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G-CSF Drives a Posttraumatic Immune Program That Protects the Host from Infection

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Traumatic injury is generally considered to have a suppressive effect on the immune system, resulting in increased susceptibility to infection. Paradoxically, we found that thermal injury to the skin induced a robust time-dependent protection of mice from a lethal Klebsiella pneumoniae pulmonary challenge. The protective response was neutrophil dependent and temporally associated with a systemic increase in neutrophils resulting from a re-prioritization of hematopoiesis toward myeloid lineages. A prominent and specific activation of STAT3 in the bone marrow preceded the myeloid shift in that compartment, in association with durable increases in STAT3-activated serum cytokines G-CSF and IL-6. Neutralization of the postburn increase in serum G-CSF largely blocked STAT3 activation in marrow cells, reversing the hematopoietic changes and systemic neutrophilia. Daily administration of rG-CSF was sufficient to recapitulate the changes induced by injury including hematopoietic re-prioritization and protection from pulmonary challenge with K. pneumoniae. Analysis of posttraumatic gene expression patterns in humans reveals that they are also consistent with a role for G-CSF as a switch that activates innate immune responses and suppresses adaptive immune responses. Our findings suggest that the G-CSF STAT3 axis constitutes a key protective mechanism induced by injury to reduce the risk for posttraumatic infection. The Journal of Immunology, 2014, 192: 000–000.

The human response to traumatic injury has recently been described as a “genomic storm,” in which the whole-blood leukocyte transcriptome is reshaped by a concomitant induction of innate immunity genes and suppression of adaptive immunity genes (1). These gene expression changes occur in both survivors and nonsurvivors, but the poorest outcomes are associated with an excessive magnitude and duration of the molecular response. Trauma, burn injury, and to some degree LPS exposure all result in a similar pattern of gene expression. The conserved molecular response to different stressors is thought to be driven by channeling of signals through shared pathways responsive to TLR4 activation, pathogen-associated molecular patterns, damage-associated molecular patterns, or alarms; however, no specific factors have been implicated in the induction of the “genomic storm.”

On a functional level, animal studies indicate that injury primes an enhanced innate immune reactivity resulting in excess production of soluble inflammatory mediators including TNF-α, IL-6, and IL-1β (2, 3), and heightened TLR-2 and TLR-4 reactivity (2, 4, 5). In trauma patients, circulating neutrophils are quantitatively increased, display a higher level of activation as measured by CD11b surface staining, and produce an increased oxidative burst upon stimulation (6). Triggering of deleterious inflammatory cascades by innate immune responses such as these is widely considered to contribute to high rates of lethal multiple organ failure in trauma patients (7). However, some animal models suggest that injury-induced augmentation of innate immunity may play a beneficial role in preventing infection. Several reports indicate that within a week of burn injury, mice display a heightened level of resistance to infection after i.p. challenge with Escherichia coli or Pseudomonas aeruginosa (8, 9) or s.c. challenge with P. aeruginosa (10–12). In studies that explored the mechanism of the protective phenomenon, resistance to infection developed with time after injury and was associated with increased numbers of functionally enhanced phagocytes. Our group recently reported that mice that were protected from s.c. infection 8 d after burn injury also exhibited increased mortality when challenged with LPS (10). This effect was abrogated after global depletion of myeloid cells with gemcitabine, suggesting that inflammatory tone and burn-induced resistance to infection are linked.

In contrast with the enhanced innate immune reactivity, several lines of evidence demonstrate that adaptive immune functions in burn-injured animals and humans are suppressed. These include prolonged skin-homograft survival, reductions in delayed-type hypersensitivity reactions, and impaired lymphocyte responses (13–15). Early after burn injury and for up to several days thereafter, quantitative reductions in T cell populations occur because of apoptosis (16, 17) and lymphocyte proliferative responses are impaired (18). Production of IL-2, IFN-γ, and IL-12 are reported to be preferentially suppressed after burn injury (19, 20), whereas production of IL-4 and IL-10 are increased (21), consistent with a Th1 to Th2
phenotypic switch. Th1 suppressing CD4+CD25+ T regulatory cells are suspected to play a role in the suppression of Th1 responses seen in injured humans and mice (22, 23). In addition, our group has previously noted that global depletion of myeloid cells with gemcitabine restores T lymphocyte proliferative function in burn-injured mice (10), suggesting that myeloid cells also play a role in the suppression of adaptive immune function after injury.

Traumatic injury also results in profound changes in hematopoiesis in mice and humans, best characterized as a hematopoietic reprogramming in which myeloid cells expand in the marrow, whereas other lineages are reduced (24, 25). The reprogramming of the marrow away from red cell production occurs despite high levels of circulating erythropoietin (EPO) in trauma patients, and in burn-injured mice, EPO administration does not restore normal reticulocytosis. Human burn victims are also refractory to treatment with EPO (26). Marrow reprogramming is thought to contribute to the EPO-resistant anemia of critical illness, which accounts for >50% of the transfusion requirements in burn patients (27).

