefore, we subjected MyD88 knockout (KO) mice to burn injury to broadly assess a role for TLRs and found that postburn G-CSF and IL-6 secretion levels were markedly suppressed in MyD88 KO mice (Fig. 4A). Follow-up studies incorporated TLR2 or TLR4 KO mice based on the postburn priming effects associated with these receptors (37), but circulating levels of G-CSF and IL-6 remained elevated in both mouse strains following burn injury (Fig. 4B, 4C). These data clearly identified MyD88-dependent receptors as candidates responsible for postburn G-CSF and IL-6 secretion but did not support TLR2 or TLR4 as the critical receptors.

G-CSF and IL-6 expression levels are increased at the site of burn injury and are associated with IL-1R1 expression

To determine whether the burn wound was a source of G-CSF and IL-6 secretion, we quantified gene expression in skin isolated from the dorsal region of sham mice and the wound margin of burn-injured mice. We detected high levels of *Csf3* and *Il6* expression in the burn skin samples compared with sham skin samples (Fig. 5A). To assess human relevance, we quantified G-CSF and IL-6 protein levels in skin tissue that we collected as surgical discard and examined public bulk RNA sequencing (RNA-seq) data from a study that collected the same tissues (40). We measured high G-CSF and IL-6 protein levels in burn eschar samples compared with normal skin (Supplemental Fig. 2A). The public data showed a consistent elevation in *CSF3* and *IL-6* gene expression, so we interrogated expression related to MyD88-dependent signaling and recognized increased expression of *IL-1R1*, *IL-1 β* , and *IL-1 α* , albeit the elevation of *IL-1 α* did not reach statistical significance (Supplemental Fig. 2B). To explore IL-1/MyD88 as a potential mechanism of G-CSF and IL-6 secretion in mice, we performed single-cell RNA-seq (scRNA-seq) on mouse skin cells isolated from the wound margin on postburn day 3. The time for analysis was selected based on a report indicating that IL-6-secreting myeloid-derived suppressor cells maximally infiltrate the wound on postburn day 3 (41). The cells isolated from postburn day 3 mouse skin segregated into 11 clusters (Fig. 5B) based on differential expression of marker genes (Supplemental Table 1). Cluster 4 cell type expression patterns were ambiguous due to low read counts (Supplemental Fig. 3A), so cell identities were only assigned for 10 clusters (Supplemental Fig. 3B). Three clusters of macrophages that were identified based on common marker genes

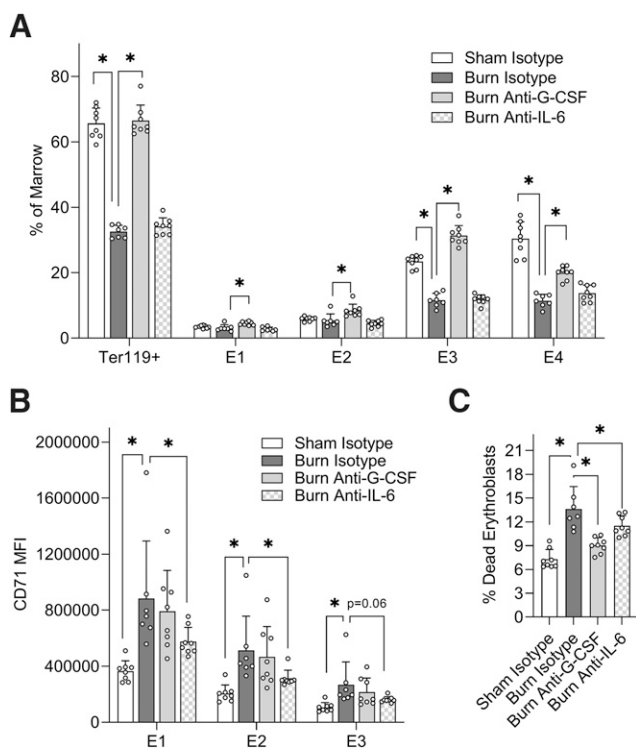


FIGURE 3. Postburn G-CSF blockade rescues bone marrow erythroid cellularity whereas IL-6 blockade attenuates elevation of the erythroid transferrin receptor. Mice were administered 100 μ g of isotype, 90 μ g of isotype + 10 μ g of anti-G-CSF, or 10 μ g of isotype + 90 μ g of anti-IL-6 Abs 12 h prior to injury, at the time of injury, and then daily until harvest on day 7 after injury for flow cytometry using the gating strategies described in Fig. 1. **(A)** Erythroid composition of the marrow. **(B)** CD71/transferrin receptor median fluorescence intensity (MFI) on erythroblast populations. **(C)** Erythroblast viability. Data are presented as mean $\pm$ SD with all points shown. * p < 0.05, ANOVA with post hoc Holm–Sidak test comparing all groups to burn isotype.

