The independent prognostic value of global epigenetic alterations: An analysis of single-cell ATAC-seq of circulating leukocytes from trauma patients followed by validation in whole blood leukocyte transcriptomes across three etiologies of critical illness

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eBioMedicine 2022;76: 103860
Published online xxx
https://doi.org/10.1016/j.ebiom.2022.103860

Summary

Background While bulk and single cell transcriptomic patterns in circulating leukocytes from trauma patients have been reported, how these relate to changes in open chromatin patterns remain unstudied. Here, we investigated whether single-cell ATAC-seq would provide further resolution of transcriptomic patterns that align with patient outcomes.

Methods We performed scATAC-seq on peripheral blood mononuclear cells from four trauma patients at <4 h, 24 h, 72 h post-injury and four matched healthy controls, and extracted the features associated with the global epigenetic alterations. Three large-scale bulk transcriptomic datasets from trauma, burn and sepsis patients were used to validate the scATAC-seq derived signature, explore patient epigenetic heterogeneity (Epigenetic Groups: EG_hi vs. EG_lo), and associate patterns with clinical outcomes in critical illness.

Findings Patient subsets with gene expression patterns in blood leukocytes representative of a high global epigenetic signature (EG_hi) had worse outcomes across three etiologies of critical illness. EG_hi designation contributed independent of the known immune leukocyte transcriptomic responses to patient prognosis (Trauma: HR=0.62 [95% CI: 0.43−0.89, event set as recovery], p=0.01, n=167; Burns: HR=4.35 [95% CI: 0.816−23.2, event set as death], p=0.085, n=121; Sepsis: HR=1.60 [95% CI: 1.10−2.33, event set as death], p=0.013, n=479; Cox proportional hazards regression).

Interpretation The inclusion of gene expression patterns that associate with global epigenetic changes in circulating leukocytes improves the resolution of transcriptome-based patient classification in acute critical illnesses. Early detection of both the global epigenetic signature and the known immune transcriptomic patterns associates with the worse prognosis in trauma, burns and sepsis.

Keywords: Single-cell ATAC-seq; Trauma; Burns; Sepsis; Critical care; Patient classification

Introduction

The immune system states associated with and underlying the heterogeneity in clinical trajectories of acute critical illness remain to be elucidated. Transcriptional profiling of circulating leukocytes has been used to classify disease trajectories of critically ill patients. Gene array studies on human whole-blood[1] or PBMC[2]

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Research in context

Evidence before this study

Transcriptomic profiling of circulating immune cells has the potential to identify distinct patient subtypes that associate with differential outcomes in acute critical illness. A large-scale study using bulk blood leukocyte transcriptomes has revealed outcome associated patterns in trauma (Xiao et al. J Exp Med 2011). Previously, using signatures extracted from single-cell RNA-seq, we were able to identify two subgroups (SG1 and SG2) in whole blood leukocyte transcriptomes of trauma patients, which were also recapitulated in sepsis and burn patients (Chen et al. JCI insight 2021). Two major transcriptomic classifications in adult sepsis have been reported: SRS1-2 (Davenport et al. Lancet Respir Med 2016) and Mars1-4 endotypes (Scicluna et al. Lancet Respir Med 2017). Mars3 aligns well with SRS2, and Mars2 can be largely mapped to SRS1. However, as highlighted in the recent reviews (Stanski et al. Nat Rev Nephrol 2020, and Reddy et al. Lancet Respir Med 2020), unresolved differences between these studies have hampered a harmonized consensus on transcriptome-informed sepsis endotypes.

We hypothesized that epigenetic profiling using single-cell ATAC-seq would provide further resolution of the transcriptomic patterns that align with patient outcomes in trauma as well as in burns and sepsis. A search for scATAC-seq analyses on cells or tissues from patients with critical illness, covering any time prior to paper submission and using the following strategy "(((single cell) AND (ATAC)) OR (scATAC)) AND ((trauma) OR (burn) OR (sepsis) OR (septic shock) OR (hemorrhagic shock))" identified no scATAC-seq studies in trauma, burns or sepsis.

Added value of this study

We provide the first scATAC-seq study of immune cells from trauma patients revealing the global epigenetic alterations induced after severe systemic injury. These global changes were linked to unique transcriptomic patterns that allowed us to define epigenetic subgroups (EG subtypes) consistently associated with differential prognosis across three etiologies of critical illness. The prognostic value of EG subtypes were independent of the immune response (including inflammation, antigen presentation, and IFN signaling pathways)-associated transcriptomic subtypes we previously characterized in trauma and others have defined in sepsis using bulk leukocyte transcriptomes. The global epigenetic alterations in acute critical illness potentially involve multiple biological processes not specific to immune responses, such as (i) de-repression of polycomb targets (non-hematopoietic developmental genes), (ii) suppression of genes involved in DNA repair and (iii) dysregulation of genes related to RNA processing.

Implications of all the available evidence

The most important implication of these findings is that immune cell transcriptomic patterns can be dramatically impacted by not only focal epigenetic regulatory mechanisms that drive the established immune responses, but also through global epigenetic changes that potentially associate with the state of cellular stress and have a broad impact on diverse biological processes. Combining the transcriptomic patterns derived from the global epigenetic changes with the canonical immune response patterns adds a new level of resolution to transcriptomic patient subtyping in acute critical illness. Subgroups that have transcriptomic patterns in circulating immune cells that exhibit extreme deviation from steady state in both the focal and global epigenetic regulatory mechanisms have the worst outcomes.

revealed transcriptional patterns that associate with outcomes after severe injury. We previously performed single-cell RNA sequencing (scRNA-seq) on PBMC from trauma patients and showed that many of the changes observed in bulk leukocytes could be localized within the myeloid compartment, notably CD14+ monocytes.[3] We applied these gene signatures to the bulk leukocyte datasets to identify two patient transcriptomic subgroups (Signature Groups: SG1 and SG2) in trauma patients, where SG1 associated with worse outcomes and had a greater induction of pro-inflammatory genes and suppression of genes involved in MHC II and IFN signaling pathways. The two SG subtypes and the association with differential prognosis were recapitulated in burn and sepsis patients.[3]

Single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq)[4] provides genome-wide analysis of open (accessible) chromatin regions within individual cells derived from heterogenous populations. Currently, leukocyte scATAC profiles have not been analyzed in the context of immune dysfunction associated with acute critical illness. We postulated that epigenetic profiling using scATAC-seq would complement transcriptional profiling and generate novel genomic features for analyzing patient heterogeneity associated with outcomes in trauma as well as in burns and sepsis. Thus, we assessed the epigenomic changes that occurred over time in PBMC isolated from patients suffering severe trauma. scATAC-seq revealed not only the expected epigenetic patterns associated with known immune cell transcriptional responses in severely injured patients but importantly uncovered global chromatin alterations across major immune cell types. We developed a computational genomics strategy to extract the gene expression signature associated with the global epigenetic alterations identified by scATAC-seq. This "global epigenetic signature" was used to explore patient heterogeneity and the associated clinical outcomes by analyzing large-scale datasets of whole-blood leukocyte transcriptomes from trauma, burn and sepsis patients. We demonstrate that combining the newly defined transcriptomic patterns revealed by global epigenetic alterations (Epigenetic Groups: EG subtypes)

with the established immune response transcriptomic patterns (SG subtypes) provides superior resolution of patient heterogeneity in acute critical illness than EG or SG subtyping alone. Patients manifesting transcriptomic patterns in circulating immune cells that are reflective of the established immune responses as well as global epigenetic alterations have the worst outcomes.

Methods

Study design and participants

Patients suffering blunt or penetrating trauma that were admitted to the intensive care unit of UPMC Presbyterian University Hospital and suffering hypotension (systolic blood pressure <90 mmHg) or tachycardia (heart rate > 108) on admission were eligible for enrollment. Four patients were randomly selected from a larger cohort enrolled between December 2018 and April 2019. Blood samples were obtained within 4 h of injury and at 24 h and 72 h after injury. Demographic characteristics of the four patients are shown in Tables S2 and S3. The healthy controls were recruited based on matching age and sex of each enrolled patient. The raw scATAC-seq datasets in the FASTQ format with filtered peak/barcode matrix have been uploaded to the Gene Expression Omnibus (GEO) database (GSE175694).

Ethics

Trauma patients and healthy volunteers were enrolled in an observational study approved by the University of Pittsburgh Institutional Review Board (IRB protocol number: 19040329). Informed consent was obtained from all the subjects or next of kin.

