SUPPLEMENTAL MATERIAL

Detailed Methods:

Animals: Male (C57BL/6J, 27 to 30g) (Jackson Laboratory, Bar Harbor, ME) mice were randomly housed (simple randomization with computer-generated random numbers), for 3 weeks, 4 animals per cage (environmental enrichment included), before surgery and 3 days after surgery in 2 different rooms, one room had standard light schedule 12 hours of light (on) during the day (7am-7pm) and 12 hours of dark (off) at night (7pm-7am) and another room had switched cycle 12 hours of dark (off) during the day (7am-7pm) and 12 hours of light (on) at night (7pm-7am). Experiments were performed under institutionally approved protocol in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and following Animals in Research: Reporting In vivo Experiments (ARRIVE) guidelines. JHP performed the randomization and provided assistance with data processing and analyses.

To avoid confounding factors our study design controlled for many variables including mouse strain, age, sex and weight, husbandry techniques, animal environment, randomization method, and blinding to treatment.

A total of 41 mice were used, 26 mice for ZT1-3 (7 cages) and 15 for active ZT13-15 (4 cages).

Focal Cerebral Ischemia

Mice were anesthetized with isoflurane (1.5%) in 30%/70% oxygen/nitrous oxide. Transient focal ischemia was induced introducing a 6-0 surgical monofilament nylon suture (Doccol) from the external carotid artery into the internal carotid artery and advancing it to the branching point of the MCA. Adequate ischemia was confirmed by laser Doppler flowmetry (LDF) (Perimed, North Royalton, OH, U.S.A.). After occluding the MCA for 60 mins the monofilament suture was gently withdrawn in order to restore blood flow. Rectal temperature was maintained at 37°C with a thermostat-controlled heating pad. Pain management after surgery was performed by Buprenorphine 0.05 mg/kg IP q12h for the first 3 days post-op. Mice that showed unacceptable signs were euthanized with deep isofluorane anesthesia followed by decapitation. Signs of unacceptable stress include inability of eat and drink, ruffled fur/inadequate grooming, excessive lack of spontaneous activity, excessive weight loss of more than 20% (mice will be weighed every day).

All surgeries were performed during the inactive (ZT1-3) or active (ZT13-15) phases. All procedures and measurements were performed in a blinded and randomized fashion, the operator did not know from which room the animals were coming from.

The exclusion criteria for tMCAO were based on LDF (animals that did not have a significant reduction to less than 30% baseline during MCAO or LDF was not recovered to 100% during reperfusion were excluded), none; surgery failure (i.e. bleeding during surgery), none; and animals euthanized for poor health conditions when suggested by veterinary, 4 mice ZT 1-3 were euthanized and none mouse ZT 13-15.

Mortality was: 3 ZT 1-3 out of 23 mice died and 1 ZT 13-15 mouse out of 15 died.

Evaluation of infarct volume

The animals underwent transcardial perfusion with saline at 72h after ischemia. Brains were quickly removed and cut in 8 coronal sections using a brain matrix. The sections were then incubated in 2% TTC in saline for 10 min at room temperature. Infarction

volumes were quantified using the "indirect" morphometric method with Image J software. All analyses were blinded.

Flow Cytometry

Single-cell suspensions were prepared from blood, spleen or brain tissues of normal or MCAO mice at day 3. Then these cells were stained with fluorochrome conjugated antibodies. All antibodies were purchased from BD Bioscience (Franklin lakes, NJ, USA) or Biolegend (San Diego, CA, USA). Antibodies were directly labeled with one of the following fluorescent tags: fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), PerCP-Cy5.5, PE-Cy7 or pacific blue. The following antibody to mouse antigens were used: CD3, CD4, CD8, CD19, CD45, CD11b, F4/80, Ly6G. Antibodies staining were performed according to their instructions. Cell phenotypes were performed on a FACS FORTESSA flow cytometer (BD Bioscience, Franklin lakes, NJ, USA). Data were analyzed with Flow Jo software version 7.6.1 (Flow J, LLC, Ashland, OR, USA).

Statistical analysis

Results were expressed as mean ± SD. All experiments were performed with randomization of group assignment. Sequentially numbered method was used to ensuring allocation concealment. Animal sample size was predetermine using the software available online: https://www.danielsoper.com/statcalc/calculator.aspx?id=47. The calculation was based on Cohen's d value where SD and average were estimated from our historical and preliminary data. Drawing on the collaborative expertise of the MGH stroke program, my lab could begin to estimate power$\alpha=0.05$, $1 - \beta=0.8$. Statistical analysis was performed using Graphpad prism 7.6.1 (Graphpad Software Inc., San Diego). Model assumptions were checked using Two-tailed unpaired t test was used for comparison between two groups, 2-Way Anova was used to determine the effect of two variables.

Supplementary Figure Legends

Figure S1. Effects of diurnal rhythm in blood in normal mice. a. Numbers of total T cells (CD3+), CD4+ T cells (CD3+CD4+) and CD8+ T cells (CD3+CD8+). **b.** Counts of CD19+ B cells (CD19+). **c.** NK cells (CD3-NK1.1+). **d.** Neutrophils (CD11bhighLy-6c+). **e.** Ly6c+ monocytes (CD11b+Ly-6c+) or Ly6c- monocytes (CD11b+Ly-6c-). (n=5-6). All values are mean±SD; comparisons via 2-tailed t-test.

Figure S2. Effects of diurnal rhythm in spleen in normal mice a. Spleen weight and total cell numbers. **b.** Numbers of total T cells (CD3+), CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+). **c.** Counts of B cells (CD19+). **d.** NK cells (CD3-NK1.1+) **e.** Neutrophils (CD11b+Ly-6G+) **f.** Macrophages (CD11b+ F4/80+). (n=5-6). All values are mean±SD; comparisons via 2-tailed t-test.

