**Supplemental Information**

# STAT6/Arg1 Promotes Microglia/Macrophage Efferocytosis and Inflammation Resolution in Stroke Mice

Wei Cai[1,*], Xuejiao Dai[1,*], Jie Chen[1,2], Jingyan Zhao[1], Mingyue Xu[1], Lili Zhang[1], Boyu Yang[1], Wenting Zhang[1], Marcelo Rocha[1], Toshimasa Nakao[3], Julia Kofler[4], Yejie Shi[1,2], R. Anne. Stetler[1], Xiaoming Hu[1,2,#], Jun Chen[1,2,#]

[1]Pittsburgh Institute of Brain Disorders and Recovery, and Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[2]Geriatric Research, Education and Clinical Center, Veterans Affairs Pittsburgh Health Care System, Pittsburgh, PA, USA

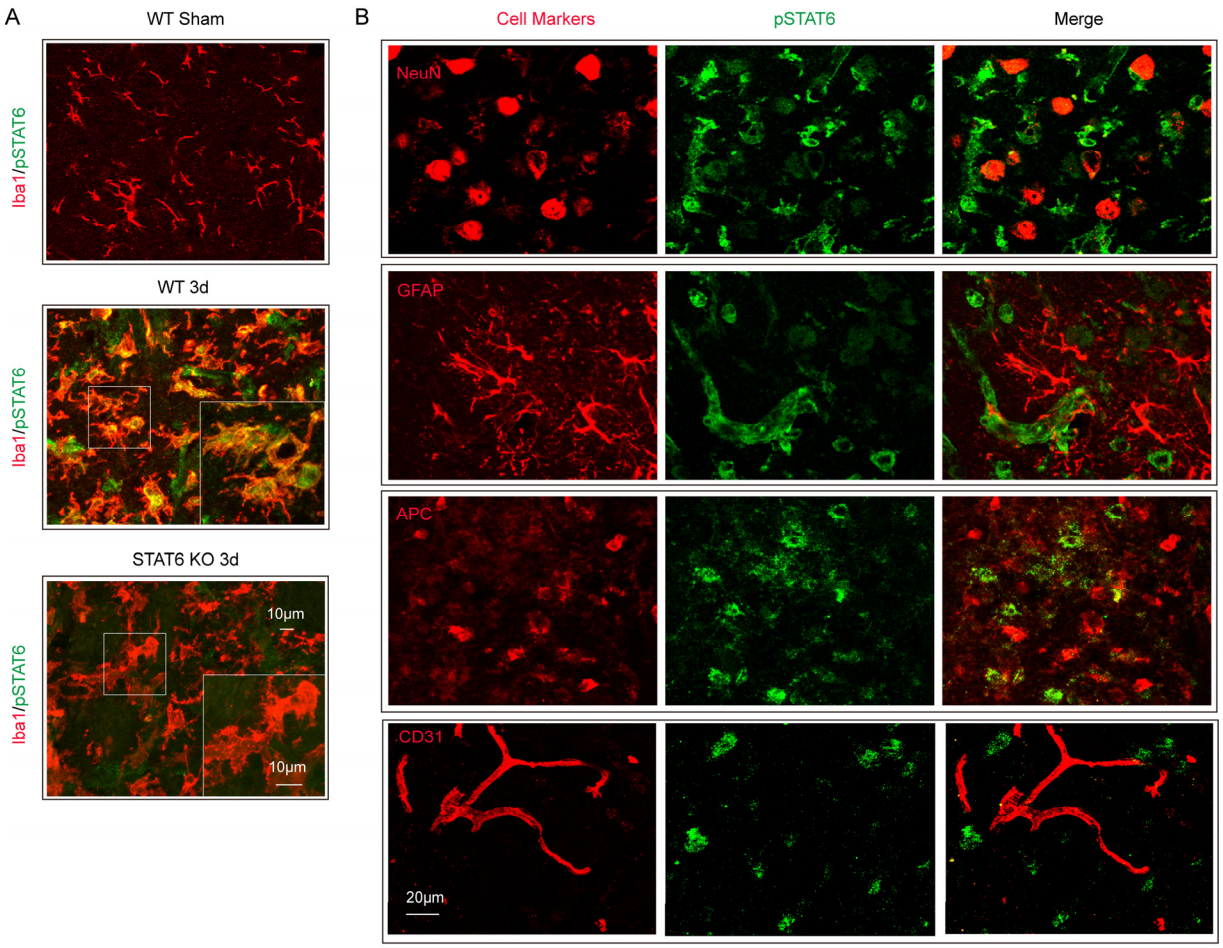
[3]T.E. Starzl Transplantation Institute and Department of Immunology, University of Pittsburgh, Pittsburgh, PA, USA

[4]Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

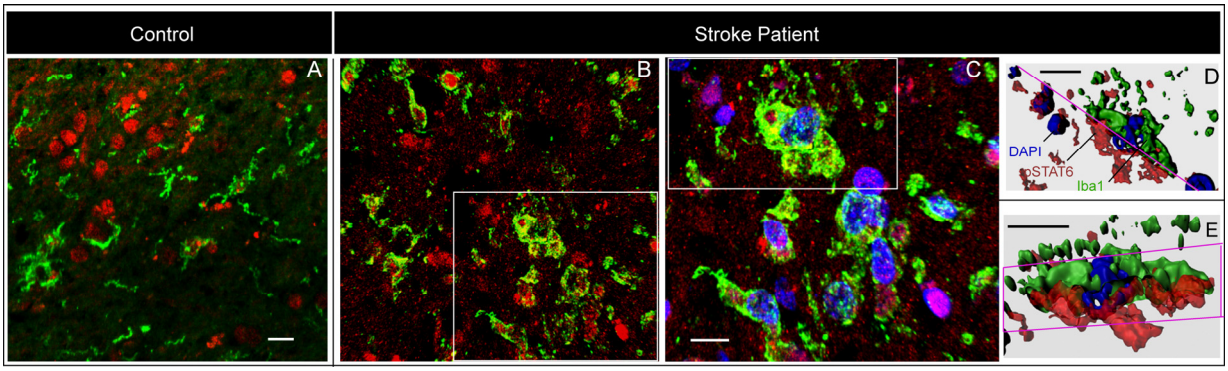
* Cai W and Dai X contribute equally to this manuscript.

