

# SYK activation promotes the expansion of myeloid-derived suppressor cells and enhances their immunosuppressive function

**Weihua Yang**

Anhui University of Science and Technology

**Haozhe Guo**

East China Normal University

**Xiya Cao**

East China Normal University

**Yuxuan Lai**

Shanghai Fengxian District Central Hospital

**Jialiang Sun**

Shanghai Fengxian District Central Hospital

**Youyuan He**

Shanghai Fengxian District Central Hospital

**Dali Li**

East China Normal University

**Zhenliang Sun**

Anhui University of Science and Technology

**Zhiyang Zeng**

**zyzeng1991@163.com**

Shanghai Fengxian District Central Hospital

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## Research Article

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# Abstract

## Background

Myeloid-derived suppressor cells (MDSCs) expand significantly under pathological conditions like cancer and exhibit immunosuppressive functions, with their abundance negatively correlated with the efficacy of immune checkpoint blockade (ICB) therapy. However, the mechanisms driving MDSC expansion and immunosuppressive function acquisition are not fully understood. Spleen tyrosine kinase (SYK), a key intracellular signaling molecule in immune cell receptor pathways, is involved in regulating multiple cellular functions, but its influence on MDSC functions remains unclear.

## Methods

A SYK gain-of-function mutation (SYK<sup>S544Y</sup>) mouse model was utilized to investigate the role of SYK activation in MDSCs. Single-cell RNA sequencing (scRNA-seq) was performed to explore the underlying signaling pathways. Additionally, pharmacological inhibition of SYK was achieved using R406 to assess its impact on MDSCs.

## Results

SYK activation was found to promote the expansion of MDSCs and enhance their immunosuppressive function. scRNA-seq revealed that SYK activation potentially augments the immunosuppressive function of MDSCs through the PI3K-AKT and MAPK signaling pathways. Furthermore, pharmacological inhibition of SYK by R406 not only reduced the number of MDSCs but also diminished their immunosuppressive function.

## Conclusion

SYK signaling plays a crucial role in regulating MDSC expansion and function. Targeting SYK may represent a novel strategy to enhance the efficacy of immunotherapy.

## Introduction

MDSCs are a heterogeneous population derived from bone marrow progenitor cells and immature myeloid cells. They expand extensively in pathological microenvironments such as cancer, infection, and autoimmune diseases where they suppress host immune responses through multiple mechanisms<sup>[1]</sup>. The classical activation pathway involves the rapid mobilization and maturation of monocytes and neutrophils in the bone marrow. These cells promote phagocytosis, the production of pro-inflammatory

cytokines, and MHC II expression, thereby swiftly eliminating potential threats to the body, playing a critical role in tumor immune escape<sup>[2–4]</sup>.

MDSCs are broadly classified into two major subsets: granulocytic or polymorphonuclear MDSCs (G-MDSCs, CD11b<sup>+</sup>Ly6G<sup>+</sup>) and monocytic MDSCs (M-MDSCs, CD11b<sup>+</sup>Ly6C<sup>+</sup>). Their expansion and immunosuppressive functions are regulated by various inflammatory mediators within the tumor microenvironment<sup>[5–8]</sup>. MDSCs suppress the immune response through multiple mechanisms, including the depletion of arginine and cysteine, which are essential for T cell activation<sup>[9, 10]</sup>, induction of oxidative stress that impairs T cell receptor signaling<sup>[11]</sup>, secretion of immunosuppressive cytokines such as IL-10 and TGF- $\beta$  to promote regulatory T cell development<sup>[12]</sup>, and expression of immune checkpoint molecules such as PD-L1<sup>[13]</sup>. In addition to immune suppression, MDSCs also promote angiogenesis through the secretion of VEGF<sup>[14]</sup>, and induction of epithelial–mesenchymal transition<sup>[15]</sup>. Consequently, MDSCs play a pivotal role in tumor immune evasion, and their abundance is closely associated with tumor stage and poor prognosis. A comprehensive understanding of the mechanisms governing MDSC expansion and function is therefore essential for developing therapeutic strategies to enhance immunotherapy efficacy.

SYK is a non-receptor tyrosine kinase widely expressed in immune cells and certain non-immune cells. As a central mediator of intracellular signal transduction, SYK is recruited and activated by binding to immunoreceptor tyrosine-based activation motifs (ITAMs) associated with immune receptors<sup>[16]</sup>. It is expressed in a variety of cell types, including T cells, B cells, natural killer (NK) cells, macrophages, dendritic cells (DCs), mast cells, intestinal epithelial cells, and certain tumor cells<sup>[17, 18]</sup>. Upon activation, SYK phosphorylates multiple downstream adaptor proteins, initiating signaling cascades such as MAPK, PI3K–AKT, and NF- $\kappa$ B pathways, thereby regulating cell survival, proliferation, differentiation, and cytokine production.

Although initially characterized as a mediator of adaptive immune receptor signaling (BCR, TCR, FcR), subsequent studies have demonstrated its involvement in innate immune processes through interactions with C-type lectins and integrins, contributing to immune recognition, cell adhesion, osteoclast maturation, and platelet activation<sup>[18–21]</sup>. Importantly, the function of SYK is highly cell type-specific and context-dependent. In solid tumors, SYK can be activated in MDSCs by multiple upstream signals, including FcR, integrins, IL-4 receptor, and TREM2. This activation promotes the secretion of immunosuppressive molecules such as IL-10, TGF- $\beta$ , Arg1, and iNOS, thereby inhibiting T cell and NK cell activity<sup>[18, 22]</sup>. In addition, SYK regulates MDSC recruitment to tumor sites through integrin signaling and chemokine axes such as CXCR1/2<sup>[23, 24]</sup>. SYK also modulates metabolic pathways in MDSCs, including glycolysis and fatty acid oxidation, thereby influencing their survival and suppressive function<sup>[23]</sup>. Therefore, inhibiting SYK can effectively weaken the metabolic adaptability and immunosuppressive capacity of MDSC, reversing its pro-tumor immune regulatory effect. Based on these complex regulatory mechanisms, SYK has gradually become an important target for the treatment of autoimmune diseases and cancer.

Emerging evidence indicates that activation of the SYK signaling pathway in the tumor microenvironment (TME) is closely associated with dysregulation of myeloid cell function<sup>[25, 26]</sup>. However, how SYK activation influences MDSC expansion and function, as well as the underlying molecular mechanisms, remains unclear. One major limitation has been the lack of clinically relevant animal models. We previously reported a clinical case of a gain-of-function SYK mutation (SYK<sup>p.Ser544Tyr</sup> hereafter denoted SYK<sup>S550Y</sup>) associated with systemic inflammatory disease, characterized by immunodeficiency, multi-organ inflammation (including colitis, arthritis, and dermatitis), and diffuse large B-cell lymphoma<sup>[27]</sup>. Correspondingly, we generated a gain-of-function mouse model carrying the homologous mutation (SYK<sup>S544Y</sup>), which provides an excellent platform to investigate the role of SYK in MDSCs.

