Development of a predictive algorithm for patient survival after traumatic injury using a five analyte blood panel

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ABSTRACT

Severe trauma can induce systemic inflammation but also immunosuppression, which makes understanding the immune response of trauma patients critical for therapeutic development and treatment approaches. By evaluating the levels of 59 proteins in the plasma of 50 healthy volunteers and 1000 trauma patients across five trauma centers in the United States, we identified 6 novel changes in immune proteins after traumatic injury and further new variations by sex, age, trauma type, comorbidities, and developed a new equation for prediction of patient survival. Blood was collected at the time of arrival at Level 1 trauma centers and patients were stratified based on trauma level, tissues injured, and injury types. Trauma patients had significantly upregulated proteins associated with immune activation (IL-23, MIP-5), immunosuppression (IL-10) and pleiotropic cytokines (IL-29, IL-6). A high ratio of IL-29 to IL-10 was identified as a new predictor of survival in less severe patients with ROC area of 0.933. Combining machine learning with statistical modeling we developed an equation ("VIPER") that could predict survival with ROC 0.966 in less severe patients and 0.8873 for all patients from a five analyte panel (IL-6, VEGF-A, IL-21, IL-29, and IL-10). Furthermore, we also identified three increased proteins (MIF, TRAIL, IL-29) and three decreased proteins (IL-7, TPO, IL-8) that were the most important in distinguishing a trauma blood profile. Biologic sex altered phenotype with IL-8 and MIF being lower in healthy women, but higher in female trauma patients when compared to male counterparts. This work identifies new responses to injury that may influence systemic immune dysfunction, serving as targets for therapeutics and immediate clinical benefit in identifying at-risk patients.

INTRODUCTION

Traumatic injury leads to a cascade of immune activation to prevent infection and scavenge debris from damaged tissues, followed by a refractory immunosuppressive response due to increases in glucocorticoids and responses to the initial inflammatory activation. This natural immune suppression can increase susceptibility to downstream infections at the injury site as well as opportunistic infections such as respiratory viruses and bacterial pneumonia. The large number of variables – both intrinsic to proteins and cells of the immune response and extrinsic to the injury itself – complicates inferences about factors affecting patient outcomes and efforts to develop therapeutic agents to improve treatment. By assembling a large, diverse cohort of trauma patients, we can elucidate the immune phenotypes associated with traumatic injury and their role in and prediction of trauma recovery.

Recent technological advances have increased our understanding of the human immune response to traumatic injury.[1] In addition to glucocorticoid release, a significant increase in interleukin-10 (IL-10) is associated with immunoregulation and immunosuppression. Increasing severity of wounds is associated with increasing systemic (peripheral blood) concentrations of IL-10.[2] Other cytokines such as IL-4, IL-6, IL-8, and transforming growth factor-beta (TGFβ) have been implicated in systemic inflammatory response after trauma.[3] Severe injury has been shown to correlate with downregulation of IL-9, IL-21, IL-22, IL-23, and IL-17E/IL-25 in blunt trauma patients, all of which are mediators of tissue repair.[4]

While tissue-based analyses often yield more insight into the immune function in response to specific injury types, peripheral blood, despite being limited to providing systemic rather than local information, is a more easily accessible analytic medium that can be collected longitudinally and less invasively. An ability to infer outcomes or inform therapeutic decision making from peripheral blood proteins can lead to potential diagnostic platforms for use in triage, allow more insight into the basic mechanistic responses to injury, and provide investigative targets to explore for downline therapeutic engineering.[5,6] Here, we present a large, 1000 patient retrospective study on human systemic immune response to trauma, evaluating 59 plasma proteins in comparison to healthy controls, and stratified by demographics, mechanism of injury, body region, and tissues injured. These plasma proteins were selected to include proteins associated with inflammation and immunity whose levels were detectable with multiplexed assays based on preliminary analyses of a small subset of samples. Furthermore, we utilize machine learning to classify proteins that

are important for predicting a trauma-associated inflammatory profile and identify a novel predictor of patient survival that can have immediate clinical impact. This work represents the largest study of plasma proteins in trauma patients and provides insight that can be used for patient diagnosis and prediction of patient outcomes, as well as a potential therapeutic pathway for further research.

RESULTS

We analyzed 1000 plasma samples collected as a part of astudy focused on drug prevalence among trauma victims treated at selected Level 1 trauma centers[7–9] and 50 samples from healthy individuals at the NIH. Trauma patient samples came from 5 centers located in Worcester MA, Baltimore MD, Charlotte NC, Jacksonville FL, and Miami FL. Of the trauma samples, 997 had complete clinical and laboratory data (**Supplementary Figure 1**). The median age of trauma patients was 39 years (**Figure 1a, Supplemental Table 1**). The majority of patients were male (71.8%) and White/European American (55.1%). Black/African Americans represented 36.9%, other races comprised 1.6%, and race was unknown for the remaining 6.2% of patients (**Figure 1b**). Many patients were seen in the emergency department and discharged (60.3%); 25% were sent to the operating room (OR), 12.3% to the intensive care unit (ICU), and 2.5% ultimately died from their injuries (**Fig. 1b**). Almost half (42%) of patients were admitted due to a motor vehicle crash (MVC) followed by falls (21.1%) and gunshot/shotgun wounds (GSW, 11.2%). Injury types included fractures (57.9%), lacerations (40.7%), abrasions (31.5%), and contusions (23.6%) with many patients reporting more than one injury type (**Figure 1c**). Additionally, we categorized injuries as “skin breaking” (avulsion, incision, laceration, open fracture, stab wound, puncture, gunshot wound, amputation, abrasion, and burns) or “internal” (hematoma, ecchymosis, contusion, seatbelt mark, swelling, fracture, deformity) to evaluate tissue-specific immune responses (**Figure 1c**). Additional details are in **Supplemental Figure 1**.

Significant immunosuppression and select protein upregulation in trauma patients

After normalization and clustering of all proteins, the healthy controls clustered together in a hierarchical dendrogram in comparison to trauma samples (**Figure 1d, Supplemental Figure 2**). Tobit regression revealed that traumatic injury led to overall increased levels of 9 proteins (IL-10, MIF, IL-29, TRAIL, IL-23, IL-16, IL-6, IL-1Ra, MIP-5) and decreased levels of 23 proteins compared to healthy controls (MCP-4, IP-10, MDC, FLT3L, Eotaxin-3, CTACK, TARC, MCP-1, Eotaxin, IL-3, TPO, MIP-1b, ENA-78, MIP-3b, I-309, IL-21, IL-12/IL-23p40, IL-17E/IL-25, MIP-1a,

VEGF-A, IL-7, IL-8, IL-2, **Figure 2a,b, Supplemental Figure 3a**). The greatest differences in protein concentration between the total trauma population and healthy controls were a strong upregulation of IL-10 (23.1% increase versus healthy control, 95% CI: 5.0 – 106.8) and a strong downregulation of IL-2 (97.8% decrease, CI: 91.8 – 99.4). The smallest increase detectable was a 1.46% increase in MIP-5/CCL15 (1.1 – 1.9) and the smallest decrease was a 29.1% decrease in MCP-4/CCL13 (14.4 – 41.3).

Most cytokines and chemokines were detectable (**Figure 1d, Supplemental Figure 4**), although several, including IL-10, IL-2, IL-5, IL-23, and IFNβ, were below the assay limit of detection (LOD) in >75% of either trauma or control samples. We therefore evaluated whether trauma influenced the probability of a protein concentration being below the LOD. We found that 30 proteins were significantly more likely to be below LOD in trauma samples versus controls (CTACK, ENA-78, IFNβ, IFNγ, I-TAC, IL-2, IL-3, IL-5, IL-7, IL-9, IL-13, IL-15, IL-17A/F, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-31, IL-33, MCP-4, MIP-3a, IL-6, IL-27, IL-17B, MIP-1a, MIP-3b, SDF-1a, TNFa, TRAIL, VEGF-A) while 4 were significantly more likely to be below LOD in healthy controls (IL-4, IL-10, IL-23, IL-29), with the largest differences of each being IL-2 (46.3% difference below LOD in trauma vs healthy, $p<0.001$) and IL-10 (32.7% difference in trauma vs healthy, $p<0.001$) (**Figure 2c, Supplemental Figure 4**). In terms of patient sex, the pattern of differences in protein concentration were similar when controlling for sex in trauma patients versus healthy controls (**Supplemental Figure 5**); however, two proteins had significantly different patterns in males and females. IL-8 and MIF were higher in female trauma patients when compared to male but the opposite in healthy controls **(Figure 2d).**

As patient age spanned 83 years, we evaluated protein levels as a function of age. When controlling for age, trauma patients and healthy controls had a similar pattern of protein concentrations (**Supplemental Figure 5**). Looking at only the population of trauma patients, the concentrations of several proteins, including YKL-40 (CHI3L1), IL-6, IL-8, IFNγ, and others, were positively correlated with patient age **(Figure 2e).** Comparatively, few protein concentrations decreased with age (ENA-78 (CXCL5), TRAIL, IL-16, and Gro-a (CXCL1)). While heterogeneity in protein concentration and a smaller sample of healthy controls prohibited a direct statistical comparison of the regression coefficients between healthy controls and trauma patients, we did observe some age-related patterns in proteins that were significantly different in trauma patients versus healthy controls overall (**Figure 2f – j**). In proteins that were significantly higher in trauma patients versus controls, increasing age had a greater impact on protein concentration (in either

direction) (**Figure 2f**). In proteins significantly decreased in trauma patients, the association with age appeared to be more pronounced in healthy controls (**Figure 2g**). The effect of age appeared negligible when protein concentration was equal in healthy controls and trauma patients, with both groups having similar age trends (**Figure 2h**). Raw data for representative analytes is shown in **Figure 2i** and **2j**. Of the proteins that exhibited significant changes as a result of traumatic injury, some variability was observed with self-reported race (IL-12/IL-23p40 and ENA-78, **Supplemental Figure 6**).

Trauma level, location, and tissue injured alter immune profile

Each patient was categorized as level of trauma activation 1 – 4 per the admitting trauma center, with 1 being the most severe (n = 177) and 4 being alert (n = 490) with a higher risk of death in Level 1 patients compared to level 4 (**Supplementary Figure 7a-g**). Several proteins were significantly associated with trauma level. Specifically, IL-1Ra and IL-16 were increased in severe trauma compared to alert patients (**Figure 3a**) and TPO, IL-21, IL-22, IL-17F, IL-31, IL-33, IL-17A/F, IL-17E/IL-25, and IL-23 were decreased in severe trauma (**Figure 3a**). While not correlated with trauma level, an increase in IL-29 in patients who survived trauma was noted and when compared to IL-10 as a ratio of concentrations, high risk patients could be identified with a low IL-29:IL-10 ratio (**Figure 3b, Supplementary Figure 7h-k**). Analyzing these data using a receiver operating curve (ROC) we found an area of 0.933 with a sensitivity of 100% (95% CI: 70.09 - 100%) and specificity of 85% (81.3 – 88.76%) suggest a potential diagnostic tool that can be used to identify patients that have sustained less severe trauma but are at risk for death (**Figure 3c**).

In addition to these differences in trauma level, we saw alterations in immune profile dependent upon the injury location (**Figure 3d,e**). Trauma location was classified as torso only, head/neck only, peripheral only, a combination, all, or unknown (**Figure 3e**). When evaluating IL-29 (an IFNλ) as a function of injury location, we found that patients that had sustained injuries in all locations (torso, head/neck, peripheral) had lower concentration when compared to those that only sustained injuries on the periphery (**Figure 3f**). As with patient survival, this pattern was inverted with IL-10 (**Figure 3g**). There was no effect with IFNγ (which was also not significantly increased in trauma patients) suggesting this is not a pan-interferon pattern (**Figure 3h**).

Variations could also be detected by mechanism of injury, whether or not it was an internal versus penetrating wound (**Figure 3i**), as well as subset by hard (bone) and soft tissue injuries (**Figure**

3j,k). These subsets were selected to enable analysis of variables that are known to affect preclinical models. As with other variables, IL-10 concentration depended upon tissue type, with combination internal soft tissue and bone injuries trending higher than bone wounds alone (4.4% higher than HC versus 2.5%, **Figure 3l**). This correlated with injury mechanisms that were more likely to induce compound injuries such as MVCs (**Figure 3m,n**) wherein IL-10 trended higher than injuries with just one factor (e.g. stab, 3.3% versus 2.0%, **Figure 3o**).

Immune response to trauma is altered in the presence of active respiratory virus infection

Given sampling occurred during the COVID-19 pandemic, a proportion of patients were tested for COVID-19 upon admission to the hospital (66%). No control samples were tested for SARS-CoV-2 and were excluded from this analysis. Using the data from patients with test results, we compared the immune profiles of trauma patients that tested positive and those that tested negative for active SARS-CoV-2 infection (**Figure 3p**). A total of 25 people tested positive for SARS-CoV-2 and were compared to 612 people who tested negative for SARS-CoV-2 infection. Those without COVID testing or with undetermined results were excluded from this analysis. SARS-Cov-2 positivity appears to be associated with a slight decrease in IL-10 and IL-6 (**Figure 3p)** which were both upregulated in trauma patients relative to HC overall **(Figure 2b).** IL-4, a protein associated with type-2 immunity, trends higher in SARS-CoV-2+ patients, while IL-13 trends lower in SARS-CoV-2+ patients. However, these changes were not statistically significant after correction for multiple comparisons.

Generation of a predictive equation for patient survival using a subset of four analytes

Given our observation that a novely described responder to trauma (IL-29) could be used in a predictive manner for patient survival in non-severe patients, we investigated further the potential to generate predictive calculations for both non-severe and other trauma patients. When comparing concentrations of proteins in trauma patients that survived to discharge (D/C) versus those that succumbed to their injuries (deceased), a number of proteins trended higher or lower in concentration in the latter group **(Figure 4a)**. Several stood out with stark differences including IL-6 **(Figure 4b)** and M-CSF **(Figure 4c)** that were higher in those that died, whereas IL-29 **(Figure 4d)** and IL-21 **(Figure 4e)** were higher in those that survived. As we had found that a ratio of IL-29 to IL-10 had been predictive of survival, we compared ratios of all cytokines to each other and found that several of the protiens that were highlighted in overall differences between D/C

and deceased patients appeared in these estimates (ex. IL-6, **Figure 4f**). Using Random Forest machine learning several of these appeared as having high importance in distinguishing a D/C blood profile from deceased (**Figure 4g**). Isolating proteins that appeared in both machine learning and ratio-based analyses, we generated ROCs of proteins that appeared predictive of survival (IL-6, VEGF-A, IL-10, IL-29, M-CSF, and IL-21) and generated ROC areas that ranged from 0.5049 (non-predictive) to 0.8677 (highly predictive, **Figure 4h**). The variability and lower area under the curve (AUC) values of M-CSF led us to create an abridged list of 5 proteins (IL-6, VEGF-A, IL-10, IL-29, and IL-21) for further analysis. Different machine learning methods generated ROC areas ranging from 0.7600 (XGBoost with class weights) to 0.8957 (Gradient Boost with class weights) with algebraic approaches including regression models ranging from 0.8638 – 0.8873 (**Figure 4i, Supplemental Table 5**). To optimize predictive power, we generated an algebraic equation that involved these five top proteins that we called "Vital Injury Protein Evaluation for Recovery" (VIPER, **Figure 4i,j**). VIPER scores were generated for all patients and subset into both Level 1 (most severe) and Level 4 (least severe) groups then evaluated by ROC (**Figure 4k**). While most predictive for survival in least severe patients (0.9695, 0.9310 – 1.000) which is beneficial due to the low alert level for trauma centers of potential life-threatening injuries, the model remained predictive for all patients, with Level 1 having the most variability but still having an ROC of 0.8431 (0.7375 – 0.9486) (**Figure 4l**).

Animal model correlates with human immunomodulation after traumatic injury

As traumatic injuries cannot be randomized in human clinical studies, many researchers rely on animal models to evaluate therapeutics for wound management and tissue reconstruction. To assess similarities and differences between human trauma response and animal models we evaluated RNAseq data following traumatic soft tissue injuries in three common model organisms: mice, rats, and pigs. When mining bulk RNAseq data,[10–13] we found that early responses to volumetric muscle injury in rats led to increases in *Il10*, *Il16*, *Il6*, *Il23a*, and *Tnfsf10* (encoding TRAIL) similar to human responses (**Figure 5a**).[10,14] We also found a modest increase in *Mif* and *Il17b*. These trends were maintained in a later timepoint of a porcine volumetric muscle loss (VML) model,[11,15] however further study of earlier timepoints with increased replicates is needed (**Figure 5b**). This was recapitulated to some degree in freeze-based muscle injuries in mice,[12,16] including upregulation of *Il10*, *Il1rn* (not seen in rat VML), *Il16*, *Il6*, but with minimal upregulation *Il23a* and *Tnfsf10* (**Figure 5c**). In skin injuries of mice,[13,17] a robust upregulation of *Il23a* was seen in multiple

injury locations including the abdomen, back/dorsum, and cheek at 3 days post-injury (**Figure 5d**).

Additionally, we performed VML surgeries on mice and evaluated peripheral blood at 24 hours post-injury for immediate responses to trauma that are detectable in the plasma of mice (**Figure 5e-g**). Using a cytokine/chemokine blot to screen these responses, we found multiple proteins that were modulated after injury (**Figure 5e**). Several were systemically altered including chemokines CXCL13, CCL6, and CCL21 (**Figure 5f**). Compared to our human data, we found a low signal to noise ratio (suggesting low concentrations), but trending increases in IL-10, IL-1ra (encoded by *Il1rn*), IL-6, and IL23 (**Figure 5g**). Unfortunately, IL-29 is a pseudogene in mice and rats and could not be evaluated mechanistically, highlighting a limitation in animal models for studying trauma immunology.