#Corresponding authors:

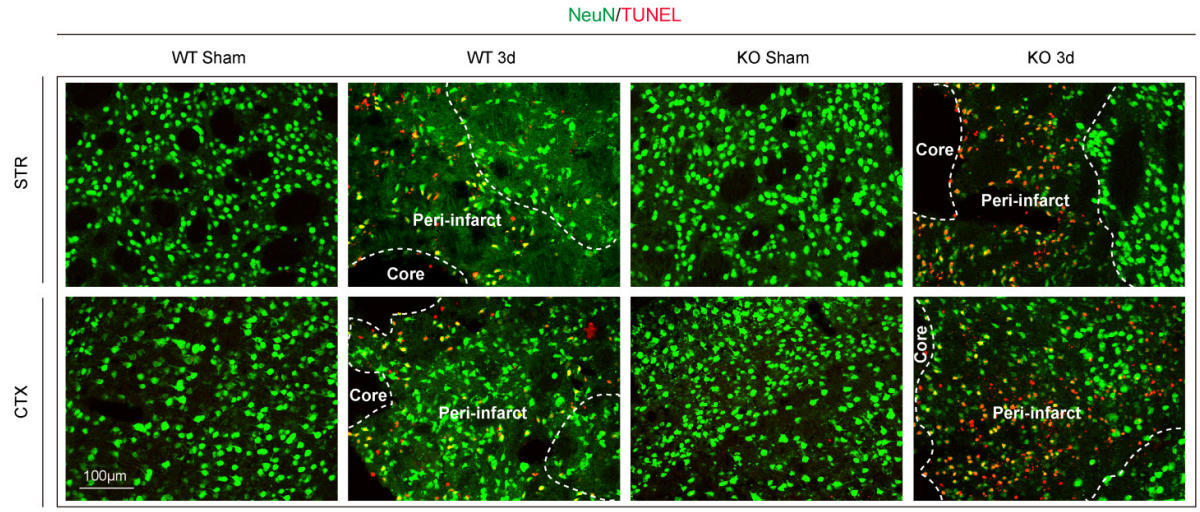
Dr. Jun Chen, E-mail: chenj2@upmc.edu or Dr. Xiaoming Hu, E-mail: hux2@upmc.edu



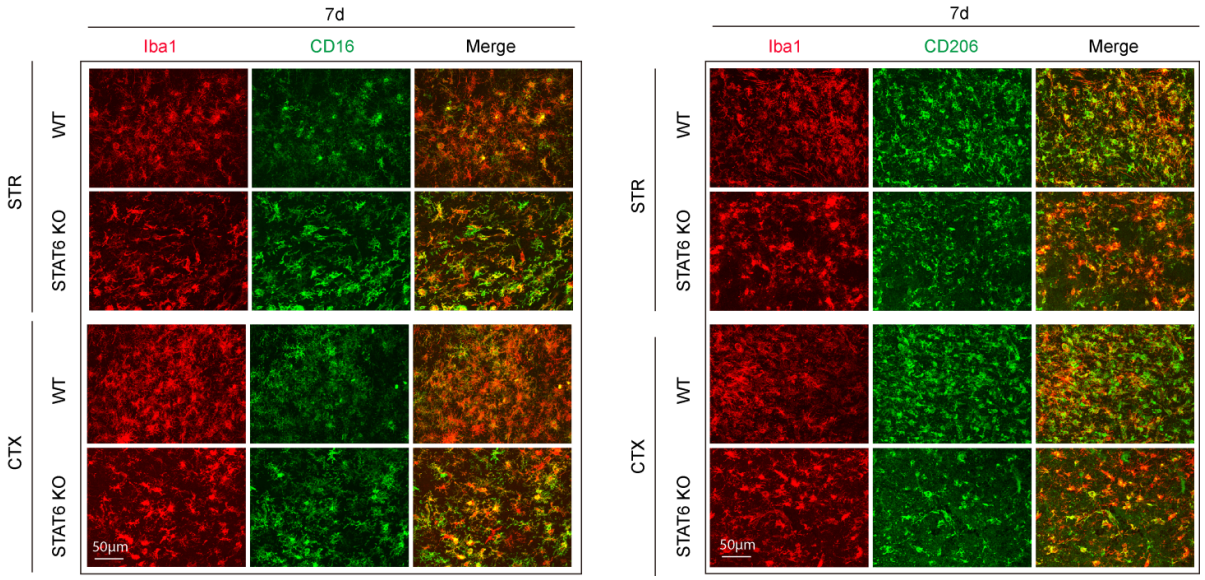

**Supplemental figure 1. Specificity of pSTAT6 antibody and STAT6 activation observed in brain cells. (A)** Characterization of the specificity of the pSTAT6 antibody (Y641). Representative images of cells double stained with a microglia/macrophage marker (Iba1, red) and pSTAT6 (green) in wild type (WT) sham, and WT or STAT6 knockout (KO) brains 3d after tMCAO. No pSTAT6 staining was detected in WT sham or STAT6 KO brain after tMCAO. **(B)** pSTAT6 (green) was not detected in neurons ($NeuN^+$, red), astrocytes ($GFAP^+$, red), oligodendrocytes ($APC^+$, red) or endothelial cells ($CD31^+$, red) in WT brain 3d after tMCAO.



