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Original Article

Glucocorticoid release is involved in myeloid-derived suppressor cell accumulation in the spleen following polytrauma

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Abstract: Myeloid-derived suppressor cells (MDSCs) accumulate in the spleen and suppress T cell activity after polytrauma. Glucocorticoid (GC) release is activated by the hypothalamic-pituitary-adrenal (HPA) axis following polytrauma and GCs have been shown to induce a monocyte subset which resembles MDSCs *in vitro*. We investigated the effect of GCs on MDSC expansion following polytrauma. Thirty-six Sprague-Dawley rats were subjected to experimental polytrauma. Animals were sacrificed at 2, 6, 12, 18, 24 or 48 h postoperatively, and blood, spleen and bone marrow were collected for analysis. Dexamethasone was given to naïve rats (n = 6), which were then sacrificed 2 h after administration. Another group of rats received RU486 30 min before polytrauma (n = 6) and were then sacrificed 2 h postoperatively. Serum concentrations of corticosterone were measured using an ELISA kit. Expansion of MDSCs was assessed by flow cytometry. Incorporation of [^{3}H]thymidine was used to measure T cell proliferation. The results showed that MDSCs were mobilized in the bone marrow, increased in the blood and accumulated in the spleen after polytrauma ($P < 0.05$) and this accumulation showed a linear correlation with the release of GCs ($P < 0.05$). MDSCs induced by polytrauma suppressed T cell proliferation. Further, dexamethasone treatment of naïve rats enhanced the MDSC expansion while RU486 administered before polytrauma attenuated it ($P < 0.05$). These results suggest that the release of GCs following polytrauma is involved in MDSC expansion, which suppresses T cell activity. These findings provide new insight into GC regulation of immune cells.

Keywords: Multiple trauma, immune suppression, bone marrow cells, spleen, glucocorticoids

Introduction

Trauma, especially polytrauma, often induces inflammatory responses and immune dysfunction leading to infections and multiple organ failure. Immune dysfunction, frequently observed as T cell dysfunction, is central to the development of infections after trauma [1].

The spleen is rich in immune cells including T cells and B cells. It has been found that myeloid-derived suppressor cells (MDSCs) accumulate in the spleen and suppress T cell function after trauma [2, 3]. MDSCs suppress T cell activation via multiple mechanisms, among which is their uptake of arginine and high intracellular level of arginase that depletes the surrounding arginine, an essential amino acid for T cell activation [4]. Reactive oxygen species (ROS) produced by MDSCs inhibit T cells by catalyzing the nitration of the T cell receptor (TCR), thereby preventing T cell-peptide-MHC interactions [5]. However, the mechanism of MDSC accumulation in the spleen after trauma remains unclear.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is an essential step in the maintenance of homeostasis following trauma and leads to release of glucocorticoids (GCs) [6, 7]. GCs regulate a wide variety of immune cell functions and expression of immune-related genes. GCs can also suppress maturation, differentiation, and proliferation of immune cells [8]. It has been reported that GCs can induce a monocyte subset, which has immune suppressive function and resembles MDSCs *in vitro* [9, 10]. Recently, Zhang *et al.* found that blockage of endogenous GCs with RU486 attenuated the

expansion of MDSCs in the spleen after trauma [11]. However, RU486 is a non-specific antagonist which also has other functions [12, 13], and these may have influenced the conclusion of the above study. Thus we investigated the effect of GCs on MDSC expansion by studying the dynamic changes of MDSCs in the blood, bone marrow and spleen after polytrauma. Serum GC levels were also examined. We found that serum GC levels showed a linear correlation with the expansion of MDSCs after polytrauma. To further confirm the findings, the agonist dexamethasone and the antagonist RU486 were used. The results showed that dexamethasone enhanced MDSC expansion in naïve rats while RU486 attenuated MDSC expansion following polytrauma.

Materials and methods

Animals

This study was approved by the animal research committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Fifty-four adult male Sprague-Dawley rats (250-300 g) were housed two per cage on a 12:12 h light-dark schedule and habituated to the laboratory environment for 1 week prior to use.