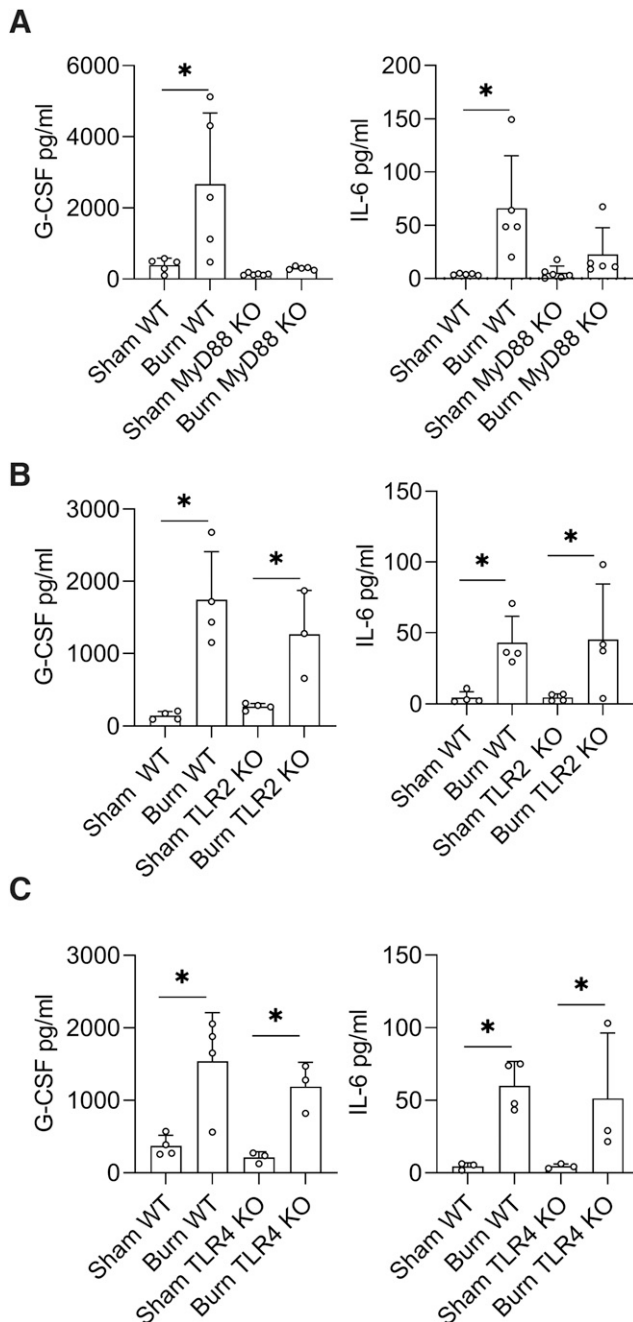


FIGURE 4. Postburn secretion of G-CSF and IL-6 is MyD88-dependent but does not require TLR4 or TLR2. Secretion of G-CSF and IL-6 was quantified in wild-type (WT) and knockout (KO) mice on postinjury day 7. **(A)** MyD88 WT and KO mice. **(B)** TLR2 WT and KO mice. **(C)** TLR4 WT and KO mice. Data are presented as mean $\pm$ SD with all points shown. * $p < 0.05$, ANOVA with post hoc Holm–Sidak test comparing sham WT versus burn WT mice and sham KO versus burn KO mice.

such as Cd68 and marker genes such as Arg1/Il10 for cluster 0, Cd209 for cluster 6, and Mki67 for cluster 7 were found to distinguish macrophages. Two fibroblast clusters were identified based on marker genes associated with matrix production, and features that distinguished the fibroblast clusters included higher levels of collagen gene expression in cluster 3 and higher levels of inflammatory gene expression in cluster 1. Monocyte cluster 2 and neutrophil cluster 5 could be identified by high Ccr2/Ly6c and Csf3r/Ly6g expression, respectively. Other clusters were identified as muscle cluster 8 expressing myosin genes, endothelial cluster 9 expressing