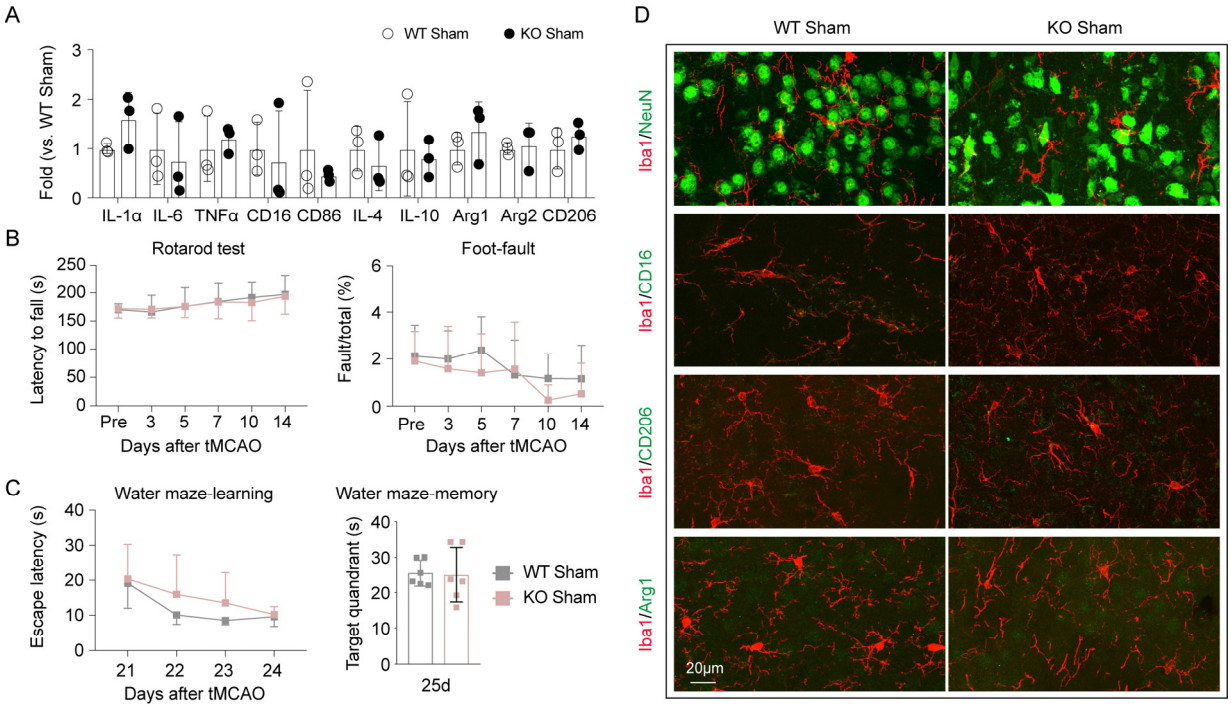

**Supplemental figure 2. STAT6 is activated in microglia/macrophages in stroke patients.** Representative images of STAT6 activation (pSTAT6, red) in Iba1$^+$ (green) microglia/macrophages in human postmortem brains of an individual with no history of stroke **(A)** and in the acute infarct area of a stroke patient **(B-C)**. The image is a representative of two stroke patients. White rectangles indicate the enlarged figure to the right. **(D-E)** Three-dimensional reconstruction of pSTAT6$^+$Iba1$^+$ cells in the ischemic brain. (D) The clipping plane (purple) is shown at the level of the intra-nucleus. The Iba1 staining left to the clipping plane was removed to expose the pSTAT6 staining (red) within and surrounding the nucleus(blue). (E) Front view of a three-dimensional reconstruction of a pSTAT6$^+$Iba1$^+$ microglia/macrophage in the ischemic brain. Blue shows the nuclear (DAPI) staining. Scale bar: 15 μm.