Groups and animal injury model

Polytrauma (PT) group (n = 36): Rats were anesthetized using inhalational isoflurane and restrained in the supine position. The polytrauma model was created as described previously [14]. In brief, for the femur fracture, careful blunt dissection of the soft tissues to expose the femur was performed and the femur was fractured with a wire saw. The bones were then realigned. In addition, the superior muscle tissue was grasped with a clamp for 30 s. The skin was closed in a single layer. For the polytrauma model with the addition of cecectomy, the cecum was identified after laparotomy, ligated twice with a 3-0 silk suture, and resected. The abdominal incision was closed in two layers. The rats were administered buprenorphine (0.2 mg/kg body weight) prior to arousal from anesthesia and every 12 h afterward until sacrifice.

The DEX group (n = 6): Dexamethasone (Sigma-Aldrich, St Louis, MO, USA) was administered by intraperitoneal (i.p.) injection at a dose of 0.05 mg/kg. The dose was chosen as described by Bergquist *et al.* [15]. The rats were then sacrificed 2 h after the administration.

The PT-RU486 group (n = 6): RU486 (Sigma-Aldrich) was diluted in corn oil and the rats received 30 mg/kg; i.p 30 min before application of polytrauma stress. The dose was chosen as described by Zhang *et al.* [11]. The rats were sacrificed 2 h postoperatively.

The Sham group (n = 6): Sham animals received anesthesia, but no trauma was applied.

Sample collection

Animals were sacrificed at 2, 6, 12, 18, 24 or 48 h postoperatively (n = 6 at each time point), and their blood, spleen and bone marrow were collected for analysis. The serum concentrations of the endogenous GC corticosterone were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

Isolation of cells

The harvested spleen, bone marrow and blood were converted into single-cell suspensions. Erythrocytes were depleted using RBC lysing buffer (Sigma-Aldrich), and splenocytes were washed in MACS buffer (1 × PBS supplemented with 2 mM EDTA and 0.5% BSA). $CD4^+$ T cells were isolated using corresponding MACS magnetic microbeads (Miltenyi Biotec, Cologne, Germany). The purity of separated cells ranged between 89% and 95%.

Flow cytometric analysis

Suspensions of 1×10^6 cells were stained with appropriately-diluted antibodies directly conjugated with FITC-His48 (anti-granulocytes) and PE-CD11b/c (OX42) (BD Pharmingen, San Diego, CA, USA) according to the standard procedure. All staining procedures were performed on ice. The stained cells were counted using a MoFlo XDP flow cytometer (Beckman Coulter, Brea, CA, USA). $CDbc^+/His48^+$ cells were selected by flow cytometry with purities of > 95%.

T cell proliferation assay

The incorporation of $[^3H]$thymidine was used as a measure of T cell proliferation as described [16]. Briefly, 1×10^6 $CD11bc^+/His48^+$ cells from polytrauma rats or 1×10^6 $CD11bc^+/His48^+$