Pecam1/Cldn5, and NK/T cell cluster 10 expressing Nkg7/Cd3e. A search for candidate MyD88-dependent receptors identified high levels of Il1r1 expression in fibroblasts that overlapped with high levels of Il6 and Csf3 expression (Fig. 5B). Furthermore, feature plots show that neutrophils and monocytes expressed high levels of Il1b and a faint level of Il1a that was most apparent in neutrophils (Fig. 5B). Overall, these data suggested wound-based IL-1/MyD88 signaling as a candidate mechanism that could lead to G-CSF and IL-6 secretion in mice and humans following burn injury.

IL-1 α / β blockade attenuates postburn G-CSF and IL-6 secretion and improves serum iron levels

IL-1 α and IL-1 β both initiate MyD88-dependent signaling through binding IL-1R1; however, IL-1 α plays a unique role as an alarmin because it is constitutively present in most cell types and is released from necrotic cells as a biologically active precursor (42). The IL-1 α precursor and the mature form IL-1 α are both capable of stimulating IL-6 secretion in a dose-dependent manner (43). Furthermore, IL-1 α blockade is sufficient to reduce IL-6 and G-CSF secretion in a model of sterile inflammation (44) and G-CSF secretion in a model of poly-trauma (45). To assess IL-1/MyD88-induced G-CSF and IL-6 secretion as a mechanism of ACI, we used a dual IL-1 α / β neutralization strategy because we detected high levels of IL- β gene expression in the skin following burn injury and because biologically active IL-1 α could be released from necrotic wound cells regardless of gene induction. The neutralization studies revealed that IL-1 α / β blockade largely abolished G-CSF and IL-6 secretion and rescued serum iron levels on postburn day 1 (Fig. 6A). Similar results were recorded on day 7 for mice subjected to postburn IL-1 α / β neutralization, albeit the reduction in G-CSF secretion afforded by IL-1 α / β blockade was not as remarkable (Fig. 6B). These results confirmed that postburn secretion of G-CSF and IL-6 is IL-1/MyD88-dependent, and the improvement in serum iron levels was consistent with the reduction in IL-6 secretion.

IL-1 α / β blockade broadly attenuates postburn anemia

To determine whether the attenuated secretion of G-CSF and IL-6 and improvement in serum iron levels in burn-injured mice subjected to IL-1 α / β blockade were meaningful, we assessed medullary erythropoiesis and the peripheral anemic response on postburn day 7. We used the same gating strategies shown in Fig. 1 to assess medullary erythropoiesis and found that postburn IL-1 α / β blockade improved the erythroid composition of the marrow (Fig. 7A), attenuated elevation of the erythroid transferrin receptor (Fig. 7B), and reduced the percentage of dead erythroblasts (Fig. 7C). Importantly, IL-1 α / β blockade significantly reversed the postburn reduction of circulating RBCs, hemoglobin, and hematocrit (Fig. 7D). All together, these results confirmed IL-1/MyD88-dependent G-CSF and IL-6 secretion as a mechanism that leads to postburn ACI.

Discussion

Iron restriction (11, 12), hemolysis (13–15), a reduced RBC lifespan (16), and an attenuated response to EPO (7–9) have been well documented as characteristic features of postburn ACI. Although these features are consistent with inflammation-driven anemia, no inflammatory pathway has been identified as a critical mechanism of postburn ACI, and whether distinct features of postburn ACI could be mediated by specific cytokines was not clear. This information is important to understand because a therapeutic strategy that only attenuates certain features of ACI may not be effective. Our study provides evidence for wound-based IL-1/MyD88-driven G-CSF and IL-6 secretion as a key mechanism that leads to postburn ACI and identifies