In this study, we demonstrated that SYK activation promotes MDSC expansion while enhancing their immunosuppressive function by utilizing the SYK gain-of-function (SYK<sup>S544Y</sup>) mouse model. Blocking SYK activity by R406 reduced the number of MDSCs and their immunosuppressive abilities. These findings provide new mechanistic insights and a theoretical basis for SYK-targeted immunotherapeutic strategies.

## Materials and Methods

### Animal Models

SPF-grade wild-type (WT) C57BL/6 mice and SYK gain-of-function mutant mice (SYK<sup>S544Y</sup>) were used in this study. Mice were housed in the Laboratory Animal Centre of East China Normal University under standard conditions (22 °C, 50% humidity, 12 h light/dark cycle) with free access to food and water. All animal experiments were performed in accordance with the guidelines of the Shanghai Association for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of East China Normal University (No. m20241002). Age- and sex-matched mice (6–8 weeks old) were used for all experiments.

### Preparation of single-cell suspensions from mouse immune organs

**Peripheral Blood:** After blood collection, red blood cells (RBCs) were lysed by adding RBC lysis buffer (BD Biosciences) and incubating for 15 minutes at room temperature on a shaking incubator at 100 rpm. The reaction was stopped by adding flow cytometry buffer (PBS containing 1% FBS). The cell suspension was filtered through a 40 µm mesh filter into a fresh 15 mL centrifuge tube, centrifuged at 500 g for 5 minutes at 4°C, and the supernatant was discarded. The pellet was resuspended in 1 mL of flow cytometry buffer, transferred to a 1.5 mL Eppendorf tube, and centrifuged again at 500 g for 5 minutes at 4°C. The supernatant was removed, and the pellet was subjected to a second RBC lysis step by adding 1 mL of RBC lysis buffer and incubating for 3 minutes at room temperature. After centrifugation at 500 g for 5 minutes at 4°C, the supernatant (containing lysed RBC debris) was carefully discarded, leaving a

white cell pellet. The cells were then gently resuspended, counted, and adjusted to a concentration of  $1 \times 10^6$  cells/mL in flow cytometry buffer for downstream applications.

**Spleen:** The spleen was placed in a 6 cm dish fitted with a 40  $\mu$ m mesh sieve and set on ice. Flow cytometry buffer was added to the mesh, and the spleen was ground using the plunger of a 2 mL sterile syringe. The spleen cells were filtered into the 6 cm dish. The filtered solution was collected into a 15 mL centrifuge tube, centrifuged at 500 g for 5 minutes at 4°C, and the supernatant was removed. Ten times the pellet volume of erythrocyte lysis buffer was added, and the cells were lysed at room temperature for 3 minutes. The lysis was terminated with flow cytometry buffer. The suspension was filtered through a 40  $\mu$ m mesh sieve into a new 15 mL centrifuge tube, centrifuged at 500 g for 5 minutes at 4°C, and the supernatant was discarded. The cells were then counted and resuspended at a concentration of  $1 \times 10^6$  cells/mL in flow cytometry buffer (PBS with 1% FBS).

**Bone Marrow:** The mouse tibia and femur were isolated, and any adherent muscle tissue was removed while on ice. Pre-chilled PBS was poured into a 10 cm dish, and the mouse bone tissue was washed. Both ends of the mouse bone tissue were trimmed, the medullary cavity was flushed with PBS, and the resulting bone marrow cell suspension was collected in a 15 mL centrifuge tube placed on ice. The suspension was centrifuged at 4°C at 500 g for 5 minutes, and the supernatant was discarded. Erythrocyte lysis buffer was added, and the cells were lysed for 30 seconds. The lysis was terminated with flow cytometry buffer, and the suspension was filtered through a 40  $\mu$ m mesh filter. The filtrate was centrifuged at 500 g for 5 minutes at 4°C, the supernatant was discarded, and the cells were counted. If flow cytometry analysis was to be performed, the cells were resuspended at a concentration of  $1 \times 10^6$  cells/mL in flow cytometry buffer (PBS with 1% FBS) for subsequent experiments.

## Flow cytometry

Add a cell viability stain and blocking solution to the cell pellet and incubate at room temperature for 15 minutes, then add the surface protein antibody and incubate at 4°C for 30 minutes; if intracellular staining is not required, centrifuge, wash, resuspend, filter and proceed to the next step; If intracellular staining is required, centrifuge and wash, then resuspend in membrane lysis buffer (True-Nuclear™ Transcription Factor Buffer Set) and incubate at room temperature for 1 hour to lyse the membranes. Add buffer to stop the reaction, wash, then incubate with intracellular antibodies at room temperature for 1 hour. Finally, wash twice, resuspend, filter, and analyze. Analyse using the LSRFortessa and FlowJo v10.7.1 (BD).

Antibodies used: BV510-Zombie Aque™ Dye (Biolegend; B324734), anti-CD45 (Biolegend; B339836), anti-CD11b (Biolegend; B337295), anti-GR1 (Biolegend; B328503), anti-ARG1 (Biolegend; 2321301).

## Isolation of MDSCs

MDSCs were isolated from tissue samples employing the EasySep™ Mouse MDSC Isolation Kit (Stemcell), following the manufacturer's instructions precisely. Subsequently, the purity of the isolated MDSC population was verified to exceed 90% through flow cytometry analysis.

## RNA Extraction and cDNA Synthesis

Trizol lysis buffer was added to the sample, followed by chloroform to homogenize the mixture. After centrifugation, the supernatant was collected and an equal volume of isopropanol was added. After precipitating the RNA, 75% ethanol was added to wash the precipitate. The ethanol was discarded, and the precipitate was air-dried before being dissolved in RNA-free water. Finally, the RNA concentration was measured. Reverse transcription was performed to obtain cDNA. The reaction mixture is as follows (20 µL)

| Component                                    | volume     |
|----------------------------------------------|------------|
| 2×Hifair® 1st Strand cDNA Synthesis SuperMix | 10µL       |
| RNA                                          | 1µg        |
| Nuclease-free Water                          | Up to 20µL |

Reverse transcription was performed in a PCR thermocycler using the following program:

| temperature | Time  |
|-------------|-------|
| 25°C        | 5min  |
| 42°C        | 30min |
| 85°C        | 5min  |
| 16°C        | ∞     |

The cDNA was diluted with Nuclease-free Water.

## Quantitative Real-time PCR

Each experiment was performed in triplicate wells. The reaction system for each well was as follows:

| Component                        | volume |
|----------------------------------|--------|
| Hieff qPCR SYBR Green Master Mix | 10 µL  |
| Primer(F/R)                      | 2 µL   |
| cDNA                             | 2 µL   |
| Nuclease-free Water              | 6 µL   |

In the qPCR instrument, select the 7500 (96 well) mode,  $\Delta\Delta C_T$  method and SYBR Green reagents, and perform qPCR. Subsequently, relative gene expression levels were calculated using the Livak ( $2^{-\Delta\Delta C_t}$ ) based on the Ct values of the internal reference gene GAPDH.