Protein profile classification using machine learning

Through t-stochastic neighbor embedding (tSNE), an unsupervised clustering method, using only the 59 protein concentrations, we identified an island of healthy controls that clustered separate from trauma patients (**Figure 6a**). While some regions had increased enrichment for various factors such as sex, age, outcome, and injury mechanism, only healthy controls could be easily identified (**Figure 6b**). Interestingly, a small cluster appeared that had significantly higher levels of YKL-40 than the rest of the trauma samples (mean log concentration 17.7 pg/mL vs 12.9 pg/mL, $p<0.001$) (**Figure 6a-b, lower left**), despite YKL-40 not being significantly associated with any trauma characteristics aside from a fall injury (**Supplemental Figure 6**). This small cluster was also older than the total trauma population (median 59 years vs 39 years) which we showed was associated with increased YKL-40 in this population (**Figure 2e**) and has been reported in the literature.[18]

Using random forest, six analytes with the highest relative importance (median permutation importance > 10) for classifying samples as trauma vs control were MIF, TRAIL, and IL-29 (higher in trauma) as well as TPO, IL-8, and IL-7 (lower in trauma) (**Figure 6c, Supplemental Figure 9, 7a**). When predicting trauma designations, IL-1Ra, TARC, and Eotaxin-2 were those with the greatest importance in distinguishing lesser (level 4) from life-threatening (level 1) trauma (**Figure 6d, Supplemental Figure 10b**).

Pathway enrichment of systemically altered proteins in trauma patients

Changes to the systemically circulating proteins in trauma patients leads to potential alterations in several signaling cascades and downstream functions (**Figure 6e**). By importing increased (**Figure 6g**) and decreased (**Figure 6h**) proteins into the STRING database, we observed several interactions that could generate a network of different downstream effects. Using gene ontology (GO) enrichment, we found upregulation of pathways associated with the negative regulation of IL-1-mediated signaling pathway and negative regulation of chronic inflammatory response which correlates with general inflammatory immunosuppression (**Figure 1i**). Positive regulation of Th2 cell cytokine production has been associated with wound healing and scar tissue deposition and was also significantly enriched via GO. In decreased protein GO enrichment, we found negative effects on the regulation of TH17 cell lineage commitment, eosinophil chemotaxis, and NK cell chemotaxis (**Figure 6j**).

DISCUSSION

While there has been significant research into cytokines and chemokines peripherally and within cerebral spinal fluid of traumatic brain injury,[19–23] less research has been focused on the broad array of other traumatic injuries that present to trauma centers. This observational cohort provided an opportunity to study a large and diverse trauma population, allowing us to characterize how the immune system responds to different trauma-related factors. Some of the proteins we detected have been observed individually in different tissue damage models, but few have been evaluated systemically. The findings present implications for systemic trauma conditions such as multi-organ dysfunction syndrome (MODS).

A robust upregulation of IL-10 was observed in trauma patients as previously described.[2,24] IL-10 is an immunoregulatory protein that dampens immune responses and inhibits over-activation of immune cells and self-reactivity. We found that IL-10 concentration was dependent upon the location of injury, types of tissues injured, and source of injury. Patients presenting with combined head/neck and torso injuries, those with internal soft tissue and bone injuries, and those injured during a MVC exhibited higher IL-10 levels. These polytrauma patients suggest that core trauma (torso/head), even if not a skin-breaking or penetrating wound, are associated with higher IL-10 and immunosuppression.

Identification of a new biomarker predictor for patient survival

IL-29 has not been previously associated with traumatic injury and we have shown its robust induction after traumatic injury and increase in patients that survived trauma when compared to those that died. Furthermore, when combining IL-29 concentrations with IL-10 data we developed a ratio that has 100% sensitivity and 85% specificity for patient death after trauma that activated a lower emergency department alert (less severe). Utilization of this ratio could inform patient care by providing a two-analyte panel to predict high risk patients. As IL-29 is an IFNλ that shares the IL-10 receptor beta chain (IL-10RB) with IL-10, the potential opposing roles of IL-10 and IL-29 in post-trauma immunosuppression can be explored to uncover mechanisms of immune response to trauma and unveil new therapeutics. Though IL-22 also utilizes the IL-10RB receptor chain and IFNγ is an interferon, neither had the same pattern as IL-29 or IL-10 suggesting a unique mechanism. If IL-29 is not only a correlate but also a causative agent in patient survival, these insights may yield a useful cytokine-based therapeutic for patients. Unfortunately IL-29 is a pseudogene in organisms used for mechanistic immunology studies and the other IFNλ that is present in mice (IL-28) has unique roles from IL-29. Further work utilizing *in vitro* models may provide some insight into mechanisms, highlighting the need for clinical study to identify mediators of human responses that are lost in preclinical animal modeling. IL-29 is a pleiotropic cytokine that is a player in cancer (regression and persistence), autoimmunity (remission and establishment), and infectious diseases such as COVID-19. This is the first identification of IL-29 having a role in traumatic injury, and this analyte is often left out of standard evaluations making data mining in existing datasets difficult to leverage.

When exploring the data further we found a number of cytokines that were altered in survivors versus those that died. Looking at the relative ratios of proteins, large differences in some protein ratios were observed for those that died compared to those that survived. Combining these results with those of Random Forest machine learning resulted in a list of 6 proteins that were important to distinguishing between survival and death. Through ROC analysis, this list was further refined to 5 proteins (IL-6, VEGF-A, IL-10, IL-29, and IL-21). Applying multiple machine learning models and algebraic methods we developed VIPER, an algebraic equation for the prediction of patient death from this 5-analyte panel. VIPER scores are simple to calculate and have an ROC AUC of 0.8957 across all samples, comparable to the AUC of the machine learning gradient boosting model. Its predictive ability was even stronger for patients that might not be under strict observation for mortality due to lower severity of injuries, with those patients resulting in an ROC

area of 0.966. This suggests that VIPER could serve in the future as a clinical tool for predicition of trauma patient death, enabling rapid and increased intervention for patients who are at increased risk of death.

Novel reporting of trauma-downregulated proteins

Several proteins downregulated in our trauma samples have not been previously reported in the literature in the context of human response to trauma, including IL-17E/IL-25, ENA-78, I-309, and IL-12/IL23p40. Proteins from the IL-17 family are typically associated with autoimmune responses and fibrosis.[25–27] Here, we observed a previously unreported downregulation of IL-17E/IL-25 in traumatic injury compared to healthy controls. The proportion of samples with undetectable IL-17B was higher in trauma patients versus healthy controls. Patients who experienced either bone wounds alone, or both bone wounds and penetrating soft tissue wounds exhibited a significant decrease in IL-17E/IL-25. These findings suggest the involvement of bone injury in downregulation of IL-17E/IL-25 and possible relationship between severe injuries and prevention of autoimmune related cascades in the early stages of post-trauma responses.

ENA-78 (CXCL5), involved in neutrophil activation and chemotaxis, is upregulated in rat models of hepatectomy[28] and ischemia reperfusion injury.[29] We observed a significant decrease in ENA-78 concentration in trauma patients compared to healthy controls. However, those experiencing only internal soft tissue wounds had ENA-78 levels consistent with healthy controls. This suggests that internal soft tissue wounds may not induce robust neutrophil recruitment, possibly because these wounds do not necessarily involve exposure to pathogens. While ENA-78 is involved in neutrophil chemotaxis, I-309 (CCL1) mediates monocyte chemotaxis and was decreased in trauma patients across all wound variables. Additionally, decreases in IL-12/IL-23p40 (promotes macrophage and dendritic cell migration[30]) were observed for all injury mechanisms, wound types, and wound locations. The consistent downregulation of these cytokines across multiple variables indicates a reduction of myeloid migration in the early hours after traumatic injury.

Novel reporting of trauma-upregulated proteins

Of the proteins we found to be upregulated in trauma, the levels of IL-29 and MIP-5 have not been previously characterized for human trauma patients. IL-29 is known to have antiviral and antitumor properties, and its role in the context of infections has been extensively studied. IL-29 levels have

been shown to be elevated in patients with periodontitis,[31] breast cancer patients with periodontitis,[32] and those infected with HPV,[33] and to be decreased in patients with Type 2 Diabetes Mellitus (T2D) and patients with diabetic foot ulcers.[34] IL-29 was upregulated across all variables, suggesting that IL-29 may be upregulated to prevent the co-incidence of viral or other infections after traumatic injuries. MIP-5, a T-cell and monocyte chemokine that has also been found to be elevated in T2D patients, was also upregulated in trauma patients compared to the control. However, the concentration of MIP-5 was dependent on injury mechanism, with a significantly higher concentration in patients experiencing falls or GSW relative to healthy samples, but not those experiencing MVC or stabbings. MIP-5 was also significantly higher in patients who had combined internal and penetrating wounds, but not for those having either in isolation. Thus, while IL-29 appears to be upregulated in trauma samples across multiple variables, MIP-5 levels appear to depend on specific injury mechanisms. These trends do not appear to be a result of trauma level, as neither MIP-5 nor IL-29 levels were significantly altered in the most severe (level 1) compared to least severe (level 4) injuries.

Sex and age-based alterations of immunity in trauma patients

Due to the large size of our trauma cohort, we were able to evaluate the correlation of sex and age with immune response to trauma. Patient sex had relatively little association with protein levels with two exceptions: IL-8 and MIF. Female trauma patients had higher levels of these two proteins than male trauma patients, while in healthy females compared to healthy males, these protein concentrations were lower. Sex-specific effects on both IL-8 and MIF levels have been previously reported.[35–39] Our observation that sex-specific IL-8 and MIF levels in trauma patients exhibit an opposite trend from what is observed in healthy controls indicates that a disruption of the immune system due to traumatic injury can also lead to a disruption of sex-specific immune responses. This should be taken into account for the development of therapeutics that incorporate knowledge of sex-specific immune responses.

Despite the large age range of the trauma patients, we observed significant correlations of age with concentration of proteins including ones previously reported in the literature[18,40–49] along with several that were not significant in published data[40,50] but reached significance in ours. Age-related increases in CTACK, I-309, IL-22, IL-2ra, IL-8, IL-9, MCP-2, MIP-1a, MIP-3a, MIP-3b, and MIP-5 have not previously been described. The majority of these proteins are involved in immune cell chemotaxis with gene ontology analysis on proteins upregulated with age having 7 of 15 top

enriched pathways involved chemotaxis or cell migration (**Supplemental Figure 11**). ENA-78, Gro-a, IL-16, and TRAIL decreased with age which has not been previously reported. TRAIL induces cell death and can be produced by regulatory T cells that are known to decrease in number with age. ENA-78 and Gro-a both have chemotactic activity for neutrophils, while IL-16 stimulates migration of eosinophils, monocytes, and CD4+ lymphocytes. These observations align with previous reports of attenuated neutrophil and lymphocyte chemotaxis with age.[51–53] These data further highlight immune complexity where a cascade of proteins that may have overlapping functions can be differentially altered in response to factors such as age or traumatic injury. Additional study is warranted to explore the immunological component to frailty commonly observed following injuries sustained by geriatric patients.

In addition to our primary findings, we also explored several differences of interest for continued investigation. The co-incidence of viral infection in trauma patients was associated with variations of their immune profile. Given the strong induction of proteins that are regulatory or can act in regulatory manners (IL-10, IL-29, IL-6, IL-1Ra), the negative correlation of severe trauma with the ability to fight off infections like pneumonia, and post-acute sequelae of both infections and trauma, these data support future studies on the networked role of responses to pathogens and traumatic injury. Prior studies revealed distinct patterns of inflammatory biomarkers that distinguish blunt trauma patients with nosocomial infections from those without infections.[54] In addition to infectious disease, a small subset of trauma patients in our cohort reported co-incident cancer which was associated with higher IL-10 levels (**Supplemental Figure 12**). Increased IL-10 has been associated with worse outcomes in both tumor clearance and trauma recovery,[55,56] highlighting the need for a deeper understanding of how traumatic injuries can differentially impact specific patient populations.

Investigation into the human immune response to trauma yields insight for evaluating downline patient outcomes, understanding the basic biologic principles of the human response to traumatic injury, and identifying targets for therapeutics. In this study we were able to confirm several findings in the literature on the effects of traumatic injury on protein levels, examine factors such as trauma location and trauma level that affected these proteins, and identify new mediators of the systemic human inflammatory response to injury.

While our cohort provides a broad look at various trauma factors, it is important to note some limitations with these data. Convenience sampling of participants from trauma sites could

introduce unknown bias into our cohort for which we were unable to control, but it is not possible to do a randomized clinical trial of traumatic injury. As we did not have complete injury severity score (ISS) information for all patients, and thus compared trauma levels. While associated with severity, trauma level (as with other metrics) can be subjective. Despite this, there was a strong correlation between trauma level and risk of death (Trauma Level 1 (0.09, 95 % CI: 0.06-0.016) versus Trauma Level 4 (0.02, 95 % CI: 0.01 – 0.05)). In addition to severity, as this study evaluates acute response to trauma, a focused longitudinal study of timepoints hours, days, and weeks after traumatic injury would provide an understanding of temporal immune dynamics. We were also limited in our ability to independently assess lower incidence trauma (e.g. severe burns) and co-morbidities due to smaller numbers. A deeper evaluation of the different immune responses in non-trauma controls with varied comorbidities (e.g., cancer) and more specific trauma cohorts is needed to to deconvolute the intricate interactions of trauma and human diseases. Despite these limitations, we believe this is largely overcome with the large and diverse cohort.

This study has identified novel trauma-associated immune changes in humans that are altered based on age, sex, trauma source, injury location, and trauma level. These differences can inform future mechanistic studies and clinical evaluations based on the function of different proteins that we have detected. While infectious diseases can take days to mount and potentially cause tissue damage, traumatic injuries cause a large disruption in homeostasis in a matter of seconds. As such, our lab and others have been interested in the prevention of damaging autoimmunity after acute tissue damage caused by traumatic injury. In this study, alterations in TRAIL, IL-29, IL-23, IL-17, IL-1Ra, IL-10, IL-6 and others involved in promoting or inhibiting autoimmune like conditions show a strong connection between immunologic self-reactivity and response to traumatic injury that must be investigated further. Identification of IL-29 as a biomarker for survival from traumatic injury can directly affect clinical care and is a topic for future evaluation, albeit with limitations in mechanistic studies due to the absence of this protein in mice. Potential for IL-29 to be adopted as a therapeutic can also be investigated to determine if this is not only a correlate of survival but also a potential route for intravenous administration in at-risk patients.

FIGURES AND LEGENDS

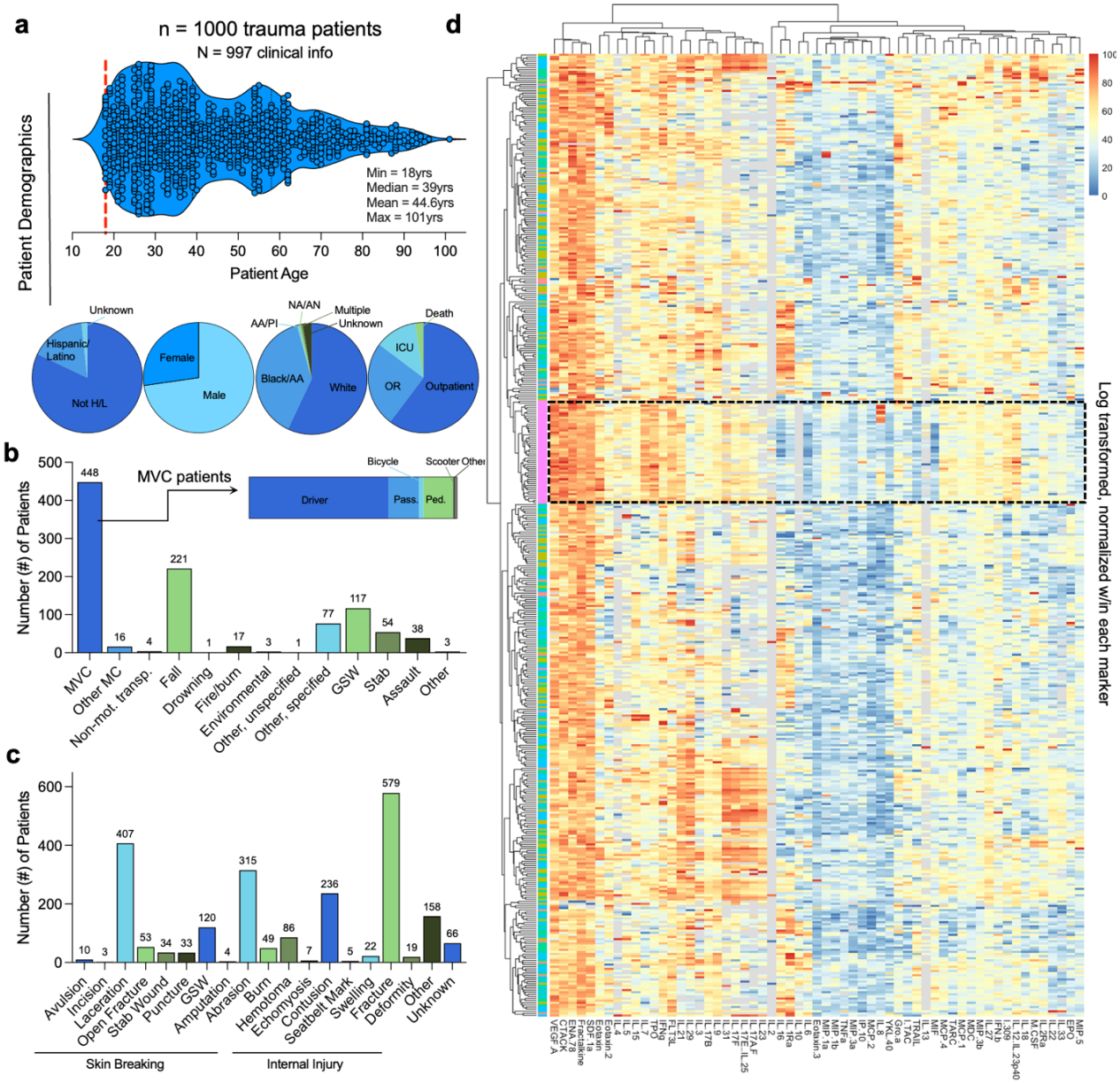

Figure 1 | Patient demographics and overall plasma immune protein profiles. (**a**) Top: Age distribution of trauma patients. Min = 18 yrs, Max = 101 yrs, Median = 36. Bottom: Demographics of patients and trauma outcome. Biologic sex, patient-reported race & ethnicity, trauma outcome. (**b**) Source of trauma. (**c**) Type of trauma. (**d**) Heatmap of protein profiling in N = 1000 trauma patients and N = 50 healthy controls (HC). HC are outlined in black dashed box; data are analyte concentration log transformed and normalized (0 to 100) within marker.

