Psychogenic Stress Prior to Burn Injury Has Differential Effects on Bone Marrow and Cytokine Responses

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It is well known that many burn patients experience psychopathological disorders prior to burn injury. However, it is not known whether individuals that have been exposed to chronic psychological stresses will respond differently than unstressed individuals when challenged by a burn injury. In this study, we assessed whether chronic psychogenic stress prior to burn injury had any significant impact on burn injury-induced alterations in the myeloid compartment in the bone marrow and serum cytokine levels utilizing a well-controlled purely psychogenic stress model (predator exposure). Mice were individually caged and exposed to a Long Evans rat for 1 hr a day on 3 consecutive days prior to a 15% total body surface area flame burn. Four days after burn injury, bone marrow and serum were collected to assess myeloid cells and cytokine levels, respectively. Bone marrow cells were cultured in granulocyte-macrophage colony-stimulating factor (GM-CSF) to assess clonogenic ability. Flow cytometry was also used to characterize the populations of myeloid cells based on Gr-1 and CD11b staining intensity and to determine the expression of the macrophage colony-stimulating factor receptor (M-CSFR). Serum was assayed for IL-6, IL-12p70, MCP-1, and IFN-γ by multiplexed sandwich enzyme-linked immunoabsorbent assay (ELISA). We found that predator exposure prior to burn injury ablated the burn-induced increase in myeloid colony formation and attenuated the burn-induced increases in immature monocytes and immature neutrophils in the bone marrow, as well as MCP-1 levels in the serum. Conversely, psychogenic stress exaggerated the burn-induced increase in the number of M-CSFR-positive cells. This study is the first to show the effects of a pure psychogenic stressor (predator exposure) on burn-induced alterations of the immune system. The clinical ramifications of our findings remain to be elucidated. Exp Biol Med 232:253–261, 2007

Key words: chronic psychogenic stress; burn injury; bone marrow; MCP-1

Introduction

Despite modern therapeutic advances, thermal injury often results in mortality. Immune system alterations are a major contributor to burn-induced fatalities. These immune irregularities manifest as immunosuppression (1–3) with a concomitant hyperinflammatory response (4–5). It has been shown in numerous studies that thermal injury alters myelopoiesis in the bone marrow (6–11). This is important because myeloid cells give rise to monocytes and neutrophils (8), which are involved in wound repair and bacterial clearance. However, excessive numbers of activated neutrophils and monocytes can cause a hyperinflammatory response that contributes to morbidity and mortality (5).

The mechanisms by which thermal injury alters myelopoiesis are currently not known, although changes in serum cytokine levels (12) and colony-stimulating factor receptor expression on the bone marrow cells have been proposed as possible mechanisms (8). For instance, Santangelo *et al*. showed that the increase in monocyte/macrophage (M) colony formation caused by burn injury was accompanied by a parallel increase in the macrophage colony-stimulating factor receptor (M-CSFR) expression at the cell membrane (8). It has been shown that IL-6 is elevated because of burn injury and enhances myelopoiesis (12).

It is well known that many burn patients experience psychopathological disorders prior to burn injury. In fact, burn patients present with a significantly higher incidence of psychological stress prior to burn compared with the general population. This can partly be explained by the fact that many psychological disorders impair mental judgment and

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reaction time. Also, certain psychological conditions increase risk-taking behavior and the desire to cause self-harm (13–15, and reviewed Ref. 16). The aforementioned studies did not ascertain the influence of prior psychological stress on the postburn immune response. The influence of stress on the immune system depends on the type of stressor and its duration and intensity, as well as the organism and immune variable analyzed (17–21). Previous studies have shown that psychogenic stress, such as predator exposure, can influence the immune system (18, 22), altering activities such as hematopoiesis (23–25) and wound healing (20, 21, 26). Psychological stress has also been shown to alter cytokine levels (27–31) and increase the susceptibility to endotoxic shock (19).

To the best of our knowledge, the impact of psychological (psychogenic) stress prior to burn injury on the burn-induced immune alterations has not been documented. Animal models of traumatic injury generally ignore the consequences of stressors other than the physical injury. Despite widespread recognition of neuroimmune interactions, there is very little rigorous experimental evidence regarding psychological stresses in relation to burn injury, which provokes complex hematopoietic and immune responses. In particular, it is not known whether individuals that have been exposed to chronic psychological stresses will respond differently than unstressed individuals when challenged by a burn injury.

Because of the variety of homeostatic adaptations that occur during stress, it is conceivable that stress causes a mixture of positive and negative influences on the immune system. Humans are known to encounter more than 1 stressor in their lives, which may result in overlapping responses that are antagonistic to one another (reviewed Ref. 20). This project examined the adaptation of mice to a combination of thermal injury and predator exposure (psychogenic stress), so as to mimic better the complex pattern of internal and external challenges that give rise to morbidity in traumatically injured humans. Specifically, we assessed whether predator exposure prior to burn injury had any significant impact on burn injury-induced alterations in the myeloid compartment in the bone marrow and serum cytokine levels.

