Pseudolycorine Chloride Ameliorates Th17-cell-mediated CNS Autoimmunity by Restraining MDSCs Expansion

Gan Zhang
Chengdu Medical College
Xinying Zhu
Chengdu Medical College
Fan Yang
Chengdu Medical College
Juan Li
Chengdu Medical College
Xiao Leng
Chengdu Medical College
Limei Li
Southwest Minzu University
Yantang Wang (yt-wang@hotmail.com)
Chengdu Medical College https://orcid.org/0000-0003-0854-4988

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Pseudolycorine chloride ameliorates Th17-cell-mediated CNS autoimmunity by restraining MDSCs expansion

Gan Zhang[a, †], Xinying Zhu[a, †], Fan Yang[a], Juan Li[a], Xiao Leng[a], Limei Li[b,*], Yantang Wang[a,*]

a Department of pharmacology, School of pharmacy, Chengdu Medical College, Chengdu, China

b College of pharmacy, Southwest Minzu University, Chengdu, China

Main corresponding author at: Department of pharmacology, School of pharmacy, Chengdu Medical College, Chengdu, China, No.783, Xindu Avenue, Xindu District, Chengdu, Sichuan, 610500, China, wangyantang@cmc.edu.cn

*These authors are co-corresponding authors

†These authors share first authorship.

E-mail addresses: zhangganliver@163.com (G. Zhang), zhuxinying06@163.com (X. Zhu), fyssg@foxmail.com (F. Yang), meme0226@foxmail.com (J. Li), lengxiao831205@163.com (X. Leng), limeili@swun.edu.cn (L. Li), yt-wang@hotmail.com (Y. Wang).

Headings

- Pseudolycorine chloride inhibited the differentiation of M-MDSCs *in vitro* and *in vivo*
- Pseudolycorine chloride inhibited the expansion of MDSCs both *in vitro* and *in vivo*
- Pseudolycorine chloride reduced CNS infiltration of inflammatory Th17 cells
- Pseudolycorine chloride alleviated inflammatory demyelination of EAE mice spinal cord

Abbreviations: PLY, pseudolycorine chloride; EAE, experimental autoimmune encephalomyelitis; MDSCs, myeloid-derived suppressor cells; M-MDSCs, monocyte-like MDSCs; G-MDSCs, granulocyte-

like MDSCs; SLE, systemic lupus erythematosus; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; LY, lycorine; LPS, lipopolysaccharide; SPF, specific pathogen-free; mAbs, monoclonal antibodies; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester; CFA, complete Freund's adjuvant; PTX, pertussis toxin; OCT, opti-mum cutting temperature; MSCs, mesenchymal stem cells; TAMs, tumor-associated macrophages; IOD, integral optical density.

Abstract

In experimental autoimmune encephalomyelitis (EAE), a large number of myeloid-derived suppressor cells (MDSCs) appear, and these cells can accelerate the development of EAE in mice. The aim of this study was to evaluate pseudolycorine chloride's (PLY) inhibitory effect on the expansion of MDSCs and differentiation of monocyte-like MDSCs (M-MDSCs), while proving that PLY can improve the severity of Th17 cell-mediated EAE by affecting MDSCs. Flow cytometry suggested that PLY significantly inhibited the proliferation and expansion of MDSCs stimulated by IL-6 and GM-CSF, especially the differentiation of M-MDSCs in a dose-dependent manner. Three concentrations of PLY (0.67, 2, and 6 μM) had no cytotoxicity *in vitro*. Hematoxylin and eosin (H&E) staining, LFB, and immunofluorescence suggested inflammatory infiltration and demyelination. MDSCs and related Th17 cell infiltration in the spinal cord were inhibited by PLY (40 mg/kg). In conclusion, PLY inhibited the differentiation and expansion of MDSCs, especially M-MDSCs, and intervened in the progression of EAE in mice.

Keywords: MDSCs, M-MDSCs, Th17 cells, experimental autoimmune encephalomyelitis/multiple sclerosis, pseudolycorine chloride

Introduction

Myeloid-derived suppressor cells (MDSCs) are derived from common myeloid progenitors (CMPs), and the expansion and differentiation of MDSCs are supported by myeloid-specific growth factors GM-CSF, G-CSF, M-CSF, and several pro-inflammatory cytokines namely IL-6 and IL-11. MDSCs were originally identified as $CD11b^{+}Gr\text{-}1^{hi}$ cells in tumor-bearing mice. The classical $CD11b^{+}Gr\text{-}1^{hi}$ MDSC cell population can be further subdivided into granulocyte-like MDSCs ($Ly6C^{med}Ly6G^{+}$, G-MDSCs) and monocyte-like MDSCs ($Ly6C^{+}Ly6G^{-}$, M-MDSCs). In recent years, MDSCs have become the focus of intense study in cancer contexts, which were originally described as a heterogeneous population with immunosuppressive functions in tumor-bearing hosts (Bronte, 2009, Gabrilovich and Nagaraj, 2009, Ostrand-Rosenberg and Sinha, 2009a, Ostrand-Rosenberg and Sinha, 2009b, Guo et al., 2016a, Groth et al., 2019). Unlike tumors, the immune response is stronger in the inflammatory environment, and in a variety of Th17 cell-mediated autoimmune diseases, MDSCs have been identified to accelerate diseases, such as systemic lupus erythematosus (SLE), which is closely related to the number of MDSCs, which are highly potent in promoting the differentiation of Th17 cells (Wu et al., 2016). In the case of collagen-induced arthritis (CIA), deletion of MDSCs relieves the severity and decreases the number of Th17 cells and serum IL-17A but does not influence other Treg cells (Zhang et al., 2015b). MDSCs also play an important role in the animal model of autoimmune disease, experimental autoimmune encephalomyelitis (EAE), and many studies have shown that MDSCs increase in EAE and can induce differentiation of Th17 cells. These contrasting findings indicated that accumulation of MDSCs *in vivo* may not exhibit suppressive activity, and possibly can even aggravate the disease as reported in autoimmune diseases. The dysfunction of MDSC promote Th17 cells polarization and IL-17 production under the inflammatory

microenvironment, substantially may be a factor driving autoimmune inflammatory pathology. Accumulation of MDSCs does not alleviate inflammation but aggravates EAE and tissue damage by promoting the development of Th17 cells (Yi et al., 2012). Thus, we speculate that the pro-inflammatory effect of MDSCs may be related to Th17 cells.