Although the described injury-induced changes in innate and adaptive immune function, as well as the alteration of marrow priorities, have been a prominent focus of trauma research over several decades, no study has systematically linked these changes to a specific factor or factors induced by injury. In this study, we demonstrate that thermal injury of the skin in mice results in a paradoxical protection against a lethal Klebsiella pneumoniae pulmonary infection, associated with a myeloid-specific activation of STAT3 in the marrow, hematopoietic reprogramming, and a systemic expansion of functionally enhanced neutrophils. We provide evidence that these processes in mice are driven by G-CSF, and that remarkably congruent human gene expression profiles for G-CSF administration and trauma are consistent with a central role for G-CSF as a regulator of the "genomic storm," driving divergent innate and adaptive immune responses after traumatic injury.

Materials and Methods

Mouse injury model

All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Non-Swiss Albino Outbred Mice Hsd: Nsa(CF-1) were purchased from Harlan Laboratories (Indianapolis, IN). The burn procedure used resulted in a full-thickness injury, as previously described (11, 28). In brief, mice were anesthetized with isoflurane and covered with a flame-resistant template exposing a dorsal skin section equivalent to 15% of total body surface area. The target area was saturated with 0.5 ml absolute ethanol, and either ignited for 3 or allowed to evaporate without ignition. Immediately after burn or sham procedure, mice received 0.5 ml saline for volume resuscitation via the i.p. route.

Infection procedure and quantitative culture

K. pneumoniae KP2 2-70, a virulent and highly encapsulated Gram-negative bacterial strain (29, 30), was grown overnight in brain heart infusion broth. The burn procedure used resulted in a full-thickness injury, as previously described (11, 28). In brief, mice were anesthetized with isoflurane and covered with a flame-resistant template exposing a dorsal skin section equivalent to 15% of total body surface area. The target area was saturated with 0.5 ml absolute ethanol, and either ignited for 3 or allowed to evaporate without ignition. Immediately after burn or sham procedure, mice received 0.5 ml saline for volume resuscitation via the i.p. route.

Isolation of whole lung and blood cells

Whole lung tissue cells were isolated in a two-step protocol using a gentleMACS Dissociator according to manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Dissociation buffer was composed of 2 ml DPBS, 150 U/ml Type IV collagenase (catalog no. L500418; Worthington Biochemical), and 50 U/ml DNase (catalog no. L5002139; Worthington Biochemical). Cell suspensions were filtered through 70-μm filters and washed with DPBS. Leukocytes were isolated by LyphoMise—M separation (Cedarlane Laboratories, Burlington, ON, Canada). Blood was collected by cardiac puncture and stored in EDTA-coated tubes before analysis using the Hemavet 950 system (Drew Scientific, Waterbury, CT).

Quantitative depletion of neutrophils

Neutrophils were depleted with 200 μg anti-Ly6G (clone 1A8; BioXcell, West Lebanon, NH), delivered i.p. in a 100-μl volume of saline 1 d pre-infection and again at the time of infection. Rat IgG2a clone 2A3 (BioXcell) was used as an isotype control, delivered at the same dose and by the same route and schedule as the neutrophil depleting Ab.

BAL cell collection and oxidative burst

Bronchial alveolar lavage (BAL) was performed by three cycles of inflation and aspiration using 0.7 ml HBSS, repeated three times. Recovery was routinely ≥80% of instilled volume. Harvested BAL cells were collected by centrifugation at 500 × g for 5 min, washed, counted, and re-suspended in FACS buffer before staining with Abs for flow cytometry. For analysis of oxidative burst, BAL cells were collected 4 h after i.n. challenge with K. pneumoniae at 4 × 103 CFU/mouse. Cells were incubated in HBSS + 5 μM DHR123 (catalog no. D-23806; Invitrogen) at 37˚C for 10 min, stimulated with 1 μM PMA (catalog no. P1585; Sigma-Aldrich) for 30 min, placed on ice, and stained for FACS analysis. Neutrophils were identified by the staining pattern CD45+ CD11b+ Ly6G− F4/80-.

Serum and BAL fluid cytokine determinations

For isolation of serum, whole blood was collected by cardiac puncture, allowed to clot on ice, and centrifuged in BD Microtainer serum separator tubes. BAL fluid for cytokine quantitation was from the first cycle of inflation and aspiration described in BAL method. Serum and BAL fluid were frozen at −70˚C until analysis using Milliplex MAP Kits (Millipore, Billerica, MA) according to manufacturer’s protocol. Plates were read on the Bio-Plex (Bio-Rad, Hercules, CA), and concentrations were calculated using recombinant protein standards. Cytokine analysis was performed by the Research Flow Cytometry Core at Cincinnati Children’s Hospital Medical Center.

