**Supplementary material**

# SOD3 suppresses early cellular immune responses to parasite infection

**Supplementary Method**

**Fluorescence-activated cell sorting (FACS) of macrophages and neutrophils**

For isolation of splenic macrophages and neutrophils, spleens from WT mice were mechanically dissected through a 70-μm cell strainer at different time points after parasite infection. Macrophages were isolated with a BD FACSAria III by gating on $CD45^+$, $F4/80^+$, and $CD11b^+$ cells; neutrophils were isolated with a BD FACSAria III by gating on $CD45^+$, $F4/80^-$and $CD11b^+$ cells. The purity of sorted cells, as verified by FACS analysis, was more than 92%, which separated intact live cells from dead cells and enucleated cellular debris. The proportion of dead cells was determined by using a hemocytometer to count the cells stained with trypan blue and was lower than 5%.

**Quantitative PCR with reverse transcription (RT–qPCR)**

RNA from the above sorted cells was extracted using TRIzol solution (Invitrogen, CA, USA). Cat# 15596026) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop2000 (Thermo Fisher Scientific). Reverse transcription reactions were performed using 5 μg of total RNA with HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China; Cat# R323).

RT–qPCR was performed with 5 ng of cDNA from sorted neutrophils and macrophages prior to infection and at different time points post-infection using a final concentration of 1 μM each for forward and reverse primers using SYBR qPCR Master Mix (Vazyme, Nanjing, China; Cat# Q711-00) on an Applied Biosystems 7500 fast Real-Time PCR System. All RT–qPCR amplicons were analyzed by agarose gel

electrophoresis to confirm the presence of a single specific band. Statistical analysis was performed using Applied Biosystems 7500 software. Target gene expression relative to that of an internal reference gene (*β-actin*) was calculated using the $2^{-\Delta\Delta Ct}$ method. Amplification primers were designed using PrimerBank (www.pga.mgh.harvard.edu/primerbank) and purchased from Sangon Biotech.

*β-actin* was amplified using the following primers:

forward primer, 5′-GGCTGTATTCCCCTCCATCG-3′

reverse primer, 5′-CCAGTTGGTAACAATGCCATGT-3′

*SOD3* was amplified using the following primers:

forward primer, 5′- CCTTCTTGTTCTACGGCTTGC-3′

reverse primer, 5′-TCGCCTATCTTCTCAACCAGG-3′

**Histopathological analysis**

The spleens and brains from WT and $SOD3^{-/-}$ mice at different time points post-infection with *P. berghei* ANKA were fixed in 4% paraformaldehyde (Servicebio, China; cat. G1101-15ML) for 24 h at room temperature, embedded in paraffin, and sectioned at 5 μm. The spleen and brain sections were stained with hematoxylin and eosin, and the slides were scanned with a Panoramic SCAN and Panoramic Viewer (3D HISTECH).

**Detection of cytokines**

Sera were collected from WT and $SOD3^{-/-}$ mice at different time points post-infection, and the levels of IL-2, IL-10, MCP-1, IFN-γ, and TNF-α were determined using the

Mouse Cytokine Assay Kit according to the manufacturer's specifications (Integrated Biotech Solutions, IBS, Shanghai, China). A total of 1500 events were recorded for each preparation.

**Western blotting**

Protein from T cells or splenocytes sorted from WT and SOD3$^{-/-}$ mice prior to infection and at different time points post-infection were extracted with RIPA Lysis Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, with a protease and phosphatase inhibitor cocktail for general use) (Biyuntian, China, Cat# P0013B), followed by denaturation with SDS–PAGE Protein Sample Loading Buffer (Biyuntian, Cat: P0286) at 100°C for 10 minutes. The total proteins were resolved by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Cat# 1620177). The membranes were blocked in 2% bovine serum albumin in PBS at 37°C for 60 min, incubated with primary antibodies overnight at 4 °C and then washed three times in 0.05% Tween-20 in PBS, followed by incubation with an HRP-conjugated goat anti-rabbit IgG secondary antibody (Biyuntian, Cat# A0208) or HRP-conjugated goat anti-mouse IgG secondary antibody (Biyuntian, Cat# A0216). Protein bands were quantified using ImageJ software. Target protein levels were normalized to that of β-actin. All WB experiments were repeated at least 3 times. Blots for phosphorylated p38 MAPK, phosphorylated JNK and phosphorylated AKT were stripped and reprobed for total p38 MAPK, total JNK and total AKT, respectively.