All qPCR primers were synthesized by Platinum Bio, with the following sequences:

| ID       | Primer sequence(5'-3')    |
|----------|---------------------------|
| Arg1-F   | GGGCAACCTGTGTCCTTTCT      |
| Arg1-R   | ATCGGCCTTTTCTTCCTTCCC     |
| Cox2-F   | TCCCATGGGTGTGAAGGGAAA     |
| Cox2-R   | ACCCAGGTCCTCGCTTATGA      |
| Nos2-F   | GCTCTAGTGAAGCAAAGCCCA     |
| Nos2-R   | TGATGGACCCCAAGCAAGAC      |
| S100a4-F | TTGTGGTTGAGCTGTGGGAG      |
| S100a4-R | GGTAACCGTTGAGACCCCTC      |
| S100a8-F | GTCCTCAGTTTGTGCAGAATATAAA |
| S100a8-R | GCCAGAAGCTCTGCTACTCC      |
| S100a9-F | ATAAATACTGGGCTTACACTGCTCT |
| S100a9-R | GCCATCTTCTTGCTCTTCAAGG    |
| Il6-F    | GACAAAGCCAGAGTCCTTCAGA    |
| Il6-R    | TGTGACTCCAGCTTATCTCTTGG   |
| Il1b-F   | TGCCACCTTTTGACAGTGATG     |
| Il1b-R   | TGATGTGCTGCTGCGAGATT      |
| Gapdh-F  | ATGTTTGTGATGGGTGTGAA      |
| Gapdh-R  | ATGCCAAAGTTGTCATGGAT      |

## scRNA-seq

When performing single-cell sequencing, the first step is to prepare a peripheral blood single-cell suspension following the protocol outlined. After collecting target cells, dead cells are removed using the Dead Cell Removal Kit. The cells are then resuspended in 1× PBS (containing 0.04% BSA) and centrifuged at 300 g for 3 minutes at 4°C, with this step repeated twice. The final cell suspension must meet the following criteria: cell viability > 85% as determined by trypan blue exclusion assay, and a cell

concentration of approximately 2,000 cells/ $\mu$ L when counted using a hemocytometer or automated cell counter. Following the preparation of the single-cell suspension, libraries are constructed using the 10X Genomics Chromium Single-Cell 3' Kit (v3) and subjected to paired-end sequencing on the NovaSeq 6000 platform, with a read length of 150 bp and a sequencing depth set at 20,000 reads per cell. This experimental work was conducted with assistance from LC-Bio Technologies (Hangzhou) Co., Ltd.

## In vitro differentiation assay

The femurs were harvested from 6-8-week-old mice, and bone marrow cells were isolated and cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), along with the cytokines interleukin-6 (IL-6, 20 ng/mL, Peprotech) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/mL, Peprotech). To assess the impact of the SYK inhibitor R406 on the generation of MDSCs, it was added to specific cultures every other day to achieve a final concentration of 2  $\mu$ M. After a 7-day incubation period, the cells were collected, stained with the appropriate antibodies, and the percentages of Gr1<sup>+</sup> cells were quantified using flow cytometry.

## Statistical Analysis

All statistical analyses and visualizations were performed using Prism 9.0 (GraphPad). Differences between two groups were assessed using Student's t-test. All experiments were independently repeated at least three times. Numerical data are shown as means  $\pm$  standard error of the mean (SEM). Throughout all figures: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Significance was concluded at  $p < 0.05$ .

## Results

### SYK Activation Promotes Expansion of MDSCs and Expression of Immunosuppressive Factors

Our previous study has demonstrated that gain-of-function mutations in SYK can lead to systemic inflammation and spontaneous arthritis in mice. A marked infiltration of CD11B<sup>+</sup> myeloid cells was also found in the joints of SYK<sup>S544Y</sup> mice [27]. We were thus prompted to explore whether SYK activation would have an impact on MDSC. Flow cytometry analysis of peripheral blood derived from WT and SYK<sup>S544Y</sup> mice was performed. Compared to WT mice, the proportion of MDSCs (CD11B<sup>+</sup>Gr1<sup>+</sup>) in SYK<sup>S544Y</sup> mice increased by approximately 10% (Fig. 1a). The increases of MDSCs proportion were also observed in the spleen (23% in SYK<sup>S544Y</sup> vs. 5% in WT mice) and bone marrow (BM) (62% in SYK<sup>S544Y</sup> vs. 50% in WT mice) of SYK<sup>S544Y</sup> mice. (Fig. 1b-c). It has been demonstrated that Arg1, Cox2 and Nos2 are key molecules mediating the immunosuppressive function of MDSCs [28, 29]. Therefore, we further examined the mRNA expression levels of MDSC-associated immunosuppressive markers in peripheral blood via

qPCR and found that SYK<sup>S544Y</sup> mice exhibited elevated expression of Arg1, Cox2 and Nos2, with Arg1 expression upregulated approximately 12-fold, Cox2 and Nos2 expression were each upregulated approximately 5-fold. (Fig. 1d). Collectively, these results indicate SYK activation not only promotes the expansion of myeloid cells but also induces their differentiation towards pro-inflammatory and immunosuppressive phenotypes.

## scRNA-seq Reveals SYK Activation Specifically Expands A Subpopulation Of MDSCs

To further investigate the effects of SYK activation on various subpopulations of myeloid cells and its underlying molecular mechanisms, we performed single-cell RNA sequencing on blood from WT and SYK<sup>S544Y</sup> mice. Following standard data processing and quality control procedures, we obtained transcriptomic data from a total of 8,207 cells derived from WT mice and 8,315 cells derived from SYK<sup>S544Y</sup> mice. Cluster analysis revealed a total of 27 cell clusters (Fig. 2a). Based on cell surface markers, they can be classified into three categories: T cells, B cells and myeloid cells. Molecular markers used for identification were Cd3d and Cd3e for T cells, Cd79a and Cd79b for B cells, and Itgam and Lyz2 for myeloid cells (Fig. 2b). This transcriptomic data were subjected to cluster analysis (Fig. 2c). Beyond the previously reported T-cell proliferation and B-cell depletion in SYK<sup>S544Y</sup> mice<sup>[18]</sup>, we observed an increased proportion of myeloid cells in these mutants relative to WT controls (Fig. 2d).