Procedures

Human peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll centrifugation (Ficoll-Paque PLUS, Cat#17-1440-03, GE Healthcare). The isolated cells were cryopreserved and thawed for analysis according to the 10x Genomics protocol (CG00039_RevC). After thawing, the cells were resuspended in calcium and magnesium free buffer (PBS with 0.04% BSA) and immediately processed for nuclei isolation, strictly following the 10x Genomics protocol (CG000169_RevD). Chromium Next GEM Single Cell ATAC Library & Gel Bead Kit v1.1 (Cat#1000175, 10x Genomics), Chromium Next GEM Chip H Single Cell Kit (Cat#1000162, 10x Genomics) and Single Index Kit N Set A (Cat#1000212, 10x Genomics) were purchased for scATAC library preparation. The experimental steps strictly followed the 10x Genomics Next GEM single-cell ATAC kit v1.1 protocol (CG000209_RevD). Libraries were pair-end and dual-indexing sequenced on an Illumina NovaSeq 6000 in the UPMC Genome Center.

The BCL files generated by an Illumina sequencer were processed by the *Cell Ranger Atac* pipeline (v1.2.0) and mapped to GRCh38 human reference genome. The generated peak-by-barcode count matrix was processed using Signac[5] (R package, v1.0.0.9000) and Seurat[6] (R package, v3.2.0). We removed the cells that failed quality control (as described in the Supplementary methods), leading to an average of 3562.5 cells/sample (57,000 cells/ 16 samples) that passed quality control. Taking advantage of 15-state ChromHMM model,[7] we generated a new count matrix, the state-by-barcode count matrix, to evaluate global epigenetic alterations in CD14 + monocytes as described in the Supplementary methods. The cells fell into two groups of clusters displaying high vs. low global epigenetic alterations (Global_hi vs. Global_lo). Differential accessible peaks were identified between the two groups. The "global epigenetic signature" is a composite of the peaks that were more accessible in Global_hi. The peaks were annotated to the nearest genes using ChIPseeker[8] (R package, v1.20.0) yielding the genes used to derive the global epigenetic signature. We calculated the signature score of global epigenetic signature in the scATAC-seq data of other immune cell types.

We queried a published, large-scale, whole-blood leukocyte transcriptomic dataset from trauma patients (GSE36809). For biological interpretation of the global epigenetic signature, we extracted the genes that showed an intermediate to high correlation with the global epigenetic signature (Spearman's correlation: $|\rho| \geq 0.4$) and performed Gene Set Enrichment Analysis (GSEA).[9]

To explore epigenetic heterogeneity of trauma patients, we used the global epigenetic signature genes to cluster each patient at the first sampled time point using hierarchical clustering based on the method of Ward. The identified patient clusters were further annotated as EG_hi or EG_lo (Epigenetic Group: high vs. low) based on the expression level of the global epigenetic signature genes. We identified differential expressed genes (DEGs) between the two EG subtypes using limma (R package, v3.40.6) and then performed GSEA. Univariate and multivariate time-to-event analysis (event = recovery) were performed using survival (R package, v3.1.8).

We also explored the epigenetic heterogeneity in published, large-scale, whole-blood leukocyte transcriptomic datasets from burn (GSE37069) and sepsis (GSE65682) patients, two other major etiologies leading to critical illness. Considering the shared features of critical illness and potential unique features from the different etiologies, we used DEGs between two EG subtypes of trauma patients (top 2000 up + top 2000 down) as the pool of epigenetic associated genes. Then, the variable epigenetic associated genes were used to cluster burn and sepsis patients within the corresponding dataset using hierarchical clustering according to

the method of Ward. Similar to the analysis in trauma patients, the identified patient clusters were annotated as EG_hi or EG_lo. We also performed survival analysis and adjusted for potential covariates.

We illustrate the full analytic work flow in Figure 1 and summarize the major conclusions in a table of the results (Table S1).

Statistical analysis

Statistical analysis was performed under R 3.6.0. To identify the DA peaks between two clusters, we selected Signac[5] (R package, v1.0.0.9000) using logistic regression and adding the total number of fragments and experimental batches as latent variables. The detected DA peaks were adjusted by the Bonferroni method (default method by Signac) for multiple testing. For enrichment analysis, raw hypergeometric p values were corrected by Benjamini-Hochberg method using the p.adjust() function (method = 'BH') for multiple testing. The adjusted p value was considered statistically significant at < 0.05. For comparison between groups, categorical variables were quantified by Fisher's Exact Test, and the continuous variables were quantified by Wilcoxon's Rank-Sum Test, and two-sided p values were computed. Univariate time-to-event analysis was done by Kaplan-Meier analysis (log-rank test). Multivariate time-to-event analysis was performed by Cox proportional hazards regression. Age, sex, other variables unevenly distributed between the two EG subtypes, and the SG subtypes we previously defined were adjusted by Cox proportional hazards model. Sample size based on feasibility and available subjects based on past experience should provide sufficient provision. Post-hoc sample size justification for the analysis of each bulk dataset is provided in Supplementary methods. Blinding was not applicable since no specific grouping was involved in signature extraction.

Outcomes

The primary goal of this study was to characterize the trauma-induced epigenetic changes. We established the epigenetic heterogeneity and corresponding prognostic value for trauma patients. We also queried burn and sepsis patents, and found that high global epigenetic alterations (EG_hi) were consistently associated with poor prognosis across three major etiologies for critical illness.

Bias and confounding

When performing scATAC-seq analysis batch effects can lead to critical confounding bias if not controlled for appropriately. To minimize the influence of batch effects, we first processed and sequenced our samples in parallel batches. Four samples for each patient + healthy control (1-paired healthy control + 3-time points for each patient) were processed in parallel. For 4 patients, this means that 16 samples were processed in 4 parallel batches. That a single cluster of dendritic cells and single cluster of CD16+ monocytes were identified across the 16 samples in contrast to identification of multiple clusters of CD14+ monocytes after trauma further confirmed that the batch effects were minimal compared with the biological changes (Figures S2a–S2c). In addition, we always added batch as a latent variable when extracting DA peaks between clusters. We also expect a selection bias for each large-scale dataset, due to study-specific inclusive and exclusive criteria. However, since we queried three bulk datasets from different studies, we reasoned that our conclusions should be generalizable.

Role of funding source

This project was supported by an R35 grant from National Institutes of Health: 1R35GM127027-01 (T.B.). The funding source did not have any role in study design, data collection, data analyses, interpretation or writing of report.

Results

To characterize the accessible chromatin regions in circulating cells after severe injury, we isolated PBMCs from four trauma patients across three time points (<4 h, 24 h, 72 h) post-injury (Tables S2 and S3). These cells were analyzed in parallel with age and sex matched healthy controls yielding a total of 16 samples that were subjected to scATAC-seq with a total of ~57,000 cells passing quality control (see Supplementary methods for the filtering criteria) (Figure S1). The epigenomic profiles comprised of open chromatin regions revealed pronounced shifts over time after trauma that were distinguishable from their steady state counterparts (Figures S1b–S1d).

Given our previous scRNA-seq analyses of CD14+ monocytes in severely injured patients,[3] we first analyzed for corresponding chromatin alterations within this immune cell type using a peak-by-barcode matrix (Supplementary analysis and Figures S2–S5). We recapitulated our previous scRNA-seq findings in scATAC-seq. Specifically, the up-regulation of inflammatory genes and the down-regulation of MHC II pathway components and IFN signaling after trauma were largely reflected with changes in differentially accessible (DA) chromatin peaks associated with enhancers (Enh), active transcription start sites (TssA) or flanking active transcription start sites (TssAFlnk) (Figure S3d, annotated in blue). We also observed an increased ratio of DA peaks associated with bivalent histone modifications at Tss or Enh regions after trauma (Figure S3c, designated with red arrows). These bivalent regions were enriched for transcription factor binding site motifs for