A detailed list of primary antibodies including pan-AKT1/2/3 Antibody (Affinity, AF6261), P-AKT(Affinity, AF3262), JNK1+JNK2+JNK3 Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AF1048 and Affinity, AF6318), Phospho-JNK1/JNK2/JNK3 (Thr183/Thr183/Thr221) Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AF1762 and Affinity, AF3318), p38 MAPK Ab (Affinity, AF6456 and Biyuntian Biotechnology Institute,AF7668), Phospho-p38 MAPK Mouse Monoclonal Antibody (Biyuntian Biotechnology Institute, AM063-1), SIRT1 (Jingjie PTM Biolabs, PTM5021), STAT5a Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AF2038) , STAT5b Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AG3329) , TLR1/Toll-like Receptor 1 Rabbit Polyclonal Antibody (Biyuntian Biotechnology Institute AF8178) ,TLR4 (Biyuntian Biotechnology Institute, AF8187) ,TLR7 (Biyuntian Biotechnology Institute, AF0300) ,TLR8 (Biyuntian Biotechnology Institute AF8190),TLR9 (Biyuntian Biotechnology Institute, AF8193) , NF-kB p65 Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AF1234) , Phospho-NF-κB p65 (Ser276) Rabbit Polyclonal Antibody (Biyuntian Biotechnology Institute, AF5875 ,Biyuntian Biotechnology Institute AF5878, Biyuntian Biotechnology Institute, AF5881) , ERK1 Rabbit Monoclonal Antibody or ERK1/2 Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AF1315, Biyuntian Biotechnology Institute, AF1051), Phospho-ERK1-Thr202/Tyr204+ERK2-Thr185/Tyr187 Rabbit Polyclonal Antibody (Biyuntian Biotechnology Institute, AF5818), β-Actin Rabbit Monoclonal Antibody (Biyuntian Biotechnology Institute, AF5003 and Affinity, BP001M and Servicebio,

GB12001).

**Flow cytometry analysis of immune cells**

Flow cytometry was performed using a BD FACSAria III. Spleens from SOD3$^{-/-}$ and WT mice at different time points after parasite infection were prepared for dissection as described in the section 'Fluorescence-activated cell sorting'. Erythrocytes in the blood and splenic single-cell suspensions were lysed in lysis buffer for 10 min, washed in 10 mL of PBS, incubated with a purified anti-mouse CD16/32 antibody, fixed with a freshly prepared True-NuclearTM Transcription Factor Buffer Set (BioLegend, USA, Cat# 424401), washed twice with True-NuclearTM Perm Buffer (BioLegend), and incubated with specific antibodies or isotype controls according to the manufacturer's guidelines. The specific antibodies included anti-CD3 (BioLegend, clone 17A2), anti-CD8 (Tonbo, China, Cat# 50-0081), anti-CD4 (BioLegend, clone GK1.5), anti-IFN-γ (BioLegend, clone XMG1.2), anti-NK1.1 (BioLegend, clone S17016D), anti-IL-2 (BioLegend, clone JES6-5H4), and anti-CD45 (BioLegend, clone 30-F11) antibodies.

**T cell isolation**

Isolation of T cells were purified from splenocytes using Mouse CD4$^{+}$ T Cell Isolation Kit (Negative Selection, M7401M, UElandy Inc, China) or Mouse CD8$^{+}$ T Cell Isolation Kit (Negative Selection, M7402S, UElandy Inc, China) followed by magnetic cell sorting. The purity of T cell populations enriched by sorting was ≥95%.

**T cell culture**

Mouse T cells were isolated from spleen 4–6 weeks old wild-type C57BL6. Cells was separated conventionally, and centrifugated and washed by PBS to prepare lymphocyte suspension. T cells were then activated with combination of 5 μg/mL of mouse anti-CD3 (Catalog No. 102115, BioLegend) and 5 μg/mL of anti-CD28 (Catalog No. 100339, BioLegend) for 24 h in RPMI-1640 medium, GlutaPlus, HEPES, sodium pyruvate (G4535-500ML; Servicebio, Wuhan, China) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (servicebio, G4003-100ml) and recombinant mouse IL-2 (carrier-free, 300U/ml, BioLegend, USA, 575402) at 37°C in an incubator.