It is well known from many studies that myeloid cells, especially MDSCs, function as key mediators of immunosuppression and tumor progression<sup>[30]</sup>. We focused on the reclassification of myeloid cell populations, using genes highly expressed in representative subtypes of myeloid cells as molecular markers to distinguish between the various subpopulations, resulting in 16 distinct myeloid subclusters (Fig. 3a, Table 1). As shown in the t-SNE clustering plot, clusters 0, 5 and 9 correspond to M-MDSCs; clusters 1, 2, 3, 7, 8, 10 and 11 correspond to G-MDSCs; clusters 4, 12 and 13 correspond to monocytes; cluster 14 corresponds to dendritic cells; cluster 15 corresponds to mast cells; and cluster 6 corresponds to megakaryocytes. (Fig. 3b-3c). Statistical analysis showed that MDSCs constituted the majority of myeloid cells regardless of genotype (WT: 74.36%; SYK<sup>S544Y</sup>: 84.72%) (Fig. 3d). In particular, the difference in the number of M-MDSCs between WT and SYK<sup>S544Y</sup> mice was more pronounced than that of G-MDSCs. There were no significant differences in the proportions of other myeloid subsets, such as monocytes and dendritic cells, between the two groups. These results confirm that SYK activation specifically promotes the expansion of MDSCs.

Table 1  
Expression levels of representative genes defining different myeloid cell subsets

| Blood myeloid cell cluster | Functional properties | Representative genes                                                                                                                     |
|----------------------------|-----------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| 0,5,9                      | M-MDSCs               | Ly6c2 S100a4 Chil3 Fn1 Ccr2 F13a1 Tmsb10 Crip1 Vcan Ahnak Ifi30 Lyz2 Plac8 Psap Aif1 Cd74                                                |
| 1,2,3,7,8,10,11            | G-MDSCs               | Ly6g S100a8 S100a9 Csf3r Mmp9 Cxcr2 Dusp1 Retnlg Slc7a11 Stfa2l1 Gm5483 Il1b Lrg1 Ifitm1 Lcn2 Wfdc21 Steap4 Igfbp6 Wfdc17 Slpi Hdc Cxcr1 |
| 4,12,13                    | Monocytes             | Ear2 Apoe Ace Pou2f2 Spn Grk3 Adgre4 Cd36 Gngt2 Fabp4 Smc6 Igkc Ighm Cd79a Cd79b Ccl5 Gm26917 Lrp1 Zeb2                                  |
| 14                         | DCs                   | Cd209a H2-Dma Bst2 H2-DMb1 Cd74 H2-Aa H2-Ab1 H2-Eb1 Cd7 Crip1                                                                            |
| 15                         | Mast cells            | Cyp11a1 Ccl3 Ms4a2 Cd63 Cd200r3 Ccl9 Gata2 Mcpt8 Ccl4 Gm12840                                                                            |
| 6                          | Megakaryocytes        | Pf4 Pbpb Clec1b Tsc22d1 Nrgn Thbs1 Itga2b Alox12 Itga6 Lsm12                                                                             |

## SYK Activation Induces Upregulation of Immunosuppressive and Pro-inflammatory Genes in MDSCs

To further investigate the effects of SYK activation on MDSC function, we performed a differential gene expression analysis on MDSCs (combining G-MDSCs and M-MDSCs) derived from WT and SYK<sup>S544Y</sup> mice. The volcano plot revealed numerous differentially expressed genes between the two groups, with approximately 50 genes upregulated and 86 genes downregulated (Fig. 4a). Compared with WT MDSCs, several immunosuppressive genes (Arg1, Cox2, Nos2) and classical pro-inflammatory genes (S100a8, S100a9, S100a4, Il1b, Il6) were significantly upregulated in SYK<sup>S544Y</sup> MDSCs (Fig. 4b). In addition, qPCR results confirmed that expression levels of Arg1, Nos2, Il1b, Il6 were significantly higher in SYK<sup>S544Y</sup> MDSCs compared with WT MDSCs, consistent with the scRNA-seq data (Fig. 4c). Taken together, these results indicate that SYK activation not only promotes the expansion of MDSCs but also enhances their pro-inflammatory and immunosuppressive functions.

## SYK Activation Drives MDSC Expansion via the MAPK Signaling Pathway

To explore the molecular mechanisms by which SYK activation promotes MDSC proliferation and function, we performed a KEGG pathway enrichment analysis on differentially expressed genes. Analysis revealed that the most significantly enriched pathways included cytokine-related signaling pathways, the PI3K-Akt signaling pathway and the MAPK signaling pathway (Fig. 4d). GSEA further revealed significant

enrichment of immune and metabolism-related gene sets (Fig. 4e). Considering that MAPK signaling is a classical pathway regulating cell proliferation and is closely linked to the pro-inflammatory function of immune cells<sup>[31]</sup>, we focused on the MAPK pathway. GSEA further confirmed that gene sets associated with the MAPK signaling pathway are significantly enriched in SYK<sup>S544Y</sup> MDSCs. In addition, GSEA analysis revealed enrichment in immune system and metabolic pathways, which are consistent with the findings of previous studies<sup>[28, 32]</sup>. These findings suggest that SYK activation may drive MDSC expansion and functional activation by upregulating MAPK and other signaling pathways.

## **SYK Inhibitor R406 Reverses SYK Activation-Induced MDSC Expansion**

To further validate the pivotal role of SYK activation in the expansion and functional activation of MDSCs, we initially conducted in vitro inhibition experiments. Specifically, BM cells from wild-type (WT) and SYK<sup>S544Y</sup> mice were induced to differentiate into MDSCs in vitro, followed by the addition of the SYK-selective inhibitor R406. The results demonstrated that the proportion of MDSCs derived from SYK<sup>S544Y</sup> mice within CD45<sup>+</sup> immune cells decreased from approximately 85% to around 70% upon treatment with the R406 inhibitor (Fig. 5a), while the immunosuppressive marker Arg1 decreased by roughly 20% (Fig. 5b).

Subsequently, we validated our findings through in vivo experiments. SYK<sup>S544Y</sup> mice were administered the selective SYK inhibitor R406 (10 mg/kg, intraperitoneally) for 7 consecutive days. Flow cytometry analysis revealed that R406 treatment significantly reduced the proportion of MDSCs among CD45<sup>+</sup> immune cells in both the peripheral blood and spleen of SYK<sup>S544Y</sup> mice. Specifically, the proportion of MDSCs in the peripheral blood decreased from approximately 8% to around 2%, while in the spleen, it declined from about 10% to roughly 5%, approaching the levels observed in WT mice (Fig. 5c-d). At the same time, their immunosuppressive function and the expression levels of related inflammatory factors were verified by qPCR. The results showed that in SYK<sup>S544Y</sup> mice treated with the R406 inhibitor, the expression levels of immunosuppressive factors (Arg1, Cox2, Nos2) and inflammatory factors (S100a8, S100a9, S100a4, IL-1b, IL-6) were significantly reduced, and in some cases even returned to wild-type levels (Fig. 5e). These results further validate the crucial role of SYK signaling in maintaining MDSC expansion and immunosuppressive function in vivo and suggest that inhibition of SYK activity can effectively reverse this process.