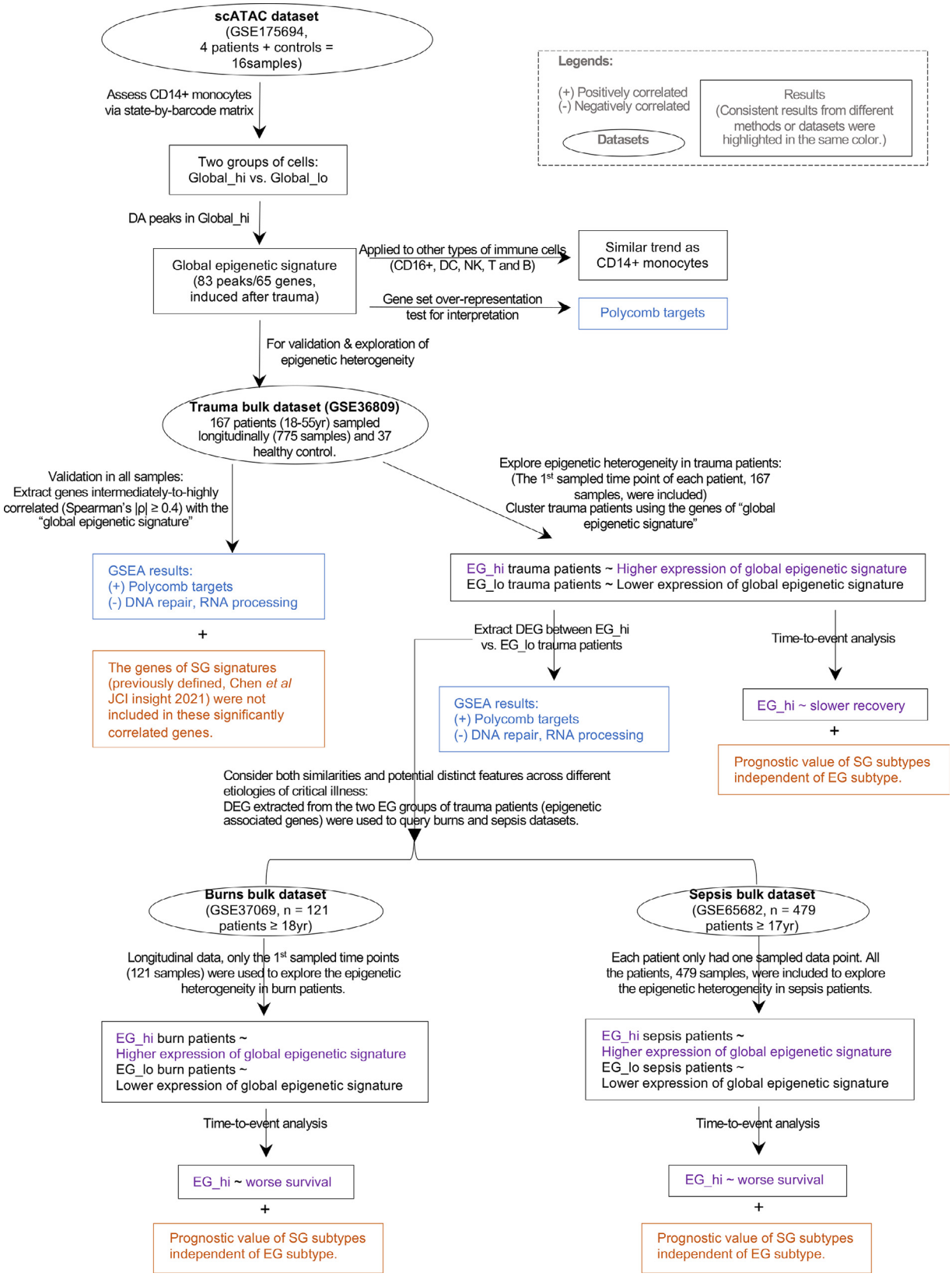

Figure 1. The whole work flow of this study.

the HOX, PAX and LMX families (Figure S3d, annotated in red), that encode embryonic regulators of developmental patterning.[10] Alterations of such developmental genes was not anticipated in mature immune cells. We hypothesized that this was due to extreme cellular stress caused by trauma and could be reflected by global epigenetic alterations.

To systematically evaluate the global epigenetic alterations within CD14+ monocytes (analysis depicted in Figure 2a), we took advantage of the chromHMM 15-state model (E124: the model for CD14+ monocytes).[7] A state-by-barcode matrix was generated by counting total cut sites for each of the 15 states in individual cells for all CD14+ monocytes. Seven clusters were identified based on the new count matrix (Figures 2b and S6a). These clusters are referred to as "State15_CC#" (Cell Cluster based on 15States) in order to distinguish the clusters from the peak-by-barcode matrix mentioned above. Cell cycle phases were evenly distributed across these seven clusters (Figure S6b). Hierarchical clustering demonstrated that the seven State15 clusters could be generally classified into two categories (Figure 2c). State15_CC4, 5, 2 and 1 aligned with the accessibility patterns in the reference epigenome. In contrast, State15_CC0, 3 and 6 exhibited greater accessibility in regions that were less accessible in the reference epigenome. This indicated that CD14+ monocytes represented by State15_CC0, 3 and 6 underwent higher global epigenetic alterations than cells from State15_CC4, 5, 2 and 1. Thus, State15_CC0, 3 and 6 were defined as "Global_hi", while State15_CC4, 5, 2 and 1 were designated as "Global_lo" (Figure 2d). Furthermore, the cells of Global_hi were prominent after trauma (Figure 2e). These results supported our hypothesis that trauma induced global epigenetic alterations in CD14+ monocytes.

Next, we sought to directly extract the features associated with the global epigenetic alterations by identifying the DA peaks between the cells of Global_hi vs. Global_lo (Figure 2a, Supplementary Spreadsheet 1). A total of 83 peaks were statistically significantly more accessible in the Global_hi cells, while no peaks were statistically significantly more accessible in the Global_lo cells. These 83 peaks were associated with 65 unique genes. Among MSigDB[11] (v5.2) C2 curated gene sets (Supplementary Spreadsheet 1), the top enriched gene sets ($p < 0.05$) associated with the global epigenetic signature largely involved polycomb targets (SUZ12 targets or domains with H3K27me3) and bivalent domains. Consistent with these targets, the top enriched GO terms were associated with neuron development or morphogenesis, which are known to involve developmental genes marked by bivalent domains and regulated by polycomb-group proteins (PcG).[10] We used these findings to define a "global epigenetic signature" that could be identified by assessing the either the 83 DA peaks or the 65 associated genes.

Because the genes associated with morphogenesis and neuron development would not be expected to be expressed in myeloid, or even in hematopoietic lineages, we next asked whether other types of immune cells underwent similar epigenetic changes after trauma. To assess this, we calculated the average accessibility of the global epigenetic signature (83 peaks) in other major circulating immune cell types using our scATAC-seq data. Notably, B cells, NK and T cells, DC and CD16+ monocytes also showed an increase in the accessibility of peaks representing the global epigenetic signature after trauma (Figure S7). Thus, the global epigenetic changes appear to be a generalized genomic feature reflected by major immune cell types in trauma patients. Independent analysis of global epigenetic alterations in T cells using the pan T cell E034 reference epigenome[7] also supported this conclusion (Supplementary analysis, Figure S8).

We next sought to validate the global epigenetic signature inferred from our profiling of chromatin states using transcriptional datasets. Since the global epigenetic alterations took place across the major circulating immune cell types after trauma, we reasoned that the transcriptional impact on the associated genes would be detectable within the bulk transcriptional profiles of whole-blood leukocytes. Thus, we queried a published, large-scale, whole-blood leukocyte transcriptomic dataset from trauma patients (n=167, Figure 3a).[1] A total of 37 of the 65 genes that comprised the global epigenetic signature were present in the trauma dataset. Compared with the healthy controls, the genes associated with the global epigenetic signature were clearly up-regulated after trauma. Furthermore, this up-regulation persisted in patients with a slow recovery based on unresolved organ dysfunction (time-to-recovery [TTR] $\geq$ 14 days) and was maintained at an even higher level in the patients that failed to recover within 28 days after injury (Figure 3b). To further interpret the global epigenetic alterations, we extracted the genes from this bulk dataset that were highly or intermediately correlated with the global epigenetic signature genes (Spearman correlation: $|\rho| > 0.4$) and performed GSEA (Gene Set Enrichment Analysis)[9] using the correlation coefficient as the rank (Figure 3a). As anticipated, the gene sets included polycomb and bivalent domain targets that were statistically significantly enriched in genes positively correlated with the global epigenetic signature (Figure 3e–3g, Supplementary Spreadsheet 2). Thus, the transcriptional signature derived from the trauma-induced global epigenetic alterations observed in single cell analysis (4 representative patients) was reflected in a bulk whole-blood leukocyte transcriptomic dataset (167 patients). In addition, we also observed enriched gene sets that were negatively correlated with the global epigenetic signature. These were associated with DNA repair and RNA processing (Supplementary Spreadsheet 2), indicating that the global epigenetic alterations