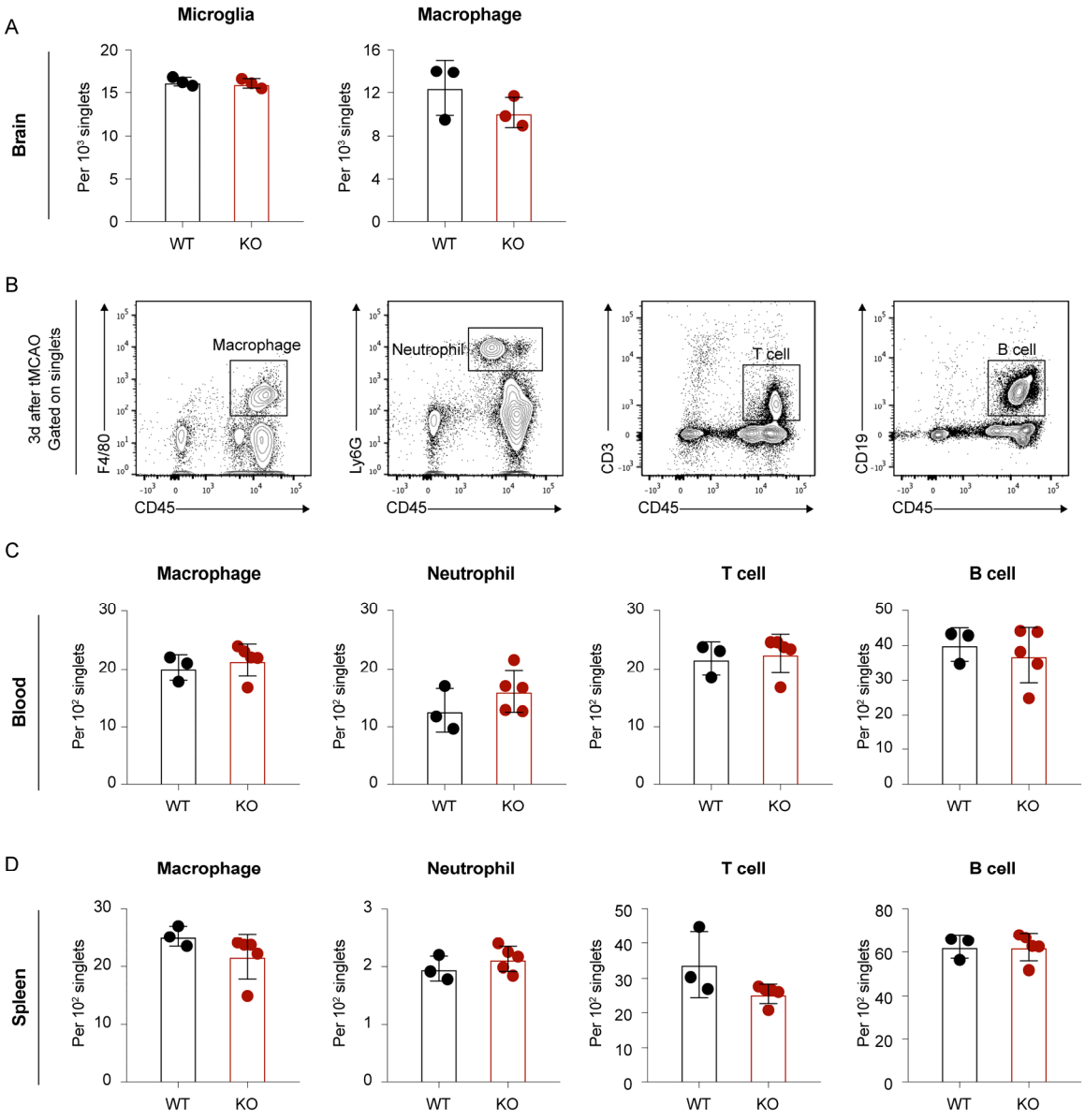

**Supplemental figure 3. STAT6 deficiency exacerbates neuronal death after tMCAO.** Neuronal death increased in STAT6 KO brains 3d after tMCAO. Representative images show TUNEL (red) co-labeling with the neuron marker NeuN (green) in ischemic areas in the striatum (STR) and cortex (CTX).




**Supplemental figure 4. STAT6 deficiency enhances pro-inflammatory phenotype of microglia/macrophages 7d after ischemic stroke.** Brains were collected from WT and STAT6 KO mice 7d after 60 min tMCAO. Representative images show double staining of microglia/macrophage marker Iba1 (red) and pro-inflammatory phenotype marker CD16 (green, left panel) or anti-inflammatory phenotype marker CD206 (green, right panel).

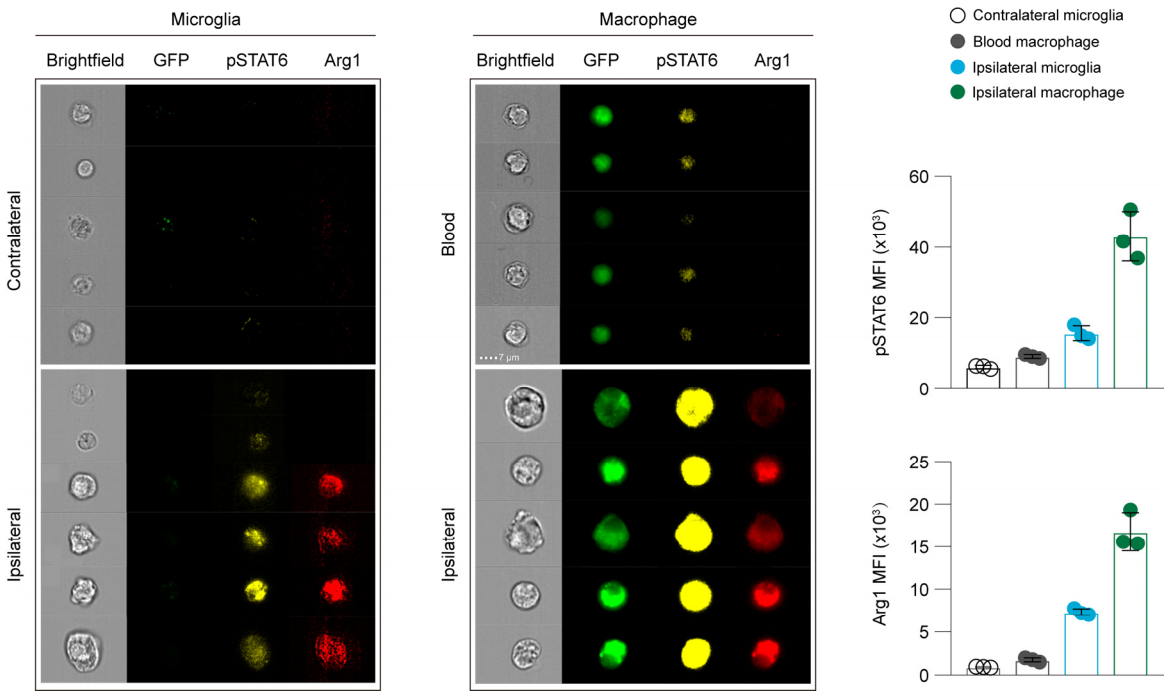



**Supplemental figure 5. Comparison between WT and STAT6 KO mice after sham operation. (A)** RT-PCR analysis of pro-inflammatory and anti-inflammatory markers in the brains from WT and STAT6 KO mice 3d after sham operation. *n* = 3 mice per group. Student's *t* test**. (B)** Sensorimotor functions were evaluated with rotarod test and foot-fault test 3-14d after sham operation. **(C)** Cognitive function was assessed with Morris water maze at 21-25d after sham operation. Latency to find the hidden platform in the cued test (spatial learning) and time spent in the target quadrant in the probe test (memory consolidation) were recorded. *n* = 6 mice per group, two-way ANOVA. **(D)** Representative images showing that Iba1$^+$ cells (red) did not engulf neurons (green) in WT or STAT6 KO sham mice. The expression levels of CD16 (green), CD206 (green) or Arg1 (green) in Iba1$^+$ microglia/macrophages (red) were barely seen in WT and STAT6 KO sham mice. *n* = 6 mice per group, two-way ANOVA or Student's *t* test.