**Cell binding assay with recombinant SOD3**

His-SOD3 fusion protein was incubated with spleen immune cells as previously described[1]. The SOD3 bound to the cell surface was detected by flow cytometry using anti-SOD3- Rabbit antibody (Affinity, DF7753) and Alexa Fluor™ 488-conjugated goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Invitrogen, A-11034). For the control staining, cells were treated with a non-specific rabbit IgG as the primary antibody, using the same AF488-conjugated goat anti-rabbit IgG as the secondary antibody. Flow cytometric analyses were performed using EV450 - conjugated anti-mouse CD45 (Elabscience, E-AB-F1136Q), Percp cy5.5-conjugated anti-mouse CD4 (Biolegend, 100433), APC-conjugated anti-mouse CD8a (Biolegend,

100711), PE-conjugated anti-mouse NK1.1 (Elabscience, E-AB-F0987D).

**Cell activation analysis**

Isolated T cells were activated with combinations of 5 μg/mL of mouse anti-CD3 and cultivated in RPMI-1640 with 5 μg/mL of anti-CD28 and 300U/mL IL-2 for 24 h. Then, the cells in the SOD3-treated group were treated with rSOD3 (100 U/mL) for 24 hours, while the control group was treated with the same dose of PBS. After 48 h of cell culture, T cells were then harvested, washed, and stained with EV450 -conjugated anti-mouse CD45 (Elabscience, E-AB-F1136Q), APC-conjugated anti-mouse CD25 (Biolegend, 102012), FITC-conjugated anti-mouse CD8 antibodies for analyzed by flow cytometry.

**T cell differentiation analysis**

Isolated T cells were activated with combinations of 5 μg/mL of mouse anti-CD3 and cultivated in RPMI-1640 with 5 μg/mL of anti-CD28 and 300U/mL IL-2 for 24 h. Then, the cells in the SOD3-treated group were treated with rSOD3 (100 U/mL) for 24 hours, while the control group was treated with the same dose of PBS. Cells were then harvested, washed and stained with EV450 -conjugated anti-mouse CD45 (Elabscience, E-AB-F1136Q), FITC-conjugated anti-mouse CD8 (Biolegend, 100706), BV605-conjugated anti-mouse CD44 (Biolegend, 103047), PE/Cy7-conjugated anti-mouse CD62L (Biolegend, 104417) antibodies for analyzed by flow cytometry.

