Supplementary Information for

Double-negative T cells remarkably promote neuroinflammation after ischemic stroke

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SI Materials and Methods

Patients. The clinical study was approved by the ethics committee of Nanjing Drum Tower Hospital, Medical School of Nanjing University. Forty-seven stroke patients and thirty-three age- and gender-matched healthy controls were included in this study with patients' consents (Table S1 and Table S2). The inclusion criteria were as follows: (1) Acute ischemic stroke within 7 days as confirmed by CT scan or MRI and (2) National Institutes of Health stroke scale (NIHSS) score ≥4. The exclusion criteria were as follows: (1) Transient ischemic attacks, (2) Brain haemorrhages, (3) Cardiogenic cerebral embolism, (4) Brain trauma or neoplasms, (5) Acute infections, and (6) Autoimmune diseases. Blood samples were collected upon hospital admission and were used for flow cytometric analysis.

Reagents and antibodies. The antibodies to mouse CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD45 (30-F11), CD86 (GL1) and anti-Armenian Hamster IgG Secondary Antibody conjugated with R-phycoerythrin (PE) (12-4112-83) for FACS were purchased from eBioscience (Frankfurk, Germany). Anti-mouse CD206 antibody (MR6F3) for FACS was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies to human CD3 (555339), CD4 (555347), CD8 (555369), CBA kit (560485) and FasL neutralizing antibody (555022) were purchased from BD Bioscience (San Diego, CA, USA). The antibodies CD3 (ab16669), CD8 (ab17147), CD86 (ab119857), CD206 (ab8918), Iba-1 (ab5076) for immunofluorescence and secondary antibody against Armenian Hamster IgG (ab5745), PTPN2 (ab129070) for WB were purchased from Abcam (Cambridge, United Kingdom). CD4 (sc-13573) antibody for immunofluorescence and ethyl-3,4-dephostatin (PTPN2 inhibitor, sc-220886) were purchased from Santa Cruz Biotechnology (Wembley, Middlesex, UK). Primary antibody against TIAL1 (8509) for western blot was purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody against β-tubulin (AP0064) was from Bioworld Biotechnology (Minneapolis, MN, USA). Recombinant mouse IL-10 (210-10), TNF-α (315-01A) were purchased from Peprotech (Rocky Hill, NJ, USA). Lenalidomide (TNF-α inhibitor, S1029) was purchased from Selleck Chemicals (LLC, Houston, TX).

Human Post-mortem Brain Tissue. Brain sections from patients with ischemic stroke were obtained from the Sun Health Research Institute (Sun City, AZ, USA). Patients or their immediate family provided informed consent for brain donation as well as for the purpose of research analysis. 9 human samples were analyzed in this study. 6 cases were achieved from ischemic stroke patients who died within 3-7 days after onset (2 males and 4 females). The locations of stroke lesions were within the cortical areas supplied by the middle cerebral artery. Another 3 cases were obtained from control patients who died from non-neurological disease and had no history of neurological or neuropsychiatric diseases (2 males and 1 female). Brain tissues were collected within 4 h after death. All 9 cases do not have other disease conditions, such as autoimmune diseases, heart failure, acute myocardial infarction, hematological system diseases or infection before stroke. Stroke patients and controls showed no significant difference in terms of their mean age (stroke patients, 88±4 years of age; controls, 82±4 years of age, mean±s.e.m; $P>0.05$, Student's t test). Detailed characteristics of human post-mortem were provided in Supplementary Table S3.

Ischemic Models in Mice. Male C57BL/6J (B6) mice, FasL mutant (gld) mice and $Rag1^{-/-}$ mice on B6 background weighing about 25 g were obtained from the Animal Model Centre of Nanjing University. All animal experiments were approved by the Animal Care and Use Committee and performed according to institutional guidelines. In middle cerebral artery occlusion (MCAO) model, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (1%) at a dose of 45 mg/kg. During the surgery, mice body temperature was maintained at 37.0±0.5 °C. A 6/0 surgical monofilament nylon suture with a heat-rounded tip was inserted through the internal

carotid artery into the beginning of the middle cerebral artery (MCA). Mice were subjected to 60 min of occlusion and then the filament was withdrawn for blood reperfusion at day 1 and day 3. Mice were included if laser Doppler reading is below 30% of baseline and no hemorrhage were found when taking out the brain. Sham-operated mice were used as controls.

FasL neutralizing antibody treatment in mice. Anti-FasL antibody (0.002 g/kg each mouse) was i.p. administered to each animal at 30 min, 24 h and 48 h after MCAO in a double-blind manner. In parallel experiments, the mice were i.p. treated with control isotype IgG with the same dose. To analyze the penetration of anti-FasL antibody to MCAO brain, saline was also used as control. Both the control and treated mice were kept in an air-conditioned room at 25 °C with a 12 h light/dark cycle, and chow and water were provided ad libitum.