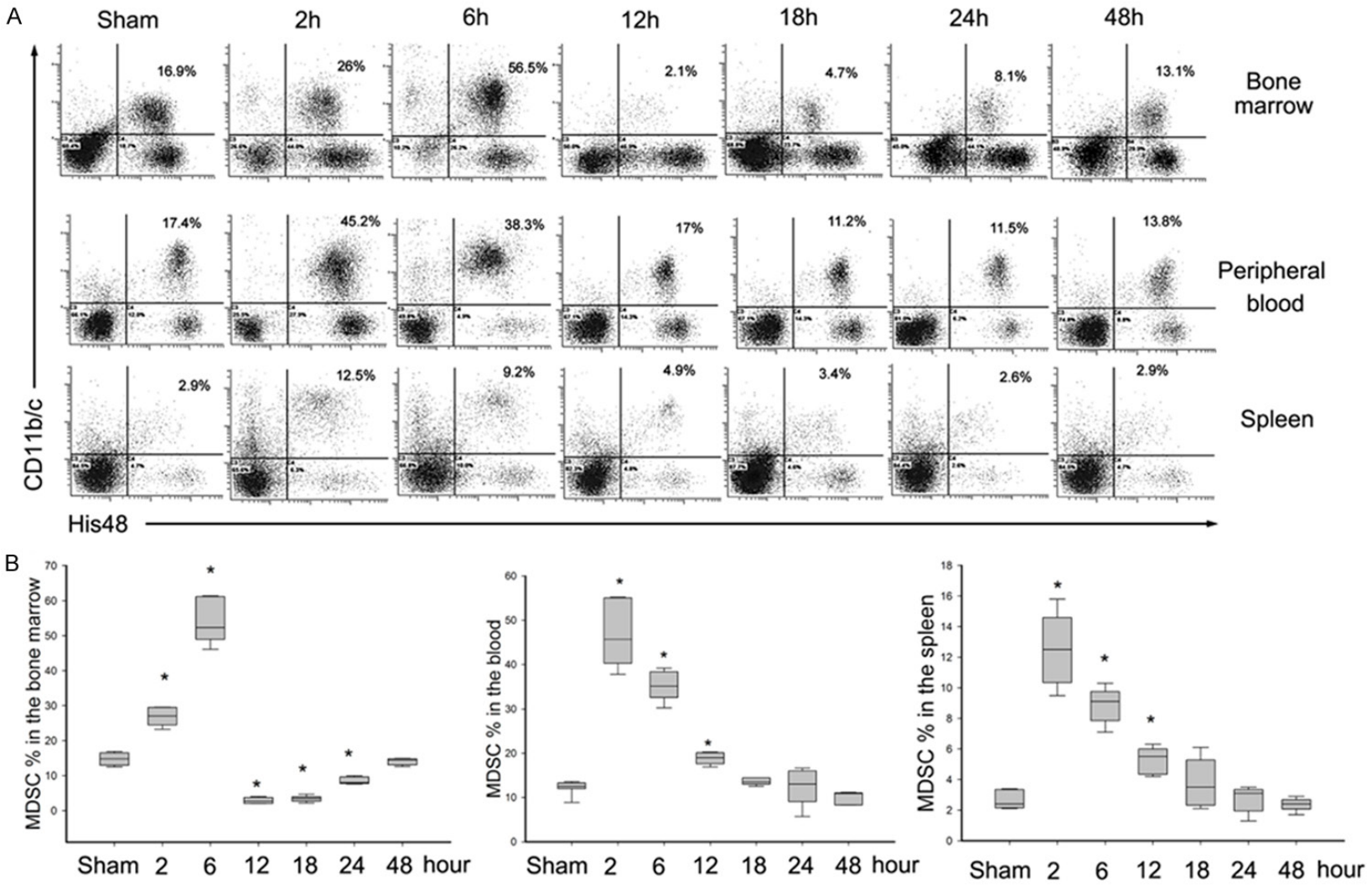

Figure 1. A. Kinetic changes in myeloid-derived suppressor cells in bone marrow, blood and spleen following polytrauma, examined by FACS. B. Asterisks in the box-plots indicate significant differences vs. sham ($P < 0.05$).

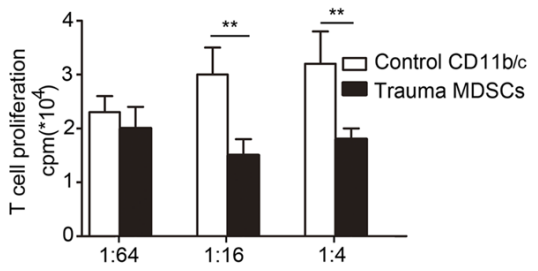

Figure 2. Myeloid-derived suppressor cells induced by polytrauma suppress T cell proliferation. T cells co-cultured in the presence of CD11bc$^+$/His48$^+$ cells induced by polytrauma exhibited a significant decrease in proliferation as measured by [^{3}H] thymidine incorporation. The most significant suppressive effect of CD11bc$^+$/His48$^+$ cells induced by polytrauma on T cell proliferation was observed at an effector:target (E:T) ratio of 1:16 ($P < 0.05$).

cells from sham-operated rats were mixed with 1×10^6 CD4$^+$ T cells. The mixed cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, HEPES (10 mM) and antibiotic/antimycotic solution (RPMI medium) and re-stimulated with 1 μg/mL anti-CD3 and 1 μg/mL anti-CD28 (BD Biosciences, San Jose, CA, USA). For the final 16 h of culture, 1 μCi [^{3}H]thymidine (GE healthcare, Little Chalfont, UK) was added. Proliferation was determined by the incorporation of [^{3}H]thymidine into the cells in co-culture.