Th17 cells are a crucial subset of activated $CD4^+$ T cells. Naïve $CD4^+$ T cells stimulated by cytokines, such as IL-6 and IL-1β, can differentiate into pathogenic Th17 cells (Lee et al., 2020). Th17 cells are thought to be a distinct lineage of Th cells that mainly produce IL-17A, which mainly causes the pathogenesis of multiple inflammatory diseases and autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and colitis (Gao et al., 2010, Liu et al., 2011). It has been previously shown that the differentiation of M-MDSCs is promoted by IL-1β, and that M-MDSCs promote IL-17 production by Th17 cells (Dumont et al., 2019). At the onset of EAE, MDSCs, especially M-MDSCs, were increased *in vivo*, and the decreased accumulation of dysfunctional MDSC at sites of inflammation, may cause a reduction in EAE pathology. If MDSCs can be selectively depleted, the severity of EAE will lead to remarkable remission.

This is mainly related to the inhibition of Th17 cells and reduction of IL-17A (Yi et al., 2012, Zhang et al., 2015a, Zhang et al., 2015b, Ji et al., 2016, Wu et al., 2016). The understanding of the relationship between MDSCs and Th17 cells has provided new perspectives on the treatment of EAE.

Pseudolycorine is an alkaloid isolated from *Narcissus tazetta* L. var. *Chinensis* Roem (**Fig. 1**). Recently, a structural analogue of pseudolycorine, lycorine (LY), was shown to have exhibited anti-tumor activity via the JAK/STAT pathway, such as against osteosarcoma cells (Kang et al., 2012, Hu et al., 2019), and it also shows anti-proliferative activity and promotes tumor apoptosis in colorectal cancer (Wu et al., 2018). STAT3 phosphorylation leads to the transcription of downstream genes, which plays

critical roles in tumorigenesis and inflammation. Myeloid-specific growth factors and inflammatory cytokines that are responsible for the differentiation of MDSCs depend on the STAT3 signaling pathway. (Kang et al., 2012, Hu et al., 2015, Ge et al., 2020). Therefore, the differentiation and function of MDSC may be related to STAT3. Although, the role of PLY and its analogues in the intervention of EAE via its effect on MDSCs remains unclear, we speculated that they have the potential to regulate MDSC, so as to intervene in autoimmune diseases through JAK/STAT pathway. And in the current study, we found that PLY attenuated the progression of Th17-cell-mediated CNS autoimmunity primarily via inhibition of MDSCs.

Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from the Shanghai Model Organisms (China). All mice were bred under specific pathogen-free (SPF) conditions in the experimental animal center of Chengdu Medical College at consistent room temperatures (20–26°C) and humidity (approximately 40–70%), with a 12 h/12 h light/dark cycle and provided with normal food and water. All animal procedures reported in this study were conducted in compliance with the protocol reviewed by the Animal Care and Use Committee of Chengdu Medical College.

Reagents and Antibodies

The following monoclonal antibodies (mAbs) to murine cell surface molecules were purchased from Biolegend (America): FITC-conjugated anti-CD11b (M1/70 clone), APC-conjugated anti-Ly6C (HK1.4

clone), PE-conjugated anti-Ly6G (1A8 clone), BV421-conjugated anti-Gr-1 (RB6-8C5 clone), and APC-conjugated anti-CD11b (M1/70 clone). The PE Annexin V Apoptosis Detection Kit with 7-AAD as well as the purified anti-CD4 (GK1.5 clone) and purified anti-Gr-1 (RB6-8C5 clone) antibodies were purchased from BD Pharmingen (America). Meanwhile, the 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 μM) and anti-IL-17 and anti-CD11b antibodies were purchased from Invitrogen (America) and Abcam (America), respectively. The following reagents were also purchased from Invitrogen: Alexa Fluor 594 goat anti-rat IgG (H+L) and Alexa Fluor 488 goat anti-rabbit IgG (H+L). Myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide was purchased from Bankpeptide (China).

Proliferation and survival of MDSCs

MDSCs from the bone marrow of 8–12 weeks C57BL/6 female mice which were euthanized by cervical disconnection, were cultured in 96-well plates (2×10^5 in 200 μL) with RPMI 1640 (Gibco, America), supplemented with 10% FBS (Gibco, America), 1% penicillin and streptomycin (Hyclone, America), 20 ng/mL GM-CSF, 20 ng/mL IL-6 (PeproTech, America), and CFSE (5 μM/L, Invitrogen, America) for 96 h. MDSCs were stained with 7AAD and PE-Annexin V, and analyzed by Novocyte flow cytometer (ACEA Bioscience, China). Cell culture was performed at 37 °C (5% CO_2).

Differentiation of MDSCs

MDSCs from the bone marrow of 8–12 weeks C57BL/6 female mice which were euthanized by cervical disconnection, were cultured in 24-well plates (1×10^6 in 1 mL) with RPMI 1640 (Gibco, America), supplemented with 10% FBS (Gibco, America), 1% penicillin and streptomycin (Hyclone, America), and

20 ng/mL GM-CSF, and 20 ng/mL IL-6 (PeproTech, America) for 48 h. MDSCs were stained with FITC-anti-CD11b, BV421-anti-Gr-1, PE-anti-Ly6G, and APC-anti-Ly6C and analyzed using a Novocyte flow cytometer (ACEA Bioscience, China). Cell culture was performed at 37 °C (5% CO_2).

Induction and assessment of EAE

MOG_{35-55} and complete Freund's adjuvant (CFA, Chondrex, America) were mixed at a ratio of 1:1 (v/v) to form a water-in-oil emulsion, and 200 μg/mouse subcutaneously injected into C57BL/6 female mice at 8–12 weeks. The mice were injected with pertussis toxin (PTX, 200 ng/mouse, Sigma-Aldrich, America) at 0 and 48 h. From day 2, PBS, 40 mg/kg Pseudolycorine chloride (PLY), and 1 mg/kg FK-506 (tacrolimus, CNSpharm, America) were intraperitoneally injected for 21 d. Clinical scores were made according to the Benson score: 0, no clinical signs; 1, paralyzed tail; 2, loss of coordinated movement, hind limb paresis; 3, both hind limbs paralyzed; 4, forelimbs paralyzed; and 5, moribund.

Histopathology and immunofluorescence

21 days after administration (d23), the mice were anesthetized (2% pentobarbital sodium, 50 mg/kg). Then the mice were perfused with saline and perfused with 4% (w/v) paraformaldehyde and embedded in paraffin. We then dissected the EAE mice, and the pathological sections of the spinal cord were stained with HE and LFB (Beyotime, China), Images were captured using an OLYMPUS BX63 (Japan).

D23, the mice were anesthetized (2% pentobarbital sodium, 50 mg/kg). Then the mice were perfused with saline and were perfused with 4% (w/v) paraformaldehyde and the spinal cord embedded in optimum cutting temperature (OCT) compound (SAKURA, America). After freezing, the spinal cord was stained with anti-mouse Ly-6G/Ly-6C (Gr-1) (1: 100) (BD Pharmingen , America, RB6-8C5 clone) and

anti-rabbit-CD11b (1: 1000) (Abcam, America), or with anti-rabbit-IL17A (1: 200) (Abcam, America) and anti-mouse CD4(1: 100) (BD Pharmingen, America, GK1.5 clone), followed by an Alexa Fluor 594 goat anti-rat IgG (H+L) (1: 500) (Invitrogen, America) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1: 2000) (Invitrogen, America). Red and green fluorescent images were captured using an OLYMPUS BX63 (Japan).

Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were examined by one-way analysis of variance (ANOVA) using PRISM software (version 8.0; GraphPad Software). Statistical significance was set at $P < 0.05$.

Results

PLY inhibit differentiation of M-MDSCs

MDSCs included $Ly6C^{+}Ly6G^{-}$ M-MDSCs and $Ly6C^{med}Ly6G^{+}$ G-MDSCs. M-MDSCs play a significant role in EAE, which can differentiate into dendritic cells and macrophages and increase IL-17A secretion by promoting the differentiation of Th17 cells. Therefore, M-MDSCs may be a potential target for the treatment of Th17-cell-mediated CNS autoimmunity. 0.67, 2, and 6 μM PLY, PBS (vehicle), and JAK-1 inhibitors (Gao et al., 2018, You et al., 2020) solcitinib (positive control) were used to intervene MDSCs for 48 h *in vitro* to explore the inhibitory effect of PLY. Specific surface markers of M-MDSCs were examined using flow cytometry. The data show that the number and percentage of $Ly6G^{-}Ly6C^{+}$ M-MDSCs decreased significantly compared with the vehicle (**Fig. 2**A, D, G). However, the number of $Ly6C^{med}Ly6G^{+}$ G-MDSCs did not decrease (**Fig. 2**A, H), and the percentage of G-MDSCs increased (**Fig.**

2A, E). Therefore, the number and percentage of MDSCs decreased (**Fig. 2**A, C, F). We speculate that PLY has an inhibitory effect on EAE by reducing the number and percentage of M-MDSCs.

PLY has no effect on survival rate and apoptosis of MDSCs

During PLY intervention in the expansion of MDSCs, as the concentration increased, the number of MDSCs decreased, and we speculated whether PLY was cytotoxic. Next, we confirmed that 0.67, 2, and 6 μM PLY can significantly influence the proliferation of MDSCs, but whether there is cytotoxicity was uncertain. 20 ng/mL GM-CSF and 20 ng/mL IL-6 were used to stimulate MDSCs for 48 h, and the indicated concentration of PLY intervened in MDSCs. Finally, the rate of survival and apoptosis of MDSCs were not significantly different between the PLY and vehicle groups (**Fig. 3**A, B, C). It is clear that PLY does not affect the survival and apoptosis of MDSCs.

PLY inhibit proliferation of MDSCs in vitro

The number of MDSCs plays an important role in the onset and development of inflammation. When inflammation occurs, a large number of pro-inflammatory factors and growth factors such as G-CSF, M-CSF, GM-CSF, and IL-6 are produced in the microenvironment of the affected area. We found that PLY inhibited MDSC and M-MDSC differentiation (**Fig.2.**). This effect was not due to cytotoxicity (**Fig.3.**). We then investigated whether PLY could suppress MDSC proliferation *in vitro*. Using CFSE, we examined the proliferation of MDSCs stimulated by 20 ng/mL GM-CSF and 20 ng/mL IL-6. Next, 0.67, 2, and 6 μM, PBS (vehicle) and solcitinib (positive control) were used to intervene the proliferation of

MDSCs. Compared with PBS, the analysis showed that proliferation of MDSCs was inhibited by PLY in a dose-dependent manner (**Fig. 4**A). Similarly, compared with solcitinib, medium-and high-dose PLY effectively restrained the proliferation of MDSCs (**Fig. 4**A). The data showed that PLY significantly inhibited the proliferation of MDSCs in a dose-dependent manner.

PLY reduces the number and percentage of M-MDSCs in EAE mice

We have shown that PLY inhibits the differentiation of M-MDSCs *in vitro* (**Fig. 2.**). However, whether PLY intervenes in the differentiation of M-MDSCs in EAE mice is uncertain. All the mice were sacrificed after treatment for 21 days. Murine peripheral blood from MOG_{35-55}-induced EAE was tested by flow cytometry. The *in vivo* results were similar to those *in vitro*. The number and percentage of MDSCs and M-MDSCs significantly decreased after treatment with PLY (**Fig. 5**A, C, D, F, G). In addition, the percentage and number of $Ly6G^{+}Ly6C^{med}$ G-MDSCs was not significantly changed by PLY (**Fig. 5**E, H). Thus, PLY mainly inhibited the differentiation of M-MDSCs in EAE mice.

Treatment with PLY attenuates EAE severity

MOG_{35-55}-induced EAE is a good animal model for human multiple sclerosis. After construction of EAE models, treatment with 40 mg/kg PLY, PBS (vehicle), and 1 mg/kg FK-506 (tacrolimus, positive control), which has been proved to inhibit the development of experimental allergic encephalomyelitis(Thomson et al., 1993, Gold et al., 2004), by intraperitoneal injection for 21 d was carried out. It was observed that PLY and FK-506 alleviated EAE progression (**Fig. 6**A). In addition, the severity of EAE in mice treated with PLY was similar to that in the positive control. Meanwhile, hematoxylin and eosin (H&E) staining

and LFB were performed to assess inflammation and demyelination, respectively. The data showed that compared with vehicle, PLY and FK-506 attenuated inflammatory infiltration and demyelination (**Fig. 6**B, C). These results confirmed that PLY alleviated the progression and severity of EAE and reduced inflammatory infiltration and demyelination.

PLY inhibits the differentiation of Th17 cells mediated by MDSCs in spinal cord of EAE mice

The infiltration of MDSCs and Th17 cells in the spinal cord of mice was detected by immunofluorescence. Th17 cells are the main pro-inflammatory T cell in various autoimmune diseases. It has been previously shown that MDSCs can inhibit the differentiation of Th17 cells and reduce the secretion of IL-17A (Yi et al., 2012). In our results, there was a positive correlation between MDSC infiltration and IL-17A levels in the spinal cord of EAE. After a 21-day treatment with drugs, the numbers of $CD11b^{+}Gr\text{-}1^{hi}$ MDSCs and Th17 cells in the spinal cord of each group were examined by immunofluorescence staining. A large number of MDSCs and Th17 cells infiltrated the vehicle group; however, in the PLY and FK-506 groups, MDSCs and Th17 cells were significantly reduced in the spinal cord (**Fig. 7**). Therefore, we suggest that PLY can reduce the number of MDSCs, thereby inhibiting the differentiation of related Th17 cells and the release of IL-17A.

Discussion

PLY is a natural alkaloid extracted from *Narcissus tazetta* L. var. *Chinensis* Roem. Their role is well established in cancer, but not in inflammatory diseases. In this study, we have shown that the reduction of MDSCs ameliorates disease activity in an animal model of EAE, and that MDSCs play an important

role in the PLY-mediated suppression of disease activity. We have shown, for the first time, that PLY can improve the progression of EAE in mice and inhibit the differentiation and infiltration of related Th17 cells by intervening in the differentiation and expansion of MDSCs, especially M-MDSCs.