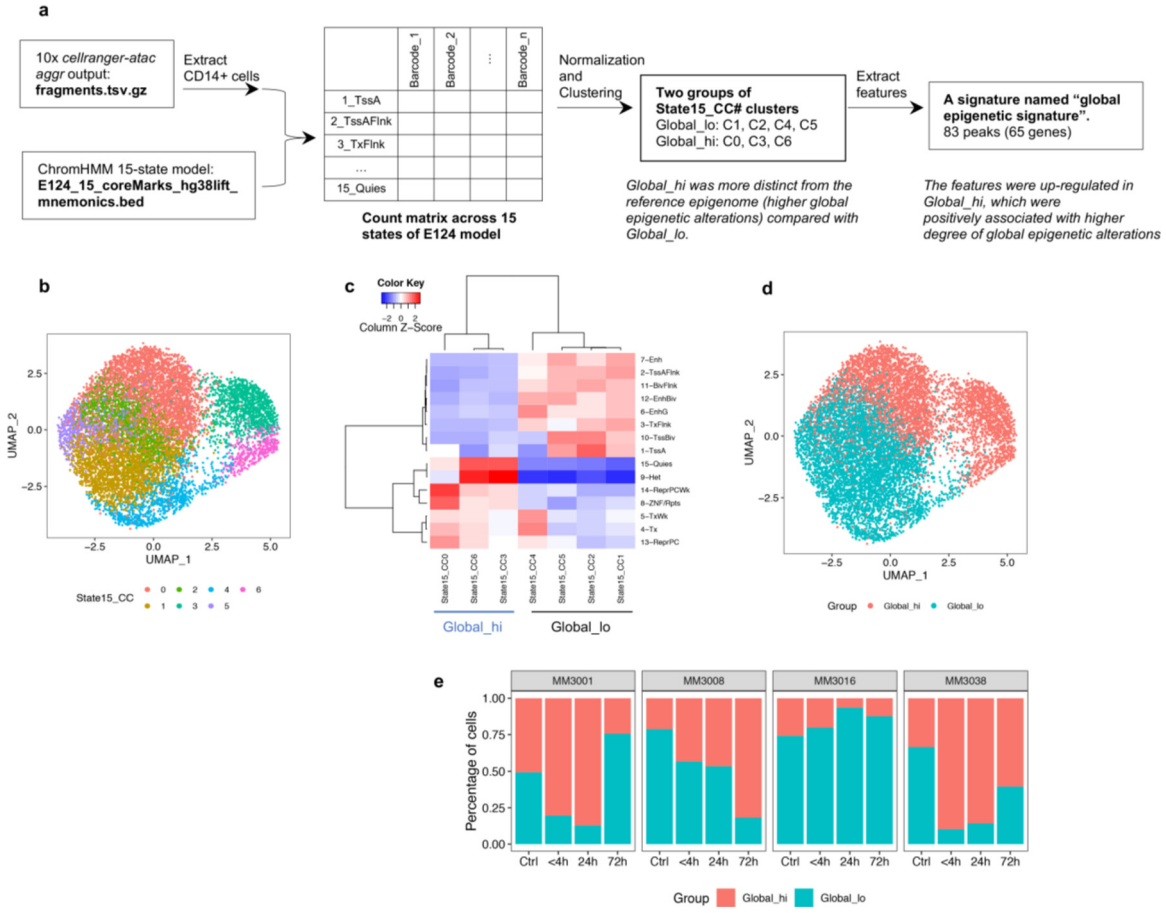

Figure 2. Characterization of global epigenetic changes across ChromHMM 15 states in CD14+ monocytes.

(a) Schematic of workflow of how the state-by-barcode count matrix was generated and the global epigenetic signature was extracted, *n*=4 patients (16 samples) (b) UMAP was performed and color coded by the cell clusters identified based on the state-by-barcode matrix. These clusters are referred to as "State15_CC#". (c) Hierarchical clustering of the State15_CC# clusters shown in (b), revealing two distinct groups of clusters showing high vs. low global epigenetic alterations: Global_hi vs. Global_lo. (d) UMAP plot as shown in (b) was color coded by the two groups revealed in (c). (e) The changes in cell composition in each of the 4 patients at each sampled time point.

could be involved in multiple biological processes including, de-repression of polycomb targets (non-hematopoietic developmental genes), deficiency in DNA repair, and dysregulation of RNA processing. Importantly, no genes from the previously identified SG transcriptomic signatures[3] had a Spearman correlation coefficient $|\rho| > 0.4$ (Figure 3c, 3d), indicating that the chromatin alterations revealed by our epigenetic profiling yielded a new set of actionable trauma-induced gene expression changes.

Next, we sought to determine if we could identify patient heterogeneity based on the genes associated with the global epigenetic response to systemic injury. Using the trauma bulk gene array from the 167 severely injured patients referenced above[1], we extracted the global epigenetic signature genes to cluster the patients. This was done based on 37 genes from the global epigenetic signature that were present in the trauma bulk gene array data. Based on the first timepoint (~12 h after injury), patients were clustered into three groups (T1–T3) (Figure 4a, Supplementary Spreadsheet 3). T3 patients showed obviously higher expression of the global epigenetic signature compared with T2 and T1. Thus, T3 patients were annotated as EG_hi, and T1 and T2 patients were annotated as EG_lo (Figure 4b). EG_hi trauma patients also tended to have a persistent up-regulation of the global epigenetic signature along the 28-day timeline (Figure S9).

To further analyze the transcriptomic profiles between trauma epigenetic subtype EG_hi vs. EG_lo, we identified the DEGs between these two groups of patients and performed GSEA (Figure S10a). The genes up-regulated in EG_hi trauma patients were largely associated with bivalent domains and polycomb targets

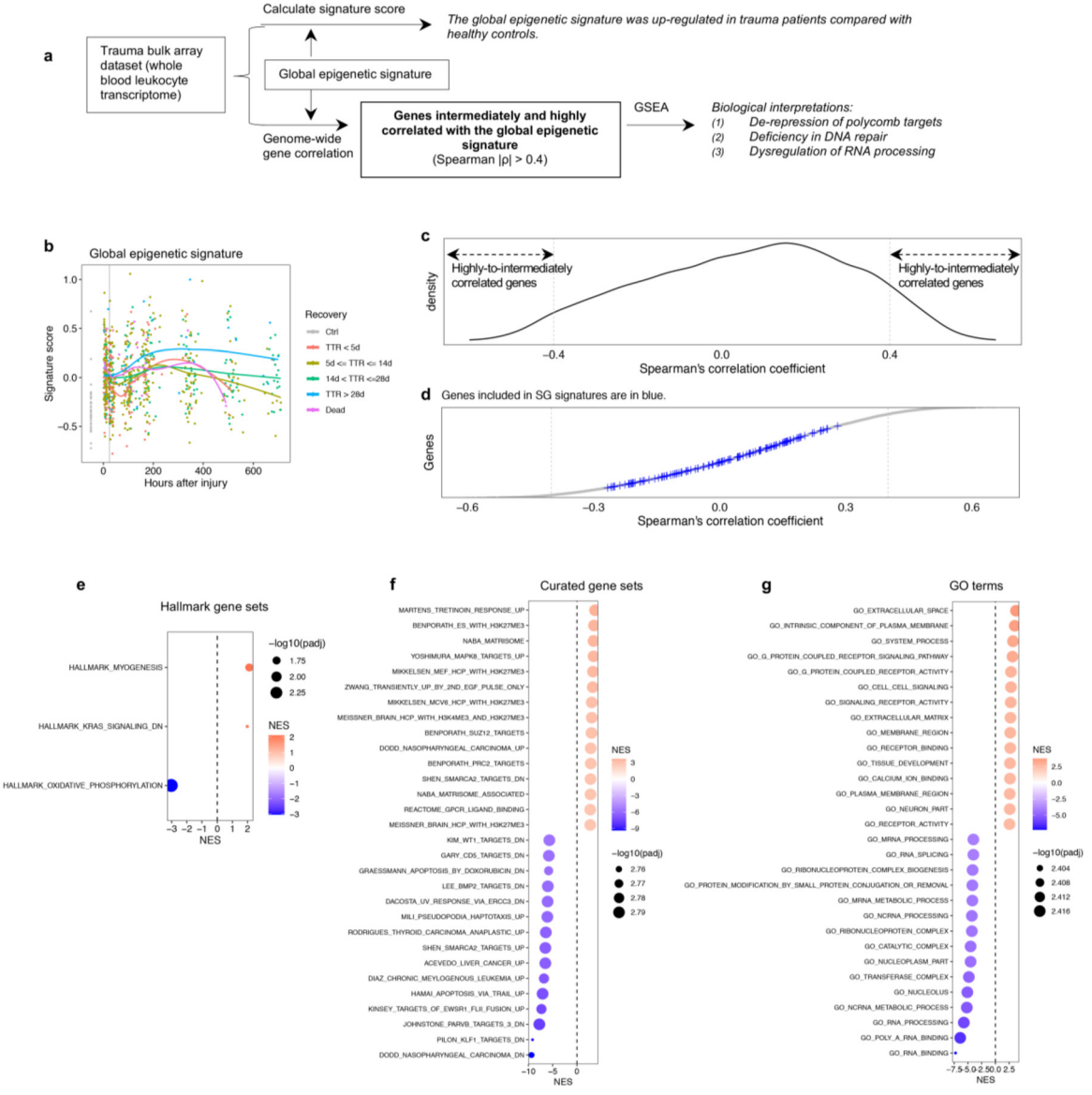

Figure 3. Validation of the global epigenetic signature in trauma bulk microarray data.