Materials and Methods

Mice. Experiments were performed on 7-week-old male C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) using barrier housing within AAALAC-accredited animal quarters under protocols approved by the Institutional Animal Care and Use Committee. There were 5–6 animals per group. The mice had free access to food and water, and all were born in our animal facility at the Shriners Hospitals for Children and handled frequently. Therefore, they were acclimated to their environment.

Predator Exposure. Mice were individually caged in a clear 11.5 × 7.5 × 5-in. cage with microfilter top securely attached. They remained individually caged throughout the duration of the experiment. The encaged mouse was transported to the rat test room. The cage was then placed inside a 24 × 17 × 8-in. cage containing 1 male Long Evans rat (>500 g). The test apparatus was assembled so that the mouse was exposed to rat odors, noises, and sight but could not actually come in contact with the rat. The unexposed mice were placed in a clean empty cage in an empty room. All test sessions lasted for 1 hr at irregular times between 0800 hrs and 1400 hrs and were repeated on 3 consecutive days.

Burn Injury. The day after the last predator exposure session, half of the mice (controls and predator exposed) received 3% isoflurane anesthesia at 2 l/min for 10 mins and had the hair removed from their back by clipping. Subsequently, their backs were covered with a fireproof template, which allowed for a controlled full-skin thickness burn on an area equivalent to 15% of the total body surface area. The burn group was doused in the treatment area with 1 ml of 100% ethanol, followed by a flame-induced burn for 10 secs. The mice then received 0.5 ml of saline intraperitoneally for fluid resuscitation. To avoid additional experimental variables such as analgesics or secondary nonburn stresses, mice that showed any signs of distress after the burn treatment (lethargy, anorexia, scratching at wound, etc.) were eliminated from the study and humanely euthanized by CO_2 asphyxiation. Half of the mice served as nonburn controls. Four days after treatment, all mice, including controls, were sacrificed by CO_2 asphyxiation and their abdomens and both legs were shaved. Both femoral bones with muscles and tissues removed, and blood from the heart were harvested from each mouse.

Bone Marrow Cells. The femoral bones were cut at both ends, and the marrow cavities were flushed with 3 ml of cold PBS. The cell suspension was filtered through a 100-μm nylon mesh (Falcon, Becton Dickinson, Bedford, MA), washed with an additional 4 ml of cold PBS, and centrifuged at 400 *g* for 10 mins at 4°C. Following centrifugation, the pellet was resuspended with 1 ml of Iscoves Modified Dulbecco's medium (IMDM) with 2% fetal bovine serum (FBS) containing penicillin/streptomycin 125 U/ml and gentamycin 125 μg/ml. Viability was determined via trypan blue exclusion, and cells were counted using a hemocytometer.

Clonogenic Assay. Bone marrow cells were suspended at 3×10^4 cells/300 μl in IMDM 2% FBS and 3 ml of Methocult M3234 (Stem Cell Technologies Inc., Vancouver, Canada), and 1 of the 3 following colony-stimulating factors (CSF): granulocyte-macrophage (GM)-CSF 20 ng/ml (R & D Systems, Minneapolis, MN), monocyte/macrophage (M)-CSF 100 ng/ml (R & D Systems), or granulocyte (G)-CSF 20 ng/ml (R & D Systems); no CSF served as the negative control. One milliliter of the mixture was subsequently plated in a 35 × 10 mm^3 vacuum gas plasma polystyrene–treated well (Falcon, Becton Dickinson). The cultures were then

incubated 5–7 days at 37°C and 5% CO_2. Following incubation, utilizing a ×20 dissecting microscope, aggregates containing 50 or more cells were counted as a colony.

Cell Surface Staining. Bone marrow cells were washed in PBS, and the total viable cells were quantified by trypan blue exclusion using a hemocytometer. After blocking the Fc receptors with mouse gamma-globulin (10 μg/7.0 $\times 10^5$ cells) (Sigma, St. Louis, MO) for 5 mins at 4°C, bone marrow cells (7.0×10^5 cells) were labeled with allophycocyanin (APC)-conjugated anti-CD11b (1 μg/7.0×10^5 cells) (BD Pharmingen, San Diego, CA), and biotin-conjugated Gr-1 (1 μg/7.0×10^5 cells) (Caltag Laboratories, Burlingame, CA), or phycoerythrin (PE)-conjugated anti-CD115 (M-CSF receptor) (1 μg/7.0×10^5 cells) (Serotec, Raleigh, NC) for 25 mins at 4°C. Labeling with the corresponding isotype controls (Caltag Laboratories) was performed under the same conditions (1 μg/7.0×10^5). The cells were then diluted in 2 ml PBS, 5% FBS, 0.1% sodium azide, and centrifuged at 300 *g* for 7 mins at 4°C. Subsequently, the biotinylated cell pellets were resuspended in 100 μl of PBS, 5% FBS, 0.1% sodium azide and incubated with Streptavidin-PerCP (1 μg/7.0×10^5 cells) (BD Pharmingen) for 30 mins at 4°C. After incubation, the cells were resuspended in 2 ml of PBS, 5% FBS, 0.1% sodium azide and centrifuged at 300 *g* for 7 mins at 4°C.