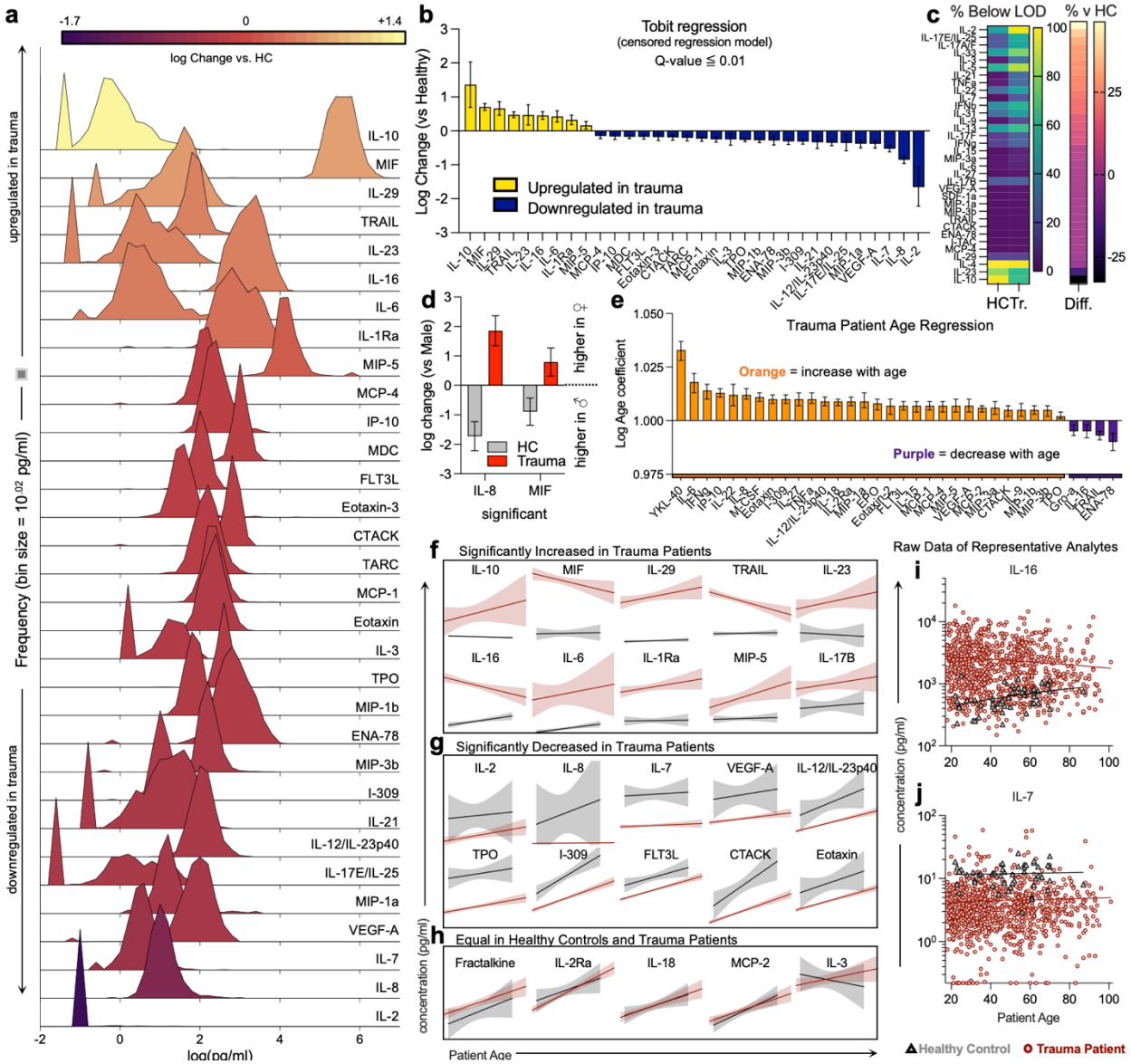

Figure 2 | Differential immune responses in trauma patients are altered by age and sex of patient. (**a**) Distribution of significantly altered proteins in trauma patients compared to healthy controls. (**b**) Tobit regression of significantly altered proteins. Yellow = increased in trauma, Blue = decreased in trauma. Data are log fold change (versus healthy control, HC). (**c**) Proteins that are different in percent (%) below limit of detection (LOD) in trauma patients versus HC (left) and % difference between HC and Trauma (Tr). (**d**) Sex dimorphisms in trauma patients (grey = HC, red = trauma). (**e**) Age coefficients of proteins tested in trauma patients (orange = positive correlation with age, purple = negative correlation with age). (**f-h**) Examples of simple linear regression of age and protein concentration for markers significantly (Tobit) (**f**) increased, (**g**) decreased, (**h**) or equal in trauma patients compared to HC. Raw data of representative analytes that are (**i**) increased and (**j**) decreased in trauma patients compared to healthy control. (**f – j**) Red = trauma patient, black/grey = healthy control. Error bars = with 95% confidence intervals.

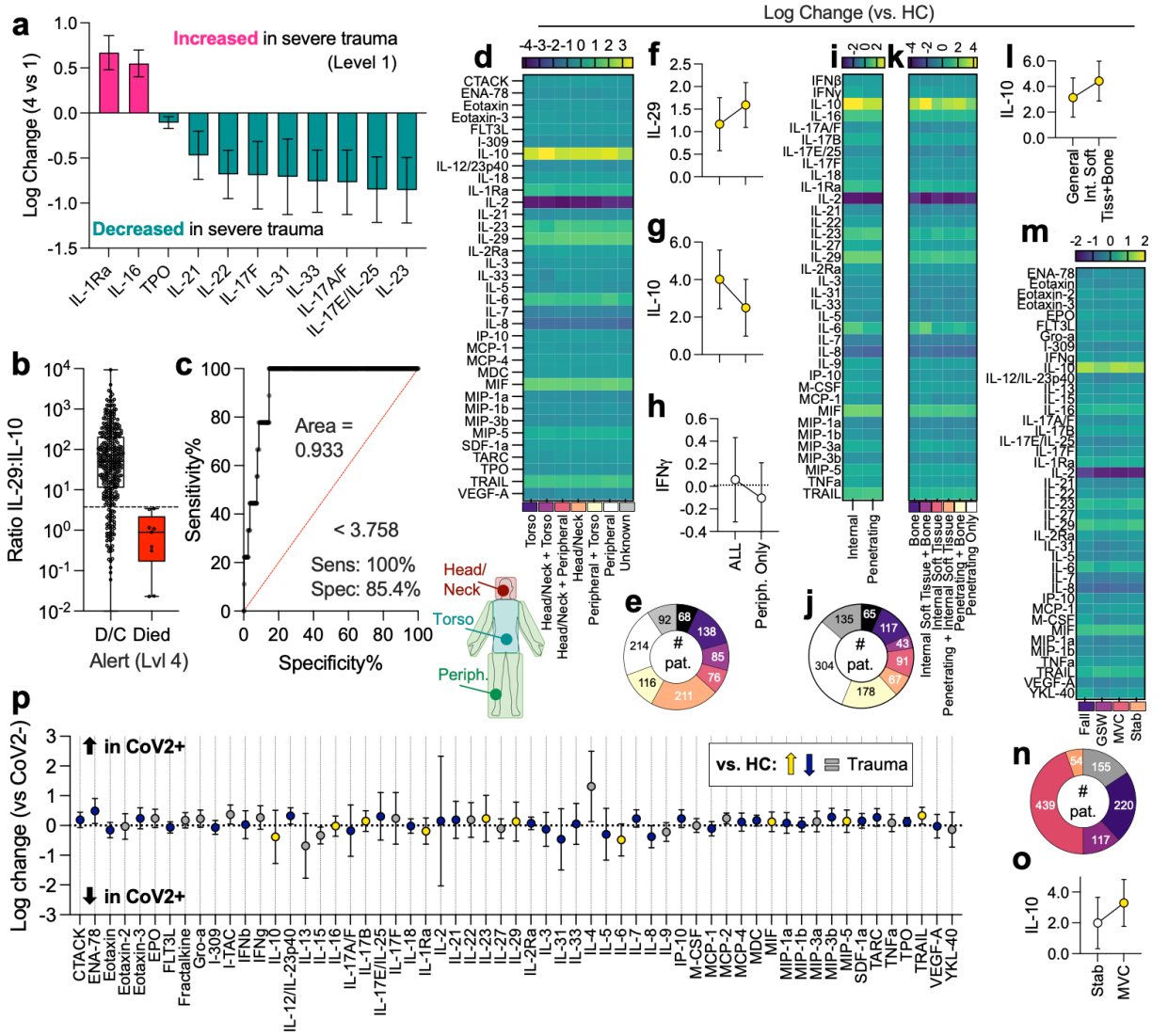

Figure 3 | Trauma level, location, and co-incidence of viral infections cause alterations in immune profiles and reveal IL-29 as a a new predictor of patient survival. (**a**) Protein differences in severe (1) vs less severe (4) traumas. (**b**) IL-29 to IL-10 ratio in survivors versus those that died from less severe trauma. (**c**) ROC of IL-29:IL10 ratio as a predictor of patient survival. (**d**) Log change of protein versus healthy control (HC) by injury location. Purple = decreased in trauma, Yellow = increased. (**e**) Incidence of injury locations. (**f-h**) Cytokine concentration in patients with injuries in all locations versus peripheral only for (**f**) IL-29, (**g**) IL-10, and (**h**) IFNγ. Data are log change ± 95% CI. (**i**) Patients with internal vs penetrating injuries. (**j**) Incidence of injury types. (**k**) Log change by injury types. (**l**) IL-10 change compared to HC in all injuries vs patients with internal soft tissue injury + bone injury. (**m**) Protein profile by trauma source. (**n**) Incidence of trauma source. (**o**) IL-10 change vs HC in stab vs MVC patients. (**p**) Log change in SARS-CoV-2+ trauma patients vs SARS-CoV-2- patients. Yellow = increased in general trauma patients vs HC, Blue = decreased in general trauma patients compared to HC, grey = proteins not significantly different between trauma patients and HC. Data are log change ± 95% CI.

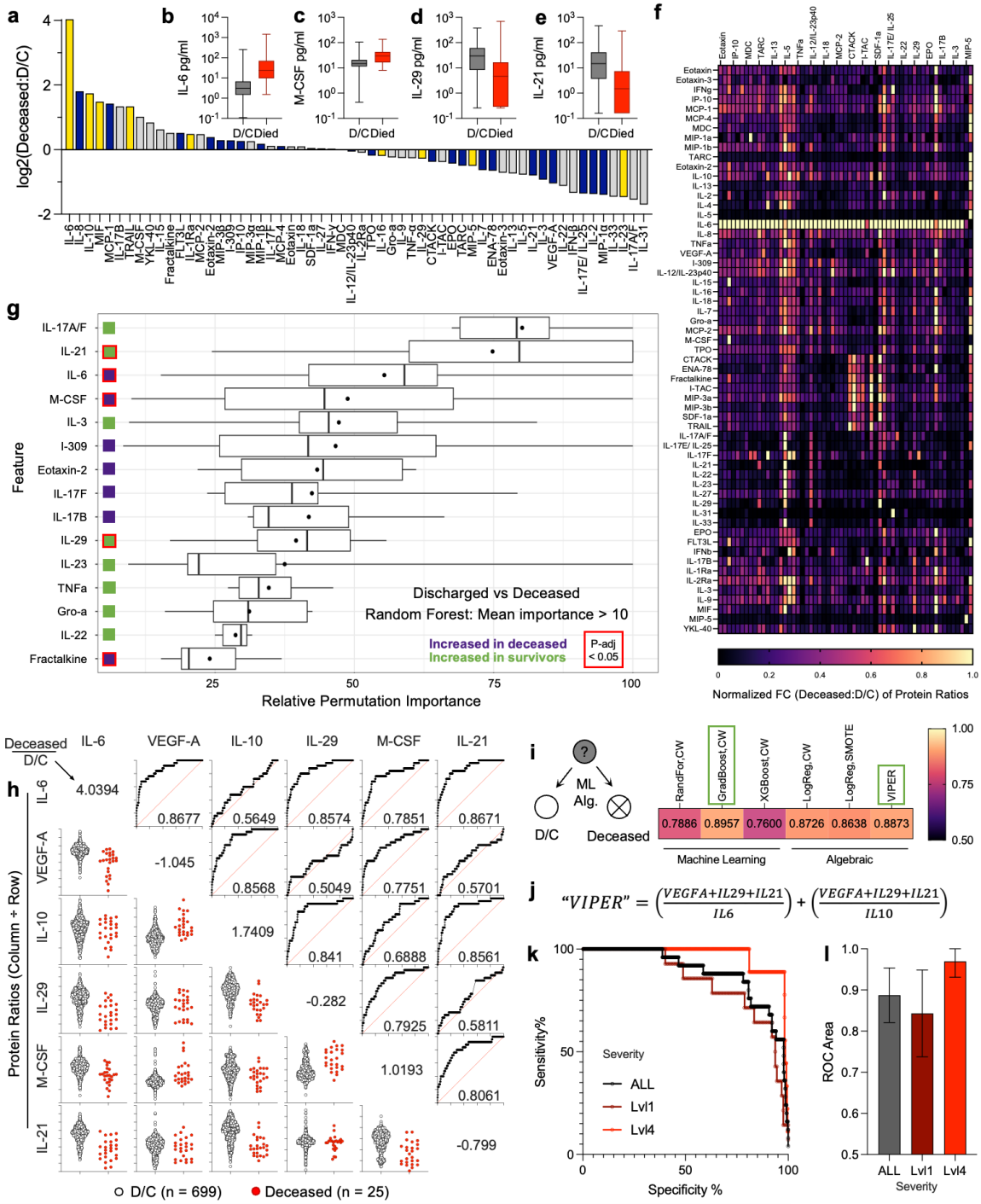

Figure 4 | Development of VIPER equation for survival prediction. (**a**) Fold change of concentration in those that died over those that were discharged home (D/C). (**b – e**) Cytokines altered in moribund patients. (**f**) Fold changes in the calculated ratios of each protein to every other protein for moribund versus D/C. (**g**) Random Forest machine learning permutation importance. Green squares = increased in D/C, Purple squares = increased in deceased, red outline = $p < 0.05$ when comparing concentrations via Mann Whitney with post-hoc correction for multiple comparisons. Data are mean (dot) and median (line) ± IQR. (**h**) Ratios and ROC curves among six selected important proteins. (**i**) AUC values for multiple machine learning and algebraic models applied to predict patient survival. (**j**) "Vital Injury Protein Evaluation for Recovery" (VIPER) algebraic equation developed using 5 proteins with predictive value for patient death. (**k**) ROC curve of VIPER equation for all patients, and those with level 1 or 4 injuries. (**l**) AUC for VIPER.

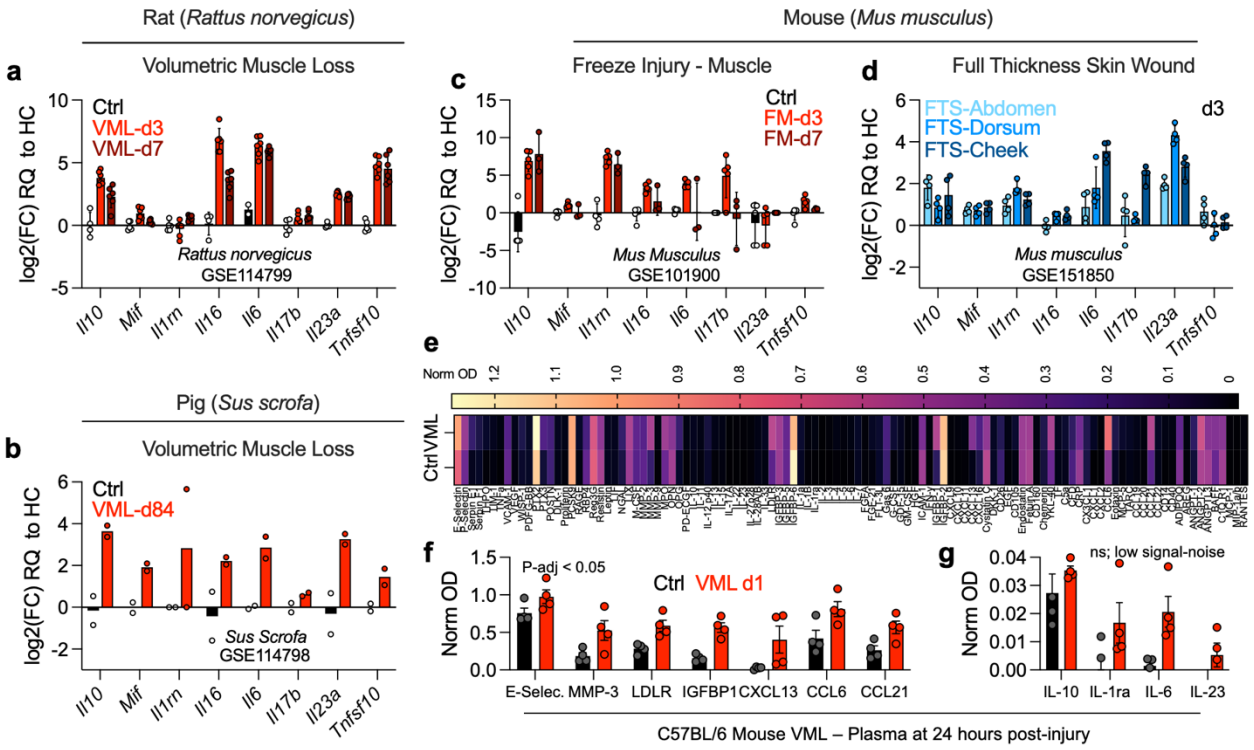

Figure 5 | Comparative animal models for trauma study with human injury response evaluation. (**a-d)** RNAseq data mining for human trauma upregulated markers in local tissue from (**a**) Rat volumetric muscle loss (VML), n = 4 - 6; GSE114799,[14] (**b**) Pig volumetric muscle loss (VML) n = 2; GSE114798,[15] (**c**) Mouse freeze injury of muscle (FM), n = 3 - 5; GSE101900,[16] and (**d**) Mouse full thickness skin wound (FTS), n = 4; GSE151850.[17] Data are fold change over uninjured/sham controls ± SD. (**e**) Mean normalized optical density (OD) from mouse cytokine/chemokine profiling blot of plasma at 24 hours post VML, n = 4. (f) Values from (**e**) that are significantly different from control (p-adj < 0.05, ANOVA with Tukey post-hoc correction for multiple comparisons). (**g**) Values from (**e**) that were present in human dataset but had low signal to noise ratio (low concentration) in mouse blot. Data are mean ± SEM, n = 4.