To understand the potential regulatory role of PLY on MDSCs, we chose to use 0.67, 2, and 6 μM PLY to intervene in the expansion and differentiation of MDSCs. We have elucidated that PLY can inhibit the differentiation of MDSCs *in vitro*. The number and percentage of MDSCs, especially M-MDSCs, significantly decreased, and we identified the cytotoxicity of PLY. Our data indicate that the percentage of surviving cells and apoptotic cells (Annexin-V^+) did not significantly change with different concentrations of PLY. Next, we further clarified that the decrease in MDSCs could be attributed to the proliferation of MDSCs inhibited by PLY.

MDSCs are a group of immature immune cells that arise from common myeloid progenitors (CMPs) and are highly plastic. MDSCs play an important role in the onset and progression of EAE. Our study showed that the number of MDSCs and related Th17 cells decreased with PLY treatment in the spinal cord and ameliorated inflammation infiltration and demyelination.

In fact, the function of MDSCs remains controversial. Under normal circumstances, immature myeloid cells (IMCs) differentiate into immune cells (e.g., DCs, macrophages, and mature granulocytes)(Groth et al., 2019); however, under abnormal conditions, such as autoimmune diseases, IMCs can differentiate into MDSCs (Condamine and Gabrilovich, 2011). Thus, we consider that in inflammatory conditions, MDSCs will appear in large numbers. In contrast to tumors, inflammation promotes the proliferation of various cells either indirectly or directly(Zhang et al., 2018). Under continuous inflammatory conditions, early granulocyte or monocyte precursors differentiate into G-MDSCs and M-MDSCs, respectively. On the one hand, a large number of anti-inflammatory mediators

are produced by MDSCs(Yang et al., 2008); on the other hand, they participate in the inflammatory response by secreted pro-inflammatory factors(Chen et al., 2021). Similarly, chronic inflammatory disease leads to persistent pro-inflammatory signals, leading to inflammatory bone marrow microenvironments, such as bone marrow mesenchymal stem cells (MSCs), leading to myeloid cell expansion and recruitment of MDSCs (Leimkuhler and Schneider, 2019). Therefore, as our results, there was a pathological increase in MDSCs under abnormal conditions.

Although the number of MDSCs increases either in tumors or in inflammation, the function of MDSCs may differ. In the tumor microenvironment, the body is in a state of immunosuppression, and the function of immunocytes (e.g., T cells, B cells, DCs, and macrophages) is restrained. Some inflammatory factors, such as IL-6, GM-CSF, and TNF-α, mainly promote tumor growth (Yang et al., 2008). Thus, the inhibitory effect of MDSCs may be dominated by differentiation into tumor-associated macrophages (TAMs), which can co-suppress NK cells, $CD8^+$ cells, and other immune cells (Zhang et al., 2018). In contrast, MDSCs may play a pro-inflammatory role in a hyperactive inflammatory environment. Scholars generally believe that M-MDSCs are pro-inflammatory compared to G-MDSCs (Zhang et al., 2015b, Chen et al., 2021). Some studies have identified that the inhibitory function of M-MDSCs is damaged during the development of SLE in MRL/LPR mice, to play a pro-inflammatory role by decreasing IL-1β produced by M-MDSCs, leading to a decrease in Th17 cell differentiation (Ji et al., 2016). In addition, MDSCs and Arg-1 of M-MDSCs have been found to increase in primary membranous nephropathy (PMN), and MDSCs promote the differentiation of Th17 cells in an Arg-1-dependent manner (Li et al., 2020). These reciprocal and distinctive actions of MDSCs and Th17 cells collaboratively perpetuate multiple pathogenic processes, including but not limited aggravated inflammation. In a study by Guo et al., MDSCs show decreased T cell inhibitory activity during the

progression of arthritis (Guo et al., 2016b). This may be determined by different inflammatory signals present in the inflamed sites, which are part of the functional plasticity of MDSC. Thus, we believe that MDSCs can alleviate the inflammatory response of EAE and MDSCs-mediated Th17-cells, producing inflammatory cytokines such as IL-17A that indirectly and directly damage the myelin sheath in the CNS (Elliott et al., 2018). Consistent with the above point, with the deterioration of EAE, the number of MDSCs increased, and after PLY treatment, the development of the Th17-cell-mediated CNS autoimmunity was improved, the proportion and number of MDSCs and M-MDSCs were reduced, and the infiltration of MDSCs and related Th17 cells also decreased in the CNS.

Myelin-specific Th17 cells are responsible for the pathogenesis of EAE and MS. Although the number of MDSCs and related Th17 cells decreased after PLY treatment in the spinal cord, naïve $CD4^+$ T cells differentiate into Th17 cells in many ways, such as naïve $CD4^+$ T cell differentiation to non-pathogenic Th17 cells stimulated by IL-6 and TGFβ, but differentiate into pathogenic Th17 cells stimulated by IL-16 and IL-1β (Chung et al., 2009, Das et al., 2009). It is widely known that IL-1β promotes the expansion of MDSCs and is more associated with Th17 cells. However, IL-1β was not chosen, one of the reasons being the differentiation of Th17 cells can also be regulated by STAT1/3/5 (Damasceno et al., 2020). IL-1β accelerates the expansion of MDSCs mainly via the activation of NF-κB or AP-1 (Wang et al., 2020). The difference is that IL-6 and GM-CSF mainly stimulate the JAK-STAT pathway. In addition, the inhibition ability of M-MDSCs was improved by increased levels of ROS via STAT3 activation. The proliferation of neutrophil T-cells is restrained by the influence of GM-CSF on M-MDSCs via the JAK-STAT pathway. Related pathway has been mentioned in a number of studies: JAK-2/STAT-3 inhibition reduced the number of MDSCs and these pathways are reported to be critical for MDSCs proliferation in cancer (Bayne et al., 2012, Nishimura et al., 2015, Sendo et al., 2019). Thus,

IL-6 and GM-CSF have been chosen to co-stimulate MDSCs, and we speculate that IL-6 and GM-CSF play important roles in MDSCs via JAK-STAT, and these pathways may be associated with targets of PLY.

These data provide new insights into the relevance of MDSCs in EAE. It would be interesting to identify the mechanism of PLY in Th17-cell-mediated CNS autoimmunity, which could help to further elucidate the pathogenesis of EAE and guide the design of new drugs and would ultimately benefit patients with safer and more effective therapeutic options for these diseases.