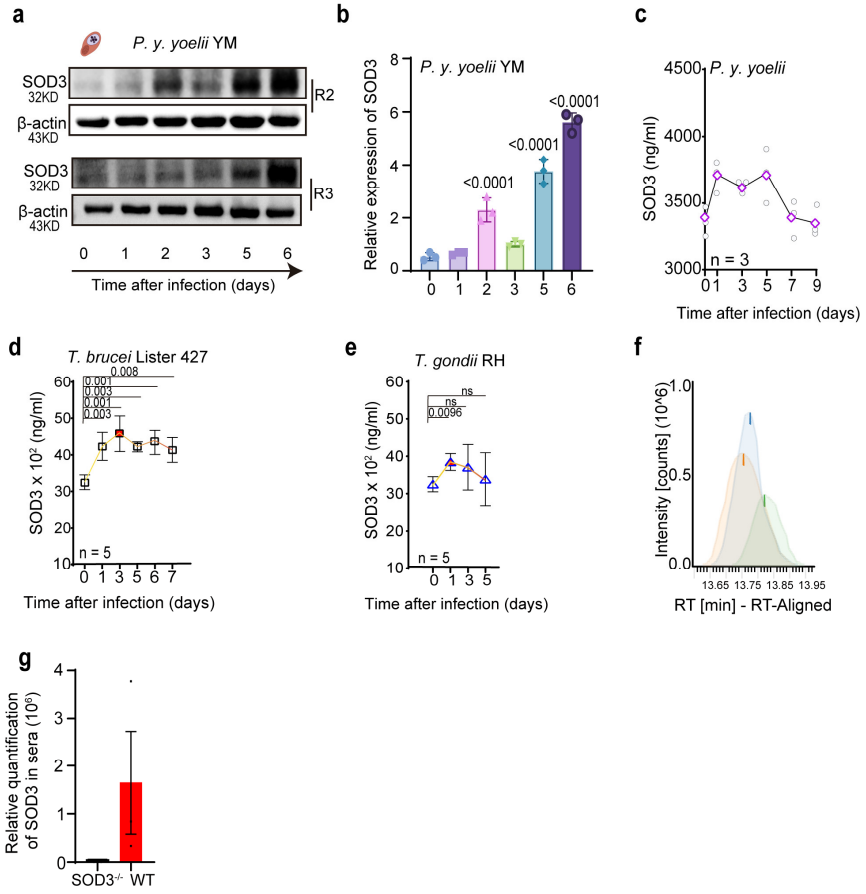
**IL-2 and IFN-γ production analysis**

T cells were cultured as before and incubated with rSOD3 (100 U/mL) or SP600125 (ATP-competitive JNK inhibitor, Selleck, S1460) for 24 h. Cells were then harvested, washed and stained with antibodies for analyzed by flow cytometry. BV605-conjugated anti-mouse IL-2, APC-conjugated anti-mouse IFN-γ, Pacific Blue™-conjugated anti-mouse CD45, FITC-conjugated anti-mouse CD8 was purchased from Biolegend.

**Mass cytometry analysis of Th1 cells in *P. y. yoelii*-infected mice**

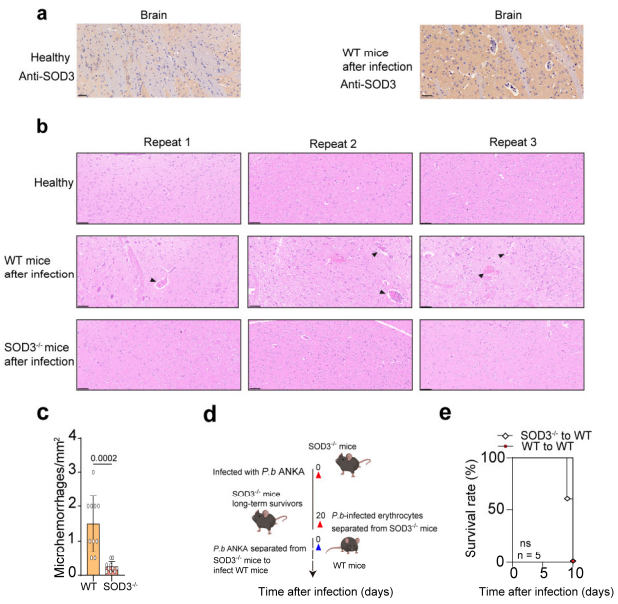
Mass cytometry (CyTOF) was performed as previously described[2]. Spleens from WT mice and $SOD3^{-/-}$ mice were mechanically dissected through a 70-μm cell strainer at different time points after *P. y. yoelii* infection. The single cells were first stained with cisplatin viability dye, and then stained with 144Nd labeling IL-2, 165Ho-labeling IFN-γ, 147Sm-labeling CD45, 168Er-labeling CD8. All antibodies were purchased from polarisbiology (China, Jiangsu).