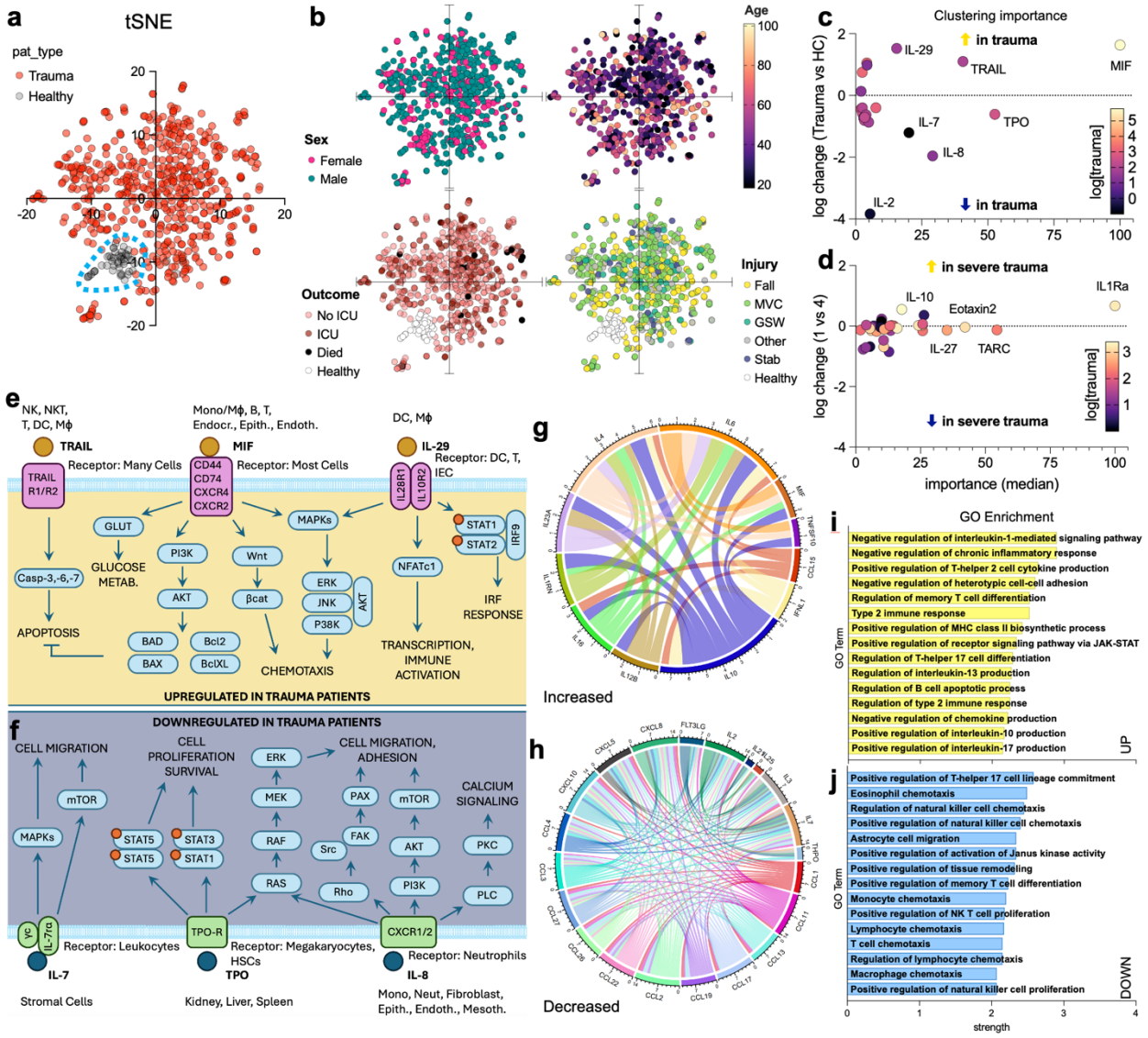

Figure 6 | Machine learning identifies patterns of conserved responses to traumatic injury. (**a**) t-Stochastic neighbor embedding of protein concentrations in trauma patients (red) versus healthy controls (black/grey). (**b**) Labeling of t-SNE diagrams by variables including sex (pink = female, teal = male), age (graded color scale, black = 18yrs, light yellow = 101 yrs), treatment outcome (white = healthy, light pink = outpatient/no ICU, red = intensive care unit (ICU), black = death), and mechanism of injury (yellow = fall, green = motor vehicle crash (MVC), blue green = gunshot wound (GSW), grey = other, blue = stab, white = healthy). (**c-d**) Median importance from machine learning clustering to distinguish (**c**) healthy control from trauma patients, and (d) severe (designation 1) versus alert (designation 4) trauma patients by blood profile. (**e-f**) Proteins (**e**) upregulated or (**f**) downregulated in trauma patients by machine learning and their signaling cascades from receptors. (**g-h**) STRING diagram of interactions of proteins (**g**) increased or (**h**) decreased in trauma patients (determined via Tobit regression). (**i-j**) STRING database gene ontology enrichment of pathways from (**i**) upregulated and (**j**) downregulated proteins

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

MATERIALS AND METHODS

Clinical study and sample collection

Clinical study and sample collection proceeded as previously described[7] (See Report No. DOT HS 813 399). Briefly, samples were collected by convenience sampling in the time period of May 9th 2020 to January 26th 2021 during an ongoing National Highway Traffic Safety Administration (NHTSA) study of drug prevalence among adult (age 18+) trauma victims who had severe enough injuries for transport by EMS and had a trauma team activated/alerted at selected Level-1 trauma centers. These specimens were collected from patients who were already having blood drawn as part of medical treatment at the trauma centers and were made available for research purposes. Serological analyses were conducted on excess plasma samples from the study when possible.

The study was conducted in accordance with Good Clinical Practice, the principles of the Belmont Report, and HHS regulations enumerated under 45 CFR 46. Five of the sites had the Chesapeake/Advarra Institutional Review Board as the central IRB (Advarra Protocol # Pro00022129), and the Jacksonville, FL site had the University of Florida Institutional Review Board as the IRB of record. De-identified samples and other data were included in the study through IRB-approved waivers of consent and authorization. Medical records or other secondary sources such as emergency medical services run reports and crash reports provided the demographic information. De-identified samples were then sent on dry ice overnight to NIH and stored at −80°C until processing. De-identified healthy volunteer blood samples were collected under clinical protocol NCT0000128 and NIH IRB-approved protocol 99-CC-0168 at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda MD.

Patient and Injury Categorization

Based on clinical data, patients were sorted into a variety of subcategories for further analysis. Patients were sorted into one of five injury mechanisms: fall, gushot/shotgun wound (GSW), motor vehicle crash (MVC), stabbing, or other. Among all injuries, the injury location or locations were also identified for each patient, with injuries categorized as occurring in the head/neck area, torso, peripheral regions, a combination of two of these, or in unknown locations. Regardless of injury mechanism or location, patient injuries were also sorted into the categories of internal (no broken skin), or penetrating (breaking the skin). A more granular understanding of the effects of tissue types on immune response was also obtained by sorting injuries based on tissue involvement, where the major tissue types were categorized as bone, internal soft tissue, and penetrating (breaking the skin), or a combination of two of these. Due to a lack of complete injury severity scores for all patients, the injury severity was analyzed using clinically-assessed trauma levels 1-4 as a proxy, where level 1 was the worst trauma level.

Electrochemiluminescence detection of protein analytes

Samples were processed and tested on a custom 59-plex MesoScale Diagnostics electrochemiluminescence assay as per manufacturer's instructions. Protein concentrations were calculated off of standard curves.

Volumetric muscle loss model

Six (6) week-old C57BL/6J female mice (Jackson Labs) were received and equilibrated in the animal facility for 7 days prior to surgery. The day preceding surgery mice were anesthetized under 4% isoflurane in oxygen and maintained under 2% isoflurane prior to removal of hair from the legs using an electric razor followed by depilatory cream. Remaining depilatory cream was removed with a gauze pad with 70% isoflurane and mice were returned to a clean cage until the following day. The day of surgery mice were anesthetized under 4% isoflurane and maintained under 2% isoflurane diluted in oxygen for the duration of the procedure (roughly 6 minutes). Perioperatively, mice received 0.5 mg/ml Buprenorphine ER (ZooPharm) subcutaneously followed by eye lubricant and surgical site was sterilized with three successive rounds of betadine followed by 70% isopropanol. A 1 cm incision was made in the skin overlying the quadriceps muscle (quad) followed by the underlying fascia. A 30 mg portion of muscle was resected from the midbelly of the quad resulting in a 3mm x 3mm injury. Skin was closed using 3 – 4 wound clips (7 mm, Roboz) and the procedure was repeated on the contralateral leg. Mice were kept warm during the procedure through hand warmers separated by sterile drapes. After surgery, animals received 100 ul warm surgical saline and were monitored under a heat lamp until ambulatory and grooming. All animal research was supervised and approved by the NIH Clinical Center ACUC under protocol NIBIB23-01.

Mouse cytokine and chemokine protein array

Peripheral blood was collected from the mouse peri-euthanasia through a submandibular bleed. Serum (100 µl) was loaded onto a Proteome Profiler Mouse XL Cytokine and Chemokine Array (R&D Systems) as per manufacturer's instructions. Briefly, assay membranes were incubated with blocking buffer for one hour before addition of samples. Each sample was diluted to a final volume of 1.5 mL prior to addition, and samples were incubated on the membranes overnight at 4 °C on a rocking platform. Membranes were washed with wash buffer on a rocking platform three times (10 minutes each), and then incubated with the diluted detection antibody cocktail for 1 hour at room temperature on a rocking platform. Membranes were then incubated with horse radish peroxidase-conjugated streptavidin for 30 minutes at room temperature on a rocking platform shaker, washed three times, and incubated with the Chemi Reagent Mix for 1 minute. Images were acquired using a BioRAD Gel Imager, and quantification was conducted using ImageJ.

STRING Analysis

The lists of upregulated and downregulated proteins were searched for in STRING, a database of known and predicted protein-protein interactions.[57] The combined score for interactions between pairs of proteins was exported and used to generate the chord diagrams in R. Lists of functional enrichments, in the form of Biological Processes (Gene Ontology), were exported and the 15 pathways with the lowest false discovery rates (FDR) were selected for each list of proteins. These were then sorted by strength.

Data Analysis and Statistics

Each analyte was subject to lower and upper limits of detection (LOD) of the assay, both for healthy control and trauma samples. Values above the LOD were set to the analyte-specific assay upper LOD + 10%. Values below the lower LOD were set to zero for data visualization and were censored for regression analysis. All protein concentrations were log-transformed.

To assess the relationship between protein concentration and independent variables of interest, we chose to use Tobit regression, which estimates linear relationships when the outcome is censored from one direction, in this case, left censoring at the lower limit of detection. Using the AER package in R,[58] we constructed individual Tobit models for each of the 59 proteins and the following independent variables: patient type (trauma or healthy control), age (mean-centered), sex, COVID-19 infection status, injury mechanism, trauma level, general and specific wound types, and wound location. Age and sex models controlled for patient type; all others were univariable. Exponentiated regression coefficients and 95% confidence intervals are reported as percent change in protein concentration per unit increase in the independent variable. When reporting whether an association between a protein and predictor was statistically significant, we separately computed q-values for the probability that the association was positive or negative[59] and controlled the FDR at 0.01 for each of the comparisons.

Exploratory cluster analysis of normalized protein concentrations was conducted using Van der Maaten's Barnes-Hut implementation of t-Distributed Stochastic Neighbor Embedding (t-SNE) in R using the default settings in the Rtsne package.[60] Random Forest from the tidymodels[61] R package was employed to determine the relative importance of features for classifying trauma vs control. All continuous variables (age, protein concentrations) were normalized, patient sex was one-hot encoded. For each variable in the model, we calculated permutation importance across

10 model iterations. Relative importance was determined by dividing each variable's permutation importance score by the largest importance score of all variables for each of the 10 iterations, then multiplying by 100. See the **Supplemental Materials** and Mayer et al for more information.[62] Tobit regression, cluster analysis, and random forest analyses were conducted using R version 4.2.x.[63]

IL-29:IL-10 concentration ratio was generated as a ratio (pg/ml:pg/ml) of the two cytokines and the resulting ratio was compared in level 4 trauma activation patients via receiver operating curve (ROC) in GraphPad Prism v10.2.2 comparing those that were discharged to home (D/C, n = 343) versus those that died (n = 9). Patients that were discharged to any other location were excluded from analysis (rehab, correctional facility, hospice, left against medical advice). Any values that were stated as below limit of detection were replaced with the concentration of the lowest point on the standard curve or lowest detected sample, whichever was smaller. Exploratory analysis of cancer data was conducted on GraphPad Prism with undetected values treated as previously stated and comparisons made without corrections due to the exploratory nature of the evaluation.

DATA AND CODE AVAILABILITY

Data and abbreviated clinical information will be made available in supplement after peer review. Not all detailed clinical information gathered during the study will be made available to prevent de-identification of samples and participants. Some values may be changed to ensure privacy of data while maintaining ability to complete any necessary meta-analyses.

Statistical comparisons with resulting estimates, p-values, and q-values, along with designation of significance will be made available in supplement along with code used for these analyses (via GitHub) after peer review.

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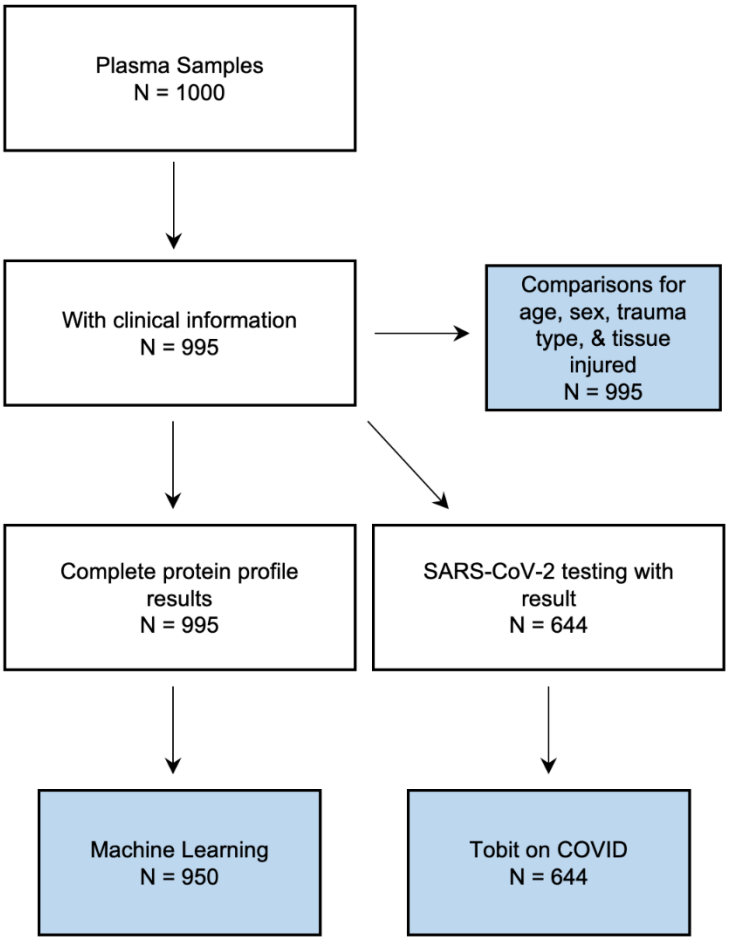