## **Discussion**

MDSCs, as a key immunosuppressive cell population within the tumor microenvironment, possess critically important mechanisms that govern their expansion and functional regulation. In this study, using a SYK gain-of-function mouse model in combination with flow cytometry, single-cell RNA sequencing, and qPCR, we systematically demonstrated that SYK activation promotes in vivo expansion

and enhances the immunosuppressive function of MDSCs, with the MAPK signaling pathway serving as an important mediator.

Previous studies have shown that SYK gain-of-function mutations can lead to spontaneous arthritis and systemic inflammation in mice, accompanied by infiltration of CD11B<sup>+</sup> myeloid cells<sup>[27]</sup>. However, these prior investigations primarily focused on the inflammatory and autoimmune phenotypes, without systematically examining the effect of SYK activation on MDSC expansion or function. In particular, the specific subsets of MDSCs (G-MDSCs vs. M-MDSCs) affected by SYK signaling, as well as the underlying molecular mechanisms, remained unexplored. Building on these findings, our flow cytometry analysis revealed that SYK<sup>S544Y</sup> mice exhibit a significant increase in myeloid cell proportions in peripheral blood, spleen, and bone marrow, particularly in G-MDSC and M-MDSC subpopulations. This indicates that SYK may be a critical regulator of myeloid cell differentiation.

Subsequently, through single-cell RNA sequencing, myeloid cells from WT and SYK<sup>S544Y</sup> mice were classified into 16 distinct subpopulations, revealing the specific expansion of G-MDSCs and M-MDSCs in SYK<sup>S544Y</sup> mice. This represents the first high-resolution characterization of SYK-driven MDSC heterogeneity and provides a clear distinction from earlier studies that only assessed bulk myeloid populations. This result not only validates the findings from flow cytometry but also precisely identifies the specific myeloid cell populations that undergo expansion, laying the foundation for subsequent research into the functional differences between various MDSC subsets.

Mechanistically, our data indicate that SYK activation promotes MDSC expansion and functional activation primarily through the MAPK signaling pathway. Both KEGG enrichment and GSEA analyses showed significant enrichment of MAPK pathway-related genes in SYK<sup>S544Y</sup> MDSCs. The MAPK signaling pathway is a critical pathway regulating cell proliferation, differentiation, and survival, and it extensively interacts with pro-inflammatory signaling pathways in immune cells. Furthermore, in this study, SYK activation simultaneously upregulated the expression of both immunosuppressive molecules and classic inflammatory cytokines in MDSCs, suggesting that MDSCs in SYK<sup>S544Y</sup> mice may be in a unique functional state characterized by both immunosuppressive and pro-inflammatory activity.

Inhibitor intervention experiments further validated the key role of the SYK signaling pathway in the regulation of MDSCs. R406, an ATP-competitive SYK inhibitor, has been demonstrated in multiple studies to alleviate various disease phenotypes. For example, R406 and its structural analogs can mitigate Parkinson's disease by enhancing SNCA-LC3B interactions, partially ameliorate systemic inflammatory disease in SYK-activated mice via bone marrow transplantation, and block SYK-dependent osteoclastogenic signaling to relieve periodontitis associated with rheumatoid arthritis<sup>[27, 33, 34]</sup>. Notably, although R406 has been used to suppress inflammation in previous models, its direct effect on MDSC expansion and function had not been examined. Our study provides the first evidence that pharmacological inhibition of SYK not only reduces the number of MDSCs but also diminishes their immunosuppressive capacity in vitro and in vivo. In this study, R406 treatment significantly reduced the proportion of MDSCs in both the peripheral blood and spleen of SYK<sup>S544Y</sup> mice. Moreover, qPCR results

demonstrated a notable decline in the expression levels of immunosuppressive functional factors and related inflammatory cytokines. These findings are consistent with our previous research, which reported that R406 could alleviate inflammation in a mouse model of arthritis<sup>[27]</sup>, and further support the potential of SYK as a therapeutic target for immune modulation. Moreover, given the critical role of MDSCs in tumor immune evasion, our findings that SYK activation promotes both MDSC expansion and immunosuppressive function underscore the potential of targeting the SYK signaling pathway to enhance anti-tumor immunity and improve immunotherapy efficacy.

However, this study has certain limitations. There is a conspicuous absence of phenotypic analysis in tumor models. This would offer strong evidence to support the notion that targeting SYK could achieve a significant alleviation of disease symptoms and progression. Additionally, the functional differences between G-MDSCs and M-MDSCs under SYK activation, as well as the precise mechanisms by which they suppress T cell responses, require further investigation. Moreover, the specific downstream target genes of the MAPK pathway that regulate MDSCs expansion and function remain to be identified.

## **Declarations**

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### **Author contributions**

The project was conceived and designed by Z.Z., Z.S., and D.L. Research and data analysis were performed by W.Y., H.G., X.C., Y.L., J.S., and Y.H. The manuscript was written by W.Y. and H.G., with contributions from all authors. All authors contributed to the article and approved the final submission.

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### **Declaration of interests**

**Ethics Approval** This study was approved by the East China Normal University Center for Animal Research (Notification m20241002).

