

Semaphorin 3A contributes to sepsis-induced immunosuppression by impairing CD4⁺ T cell anergy

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Abstract. Semaphorin 3A (Sema3A), a member of the Sema family of proteins, appears to serve an important role in sepsis and sepsis-induced immunosuppression and has been regarded as a crucial regulator involved in cellular immune response. However, the role of Sema3A in CD4⁺ T cell anergy during sepsis remains to be elucidated. In the present study, the cecal ligation and perforation model and lipopolysaccharide (LPS) were used to simulate sepsis and the role of Sema3A in sepsis-induced CD4⁺ T cell anergy was investigated *in vivo* and *in vitro*. *In vivo*, the serum concentration of Sema3A was enhanced and exacerbated sepsis-induced T cell immunosuppression and multiple organ dysfunction syndromes (MODS). Administration of (-)-epigallocatechin-3-gallate, an inhibitor of Sema3A, markedly improved sepsis-induced T cell immunosuppression and MODS. *In vitro*, both lymphoid and myeloid lineages secreted high concentration of Sema3A in LPS-induced sepsis, especially in the lymphoid lineage. Inhibition of Sema3A alleviated T cell anergy. The NF-κB signaling pathway was involved in Sema3A-mediated auto-crine loop aggravating T cell immune dysfunction during LPS-induced sepsis. Inhibiting Sema3A exerted significant improvement of sepsis-induced immunosuppression and MODS, which was associated with improvement of CD4⁺ T cells anergy via regulation of the NF-κB signaling pathway.

Introduction

Sepsis is defined as a syndrome characterized by ‘life-threatening organ dysfunction caused by the dysregulated host response to an infection,’ with emphasis on multiple organ

dysfunction syndromes (MODS) (1). In China, sepsis affects one-fifth of patients admitted to intensive care units in mainland China with a 90-day mortality rate of 35.5% (2). In 2015, a total of 1,937,299 deaths occurred across the 605 mainland Chinese disease surveillance points and the standardized sepsis-related mortality rate was 66.7 deaths per 100,000 population (3). The immunological responses in patients with sepsis are complex, comprising concurrent pro- and anti-inflammatory responses (4). Compelling experimental and clinical evidence has indicated that immunosuppression is the cause of the aggravation, complicated with MODS and even mortality of patients with sepsis (5).

Immune activation is accompanied by functional impairment of innate and adaptive immune cells, such as apoptosis of a large number of immune cells, CD4⁺ T cell anergy and enhancement of negative immunomodulatory cells (5,6). It has become more evident that the majority of patients with sepsis succumb not to the early, overwhelming pro-inflammatory response, but to the immunosuppression-related complications that occur later in their disease trajectory (7,8). Sepsis-induced immunosuppression prevents patients from removing pathogenic microorganisms and increases their susceptibility to secondary infections, especially caused by opportunistic pathogens (9). CD4⁺ T cell anergy is defined by three features in sepsis-induced immunosuppression: Apoptosis-induced depletion leading to a decrease in the number of CD4⁺ T cells, a decrease in the CD4⁺ T cell proliferative response and even immunological paralysis and a shift from a helper T cells (Th)1 to a Th2 cell profile, defined as Th2 immune polarization (10).

Semaphorin 3A (Sema3A) is a ligand of Neuropilin-1 (Nrp-1) and serves a key role in neuraxon development (11). Using lipopolysaccharide (LPS)-induced acute kidney injury model, Sema3A has been identified in tubular epithelial cells (12). Anti-Sema3A antibodies have been reported to be effective in improving the survival rate in LPS-induced sepsis in mouse models (13). By contrast, the suppression of Sema-3A in tumor cells with a small interfering (si)RNA augments T cell activation (14). Thus, the objective of the present study was to investigate the effect of Sema3A on the CD4⁺ T cell anergy. This information will provide a potential novel target for the study of immune regulation in sepsis. The present study demonstrated

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that inhibition of Sema3A significantly relieved CD4⁺ T cell anergy via regulation of the NF- κ B signaling pathway in sepsis.

Materials and methods

Animals and ethics statement. A total of 120 inbred male C57BL/6J mice (obtained from the Laboratory Animal Center of the Chinese Academy of Medical Sciences, no. SCXK-Jing-2014-0004), 6-8 weeks old, 20 \pm 2 g were used for the *in vivo* study. Mice received standard care under a 12-h dark/light cycle (25°C with an atmosphere of 60%) and given free access to food and water, in accordance with the animal care guidelines of the Tianjin Medical University General Hospital (Tianjin, China). Before the experiment, adaptive feeding was carried out for 1 week. Before surgery, food and water was not given for 12 h. All experimental procedures complied with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (15) and were approved by the scientific investigation review board of Tianjin Medical University General Hospital (approval no. ZYY-DWFL-IRB-001F-01).

Sepsis model. After being anesthetized, a 0.5-cm incision was made on the abdomen of mice and the cecum was exposed. Briefly, mice were anesthetized with isoflurane inhalation at the concentration of 2.5% for anesthetic induction and then at 1% for anesthetic maintenance until the end of the cecal ligation and perforation (CLP). The diameter of the puncture needle was 0.6 mm and it was used to induce CLP in the experiment. The mice were given a subcutaneous injection of 0.9% sterile saline solution in a volume of 40 ml/kg body weight following CLP.

Experimental design. The mice were randomly divided into six groups (n=10): Control, sham, CLP and the different epigallocatechin-3-gallate (EGCG; cat. no. 989-51-5; Beijing Solarbio Science & Technology Co., Ltd.)-treatment groups. In the control group, the mice were anesthetized, but no surgery was carried out. For mice in the sham group, the cecum was exteriorized without ligation and puncture. In the CLP group, a midline laparotomy was performed according to the sepsis model described above. After CLP was obtained, the different EGCG-treatment groups were immediately administered increasing concentrations (25, 50 and 100 mg/kg, intravenously) of EGCG, a strong inhibitor of Sema3A activity.