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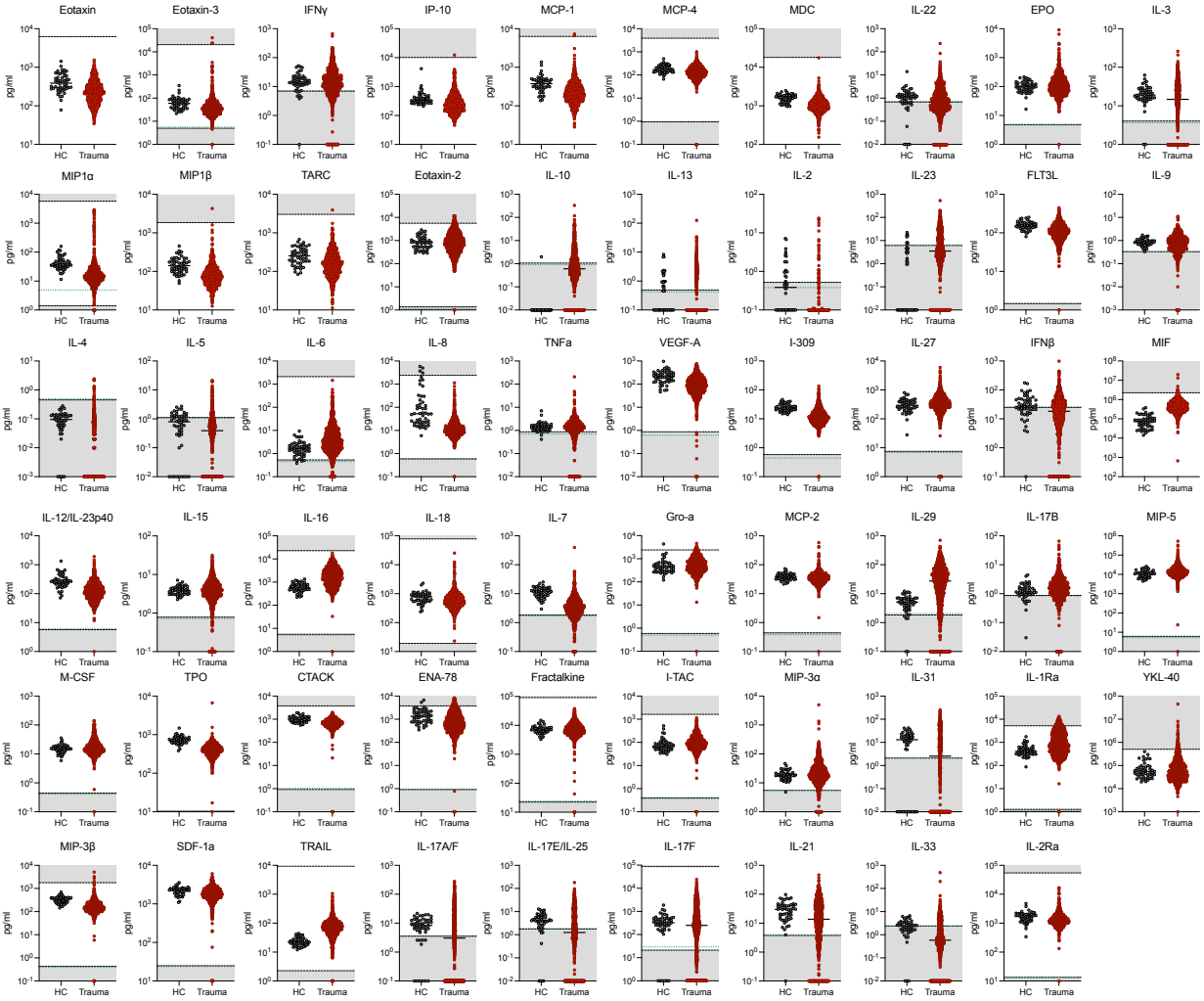
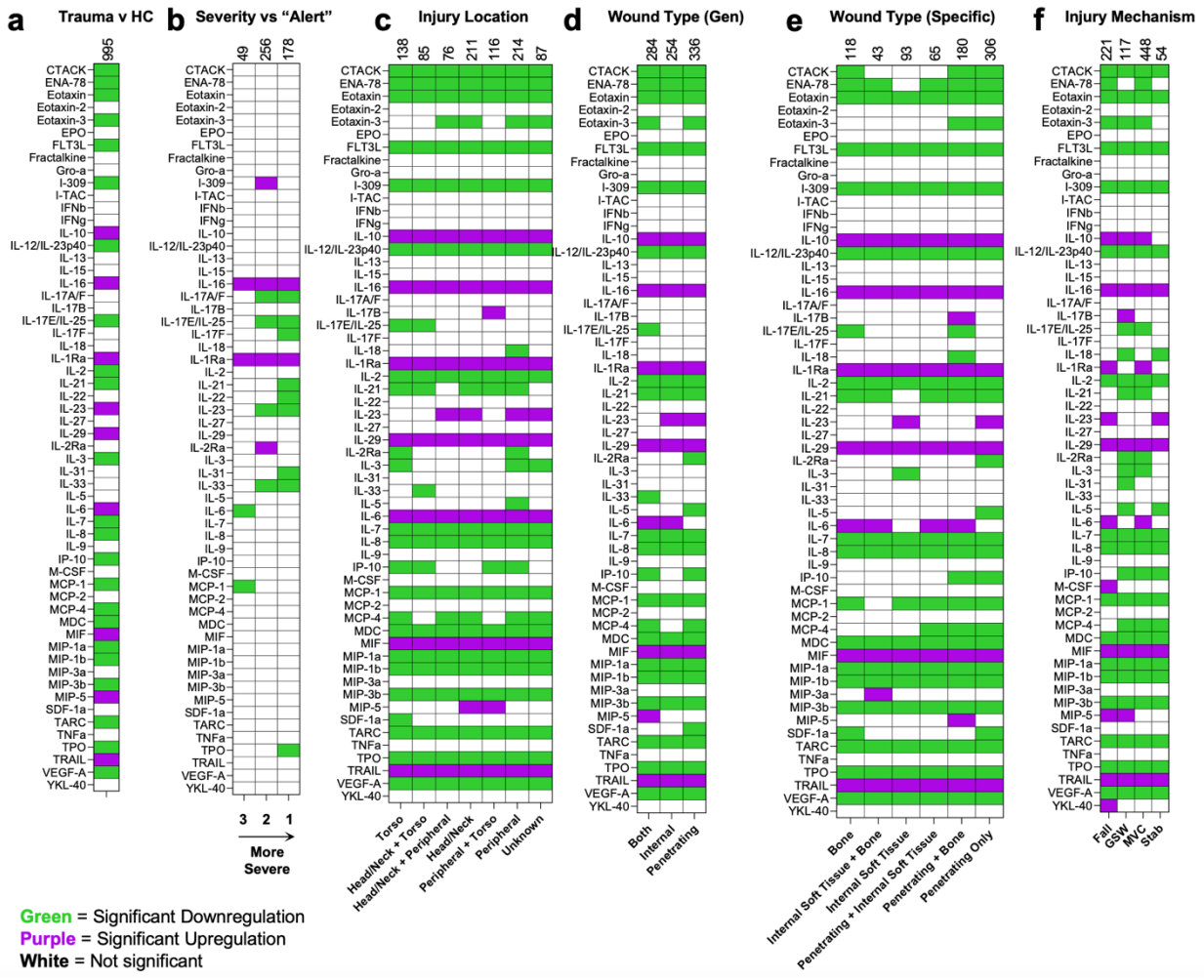
926 **Supplemental Figure 1 | Sample and data analysis workflow.** White boxes = sample input. Blue boxes

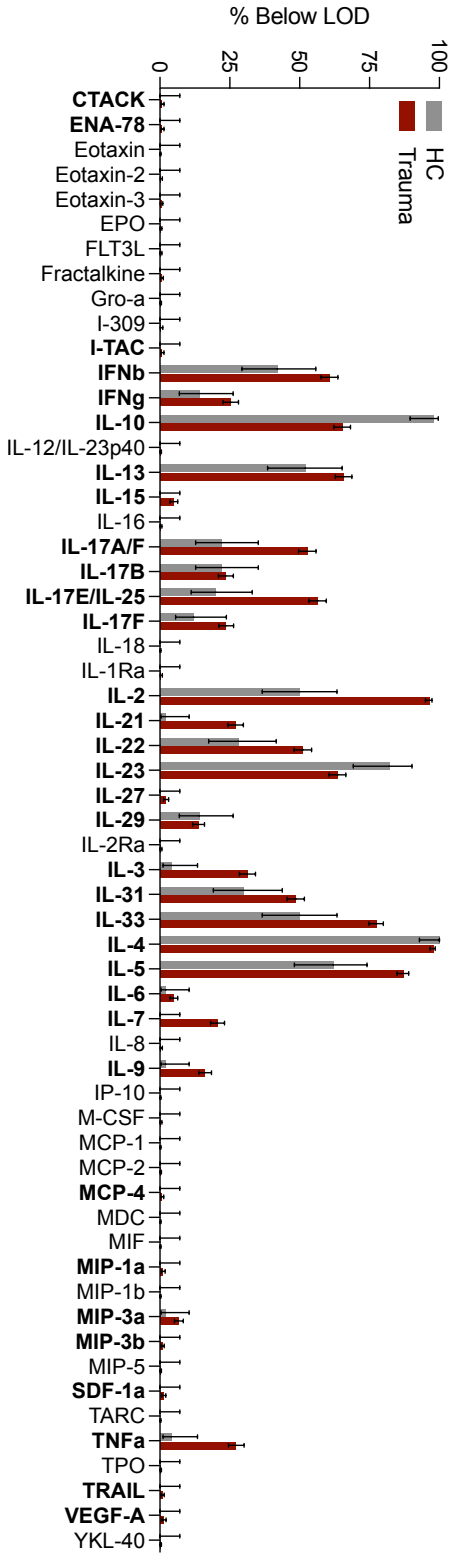
927 = analyses.



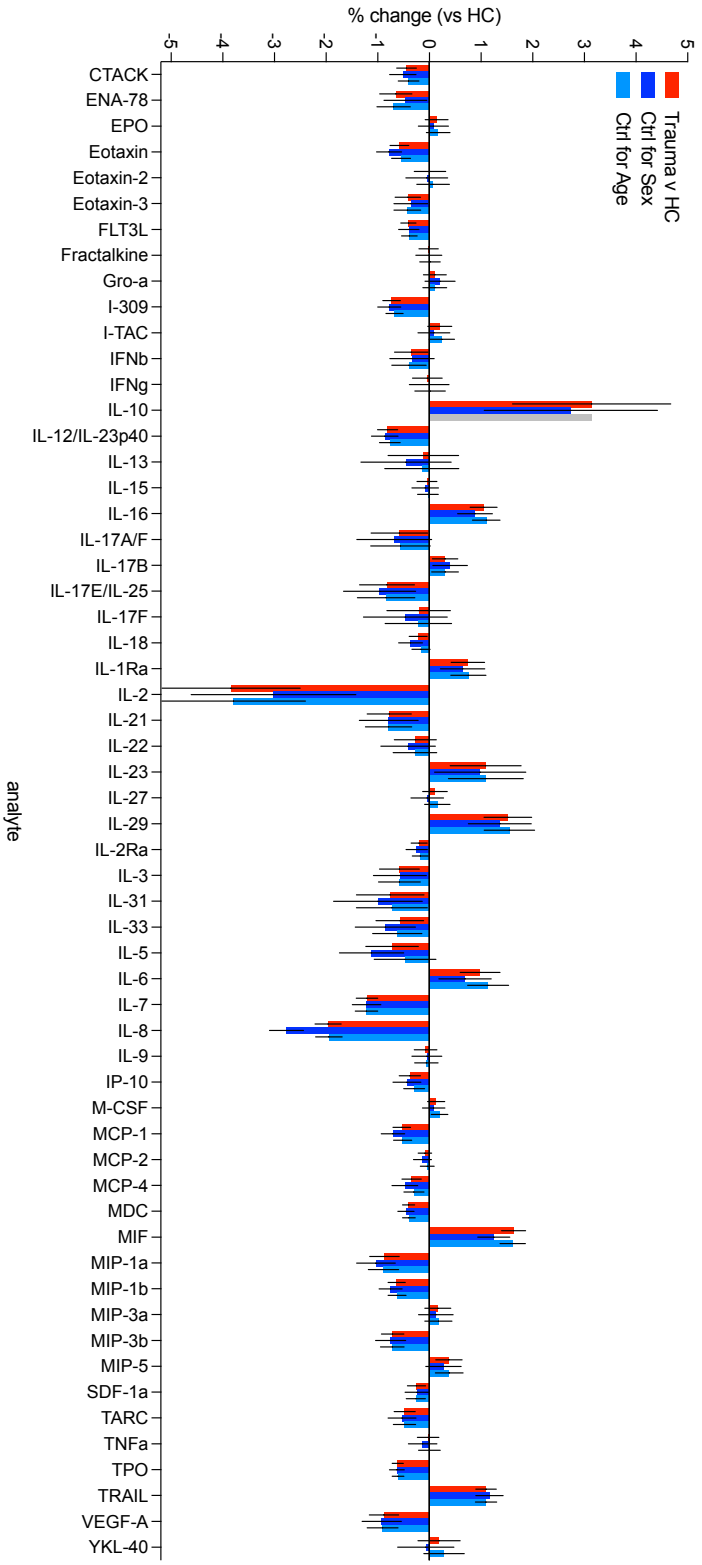
Supplemental Figure 2 | Raw data display for all analytes measured in full cohort. Grey = Healthy controls (HC); Red Points = Trauma Patients. Green Iline = threshold for healthy control samples, Black line = threshold for trauma samples. Grey shaded areas = above or below highest known standard curve value.



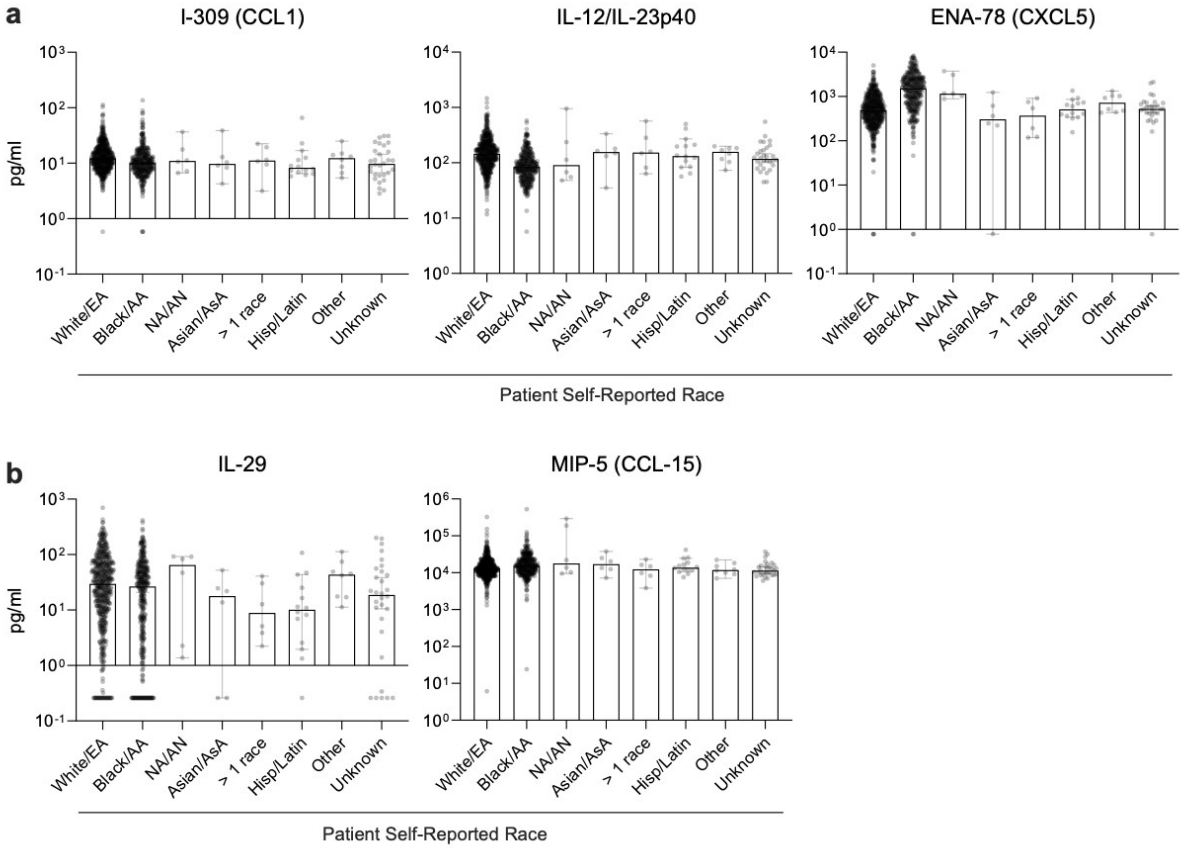
Supplemental Figure 3 | Binary Heatmaps of Significant Tobit Estimates. Heatmaps of statistically significant Tobit estimates for (a) Trauma versus healthy controls, (b) Different trauma levels compared to trauma level 1, (c) Injury location, (d) general wound type, (e) Specific wound type, and (f) injury mechanism.



Supplemental Figure 4 | Proportion below limit of detection. Proportion of samples that were below the limit of detection (LOD) for the assay. Grey = Healthy controls, Red = trauma patients. Data are % below LOD ± 95% confidence intervals (Wilson).



Supplemental Figure 5 | Estimates of change from healthy volunteers when controlled for sex and age. Tobit estimates for trauma vs healthy controls (HC) with overall (red), controlled for sex (blue), and controlled for age (light blue). Data are log change ± 95% confidence intervals.



