

Endogenous glucocorticoids promote the expansion of myeloid-derived suppressor cells in a murine model of trauma

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Abstract. Stress-dose of glucocorticoid has been demonstrated to be beneficial for trauma patients in clinical studies. Recently, a heterogeneous population of myeloid cells with immunosuppressive activity named myeloid-derived suppressor cells (MDSCs) has been found to accumulate in the trauma host and can be induced by glucocorticoids *in vitro*. In order to explore the effect of endogenous glucocorticoids on MDSCs under trauma conditions, we blocked the glucocorticoid signal in a murine trauma model using the antagonist of the glucocorticoid receptor RU486 (mifepristone). We found for the first time that RU486 not only blunted MDSC expansion induced by trauma in the spleen, peripheral blood and bone marrow especially at 6 h after traumatic stress but also decreased the survival rate from 100 to 20% in traumatic mice within 7 days. Moreover, neither MDSCs producing arginase-1 nor the morphological characterization of trauma-induced MDSCs was affected by the blockage of the glucocorticoid receptor. Our results suggest that endogenous glucocorticoids may promote MDSCs expansion in a murine trauma model and MDSCs may be beneficial for the trauma host.

Introduction

Trauma patients are often diagnosed as having systemic inflammatory response syndrome (SIRS), which usually leads to nonreversible multi-organ dysfunction syndrome (MODS) and eventually to patient death. Thus, controlling the SIRS after trauma is of high value in the care of trauma patients (1-5). Glucocorticoids, which have been widely used in treating inflammatory disorders, are well known for their regulation of the immune system. Due to their suppressing effects on excessive immune response and SIRS after trauma, glucocorticoids

are supposed to be theoretically beneficial in treating trauma patients (6,7). Moreover, a clinical study has demonstrated that a stress-dose of hydrocortisone improves outcome in trauma patients, especially in patients with trauma-related corticosteroid insufficiency (6,8). However, the antiinflammatory action of glucocorticoids is very complicated (9). In this study, we mainly focused on the relation between glucocorticoid and the immunoregulatory myeloid derived suppressor cells (MDSCs) under traumatic conditions.

MDSCs are a heterogeneous population of cells that consists of mature or immature myeloid cells, which express CD11b⁺/Gr-1⁺ markers and exert immune regulatory function in cancer, autoimmune diseases and chronic or acute inflammation (10-14). It has been reported that MDSCs expansion can be induced by physical injury in the spleen of a trauma model (15,16). Moreover, trauma-induced MDSCs (TIMDSCs) are considered to take responsibility for immune suppression and high susceptibility after trauma via producing arginase-1 which mediates arginine depletion and results in T-cells dysfunction (15,17). However, the precise role of MDSCs in trauma remains unclear. Some studies advocate that MDSCs expansion may play a positive role and be a part of the self-protection in trauma (18).

Recent studies have reported that glucocorticoids can induce a monocyte subset, which has immune suppressive function and resembles MDSCs *in vitro* (19-21). However, the effects of endogenous glucocorticoid on TIMDSCs *in vivo* are still unknown. Therefore, we hypothesized that endogenous glucocorticoids might be involved in TIMDSCs expansion, which may be a mechanism underlying the protective role of endogenous glucocorticoids under traumatic condition. In this study, we found that a glucocorticoid receptor blocker RU486 inhibited TIMDSCs expansion in the spleen, peripheral blood and bone marrow without affecting their characterization. Our study provides the first evidence that endogenous glucocorticoids are involved in TIMDSCs expansion in a murine trauma model and suggests that TIMDSCs may correlate with the protective role of endogenous glucocorticoids in trauma host.

Materials and methods

Mice. Male BALB/c mice, 6 to 8 week of age, were obtained from the Center of Medical Experimental Animals of Hubei

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Province (Wuhan, China) and the study was approved by the Animal Care and Use Committee of Tongji Medical College. Mice were housed four per cage and were fed with food and water *ad libitum* under a 12-h light/dark cycle at 20–22°C in a pathogen-free facility. Mice underwent an acclimation period of 2 weeks before experiment.