Isolation and culture of splenic lymphocytes. The mice were sacrificed by cervical dislocation. Spleens were harvested and prepared as single-cell suspensions and then subjected to Ficoll-Paque density gradient centrifugation (cat. no. LTS1092P; Tianjin TBD Science) at 4°C, 1,500 x g for 15 min. Lymphocytes [CD4⁺ T cells, CD4⁺CD25⁺ regulatory T cells (Tregs)], B cells and natural killer (NK) cells were isolated from mononuclear cells using a CD4⁺ T cell Isolation kit, mouse (cat. no. 130-104-454), a CD4⁺CD25⁺ regulatory T cell Isolation kit, mouse (cat. no. 130-091-041), a Pan B Cell Isolation kit II, mouse (cat. no. 130-104-443), and a NK Cell Isolation kit, mouse (cat. no. 130-115-818; all from Miltenyi Biotec GmbH) and autoMACS[®] Columns (Miltenyi Biotec GmbH) according to the manufacturer's instructions. Cells of the myeloid lineage, including macrophage (M ϕ) cells and

dendritic cells (DC), were isolated from the abdominal cavity and femur, respectively, using a M ϕ Isolation kit (Peritoneum), mouse (cat. no. 130-110-434) and a Pan DC Isolation kit, mouse (cat. no. 130-100-875; both from Miltenyi Biotec GmbH) and autoMACS[®] Columns (Miltenyi Biotec GmbH) according to the manufacturer's instructions. Isolated cells were cultured in RPMI-1640 medium (Nanjing KeyGen Biotech Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) and incubated at 37°C with 5% CO₂, following treatment with anti-CD3e (5 μ g/ml; cat. no. 564378; BD Pharmingen; BD Biosciences) and anti-CD28 (2 μ g/ml; cat. no. 562767; BD Pharmingen; BD Biosciences) antibodies for polyclonal activation of T cells.

In vitro studies. LPS (100 ng/ml; *Escherichia coli* 0111:B4; cat. no. L2630; Sigma-Aldrich; Merck KGaA) induction was used for septic simulation. Subsequently, splenic CD4⁺ T cells were seeded on 96-well cell-culture plates (2x10⁵ cells/well) and treated with anti-CD3e and anti-CD28 antibodies for polyclonal activation of T cells and treated with different doses of recombinant Sema3A polyclonal antibody (anti-Sema3A antibody, 0.5, 2 and 4 μ g/ml; cat. no. 201253-T10; Sino Biological, Inc.), recombinant Sema3A (rSema3A, 10, 100 and 1,000 ng/ml; cat. no. 7201; BioVision, Inc.) and EGCG (1, 10 and 100 μ mol/l) for 24 h. The proliferative activity, apoptotic rate, cytokine secretion (including IFN- γ and IL-4) and Foxp-3 expression of CD4⁺ T cells were determined. The serum levels of aspartate aminotransferase (AST), alanine transaminase (ALT) and creatinine (Cr), as well as arterial blood gas (ABG), were determined by The Central Laboratory of Tianjin Medical University General Hospital.

Immunofluorescence analysis. The expression of phosphorylated (p)- $\text{I}\kappa\text{B}\beta/\text{I}\kappa\text{B}\beta$ and p-P65/P65, the main molecules of the NF- κ B signaling pathway, were examined by immunofluorescence microscopy (Olympus Corporation). CD4⁺ T cells were suspended in 1 ml fixation/permeabilization solution (cat. no. 88-8824-00; eBioscience; Thermo Fisher Scientific, Inc.) at 25°C for 30 min, and seeded on glass coverslips, washed with phosphate buffer saline (PBS), fixed in 4% paraformaldehyde (Nanjing KeyGen Biotech Co., Ltd.) at 25°C for 15 min, and then blocked with 10% normal goat serum (cat. no. SL038; Beijing Solarbio Science & Technology Co., Ltd.) at 25°C for 30 min. Following which, cells were incubated at 4°C for 12 h with rabbit anti-mouse p- $\text{I}\kappa\text{B}\beta$ (1:100; cat. no. ab194845), $\text{I}\kappa\text{B}\beta$ (1:100; cat. no. ab97406), p-P65 (1:100; cat. no. ab222494) and P65 (1:100; cat. no. ab32536) antibodies (all purchased from Abcam). The cells were subsequently washed and incubated at 25°C for 60 min with FITC/APC-conjugated goat anti-rabbit IgG (1:200; cat. no. 4030-02; SouthernBiotech). Immunofluorescence was assessed by fluorescence microscopy. Data were collected and processed with ImageJ (v1.51j8; National Institutes of Health).

Flow cytometric analysis. To investigate the expression of Foxp-3, CD4⁺ T cells (1x10⁶ cells/ml) were obtained post-treatment according to the flow cytometry kit protocol. CD4⁺ T cells were suspended in 1 ml fixation/permeabilization solution and incubated at 25°C for 30 min. After washing cells with fixation/permeabilization buffer twice, CD4⁺ T cells were stained with FITC-conjugated anti-mouse/rat-Foxp-3