Conclusion

Our data indicated that the mitigation of EAE with PLY comes from its ability to target a range of anti-inflammatory pathways, including (a) inhibition of the differentiation of M-MDSCs and proliferation of MDSCs both *in vitro* and *in vivo*, (b) decrease inflammatory infiltration and demyelination in the spinal cord, and (c) preventing the migration of MDSCs and related Th17 cells to the CNS. Our studies suggest that PLY may be an excellent candidate for the treatment of MS and other autoimmune diseases. In future research, it is crucial to clarify the mechanism of PLY in the treatment of EAE. Therefore, our findings may provide new insights into the importance of MDSCs in autoimmune diseases and aid in the design of new therapies for MS.

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Authorship Contributions and statement L.-M.L. and Y.-T.W. conceived and designed the experiments; X.-Y.Z., G.Z., F.Y. and J.L. performed the experiments; X.-Y.Z., G.Z. and Y.-T.W.

analyzed the data; L.-M.L. and X.L. contributed the reagents/materials/analysis tools; G.Z. and Y.-T.W. wrote the paper.

All the authors declare that all data were generated in-house and that no paper mill was used. All authors have read and approved the content, and agree to submit it to your journal.

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Compliance with ethical standards All animal procedures reported in this study were conducted in compliance with the protocol reviewed by the Animal Care and Use Committee of Chengdu Medical College.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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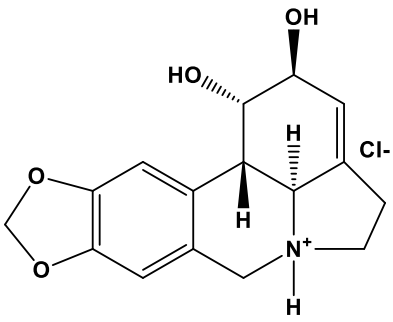


Chemical Formula:$C_{16}H_{18}ClNO_4$
Exact Mass:323.77

Fig. 1. The chemical structure of pseudolycorine chloride (PLY).

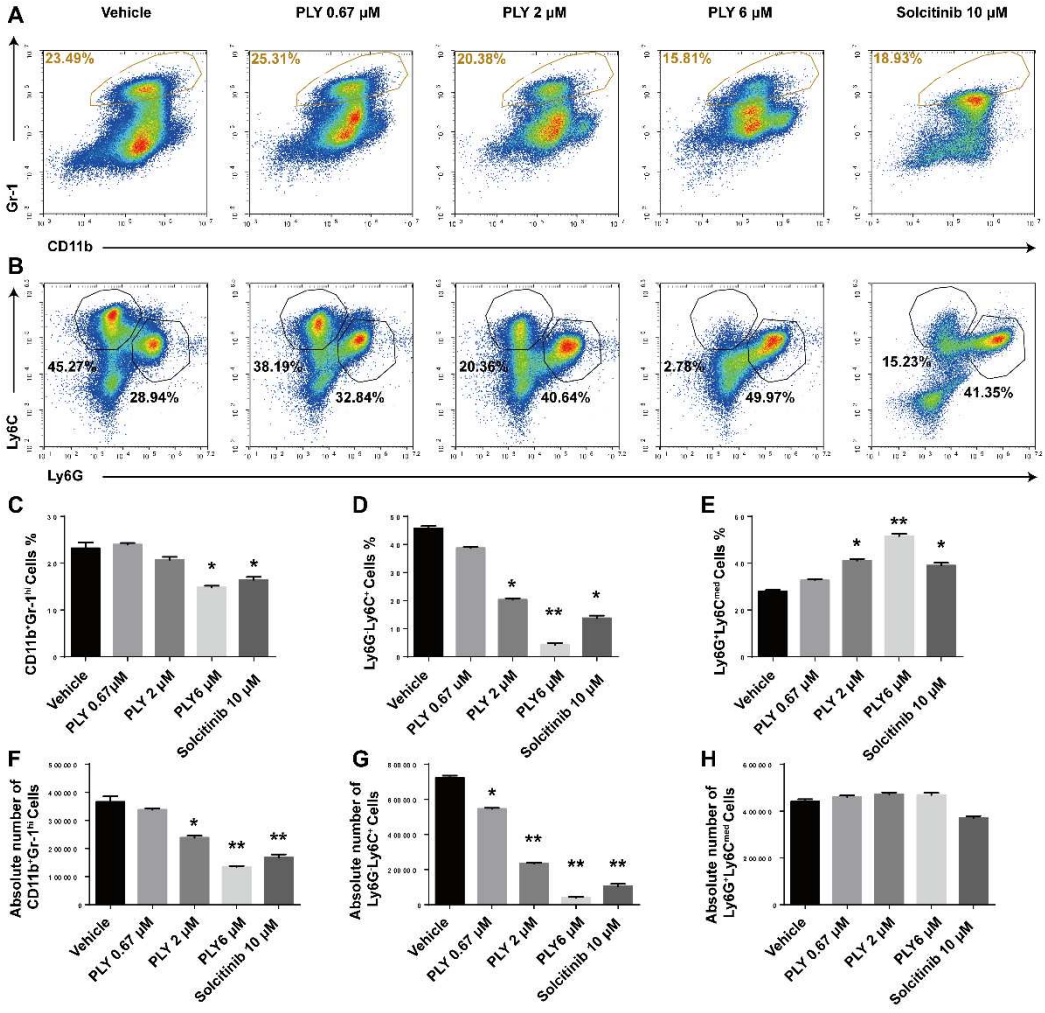


Fig. 2. The differentiation of MDSCs. PLY effects on MDSCs differentiation stimulated by GM-CSF and

IL-6 for 48 h. The cells were stained with anti-Gr-1, anti-CD11b, anti-Ly6C, anti-Ly6G antibodies and examined by flow cytometry. (A) The percentage of Gr-1^{hi}CD11b^{+} MDSCs, after intervened by 0.67 μM, 2 μM, 6 μM PLY, PBS（vehicle）and solcitinib (positive control). (B) In the Gr-1^{hi}CD11b^{+} MDSCs, two subsets were identified by anti-Ly6C and anti-Ly6G (Ly6G^{-}Ly6C^{+} M-MDSCs; Ly6C^{med}Ly6G^{+} G-MDSCs). The percentage of MDSCs (C), M-MDSCs (D), G-MDSCs (E). And the absolute number of MDSCs (F), M-MDSCs (G), G-MDSCs (H). The data are expressed as the mean ± SEM of six independent experiments (*$P < 0.05$, **$P < 0.01$).