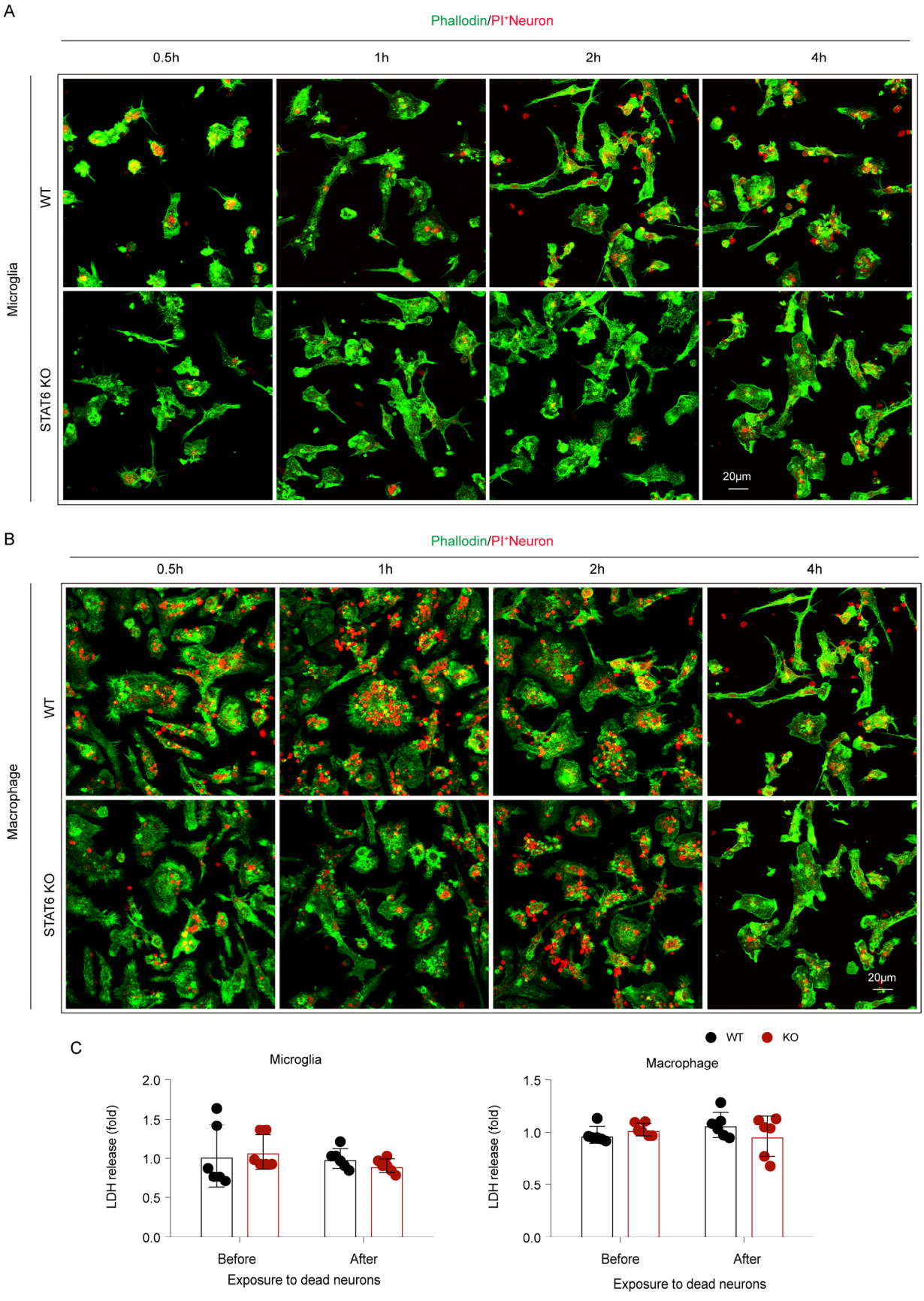



**Supplemental figure 6. STAT6 deficiency does not change the numbers of microglia/macrophages in the brain or the numbers of peripheral immune cells 3d tMCAO.** Brains, blood and spleens were collected from WT and STAT6 KO mice 3d after 60 min tMCAO. **(A)** The numbers of $CD45^{intermediate}CD11b^{+}$ microglia and $CD45^{high}CD11b^{+}$ macrophages in the ischemic brain were quantified. N=3 per group, Student's *t* test. **(B)** Gating strategy for $CD45^{+}F4/80^{+}$ monocytes/ macrophages, $CD45^{+}Ly6G^{+}$ neutrophils,