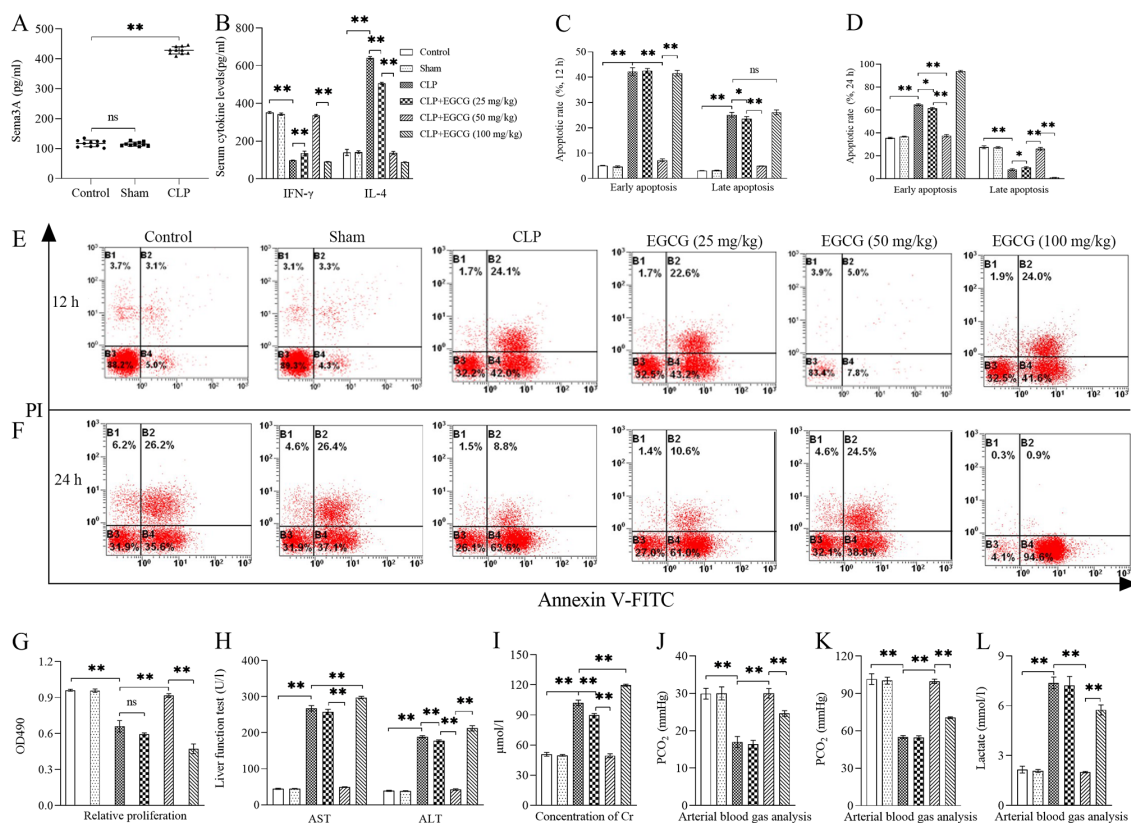


Figure 1. Treatment with EGCG improves sepsis-induced T cell immunosuppression and MODS. (A) The serum concentration of Sema3A was significantly enhanced in CLP-induced sepsis (n=10). (B) Serum concentrations of IFN- γ and IL-4. Statistical analysis and representative flow cytometry images of proportions of early and late apoptotic stage CD4⁺ T cells from (C and E) 12 h to (D and F) 24 h. PI⁻Annexin V-FITC⁺ represent early apoptosis, while PI⁺Annexin V-FITC⁺ represent late apoptosis. (G) The proliferation of CD4⁺ T cells. Serum concentrations of (H) AST and ALT, (I) Cr, (J) PCO₂, (K) PO₂ and (L) Lactate. Data are presented as the mean \pm standard deviation, n=4 per group. One-way ANOVA was performed for data analysis. *P<0.05, **P<0.01. EGCG, (-)-epigallocatechin-3-gallate; MODS, multiple organ dysfunction syndromes; ns, not significant; CLP, cecal ligation and perforation; AST, aminotransferase; ALT, alanine transaminase; Cr, creatinine; Sema3A, semaphorin 3A.

(cat. no. MA1-41628; eBioscience; Thermo Fisher Scientific, Inc.) and incubated at 25°C for 15 min. Cells were analyzed using a flow cytometer (BD FACSCalibur™; cat. no. 342975; BD Biosciences) and FlowJo software (version 7; FlowJo LLC).

To investigate CD4⁺ T cell apoptosis, cells (1x10⁶ cells/ml) were obtained post-treatment according to the flow cytometry kit protocol. CD4⁺ T cells were suspended in 200 μ l binding buffer, followed by FITC-conjugated Annexin V and propidium iodide (cat. no. CA1020; Beijing Solarbio Science & Technology Co., Ltd.) in the dark according to the manufacturer's protocol, and then subjected to flow cytometric analysis using a flow cytometer (BD FACSCalibur) and FlowJo software (version 7).

Enzyme-linked immunosorbent assay (ELISA). Supernatants or serum were collected for the measurement of Sema3A (cat. no.011592), IFN- γ (cat.no.HS4595) and IL-4 (cat.no.BH-E0800) levels, using ELISA kits (Shanghai Xin Fan Biotechnology Co., Ltd.), according to the protocols specified by the manufacturer. The absorbance was read in a microplate reader (Spectra MR; Dynex Technologies) at a wavelength of 450 nm.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay. The present study used a one-step TUNEL Apoptosis Assay kit (cat. no. T2190; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. CD4⁺ T cells were suspended in

1 ml fixation/permeabilization solution at 25°C for 30 min, and seeded on glass coverslips, washed with PBS, fixed in 4% paraformaldehyde at 25°C for 15 min, and then incubated with 20 μ g/ml Proteinase K (cat. no. P9460; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 30 min. Cells were then blocked with 10% normal goat serum at 25°C for 30 min. Apoptotic cells were observed and the images from five random fields were analyzed by immunofluorescence microscopy (Olympus Corporation) at x200 magnification. The emission wavelength of the green fluorescence was 525 nm. Data were collected and processed using ImageJ software (v1.51j8; National Institutes of Health).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay (Ameresco, Inc.) was used to evaluate the proliferative ability of CD4⁺ T cells. Growth medium was removed and replaced with fresh medium. The assays were initiated by adding 10 ml MTT reagent (10 mg/ml) to each well and incubating the cells for 4 h at 25°C. Finally, the medium was removed and 100 μ l DMSO (Ameresco, Inc.) was added to each well. The absorbance was read in a microplate reader (Spectra MR; Dynex) at a wavelength of 490 nm.

Statistical analysis. Data are presented as the mean \pm standard deviation. All experiments were performed at least four times. Data were analyzed by one-way ANOVA and Tukey's post hoc