Bone marrow cell harvest and phoxflow cytometry

Bone marrow isolation was performed by flushing the femurs of mice with 1.5 ml HBSS, then filtering the eluate through 70-μm filters, and all subsequent processing was conducted in the cold. The samples were filtered through a 70-μm filter, pelletted at 400 × g for 10 min, and then resuspended to a volume of 0.2 ml. Two milliliters of 100% ice-cold methanol was added to the pellet with vortexing. The samples were kept at −20˚C until staining, then washed with FACS buffer and stained with Abs to surface markers to phenotype the cells, as well as with Abs to characterize the phosphorylation state of intracellular signaling molecules. In the initial screening assay, Fluorescence Minus One (FMO) controls were used to set backgrounds and control for interference among fluorescence channels (32). Cells were interrogated for phosphorylation of the following proteins (resides): PE-ACKT(pT308), PE- pS38(pY182/pT180), AF647-ERK(pY202/pT204), AF647-STAT3(pY705), PE-STAT3(pY694), and AF647-STAT1(pY701) (BD Biosciences, San Jose, CA). For the time course of p-STAT3, background staining was evaluated with labeled p-STAT3 Ab in the presence of a 10-fold excess of unlabeled p-STAT3 Ab. In these experiments, identification of bone marrow cell subtypes was described as described in the following section.

Flow cytometry and identification of cell subtypes

Cells isolated from lung or bone marrow were resuspended in FACS buffer (DPBS, 0.1% sodium azide, and 1% BSA). Non-specific binding was blocked by using 2μl Fc Block (BD Biosciences) and 5 μl rat serum per 100 μl cell staining volume. Bone marrow cells were stained with fluoro-rescently labeled Abs to CD45 (leukocytes), CD45R (B cells), TER119 (red cells), CD117 (progenitors), CD11b (myeloid cells), and CD34 (myeloid progenitors; Biolegend or eBiosciences, San Diego, CA). Abs to LY6G and LY6C were used to identify CD11b+ LY6G− neutrophils and CD11b+ LY6C− monocytes (BD Biosciences, San Jose, CA). All Abs were directly conjugated with FITC, PE, PerCP CY 5.5, allophycocyanin, E450, or Cy5.9. Mouse IgG isotype controls were used as negative controls, with FACS analysis of CD45+ cells. Markers used to differentiate leuko-cyte subsets were as follows: T cells (CD3+), B cells (CD45R+), alveolar macrophages (CD11c+, F4/80+, CD11b+/dim), dendritic cells (CD11c+,...
Neutralization of G-CSF with rG-CSF treatment

Mice were injected i.p. twice daily with 7.5 μg/kg carrier free murine G-CSF (R&D Systems) in a volume of 100 μl PBS or with PBS alone for 6 consecutive days. On day 7, mice were sacrificed for determination of bone marrow composition by flow cytometry or infected with K. pneumoniae i.n. at 4 × 10^4 CFU/mouse. The vital status of infected mice was followed for 2 wk.

Comparison of whole-blood leukocyte gene expression profiles of G-CSF–treated humans and trauma victims

Gene Expression Omnibus (GEO) experiments GSE7400 (G-CSF mobilized leukocytes) and GSE36809 (A genomic storm in critically injured humans) were analyzed in GeneSpring 12.5, using robust multi-array average normalization and baselining each experiment’s data to the median of controls. In both data sets, we performed ANOVA to generate gene lists with differential regulation between controls and posttrauma (each time point was assessed) and post–G-CSF administration with rG-CSF treatment compared.

Results

Thermal injury of the skin induces a time-dependent reduction in K. pneumoniae–induced mortality, associated with enhanced pulmonary bacterial clearance

To determine whether mice develop resistance to a lethal pulmonary infection with time after thermal injury, as has been reported in models of postburn skin and i.p. infection, we challenged mice i.n. with 4 × 10^4 CFU K. pneumoniae at 2 h or 7 d after administering a sham injury or 15% total body surface area burn. Vital status was monitored over a 2-wk period postinfection. The survival curves of the burn and sham groups were very similar when the infectious challenge was delivered 2 h after injury, with ~50% of mice dying by day 6 (Fig. 1A), but the mortality of the groups diverged substantially when mice were challenged at 7 d after injury (Fig. 1B). The burned animals had a marked survival advantage compared with their sham counterparts at that late time point (p < 0.01 burn versus sham). Quantitative culture of homogenized lung and spleen tissues was performed at 24 and 48 h postinfection in mice challenged at day 7 after injury (Fig. 1C, 1D). At 24 h, the burden of organisms in the lungs of the burned animals was very similar to that of the original inoculum, and fell two logs by 48 h, consistent with progressive clearance of the organism from the lung. In the sham group, the burden of organisms at 24 h was about one log higher than that of the original inoculum and that of the burn group, and did not fall by 48 h, indicating failure of clearance. Splenic CFUs were also quantified as an index for systemic bacterial dissemination. No bacterial colonies were detected in homogenized splenic tissue at 24 h, but by 48 h, CFUs were two logs greater in the sham group than in the burn group.