Figure S3. Numbers of CD86 and CD206 expressing macrophage in brain after MCAO (n=6). All values are mean±SD; comparisons via 2-tailed t-test.

Table S1. All mean±SD values

Figure S1

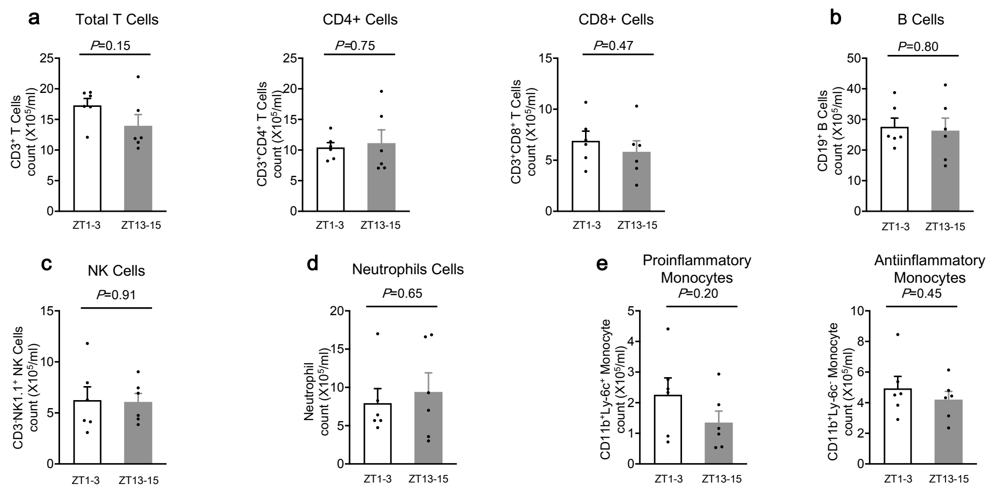

Figure S2

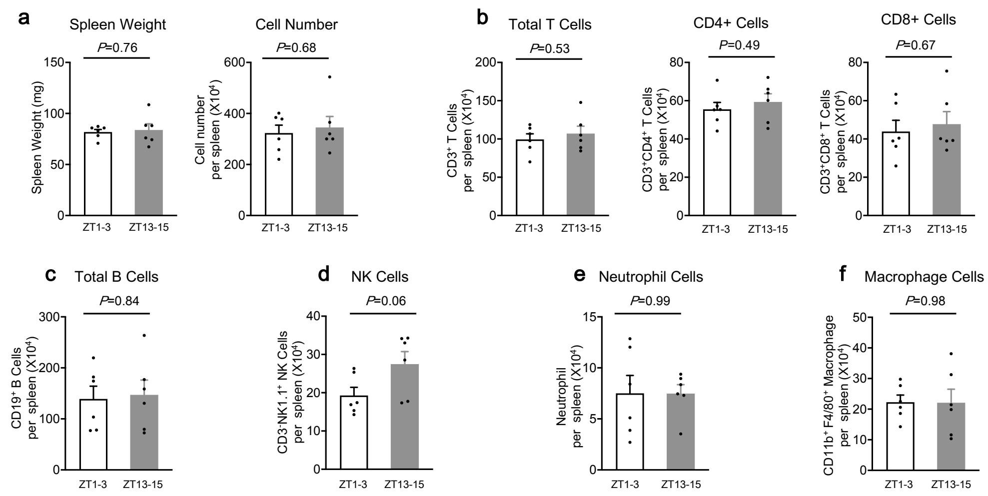

Figure S3

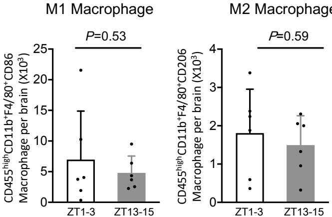

Table S1

Ischemic Volume	**ZT1-3 MCAO**	**ZT13-15 MCAO**
	60.78±7.98	38.86±5.34
Brain	**ZT1-3 MCAO**	**ZT13-15 MCAO**
macrophage	25.84±18.36	17.56±11.27
macrophage-CD86	6.99±3.22	4.79±1.12
macrophage-CD206	1.80±0.47	1.49±0.31
microglia	41.54±12.9	41.46±10.2
B Cell	1.63±0.42	1.66±0.43
NK	1.17±0.32	1.23±0.32
Neutrophil	33.87±3.17	31.73±6.83
T cell	5.94±1.51	2.84±0.16
T cell-CD69	0.91±0.29	0.29±0.07
Blood	**ZT1-3 MCAO**	**ZT13-15 MCAO**
B cell	28.79±10.12	12.18±3.19
NK	9.39±2.97	4.77±1.68
Neutrophil	21.08±6.14	12.37±2.77
Ly6c+ monocyte	1.56±0.35	0.91±0.21
Ly6c- monocyte	1.00±0.23	1.39±0.39
T cell	14.85±1.83	10.13±1.27
CD4 T cell	9.92±2.82	6.18±0.88
CD8 T cell	17.63±5.58	5.19±1.30
Spleen	**ZT1-3 MCAO**	**ZT13-15 MCAO**
spleen weight	41.8±2.59	54.75±4.31
cell number	145.92±17.87	218.1±84.45
B Cell	36.36±10.40	57.8±20.32
Neutrophil	6.42±1.40	5.68±1.00
macrophage	6.86±1.38	10.35±1.78
NK	4.58±0.95	8.00±1.05
T cell	22.92±2.69	41.67±8.97
CD4 T cell	17.23±4.08	21.31±5.37
CD8 T cell	8.27±1.67	17.69±4.43