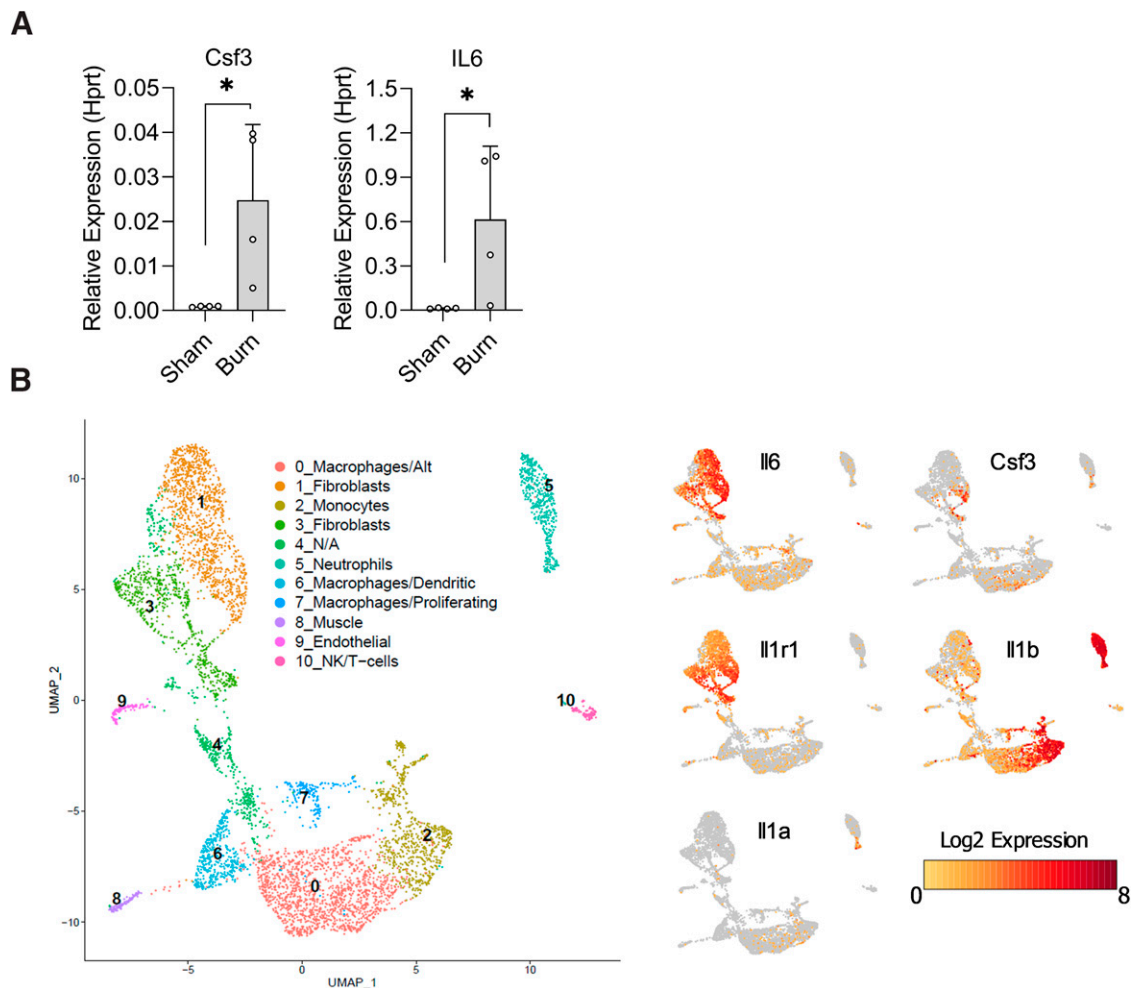


FIGURE 5. Expression of G-CSF and IL-6 is increased at the site of burn injury and associated with IL-1R1 expression. Skin was harvested from the dorsal region of sham mice or the wound margin of burn mice on postinjury day 1 for RT-PCR and from the wound margin on postburn day 3 for scRNA-seq. **(A)** RT-PCR presented as mean $\pm$ SD with all points shown. * $p < 0.05$, t test. **(B)** scRNA-seq UMAP clustering and feature plots.

distinct features of postburn ACI that are mediated by G-CSF or IL-6 secretion.