Thermal injury induces a neutrophilic response in lung and blood after 7 d

To evaluate the role of inflammation in the time-dependent, burn-induced protection from infection, we examined the leukocyte composition of lung tissue and peripheral blood harvested from uninfected, day 7 after burn- and sham-injured mice. FACS analysis of whole-lung tissue from burned mice revealed a significant increase in lung-associated neutrophils, an increase in inflammatory monocytes, and a reduction in B cells (Fig. 2A), relative to sham injured controls. Proportions of lung tissue–associated T cells, alveolar macrophages, dendritic cells, and monocytes were not different between the groups. Quantitation of peripheral blood leukocytes revealed an increase in circulating neutrophils in the burn group compared with the sham group, but no differences in total WBC counts, lymphocytes, or monocytes were detected in circulating cells (Fig. 2B). The systemic neutrophilic response was accompanied by a reduction in RBC numbers, as well as in hemoglobin and hematocrit (Fig. 2C).

Protection from pulmonary infection induced by thermal injury is neutrophil dependent

The role of the neutrophilic response in thermal injury–induced resistance to infection was assessed by quantitative depletion of neutrophils in burn- and sham-injured mice before infectious challenge. The neutrophil-depleting Ab, anti-Ly6G (clone 1A8), or an isotype control Ab was administered i.p. on day 6 after injury and at the time of infection on day 7 after injury. The protection from pulmonary infection induced by injury was apparent in isotype-treated mice, with a substantial mortality reduction in the burn-injured group compared with sham controls (p < 0.01 burn isotype versus sham isotype). The survival curves for the anti-Ly6G sham and burn groups were overlapping with each other and with the isotype–Ab–treated sham control, indicating that protection afforded by burn injury was completely reversed by neutrophil depletion (Fig. 3).

Assessment of airspace neutrophil recruitment, oxidative burst, and BAL cytokine levels indicates neutrophils from thermally injured mice are primed for an enhanced oxidative response

To quantify neutrophil recruitment, we challenged mice 7 d after burn or sham injury with 4 × 10^4 K. pneumoniae and collected BAL cells for neutrophil quantitation by FACS at 0, 4, and 24 h after challenge (Fig. 4A). Preinfection, F4/80^+ Ly6G^+ cells (neutrophils) comprised <1% of airway cells. Upon administration of the bacterial inoculum, a similar increase in neutrophils was seen in sham- and burn-injured mice, reaching ~10% of airway cells at 4 h postchallenge. After 24 h of infection, a trend toward greater increases in neutrophils in the sham-injured mice appeared to be developing; however, statistically significant differences between the groups were not seen at any time point.
To assess the effect of thermal injury on the functional state of neutrophils recruited to the airspace early postinfection, we challenged mice 7 d after burn or sham injury i.n. with \(4 \times 10^4\) K. pneumoniae. Four hours later, BAL cells were isolated and stimulated with PMA, then subjected to FACS analysis to quantify the oxidation-driven conversion of DHR123 to fluorescent R123, as well as CD11b surface expression, measures of oxidative burst and degranulation, respectively (33). BAL neutrophils from burn-injured mice responded to PMA stimulation with a more robust oxidative response and greater degree of degranulation (Fig. 4B) than those from sham mice.

To evaluate potential differences in airspace inflammatory tone between sham- and burn-injured mice, we quantified soluble inflammatory mediators present in BAL fluid collected 0, 4, and 24 h postinfection of mice challenged with K. pneumonia on day 7 after thermal injury. Preinfection, all analytes tested were below the detectable limit of our assay (data not shown). At 4 h postinfection, the time at which cells were collected for functional assays, levels of G-CSF, IL-6, KC, MIP-2, and TNF-α were all found to be increased to levels that were similar between burn- and sham-treated groups (Fig. 4C). After 24 h of infection, there was a trend toward increased levels of several inflammatory cytokines and chemokines in sham-injured mice compared with the burned animals, although these between-group differences did not reach statistical significance (Fig. 4D).

**FIGURE 1.** Time-dependent survival of burn and sham-treated mice after early and late infectious pulmonary challenge. Burn- or sham-treated mice were infected i.n. with \(4 \times 10^4\) CFU K. pneumoniae/mouse (A) 2 h or (B) 7 d after thermal injury. Vital status was monitored for 14 d postinfection. Kaplan–Meier survival curves are shown for infectious challenges delivered 2 h after injury (n = 8 per group) and 7 d after injury (n = 16 per group). Quantitative culture of mice infected at 7 d postinjury. (C) Lung and (D) splenic tissues were harvested for quantitative culture at 24 and 48 h postinfection, and microbial burden was quantified as log transformed counts per whole tissue. Line represents the geometric mean of CFUs for n = 8 per group. *p < 0.05, **p < 0.01.