Intracellular Staining. Each of the above tubes containing the conjugated antibodies or isotype controls were incubated in fixative medium (Fix & Perm Kit, Caltag Laboratories) for 15 mins at room temperature. After incubation, the cells were resuspended in 3 ml of PBS, 5% FBS, 0.1% sodium azide and centrifuged at 300 *g* for 7 mins at 20°C. Supernatants were aspirated off, and the cells were incubated with 20-μg rabbit γ globulin (Sigma) and permeabilization medium (Fix & Perm Kit, Caltag Laboratories) for 5 mins at room temperature. Subsequently, cells were incubated with either 2 μg of FITC isotype control (Sigma) or 2 μg of FITC-conjugated anti-active caspase 3 (BD Pharmingen) for 20 mins at room temperature. After incubation, the cells were washed in 3 ml of PBS, 5% FBS, 0.1% sodium azide and centrifuged at 300 *g* for 5 mins at 20°C. Supernatants were removed by aspiration, and the cell samples were resuspended in 1 ml of PBS, 1% paraformaldehyde in preparation for flow cytometric analysis. Samples were analyzed on a LSRI flow cytometer (BD Pharmingen) using the 488 line of an argon ion laser. Data were collected and analyzed using CellQuest Pro software (BD Pharmingen). As a second confirmation of apoptosis, a separate tube of cells (7.0×10^5 cells) from each mouse was incubated with anti-CD11b and anti-Gr-1 as described above for cell surface staining, and suspended in 100 μ*M* of H33342 and 100 μl of PBS, 1% paraformaldehyde and incubated overnight at 4°C. The next day, 10 μl of cells was loaded onto a hemocytometer and viewed under a fluorescent microscope to detect fragmented DNA for apoptosis. Jurkat T cells and bone marrow cells from a nonburn control mouse (4.0×10^6 cells) were treated with camptothecin (4 μg/10^6 cells) for 3 hrs at 37°C to induce apoptosis. Cells were subsequently assayed for caspase 3 activation and DNA fragmentation by H33342 staining as stated above and served as positive controls.

Table 1. Total Number of Viable Bone Marrow Cells ($\times 10^6$) in Mice Exposed to Burn Injury With or Without Prior Psychogenic Stress (Predator Exposure)[a]

		Burn	
		−	+
Predator	−	30.5 ± 3.30	23.9 ± 3.25
	+	21.3 ± 2.69	28.5 ± 2.21

[a] Bone marrow cells were harvested at the time of sacrifice on postburn day 4. Viable bone marrow cells from both femurs were assessed by trypan blue exclusion and counted using a hemocytometer. The values are the mean ± SEM 5–6 animals per group. Differences between mean bone marrow cellularities were not significantly different ($P = 0.14$; one-way ANOVA).

Serum Cytokines. Harvested blood serum, 50 μl per mouse, was analyzed in duplicate for monocyte chemoattractant protein–1 (MCP-1 CCL2), interferon gamma (IFN-γ), interleukin (IL)-6, and IL-12p70 utilizing Search-Light Proteome Array (Pierce, Woburn, MA), which is a multiplexed sandwich enzyme-linked immunoabsorbent assay (ELISA). Sensitivities are as follows: MCP-1 (2.0 pg/ml), IFN-γ (7.8 pg/ml), IL-6 (5.5 pg/ml), and IL-12p70 (0.8 pg/ml).

Statistical Analysis. Means were reported as ± SEM. Comparisons between multiple treatment groups were made by one-way ANOVA with Tukey's test to assess differences among the means. Significance was defined as $P < 0.05$.

Results

Behavioral Response. Behavioral activities during and after predator exposure were done based on published studies (18, 22, 32) to ascertain the overt responses of the exposed mice. As in the previous studies, predator-exposed mice exhibited anxiety-related behaviors, including startling, cowering, and burrowing during all 3 exposure sessions. When the mice were returned to the holding room, anxiety-related behaviors ceased rapidly. Mice placed in an unoccupied rat cage did not exhibit any anxiety-related behaviors during any of the 3 test sessions. There were no observable differences in feeding and/or drinking behavior among any of the groups studied.

Burn Injury-Induced Increase in Colony Formation Was Reduced in Predator-Exposed Mice. Total bone marrow cellularities for all 4 groups are shown in Table 1. Although no significant differences in total bone marrow cellularities were observed among the 4 groups, we did see a trend toward lower cell counts in burn injury and predator-exposed mice relative to nonexposed, nonburn controls. Mice subjected to both psychogenic stress (predator