## Supplemental Figures

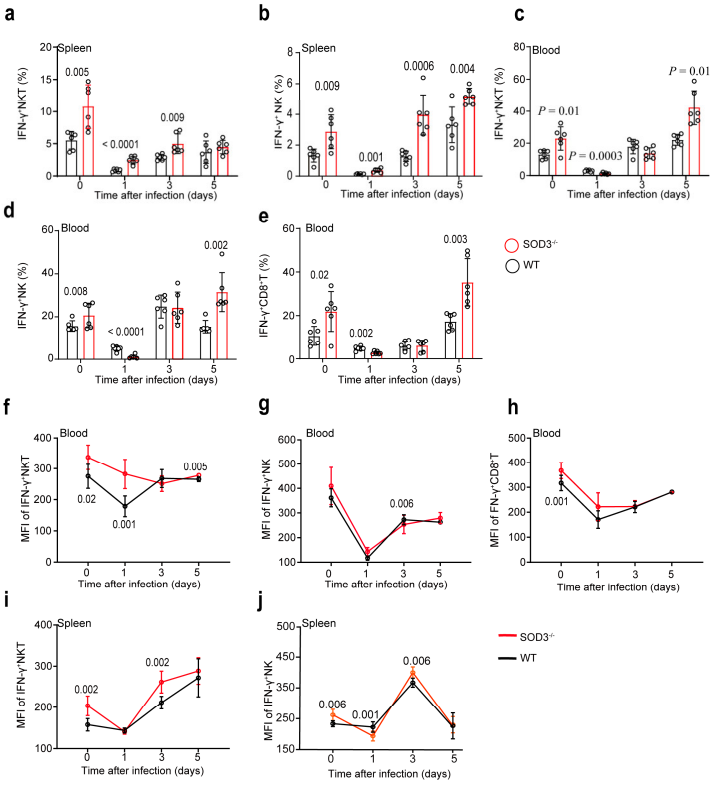



**Supplementary Figure 1. Impact of SOD3 knockout on parasite infection. (a** and **b)** SOD3 expression was determined in WT mice at different time points post-infection with *P. y. yoelii* YM. Two other independent repeats of Figure 1a are shown. **(c)** SOD3 levels in sera were elevated after infection with *P. y. yoelii* at different time points post-infection, with peak effects observed at 3 days and 7 days post-infection. **(d)** Serum SOD3 expression was elevated after infection with *T. b. brucei*, with peaks observed at 3 days post-infection, respectively. To test the levels of SOD3 in WT mice infected with *T. b. brucei*, serum was collected from mice on days 1, 3, 5, 6 and 7 post-challenge *T. b. brucei*. Average survival time of WT mice was 8 days from the time of challenge with *T. b. brucei*. **(e)** Serum SOD3 expression was elevated after infection with *T. gondii* RH, with peaks observed at one day post-infection, respectively. To test the levels of SOD3 in WT mice,

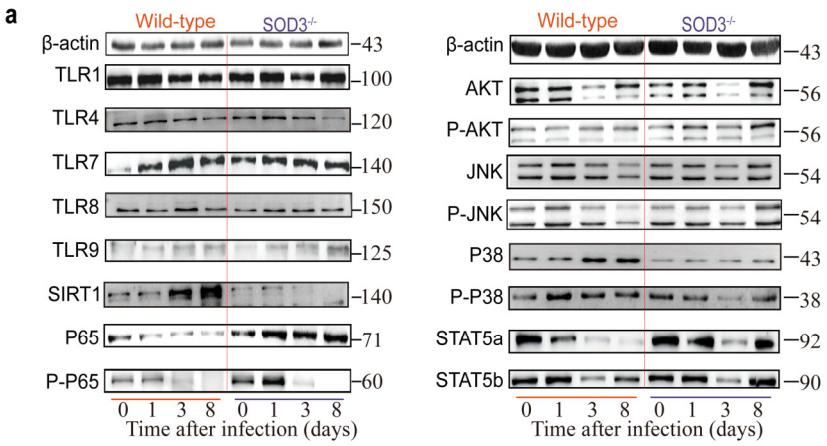
serum was collected from mice on alternate days from day 1 to 5 post-infection of *T. gondii* RH. Average survival time of WT mice was 7 days from the time of challenge with *T. gondii* RH. Due to the death of these mice after *T. gondii* RH infection, mouse serum samples could not be collected at days 7 post -infection. **(f** and **g)** Serum SOD3 levels were quantified using an Orbitrap Exploris 480 mass spectrometer for both $SOD3^{-/-}$ mice and WT mice after *P. berghei* ANKA infection. $SOD3^{-/-}$ mice completely lack SOD3 expression. Source data are provided as a Source Data file.