IL-1 α and/or IL-1 β binding to IL-1R1 leads to recruitment of MyD88 to Toll/IL-1R (TIR) domains of IL-1R1 and the coreceptor IL-1R3, activation of IRAK1/4, and production of inflammatory cytokines (39). We found that IL-1 α/β blockade or MyD88 deficiency significantly reduced postburn G-CSF and IL-6 secretion whereas postburn G-CSF or IL-6 neutralization specifically reduced the cytokine that was targeted. Taken together, these results indicate that G-CSF and IL-6 are secreted independently through an IL-1/MyD88-dependent mechanism. Our results also show that G-CSF and IL-6 secretion are not attenuated in TLR2- or TLR4-deficient mice subjected to burn injury. This result is consistent with an IL-1R1/MyD88-dependent sterile inflammatory response triggered by cell death in which TLR signaling was largely ruled out (46). Our results are also consistent with prior studies that found that IL-1R1/MyD88 induces IL-6 and G-CSF secretion in a model of sterile inflammation (44) or G-CSF secretion in a model of polytrauma (45), together suggesting that the IL-1-driven G-CSF and IL-6 secretion levels we recognized following burn injury are a shared response to different forms of sterile tissue injury.

Transferrin receptor (CD71) levels can be elevated on erythroid cells when iron availability is limited (47–50), and we previously identified increased CD71 staining on bone marrow erythroid cells

as an indicator of iron-restricted erythropoiesis on postburn day 7 (28). In this study, we show that the postburn elevation of erythroid CD71 is preceded by a marked reduction in circulating iron levels, which further validates the increase of CD71 as a marker of iron-restricted erythropoiesis. Neutralizing IL-6 increased circulating iron levels and reduced the elevation of erythroid CD71 following burn injury whereas postburn G-CSF blockade did not improve either measure. As in our prior studies (26, 27), postburn G-CSF neutralization largely rescued erythroid cellularity in the bone marrow following burn injury whereas postburn IL-6 blockade did not. These results demonstrate that secretion of IL-6 and G-CSF mediates distinct features of postburn ACI; IL-1/MyD88-driven IL-6 secretion plays a role in limiting the access of erythroid cells to iron, and IL-1/MyD88-driven G-CSF secretion plays a key role in reducing erythroid cellularity of the marrow.

Our study does not determine exactly how G-CSF and IL-6 secretions mediate features of postburn ACI, but other investigators have directly administered the cytokines in recombinant form to interrogate mechanisms. G-CSF-mediated impairment of medullary erythropoiesis has been attributed to a reduction in erythroblastic island (EBI) macrophages (51, 52), and IL-6-mediated iron restriction has been attributed to induction of hepcidin (29). The EBI is a hematopoietic niche composed of a central macrophage surrounded by developing erythroblasts in which the macrophages support the survival,

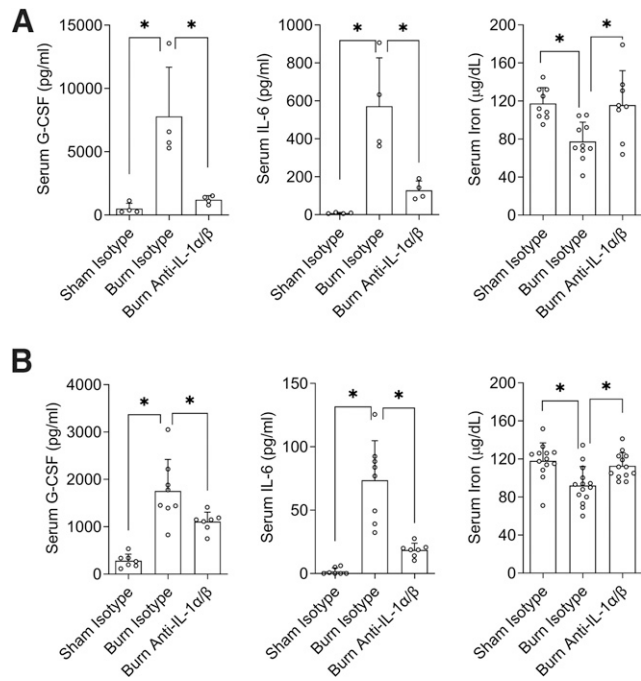


FIGURE 6. IL-1 α/β blockade attenuates postburn G-CSF and IL-6 secretion and improves serum iron levels. Mice were administered 400 μ g of isotype Abs or 200 μ g of anti-IL-1 α + 200 μ g anti-IL-1 β Abs 12 h prior to injury, at the time of injury, and then daily until the day prior to harvest. **(A)** Postinjury day 1 serum levels of G-CSF, IL-6, and iron. **(B)** Postinjury day 7 serum levels of G-CSF, IL-6, and iron. Data are presented as mean $\pm$ SD. * p < 0.05, ANOVA with post hoc Holm-Sidak test comparing all groups to burn isotype.