**Competing Interests** The authors declare no competing interests.

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## Figures

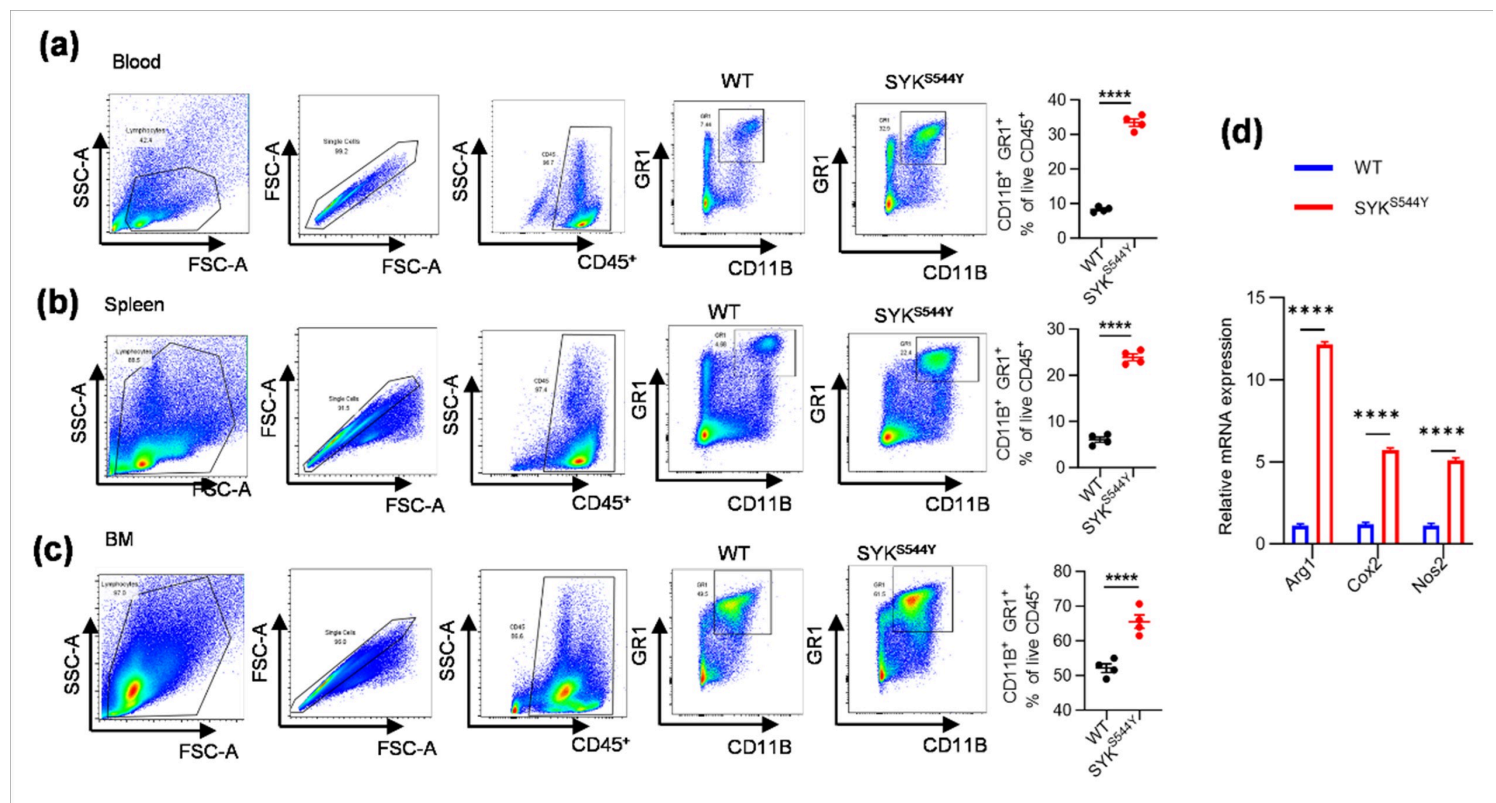


**Figure 1**

**Flow cytometry analysis of MDSC numbers in various immune organs from WT and SYK-GOF mice.** (a) Schematic diagram and statistical graph of flow cytometry gating for MDSCs in the Blood of WT and SYK-GOF mice (n=4). (b) Schematic diagram and statistical graph of flow cytometry gating for MDSCs in the Spleen of WT and SYK-GOF mice (n=4). (c) Schematic diagram and statistical graph of flow cytometry gating for MDSCs in the BM of WT and SYK-GOF mice (n=4). (d) Statistical graph of mRNA expression levels of classical immunosuppressive factors (Arg1, Cox2, Nos2) in Blood (n=4). In all panels, NS, was not significant, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001.

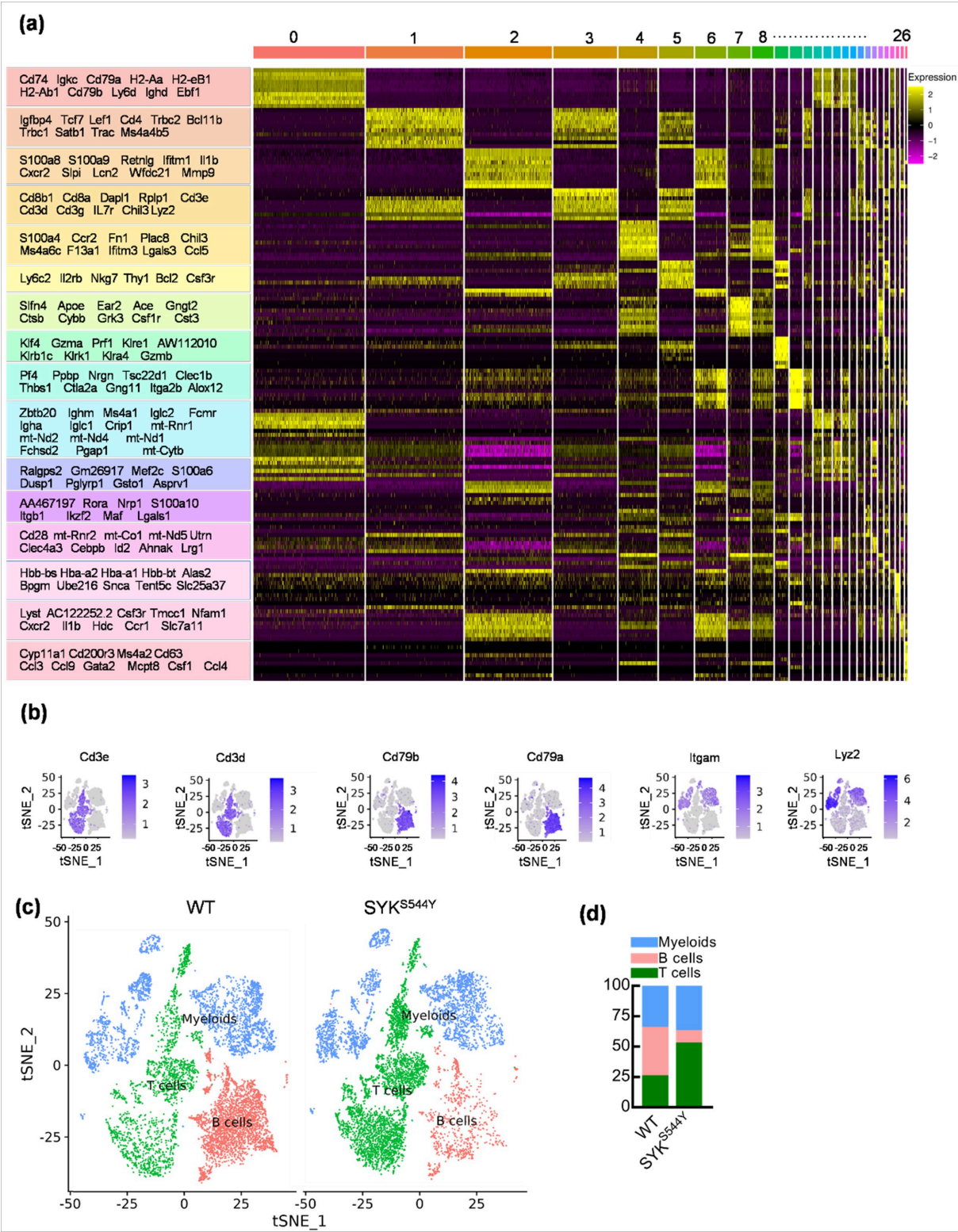


**Figure 2**

**Identification of cell types in WT and SYK<sup>S544Y</sup> blood by scRNA-seq** (a) Gene expression heatmap analyzed by scRNA-seq. (b) Expression of marker genes for the cell types defined above each panel. (c) t-SNE of single-cell profiles with each cell color-coded for sample type and associated cell type. (d) The fraction of cells for three cell types in WT and SYK<sup>S544Y</sup>.