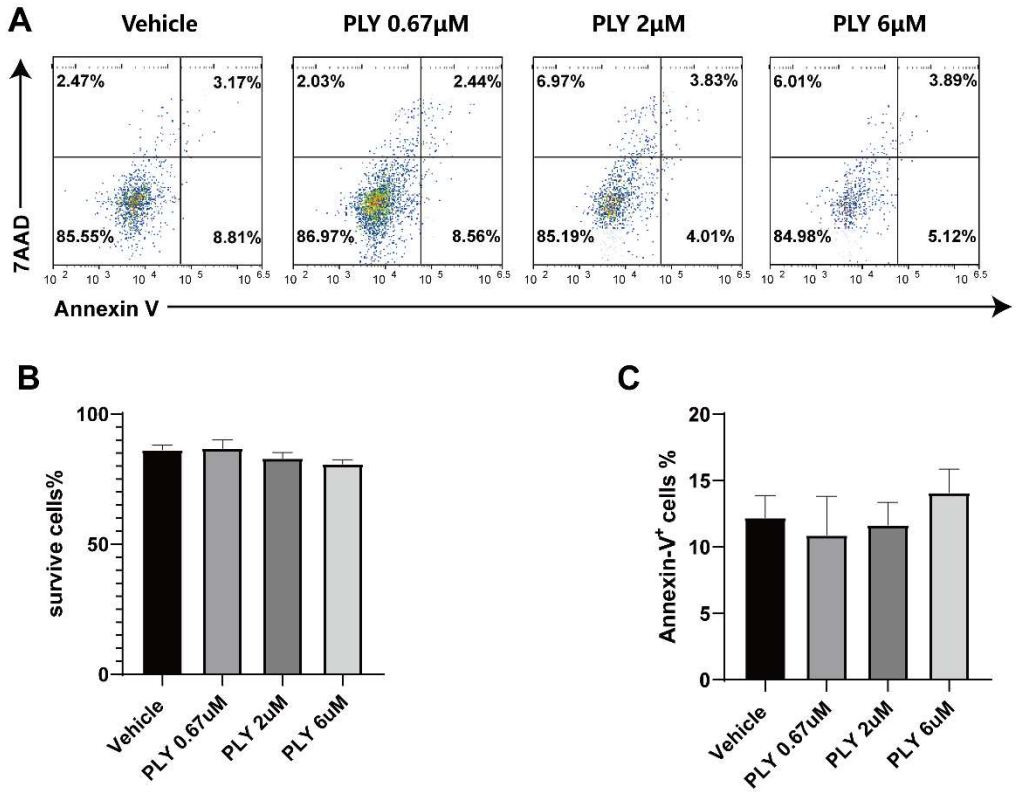


Fig. 3. The apoptosis of MDSCs. PLY effects on MDSCs survival rate and apoptosis stimulated by GM-CSF and IL-6. The cells were stained with 7-AAD, annexin-V and tested by flow cytometry. (A) After 48 h, MDSCs survival and apoptosis between vehicle and PLY group shows no significant difference. The percentage of MDSCs that survive (B) and undergo apoptosis (C). The data are expressed as the

mean ± SEM of six independent experiments (P＞0.05).

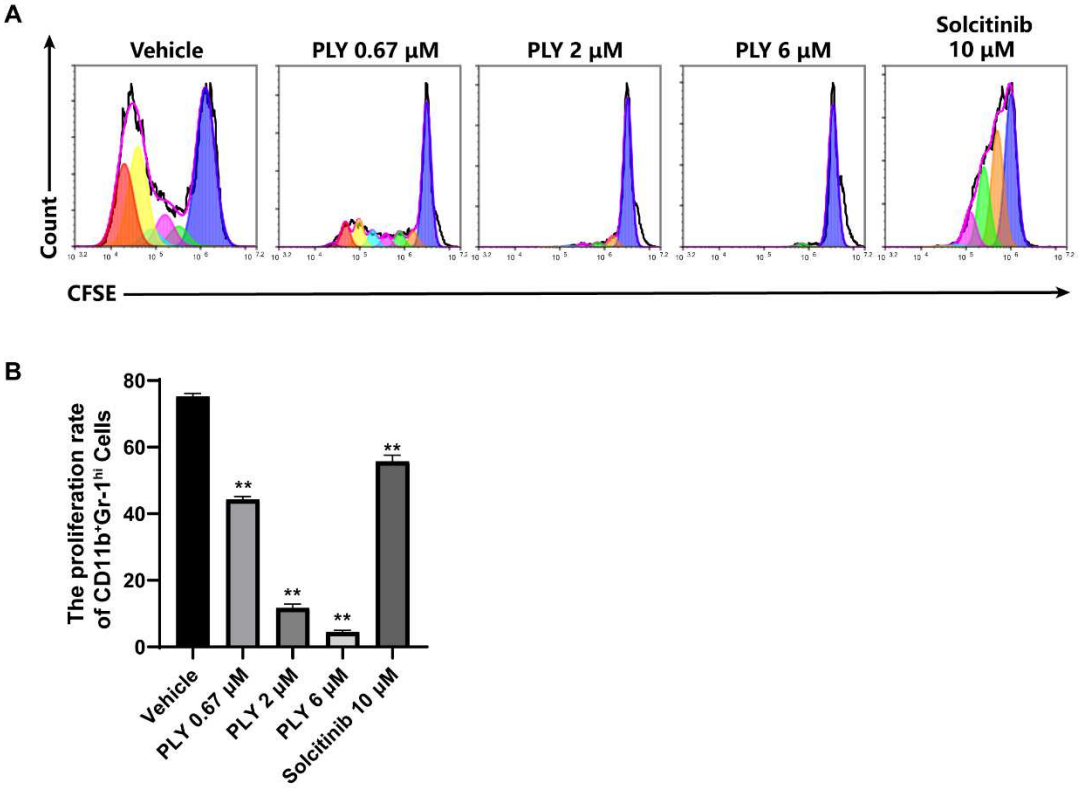


Fig. 4. The proliferation rate of MDSCs. PLY effects on MDSCs proliferation stimulated by GM-CSF and IL-6. GM-CSF and IL-6 were used to stimulate proliferation in the presence or absence of increasing concentrations of PLY compared with the positive control. (A) After 96 h, CFSE-stained cells were collected for cell division assay by flow cytometry. (B) The percentage of Gr-1^{hi}CD11b^{+} MDSCs proliferation. The data are expressed as the mean ± SEM of six independent experiments (*$P < 0.05$, **$P < 0.01$)