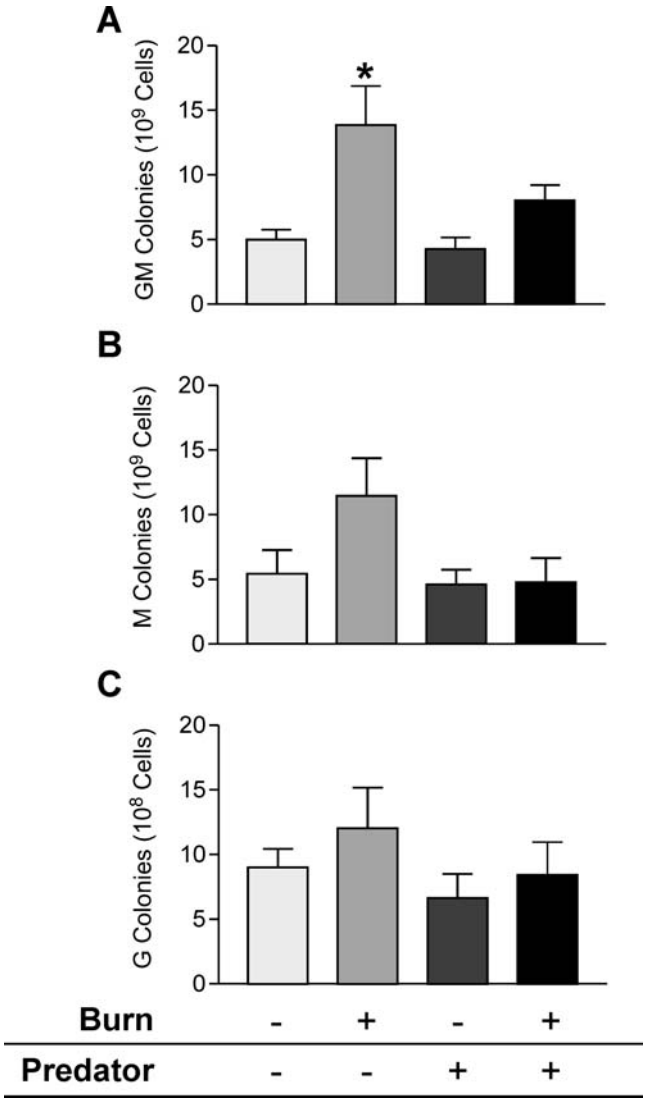

Figure 1. The burn injury-induced increase in colony formation is reduced in the predator-exposed mice. Bone marrow cells (3.0×10^4) harvested at the time of sacrifice on postburn day 4 were cultured in 3 ml Methocult containing 20 ng/ml of GM-CSF, 100 ng/ml of M-CSF, or 20 ng/ml of G-CSF and incubated for 5–7 days. Aggregates of 50 or more cells were counted as a colony utilizing a 20 ×-dissecting microscope, and total colony numbers were enumerated by multiplying the number of colonies and the total number of viable bone marrow cells. (A) GM colony number (mixture of cells from the monocyte and granulocyte lineages). In the absence of prior predator exposure, burn injury led to a significant increase in the number of GM colonies relative to the nonburn groups (*$P < 0.01$). No significant difference was seen between the nonexposed burn and the predator-exposed burn groups ($P > 0.05$). The GM colony number in the predator-exposed controls was not significantly different relative to the nonexposed controls ($P > 0.05$). (B) M colony number (mostly cells of the monocyte lineage). There were no significant differences among the means ($P > 0.05$). (C) G colony number (mostly cells of the granulocyte lineage). There were no significant differences among the means ($P > 0.05$). Data in each panel were analyzed by ANOVA with Tukey's test for differences among the means.

exposure) and burn injury had bone marrow cellularities more closely resembling that of the controls.

GM colonies consist of a mixture of cells from the monocyte and granulocyte lineages, M colonies consist of mostly cells of the monocyte lineage, and G colonies consist mostly cells of the granulocyte lineage (8). Overall, colony formation was higher in bone marrow from burned mice that did not receive prior predator exposure relative to unburned groups. However, this burn-induced increase was statistically significant only for GM colony formation ($P < 0.01$; Fig. 1A). Predator exposure prior to burn injury resulted in GM, M, and G colony numbers that were similar to the nonburn groups. Therefore, prior predator exposure reduced the burn-induced increases in myeloid colony formation. GM colony number was very similar in predator-only mice relative to the nonexposed controls. Although the patterns were similar to that seen for GM colonies, the M and G colony values were smaller and not significant at the $P \leq 0.05$ level by one-way ANOVA (Figs. 1B and 1C).

Effects of Burn Injury and Predator Exposure on the Myeloid Populations in the Bone Marrow. We used fluorescently labeled monoclonal antibodies (Gr-1 and CD11b), which recognize cell surface markers expressed on cells of the myeloid lineage (33–35) in conjunction with flow cytometry. With dead cells, aggregates, and debris gated out, relative fluorescence intensities were used to identify the cell populations. These values were used to ascertain differences among the means by one-way ANOVA and Tukey's test between groups. We found that there was an increase in the percentage of immature monocytes and immature neutrophils (Gr-1^{dim}CD11b^+) in response to burn injury relative to the control group ($P < 0.01$; Fig. 2A). However, the burn-induced increase was attenuated by prior predator exposure such that the difference was not significant between the predator-exposed burn group and the respective unburned group. Again, we found that predator exposure by itself resulted in values that were similar to the untreated controls. When we looked at the total number of immature monocytes and immature neutrophils (Gr-1^{dim}Cd11b^+), we found that the values after burn injury, though higher than the respective unburned values, were not significantly different ($P > 0.05$) in either the unexposed or predator-exposed groups (Fig. 2B). For the mature neutrophils (Gr-1^{bright}CD11b^+) and mature monocytes (Gr-1^-CD11b^+), we did not see any significant differences among the 4 groups with respect to either the percentage or the absolute cell number of these populations ($P > 0.05$) (data not shown).