(a) Schematic of the workflow of the analysis shown in this figure, including 167 trauma patients sampled longitudinally (775 samples) and 37 healthy controls. (b) The changes in global epigenetic signature scores after trauma, color coded by different clinical trajectories (TTR: Time-To-Recovery). Curves were fitted by Loess regression. (c,d) Spearman's correlation coefficients were computed for a genome-wide gene correlation with the global epigenetic signature score. Density plot of correlation coefficients ρ were plotted in (c). $|\rho|= 0.4$ was labeled as the vertical dashed lines. The genes from the CD14+ signatures we previously used to define SG subtypes (called SG signatures and largely associated with inflammation, MHC II expression and IFN signaling) were color coded in blue as shown in (d). (e−g) GSEA results of hallmark gene sets (e), curated gene sets (f) and GO terms (g) (MSigDB gene sets v5.2) using the highly-to-intermediately correlated genes identified above ($|\rho| > 0.4$). The enrichment p value was computed by fgsea (R package) with 10,000 permutations, and corrected by Benjamini-Hochberg method for multiple testing. The statistically significantly enriched pathways were shown (adjusted p-value < 0.05) and sorted by normalized enrichment scores (NES). NES > 0 indicates that the enriched gene set is positively associated with global epigenetic signature, and NES < 0 indicates a negative correlation. If the number of significantly enriched pathways was more than 15, only the top 15 pathways were shown in the figure.

(targets of PRC2, EED, SUZ12 or domains with H3K27me3) (Figure S10b−S10d, Supplementary Spreadsheet 4). The genes down-regulated in EG_hi trauma patients were generally enriched in RNA processing and DNA repair. This was consistent with the GSEA analysis described above using the genes found

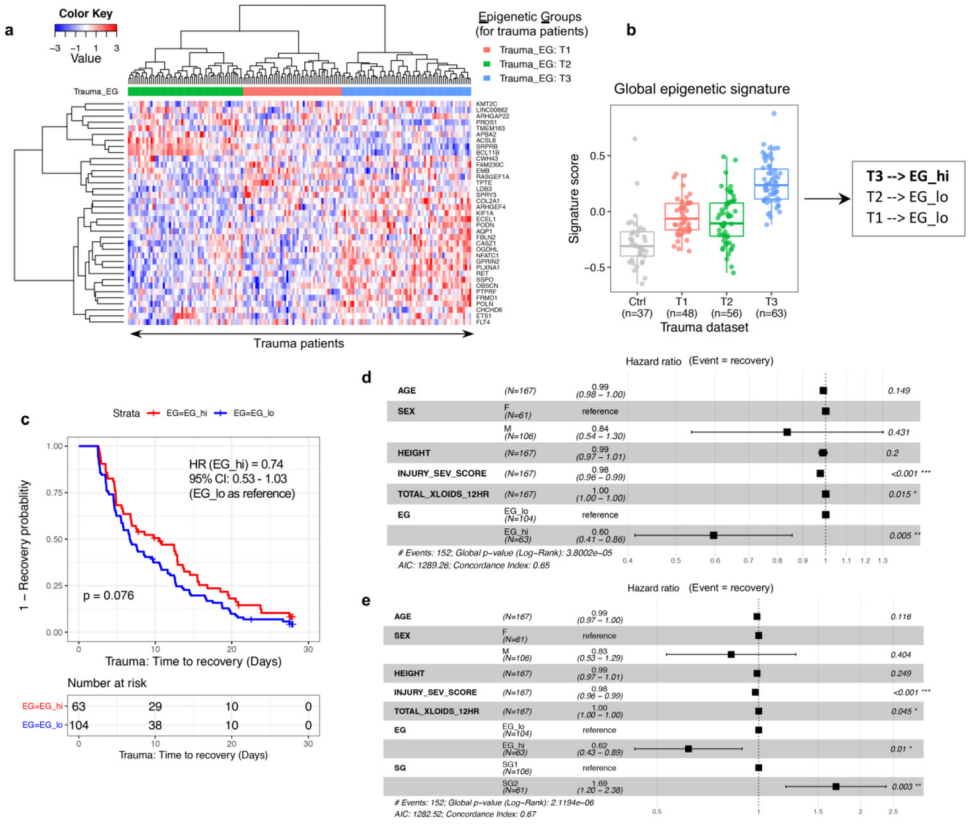

Figure 4. Epigenetic subtypes and their prognostic value in trauma patients.

(a) Hierarchical clustering of trauma patients by global epigenetic signature genes with an available expression value in the trauma dataset. The 1st sampled time point (~12 h of injury) for each patient (n = 167 samples) was extracted for the clustering analysis. Three distinct clusters of trauma patients (Epigenetic groups: T1–T3) were observed. (b) Identified patient clusters were further annotated as EG_hi or EG_lo, based on the global epigenetic signature scores. The boxes span from the Q1 to the Q3, with the centerline showing the median. Lower whiskers represent Q1 - 1.5*IQR, and upper whiskers represent Q3 + 1.5*IQR (Q1: the first quantile, Q3: the third quantile, IQR = Q3 - Q1). (c–e). Time-to-event analysis between EG_hi vs. EG_lo trauma patients. The event was set as recovery (absence of organ dysfunction). (c) Univariant analysis by Kaplan-Meier estimate. Log-rank p value is shown. (d) Multivariant analysis using Cox model to adjust potential co-variants of EGs. (e) Multivariant analysis using Cox model to further adjust for SG subtype designations.

to highly correlate with the global epigenetic signature (Figure 3e–g, Supplementary Spreadsheet 2) and the gene set over-representation test directly using global epigenetic signature genes (Supplementary Spreadsheet 1). Therefore, the 37 gene subset derived from the published bulk leukocyte gene array data are likely to be representative of the global epigenetic alterations and sufficient to define epigenetic subtypes in trauma patients.

We next compared clinical outcomes between the EG_hi versus the EG_lo trauma patients. EG_hi trauma patients showed a trend towards slower recovery (Kaplan-Meier analysis: log-rank p value = 0.076, Hazard Ratio [HR] = 0.74 [95%CI: 0.53–1.03], Figure 4c), compared with EG_lo patients. Sex, height, ISS (Injury Severe Score) and total crystalloids received within 12 h after injury (TOTAL_XLOIDS_12HR, a marker of worse disease state) were statistically significantly and differentially distributed between EG_hi vs. EG_lo (Table S4). After adjusting for age, sex and the other unevenly distributed co-variants between two EG groups, the p value for EG classification became statistically significant (Cox regression: p = 0.005, HR = 0.6 [0.41–0.86] Figure 4d). We then added the SG designation,[3] the transcriptomic subtypes that define the well-established immune response transcriptomic patterns in myeloid cells after trauma, into the Cox model. Both SG1 (more pronounced immune response) and EG_hi were statistically significantly and independently associated with slower recovery (EG_hi: p = 0.01, HR = 0.62 [0.43–0.89]; SG2: p = 0.003, HR = 1.69 [1.20–2.38], Cox regression, Figure 4e). This analysis suggests there are two distinct mechanisms that contribute to the differential prognosis following systemic injury that are reflected by the SG and EG subtypes.

We have previously shown that the SG subtypes and their association with differential prognosis can be recapitulated using leukocyte transcriptomic datasets from burn and sepsis patients.[3] To determine if our findings on global epigenetic heterogeneity in trauma could also be identified in burn and sepsis patients, we first sought to use the global epigenetic signature genes that were used to cluster trauma patients to cluster burn and sepsis patients using published gene array datasets (Burns: GSE37069; Sepsis: GSE65682). However, the patient clusters based on these signature genes did not show an obvious association with survival, the outcome endpoint provided in these studies. This led us to hypothesize that while these other etiologies for acute critical illness are likely to be influenced by global epigenetic processes, the gene co-expression patterns may vary by etiology. Hence, the limited number of genes in the global epigenetic signature derived from trauma patients may not be sufficient to define the full profile of heterogeneity for other etiologies. Therefore, we took two steps to identify epigenetic-associated genes for clustering burn and sepsis patients. In step 1, we extracted the top 2000 up-regulated and top 2000 down-regulated genes in EG_hi vs. EG_lo trauma patients to obtain a broad set of initial features of potential relevance. These 4000 top DEGs are displayed by heatmap and is comprised of three Gene Clusters (GC), including: Trauma_GC3 (highly expressed in EG_hi trauma patients), Trauma_GC2 (markedly suppressed in EG_hi trauma patients) and a small fraction of relatively less variable genes (Trauma_GC1) (Figure S10e). In step 2, we used only the variable genes (standard deviation of scaled expression $\geq$ 0.5 among the analyzed samples) from the top 4000 DEGs to cluster the burn and sepsis patients.