Time-dependent, injury-induced protection from pneumonia is temporally associated with hematopoietic reprioritization

Experiments were performed to test the hypothesis that postburn alterations in hematopoiesis may explain time-dependent quantitative and functional neutrophil responses and resistance to infection. In the marrow, we found a myeloid expansion (CD11b+ cell percentage) in the burn group compared with sham controls that was evident by 3 d after injury and was further increased by day 7 after injury (Fig. 5A), largely attributable to a rise in neutrophils and monocyte numbers (Fig. 5B). Concurrent with the increase in myeloid cells, there was a reduction in B cells and RBC lineages. Total bone marrow cell counts were not significantly different between burn- and sham-injured groups (Supplemental Fig. 1A), indicating that an expansion of myeloid populations was counterbalanced by a reduction in other lineages. This reprioritization of the marrow was consistent with postinjury day 7 changes in cellular compositions of blood and whole lung tissue, including systemic increases in neutrophils and inflammatory monocytes, and reductions in circulating RBCs and B cells in whole lung tissue. To assess differences in progenitor populations, we isolated bone marrow from mice 7 d after sham and burn injuries, and determined the proportion of CD117+CD34+ that are primarily composed of myeloid progenitors (80–90%) relative to CD117+CD34+ megakaryocyte/erythrocyte progenitors. We found
that changes in the progenitor populations mirrored those of the mature cells in the marrow and the circulation, exhibiting a shift toward myeloid lineages (Fig. 5C).

Cytokine response and bone marrow signaling suggest STAT3 activation is a primary driver of thermal injury–induced hematopoietic reprioritization

Experiments were performed to explore the factors that drive marrow reprioritization and resistance to pulmonary infection after thermal injury. Serum was collected before insult and at 6 h, 1 d, 3 d, and 7 d after burn or sham treatment, and samples were screened for 42 cytokines by multiplex analysis. Serum concentrations of G-CSF, IL-6, and KC peaked sharply within 1 d in the burn group relative to the sham group, with persistent elevation of G-CSF and IL-6 (albeit at declining levels) for 7 d (Fig. 6A). Remarkably, there were no consistent elevations in other serum cytokines in the multiplex panel.

We used phosflow to detect injury-activated phosphorylation of key nodes in the Akt, STAT, MAPK, and ERK signaling pathways in mature (CD11b+) and progenitor (CD117+) bone marrow cells. Although constitutive activation of several pathways was suggested in our initial screen of bone marrow cells from burn- and sham-injured mice, STAT3 was the only pathway that was found to be differentially activated in response to burn injury, in both progenitors and mature myeloid cells (Fig. 6B).

Bone marrow STAT3 activation is durable and specific to myeloid cells and myeloid progenitors following burn injury

We next investigated the durability and cellular specificity of STAT3 activation. Bone marrow cells were harvested for phosflow before insult and at 6 h, 1 d, 3 d, and 7 d after burn or sham treatment. The STAT3 response was interrogated over time by gating on (CD11b+Ly6G+) neutrophils, (CD11b+Ly6G2) monocytes, (CD11b2Ly6G2) nonmyeloid cells, (CD117+CD342) megakaryocyte erythrocyte progenitors, and (CD117+CD34+) myeloid progenitors (Fig. 7A). Histograms from representative bone marrow samples isolated at 1 d after burn or sham treatment demonstrate that STAT3 activation is specifically increased in neutrophils, monocytes, and myeloid progenitors after burn injury, because activation of this signaling pathway is greater in progenitors than in mature myeloid cells.
intermediate was not seen in nonmyeloid bone marrow cells or in megakaryocyte erythrocyte progenitors (Fig. 7B). STAT3 activation in the mature and progenitor myeloid cells from burned animals was sustained above baseline for up to a week (Fig. 7C). The patterns of increased STAT3 activation in the burned animals and elevation of serum G-CSF and IL-6 were strikingly similar, suggesting a possible causal role for the cytokines in driving STAT3 signaling in the marrow (34, 35). These findings led us to postulate that G-CSF, IL-6, and STAT3 constitute an axis that is responsible for the postburn hematopoietic reprioritization and protection from pulmonary infection.

Neutralization of postburn serum G-CSF prevents myeloid-specific STAT3 activation, bone marrow reprioritization, and neutrophilia

We used an in vivo neutralization strategy to determine whether G-CSF is necessary for the postburn bone marrow reprioritization and neutrophilia. Anti–G-CSF Ab blocked nearly 80% of the STAT3 activation induced by thermal injury in CD117+CD34+ bone marrow progenitors at 6 h after burn (Fig. 8A, 8B). Similar reductions in STAT3 activation were seen in bone marrow neutrophils and monocytes upon G-CSF neutralization (data not shown).