Apoptosis in the Myeloid Population Was Unaffected by Burn Injury and/or Predator Exposure. Little is known about the impact of burn injury on the level of apoptosis in the bone marrow (36). Likewise, research investigating the influence of psychogenic stress on bone marrow cell apoptosis is limited (37, 38). Therefore, we wanted to determine whether differences in the level of

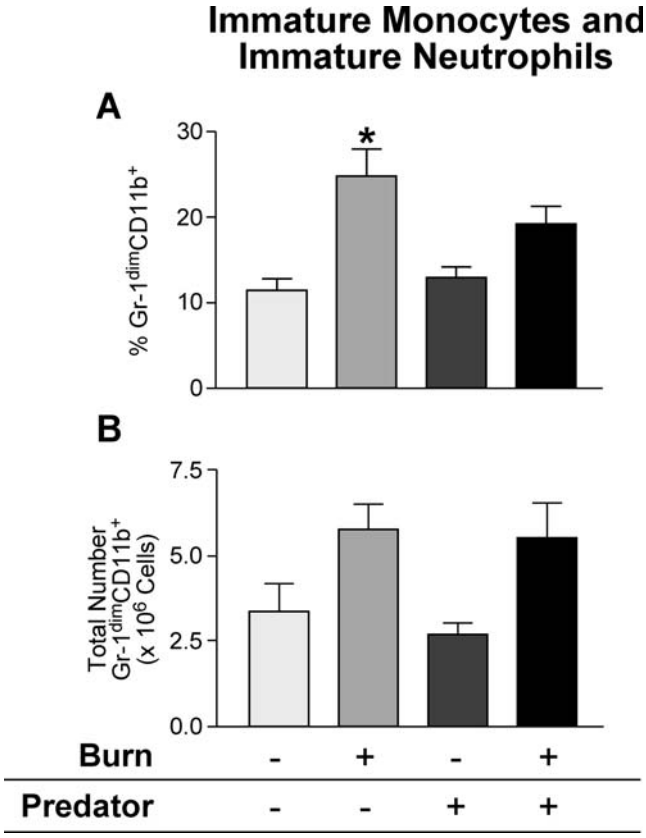

Figure 2. Effects of burn injury and predator exposure on the myeloid populations in the bone marrow. (A) Bone marrow cells (7.0×10^5) harvested at the time of sacrifice on postburn day 4 were stained for dual color flow cytometry with 1 μg of APC-conjugated anti-CD11b and biotin-conjugated anti-Gr-1 monoclonal antibodies. Cells were then incubated with 1 μg of streptavidin PerCP. The percentage of mature neutrophils ($Gr\text{-}1^{bright}CD11b^{+}$), immature monocytes and immature neutrophils ($Gr\text{-}1^{dim}CD11b^{+}$), and mature monocytes ($Gr\text{-}1^{-}CD11b^{+}$) in the bone marrow obtained by flow cytometry were analyzed by one-way ANOVA with Tukey's test to ascertain differences among the means. Burn injury in the absence of prior predator exposure led to a significant increase in the percentage of immature monocytes and immature neutrophils ($Gr\text{-}1^{dim}CD11b^{+}$), relative to the nonburn control groups (*$P < 0.01$). The percentage of immature monocytes and immature neutrophils was not significantly different between the nonexposed burn and the predator-exposed burn groups or between the predator-exposed controls and the nonexposed controls ($P > 0.05$). (B) The total number of immature monocytes and immature neutrophils was enumerated by multiplying the percentage of the population in the bone marrow and the total number of viable bone marrow cells. The absolute number of immature monocytes and immature neutrophils in the bone marrow was increased by burn injury, but this was not significantly different than their respective nonburn controls ($P > 0.05$). No significant differences were seen between the nonexposed burn and predator-exposed burn groups or between the nonexposed control and the predator-exposed control groups ($P > 0.05$).

apoptosis could explain the differences in colony number among the 4 groups studied.

We assessed the level of apoptosis in the $Gr\text{-}1^{+}CD11b^{+}$ (myeloid cells) in the 4 groups by flow cytometry and intracellular staining with active caspase 3 monoclonal antibody. Jurkat T cells and mouse bone marrow cells were treated with camptothecin (4 $\mu g/10^6$ cells) for 3 hrs (to induce apoptosis) to confirm the assay. Jurkat cells had 68% active caspase 3-positive cells, while bone marrow cells treated with the same concentration of camptothecin had only 4% active caspase 3-positive cells (Fig. 3), indicating that the bone marrow cells are very resistant to apoptosis, relative to tissue culture cells. We found that the intrinsic level of apoptosis in marrow myeloid cells (i.e., the percentage active caspase 3-positive) was <1% in all of the groups, with no significant differences among the means. The percentage of active caspase 3-positive cells in the total bone marrow cells was also <1%, with no significant differences among the means (data not shown). Because proliferation of certain immune cells has been shown to activate caspase 3, and apoptosis has been shown to occur independent of caspase 3 activation (38), H33342 staining, which detects DNA fragmentation (indication of apoptosis), was performed. A very low level of H33342 staining in the total bone marrow and myeloid population (<1%) was in agreement with the low (<1%) level of active caspase 3 detected. The absence of substantial numbers of apoptotic cells or differences among the groups indicates that apoptosis is not likely to account for the differences in colony number among the groups.