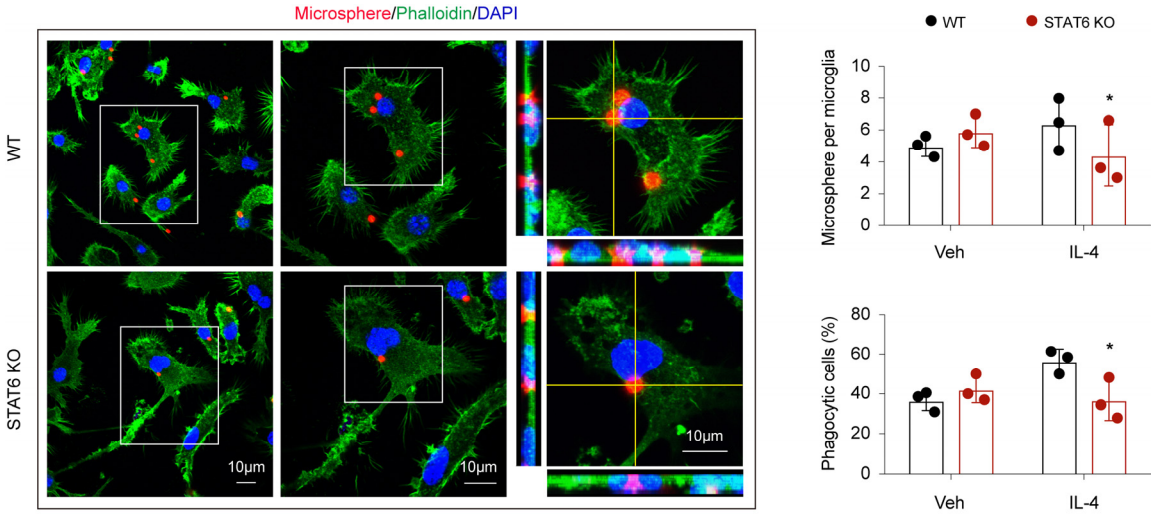
$CD45^+CD3^+$ T cells, or $CD45^+CD19^+$ B cells. **(C-D)** The numbers of monocytes/ macrophages, neutrophils, T cells, and B cells in the blood (C) and spleen (D) were quantified. N=3-5 per group, Student's *t* test.



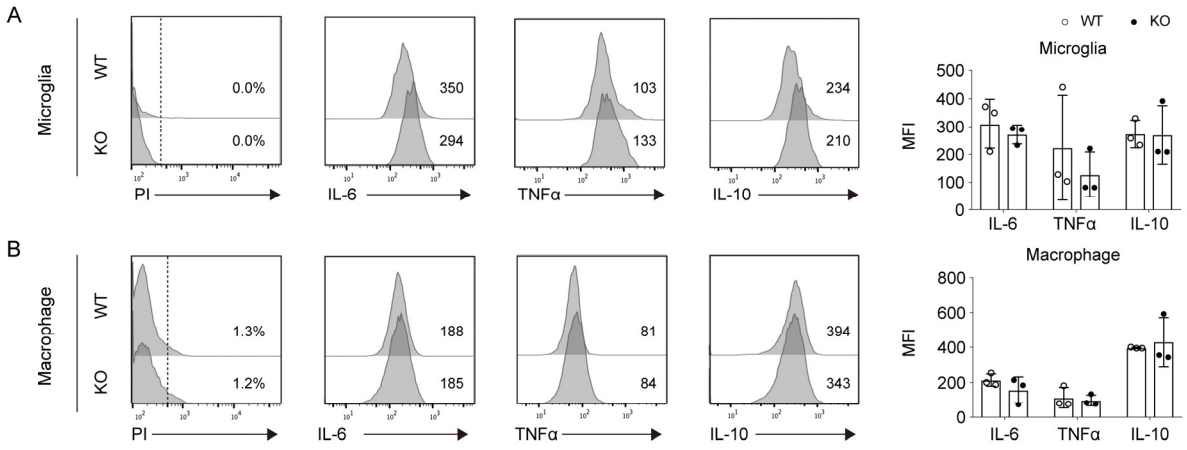

**Supplemental figure 7. pSTAT6 and Arg1 expression in microglia and macrophages from WT/CX3CR1-GFP chimeric mice.** WT/CX3CR1-GFP chimeric mice were constructed as illustrated in Figure 6A. Brains and blood were collected 3d after tMCAO. ImageStream was used to analyze the expression of pSTAT6 and Arg1 in macrophages ($CD45^+CD11b^+GFP^+$) and $CD45^+CD11b^+GFP^-$ cells. Microglia from contralateral brain and blood macrophages showed low levels of pSTAT6 and Arg1 expression. The expression of pSTAT6 and Arg1 increased in both $CD45^+CD11b^+GFP^-$ cells and macrophages in the ipsilateral hemisphere. Data represents three independent experiments.


A
Phallodin/PI+Neuron
0.5h
1h
2h
4h
Microglia
WT
STAT6 KO
20μm
B
Phallodin/PI+Neuron
0.5h
1h
2h
4h
Macrophage
WT
STAT6 KO
20μm
C
WT
KO
Microglia
LDH release (fold)
2.0
1.5
1.0
0.5
0.0
Before
After
Exposure to dead neurons
Macrophage
LDH release (fold)
1.5
1.0
0.5
0.0
Before
After
Exposure to dead neurons

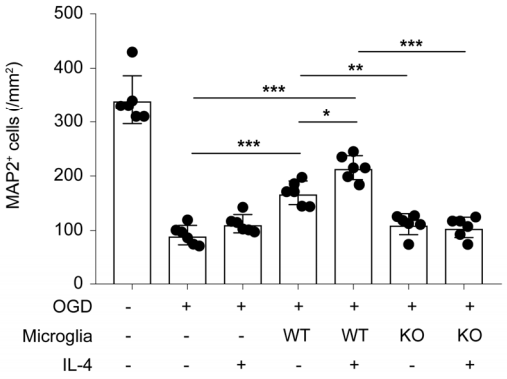
**Supplemental figure 8. STAT6 signaling is essential for efferocytosis of dead neurons in both microglia and macrophages. (A-B)** Representative images show efferocytosis of PI-labeled dead neurons (red) by phalloidin-labeled WT and STAT6 KO microglia (green) (A) or macrophage (green) (B) at indicated time points. **(C)** Cell death of WT and STAT6 KO microglia or macrophages was quantified by the LDH assay. No change in LDH production was detected in WT or STAT6 KO microglia and macrophages before and 6h after co-culturing with PI-labeled neurons. Data represents six independent experiments in duplicate. Student's *t* test.