Mouse traumatic stress model. Mice were divided into three groups randomly: Group 1 received anesthesia only, Group 2 underwent traumatic stress, Group 3 received RU486 (Sigma-Aldrich) (30 mg/kg; i.p.) 30 min before traumatic stress. The traumatic stress was mimicked using an abdominal surgery reported by Makarenkova *et al.* (15). Animals were sacrificed at 6, 12 and 24 h after surgery, and spleen, bone marrow and peripheral blood were taken for cell harvest.

Isolation of Gr-1⁺ cells. The spleen, bone marrow and peripheral blood derived from the mice of the three groups were made into a single-cell suspension, respectively. Erythrocytes were depleted by RBC lysing buffer (Sigma-Aldrich), and all the cells were washed and resuspended in MACS buffer (1X PBS supplemented with 2 mM EDTA and 0.5% BSA). Gr-1⁺ cells were sorted using corresponding MACS magnetic microbeads (Miltenyi Biotec). The purity of Gr-1⁺ cells ranged between 85 and 95%.

Flow cytometry analysis. Harvested cells were suspended in FACS medium (1X PBS supplemented with 0.1% BSA and 0.1% NaN₃) and stained with FITC-labeled anti-mouse CD11b, and PE-labeled GR1 (eBioscience) according to the standard procedure. All staining procedures were performed on ice. The stained cells were counted using a FACScan flow cytometer (BD Biosciences).

Western blot analysis. Total protein from Gr-1 cells was separated on 10–12% SDS-PAGE gels and electro blotted onto nitrocellulose membranes. The protein of the mouse liver served as a positive control. The nitrocellulose membrane was blocked in TBST (Tris-buffered saline with 0.5% Tween-20) containing 5% non-fat milk, and incubated with rabbit IgG anti-mouse arginase-1 primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. The membrane was washed and incubated with a secondary antibody for 1 h. Then the membrane was stripped, washed and incubated with goat anti-mouse β-actin Ab (Santa Cruz Biotechnology, Inc.), as an internal control for total protein concentration. After being washed, the membrane was incubated with secondary antibody for another 1 h. ECL substrate (Pierce) was used for signal detection of both arginase-1 and β-actin.

Morphological analysis of MDSCs. To observe the morphology of MDSCs, 10⁵ enriched Gr-1⁺ splenocytes were stained by One Step II Wright-Giemsa Stain Solution (Criterion Sciences). The Gr-1⁺ splenocytes suspension was dropped onto a slide air-dried at room temperature.

Immunohistochemical staining. For immunohistochemistry, paraffin sections of spleens were deparaffinized and rehydrated by successive washes with xylene and graded ethanol. To block endogenous peroxidase, 3% hydrogen peroxide was

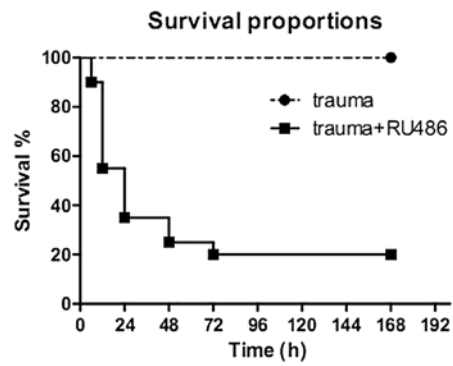


Figure 1. Survival proportions after trauma (n=20 per group).

used. Antigen retrieval was performed in a microwave oven in 100 mM sodium citrate buffer (pH 6.0) for 10 min and then cooled to room temperature. Subsequently, slides were incubated with 10% normal serum followed by the primary anti-Gr-1 antibody (eBioscience) incubated overnight at 4°C. Then the slides were incubated with biotinylated secondary antibody for 30 min, and streptavidin-conjugated horseradish peroxidase (Santa Cruz Biotechnology, Inc.) was used for immunohistochemical staining. Digital images were acquired using an Olympus Microscope.

Statistical analysis. The significance of differences between the experimental groups was evaluated using one-way ANOVA (SigmaStat software; Jandel Scientific). Student's t-test was used for a single comparison of the two groups. P-value <0.05 was considered significant. Data are presented as the mean ± SEM. All experiments were performed at least three times.