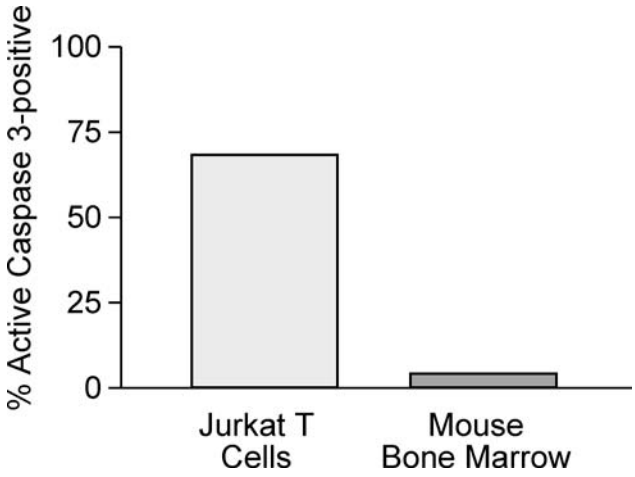

Figure 3. Active caspase 3-positive controls. Jurkat T cells and bone marrow cells from an unstressed control mouse (4.0×10^6 cells) were treated with camptothecin (4 $\mu g/10^6$ cells) for 3 hrs at 37°C. Cells were subsequently fixed and permeabilized followed by incubation with 2 μg of FITC-conjugated anti-active caspase 3 monoclonal antibody. Jurkat T cells had 68% active caspase 3-postive cells, and the mouse bone marrow had 4% active caspase 3-positive cells.

Predator Exposure Prior to Burn Injury Enhanced the Burn-Induced Increase in M-CSF Receptor-Positive Cells. Previous work has shown that burn injury increases the expression of the M-CSF receptor (8). We explored this phenomenon by assessing the density of CD115 (M-CSFR) at the cell surface. Mean channel values from our flow cytometric analysis allowed us to determine the density of the M-CSFR per cell and showed that there were no differences in M-CSFR density among the 4 groups (data not shown). We did find that burn injury led to an increase in the percentage and absolute number of

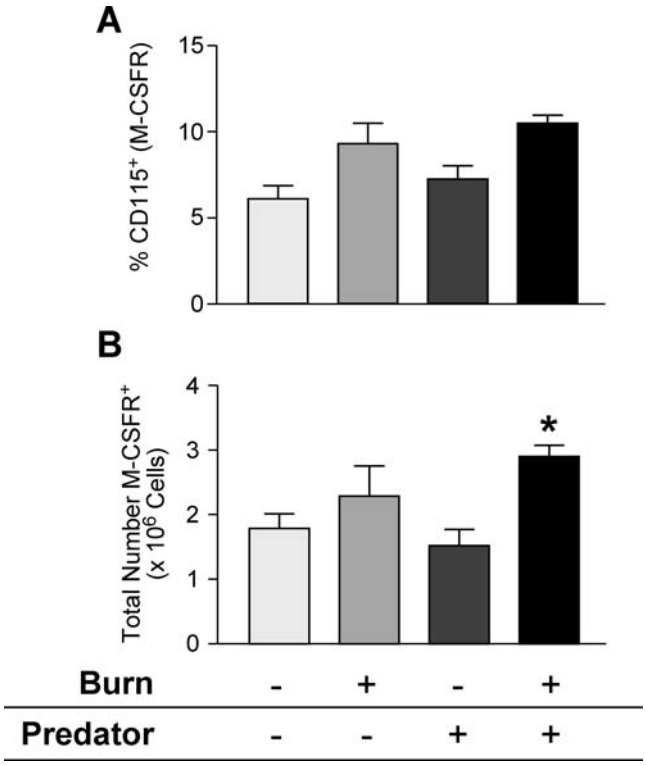

Figure 4. Predator exposure prior to burn injury enhanced the burn-induced increase in M-CSFR-positive cells in the bone marrow. (A) Bone marrow cells (7.0×10^5) harvested at the time of sacrifice on postburn day 4 were stained with 1 μg of PE-conjugated anti-CD115 (M-CSFR) monoclonal antibody. The percentage of $CD115^+$ (M-CSFR)-positive cells obtained by flow cytometry was analyzed by one-way ANOVA and Tukey's test to ascertain the differences among the means. Burn injury increased the percentage of M-CSFR-positive cells relative to the nonburn groups, and this increase was enhanced if burn injury was preceded by predator exposure. The percentage of M-CSFR-positive cells was not significantly different between the 2 burn groups ($P > 0.05$). There was no significant difference caused by predator exposure solely relative to the control group that received no predator exposure ($P > 0.05$). (B) The total number of M-CSFR-positive cells was enumerated by multiplying the percentage of the population in the bone marrow and the total number of viable bone marrow cells. The absolute number of M-CSFR-positive cells was significantly increased in the burn-injured group that received prior predator exposure relative to predator-exposed controls (*$P < 0.05$). The predator-exposed burn group had an absolute number of immature monocytes and immature neutrophils that was not significantly different than the nonexposed burn group ($P > 0.05$). Predator exposure alone produced no significant differences relative to the nonexposed controls ($P > 0.05$).