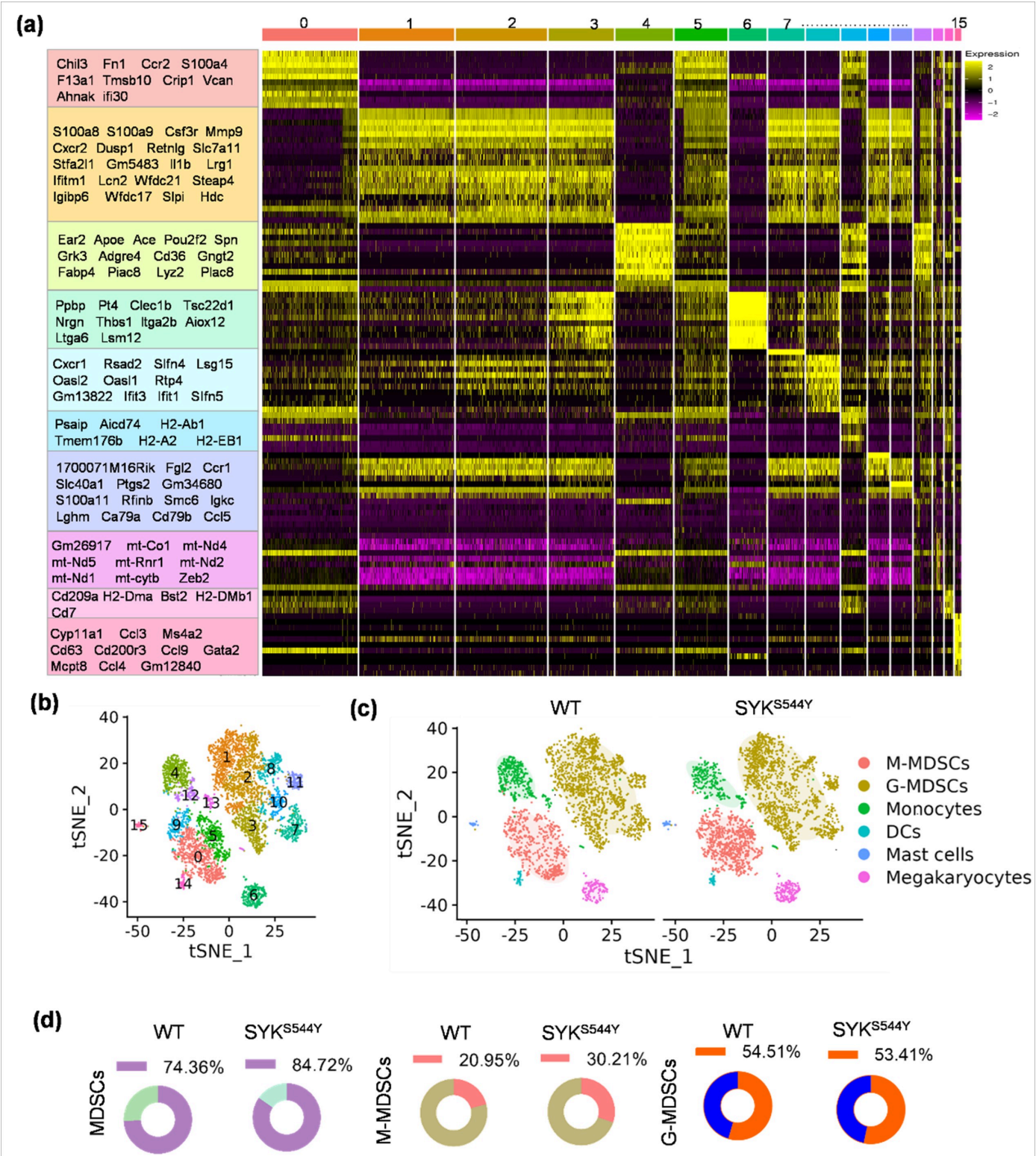


**Figure 3**

**Characterization of myeloid cells by scRNA-seq in mouse blood.** (a) Combined t-SNE plot showing clusters of all myeloid cells. (b) Reclustering analysis of myeloid cells on the t-SNE. (c) Myeloid cell clusters from WT and SYK-GOF mice on the t-SNE. (d) Proportions of total MDSCs and different MDSC subsets within myeloid cells from WT and SYK-GOF mice.