Results

RU486 decreases the survival rate of mice after trauma stress. Fourty mice were divided into two groups randomly and equally. Mice in Group 1 merely received surgery, while mice in Group 2 were treated with RU486 before surgery. After surgery, no death was observed in Group 1. However, in Group 2, the survival rate was 90% within 6 h after surgery and descended to 55% after 12 h. Nearly two-thirds of the mouse in Group 2 died on Day 1, and no more death was observed beyond Day 3. The overall survival rate in Group 2 was significantly lower than that in Group 1 during 7 days of observation (20 vs. 100%, respectively) (Fig. 1). These results indicate the protective role of endogenous glucocorticoids in the trauma mouse model.

RU486 attenuates the expansion of CD11b⁺/Gr-1⁺ cells after trauma in the spleen. It has been demonstrated that an expansion of CD11b⁺/Gr-1⁺ cells in the spleen is induced by traumatic stress (15), and the activation of the hypothalamic-pituitary-adrenal axis (HPA) after trauma results in massive glucocorticoid release (22). In order to reveal the effect of endogenous glucocorticoids on TIMDSCs, CD11b⁺/Gr-1⁺ splenocytes were analyzed in Group 2 (traumatic stress) and Group 3 (traumatic stress plus RU486) using flow cytometry. We found that the expansion of CD11b⁺/Gr-1⁺ splenocytes was