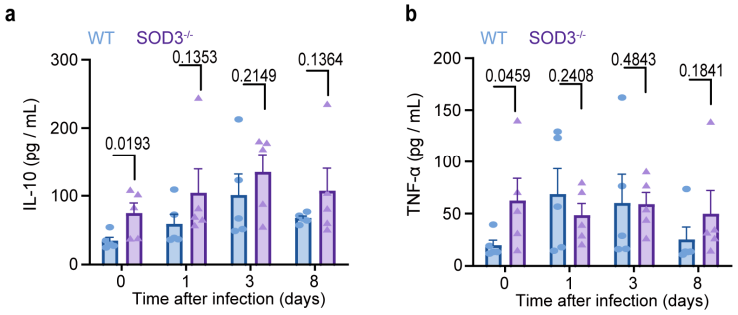

**Supplementary Figure 2. Brain damage and inflammation were reduced in infected SOD3$^{-/-}$ mice. (a)** Representative images of brain tissues from healthy and *P. berghei* ANKA-infected mice immunohistochemically stained for SOD3. **(b)** Representative H&E staining of the cerebral cortex at 63× magnification. Scale bars = 50 µm. **(c)** Statistical evaluation of microvessel density (MVD). An increase in MVD was observed in WT mice after *P. berghei* ANKA infection. Means ± SEMs, unpaired *t* test. **(d)** Schematic representation of *P. berghei* ANKA isolation from both SOD3$^{-/-}$ mice and WT mice. The mice illustration was created with http://BioRender.com (publishing license: XZ26UIL8LK). **(e)** *P. berghei* isolated from SOD3$^{-/-}$ mice and WT mice displayed similar infectivity in naïve micen = 5. Kaplan–Meier survival curves were calculated using the survival time for each mouse in all groups, and significance was determined by the log-rank test. Source data are provided as a Source Data file.



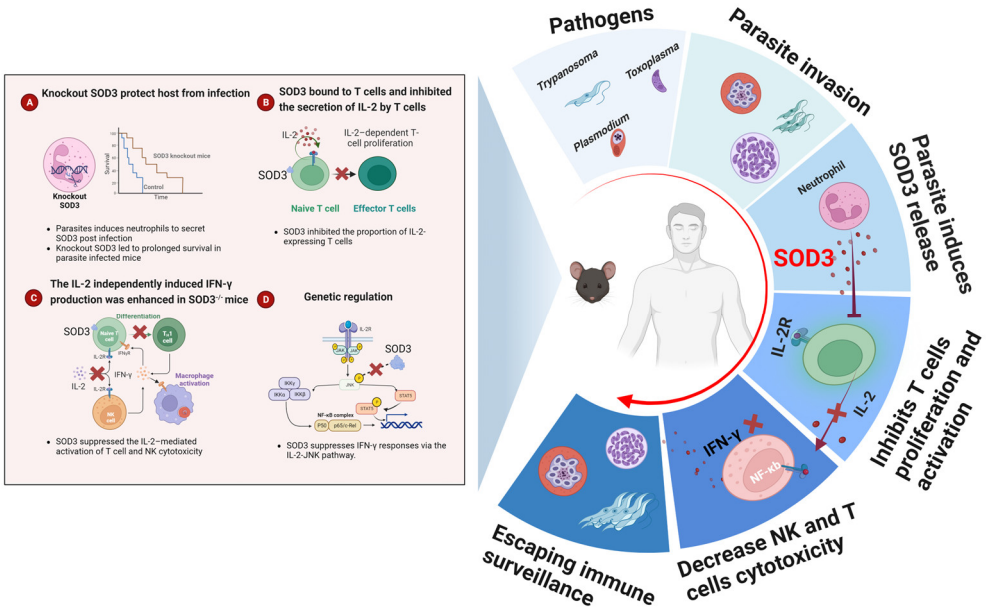

**Supplementary Figure 3. The proportion of IFN-γ-producing T cells significantly increased in SOD3$^{-/-}$ mice after parasite infection. (a** and **b)** The proportions of IFN-γ$^{+}$ NKT cells and IFN-γ$^{+}$ NK cells were quantified in both WT and SOD3$^{-/-}$ mice (n = 6) at different time points after infection with *P. berghei*. **(c-e)**, Quantification of the frequencies of splenic and blood IFN-γ$^{+}$ NKT cells, IFN-γ$^{+}$ NK cells and IFN-γ$^{+}$ CD8$^{+}$ T cells in both WT and SOD3$^{-/-}$ mice (n = 6) at different time points after infection with *P. berghei* is shown. **(f-**H**)** The relative mean fluorescence intensity (MFI) of IFN-γ in blood NKT cells, NK cells and NK cells was determined. **(i** and **j)** The MFI of IFN-γ in splenic NKT cells and NK cells is also shown. Student's *t* test was used to test for significant differences between two groups. Source data are provided as a Source Data file.