proliferation, and enucleation of erythroid cells (53). A reduction or functional alteration of EBI macrophages provides a plausible explanation for how postburn G-CSF secretion led to impairment of EPO signaling in our prior study (26) because these macrophages express factors such as insulin-like growth factor-1 (54), which is known to enhance EPO signaling (55). Heparin is a systemic iron-regulatory hormone predominantly expressed in the liver that degrades ferroportin on the surface of iron-exporting cells and in turn leads to a reduced flow of iron into plasma (56). In our prior study we only found evidence for hepatic hepcidin induction on postburn day 1 unless mice were subjected to postburn M-CSF blockade, which further elevated IL-6 (28). An early hepcidin induction could play a role in the postburn hypoferric response as serum iron levels remain low in mice for 2–3 d beyond the administration of a single hepcidin injection, a delay that is likely explained by the time required for ferroportin recovery on the surface of iron-exporting cells (57). Furthermore, IL-6 secretion could play a role in maintaining hepcidin at an inappropriate level, as opposing mechanisms exist to sense low iron levels and limit hepcidin secretion (58). Examining the impact of burn injury on EBI macrophages and incorporating models of hepcidin deficiency in future studies has potential to define the direct actions of G-CSF and IL-6 in postburn ACI.

To our knowledge there is only one study that used IL-1R1-deficient mice in a model of burn injury. These investigators found that IL-1R1 signaling in hematopoietic cells contributed to lung injury early (4–15) after burn injury but did not quantify G-CSF and IL-6 secretion (59). Myeloid-derived suppressor cells have also been reported to infiltrate burn wounds maximally on day 3 after injury and were shown to constitutively produce IL-6 in culture (41). We used scRNA-seq as part of a discovery process and found that the highest levels of *Il1r1* expression in fibroblasts and the *Il1r1*

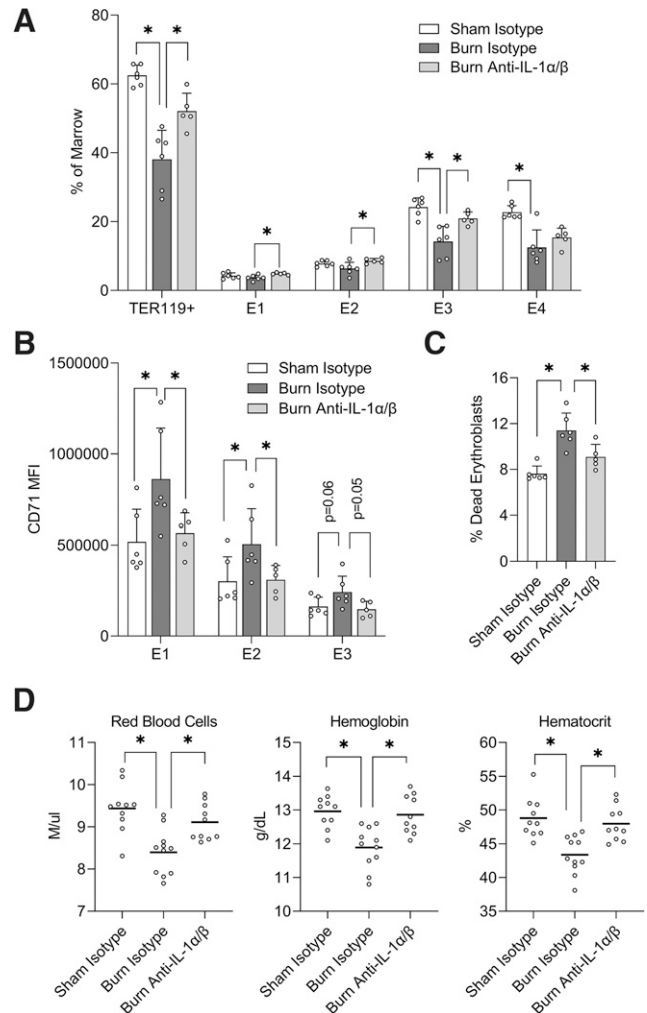


FIGURE 7. IL-1 α/β blockade broadly attenuates postburn anemia. Mice were administered 400 μ g of isotype Abs or 200 μ g of anti-IL-1 α + 200 μ g of anti-IL-1 β Abs 12 h prior to injury, at the time of injury, and then daily until harvest on day 7 after injury. **(A)** Erythroid composition of the marrow. **(B)** CD71/transferrin receptor median fluorescence intensity (MFI) on erythroid populations. **(C)** Circulating RBC measures determined on an automated hematology system. Data are presented as mean $\pm$ SD with all points shown or dot plots with line at mean. * p < 0.05, ANOVA with post hoc Holm-Sidak test comparing all groups to burn isotype.