M-CSFR-positive cells. When expressed as a percentage of M-CSFR-positive cells, we found that the combination of predator exposure and burn injury led to an increase in the percentage of M-CSFR-positive cells relative to the nonexposed burn group (Fig. 4A). This appears to have resulted from the additive effects of the 2 stressors, which separately had small effects that were not statistically significant. The total number of M-CSFR-positive cells in the marrow was significantly higher in the predator-exposed burn group relative to predator-exposed controls ($P < 0.05$; Fig. 4B). It should be noted that the enhancing effect of prior predator exposure on the burn-induced increase in M-CSFR-positive cells is opposite of the attenuating effects seen in the colony assays and the $Gr\text{-}1^{dim}CD11b^+$ cell population, which suggests that the suppressive effect of predator exposure is downstream of the receptor.

Prior Predator Exposure Attenuated the Burn-Induced Increase in Serum MCP-1 Levels. Because serum cytokine levels are known to be altered after burn injury and psychogenic stress, we wanted to determine if predator exposure prior to burn injury influenced the burn-induced effect on serum MCP-1, IFN-γ, IL-6, and IL-12p70 levels, which we (6) and others have found to be altered by burn injury (1, 4, 39). The values for all treatment groups are shown in Table 2. Burn injury led to a significant increase in the level of MCP-1 in the unexposed animals. The effect of burn injury was attenuated by prior predator exposure, which agrees with the results seen in our GM colony and $Gr\text{-}1^{dim}CD11b^+$ data. There was no significant difference in the level of MCP-1 caused by predator exposure solely. No significant differences in the levels of IFN-γ, IL-6, and IL-12p70 were seen among the 4 groups (data not shown). Why we failed to observe any significant differences in the serum IFN-γ, IL-6, and IL-12p70 levels among the groups may have to do with the fact that many of the values were below the detection limit. The sensitivities of this assay for each cytokine are listed in the methods. Based on previously published concentrations, including our own (4, 6), which show concentrations below the sensitivity of this assay, the lack of significant influence by burn injury is most likely the result of different methods used for assaying the cytokines (i.e., traditional single sandwich ELISA, bead arrays).

Discussion

In this study, we examined the adaptation of mice to a combination of burn injury and psychogenic stress (predator exposure), so as to mimic better the complex pattern of internal and external challenges that give rise to morbidity and mortality in traumatically injured humans. We showed that psychogenic stress (predator exposure) repeated chronically prior to burn injury was able to influence the burn-induced responses in the bone marrow and serum cytokine levels. We found that the influence of prior predator

Table 2. Serum MCP-1 Levels (pg/ml) in Mice Exposed to Burn Injury With or Without Prior Psychogenic Stress (Predator Exposure)[a]

		Burn	
		−	+
Predator	−	41.5 ± 6.05	66.6 ± 5.28*
	+	40.9 ± 5.11	60.2 ± 7.54

[a] Serum was harvested at the time of sacrifice on postburn day 4. All values expressed as ± SEM 5–6 animals per group.
* $P < 0.05$ compared with the levels from both nonburn groups.

exposure on the burn-induced effects depended on the variable studied. For instance, we showed a robust attenuation of the burn-induced increases in myeloid colony formation, with more modest attenuation of the burn-induced increases in the serum MCP-1 level, and in the numbers of immature monocytes and immature neutrophils in the bone marrow. Conversely, prior psychogenic stress enhanced the burn-induced increase in the number of M-CSFR-positive cells in the bone marrow. Therefore, the impact of prior chronic psychogenic stress on burn-induced immune alterations is not always the same, not even within the same immune compartment (myeloid cells in the bone marrow).

Many previous studies have shown that burn injury increases myeloid cell colony formation (6–9). Burn-induced alterations in serum cytokine levels and bone marrow cell colony-stimulating factor receptor expression have been suggested as possible mechanisms (8, 12). Dysfunction of myelopoiesis attributable to burn injury is most pronounced beginning at postburn day 3 and is attenuated by 7 days postburn (8, 9). Therefore, we chose to study the effects of prior predator exposure on the burn-induced effects on postburn day 4. We did observe a burn-induced increase in colony formation, which is in agreement with others (8, 9).