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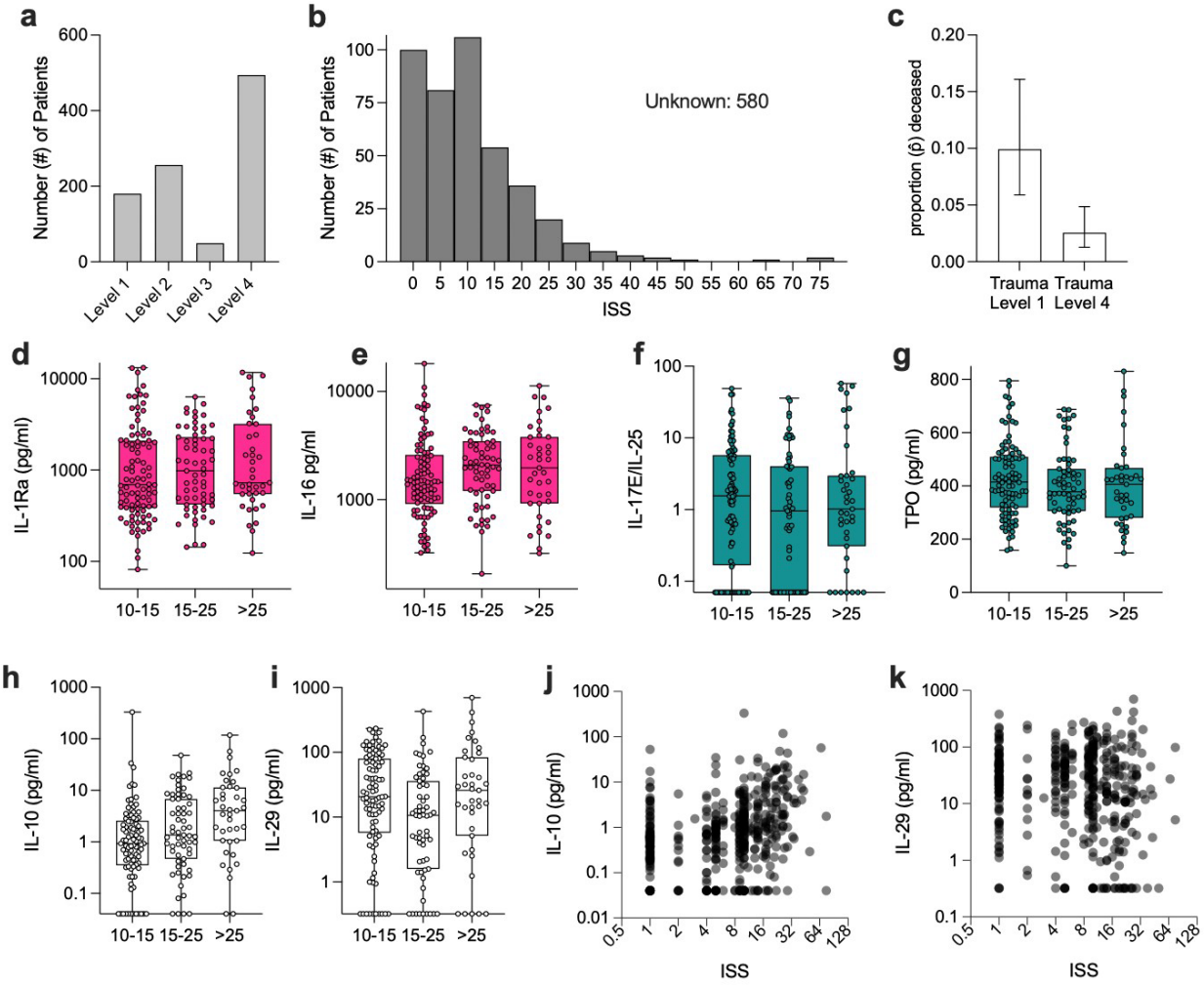
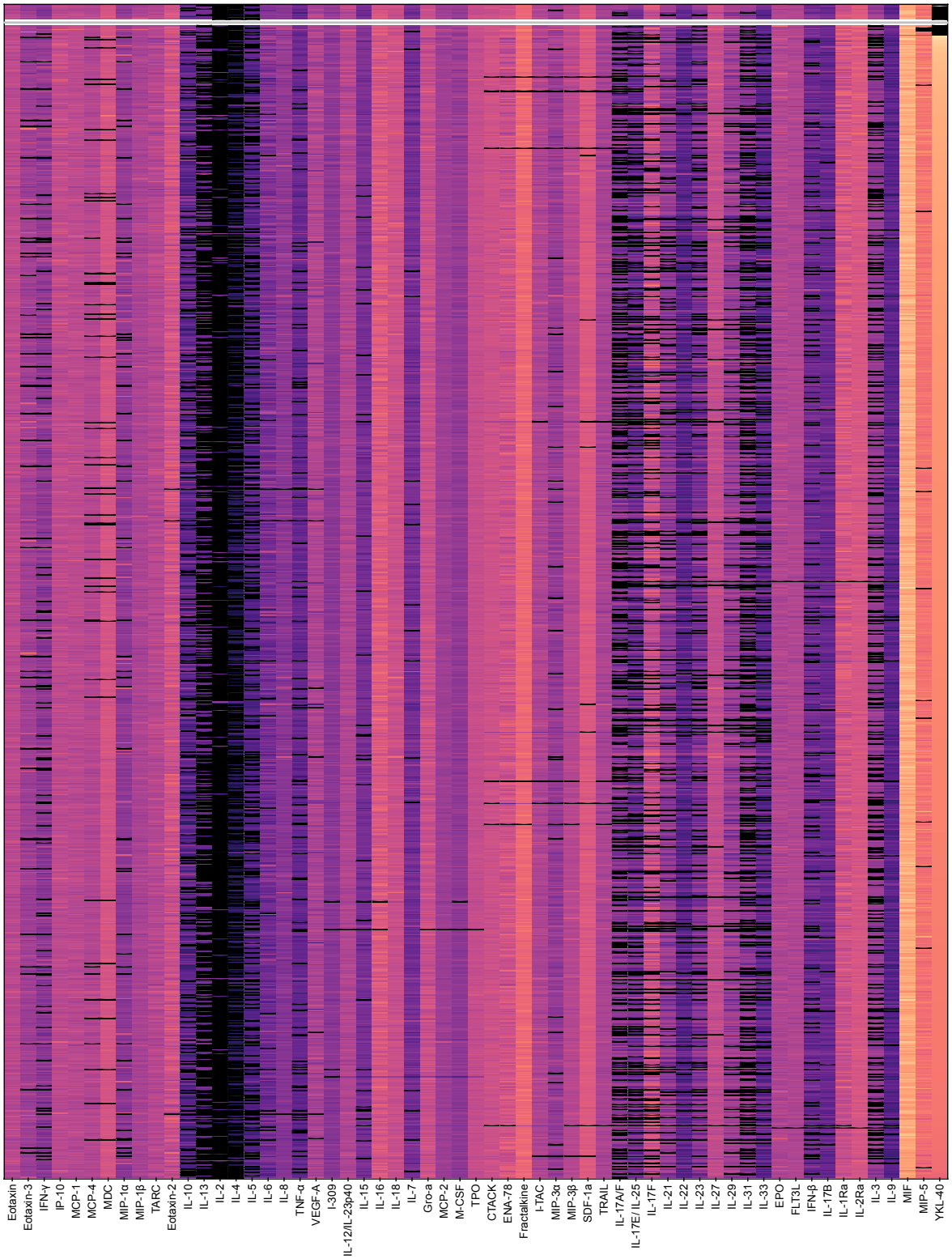
947 **Supplemental Figure 6 | Effect of self-reported race on concentration of cytokines and chemokines**
948 **in the blood of trauma patients**. (a) Novel downregulated proteins as a function of self-reported race. (b)
949 Novel upregulated proteins as a function of self-reported race. EA = European American, AA = African
950 American, NA/AN = Native American/Alaska Native, AsA = Asian American. Data are mean ± standard
951 deviation.



Supplemental Figure 7 | Trauma level correlates with risk of death and protein concentration patterns are conserved with those that have an injury severity score (ISS). (a) Number of patients categorized into different trauma levels (Level 1 = most severe trauma center activation, Level 4 = least severe trauma center activation). (b) Distribution of ISS including those listed with ISS (including those with one injury). (c) Probability of death when admitted under trauma activation level 1 versus trauma activation level 4. (d – g) Distribution of cytokine/chemokine concentration of upregulated (d, e) IL-1Ra and IL-16 respectively, and downregulated (f, g) IL-17E/IL-25 and TPO respectively, proteins as determined by Level 1 versus Level 4 concentration. (h, i) Concentration of (h) IL-10 and (i) IL-29 by ISS level and (j-k) Correlation of (j) IL-10 and (k) IL-29 concentrations as a function of ISS.



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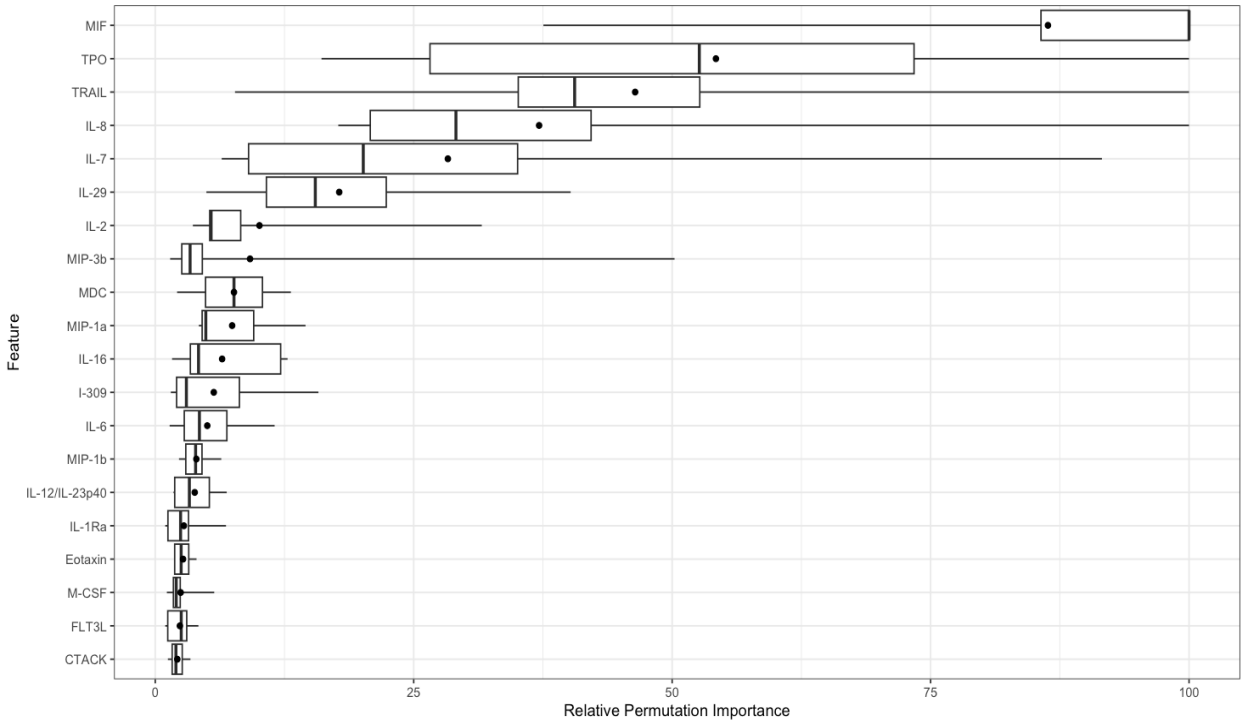
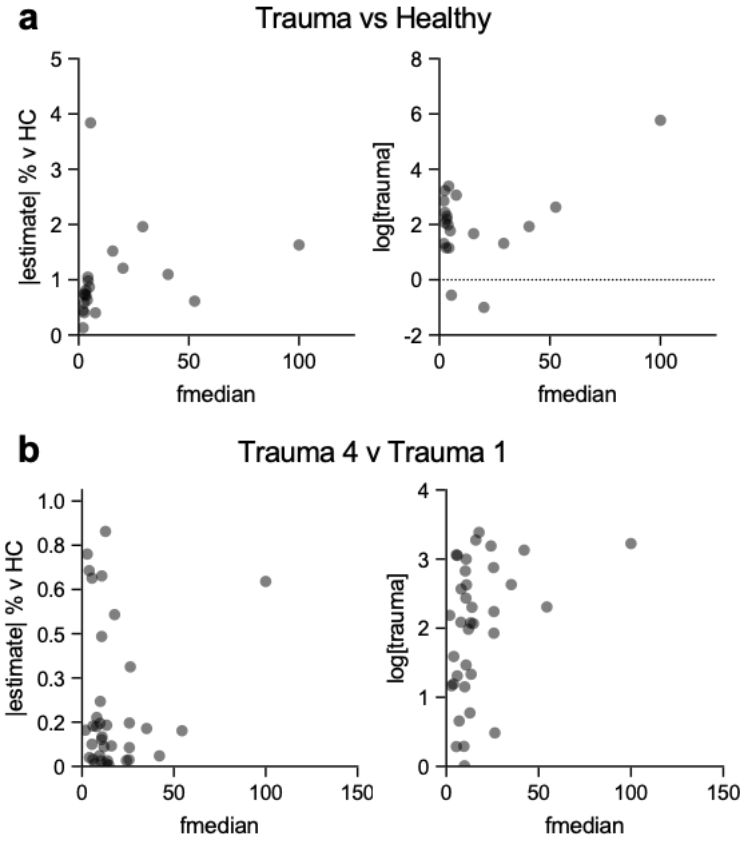
963 **Supplemental Figure 8 | Phenotypes of sub-cluster of patients with variable cytokine/chemokine**

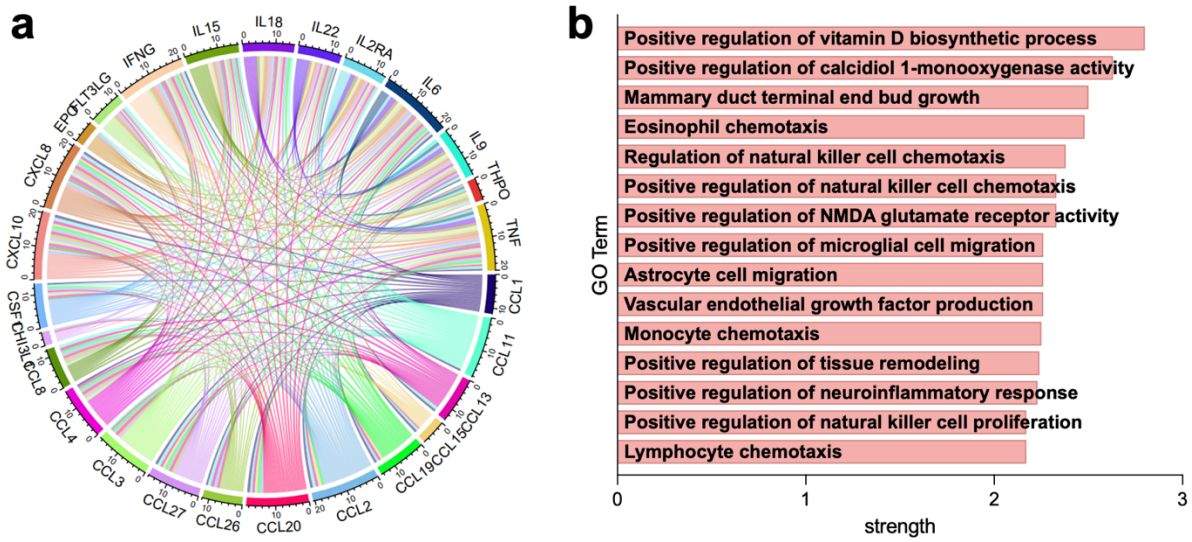
964 **levels**. Log-normalized concentration (pg/ml), black = above or below limit of detection (LOD).



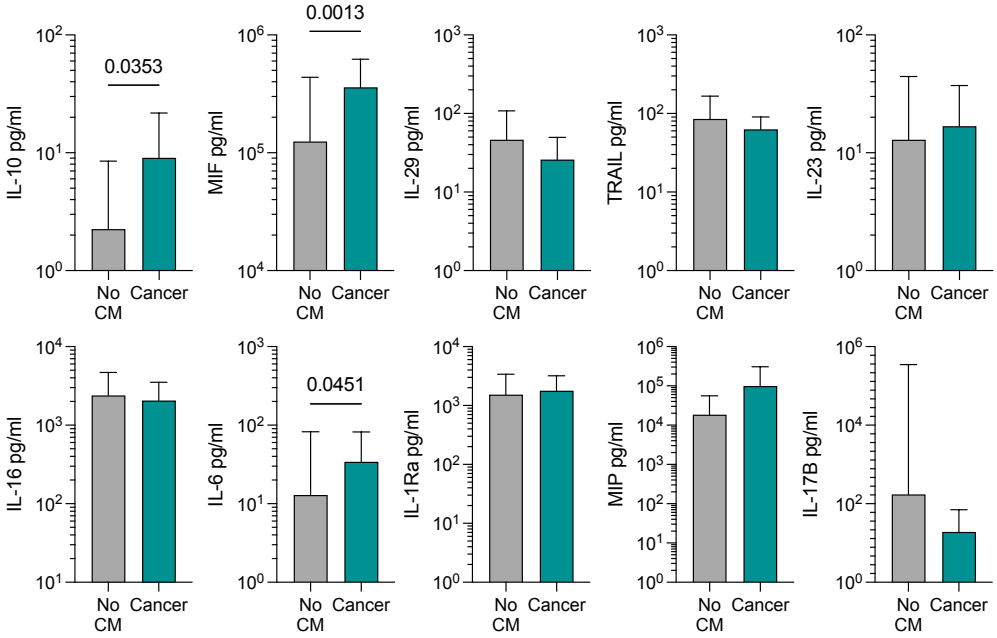
Supplemental Figure 9 | Relative permutation importance of different proteins on identification of trauma versus healthy control via machine learning. Boxplots are the protein's minimum, first quartile, median, mean (black dot), third quartile, and maximum relative importance values across 10 model iterations. Importance is ordered by average relative importance across all iterations.



Supplemental Figure 10 | Association of median relative importance with absolute percent change and analyte concentration. (a) Median relative importance (fmedian) of proteins across 10 model iterations versus its absolute percent change of concentration in trauma patients versus healthy controls (left) and concentration of analyte (right) in comparison between healthy controls and trauma. (b) Median relative importance (fmedian) of proteins across 10 model iterations versus its absolute percent change of concentration from trauma level 1 to trauma level 4 (left) and concentration of analyte (right) in comparison between trauma level.