expression overlapped with high levels of *Csf3* and *Il6* expression. Our results are consistent with fibroblasts being a key sensor of IL-1 in models of sterile skin injury (60, 61) but cannot exclude a role for IL-1/MyD88 signaling in hematopoietic or myeloid cells as a driver of postburn ACI.

In summary, our results are consistent with a hierarchical mechanism in which wound-based IL-1/MyD88 signaling drives postburn G-CSF and IL-6 secretion; in turn, secretion of G-CSF and IL-6 mediates nonoverlapping features of postburn ACI. Although the role of IL-1 in postburn ACI has not been appreciated, the burn wound has long been recognized as a primary source of IL-1 activity (62), and prior studies demonstrate that IL-1 signaling can drive anemia in humans. Anakinra (IL-1R1 antagonist) or rilonacept (IL-1 trap) markedly improve hemoglobin levels in patients suffering from IL-1R1 antagonist deficiency (63). Furthermore, exploratory analysis of a randomized clinical trial found that canakinumab (anti-IL-1 β) reduces the incidence of anemia and improves hemoglobin in humans with a history of myocardial injury (64). In both studies, targeting the IL-1 pathway was associated with a significant reduction

of C-reactive protein, which is a well-known inflammatory marker induced by IL-6. Collectively, studies demonstrate that the IL-1 pathway is sufficient to drive anemia and that therapeutic targeting of the pathway can be effective.

Disclosures

The authors have no financial conflicts of interest.

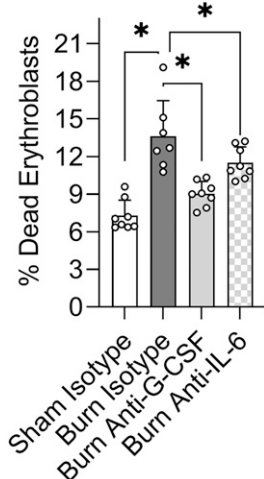
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G-CSF and IL-6 Drive Postburn Anemia

In this Top Read, Noel et al. (p. 972) showed that postburn anemia of critical illness (ACI) is enhanced by G-CSF and IL-6 secretion and is driven by IL-1/MyD88 signaling at the site of the burn wound. Using a previously described mouse model of ACI, G-CSF reduced erythroid composition in the bone marrow, whereas IL-6 reduced access of erythroid cells to iron via the transferrin receptor. MyD88, TLR2, and TLR4 individual knockout mice, and IL-1 neutralization, showed that G-CSF and IL-6 secretion was MyD88- and IL-1-dependent. IL-1 α / β blockade improved serum iron levels and attenuated postburn anemia, thereby ameliorating ACI. Finally, skin samples from both the ACI mouse model and human eschar skin samples represented in a public RNA sequencing repository showed that high G-CSF and IL-6 protein secretion originates from burned skin. Altogether, the data provide a mechanism by which increased cytokines produced by severely burned skin drives ACI, and it suggests blocking IL-1 signaling as a future therapeutic.



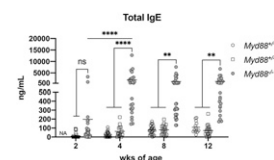
SARS-CoV-2 Vaccine Responses in Persons with HIV

Persons with HIV (PWH) have high risk for severe COVID-19 disease, perhaps in part due to persistent immune dysfunction, even with effective antiretroviral therapy (ART). In this Top Read, Quinn et al. (p. 947) examined the magnitude and heterogeneity of immune responses following SARS-CoV-2 vaccination in PWH on ART. Of the 11 patients analyzed, 2 received mRNA vaccines and 9 received replication-incompetent vector vaccines. Spike-specific Ab was increased in all patients following vaccination; however, the highest Ab titers were in patients receiving the mRNA vaccine and in a

patient with a previous SARS-CoV-2 infection. The neutralization potential also correlated with the magnitude of the spike-specific Ab response. The mRNA-vaccinated patients and the previously infected patient also showed the most robust virus-specific T and B cell responses. Virus-specific B cells were absent in only two patients, who also had the lowest Ab responses. Interestingly, spike-specific germinal centers (GC) were absent in all but one of the vector-vaccinated patients, although vector vaccination of an HIV-negative patient resulted in spike-specific GC formation. These data indicate that PWH on ART can mount an immune response to SARS-CoV-2 vaccination; however, these responses are varied and require additional investigation.