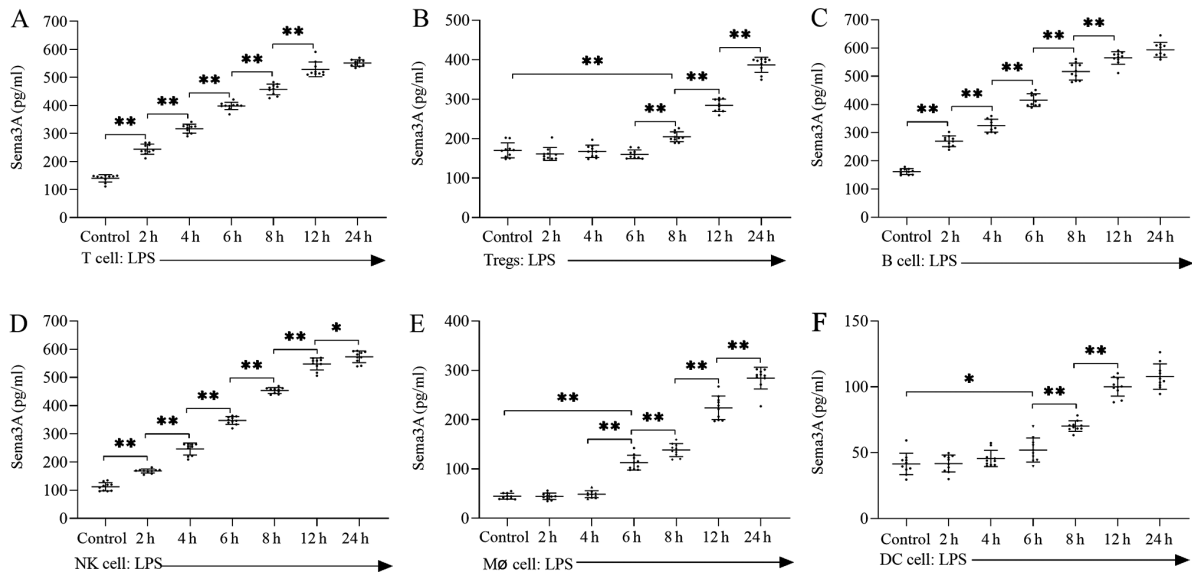


Figure 2. LPS increases the concentration of Sema3A in both lymphoid and myeloid lineages. Secretion levels of Sema3A from (A) T cells, (B) Tregs, (C) B cells, (D) NK cells, (E) M ϕ cells and (F) DC. Data are presented as the mean \pm standard deviation, n=10 per group. One-way ANOVA was performed for data analysis. *P<0.05, **P<0.01. LPS, lipopolysaccharide; Tregs, regulatory T cells; M ϕ , macrophage; DC, dendritic cells; NK, natural killer; Sema3A, semaphorin 3A.

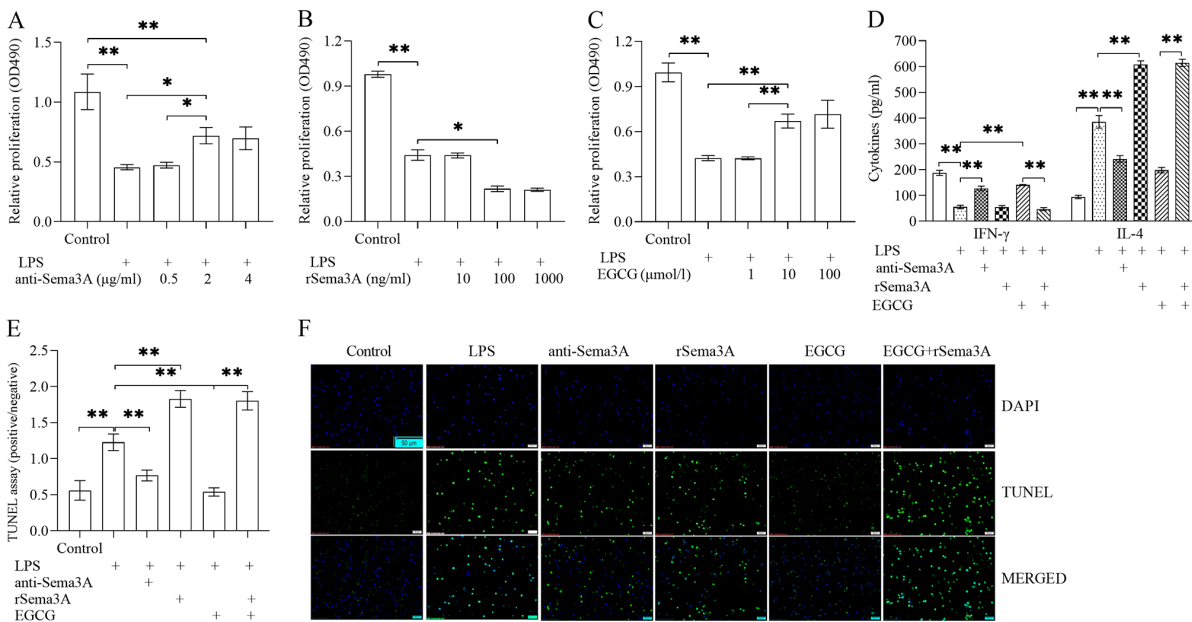


Figure 3. Sema3A-mediated autocrine loop impairs T cell immune dysfunction during LPS-induced sepsis. (A) Anti-Sema3A antibodies dose-dependently improved the proliferation of CD4⁺ T cells. (B) rSema3A dose-dependently impaired the proliferation of CD4⁺ T cells. (C) EGCG treatment dose-dependently improved the proliferation of CD4⁺ T cells. (D) Secreted levels of IFN- γ and IL-4 from CD4⁺ T cells. (E) Statistical analysis and (F) representative immunofluorescence images of the apoptotic CD4⁺ T cells (scale bar, 50 μ m). Data are presented as the mean \pm standard deviation, n=4 per group. One-way ANOVA was performed for data analysis. *P<0.05, **P<0.01. LPS, lipopolysaccharide; EGCG, (-)-epigallocatechin-3-gallate; OD, optical density; Sema3A, semaphorin 3A.

test using SPSS version 24 (IBM Corp.). Unpaired Student's t-test was used to evaluate significance between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Sema3A exacerbates sepsis-induced T cell immunosuppression and MODS. *In vivo*, the serum concentration of Sema3A

was significantly enhanced in CLP-induced sepsis by >4-fold at 24 h after CLP (P<0.01; Fig. 1A). CLP was immediately followed by intravenous administration of EGCG at different concentrations. The 50 mg/kg dose, in particular, had a clear ability to improve sepsis-induced T cell immunosuppression and MODS compared with the CLP-induced sepsis group. The serum concentrations of IFN- γ and IL-4 produced by CD4⁺ T cells were measured to identify the polarization of Th1/Th2 cells (Fig. 1B). Following treatment with 25 and 50 mg/kg