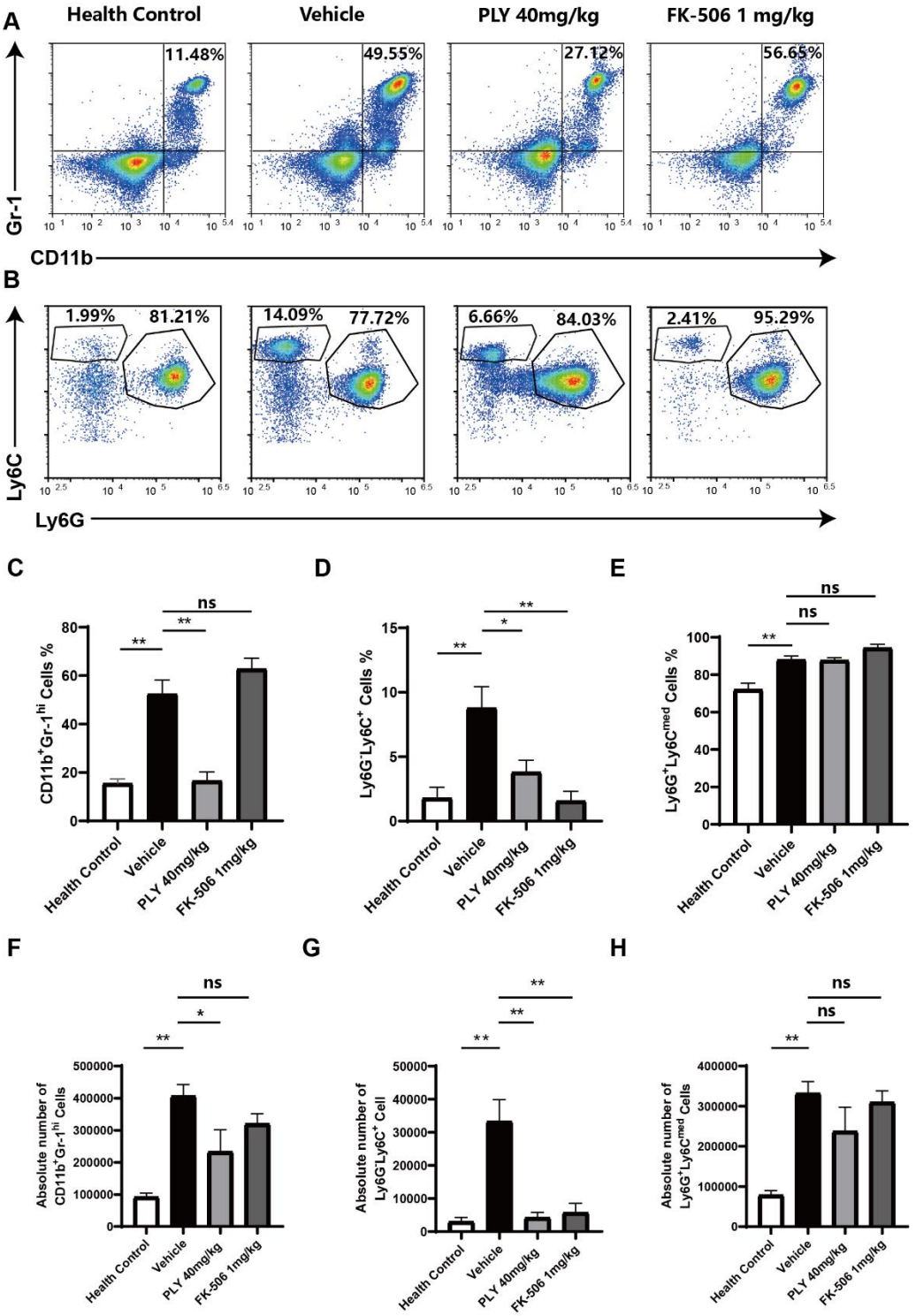
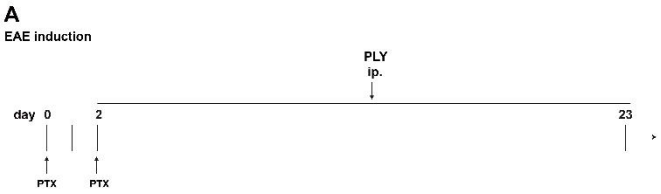


Fig. 5. The number and percentage of MDSCs in EAE mice. After 21-day treatment with 40 mg/kg PLY, 1 mg/kg FK-506 and PBS, mice peripheral blood of MOG_{35-55}-induced EAE were stained with anti-Gr-1, anti-CD11b, anti-Ly6C, anti-Ly6G antibodies and examined by flow cytometry. The percentage of MDSCs (A), G-MDSCs and M-MDSCs (B) in peripheral blood. Statistics showing the mean percentage

of MDSCs (C), M-MDSCs (D), G-MDSCs (E), and number of MDSCs (F), M-MDSCs (G), G-MDSCs (H). The data are expressed as the mean ± SEM. ($n \geq 4$ mice/group) (*$P < 0.05$, **$P < 0.01$).


A
EAE induction
PLY
ip.
day 0
2
23
PTX
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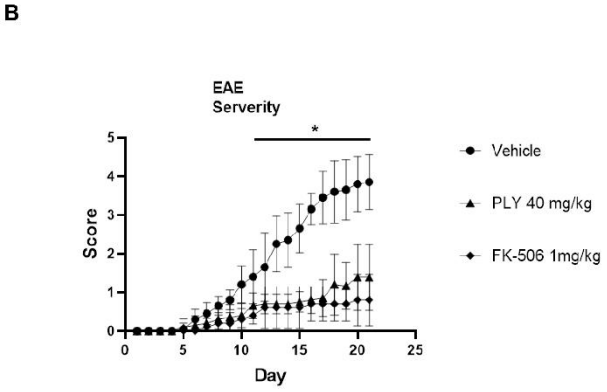
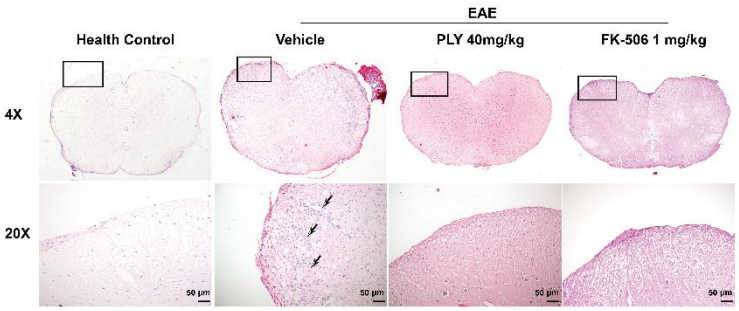
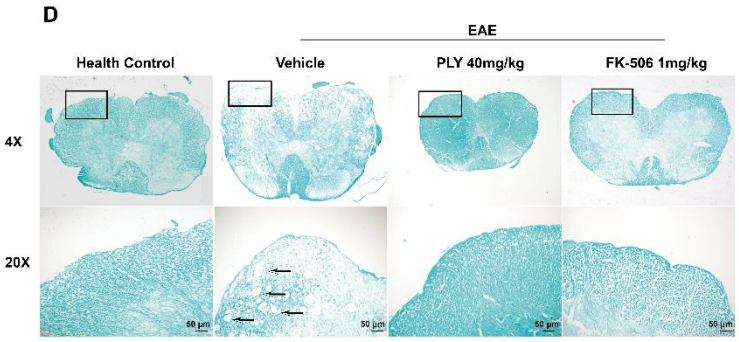