Mice were treated with anti–G-CSF or isotype Ab before and at the time of injury, then for 6 d after injury. On day 7 after burn or sham treatment, bone marrow and blood were obtained for analysis. Neutralization of G-CSF prevented hematopoietic reprioritization after burn injury (Fig. 8C). Thermal injury–induced reductions in B cell and red cell numbers in the marrow were significantly blocked by neutralizing serum G-CSF after burn, as were burn-induced increases in marrow neutrophils and monocytes. These changes represented a compositional shift of the bone marrow because total cell counts were not found to be different (Supplemental Fig. 1B). Neutralization of G-CSF also prevented the neutrophilia at 1 wk after burn injury (Fig. 8D). Although administration of anti–G-CSF blocked the postburn reduction of red cells in the bone marrow, the peripheral reduction in red cells shown in Fig. 2C was still evident in these mice (data not shown).

rG-CSF is sufficient to recapitulate hematopoietic alterations induced by injury and protection from a lethal K. pneumoniae infection

Experiments were performed to test the hypothesis that G-CSF is sufficient for bone marrow reprioritization and protection from a lethal K. pneumoniae pulmonary infection, as is seen after burn injury. We administered rG-CSF i.p. in two daily doses of 7.5 μg/kg for a total daily dose of 15 μg/kg. A control group of mice was given an equal volume of saline on the same schedule. After 6 consecutive days of treatment, bone marrow was harvested on day 7 to assess cellularity. Total bone marrow cellularity was not different in G-CSF– versus PBS-treated mice (Supplemental Fig. 1C), and alterations reflected a change in bone marrow composition with an increase in myeloid cells at the expense of other lineages (Fig. 9A). Similar to the marrow changes seen in post-
burn day 7 mice, neutrophils and monocytes were increased, whereas B cells and red cells were decreased as a percentage of bone marrow cells. Daily G-CSF treatment for 6 d was also found to provide a survival advantage (\(p = 0.054\), G-CSF versus PBS) in mice that were given a lethal \(K.\) pneumoniae pulmonary inoculum 24 h after the final G-CSF dose (Fig. 9B).

In humans, \(rG\)-CSF largely reproduces the “genomic storm” that follows traumatic injury

Having determined that G-CSF plays an important role in the murine response to traumatic burn injury and infection, we postulated that it may be a critical factor in the human response to trauma as well, based on analogous biological function and receptor binding cross-reactivity in mice and humans (36). To explore the role of G-CSF in the human response to trauma, we compared the whole-blood leukocyte transcriptional response that follows traumatic injury (1) with the response that is seen in humans treated with G-CSF for the mobilization of hematopoietic stem cells (37). In this comparison, we found that of the genes with expression changes of \(\geq 2\)-fold after trauma, 92% had the same direction of effect after consecutive doses of G-CSF (Fig. 10A). Correlation analysis of fold changes after trauma and G-CSF, using genes with at least a 2-fold change after trauma, indicated high correlation \((R = 0.65)\) of expression patterns between trauma victims and those treated with G-CSF (Fig. 10B). Most importantly, many of the genes upregulated in response to trauma and G-CSF treatment were associated with innate immunity, whereas genes that were commonly downregulated in response to trauma and G-CSF treated are associated with adaptive immunity (Fig. 10C).

**Discussion**

We have found that thermal injury of the skin induces a robust protection against a lethal \(K.\) pneumoniae pulmonary challenge delivered to mice 7 d later, whereas no protection is afforded by injury if the infection is delivered 2 h after the initial trauma. Our data demonstrate that the time-dependent resistance to infection is associated with enhanced ability to clear bacteria from the lung and reduced systemic dissemination. We found that the protective response coincided with a systemic increase in neutrophils and was neutrophil dependent.

We determined neutrophil recruitment to the airspace was not increased in burn-injured mice postinfection despite the systemic increase in neutrophils preinfection. BAL neutrophils isolated from mice 7 d after burn injury, early after \(K.\) pneumoniae challenge, were found to respond to stimulus with a greater respiratory burst and degranulation response, consistent with enhanced antimicrobial capacity. BAL cytokine and chemokine levels in burn-injured mice were not found to be different postinfection; indeed, there was a trend toward lower levels of BAL neutrophils and inflammatory mediators in that group, most likely reflecting the lower bacterial burden in burn-injured mice. Together, these results suggest the posttraumatic neutrophil-dependent protection from infection is more likely attributable to cellular antimicrobial function enhancement than to quantitative increases in neutrophils.
An unbiased screen of postburn serum cytokines identified a durable and highly specific increase in the STAT3-activating cytokines G-CSF and IL-6, which preceded myeloid expansion in the bone marrow and a systemic increase in neutrophils. Neutralization experiments indicate that G-CSF is the primary activator of STAT3 in mature myeloid cells and myeloid progenitors in the marrow after thermal injury. In addition, our data suggest that injury-induced activation of STAT3 in the marrow was highly restricted, because there was no clear evidence of differential activation of STAT1, ERK, AKT, p38, or STAT5 pathways in burn-injured mice compared with sham-injured mice. It was surprising that increased activation of STAT1 or STAT5 in burn-injured mice did not occur despite high levels of G-CSF, which has been reported to also act through these signaling intermediates in vitro (38).