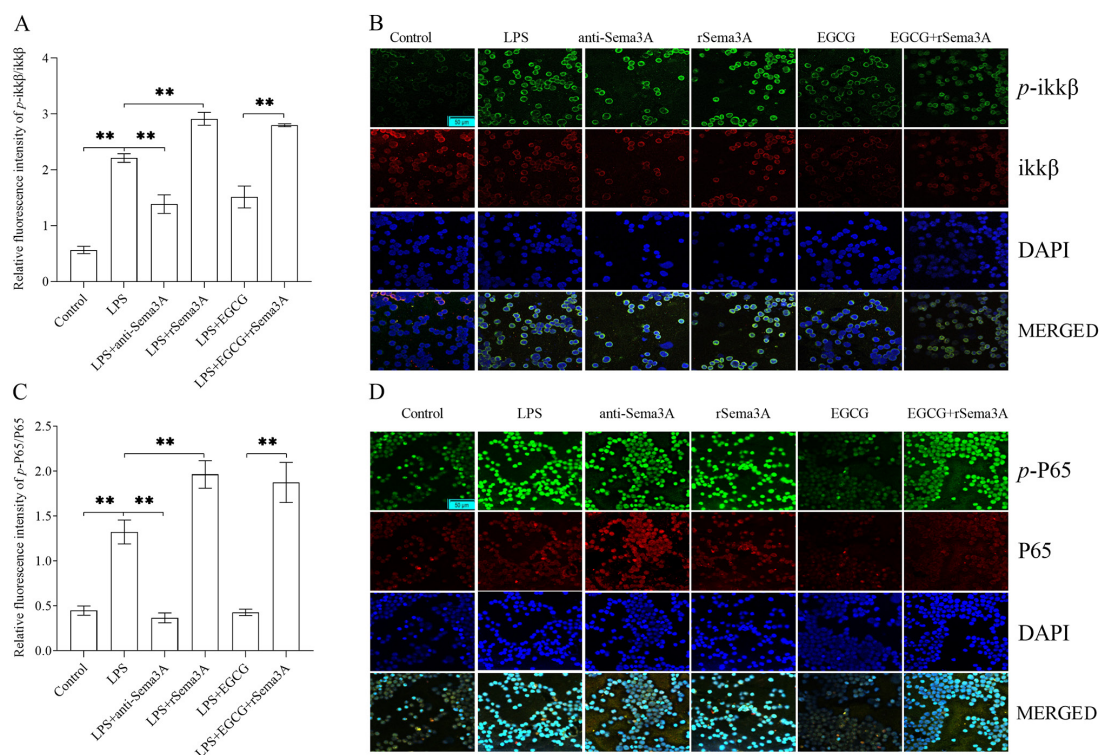


Figure 4. Sema3A-mediated autocrine loop impairs T cell immune dysfunction during LPS-induced sepsis via the NF- κ B signaling pathway. Statistical analysis and representative immunofluorescence images of the phosphorylation levels of (A and B) ikk β and (C and D) P65 in CD4⁺ T cells. Scale bar, 50 μ m. Data are presented as the mean \pm standard deviation, n=4 per group. One-way ANOVA was performed for data analysis. **P<0.01. LPS, lipopolysaccharide; p-, phosphorylated; Sema3A, semaphorin 3A; EGCG, (-)-epigallocatechin-3-gallate.

EGCG, IFN- γ levels were significantly enhanced (P<0.01), while IL-4 levels were significantly lowered (P<0.01), but the serum concentrations of IFN- γ and IL-4 were both significantly reduced following treatment with 100 mg/kg EGCG (P<0.01). The early apoptotic rate of CD4⁺ T cells was significantly decreased when animals were treated with 50 mg/kg EGCG (P<0.01) from 12 to 24 h, but 100 mg/kg EGCG significantly increased the early apoptotic rate of CD4⁺ T cells, especially at the 24 h time point, with the early apoptotic rate reaching >90% (P<0.01), there were no significant differences in early and late apoptosis between the control and 50 mg/kg EGCG groups (P>0.05) at the 12 to 24 h time points (Fig. 1C-F). The proliferative ability of CD4⁺ T cells was significantly enhanced with 50 mg/kg EGCG treatment compared with the CLP group (P<0.01) and was similar to that of the control group. CD4⁺ T cell proliferation of the 100 mg/kg EGCG treatment group was significantly lower than that of the LPS-treatment group and even of the control group (P<0.01; Fig. 1G). CLP-induced sepsis impaired organ function, as indicated by the significantly increased serum levels of AST (Fig. 1H), ALT (Fig. 1H), Cr (Fig. 1I) and Lac (Fig. 1L), but the levels of PCO₂ (Fig. 1J) and PO₂ (Fig. 1K) were significantly decreased (P<0.01), administration of 50 mg/kg EGCG following CLP significantly improved the appellate quotas of organ function (P<0.01), however, 100 mg/kg EGCG aggravated organ dysfunction when compared with the CLP group (P<0.01).

Lymphoid and myeloid lineages secrete high concentration of Sema3A during LPS-induced sepsis. Cells of the

lymphoid lineage (including T cells, Tregs, B cells and NK cells) were isolated from normal spleen tissue and cultured under LPS-induced (100 ng/ml) septic conditions for 2-24 h. LPS treatment significantly upregulated the concentration of Sema3A in T cells (Fig. 2A), Tregs (Fig. 2B), B cells (Fig. 2C) and NK cells (Fig. 2D), especially after 24 h (P<0.05 or 0.01). Tregs did not secrete Sema3A significantly until 8 h (P<0.01). Cells of the myeloid lineage, including M ϕ cells and DC, were isolated and cultured under LPS-induced (100 ng/ml) septic conditions from 2 to 24 h. Compared with cells of the lymphoid lineage, the time required for the myeloid lineage to secrete high concentrations of Sema3A was significantly delayed by LPS-induced sepsis, M ϕ cells did not secrete Sema3A significantly until after 6 h (P<0.01; Fig. 2E). DC did not secrete Sema3A significantly until after 6 h of LPS induction (P<0.01; Fig. 2F).

Inhibition of Sema3A mediates autocrine loop-alleviated T cell immune dysfunction during LPS-induced sepsis. T cells were isolated, treated with LPS (100 ng/ml) to induce sepsis and further exposed to anti-Sema3A antibody, rSema3A and/or EGCG for 24 h. Compared with LPS induction alone, the proliferative ability of CD4⁺ T cells was enhanced following anti-Sema3A antibody treatment (P<0.05; Fig. 3A) and was significantly enhanced with EGCG treatment (P<0.01; Fig. 3C), but T cells were unable to reach the level of the control group. The proliferative ability of CD4⁺ T cells was further inhibited following treatment with rSema3A (P<0.05; Fig. 3B). Compared with LPS induction alone, treatment with anti-Sema3A antibody or EGCG could alleviate the polariza-