Statistical analysis

Results are presented as mean ± SD. A one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test as a post-hoc test for multiple comparisons was performed to identify significant differences among experimental means. For a single comparison of two groups, Student's *t* test was used. The Pearson's product-moment correlation coefficient was used for correlation analysis. For all analyses, $P < 0.05$ was considered statistically significant.

Results

MDSCs were mobilized in the bone marrow, increased in the blood and accumulated in the spleen after polytrauma

In the present study, MDSCs were mobilized in the bone marrow in the first 6 hours. The percentage of MDSCs in the bone marrow increased to 27 ± 2.7% by 2 h and reached a peak of 54.5 ± 6.6% by 6 h before decreasing precipitously to 2.9 ± 0.9% by 12 h, while the baseline was 14.8 ± 1.8% (2 h vs. sham, $P < 0.001$; 6 h vs. sham, $P < 0.001$; 12 h vs. sham, $P < 0.001$). As MDSCs were mobilized in the bone marrow, an increase of the number of MDSCs in the blood was found. The percentage of MDSCs in the blood reached a peak of 47.3 ± 7.7% by 2 h, decreased slightly to 35.4 ± 3.4% by 6 h and to 18.9 ± 1.4% by 12 h, finally returning to baseline values of 13.7 ± 0.8% by 18 h, while the percentage of MDSCs in the sham group was 12.3 ± 1.6% (2 h vs. sham, $P < 0.001$; 6 h vs. sham, $P < 0.001$; 12 h vs. sham, $P = 0.018$; 18 h vs. sham, $P = 0.776$). The accumulation of MDSCs in the spleen showed a similar pattern to that in the blood. The percentage of MDSCs increased to a peak of 12.5 ± 2.4% by 2 h. This peak number of MDSCs was about five times the number in the sham group (12.5 ± 2.4% vs. 2.7 ± 0.6%, $P < 0.001$) (**Figure 1**).

MDSCs induced by polytrauma suppress T cell proliferation

T cells co-cultured in the presence of CD11bc$^+$/His48$^+$cells induced by polytrauma exhibited a significant decrease in proliferation as measured by [^{3}H]thymidine incorporation, compared with cells co-cultured with control CD11bc$^+$/His48$^+$ cells. The most significant suppressive effect of CD11bc$^+$/His48$^+$ cells induced by polytrauma on T cell proliferation was observed at a 1:16 effector:target (E:T) ratio with a more than two-fold inhibition of T cell proliferation (30,333 ± 2517 cpm for controls vs. 14,333 ± 1528 cpm for polytrauma, $P = 0.007$) (**Figure 2**).

GCs are involved in the mobilization of MDSCs in the bone marrow and the accumulation of MDSCs in the spleen following polytrauma

The activation of the HPA axis following polytrauma results in massive GC release. We tested the kinetics of GC release after polytrauma and found that its release had a linear correlation with the accumulation of MDSCs in the spleen after polytrauma ($r = 0.927$, $P = 0.008$) (**Figure 3**). To examine the effect of GCs on the accumulation of MDSCs in the spleen following polytrauma, the agonist dexamethasone (DEX: Sigma-Aldrich) and the antagonist RU486