Recently, STAT3 has been found to play an essential role in G-CSF–induced emergency granulopoiesis, regulating proliferation, maturation, and mobilization of neutrophils (39–41). STAT3 is also a signaling intermediate involved in the expansion of myeloid-derived suppressor cells (MDSCs) (42), a mixed group of immature monocytes, neutrophils, and their progenitors that are found in a broad range of inflammatory processes. Our group has previously shown that myeloid cells arising after burn injury are functionally and phenotypically consistent with MDSCs, in that they inhibit lymphocyte functions, produce increased levels of reactive oxygen species and NO, and display an altered inflammatory tone with increased ability to produce TNF-α and IL-10 (10, 43, 44). Other pathological processes in which MDSCs expand include infections, in which MDSCs have been found to exhibit antimicrobial activity, and in cancer, where MDSCs are thought to suppress tumor killing (45). Tumors have been observed to grow more rapidly in burn-injured mice because of defects in cell-mediated immune responses (46, 47), and G-CSF–driven expansion of granulocytic MDSCs has recently been directly implicated in uncontrolled tumor growth (48). Together, these data suggest that myeloid cells that expand after injury because of G-CSF–driven STAT3 activation have unique infection-fighting properties and suppress adaptive immune function.

We found that G-CSF was necessary and sufficient for the hematopoietic reprioritization that follows burn injury. Neutralization of G-CSF blocked STAT3 activation, hematopoietic reprioritization, and systemic neutrophilia. Exogenous administration of rG-CSF was sufficient to drive myeloid cell expansion in the marrow at the expense of other lineages, similar to burn injury, and protected the animals from a lethal K. pneumoniae pulmonary infectious challenge. These data provide strong support that the G-CSF STAT3 axis drives the myeloid shift in the bone marrow and resistance to infection that follows thermal injury.

A similar myeloid shift in the bone marrow occurs in both mice and humans after trauma (24, 25). Our findings in mice and correlative evidence in humans support a prominent role for G-CSF in these hematopoietic changes (49). In the correlative study performed by Cook et al. (49), trauma patients with the highest G-CSF levels were found to have greater reductions in hemoglobin and increased transfusion requirements, supporting their conclusion that G-CSF contributes to posttraumatic anemia. Recombinant human G-CSF has previously been shown to suppress bone marrow erythropoiesis in mice, although the mechanism of this suppression was not explored (50). Our data indicate that G-CSF is a primary factor produced after injury that reduces the red cell composition of the marrow, although neutralization of G-CSF did not reverse a reduction in red cells we detected in the blood 7 d after burn injury. These data indicate that mechanisms other than deficits in bone marrow erythropoiesis contribute to the low peripheral RBC counts observed in our model. Interestingly, Cook et al. (49) also reported that trauma patients with the highest G-CSF levels had increased rates of infection, shock, acidosis, arterial lactate, or early transfusion requirements. Although no attempt to correlate the severity of injury with G-CSF levels or the susceptibility to infection was made in our study, it is
reasonable to speculate that there is an optimal G-CSF response and a threshold above which a beneficial response becomes harmful or overwhelmed. In this regard, it is important to note that the incidence of infections, multiple organ failure, and death all increase with burn size (51), and the 15% of total body surface area used in our model may not reflect changes that would be seen after larger burns.

We also found that IL-6 was increased for several days after injury. IL-6 is known to signal through STAT3 and to contribute to emergency granulopoiesis, independent of G-CSF (52). Infection-stimulated increases in IL-6 enhance myeloid production and reduce erythroid production in the bone marrow (53). IL-6 and G-CSF have both been reported to arrest B lymphopoiesis (35, 54) and to cooperatively enhance STAT3 activation in neutrophils (55). Finally, IL-6 may activate hepatic STAT3 and indirectly promote MDSC expansion and recruitment through the upregulation of the acute-phase proteins, a mechanism that has been found to play a critical role in surviving sepsis (56). The acute-phase response driven by hepatic STAT3 has also been found to play a critical role in preventing systemic dissemination of infection and mortality after pulmonary infection (57).

We compared the molecular responses of whole-blood leukocytes from G-CSF–treated humans and trauma patients using data sets from the GEO repository, and found the molecular responses to be remarkably similar, with both G-CSF and trauma inducing upregulation of innate immunity genes and downregulation of adaptive immunity genes. G-CSF is known to enhance the phagocytic and bactericidal activity of human neutrophils (60). Neutrophils from G-CSF–treated humans exhibit increased surface expression of CD11b/CD18 Ag (61) and enhanced oxidative function after stimulation (62), consistent with a heightened state of activation. G-CSF–mediated expansion of MDSCs may represent one mechanism of suppression of adaptive immune functions after injury, although a substantial body of literature indicates that G-CSF promotes a functionally tolerant adaptive immune response through a variety of mechanisms (63, 64). The functional effects of G-CSF on adaptive immunity include preferential suppression of lymphocyte Th1 responses in association with a shift toward the Th2 phenotype (65–67). Proliferative responses after exposure to mitogens or anti-CD3 are impaired in lymphocytes collected from humans given consecutive doses of G-CSF. Finally, human bone marrow contains large numbers of highly functional CD4⁺CD25⁺ T regulatory cells that are mobilized into circulation after consecutive doses of G-CSF and are

![Image](http://www.jimmunol.org/)