Supplemental Figure 11 | STRING analysis of proteins upregulated with age. (a) Chord diagram generated based on STRING analysis of interactions among proteins upregulated with age. (b) STRING database gene ontology enrichment of biological processes from proteins upregulated with age (top 15 in strength of enrichment, FDR < 0. 05).



Supplemental Figure 12 | Exploratory analyses of trauma patients with no comorbidities versus those with reported cancer incidence. Grey = no comorbidities (CM), Teal = cancer patients (cancer). Data are mean ± standard deviation, p = student's T test (without correction for multiple comparisons).

	Count	%
Location		
Worcester	154	15.45
Charlotte	219	21.97
Baltimore	208	20.86
Jacksonville	223	22.37
Miami	193	19.36
Sex		
Male	725	72.50
Female	272	27.20
Unknown	3	0.30
Race		
White or EA	552	55.20
Black or AA	375	37.50
Asian or AsA	6	0.60
Native American/Alaska Native	6	0.60
Other	28	2.80
Unknown	33	3.30
Ethnicity		
Hispanic/Latino	162	16.20
Not H/L	818	81.80
Unknown	20	2.00
Age		
18 - 39	499	50.51
40 - 70	373	37.75
> 70	113	11.44
Unknown	3	0.30
Mechanism of Injury		
MVC	448	37.87
Fall	221	18.68
GSW	117	9.89
Assault	221	18.68
Stab	54	4.56
Fire/burn	17	1.44
Nature/environment	3	0.25
Non-Motorized Transport	4	0.34
Drowning	1	0.08
Other Motorized Transport	16	1.35
Other, specified	77	6.51
Other, unspecified	1	0.08
Unknown	3	0.25
Tested for COVID?		
Yes	652	65.20
No	332	33.20
Unknown/NA	16	1.60

Supplemental Table 1 | Demographics and basic trauma information for patients. Sex = biologic sex. Race = Self-reported race (EA = European American, AA = African American, AsA = Asian American). Ethnicity = Self-reported ethnicity (H/L = Hispanic/Latino). MVC = motor vehicle crash, GSW = gunshot wound or shotgun wound.

Analyte	Aliases	Reported in humans systemically?	Trauma/Surgery/ Sepsis/TBI/Model Organism?	Reported Trend	This Study	References (PMID)	Notes
CTACK	CCL27	No	In vitro	None reported	DOWN	-	No literature on traumatic injury, only cell culture
ENA-78	CXCL5	No	Surgery	Increased (local tissue)	DOWN	8961388	Hepatectomy
Eotaxin	CCL11	Yes	Surgery	Decreased with trauma	DOWN	24602333	Muscle surgical trauma
Eotaxin-3	CCL26	No	Trauma	Increased (local tissue)	DOWN	29764285	Subdural hematoma fluids
FLT3L		No	TBI	Increased (local tissue)	DOWN	31017044, 25672780	TBI, Protective in MODS
I-309	CCL1	No	TBI	Increased (local tissue)	DOWN	23788036	Neural tissue, mouse
I-TAC	CXCL11	No	TBI	Increased (local tissue)	DOWN	33189916	TBI
IFNb		No	Trauma	Increased (local tissue)	DOWN	27438769	Skin tissue
IFNg		No	Trauma	Decreased (isolated cells)	DOWN	20100328	T cells isolated from trauma patients
IL-10		Yes	Trauma	Increased with trauma	UP	19317866, 11030163, 18092384, 10580806	Multiple trauma types & severities
IL-12/IL-23p40		No		None reported	DOWN	-	No literature on traumatic injury
IL-13		Yes	TBI	No change (plasma)	DOWN	11089904	TBI
IL-15		Yes	Sepsis	Decreased (plasma)	DOWN	36915009	Sepsis in blunt trauma patients
IL-16		Yes	Trauma	Increased (plasma)	UP	15706184	General trauma patients
IL-17A/F		No	OA (Mouse)	Increased (local tissue)	DOWN	32955487	PTOA, mouse model
IL-17B		No		None reported	DOWN	-	No literature on traumatic injury, only autoimmune
IL-17E/IL-25	IL25	No		None reported	DOWN	-	No literature on traumatic injury, only autoimmune
IL-17F		No	OA (Mouse)	Increased (local tissue)	DOWN	32955487	PTOA, mouse model
IL-1Ra		Yes	Trauma	Increased (plasma, PBMCs)	UP	7474003, 7606873	Surgical and traumatic injury
IL-2		Yes	Trauma	Decreased (plasma)	DOWN	3877497, 2956432	Traumatic injury
IL-21		Yes	Trauma	Decreased (plasma)	DOWN	33748428	Down in severe trauma
IL-22		Yes	Trauma	Decreased (plasma)	UP	33748428	Down in severe trauma
IL-23		Yes	Trauma	Decreased (plasma)	UP	33748428	Down in severe trauma
IL-27		Yes	Sepsis	Increased (local tissue, serum)	DOWN	23842867, 31507598	BAL of lung injury, serum of sepsis patients
IL-29	IFNL1	No		None reported	UP	-	No literature on traumatic injury, only autoimmune
IL-3		Yes	Trauma	Decreased (plasma)	DOWN	9126191	Trauma hemorrhage
IL-31		No		None reported	DOWN	-	No literature on traumatic injury, only itch
IL-33		Yes	Trauma	Increased (plasma)	DOWN	33771098	Early in crtically injured patients
IL-4		Yes	Trauma	Decreased (serum)	DOWN	24402554	Orthopedic trauma
IL-5		No		None reported	DOWN	-	No literature on traumatic injury
IL-6		Yes	Trauma	Increased (serum)	UP	24402554	Orthopedic trauma
IL-7		Yes	Trauma	Decreased (serum)	DOWN	31246919	Chest wound + TBI versus TBI alone
IL-8	CXCL8	Yes	Trauma	Conflicting data (serum)	DOWN	24402554, 32837068	Increased in orthopedic trauma, decreased in thoracic trauma
IL-9		Yes	Trauma	Decreased (plasma)	DOWN	33748428	Decreased in severe blunt trauma patients
IP-10	CXCL10	Yes	Trauma	Increased (plasma)	DOWN	19717036	Increased in multi organ failure patients versus other trauma patients
MCP-1	CCL2	Yes	Trauma	Increased (serum)	DOWN	37562231	Greater in nonsurvivors of trauma
MCP-4	CCL13	Yes	TBI	Increased (plasma)	DOWN	29685283	Brain injury (concussed)
MDC	CCL22	No	OA (Rat)	Increased (serum)	DOWN	30623804	Rat model of OA
MIF		Yes	Trauma	Increased (serum)	UP	20499270	Higher in post-trauma MODS
MIP-1a	CCL3	No	Surgery	Increased (local tissue)	DOWN	26543677, 21371163	Increased in wound fluid of surgical wounds
MIP-1b	CCL4	No	Surgery	Increased (local tissue)	DOWN	26543677, 21371163	Increased in wound fluid of surgical wounds
MIP-3a	CCL20, LARC	No		None reported	DOWN	-	No literature on traumatic injury
MIP-3b	CCL19, ELC	No		None reported	DOWN	-	No literature on traumatic injury, only autoimmune
MIP-5	CCL15	No		None reported	UP	-	No literature on traumatic injury
SDF-1a		No	Trauma	Increased (local tissue)	DOWN	28272352	Produced at the site of injury
TARC		Yes	Trauma	Increased (plasma)	DOWN	37120600	Trauma hemorrhagic shock
TNFa		Yes	Trauma	No change (plasma)	DOWN	8230332	General trauma patients
TPO	THPO, MGDF	Yes	Sepsis	Increased (serum)	DOWN	10997881	Sepsis
TRAIL	CD253, TNFSF10	No	Trauma	Increased (local tissue)	UP	21760914	Gene expression, local tissue of elderly bone fractures
VEGF-A	VEGF	Yes	Trauma	Increased (serum)	DOWN	9711425	Polytrauma and burns

Supplemental Table 2 | Literature review of cytokines analyzed and their reported trends in trauma and associated conditions. Review on both GoogleScholar and PubMed search engines using key words "trauma". "injury", "wound", "traumatic injury", with or without "human". Publications are only listed that are indexed in PubMed.

Analyte	Aliases	Reported Trend with Age	This Study	References (PMID)	Notes
CTACK	CCL27	None reported	UP	-	
ENA-78	CXCL5	None reported	DOWN	-	
EPO		Increase with age	UP	25915923	
Eotaxin	CCL11	Increase with age	UP	26080062	
Eotaxin-3	CCL26	Decrease with age	UP	30685456	Dermatitis patients
FLT3L		Increase with age	UP	26080062	
Gro-a	CXCL1	None reported	DOWN	-	
I-309	CCL1	None reported	UP	-	
IFNg		Increase with age	UP		In children
IL-12/IL-23p40		Increase with age	UP	10671301	Evaluated total IL-12
IL-15		Increase with age	UP	16192677	
IL-16		None reported	DOWN	-	
IL-18		Increase with age	UP	21571262	Secreted from dendritic cells (not total)
IL-22		None reported	UP	-	
IL-27		Trended increase	UP		
IL-2Ra	CD25	None reported	UP	-	
IL-6		Debated	UP	1453878, 11213271	Some reports of increase, some decrease with age
IL-8	CXCL8	None reported	UP	-	
IL-9		None reported	UP	-	
IP-10	CXCL10	Increase with age	UP	26080062	Trauma Patients
M-CSF	CSF1	Trended increase	UP	26080062	
MCP-1	CCL2	Increase with age	UP	23039889	Study incorrectly calls MCP-1 as CCL1, cited here under MCP-1
MCP-2	CCL8	None reported	UP	-	
MCP-4	CCL13	Increase with age	UP	26080062	
MIP-1a	CCL3	None reported	UP	-	
MIP-1b	CCL4	Decrease with age	UP	30448299	Trauma Patients
MIP-3a	CCL20, LARC	None reported	UP	-	
MIP-3b	CCL19, ELC	None reported	UP	-	
MIP-5	CCL15	None reported	UP	-	
TNFa		Increase with age	UP	10931139	
TPO	THPO, MGDF	None reported	UP	-	
TRAIL	CD253, TNFSF10	None reported	DOWN	-	
VEGF-A	VEGF	Increase with age	UP	26080062	
YKL-40	CHI3L1	Increase with age	UP	18070151	

Supplemental Table 3 | Literature review of cytokines analyzed and their reported trends with age in human subjects. Review on both GoogleScholar and PubMed search engines using key words “age”, “increase”, “decrease”, “correlate with age”, and “human” or “patient”. Publications are only listed that are indexed in PubMed.