The ability of bone marrow cells to respond to colony-stimulating factors in clonogenic assays provides a functional indication of the cell lineage as well as the proliferative ability of the bone marrow cells. Previous studies have characterized the populations of bone marrow cells that are responsive to specific colony-stimulating factors. Cells that form colonies in response to M-CSF typically are those of the monocyte lineage, while G-CSF-responsive cells consist mostly of granulocytes. Colonies that form in response to GM-CSF are a mixture of cells from the monocyte and granulocyte lineages (8, 40). Therefore, we next characterized the populations of myeloid cells by flow cytometry. We found that burn injury led to an increase in the percentage and absolute number of immature monocytes and immature neutrophils, which is in agreement with Rosinski *et al.* (41), who, using the same antigenic markers, showed an increase in the immature populations in the bone marrow by 65 hrs postburn. Generally, the pattern of responses seen in this cell population (Fig. 2) resembles that seen in the colony assays (Fig. 1). This is consistent with the fact that the immature populations are more responsive to colony-stimulating factors (8, 42).

We found that both mature neutrophils and mature monocytes were not significantly different among the 4 groups. This agrees with the findings of Rosinski *et al.* (41), who found that early postburn (17 hrs) mature populations were decreased, but by 65 hrs postburn had returned to nonburn levels. It has been documented that the mature myeloid cells (monocytes, macrophages, and neutrophils) emigrate to the burn wound (43, 44). The production of new myeloid cells often exceeds the number of cells that have emigrated, producing a net gain in the myeloid cells in the bone marrow, especially immature cells (41). Multiple time points postburn would need to be performed to see if predator exposure influences earlier burn-induced events, such as altered emigration.

Glucocorticoids and catecholamines increase myelopoiesis and decrease lymphopoiesis in the bone marrow (11, 45, 46). The reason why the myeloid population production goes up at the expense of the lymphoid population is postulated to be an evolutionary conserved mechanism that mobilizes the innate immune system first in times of stress to prepare for a predator attack that may cause injury and infection (37, 47). Elevated glucocorticoids and catecholamines after a burn injury (48, 49) are likely to be partially responsible for the burn-induced increases in myeloid cells and colony formation. We speculate that chronic psychogenic stress results in a degree of glucocorticoid resistance (50–52), which (in addition to other mechanisms) attenuates the burn responses. This kind of adaptation would be very advantageous to limit the potential summing effect of multiple stressors in environments that typically are more stressful than controlled laboratory conditions. In this study, we did not measure either the glucocorticoid or catecholamine levels, and future studies will be needed to address this possible mechanism.

In a previous study, burn and sepsis caused a shift toward monocytopoiesis in the bone marrow (8), and our results are generally consistent with this, though different in some details. In particular, the Santangelo *et al.* (8) study noted an increase in M-CSFR density, where our observations suggest an increase in the frequency and absolute number of M-CSFR-positive cells. Since their studies used ligand binding, whereas our studies relied on antibody binding, it is possible that this apparent difference is attributable to the methods used. The reason why the percentages of M-CSFR-positive cells and the density of the receptor per cell are not congruent with the M colony data may have to do with the fact that the cells express receptors that have heterogeneous affinities for the ligand (53). It has been reported that receptors with the highest affinity for ligand were the most likely to proliferate and form colonies (8, 53, 54). If we had discerned the high affinity population of receptors, we may have found that the M-CSFR results more closely resembled the clonogenic ability of the M colony cells; however, this cannot be deduced from the assays and results of the current study.

A consistent finding in this study was that predator stress did not, by itself, alter any of the variables we studied relative to unstressed controls. We used a well-controlled psychogenic stress model that involved no physical contact so as to clearly separate psychogenic stress from the physical stress. This approach is an important complement to other studies that used models in which physical stress, such as biting, occurs (25). Not only is the modality of stressor important (physical vs. psychogenic), the timing of stressors is an important variable to consider. Previous

studies showed effects of psychogenic or physical stress on immune function when the animals were killed by 24 hrs after the last stress session (24, 25, 27). Because we wanted to observe whether psychogenic stress interacted with burn injury, we chose 24 hrs after the last predator exposure to perform the burn treatment. It was expected that the effects of predator exposure could interact with the burn at 24 hrs but be resolved in the unburned animals by the time of sample collection.

The fact that the total cellularity was decreased in the nonexposed burn group but the myeloid populations (immature monocytes and immature neutrophils) were increased in this group of animals indicates that another cell type(s) was/were decreased to a stronger degree than the increases in myeloid cells. The effect of burn injury, with or without predator exposure, on other bone marrow cell populations such as lymphocytes remains to be elucidated.

This study provides evidence, for the first time in a carefully controlled animal model, that a pure psychogenic stressor interacts with the effects of a traumatic injury and that those effects are manifest in the bone marrow and cytokine responses. It should be noted that the immune response to stressors depends on the strain of mouse utilized (22), and the results observed in this study may not necessarily occur in a different strain of mouse. The general pattern of interaction suggests that homeostatic mechanisms may prevent the summation of multiple stressors and limit the myelopoietic response to burn injury. Whether these changes in the myeloid cells are caused by altered proliferation and/or emigration, as well as their impact on postburn sequelae such as wound infection and systemic inflammatory response syndrome, warrants further investigation. Given that psychogenic stress is common in general and likely to be more common among burn patients (13–15), understanding the potential for positive and negative interactions between these modalities of stress will be important.

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