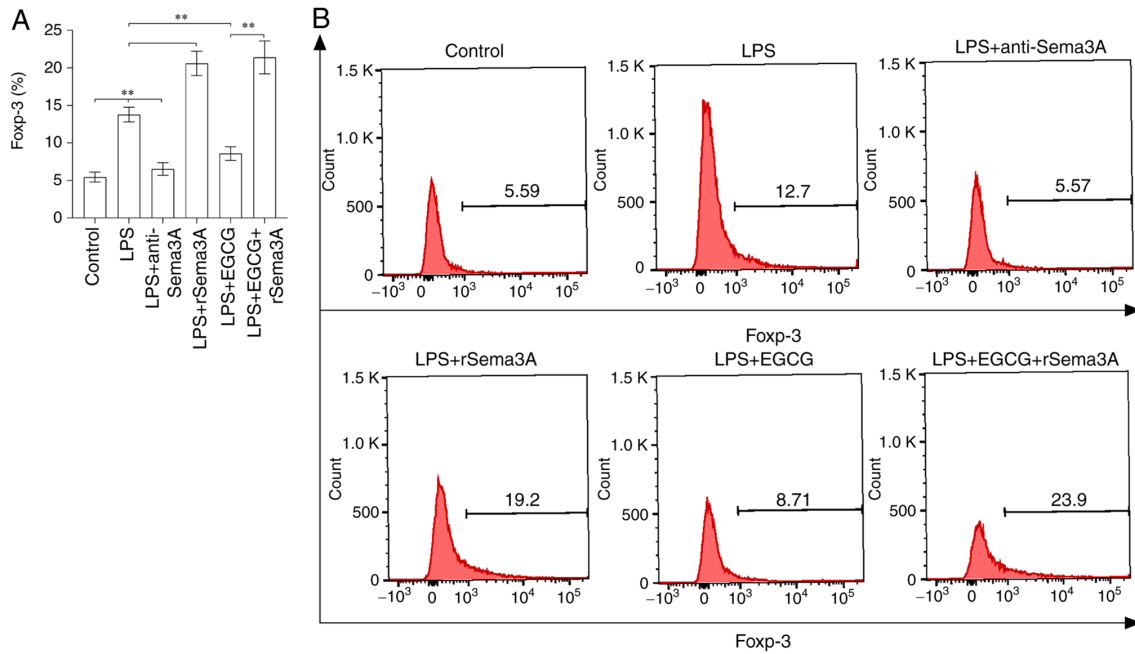


Figure 5. Sema3A-mediated autocrine loop enhances the expression of Foxp-3 in CD4⁺ T cells during LPS-induced sepsis. (A) Statistical analysis and (B) representative flow cytometry images protein levels of Foxp-3. Data are presented as the mean \pm standard deviation, n=4 per group. One-way ANOVA was performed for data analysis. **P<0.01. LPS, lipopolysaccharide; Sema3A, semaphorin 3A; EGCG, (-)-epigallocatechin-3-gallate.

tion of Th1/Th2 cells (Fig. 3D) and apoptosis (Fig. 3E and F) of T cells, specifically promoting IFN- γ secretion (P<0.01; Fig. 3D), as well as inhibiting IL-4 secretion (P<0.01; Fig. 3D) and apoptosis (P<0.01; Fig. 3E and F). Compared with LPS induction alone, Sema3A could further impair polarization of Th1/Th2 cells and apoptosis of T cells, including inhibiting IFN- γ secretion (P<0.01; Fig. 3D), as well as promoting IL-4 secretion (P<0.01; Fig. 3D) and apoptosis (P<0.01; Fig. 3E and F) until they reached the levels of the LPS induction alone.

NF- κ B signaling pathway is involved in Sema3A-mediated autocrine loop aggravating T cell immune dysfunction during LPS-induced sepsis. *In vitro*, CD4⁺ T cells were treated with LPS (100 ng/ml)-induced sepsis for 24 h, the ratio of p- $\text{Ikk}\beta/\text{Ikk}\beta$ (Fig. 4A and B) and p-P65/P65 (Fig. 4C and D) were significantly enhanced when compared with the control group (P<0.01). The ratio of p- $\text{Ikk}\beta/\text{Ikk}\beta$ and p-P65/P65 were significantly decreased with anti-Sema3A or EGCG treatment when compared with the LPS-induced sepsis group (P<0.01), but were unable to reach the level of the control group. The ratio of p- $\text{Ikk}\beta/\text{Ikk}\beta$ and p-P65/P65 were further significantly enhanced following rSema3A with or without EGCG treatment when compared with the LPS induction alone group (P<0.01).

Sema3A-mediated autocrine loop enhances the expression of Foxp-3 during LPS-induced sepsis. *In vitro*, CD4⁺ T cells were treated with LPS (100 ng/ml)-induced sepsis for 24 h, the expression of Foxp-3 (Fig. 5A and B) was significantly enhanced when compared with the control group (P<0.01). The expression of Foxp-3 was significantly decreased with anti-Sema3A antibody or EGCG treatment when compared with the LPS-induced sepsis group (P<0.01), but was unable to reach the level of the control group, especially following

exposure to EGCG. The expression of Foxp-3 was further significantly enhanced following treatment with rSema3A with or without EGCG treatment when compared with the LPS induction alone (P<0.01).

Discussion

Sepsis is a life-threatening disease that causes severe MODS and leads to mortality in the majority of patients with severe sepsis-induced MODS (1). Yamashita *et al* (13) generated a specific Sema3A monoclonal antibody that could successfully neutralize Sema3A activity and showed that administration of this specific antibody before LPS injection significantly increased the survival rate of LPS-treated mice (16). Treatment with inhibitor neutralization approaches to reduce Sema3A, such as exposure to EGCG or siRNA interference, has been shown to alleviate acute organ damage (12,17). In the current study, it was demonstrated that administration of EGCG markedly improved sepsis-induced MODS, as evidenced by a reduction in the serum levels of AST, ALT, Cr and Lac and an increase in the levels of PCO₂ and PO₂. Pasterkamp *et al* (18) showed that the selective monooxygenase inhibitor EGCG, is not a specific Sema3A inhibitor, but attenuated the repellent effects of Sema3A and Sema3F *in vitro* in a dose-dependent manner. *E. coli* sepsis is associated with a marked upregulation of sema3A and down-regulation of Sema3F expression (19). Thus, EGCG was used to perform *in vivo* experiments that may have some limitations, such as the role of Sema3F may be further weakened.