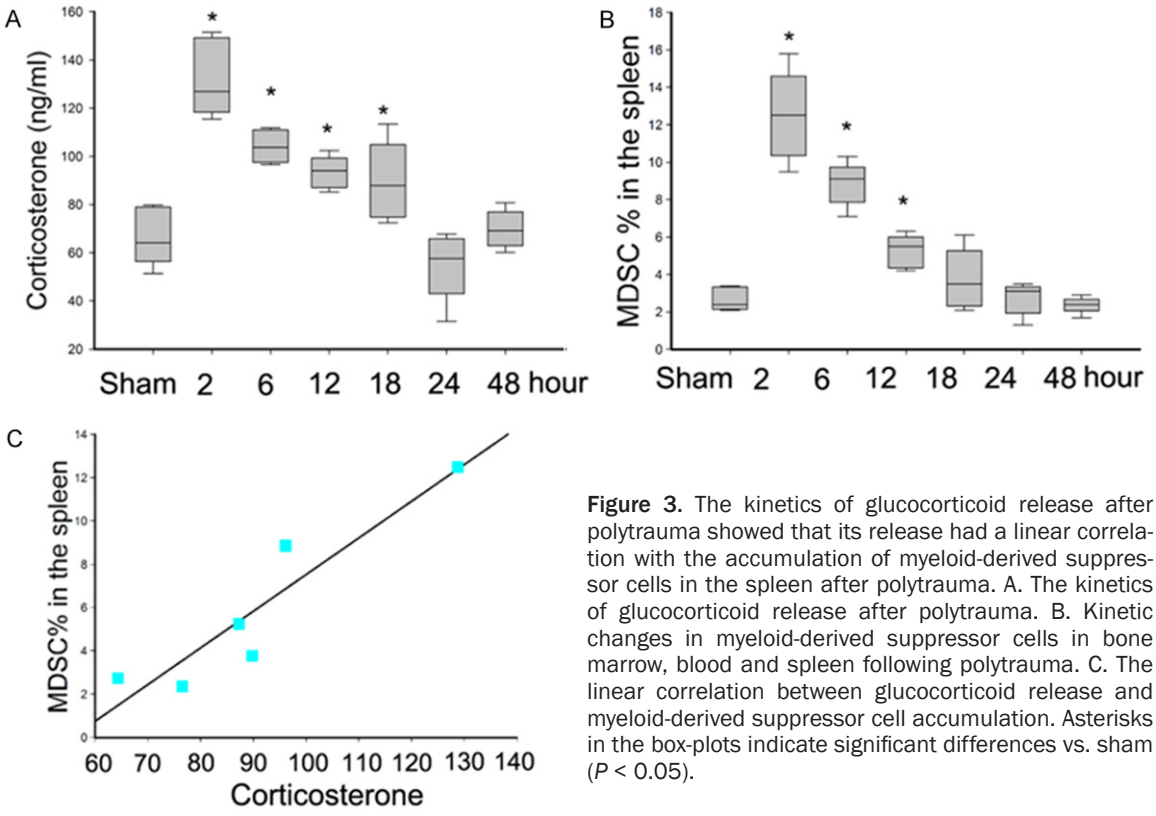

Figure 3. The kinetics of glucocorticoid release after polytrauma showed that its release had a linear correlation with the accumulation of myeloid-derived suppressor cells in the spleen after polytrauma. A. The kinetics of glucocorticoid release after polytrauma. B. Kinetic changes in myeloid-derived suppressor cells in bone marrow, blood and spleen following polytrauma. C. The linear correlation between glucocorticoid release and myeloid-derived suppressor cell accumulation. Asterisks in the box-plots indicate significant differences vs. sham ($P < 0.05$).

(Sigma-Aldrich) were administered as described above. The percentage of MDSCs in the spleen increased to 10.5 ± 1.2% which was similar to that in the PT group 2 h after dexamethasone administration (10.5 ± 1.2% vs. 12.5 ± 2.4%, DEX vs. PT, $P = 0.064$). At the same time, the mobilization of MDSCs in the bone marrow was also observed (19.7 ± 2.7% vs. 14.8 ± 1.8%, DEX vs. sham, $P = 0.007$). In the PT-RU486 group, the accumulation of MDSCs in the spleen after polytrauma was attenuated (5.4 ± 1.6% vs. 12.5 ± 2.4%, PT-RU486 vs. PT, $P < 0.001$) and the mobilization of MDSCs in the bone marrow was inhibited (6.9 ± 1.1% vs. 26.4 ± 2.5%, PT-RU486 vs. PT, $P < 0.001$) (**Figure 4**).