Analyte	ALIAS	Description in STRING
CTACK	CCL27	C-C motif chemokine 27; Chemotactic factor that attracts skin-associated memory T- lymphocytes. May play a role in mediating homing of lymphocytes to cutaneous sites. Binds to CCR10. [a.k.a. R-HSA-388396, IL-11 R-alpha-locus chemokine, R-HSA-162582, CTACK]
ENA-78	CXCL5	C-X-C motif chemokine 5; Involved in neutrophil activation. In vitro, ENA-78(8-78) and ENA-78(9-78) show a threefold higher chemotactic activity for neutrophil granulocytes; Belongs to the intercrine alpha (chemokine CxC) family. [a.k.a. R-HSA-388396, CAC42884.1, chemokine (C-X-C motif) ligan
Eotaxin	CCL11	In response to the presence of allergens, this protein directly promotes the accumulation of eosinophils, a prominent feature of allergic inflammatory reactions. Binds to CCR3. [a.k.a. eotaxin-1, R-HSA-162582, ENST00000305869, eotaxin, Eotaxin, ...]
Eotaxin-2		C-C motif chemokine 24; Chemotactic for resting T-lymphocytes, and eosinophils. Has lower chemotactic activity for neutrophils but none for monocytes and activated lymphocytes. Is a strong suppressor of colony formation by a multipotential hematopoietic progenitor cell line. Binds to CCR3. [a.k.a.
Eotaxin-3	CCL26	C-C motif chemokine 26; Chemoattractant for eosinophils and basophils. Acts as a ligand for C-C chemokine receptor CCR3 which triggers Ca(2+) mobilization in eosinophils. [a.k.a. Q92LV8, NP_001358867.1, ENST00000005180, Eotaxin-3, eotaxin-3, ...]
EPO		Erythropoietin; Hormone involved in the regulation of erythrocyte proliferation and differentiation and the maintenance of a physiological level of circulating erythrocyte mass. Binds to EPOR leading to EPOR dimerization and JAK2 activation thereby activating specific downstream effectors, including
FLT3L		Fms-related tyrosine kinase 3 ligand; Stimulates the proliferation of early hematopoietic cells by activating FLT3. Synergizes well with a number of other colony stimulating factors and interleukins. [a.k.a. AAB64685.1, B9EGH2, XP_011524980.1, FLT3L, FIGL, ...]
Fractalkine		Processed fractalkine; Acts as a ligand for both CX3CR1 and integrins. Binds to CX3CR1. Binds to integrins ITGAV/ITGB3 and ITGA4/ITGB1. Can activate integrins in both a CX3CR1-dependent and CX3CR1-independent manner. In the presence of CX3CR1, activates integrins by binding to the c
Gro-a		Growth-regulated alpha protein; Has chemotactic activity for neutrophils. May play a role in inflammation and exerts its effects on endothelial cells in an autocrine fashion. In vitro, the processed forms GRO-alpha(4-73), GRO- alpha(5-73) and GRO-alpha(6-73) show a 30-fold higher chemotactic activi
I-309	CCL1	C-C motif chemokine 1; Cytokine that is chemotactic for monocytes but not for neutrophils. Binds to CCR8; Belongs to the intercrine beta (chemokine CC) family. [a.k.a. R-HSA-388396, R-HSA-162582, NM_0029812, I-309]
ITAC	CXCL11	C-X-C motif chemokine 11; Chemotactic for interleukin-activated T-cells, but not unstimulated T-cells, neutrophils or monocytes. Induces calcium release in activated T-cells. Binds to CXCR3. May play an important role in CNS diseases which involve T-cell recruitment. May play a role in skin immune
IFNb		Interferon beta; Has antiviral, antibacterial and anticancer activities; Belongs to the alpha/beta interferon family. [a.k.a. uc003zxk.4, ENST00000380232, Interferon beta 1, IFNB]
IFNg		Interferon gamma; Produced by lymphocytes activated by specific antigens or mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the ar
IL-10		Interleukin-10; Major immune regulatory cytokine that acts on many cells of the immune system where it has profound anti-inflammatory functions, limiting excessive tissue disruption caused by inflammation. Mechanistically, IL10 binds to its heterotetrameric receptor comprising IL10Ra and IL10RB le
IL-12/IL-23p40		N/A
IL-13		Interleukin-13; Cytokine. Inhibits inflammatory cytokine production. Synergizes with IL2 in regulating interferon-gamma synthesis. May be critical in regulating inflammatory and immune responses. Positively regulates IL31RA expression in macrophages (By similarity). [a.k.a. ENST00000304506.7, N
IL-15		Interleukin-15; Cytokine that stimulates the proliferation of T-lymphocytes. Stimulation by IL15 requires interaction of IL15 with components of the IL2 receptor, including IL2RB and probably IL2RG but not IL2RA. In neutrophils, stimulates phagocytosis probably by signaling through the IL15 receptor, co
IL-16		Pro-interleukin-16; Interleukin-16 stimulates a migratory response in CD4+ lymphocytes, monocytes, and eosinophils. Primes CD4+ T-cells for IL-2 and IL-15 responsiveness. Also induces T-lymphocyte expression of interleukin 2 receptor. Ligand for CD4. Isoform 3 is involved in cell cycle progressi
IL-17A/F		Interleukin-17A; Ligand for IL17RA and IL17RC. The heterodimer formed by IL17A and IL17F is a ligand for the heterodimeric complex formed by IL17RA and IL17RC. Involved in inducing stromal cells to produce proinflammatory and hematopoietic cytokines. ; Belongs to the IL-17 family.Interleukin-1
IL-17B		Interleukin-17B; Stimulates the release of tumor necrosis factor alpha and IL- 1 beta from the monocytic cell line THP-1. [a.k.a. Interleukin-20, AF212311, AAK60336.1, IL-17B]
IL-17E/IL-25	IL25	Interleukin-25; Induces activation of NF-kappa-B and stimulates production of the proinflammatory chemokine IL-8. Proinflammatory cytokine favoring Th2-type immune responses; Belongs to the IL-17 family.
IL-17F		Interleukin-17F; Ligand for IL17RA and IL17RC. The heterodimer formed by IL17A and IL17F is a ligand for the heterodimeric complex formed by IL17RA and IL17RC. Involved in stimulating the production of other cytokines such as IL6, IL8 and CSF2, and in regulation of cartilage matrix turnover. Als
IL-18		Interleukin-18; A proinflammatory cytokine primarily involved in polarized T-helper 1 (Th1) cell and natural killer (NK) cell immune responses (Probable). Upon binding to IL18R1 and IL18RAP, forms a signaling ternary complex which activates NF-kappa-B, triggering synthesis of inflammatory mediati
IL-1Ra		Interleukin-1 receptor antagonist protein; Inhibits the activity of interleukin-1 by binding to receptor IL1R1 and preventing its association with the coreceptor IL1RAP for signaling. Has no interleukin-1 like activity. Binds functional interleukin-1 receptor IL1R1 with greater affinity than decoy receptor IL1R
IL-2		Interleukin-2; Produced by T-cells in response to antigenic or mitogenic stimulation, this protein is required for T-cell proliferation and other activities crucial to regulation of the immune response. Can stimulate B-cells, monocytes, lymphokine-activated killer cells, natural killer cells, and glioma cells. [a
IL-21		Interleukin-17C; Cytokine that plays a crucial role in innate immunity of the epithelium, including to intestinal bacterial pathogens, in an autocrine manner. Stimulates the production of antibacterial peptides and proinflammatory molecules for host defense by signaling through the NF-kappa-B and MAF
IL-22		Interleukin-22; Cytokine that contributes to the inflammatory response in vivo. [a.k.a. 1M4R, Q9GZX6, BC069308, IL-22]
IL-23		Interleukin-23 subunit alpha; Associates with IL12B to form the IL-23 interleukin, a heterodimeric cytokine which functions in innate and adaptive immunity. IL-23 may constitute with IL-17 an acute response to infection in peripheral tissues. IL-23 binds to a heterodimeric receptor complex composed o
IL-27		Myeloid-derived growth factor; Bone marrow-derived monocyte and paracrine-acting protein that promotes cardiac myocyte survival and adaptive angiogenesis for cardiac protection and/or repair after myocardial infarction (MI). Stimulates endothelial cell proliferation through a MAPK1/3-, STAT3- a
IL-29	IFNL1	Interferon lambda-1; Cytokine with antiviral, antitumour and immunomodulatory activities. Plays a critical role in the antiviral host defense, predominantly in the epithelial tissues. Acts as a ligand for the heterodimeric class II cytokine receptor composed of IL10RB and IFNLR1, and receptor engageme
IL-2Ra		Interleukin-2 receptor subunit alpha; Receptor for interleukin-2. The receptor is involved in the regulation of immune tolerance by controlling regulatory T cells (TREGs) activity. TREGs suppress the activation and expansion of autoreactive T-cells.
IL-3		Interleukin-3; Granulocyte/macrophage colony-stimulating factors are cytokines that act in hematopoiesis by controlling the production, differentiation, and function of 2 related white cell populations of the blood, the granulocytes and the monocytes-macrophages. [a.k.a. NP_0005792, R-HSA-893924
IL-31		Interleukin-31; Activates STAT3 and possibly STAT1 and STAT5 through the IL31 heterodimeric receptor composed of IL31RA and OSMR. May function in skin immunity. Enhances myeloid progenitor cell survival in vitro (By similarity). Induces RETNLA and serum amyloid A protein expression in mi
IL-33		Interleukin-33 (109-270); Cytokine that binds to and signals through the IL1RL1/ST2 receptor which in turn activates NF-kappa-B and MAPK signaling pathways in target cells. Involved in the maturation of Th2 cells inducing the secretion of T-helper type 2-associated cytokines. Also involved in activat
IL-4		Interleukin-4; Participates in at least several B-cell activation processes as well as of other cell types. It is a costimulator of DNA-synthesis. It induces the expression of class II MHC molecules on resting B-cells. It enhances both secretion and cell surface expression of IgE and IgG1. It also regulates th
IL-5		Interleukin-5; Factor that induces terminal differentiation of late- developing B-cells to immunoglobulin secreting cells; Belongs to the IL-5 family. [a.k.a. R-HSA-912526, IL5-202, XM_011543373.2, IL-5]
IL-6		Interleukin-6; Factor that induces terminal differentiation of late- developing B-cells to immunoglobulin secreting cells; Belongs to the IL-6 family. [a.k.a. R-HSA-912526, IL6-202, XM_011543373.2, IL-6]
IL-7		Interleukin-7; Hematopoietic growth factor capable of stimulating the proliferation of lymphoid progenitors. It is important for proliferation during certain stages of B-cell maturation. [a.k.a. 3574, ENST00000639719.1, 3D8, IL-7]
IL-8	CXCL8	Interleukin-8; IL-8 is a chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation. It is released from several cell types in response to an inflammatory stimulus. IL-8(6-77) has a 5–10-fold higher activity on neutrophil activation, IL-8(5-7
IL-9		Interleukin-9; Supports IL-2 independent and IL-4 independent growth of helper T-cells. Belongs to the IL-7/IL-9 family. [a.k.a. ENST00000274320.1, X17543, M30135, IL-9]
IP-10	CXCL10	C-X-C motif chemokine 10; Pro-inflammatory cytokine that is involved in a wide variety of processes such as chemotaxis, differentiation, and activation of peripheral immune cells, regulation of cell growth, apoptosis and modulation of angiostatic effects. Plays thereby an important role during viral infi
M-CSF		Processed macrophage colony-stimulating factor 1; Cytokine that plays an essential role in the regulation of survival, proliferation and differentiation of hematopoietic precursor cells, especially mononuclear phagocytes, such as macrophages and monocytes. Promotes the release of proinflammator
MCP-1	CCL2	C-C motif chemokine 2; Acts as a ligand for C-C chemokine receptor CCR2. Signals through binding and activation of CCR2 and induces a strong chemotactic response and mobilization of intracellular calcium ions. Exhibits a chemotactic activity for monocytes and basophils but not neutrophils or e
MCP-2		C-C motif chemokine 8; Chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils. May play a role in neoplasia and inflammatory host responses. This protein can bind heparin. The processed form MCP-2(6-76) does not show monocyte chemotactic activity, but inhibits the
MCP-4	CCL13	C-C motif chemokine 13; medium chain; Chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils, but not neutrophils. Signals through CCR2B and CCR3 receptors. Plays a role in the accumulation of leukocytes at both sides of allergic and non-allergic inflammation. May
MDC	CCL22	Disintegrin and metalloproteinase domain-containing protein 11; Probable ligand for integrin in the brain. This is a non catalytic metalloprotease-like protein. [a.k.a. BAA04213.1, NP_001305862, ENSP00000220557, MDC]
MIF		Macrophage migration inhibitory factor; Pro-inflammatory cytokine. Involved in the innate immune response to bacterial pathogens. The expression of MIF at sites of inflammation suggests a role as mediator in regulating the function of macrophages in host defense. Counteracts the anti-inflammatory
MIP-1a	CCL3	C-C motif chemokine 3; Monokine with inflammatory and chemokinetic properties. Binds to CCR1, CCR4 and CCR5. One of the major HIV-suppressive factors produced by CD8+ T-cells. Recombinant MIP-1 alpha induces a dose- dependent inhibition of different strains of HIV-1, HIV-2, and simian
MIP-1b	CCL4	C-C motif chemokine 4; Monokine with inflammatory and chemokinetic properties. Binds to CCR5. One of the major HIV-suppressive factors produced by CD8+ T-cells. Recombinant MIP-1 beta induces a dose-dependent inhibition of different strains of HIV-1, HIV-2, and simian immunodeficiency \
MIP-3a	CCL20, LARC	C-C motif chemokine 20; Acts as a ligand for C-C chemokine receptor CCR6. Signals through binding and activation of CCR6 and induces a strong chemotactic response and mobilization of intracellular calcium ions. The ligand- receptor pair CCL20-CCR6 is responsible for the chemotaxis of dend
MIP-3b	CCL19, ELC	C-C motif chemokine 19; May play a role not only in inflammatory and immunological responses but also in normal lymphocyte recirculation and homing. May play an important role in trafficking of T-cells in thymus, and T-cell and B-cell migration to secondary lymphoid organs. Binds to chemokine rec
MIP-5	CCL15	C-C motif chemokine 15; Chemotactic factor that attracts T-cells and monocytes, but not neutrophils, eosinophils, or B-cells. Acts mainly via CC chemokine receptor CCR1. Also binds to CCR3. CCL15(23-92), CCL15(25-92) and CCL15(29-92) are more potent chemoattractants than the small-indu
SDF-1a		Stromal cell-derived factor 1; Chemoattractant active on T-lymphocytes and monocytes but not neutrophils. Activates the C-X-C chemokine receptor CXCR4 to induce a rapid and transient rise in the level of intracellular calcium ions and chemotaxis. SDF-1-beta(3-72) and SDF-1-alpha(3-67) show a
TARC		C-C motif chemokine 17; Chemotactic factor for T-lymphocytes but not monocytes or granulocytes. May play a role in T-cell development in thymus and in trafficking and activation of mature T-cells. Binds to CCR4. [a.k.a. D43767, 1NR4, C-C motif chemokine ligand 17, TARC]
TNFa		Tumor necrosis factor, membrane form; Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is i
TPO	THPO, MGDF	Thrombopoietin; Lineage-specific cytokine affecting the proliferation and maturation of megakaryocytes from their committed progenitor cells. It acts at a late stage of megakaryocyte development. It may be the major physiological regulator of circulating platelets. [a.k.a. ENSG00000090534, ENST0
TRAIL	CD253, TNFSF	Tumor necrosis factor ligand superfamily member 10; Cytokine that binds to TNFRSF10A/TRAILR1, TNFRSF10B/TRAILR2, TNFRSF10C/TRAILR3, TNFRSF10D/TRAILR4 and possibly also to TNFRSF11B/OPG. Induces apoptosis. Its activity may be modulated by binding to the decoy receptors T
YKL-40		Chitinase-3-like protein 1; Carbohydrate-binding lectin with a preference for chitin. Has no chitinase activity. May play a role in tissue remodeling and in the capacity of cells to respond to and cope with changes in their environment. Plays a role in T-helper cell type 2 (Th2) inflammatory response and
VEGF-A	VEGF	N/A

Supplemental Table 4 | STRING description of each protein. Protein descriptions from STRING database.

	True Negative	False Positive	False Negative	True Positive
Logistic Regression with Class Weights	121	19	2	3
Logistic Regression with SMOTE	114	26	1	4
Random Forest with Class Weights	137	3	4	1
Gradient Boosting with Class Weights	138	2	2	3
XGBoost with Class Weights	132	8	2	3

Supplemental Table 5 | Confusion Matrices for Models Applied to Determine Survival. Number of true negatives, false negatives, true positives, and false positives from a sample subset used to test the accuracy of machine learning models predicting death based on the expression levels of the 5-analyte panel.

SUPPLEMENTAL METHODS

Random Forest Model Tuning

Data was split 70/30 into training/test datasets, maintaining the ratio of trauma:healthy patients in each set. Hyperparameters were tuned with tidymodels grid search (n=20) to determine the appropriate number of variables to randomly sample at splitting (mtry) and minimum amount of data needed to split (min_n). Tuning was done with 10 trees using 5-fold cross validation to select the model with the highest AUC-ROC as the best model. This model was trained on the training set, then applied to test data. It was then validated on both training and test data separately with the same tuned mtry and min_n parameters and 200 trees. Predictions, model metrics, and variable permutation importance values from these final models were collected.

Random Forest Variable Importance

Permutation importance calculates the influence of each variable on model prediction based on how much a change in the variable's values affects the model's predictive error.[43] To derive summary statistics of variable importance values and to minimize the chance of a variable erroneously showing up as important, the model training and validation steps were repeated on the dataset 10 times from different seeds and the permutation importance for each variable was ranked for each iteration. The raw variable importance values were then converted to relative importance by dividing each variable importance score by the largest importance score of the variables for each of the 10 iterations and multiplied by 100.[54] Variables whose permutation importance ranked in the top 10 in at least 5 of the iterations were plotted, along with their minimum, first quartile, median, mean, third quartile, and maximum importance values.

SUPPLEMENTARY DISCUSSION

Confirmation of literature findings:

Our observation of decreased IL-2 levels, with many below the LOD, in trauma patients versus control samples is consistent with reports of reduced IL-2 production (associated with T cell activation) in response to traumatic injury.[64,65] In agreement with the literature, trauma patients also exhibited increased levels of several other interleukins, including IL-16,[66,67] IL-1Ra,[68,69] and

IL-6,[3] as well as a decrease in IL-21[4] and IL-3.[70] We observed several other patterns consistent with previous literature. These include MIF (upregulated[71]), TRAIL (upregulated and previously reported to be increased in elderly osteoporotic fractures[72]), and Eotaxin (downregulated, and previously reported to be downregulated in musculoskeletal surgical trauma[67,73]).

Downregulated proteins that are contrary to literature findings:

We also observed some trends that differ from those reported in the literature. The downregulation of VEGF-A, which is involved in angiogenesis, in trauma samples compared to healthy samples is especially interesting due to the involvement of angiogenesis in tissue repair. Although previous studies have demonstrated increased serum VEGF levels on day of arrival for both trauma and burn patients,[74] we observed a lower level of VEGF-A in trauma samples, regardless of injury mechanism, general wound type, specific wound type, wound location, or trauma level. This suggests a potential decrease in angiogenesis in the immediate aftermath of injury, possibly as a mechanism to control blood loss.

Of the other proteins for which we found trends that are contrary to what was previously published in the literature, evaluation of many of these proteins in human patients had primarily been limited to TBI. Increased levels of FLT3L, which activates FLT3 to stimulate proliferation of hematopoietic cells, have been reported in a study of the cerebral fluid of 10 patients that had experienced severe TBI.[20] In mice, FLT3L has been shown to exhibit protective effects in ischemia-reperfusion injury, volumetric muscle loss, and multi-organ dysfunction syndrome.[75–77] We observed significant decreases in FLT3L levels in trauma, as well as for each injury mechanism, general wound type, specific wound type, and wound location, versus healthy controls. This was also true for patients who had head/neck wounds, although it cannot be assumed that those patients would have TBI. Thus, the type of injury (ex. TBI vs. Non-TBI) likely played a role in the differences in our observation compared to the previous reports. In a study of brain tissue samples from 12 patients with severe TBI, I-TAC, which is chemotactic for IL-activated T-cells, was found to be upregulated. Although we observed an upregulation of I-TAC in trauma patients compared to healthy controls, this difference was not statistically significant. Nevertheless, other factors such as the use of systemic fluids (I.e. blood) versus localized fluids or tissues should also be taken into consideration, as it is possible that local elevation of proteins would not be reflected in systemically-sampled fluid.

Levels of IL-7, which is involved in stimulation of lymphoid progenitors, have previously been reported to be elevated with increasing injury severity for trauma patients,[4] although we observed a significantly lower level of IL-7 for trauma patients versus controls, and no significant difference between IL-7 levels in the most severe (level 1) cases compared to less severe (levels 2, 3, or 4) cases. However, this is likely a result of our inclusion of non-ICU patients, while the Cai et al. study included only trauma patients who were admitted to the ICU and survived to discharge. Another study found that TBI patients with chest injury had lower mean IL-7 levels than TBI patients without chest injury.[19] Although we observed a significant decrease in IL-7 levels across all wound types, wound locations, and injury mechanisms, these findings altogether suggest the importance of injury characteristics on IL-7 levels. This is further supported by conflicting reports on the levels of a number of other cytokines, which is likely a result of differences in patient characteristics. For example, IL-8 levels were shown to be higher in patients with orthopedic injures compared to healthy controls,[3] while serum IL-8 levels were shown to be higher in healthy controls compared to chest trauma patients.[78] Meanwhile, we observed lower IL-8 levels for trauma patients across all injury mechanisms, wound types, and wound locations, compared to healthy controls. TPO also has conflicting reports in the literature, with one study finding that patients with multiple trauma have elevated serum TPO in the days following injury,[79] while another study found that TPO levels were not measurable for patients with major lower extremity trauma.[80] Lower TPO levels have also been associated with nonsurvival in patients that have experienced major trauma.[81] We observed significantly lower TPO in trauma patients compared to healthy controls, regardless of injury mechanism, wound type, and wound location, However, patients with the most severe injuries exhibited higher TPO levels than patients with the least severe injuries, which appears to support prior findings of elevated TPO after severe injury.

A statistically significant decrease in concentration of IL-17E/IL-25 was observed for injury mechanisms of GSW and MVC but was not statistically significant for the other injury mechanisms. Additionally, the wound type played a role in the concentration of IL-17E/IL-25. Patients who experienced only internal wounds or only penetrating wounds did not exhibit a significant decrease in this protein, but those who experienced both wound types did. We observed that reduced IL-17E/IL-25 concentration was statistically significant for patients who experienced central wounds or combined head/neck and central wounds, but not for patients who experienced combined head/neck and peripheral wounds, head/neck wounds only, combined peripheral and central wounds, or peripheral wounds only.

Novel reporting of proteins with changes in levels below the LOD:

The concentration of several proteins (IL-31, IL-5, MIP-3a) previously unreported in this setting were not significantly associated with trauma; however, there were significant differences in our assay's ability to detect a signal. Each of these proteins were significantly less likely to be detected in trauma samples compared to healthy controls. This suggests an exceptional potential reduction in the concentrations of these proteins. IL-31 is linked both to Th2 immunity associated with wound healing and to dermatitis. IL-5 is also associated with type-2 immunity and eosinophil inflammation. MIP-3a has been associated with autoimmunity in mouse models, and also downregulated with IL-10 which correlates with trauma that induces IL-10. Type-2 immune proteins have important impact on wound closure and collagen deposition, which are critical in long-term recovery of injuries. Disruption of these pathways presents another mechanism by which severe trauma limits healing ability by decreasing the induction of collagen stimulating immune responses to close wounds and strength those closures. Induction of these responses locally may provide a therapeutic window to assist in wound healing that may be compromised in patients sustaining severe trauma.

Upregulated proteins that are contrary to literature findings:

While levels of both IL-22 and IL-23 have been reported to decrease in severe trauma,[4] we did not observe a significant change in IL-22 levels between trauma and healthy control patients. However, we found a significant increase in IL-23 concentration of trauma samples compared to healthy controls, which held true for patients injured through falls or stabbings, but not those who were injured in MVC or GSW. IL-23 levels are significantly higher in more severe (level 1) injuries, compared to less severe (level 3, level 4) injuries, and IL-22 levels were also significantly higher in level 1 versus level 4 injuries. Increased IL-23 levels were observed for patients who experienced only internal soft tissue wounds, or only penetrating soft tissue wounds, but not for patients with bone involvement. The observed differences between trends of IL-22 and IL-23 levels in our study compared to the previous report may be attributed to differences in patient cohorts, as the Cai et al. cohorts were comprised of only patients admitted to the ICU and who survived to discharge.