Fresh specimens from liver, kidney and lung, as well as the cells in the circulatory system of septic patients who succumb in intensive care units, show a progressive, profound, apoptosis-induced loss of adaptive immunocytes and especially a rapid decrease of T-lymphocytes levels, resulting in an decreased ability to clear life-threatening pathogens (8,20,21).

The highest level of Sema3a mRNA can be observed in T, B and NK cells of the lymphoid lineage (13,14,16,22,23). Myeloid and monocytic cells also express Sema3a, but at a lower level (22,23). The *in vitro* findings of the present study showed that both lymphoid and myeloid lineages secreted high concentrations of Sema3A during LPS-induced sepsis. The concentrations of Sema3A of the T, B and NK cells were significantly upregulated with more rapidity compared with Tregs, M ϕ cells and DC. *In vivo*, the serum concentrations of Sema3A were significantly enhanced in CLP-induced sepsis. Administration of EGCG had the ability to improve the immune dysfunction of CD4⁺ T cells.

Toll-like receptor (TLR) engagement can induce Sema3A expression, thus completing an autocrine loop in LPS-induced sepsis (23). Sema3A is expressed by activated T cells and downmodulated T cell activation *in vitro* (22). Nrp-1, primarily regarded as a receptor for Sema proteins (such as Sema3A) and as a co-receptor for vascular endothelial growth factor family proteins, is expressed by neuronal and endothelial cells and has been reported to serve an essential role in the establishment of both the nervous system and the endothelial network during embryogenesis (24). NP-1 is also involved in initial cell-cell contact between T cells and antigen presenting cells (25). Tumor and T cells both express NP-1 and the cluster formation between tumor and T cells increases with reduced Sema-3A expression in tumor cells; this effect is rescued by adding a blocking anti-NP-1 antibody (14). The present study demonstrated that administration of the inhibitor alleviated T cell anergy during LPS-induced sepsis. The proliferative ability, polarization of Th1/Th2 cells and apoptosis of CD4⁺ T cells was improved with anti-Sema3A and EGCG treatment, but they were unable to recover normal levels. Using a mouse model of collagen-induced arthritis, Sema3A can increase the proportion of CD4⁺NP-1⁺ T cells and this group of cells is able to inhibit the growth of CD4⁺ T cells as well as Tregs (26), a full-time negative immunomodulatory type of the CD4⁺ T lymphocyte subpopulation (27,28). The characteristic transcription factor, Foxp-3, is certainly an crucial determinant for the stability of Tregs (29). Previous studies have demonstrated that increased expression of Foxp-3 in Tregs is positively associated with the mortality of septic mice (6,21,23,29). The present study demonstrated that the Foxp-3 expression of CD4⁺ T cells was impaired by rSema3A treatment, while anti-Sema3A or EGCG treatment inhibited the expression of Foxp-3, especially EGCG. Catalano *et al* (26) showed that treatment of Sema3A increases IL-10 concentration in serum. IL-10 serves a key role in induced immunosuppression, including sepsis. Therefore, IL-10 may also be involved, whereby rSema3A may enhance the stability of Tregs by increasing the secretion of IL-10.

TLR engagement by pathogen-associated molecular pattern is a key mechanism of cellular immune responses requiring NF- κ B activation (30). Previous studies have demonstrated that the NF- κ B signaling pathway and its transcription factors [in particular, the canonical signaling subunits, ikk β , c-Rel and P65 (RelA)], occupied a critical role in CD4⁺ T cell biology (31,32). PlexinA4, as a negative regulator in CD4⁺ T cell activation, is a receptor of Sema3A that guides the development of the nervous system and regulates the function of the immune system (23). Plexins resemble TLRs in their evolutionary conservation from flies to mammals, a plexina4

knockout cell model exhibits defective inflammatory cytokine production upon activation by a spectrum of TLR agonists and bacteria (23). Sema3A-plexinA4 interaction affects the activation of Rac1, which is known to promote NF- κ B activation and cytokine production in TLR-stimulated macrophages (33). Unfortunately, the present study did not delve into specific receptors on the cell surface.