**FIGURE 7.** Cell-specific STAT3 activation in bone marrow cells of burn and sham-treated mice. Bone marrow cells were isolated from burn and sham-treated mice 7 d after injury and analyzed by FACS. (A) The gating strategy is depicted by arrows, and numbers indicate cell type; 1 = neutrophils, 2 = monocytes, 3 = nonmyeloid, 4 = megakaryocyte erythrocyte progenitors, and 5 = myeloid progenitors. (B) Representative 24-h population-specific STAT3 activation in burn (solid line) and sham (segmented line) mice, and pooled population background (shaded area). (C) Time course of STAT3 activation in neutrophils, monocytes, and myeloid precursors 0.25, 1, 3, and 7 d after burn or sham treatment. Data are expressed as mean ± SEM for n = 4–6 per time point. **p < 0.01.
found to be more potent inhibitors of Th1 cytokine production and lymphocyte proliferation than their counterparts that normally reside in the blood (68). G-CSF–mediated mobilization may be relevant to human trauma victims, in that Th1-suppressing CD4+ CD25+ T regulatory cell numbers and activity are increased in the peripheral blood of these patients (22).

FIGURE 8. Postburn neutralization of serum G-CSF prevents STAT3 activation, bone marrow reprioritization, and neutrophilia. Mice were given a G-CSF–neutralizing Ab. (A) Representative histograms of p-STAT3 in CD117+CD34+ bone marrow progenitors 6 h after sham injury (gray line), burn injury (black line), burn injury + 10 μg anti–G-CSF (segmented black line), burn injury + 50 μg anti–G-CSF (segmented gray line), and background control staining (shaded area). Anti–G-CSF was delivered i.p. 12 h before injury and at time of injury. (B) Comparative effects of 10 and 50 μg doses of G-CSF neutralizing Ab on 6 h after injury p-STAT3. (C) Bone marrow composition 7 d after sham or burn injury in mice treated with daily 10 μg doses of anti–G-CSF (AG) or isotype (Iso) Ab as described in Materials and Methods. (D) Peripheral blood neutrophil counts at 7 d after injury determined in sham isotype (SH+Iso), burn isotype (B+Iso), sham anti–G-CSF (SH+AG), and burn anti–G-CSF (B+AG)–treated mice. Data represent mean ± SEM for n = 3 per group in (B) and n = 6 per group for (C) and (D). Significance determined by ANOVA comparing selected pairs of groups. Significance of burn versus sham: *p < 0.05, **p < 0.01. Significance of burn versus burn: #p < 0.05, ##p < 0.01. ns, Not significant burn versus sham.

FIGURE 9. rG-CSF recapitulates postinjury reprioritization and protection from infection. Mice were treated with recombinant mouse G-CSF or PBS as described in Materials and Methods for 6 consecutive days before bone marrow analysis or infection on day 7. (A) Bone marrow composition was determined by FACS analysis of n = 4 per group. **p < 0.01. (B) Mortality of G-CSF– versus PBS-treated mice infected i.n. with 4 × 10⁷ CFU K. pneumoniae/mouse. Kaplan–Meier survival curves are shown for n = 11/group. p = 0.054.
The use of G-CSF for patients suffering from pneumonia and sepsis has been assessed in numerous clinical studies and a benefit has not been proved (69). In our model of injury, the protection against pulmonary infection afforded by G-CSF is time dependent, suggesting that studies designed to assess benefit of prophylactic administration of G-CSF on pneumonia may be more likely to show an effect. Animal models generally support this idea, demonstrating that the pre-emptive use of G-CSF is protective against pneumonia, whereas more inconsistent results are seen when G-CSF is administered post pulmonary infection (70). However, conflicting reports indicate that prophylactic G-CSF may also enhance mortality post pulmonary infection. In one study, a strain of \textit{K. pneumoniae} was able to use G-CSF to enhance its own capsule production, resulting in reduced phagocytic uptake by neutrophils and enhanced mortality (71). Another group reported that G-CSF was protective against \textit{Staphylococcus aureus} pulmonary infection, whereas lung injury and death were enhanced by G-CSF after an \textit{E. coli} challenge, possibly related to a heightened inflammatory response (72). Together, these studies suggest the G-CSF response can be both beneficial and harmful, and underscore the need for a better understanding of the role of G-CSF in modulating inflammatory tone in patients with severe infection.

In summary, we find that G-CSF plays a central role in post-traumatic resistance to infection and prioritization of bone marrow responses. Although it is widely appreciated that serum G-CSF is increased in traumatized humans, we find it surprising that a single hematopoietic cytokine appears to drive much of the transcriptional signature of injury and function as a node that activates innate immune responses and suppresses adaptive immune responses. Recent reports that the transcriptional response to injury in mice and humans is substantially different (73) notwithstanding, our findings and those of others support a larger role for G-CSF in the response to injury of both species than has formerly been recognized. We submit that G-CSF plays a determinative role in the survival of postinjury infection, and that future studies of the postinjury G-CSF STAT3 axis are warranted.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