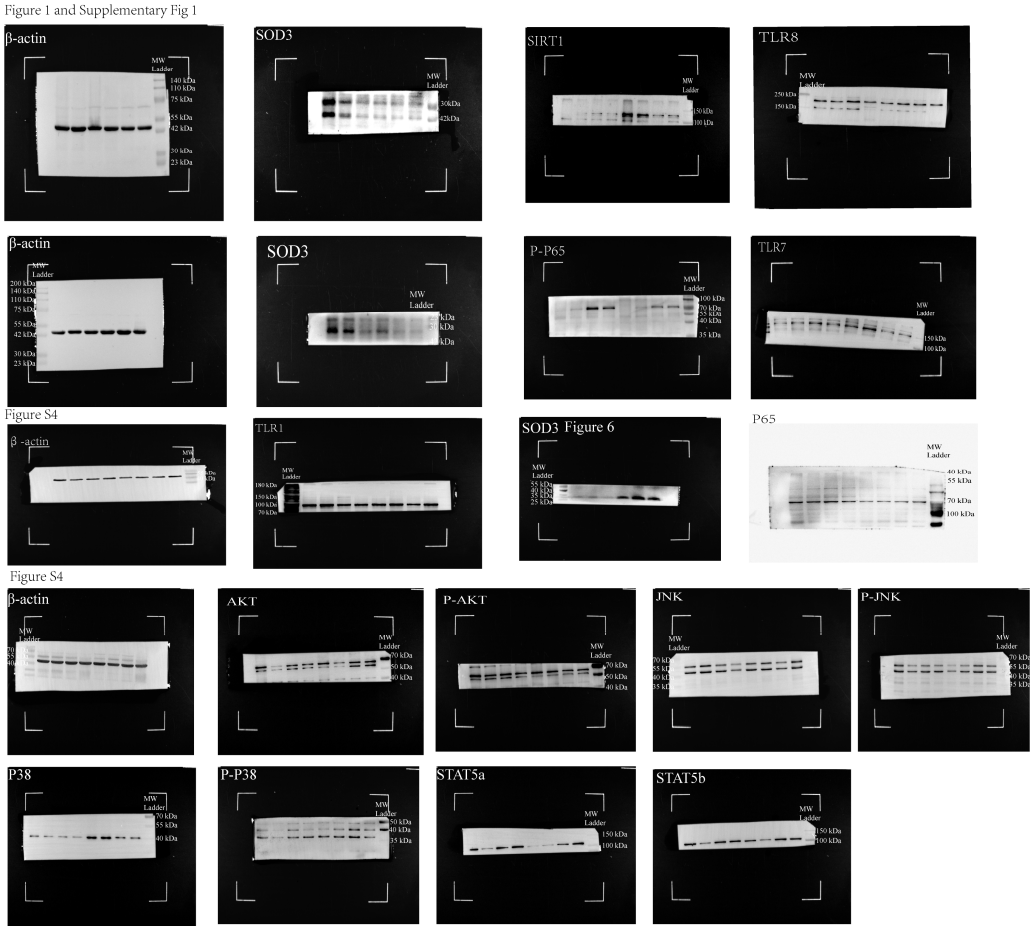

**Supplementary Figure 4. IFN-γ signaling-related proteins were quantified by Western blotting in spleen at different time points post infection**. **(a)** Representative immunoblots using antibodies against β-actin, phospho-AKT, AKT, phospho-JNK, JNK, phospho-p38, p38, TLR1, TLR4, TLR7, TLR8, TLR9, SIRT1, P65, P-P65, STAT5a and STAT5b are shown.