Using a longitudinal burn whole blood transcriptomic dataset we extracted the 1st time point from all adult burn patients ($\geq$ 18 y, n = 121). Based on the two steps described above, 1482 genes were used to cluster burn patients (Figure 5). There were three easily distinguishable subgroups of burn patients (B1–B3) (Figure 5a). B2 was the only subgroup with a global epigenetic signature higher than healthy controls (Figure 5b). Thus, B2 patients were annotated as EG_hi, and B1 and B3 were annotated as EG_lo (Supplementary Spreadsheet 5). EG_hi burn patients had worse survival (K-M analysis: log-rank p = 0.019, HR = 4.31 [1.14–16.52], Figure 5d). For co-variants, BAUXSCORE and MAX_DENVER_2_SCORE (two burn and trauma severity scores) were unevenly distributed between EG_hi vs. EG_lo (Table S5). After adjusting for age, sex, the unevenly distributed co-variants and SG classification, EG_hi burn patients still showed a trend towards worse survival compared with the other burn patients (Cox regression: p = 0.085, HR = 4.35 [0.816–23.2], Figure 5e,f). Furthermore, the gene expression profile of EG_hi burn patients (gene cluster: Burn_GC2, Figure 5a and Supplementary Spreadsheet 6) can be generally mapped to EG_hi trauma patients (gene cluster: Trauma_GC3) (Figure 5c). Unlike the adult blunt trauma group, the complete burn dataset contained a large number of young children and infants (n = 120). The prognostic value of the EG subtypes showed a different pattern between the patients $\geq$ 18 y vs. <18 y (Figure S11). In contrast, the prognostic value of SG subtype designation exhibited a similar pattern between the two burn patient age groups. This finding suggests a lower influence of global epigenetic alterations on the outcomes of young children after burns.

The sepsis dataset[12] contained a single sampled time point for each patient within 24 h of ICU admission. All but one patient in this dataset were $\geq$ 18 y (one patient was 17 y) and we included all patients from both the discovery and validation cohorts, for a total of 479 patients (Figure 6). After applying our two-step process, 976 genes were used to cluster these 479 patients. Sepsis patients were generally clustered into three epigenetic groups (S1–S3, Figure 6a). S3 patients were the only subgroup with a global epigenetic signature higher than healthy controls. Thus, S3 patients were annotated as EG_hi, and S1 and S2 patients were annotated as EG_lo (Figure 6b, Supplementary Spreadsheet 5). Consistent with the burn and trauma findings, EG_hi sepsis patients also experienced worse survival compared to the other sepsis patients (K-M analysis: log-rank p = 0.012, HR = 1.6 [1.11–2.33], Figure 6d). No baseline variables were unevenly distributed between the two EG groups (Table S6). Thus, after adjusting for age, sex and SG classification by Cox model, the EG_hi sepsis subtype still statistically significantly associated with worse survival (Cox regression: p = 0.013, HR = 1.60 [1.10–2.33], Figure 6e,f).

EG_hi sepsis patients highly expressed the gene cluster Sepsis_GC1 (Figure 6a and Supplementary Spreadsheet 6). Surprisingly, Sepsis_GC1 was more enriched in genes found in Trauma_GC1 (relatively low variable DEG in trauma) compared with Trauma_GC3 (highest expression in EG_hi trauma patients) (Figure 6c). This indicates that sepsis patients exhibit a down-stream co-expression pattern somewhat distinct from trauma and that a single cell epigenomic analysis is warranted to identify the sepsis-specific epigenetic changes. Despite these differences, the GSEA based on the DEGs between sepsis EG_hi and EG_lo (Figure S12, Supplementary Spreadsheet 7) generally showed a pattern similar to the results obtained between the two EG groups in trauma patients (Figure S10b–S10d). Thus, by querying three large-scale bulk transcriptomic datasets, we were able to demonstrate the global epigenetic alterations based on EG subtypes, as a shared prognostic factor across different etiologies of critical illness.

As a proof-of-concept analysis, we performed LASSO regression to extract a group of genes as a classifier to distinguish EG_hi vs. EG_lo trauma patients. We randomly sampled the 167 patients into training set (80%)

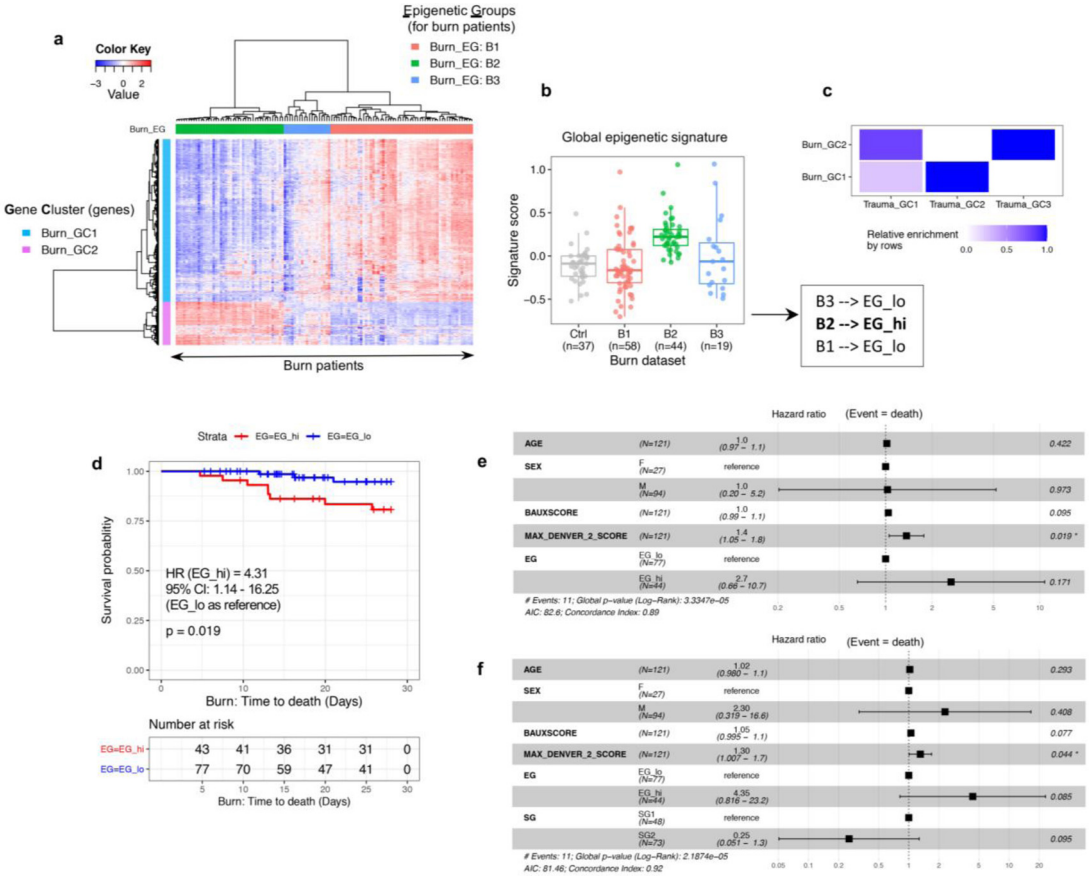

Figure 5. Epigenetic subtypes and their prognostic value in burn patients.

(a) Epigenetic subtypes in adult burn patients. The 1st sampled time point for each patient was extracted for the clustering analysis. The top 2000 up and top 2000 down DEGs between trauma EG_hi and EG_lo patients that showed standard deviation of scaled expression $\geq$ 0.5 among the 121 burn samples were used to cluster burn patients (Epigenetic groups: B1−B3). These genes largely fell into two gene clusters Burn_GC1-GC2. (b) Identified burn patient clusters were further annotated as EG_hi or EG_lo, based on the global epigenetic signature scores. The boxes span from the Q1 to the Q3, with the centerline showing the median. Lower whiskers represent Q1 - 1.5*IQR, and upper whiskers represent Q3 + 1.5*IQR (Q1: the first quantile, Q3: the third quantile, IQR = Q3 - Q1). (c) Mapping of gene clusters (Burn_GC#) derived from burn patients to those identified in trauma patients (Trauma_GC#). Fold enrichment was computed between each Burn_GC# and each Trauma_GC# and then scaled between 0 to 1 for each Burn_GC#. (d−f) Survival analysis between EG_hi vs. EG_lo burn patients. (d) Univariant analysis by Kaplan-Meier estimate. Log-rank p value is shown. (e) Multivariant analysis using Cox model to adjust potential co-variants. (f) Multivariant analysis using Cox model to further adjust for SG subtype designation.

and test set (20%). A total of 40 genes were selected using the training set and these achieved an accuracy of 0.97 in the test set (Figure S13). This analysis demonstrates the feasibility of classifying EG subsets by assessing the expression levels of a small set of genes in blood samples. The classifier may need to be tuned based on the transcriptomic platform and it will be necessary to establish etiology-specific classifiers for burn and sepsis patients.