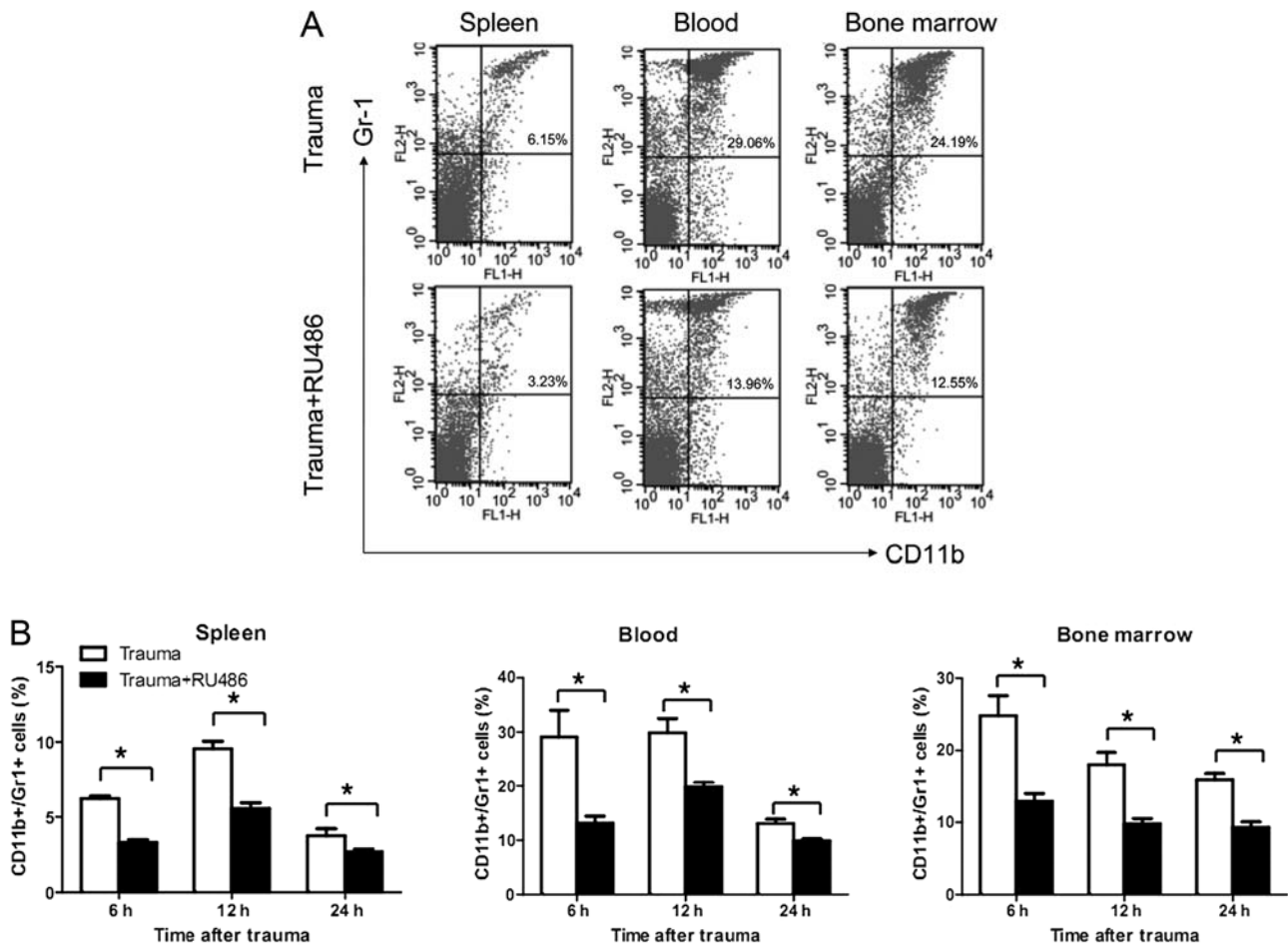


Figure 2. Expansion of CD11b⁺Gr1⁺ MDSCs is impaired in traumatic mice treated with RU486. (A) Representative flow cytometric analysis. (B) Graphic analysis of CD11b⁺Gr1⁺ MDSCs proportions in spleens, blood and bone marrow in traumatic mice treated with or without RU486 at different time points after trauma (n=4 per time point per group). Data are expressed as means ± SD. *P<0.05.

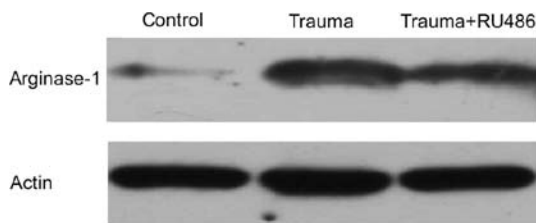


Figure 3. Arginase-1 expression in Gr-1⁺ splenocytes analyzed by western blot analysis. Liver protein lysate was used as a positive control and β-actin expression was used as a semi-quantitative control. Arginase-1 expression in Gr-1⁺ splenocytes was not obviously altered by RU486 administration in traumatic mice.

attenuated by the administration of RU486 (Fig. 2). The data suggests that massive glucocorticoid release after trauma may be involved in the expansion of CD11b⁺Gr-1⁺ cells in spleen. Besides the above-mentioned experiments, immunohistochemistry was also employed to examine the CD11b⁺Gr-1⁺ cells in the spleen of mice and showed results in line with the flow cytometry data mentioned above. An apparent decrease of Gr-1⁺ cells, which was located around the splenic corpuscle, was observed in the spleen of the mice treated by RU486 (Fig. 4). These results suggest that RU486 might attenuate the expansion of trauma-induced CD11b⁺Gr-1⁺ cells in the spleen.

RU486 inhibits the expansion of CD11b⁺Gr-1⁺ cells after trauma in both peripheral blood and bone marrow. In our study, bone marrow cells and peripheral blood cells were also determined by flow cytometry. Bone marrow cells and peripheral blood cells were harvested and stained with anti-Gr-1 (PE) and anti-CD11b (FITC) Abs, and then were determined at 6, 12 and 24 h after trauma stress using flow cytometry. We found that the expansion of trauma-induced CD11b⁺Gr-1⁺ cells was inhibited by RU486 in both peripheral blood and bone marrow with the most obvious inhibition at 6 h after trauma stress (Fig. 2). These results were consistent with the relative data observed in the spleen and indicated that endogenous glucocorticoids might be involved in TIMDSCs expansion.

RU486 does not affect the arginase-1 expression in CD11b⁺Gr-1⁺ cells after trauma. Some reports have indicated that TIMDSCs suppressed the function of T cells by upregulation of arginase-1 expression, which would deplete arginine from the immune microenvironment (15,17). To further determine whether RU486 can modify the characteristic of TIMDSCs, the arginase-1 expression of TIMDSCs was evaluated using western blotting. Contrary to our prediction, we found no obvious difference in arginase-1 expression of Gr-1⁺ splenocytes between Group 2 and Group 3 (Fig. 3). This result indicates that blockage of the glucocorticoid receptor may