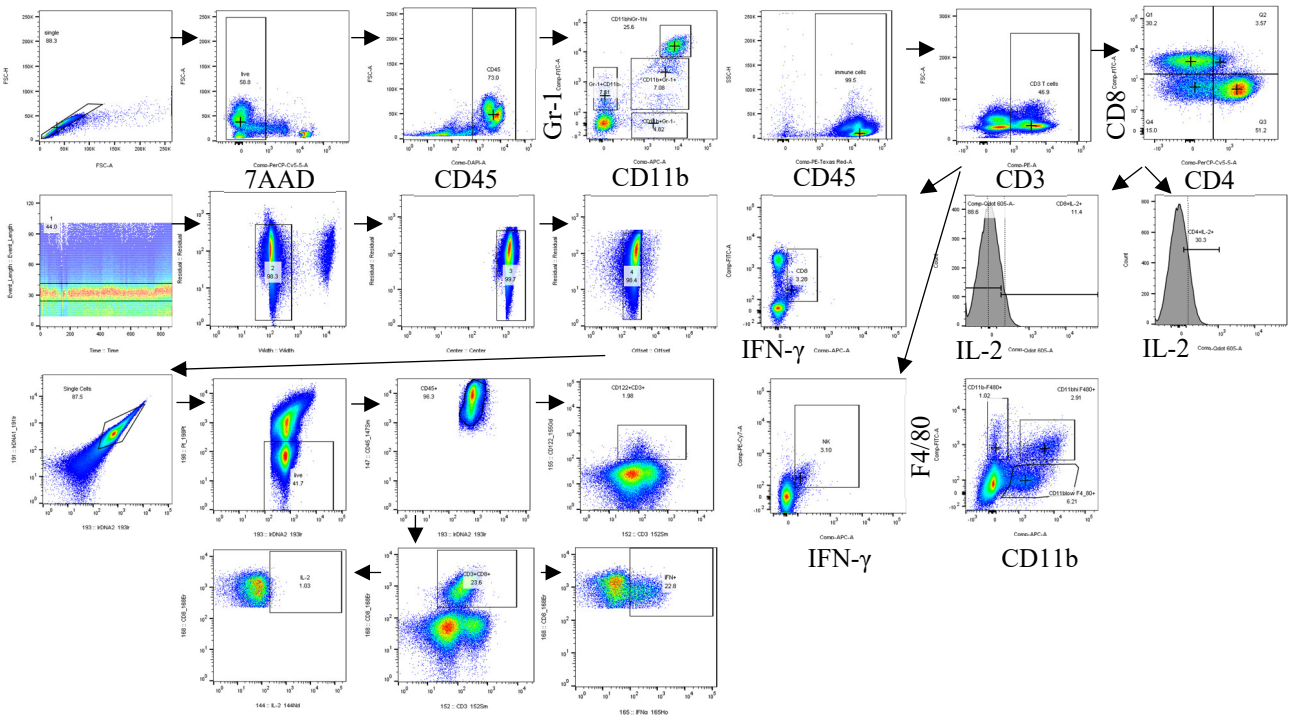

**Supplementary Figure 5. SOD3 had minimal effect on the production of IL-10 and TNF-α in *P. berghei*-infected mice. (a and b)** The levels of IL-10 and TNF-α in the sera were detected in both WT and SOD3$^{-/-}$ mice at different time points after *P. berghei* infection.




**Supplementary Figure 6. SOD3 is a critical immune factor during parasite infection in both humans and mice.** SOD3 secreted by neutrophils efficiently suppressed IL-2 production by T cells and consequently reduced the recruitment and responses of IFN-$\gamma^+$ T cells and NK cells to invading parasites. The illustration was created with http://BioRender.com (publishing license: UW26UICE18).




**Supplementary Figure 7. Uncropped Western blots.**




**Supplementary Figure 8. Gating strategy**

**Supplemental References**


1. Hu, Lili et al. “The dynamic uptake and release of SOD3 from intracellular stores in macrophages modulates the inflammatory response.” *Redox biol* **26**, 101268 (2019).
2. Leipold MD, Newell EW & Maecker HT Multiparameter phenotyping of human PBMCs using mass cytometry. *Methods Mol. Biol* **1343**, 81–95 (2015)