Discussion

This study characterized the open chromatin patterns using scATAC-seq on PBMC from humans undergoing the acute systemic stress of trauma. Using standard ATAC seq analytic methods combined with the ChromHMM 15-state epigenome model[7] within CD14+ monocytes, we show that known changes in transcriptomic patterns associated with the immune dysfunction of critical illness largely relate to established focal changes in active chromatin states (TssA/TssAFlnk/Enh) of the reference monocyte epigenome (Figure S3d). Unexpectedly, we also found that trauma induced global epigenetic alterations and that these changes were observed across major immune cell types. The transcriptomic patterns with higher expression of the global epigenetic signature extracted from the scATAC-seq data was associated with worse outcomes in trauma

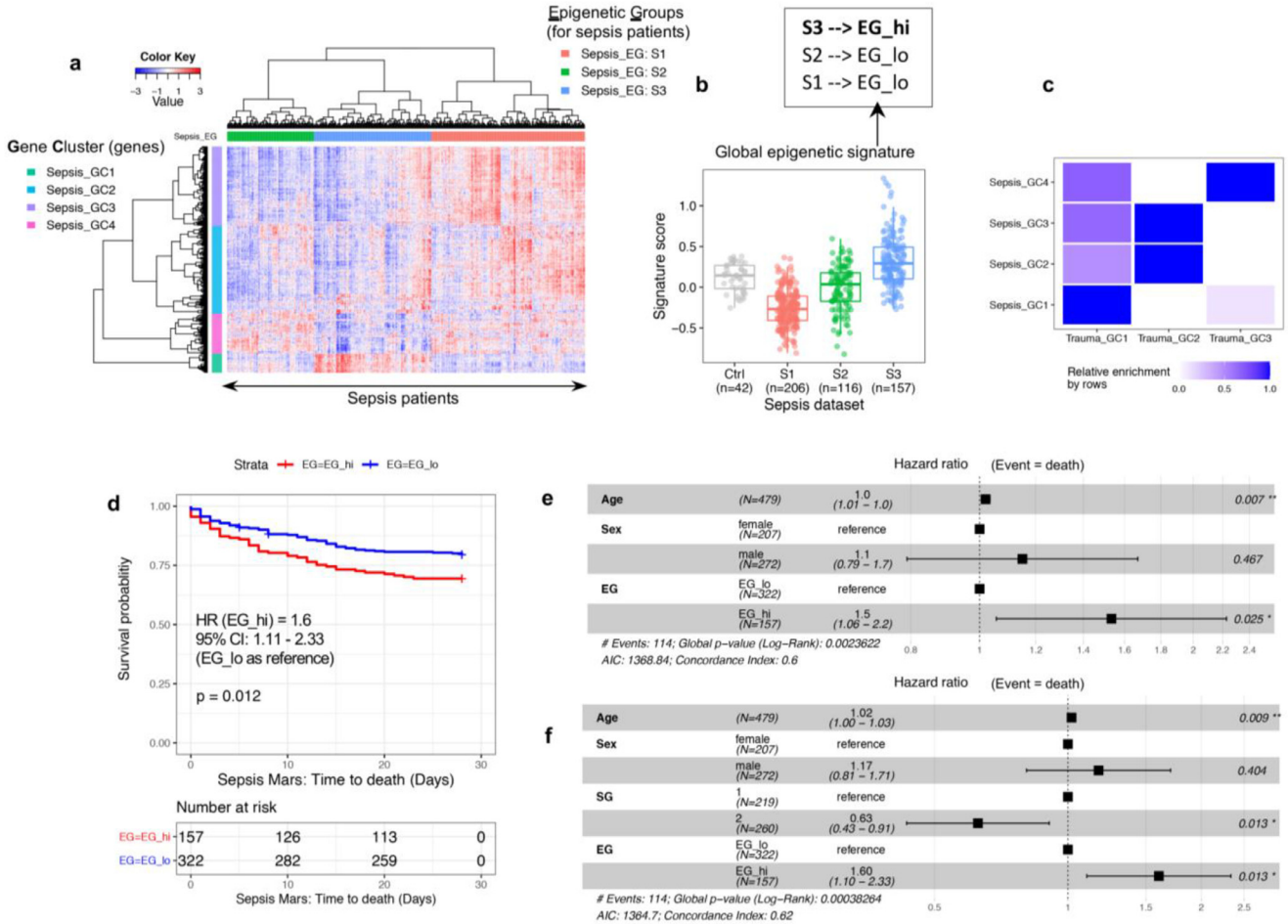

Figure 6. Epigenetic subtypes and their prognostic value in sepsis patients.

(a) Epigenetic subtypes in sepsis patients. The top 2000 up and top 2000 down DEGs between trauma EG_hi and EG_lo patients that also showed standard deviation of scaled expression $\geq$ 0.5 among the analyzed sepsis samples were used to cluster sepsis patients (Epigenetic groups: S1−S3). These genes fell into four gene clusters Sepsis_GC1-GC4. (b) Identified sepsis patient clusters were further annotated as EG_hi or EG_lo, based on the global epigenetic signature scores. The boxes span from the Q1 to the Q3, with the centerline showing the median. Lower whiskers represent Q1 - 1.5*IQR, and upper whiskers represent Q3 + 1.5*IQR (Q1: the first quantile, Q3: the third quantile, IQR = Q3 - Q1). (c) Mapping of gene clusters (Sepsis_GC#) derived from sepsis patients to those identified in trauma patients (Trauma_GC#). Fold enrichment was computed between each Sepsis_GC# and each Trauma_GC# and then scaled between 0 to 1 for each Sepsis_GC#. (d-f) Survival analysis between EG_hi vs. EG_lo sepsis patients. (d) Univariant analysis by Kaplan-Meier estimate. Log-rank p value is shown. (e) Multivariant analysis using Cox model to adjust potential co-variants. (f) Multivariant analysis using Cox model to further adjust SG subtype designations.

patients, a finding recapitulated in independent transcriptomic datasets from burn and sepsis patients. Thus, global epigenomic changes represent a previously unrecognized contributor to transcriptomic patterns in acute critical illness.

From our previous studies[3] and the studies of others[13,14] using single cell RNA sequencing of PBMC from critically ill patients, a common finding across trauma and sepsis is the up-regulation of pro-inflammatory genes and suppression of genes associated with MHC and interferon signaling in myeloid cells. We previously characterized two patient subtypes referred to as SG1 or SG2[3] that are distinguished by the magnitude in these prototypic changes. SG1 designation (higher magnitude changes) early in the clinical course aligned with delayed recovery after trauma, and higher mortality in burns and sepsis. In this scATAC study, we go beyond the information captured by single cell transcriptomics to identify EG (epigenetic group) subtypes representing different degrees of global epigenetic alterations and the associated transcriptomic features. The patients with the highest levels of global epigenetic alterations (referred as EG_hi) in whole blood leukocytes were associated with the worst prognosis across the three etiologies of critical illness. Furthermore, the designation of EG subtype had prognostic value independent of categorizing patients based on SG subtypes.

The well-described inflammatory and immune suppressive transcriptomic changes during acute critical illness largely associate with focal epigenetic changes regulating immune response-associated genes, mainly involving Tss or Enh regions. By comparison, the transcriptomic changes that associate with the global epigenetic alterations associated with several biological processes not known to be associated with immune responses, including de-repression of polycomb targets, and suppression of genes involved in DNA repair, and RNA processing. Polycomb group proteins are a well-characterized system for controlling developmental genes. Polycomb repressive complexes 2 (PRC2) catalyzes the methylation of histone H3K27 and facilitate the binding of PRC1, which is associated with gene silencing and chromatin compaction.[15] The aberrant de-repression of polycomb targets caused by acute stress of trauma, sepsis or burns may interfere with immune cell functions. Deficient DNA repair could also contribute to dysfunctional immune responses due to a failure to rescue activated cells from DNA damage.[16] Non-coding RNAs (ncRNA) act as vital epigenetic mediators to coordinate gene expression.[17] The factors and molecular mechanisms that drive dysregulation of epigenetic regulators during acute cellular stress and the ensuing alterations in gene expression remain to be elucidated.

Transcriptomic subtypes based on bulk mRNA analysis of whole blood leukocytes have been characterized in adult sepsis patients, yielding two major patient classifications: SRS1-2[18] and Mars1-4.[12] The Mars3 transcriptomic pattern align well with SRS2, and Mars2 can be largely mapped to SRS1 (2nd and 3rd bar in the original Figure S7D of Mars study[12]). The SRS study demonstrated that SRS1 designation was associated with a worse prognosis than SRS2. The Mars study demonstrated that the Mars1 endotype consistently associated with poor prognosis, while classification as Mars2-4 was not as clinically useful for prognosis. However, 60–70% Mars1 patients were classified as SRS2 rather than SRS1 (1st bar in original Figure S7D of Mars study[12]). We had demonstrated in our previous report the following[3]: (i) SG and SRS classifications largely overlap (SG1 ≈ SRS1, SG2 ≈ SRS2); (ii) three out of four Mars subtypes can be generally mapped to SG subtypes (Mars2 ≈ SG1, Mar3 & 4 ≈ SG2); (iii) Mars1 has characteristics distinct from SG or SRS subtyping. Taken together, this indicates that classification of sepsis patient heterogeneity based on leukocyte transcriptomic patterns has significantly advanced the field but has not led a complete consensus picture. Mars1 patients were reported to exhibit a pronounced decrease in both innate and adaptive immune responses with a selective increase in genes associated with heme metabolism.[12] Our examination of the relationship between Mars endotypes and the epigenetic subtypes defined in the current study found that the majority of Mars1 patients would be classified as EG_hi sepsis patients (Figure S14). This raises the possibility that Mars1 is distinguished from other sepsis outcome groups, in part, by greater global epigenetic alterations.