Lung Microbiota Induces IgE in *MyD88*^{-/-} Mice

In this Top Read, Amano et al. (p. 959) demonstrated that memory B cells (MBC) spontaneously produce natural IgE in response to commensal lung *Streptococcus azizii* in *MyD88*^{-/-} mice. Plasma cells and B cells were differentially depleted in *MyD88*^{-/-} mice, resulting in transient and sustained decreases in serum IgE, respectively, suggesting that MBC are responsible for IgE production. Dysbiosis in the lung was noted, wherein *MyD88*^{-/-} mice had increased *S. azizii* colonization compared with separately housed *MyD88*^{+/+} mice. Serum IgE levels decreased in the *MyD88*^{-/-} mice following antibiotic treatment. Additionally, *S. azizii* infection increased serum IgE levels in *MyD88*^{-/-} mice but had no effect on IgE levels in *MyD88*^{+/+} mice. Lung exudate cells from *MyD88*^{-/-} mice stimulated in vitro with *S. azizii* produced high levels of IL-4 and IL-13, suggesting that the bacteria primed a Th2 response supporting IgE production by MBC. Finally, excessive CSF1 produced by nonhematopoietic cells in the lung was responsible for the natural IgE production in *MyD88*^{-/-} mice. Because spontaneously produced natural IgE can increase risk for allergic diseases, these data provide insight into mechanisms that may lead to high levels of serum IgE and provide potential targets for treatment.



Concomitant Mild Traumatic Brain and Burn Injury Elicits a Unique Gene Expression Profile within the Brain

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Mild Traumatic Brain Injuries (mTBI) and Burn Injuries (BI) present monumental public health burdens that have been independently linked to long term nervous system-related morbidity and negative patient outcomes. Military service members in active combat theaters are among the most at risk for sustaining concomitant mTBI and BI, in most cases due to detonation of improvised explosive devices. Previous studies have provided evidence that mTBI and BI independently induce inflammation, through the induction of innate inflammatory cascades, however the concomitant effects of simultaneous mTBI and BI on early post-injury molecular processes, gene expression alterations, neuronal signaling within the brain and ultimately outcomes are currently unknown. This lack of data, coupled with an overt lack of studies conducted in human subjects, has left large gaps in the knowledge required and the available capability to successfully treat complicated polytrauma cases involving mTBI and BI. We hypothesize that concomitant mTBI and BI act in exaggerating early neuroinflammatory responses within the CNS, an effect that leads to worse long-term health and behavioral outcomes, when compared to mTBI or BI alone. To test this hypothesis, adult, wild type C57Bl/6J mice were subjected to a single, blast induced mTBI and full thickness BI (mTBI/BI), a single, blast induced mTBI alone, a full thickness BI alone, or sham treatment followed by RNA-sequencing of ipsilateral brain parenchyma conducted at six hours post-injury (hpi). Bioinformatic analysis of the RNA-sequencing results and subsequent pathway, overrepresentation, and protein-protein interaction analyses identified a large array of unique gene expression profiles specific to the combined mTBI/BI condition and identified gene-sets relevant to neuroinflammation, extracellular matrix organization and vascular integrity that are highly enriched within the data. Notably, significant, exclusive, upregulation of genes canonically expressed during mesenchymal-epithelial transition occurred within the brains of mTBI/BI subjects. Cumulatively, these data suggest that concomitant mTBI/BI results in a profound, combinatorial exacerbation of modality-specific disruptions in the blood-brain-barrier and neuro-vascular stability and may provide viable mechanism derived targets for pharmacotherapeutic development. Studies contained herein provide the first characterization of a synergistic effect of concomitant mTBI and BI on early transcription and inflammation within the CNS and afford insight into the specific molecular events involved in observed synergism. Further, our studies are anticipated to identify potential targets for the development of pharmacotherapeutics to improve neurologic outcomes in those subjected to polytrauma.

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