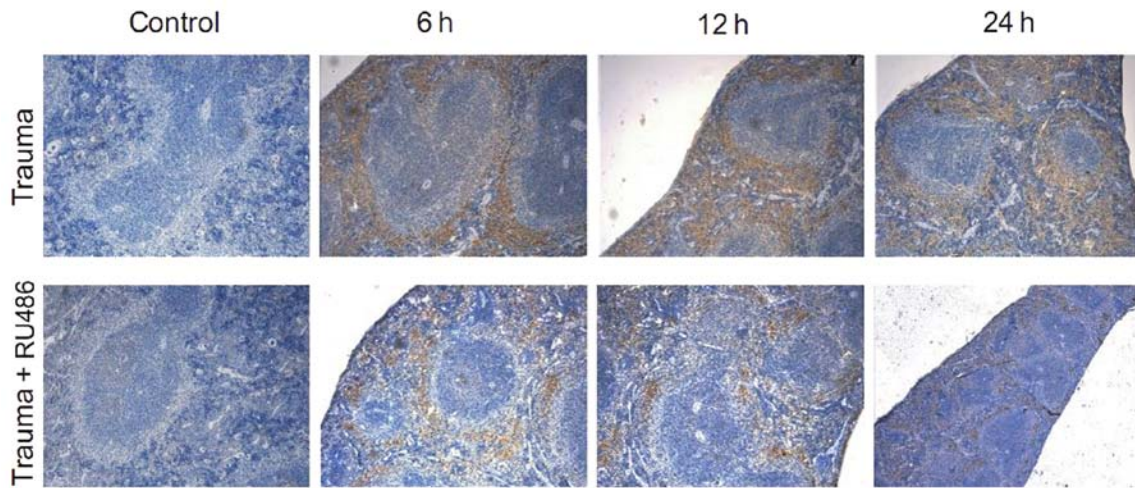


Figure 4. Accumulation of Gr-1⁺ cells in the spleen is blunted in traumatic mice treated with RU486. Paraffin sections were stained with Gr-1 antibody. The accumulation of Gr-1⁺ cells in the spleen was observed in traumatic mice within 24 h after trauma and was blunted by treating with RU486. Gr-1⁺ cells (yellow) were located at the periaarteriolar lymphatic sheaths and marginal zones. There are few Gr-1⁺ cells observed in the red pulp. Magnification, $\times 100$.

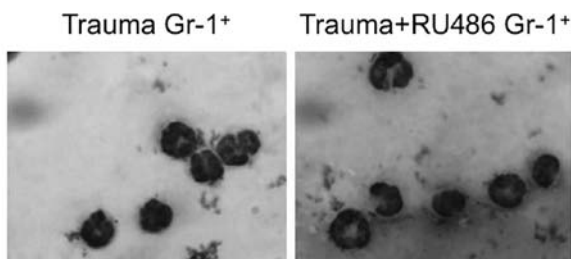


Figure 5. RU486 does not alter the morphological characterization of trauma induced MDSCs. Gr-1⁺ cells isolated from spleen (purity >95%) were stained using One Step II Wright-Giemsa Stain Solution and dropped onto a slide. These cells represent a morphology resembling granulocytes or ringlike nuclei. No matter whether the traumatic mice was treated with RU486 or not, there was no difference observed in the morphological characterization of Gr-1⁺ splenocytes. Magnification, $\times 400$.

merely attenuate the expansion of CD11b⁺/Gr-1⁺ cells induced by trauma without affecting their suppressive nature.

RU486 dose not alter the morphological characterization of trauma induced CD11b⁺/Gr-1⁺ cells. In order to further characterize the effect of RU486 on TIMDSCs, we also investigated the morphological characterization of Gr-1⁺ splenocytes sorted by Gr-1 magnetic microbeads. Enriched Gr-1⁺ splenocytes were stained by One Step II Wright-Giemsa Stain Solution (Criterion Sciences). There was no obvious difference in the morphological characterization of Gr-1⁺ splenocytes between Group 2 and Group 3, and these cells either displayed a classical morphology resembling neutrophils or showed ringed-shaped nuclei (Fig. 5), which was consistent with a previous study (15,18). This result suggests that endogenous glucocorticoids may have no effect on the morphological characterization of TIMDSCs.

Discussion

Our study provided the first evidence that blockage of glucocorticoid receptors could blunt TIMDSCs expansion, which

was observed in the spleen as well as in the bone marrow and peripheral blood in a murine trauma model. In addition, the arginase-1 expression and the morphological characterization of TIMDSCs was not affected by RU486 administration. Moreover, blockage of glucocorticoid receptors decreased the survival rate of traumatic mice while it attenuated TIMDSCs expansion. These results indicate that endogenous glucocorticoids may promote TIMDSCs expansion without changing their characteristics and TIMDSCs may exert protective effects on trauma host.