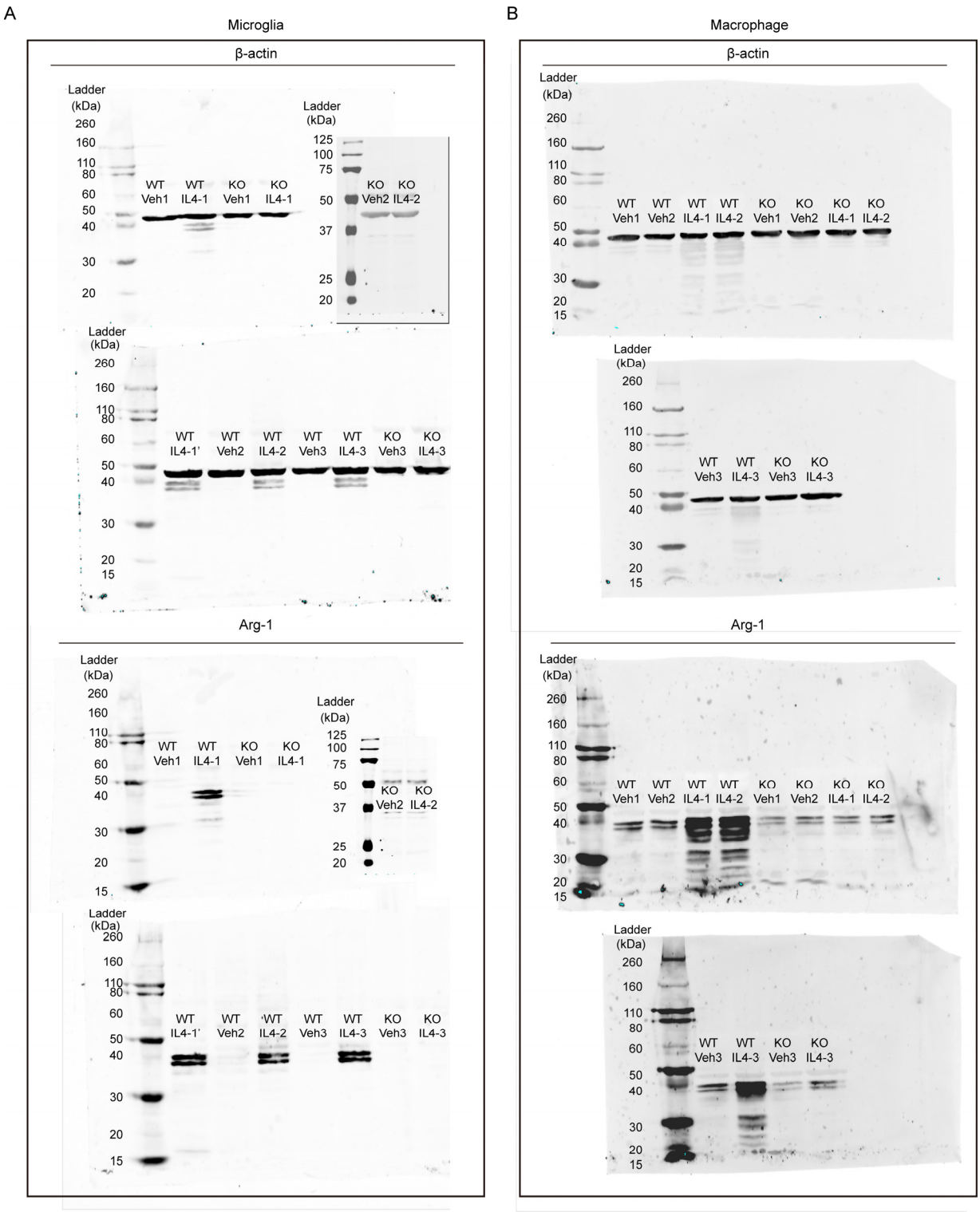

**Supplemental figure 9. STAT6 signaling is essential for microglial phagocytosis of latex beads under IL-4 stimulation.** WT and STAT6 KO microglia with or without IL-4 pre-treatment (20 ng/ml, 24h) were incubated with fluorescent beads for 4h. Left: Representative images show phagocytosis of fluorescent beads (red) by IL-4 treated WT and STAT6 KO microglia (green) after 4h incubation. Right: Microglia phagocytic capacity was quantified as number of microspheres per microglia and percentages of phagocytic cells. Data represents three independent experiments in duplicate. *$p \leq 0.05$ STAT6 KO *vs* WT, Student's *t* test.