Discussion

MDSCs were first observed more than 30 years ago in cancer patients [17]. MDSCs are a heterogeneous population of cells consisting of precursors of granulocytes, macrophages, dendritic cells and myeloid cells at early stages of differentiation [18, 19]. They function as negative regulators of immune responses, possessing a remarkable ability to suppress T cell responses in cancer and in other diseases. Recently, accumulating evidence has shown that MDSCs induced by trauma also suppress T cell function [2, 20], which is central to the development of infections after traumatic stress [1, 21, 22]. In mice, MDSCs are characterized by the co-expression of the myeloid lineage differentiation antigen Gr1 with CD11b [23]. In humans, MDSCs are most commonly defined as $CD14^-$ $CD16^+$ cells with low expression of the MHC-class-II molecule HLA-DR [24, 25]. Graf *et al.* defined cells which express both monocyte (CD11b/c) and granulocyte (His48)-associated lineage markers as MDSCs in rats [26]. Accordingly, we used FITC-His48 and PE-CD11b/c^+ to test changes in the MDSCs of bone marrow, blood and the spleen after polytrauma. To further test MDSC function, we co-cultured MDSCs following polytrauma with T cells and found that the proliferation of T cells was suppressed. This demonstrated that MDSCs induced by polytrauma in this study present a negative immunoregulatory function.

MDSCs are increased in many pathological conditions, including cancer, bacterial and par-