B
EAE
Serverity
Score
Day
Vehicle
PLY 40 mg/kg
FK-506 1mg/kg


C
EAE
Health Control
Vehicle
PLY 40mg/kg
FK-506 1 mg/kg
4X
20X


D
EAE
Health Control
Vehicle
PLY 40mg/kg
FK-506 1mg/kg
4X
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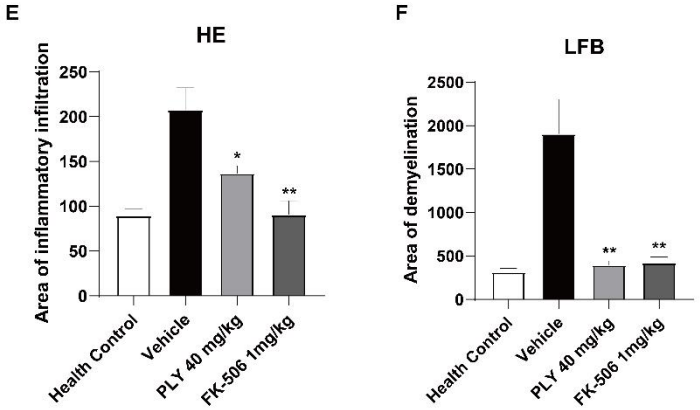
E
HE
Area of inflammatory infiltration
Health Control
Vehicle
PLY 40 mg/kg
FK-506 1mg/kg
F
LFB
Area of demyelination
Health Control
Vehicle
PLY 40 mg/kg
FK-506 1mg/kg

Fig. 6. Pathology of EAE mice. EAE induced by s.c. injection of 200 μg $MOG_{35–55}$ emulsified in 50% complete Freud's adjuvant (CFA). Individual mice were injected intraperitoneally with pertussis toxin (PTX) on day 0 and 2. From d2 to d23, PLY and FK-506 were intraperitoneally injected once a day. The severity of EAE was evaluated every day. (B) 40 mg/kg PLY and FK-506 significantly decrease the severity of EAE. (C) HE staining (×4 in the first row; ×20 in the second row) of mice spinal cords in each group. Blue arrow represented inflammatory cell infiltration. (D) LFB staining (×4 in the first row; ×20 in the second row) of the spinal cords in each group. (E) Bar graphs indicate the area of inflammatory infiltration and (F) demyelination. The data are expressed as the mean ± SEM. ($n \geq 3$ independent experiments/group) (*$P < 0.05$, **$P < 0.01$).

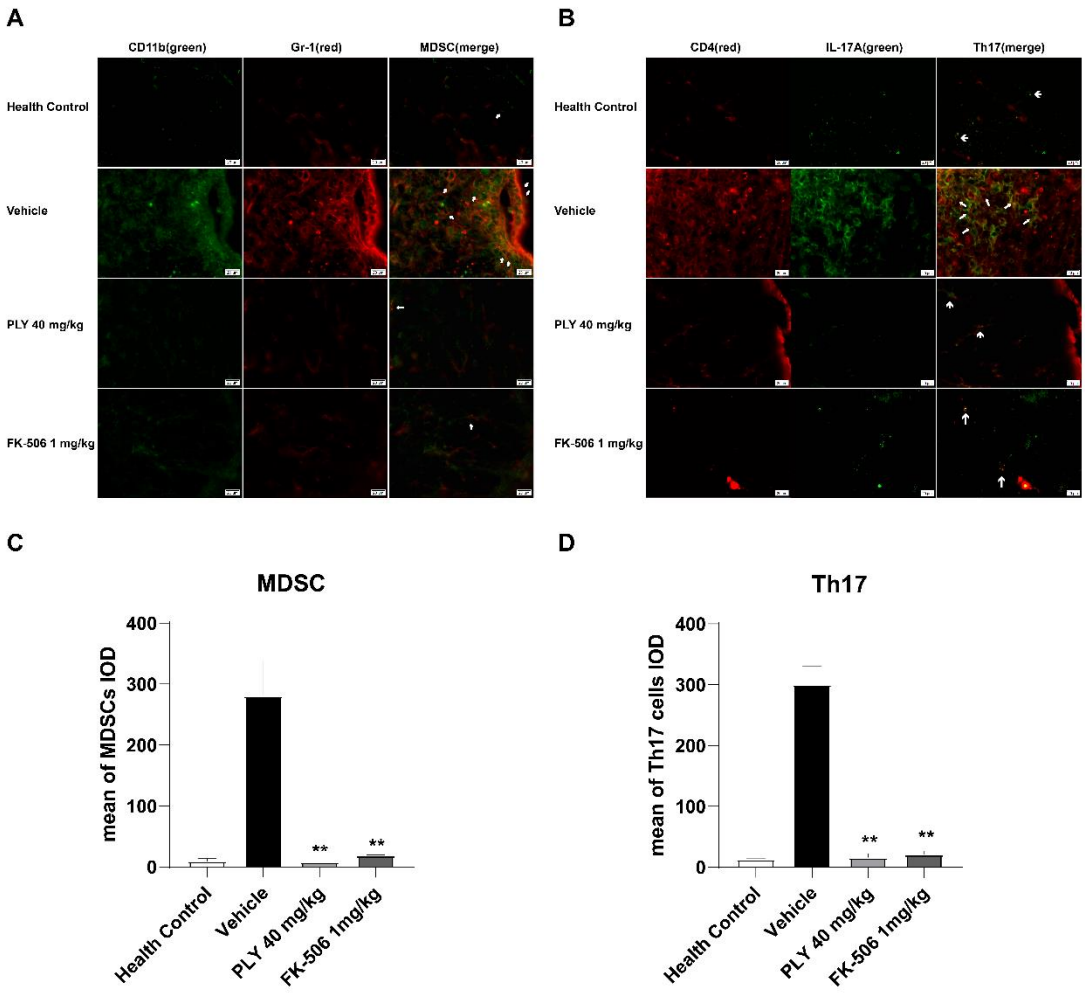

Fig. 7. Infiltration of MDSCs and related Th17 cells in spinal cord. Infiltration of MDSCs in spinal cord of EAE mice were stained by anti-rabbit-CD11b and anti-mouse Ly-6G/Ly-6C (Gr-1); Th17 cells were stained by anti-rabbit-IL17A and anti-mouse CD4. Then they were stained by Alexa Fluor 594 Goat anti-rat IgG (H+L) and Alexa Fluor 488 Goat anti-rabbit IgG (H+L) respectively. (A) The left represents CD11b(green), medium represent Gr-1 (red), and the right represent MDSCs (yellow) in spinal cord. (B) The left represents CD4 (red), medium represent IL-17A (green), and the right represent Th17 (yellow) in spinal cord. (C) The mean of MDSCs IOD (Integral optical density) and (D) Th17 cells IOD. The data are expressed as the mean ± SEM. ($n \geq 3$ independent experiments/group) (*$P < 0.05$, **$P < 0.01$)