Some consistencies emerge from our previous and current analyses of the transcriptomic data from leukocytes derived from trauma, burn and sepsis patients. These include the following: (i) For SG subtypes, SG1 (high inflammation and suppressed MHC II and IFN signaling) always associates with worse prognosis; (ii) For EG subtypes, EG_hi associates with worse prognosis; (iii) The prognostic value of EG or SG designation are independent. We also note that the co-occurrence relationship between SG1 and EG_hi did not show a consistent pattern across these three datasets (Trauma: OR = 2.52 [95%CI: 1.21–5.49], p = 0.008; Burn: OR = 0.9 [95%CI: 0.41–2.13], p = 1; Sepsis: OR = 0.77 [95%CI: 0.51–1.15], p = 0.204, Figure S15, two-sided Fisher's exact test). This supports the notion that the factors that drive the pathologic SG and EG gene signatures are part of distinct biologic processes. Differential global epigenetic heterogeneity, which reflects processes distinct from inflammation, may also be a factor in the failure of trials targeting the inflammatory response in human sepsis.[19]

There are limitations to our study. First, the scATAC cohort was derived from a limited number of trauma patients with differences in cell viability (13 samples with viability > 80% and 3 samples with viability 65–80%). However, we were able to characterize and confirm the single-cell derived signatures in whole

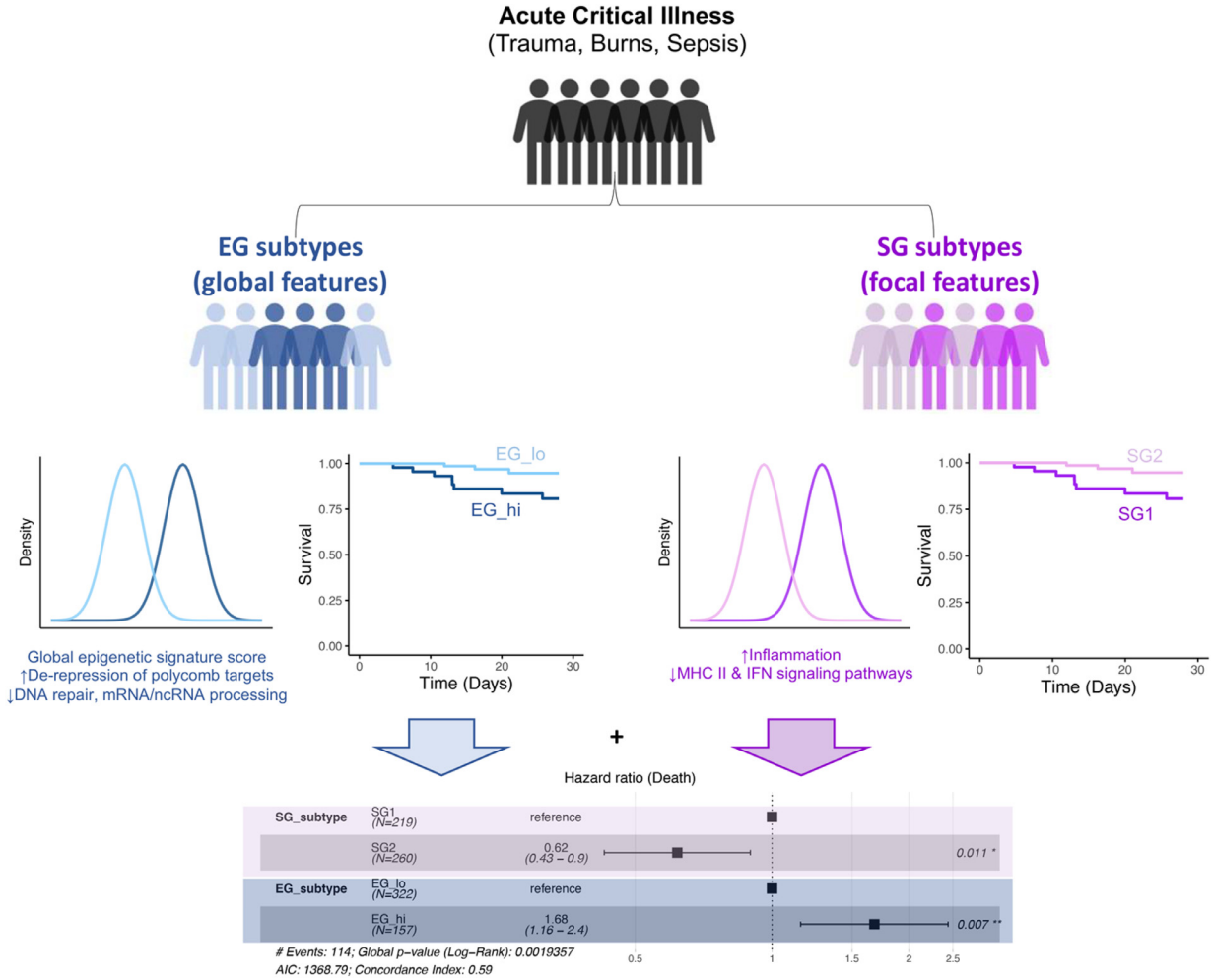

Figure 7. Proposed new model for transcriptome-based patient classifications during critical illness. Schematic depicts the independent contribution of the EG and SG designation based on blood leukocyte transcriptomic patterns measured during acute critical illness.

blood leukocyte transcriptomes in hundreds of patients across three etiologies of critical illness, demonstrating the reproducibility of our findings. Second, it will be necessary to generate scATAC-seq data from cells derived from burn and sepsis patients to directly extract the specific global epigenetic features and to confirm and refine the patient classification for each etiology. Last, even though our study highlighted the potential prognostic value of assessing epigenetic heterogeneity, the underlying biological processes still need to be elucidated.

By introducing epigenetic features into the patient classification systems in acute critical illness we provide evidence that the magnitude of global epigenetic alterations is an important contributor to patient heterogeneity. We propose a new model for patient classification in critical illness that includes epigenetic features shown in Figure 7. The combination of the transcriptomic patterns derived from the global epigenetic alterations (EG subtypes) with the focal regulatory mechanisms driving canonical immune response patterns (SG subtypes) adds a new level of resolution to transcriptomic patient subtyping in acute critical illness. Subgroups that have transcriptomic patterns in circulating immune cells that exhibit extreme deviation from steady state in both the focal and global epigenetic regulatory mechanisms have the worst outcomes.

Declaration of interests

Y.V. is a field chief editor of Frontiers in Systems Biology. Y.V. is also a co-founder of, and stakeholder in Immunetrics, Inc. The other authors declare no competing interests.

Acknowledgments

This research was supported in part by the University of Pittsburgh Center for Research Computing through the resources provided. We specifically acknowledge the assistance of Dr. Fangping Mu. We thank UPMC Genome Center for the support of sequencing. This project was supported by an R35 grant from National Institutes of Health: 1R35GM127027-01 (T.B.).

Contributors

T.B., H.S. and T.C conceptualized the study. T.C. performed all computational analyses. J.C. prepared all scATAC libraries. X.W. and T.C. performed resampling analysis. X.W. generated the classifier to distinguish EG_hi vs. EG_lo trauma patients. R.A. and Y.V. organized collection of human samples and the clinical database. M.S. extract the clinical information for the patients subjected to scATAC-seq. T.C., T.B. and H.S. interpreted the data and wrote the manuscript with the feedback of all of the authors who have read and approved the manuscript. W.C. supervised the computational analyses. T.B. and H.S. supervised the project. T. B. is responsible for funding. T.C., X.W., W.C., H.S. and T.B. have verified the underlying data.

Data sharing statement

The raw scATAC-seq datasets in the FASTQ format with filtered peak/barcode matrix have been uploaded to the Gene Expression Omnibus (GEO) database: GSE175694. The analyzed published datasets can be accessed via GEO (Trauma bulk data: GSE36809, Burn bulk data: GSE37069, Sepsis bulk data: GSE65682). To access the clinical information for trauma and burn data, please contact the lead author of the original paper (*Xiao et al. J Exp Med 2011*). The processing of scATAC peak-by-barcode matrix, hierarchical clustering and survival analysis were performed using available R packages as mentioned in the methods. The customized code (including generating and analyzing state-by-barcode matrix, chromatin state enrichment analysis) has been uploaded to https://github.com/TChen0730/scATAC-paper-2022.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103860.

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