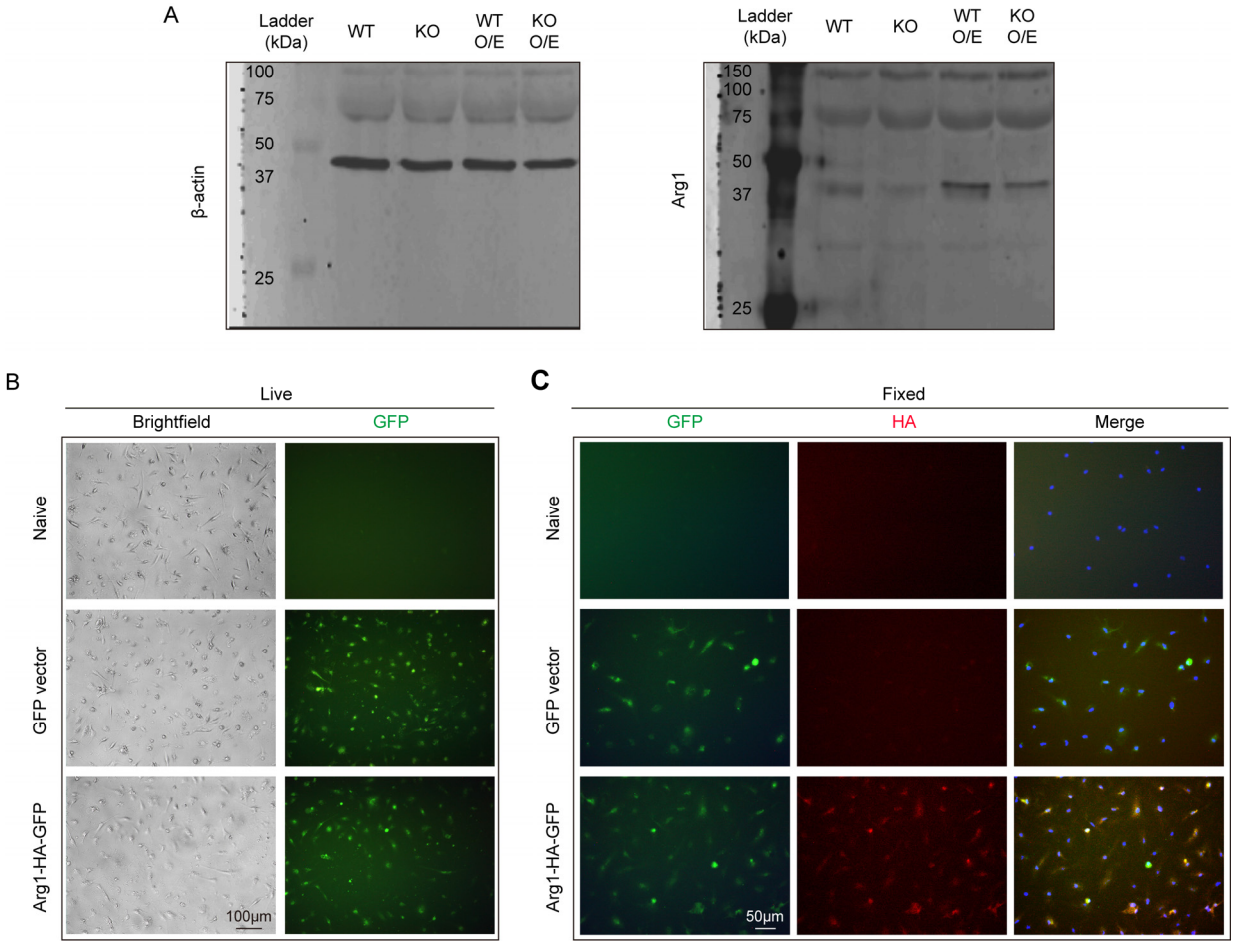

**Supplemental figure 10. STAT6 KO microglia/macrophages express comparable basal levels of cytokine expression *versus* WT microglia/macrophages without stimulation.** Flow cytometry analysis of protein expression of pro-inflammatory (IL-6 and TNFα) and anti-inflammatory (IL-10) factors in unstimulated microglia **(A)** and macrophages **(B)** at basal level. Data represents three independent experiments in duplicate. Student's *t* test.




**Supplemental figure 11. STAT6 is essential for the neuroprotective effects of IL-4-treated microglia.** Primary neurons were subjected to 90-min OGD and then co-cultured with WT or STAT6 KO microglia with or without IL-4 pre-treatment (20 ng/ml for 24h). Neuronal survival was measured 24h after co-culture by MAP2 immunostaining. Data represents six independent experiments in duplicate. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, one-way ANOVA.




**Supplemental figure 12. Whole gel scanning for western blot analysis of Arg1 and β-actin in microglia (A) and macrophages (B) in Figure 10D.**




**Supplemental figure 13. Arg1 overexpression in WT and STAT6 KO microglia. (A)** Representative western blot images of Arg1 protein overexpression in WT and STAT6 KO microglia infected with Lenti-Arg1-HA or Lenti vector. **(B)** Representative images of live microglia infected with Lenti-Arg1-HA-GFP or Lenti-GFP viral vector. **(C)** Representative images of HA staining (red) in microglia infected with Lenti-Arg1-HA-GFP or Lenti-GFP viral vector. Cells were counterstained with DAPI (blue) for nuclear labeling.

**Supplemental Table 1. Primers for Real-Time Polymerase Chain Reaction**

| | Forward | Reverse |
|---|---|---|
| IL-1α | AAGACAAGCCTGTGTTGCTGAAGG | TCCCAGAAGAAAATGAGGTCGGTC |
| IL-6 | TCCTACCCCAACTTCCAATGCTC | TTGGATGGTCTTGGTCCTTAGCC |
| TNFα | AGAAGTTCCCAAATGGCCTC | CCACTTGGTGGTTTGCTACG |
| CD16 | TTTGGACACCCAGATGTTTCAG | GTCTTCCTTGAGCACCTGGATC |
| CD86 | GACCGTTGTGTGTGTTCTGG | GATGAGCAGCATCACAAGGA |
| IL-4 | GGTCTCAACCCCCAGCTAG | GCCGATGATCTCTCTCAAGTGAT |
| IL-10 | CCAAGCCTTATCGGAAATGA | TTTTCACAGGGGAGAAATCG |
| Arg1 | TCACCTGAGCTTTGATGTCG | CTGAAAGGAGCCCTGTCTTG |
| Arg2 | GGATCCAGAAGGTGATGGAA | AGAGCTGACAGCAACCCTGT |
| CD206 | CAAGGAAGGTTGGCATTTGT | CCTTTCAGTCCTTTGCAAGC |
| GAPDH | AAGATGGTGAAGGTCGGTG | GTTGATGGCAACAATGTCCAC |