It is well known that the hypothalamic-pituitary-adrenal (HPA) axis is activated after trauma, and then, massive endogenous glucocorticoid is released and plays a protective role in trauma host (22-24). The underlying mechanisms involved in this protective effect include inhibiting pro-inflammatory cytokines and promoting anti-inflammatory cytokines (25,26). Moreover, glucocorticoids exert an effect on some immunoregulatory cells, such as immunoregulatory T cell (Treg) and MDSCs (19,26,27). Previous studies have demonstrated an accumulation of MDSCs in spleen after trauma (15,16,28,29). However, the effect of endogenous glucocorticoids on MDSCs in the trauma host has not been presented to date.

In this study, a murine trauma model was used to determine the effect of RU486 on TIMDSCs. We found that TIMDSCs accumulation in the spleen was attenuated by RU486 administration, and TIMDSCs were in the same location of the spleen as described by previous reports (14,15,30) (Figs. 2 and 4). Moreover, exogenous glucocorticoids have been reported to induce MDSCs proliferation in both human and mouse *in vitro* (19-21). Furthermore, *in vivo* studies have demonstrated the promotive effect of glucocorticoids on MDSCs in endotoxin immunosuppressed mice (31). In our study, being consistent with previous studies, endogenous glucocorticoids exerted similar effects on MDSCs in the spleen of a murine trauma model (Figs. 2 and 4). However, the majority of previous studies about TIMDSCs focus on the spleen, while little research has been conducted on TIMDSCs in other organs, such as in the peripheral blood and bone marrow. Therefore, we further determined the percentage of TIMDSCs in the

bone marrow and peripheral blood. We observed similar effects of endogenous glucocorticoids on TIMDSCs in the bone marrow and peripheral blood as we found in splenocytes (Fig. 2). It is of note that endogenous glucocorticoids are not the only responsible factor for TIMDSCs expansion. Previous studies in tumor-bearing host demonstrated that some trauma related inflammatory factors including S100A9/8 (32-34) and prostaglandin E2 (PGE2) (35-37) played an important role in MDSCs expansion. Therefore, these factors may also promote TIMDSCs expansion in trauma host, besides glucocorticoids.

Under traumatic conditions, excess neutrophils as well as neutrophil-like TIMDSCs (Fig. 5) are mobilized and activated (1). However, inappropriate neutrophil sequestration in the lung often leads to hospital-acquired pneumonia and finally to pneumonia or even acute respiratory distress syndrome (ARDS) in trauma patients (6,38,39). In a multicenter, randomized, double-blind, placebo-controlled study, investigators have elucidated that stress-dose of corticosteroid decreases the risk of hospital-acquired pneumonia in trauma patients with corticosteroid insufficiency (6). In our study, mimicking corticosteroid insufficiency, blockage of the glucocorticoid receptor with RU486 decreased the survival rate of traumatized mice while it inhibited TIMDSCs expansion (Figs. 1 and 2). We thus infer that TIMDSCs may limit SIRS after trauma via expressing immunosuppressive factors such as arginase-1. In addition, the expansion of neutrophil-like TIMDSCs may lead to the descent of pro-inflammatory neutrophils, since both TIMDSCs and neutrophils may be derived from the same progenitor. Taken together, it seems that TIMDSCs expansion promoted by endogenous glucocorticoids may play a beneficial role in the trauma host.

Since TIMDSCs regulate the immune response via producing arginase-1, which depletes arginine within the immune environment and results in arginine deficiency followed by T cell dysfunction (15), we further determined the production of arginase-1 in TIMDSCs. Western blotting found no effect of RU486 on arginase production (Fig. 3), indicating that endogenous glucocorticoids may not alter the suppressive activity of TIMDSCs though affecting their number and percentage. This discrepancy may be due to the difference between the signal pathways for MDSCs expansion and those for activation (11,40).

In summary, this study presents the first evidence that RU486 can blunt TIMDSCs expansion, which may reveal the relation between endogenous glucocorticoids and TIMDSC *in vivo*. The present study shows that endogenous glucocorticoids promote TIMDSCs expansion, and TIMDSCs may play a beneficial role in trauma host. Our study provides a possible mechanism underlying the protective effects of endogenous glucocorticoids in trauma patients.

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