The present study demonstrated that the NF- κ B signaling pathway is involved in a Sema3A-mediated autocrine loop, aggravating T cell immune dysfunction during LPS-induced sepsis. *In vitro*, the ratios of p-ikk β /ikk β and p-P65/P65 of CD4⁺ T cells were significantly enhanced when treated with LPS and decreased with anti-Sema3A antibody or EGCG treatment. The phosphorylation levels should be detected as early as possible, especially within 24 h or even a few minutes after the test. The present study conducted a preliminary 24 h phosphorylation detection and future studies will explore the phosphorylation level in depth.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YG, CW and YC designed the study, wrote the protocol, collected the data, performed statistical analyses and contributed to writing the manuscript. ZW and WL performed the technical work. ZW, YL and SS helped with data collection, study design and coordinated the study. YC participated in the study design and helped to critically revise the manuscript. YG and YC were responsible for confirming the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were undertaken following the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the scientific investigation board, Tianjin Medical University General Hospital (approval no. ZYY-DWFL-IRB-001F-01; Tianjin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Raith EP, Udy AA, Bailey M, McGloughlin S, MacIsaac C, Bellomo R and Pilcher DV; Australian and New Zealand Intensive Care Society (ANZICS) Centre for Outcomes and Resource Evaluation (CORE): Prognostic accuracy of the SOFA score, SIRS criteria, and qSOFA score for in-hospital mortality among adults with suspected infection admitted to the Intensive Care Unit. *JAMA* 317: 290-300, 2017.
- Xie J, Wang H, Kang Y, Zhou L, Liu Z, Qin B, Ma X, Cao X, Chen D, Lu W, *et al*; Chinese Epidemiological Study of Sepsis (CHES) Study Investigators: The epidemiology of sepsis in Chinese ICUs: A National Cross-Sectional Survey. *Crit Care Med* 48: e209-e218, 2020.
- Weng L, Zeng XY, Yin P, Wang LJ, Wang CY, Jiang W, Zhou MG and Du B; China Critical Care Clinical Trials Group (CCCCTG): Sepsis-related mortality in China: A descriptive analysis. *Intensive Care Med* 44: 1071-1080, 2018.
- Taeb AM, Hooper MH and Marik PE: Sepsis: current definition, pathophysiology, diagnosis, and management. *Nutr Clin Pract* 32: 296-308, 2017.
- Stolk RF, Kox M and Pickkers P: Noradrenaline drives immunosuppression in sepsis: Clinical consequences. *Intensive Care Med* 46: 1246-1248, 2020.
- van der Poll T, van de Veerdonk FL, Scicluna BP and Netea MG: The immunopathology of sepsis and potential therapeutic targets. *Nat Rev Immunol* 17: 407-420, 2017.
- Hotchkiss RS, Monneret G and Payen D: Sepsis-induced immunosuppression: From cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 13: 862-874, 2013.
- Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman SD II, Kreisel D, Krupnick AS, *et al*: Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA* 306: 2594-2605, 2011.
- Otto GP, Sossdorf M, Claus RA, Rödel J, Menge K, Reinhart K, Bauer M and Riedemann NC: The late phase of sepsis is characterized by an increased microbiological burden and death rate. *Crit Care* 15: R183, 2011.
- Delano MJ, Scumpia PO, Weinstein JS, Coco D, Nagaraj S, Kelly-Scumpia KM, O'Malley KA, Wynn JL, Antonenko S, Al-Quran SZ, *et al*: MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med* 204: 1463-1474, 2007.
- Morita A, Yamashita N, Sasaki Y, Uchida Y, Nakajima O, Nakamura F, Yagi T, Taniguchi M, Usui H, Katoh-Semba R, *et al*: Regulation of dendritic branching and spine maturation by semaphorin3A-Fyn signaling. *J Neurosci* 26: 2971-2980, 2006.
- Tian X, Gan H, Zeng Y, Zhao H, Tang R and Xia Y: Inhibition of semaphorin-3a suppresses lipopolysaccharide-induced acute kidney injury. *J Mol Med (Berl)* 96: 713-724, 2018.
- Yamashita N, Jitsuki-Takahashi A, Ogawara M, Ohkubo W, Araki T, Hotta C, Tamura T, Hashimoto S, Yabuki T, Tsuji T, *et al*: Anti-Semaphorin 3A neutralization monoclonal antibody prevents sepsis development in lipopolysaccharide-treated mice. *Int Immunol* 27: 459-466, 2015.
- Catalano A, Caprari P, Moretti S, Faronato M, Tamagnone L and Procopio A: Semaphorin-3A is expressed by tumor cells and alters T-cell signal transduction and function. *Blood* 107: 3321-3329, 2006.
- National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals. 8th edition. National Academies Press (US), Washington, DC, 2011.
- Liu LN, Li XM, Ye DQ and Pan HF: Emerging role of semaphorin-3A in autoimmune diseases. *Inflammopharmacology* 26: 655-665, 2018.
- Körner S, Bösel S, Wichmann K, Thau-Habermann N, Zapf A, Knippenberg S, Dengler R and Petri S: The Axon Guidance Protein Semaphorin 3A Is Increased in the Motor Cortex of Patients With Amyotrophic Lateral Sclerosis. *J Neuropathol Exp Neurol* 75: 326-333, 2016.
- Pasterkamp RJ, Dai HN, Terman JR, Wahlin KJ, Kim B, Bregman BS, Popovich PG and Kolodkin AL: MICAL flavo-protein monooxygenases: Expression during neural development and following spinal cord injuries in the rat. *Mol Cell Neurosci* 31: 52-69, 2006.
- Loron G, Olivier P, See H, Le Saché N, Angulo L, Biran V, Brunelle N, Besson-Lescure B, Kitzis MD, Pansiot J, *et al*: Ciprofloxacin prevents myelination delay in neonatal rats subjected to *E. coli* sepsis. *Ann Neurol* 69: 341-351, 2011.
- Kaukonen KM, Bailey M, Pilcher D, Cooper DJ and Bellomo R: Systemic inflammatory response syndrome criteria in defining severe sepsis. *N Engl J Med* 372: 1629-1638, 2015.
- Darcy CJ, Minigo G, Piera KA, Davis JS, McNeil YR, Chen Y, Volkheimer AD, Weinberg JB, Anstey NM and Woodberry T: Neutrophils with myeloid derived suppressor function deplete arginine and constrain T cell function in septic shock patients. *Crit Care* 18: R163, 2014.
- Lepelletier Y, Moura IC, Hadj-Slimane R, Renand A, Fiorentino S, Baude C, Shirvan A, Barzilai A and Hermine O: Immunosuppressive role of semaphorin-3A on T cell proliferation is mediated by inhibition of actin cytoskeleton reorganization. *Eur J Immunol* 36: 1782-1793, 2006.
- Wen H, Lei Y, Eun SY and Ting JP: Plexin-A4-semaphorin 3A signaling is required for Toll-like receptor- and sepsis-induced cytokine storm. *J Exp Med* 207: 2943-2957, 2010.
- Gu Z, Ueno M, Klinefelter K, Mamidi M, Yagi T and Yoshida Y: Skilled movements in mice require inhibition of corticospinal axon collateral formation in the spinal cord by semaphorin signaling. *J Neurosci* 39: 8885-8899, 2019.
- Tordjman R, Lepelletier Y, Lemarchandel V, Cambot M, Gaulard P, Hermine O and Roméo PH: A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat Immunol* 3: 477-482, 2002.
- Catalano A: The neuroimmune semaphorin-3A reduces inflammation and progression of experimental autoimmune arthritis. *J Immunol* 185: 6373-6383, 2010.
- Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C and Rudensky AY: Stability of the regulatory T cell lineage in vivo. *Science* 329: 1667-1671, 2010.
- Ramsdell F and Rudensky AY: Foxp3: A genetic foundation for regulatory T cell differentiation and function. *Nat Immunol* 21: 708-709, 2020.
- Gao YL, Chai YF, Dong N, Han S, Zhu XM, Zhang QH and Yao YM: Tuftsin-derived T-peptide prevents cellular immunosuppression and improves survival rate in septic mice. *Sci Rep* 5: 16725, 2015.
- Gelman AE, Zhang J, Choi Y and Turka LA: Toll-like receptor ligands directly promote activated CD4⁺ T cell survival. *J Immunol* 172: 6065-6073, 2004.
- Zaph C, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du Y, Yost EA, Gruber AD, May MJ, Greten FR, *et al*: Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* 446: 552-556, 2007.
- Kilpinen S, Henttinen T, Lahdenpohja N, Hulkkonen J and Hurme M: Signals leading to the activation of NF-kappa B transcription factor are stronger in neonatal than adult T lymphocytes. *Scand J Immunol* 44: 85-88, 1996.
- Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ and Knaus UG: Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat Immunol* 1: 533-540, 2000.



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