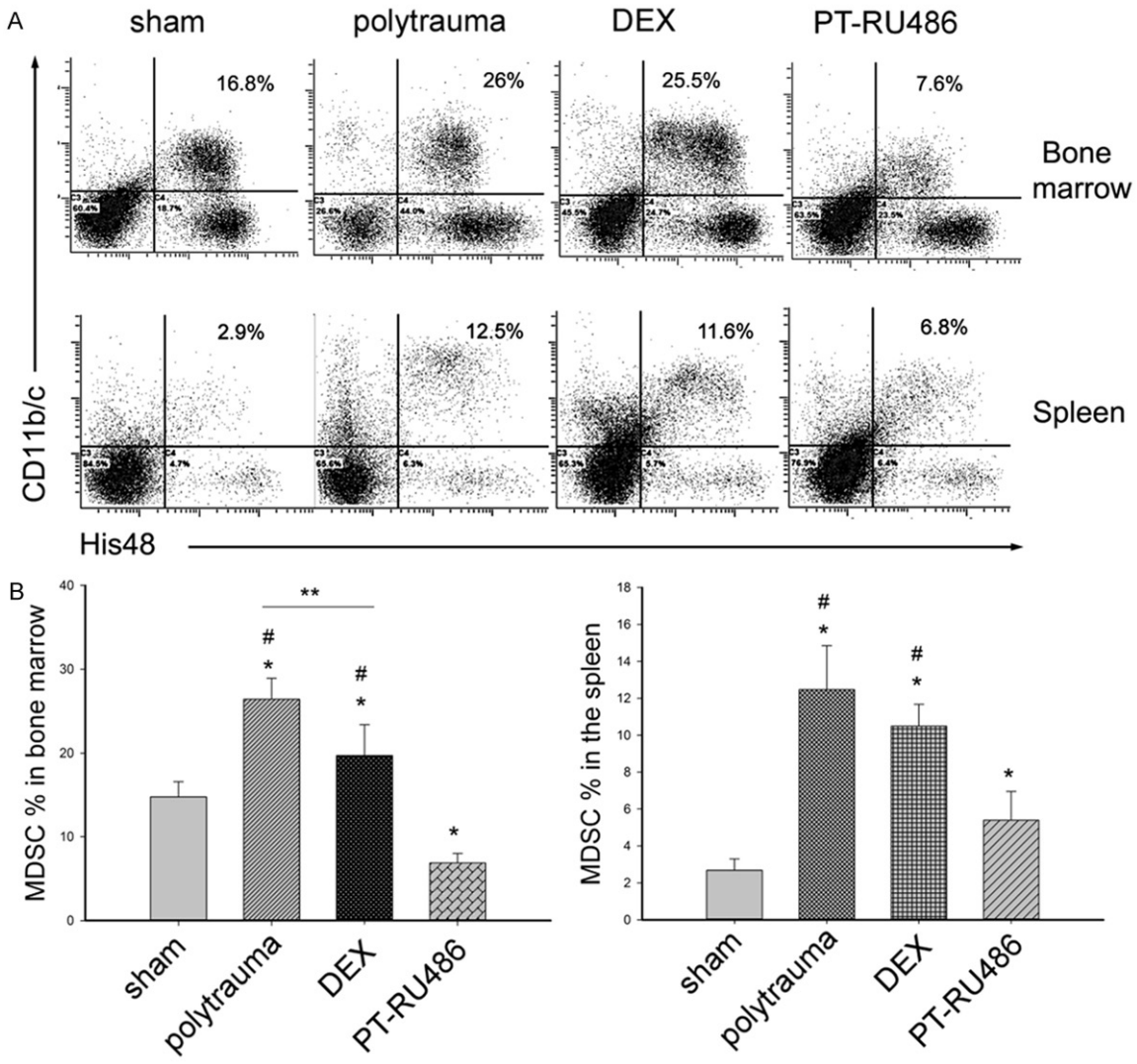

Figure 4. Dexamethasone enhanced myeloid-derived suppressor cell mobilization in the bone marrow and accumulation in the spleen, while PT-RU486 attenuated myeloid-derived suppressor cell mobilization in the bone marrow and accumulation in the spleen after polytrauma. A. Representative flow cytometric analysis. B. Graphical analysis of the proportions of myeloid-derived suppressor cells in the bone marrow and spleen after polytrauma. Asterisks indicate significant differences (PT, DEX, PT-RU486 vs. sham, $P < 0.05$). Pound sign indicates significant differences (PT, DEX vs. PT-RU486, $P < 0.05$). Double asterisks indicate significant differences (PT vs. Dex, $P < 0.05$).

asitic infections, acute and chronic inflammation, traumatic stress, surgical sepsis and transplantation [2, 16, 27-34]. There are many factors that induce the expansion of MDSCs, including cyclooxygenase-2 (COX2), prostaglandins [35-37], stem-cell factor [37], macrophage colony-stimulating factor (M-CSF), IL-6 [38], granulocyte/macrophage colony-stimulating factor (GM-CSF) [36] and vascular endothelial growth factor (VEGF) [39]. The signaling pathways in MDSCs that are triggered by most of these factors converge on Janus kinase (JAK) protein family members and members of the signal transducer and activator of transcription (STAT) protein family, which are signaling molecules that are involved in cell survival, proliferation, differentiation and apoptosis [40]. However, these findings mostly pertain to MDSCs expanded in cancer. In this study, we found that there was a linear correlation between the levels of endogenous GCs and the accumulation of MDSCs in the spleen. Application of the agonist dexamethasone in naïve rats enhanced MDSC mobilization in the bone marrow and

accumulation in the spleen, while the antagonist RU486 used before polytrauma stress attenuated MDSC mobilization in the bone marrow and accumulation in the spleen. Although GC levels were slightly higher in polytrauma model rats than in sham rats by 18 h postoperatively (P = 0.011), there was no significant difference in the accumulation of MDSCs in the spleen between the polytrauma and sham groups. This may be because MDSCs in the bone marrow were depleted. These findings suggest that GC release-caused by the activation of the HPA axis following polytrauma-mediate MDSC expansion.

GCs regulate gene expression by binding to the glucocorticoid receptor (GR). GCs play an anti-inflammatory and immunosuppressive role via the interaction of the GR with the proinflammatory transcription factors AP-1 (activator protein-1) and NF-kB [41, 42]. GCs also are involved in the signaling pathway of the STAT family [43]. Munera *et al.* found that the STAT6 pathway and T_H2 cytokines played important roles in the accumulation of trauma-induced MDSCs in the spleen [3]. Thus, it is possible that GCs mediate MDSC mobilization in the bone marrow and accumulation in the spleen through the STAT6 pathway.

In conclusion, we have demonstrated that GC release caused by activation of the HPA axis following polytrauma shows a linear correlation with the expansion of MDSCs. An agonist and an antagonist enhanced and attenuated, respectively, the mobilization of MDSCs in the bone marrow and their accumulation in the spleen. Thus, the present study provides new insight into GC regulation of immune cells.

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Disclosure of conflict of interest

None.

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