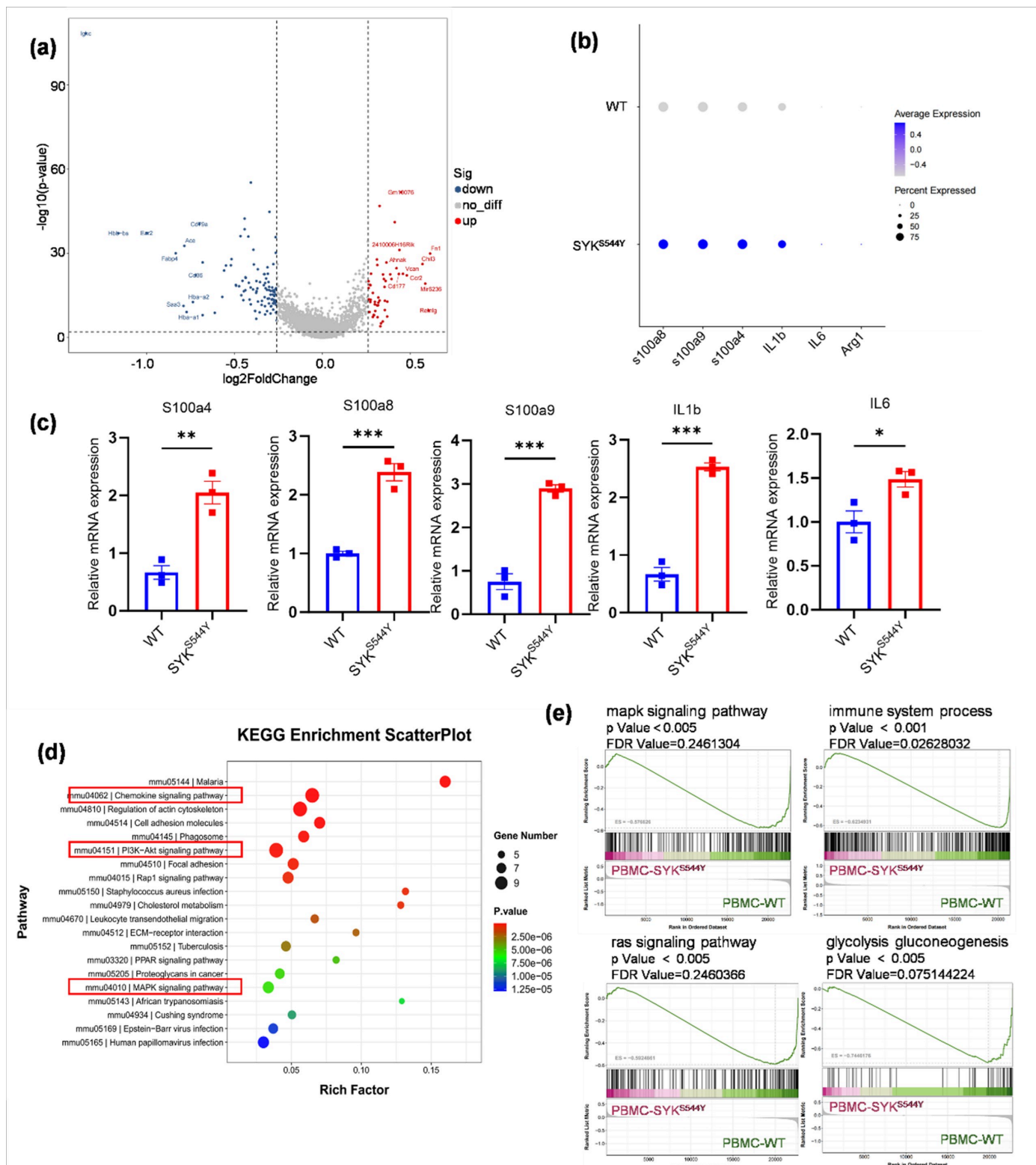


Figure 4

**Differential expression analysis and pathway enrichment results of MDSCs genes in WT and SYK<sup>S544Y</sup>**

(a) Volcano plot of differentially expressed genes between WT and SYK<sup>S544Y</sup>. (b) Schematic diagram of RNA expression levels of pro-inflammatory factors and immunosuppressive factors of MDSCs in the blood. (c) Statistical graph of qPCR results of related inflammatory factors in MDSCs. (d) KEGG

enrichment analysis results (n=3). (e) GSEA enrichment analysis of signaling pathway-related genes. In all panels, NS, was not significant, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001.

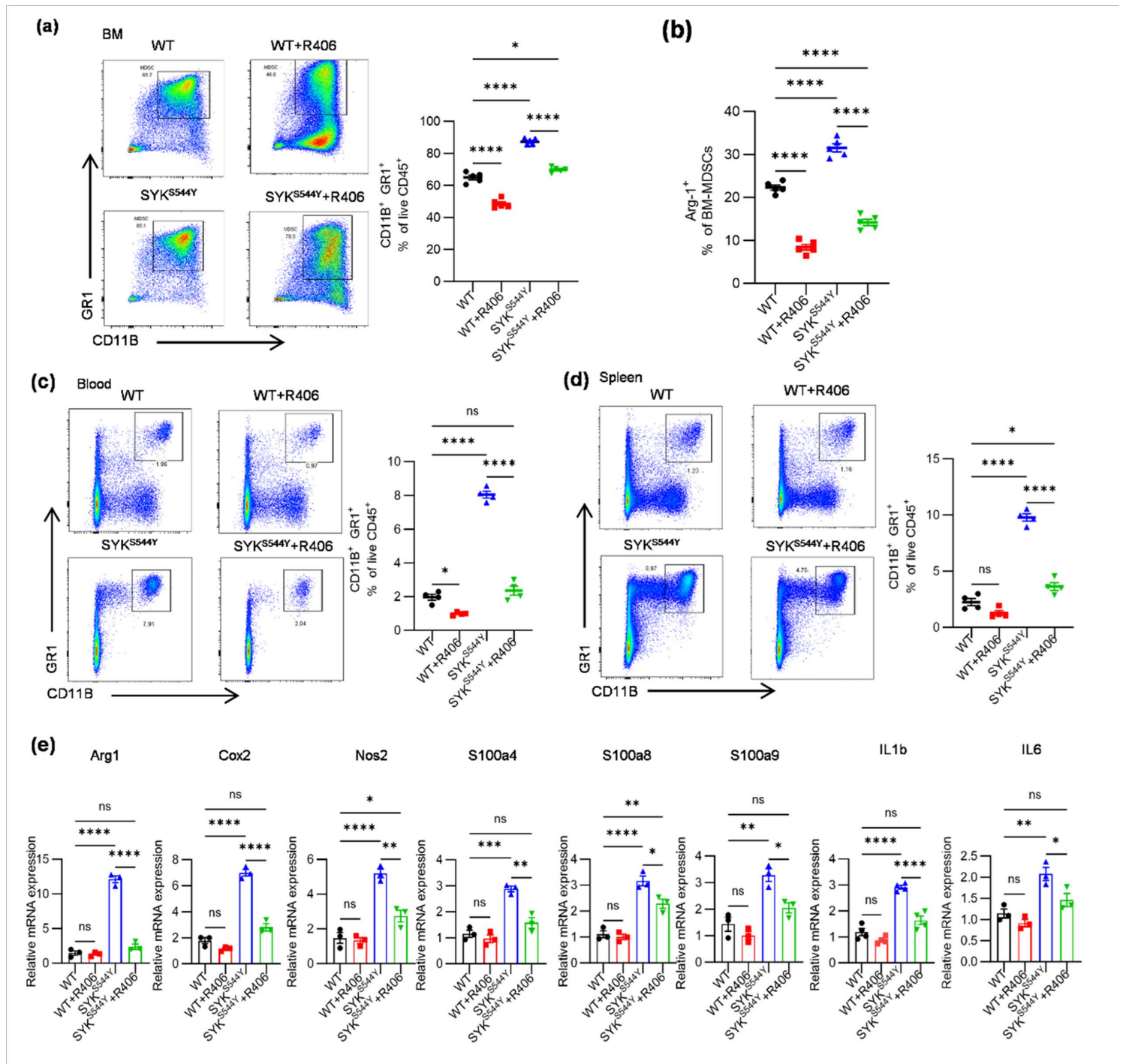


**Figure 5**

**Effects of the SYK inhibitor R406 on the number and immunosuppressive function of MDSCs in different immune organs of wild-type and SYK-GOF mice.** (a) Flow cytometry gating diagrams and statistical graphs of MDSC numbers in BM cells from WT and SYK<sup>S544Y</sup> mice treated with R406 in vitro (n=5). (b) Statistical graph of Arg1 numbers in bone marrow of WT and SYK<sup>S544Y</sup> mice treated with R406 in vitro (n=5). (c) Flow cytometry gating diagrams and cell number statistical graphs of MDSCs in the

blood of WT and SYK<sup>S544Y</sup> mice treated with R406 in vivo (n=4). (d) Flow cytometry gating diagrams and cell number statistical graphs of MDSCs in the Spleen of WT and SYK<sup>S544Y</sup> mice treated with R406 in vivo (n=4). (e) Statistical graph of qPCR results for MDSC immunosuppressive function markers and inflammatory factors (n=3). In all panels, NS, was not significant, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001.