Blood Brain Barrier (BBB) Permeability

BBB permeability was determined by the extravation of Evans Blue (EB) in the brain 3 d after MCAO. Briefly, the saline containing 2% Evans Blue (6 ml/kg) was injected via tail vein 2 h before the end of the experiment. Mice were euthanized 3 d after MCAO and transcardially perfused with saline until the fluid exiting the right atrium was clear. The brains were removed and cut into slices for analysis. The extravasation of EB indicated impaired BBB in mouse brain.

Rag1$^{-/-}$mice reconstituted with DNTs. DNTs were isolated from splenocytes using CD3, CD4 and CD8 beads according to the manufacturer's instructions (Miltenyi Biotec, Inc., Auburn, CA). The purity of DNTs was further checked by flow cytometry. Rag1$^{-/-}$ mice were randomly distributed into three groups (PBS, B6 DNT and gld DNT). For adoptive transfer DNTs to Rag1$^{-/-}$ mice, 5×10^5 isolated cells in 0.1 ml PBS were intravenously injected into Rag1$^{-/-}$ recipients. DNTs-reconstituted mice were subjected to MCAO 30 min after cell transfer. The infarct volumes, behavioral test and activation of microglia were evaluated 3 d after MCAO.

TTC Staining. The brains collected from mice 3 d after MCAO were excised, cut into seven slices, and incubated with 2% (wt/vol) TTC (Sigma-Aldrich) at 37 °C for 20 min to determine the infarcted area (1). Images were obtained using a digital camera and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Infarct volume was determined by Image-Pro Plus 6.0 software (Meida Cybernetics). The value of the infarct volume is presented as a percentage of the contralateral side.

Behaviour Tests. The mNSS, corner test and rotarod test were used to evaluate the neurological function of the mice 1 d and 3 d after MCAO. **(1) mNSS score.** Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). mNSS includes motor, sensory, reflex and balance tests. In the severity scores of injuries, one point is awarded for the inability to perform a test or for the lack of a tested reflex (1). Higher score indicated severer injury. **(2) Corner test**. During this test, a corner was made by attaching two boards (30 cm × 20 cm × 1 cm) at an angle of 30°. A small opening was made along the joint to encourage entry into the corner. Animals were placed midway from the corner. When the animals reached the corner and their vibrissae were stimulated, they reared upward and then turned to either side. The number of left (ipsilateral side) turns during 10 trials was recorded. The mice with damage to the left cortex are expected to turn more toward the left (ipsilateral) side (2). In normal conditions, the rate of turning right is 50%. Higher score suggested severer injury. **(3) Rotarod test.** The rotarod test was used to assess loss of balance and sensorimotor coordination (3, 4). Latency to fall on a rotating rod was recorded by a five-lane rotarod device (IITC Life Science). The rotating rod was placed horizontally, accelerating from 4 rpm to 40 rpm for 5 min. The mice were trained 3 d before the operation, 3 times a day, and each training lasts for 5 min with a 15-min training interval for rest. On the third

day of training, the mice that could not adhere to the fatigue rotating rod for 5 min were excluded. The mice were placed on the rod in turn, and the average time of latency to fall for three rounds of experiments was recorded.

Preparation of single-cell suspensions. Peripheral blood was obtained from the mouse tail vein, and RBCs were lysed using ACK lysis buffer (Sigma-Aldrich). Spleen lymphocytes were isolated via mechanical homogenization followed by RBC lysis. Isolated cells were resuspended at a density of 1×10^6/ml. The brains were washed with 1× cold PBS and chopped into fine pieces in 4 ml of RPMI 1640 supplemented with 10% fetal calf serum. The tissue was then incubated in 10 ml of digestion buffer for 1 h in a 37°C water bath. The suspension was filtered through a 70 μm cell strainer, resuspended in 40 ml of RPMI 1640 and centrifuged at 300×g for 10 min at 4 °C. The supernatant was discarded, and the cells were resuspended in 37% Percoll (GE Healthcare). Then, the cell suspensions were carefully placed on 70% Percoll and centrifuged at 1000×g for 30 min. Finally, the cells found between the 37% and 70% Percoll were collected. In the following FACS sorting experiments, $CD3^+CD4^-CD8^-$ cells were sorted by a BD FACSAria™ III (BD Biosciences).

Flow cytometric analysis of T cells and microglia. Single-cell suspensions were incubated with antibodies against cell surface markers (CD3, CD4, CD8, CD45, CD11b, CD86, CD206, anti-Armenian Hamster IgG) at RT for 15 min. Cells were washed with 1×PBS and centrifuged at 300 g for 5 min. The supernatant was discarded and cells were resuspended with 1×PBS. Isotype antibody was used as control. $CD11b^+CD45^{int}$ cells were gated as microglia. All the samples were measured by Accuri C6 Flow Cytometer (BD Bioscience). FlowJo v 10 (Tree Star, Ashland, OR) was used to analyze the data.

Primary microglia culture. Primary microglia were prepared from the cerebral cortices of 1~2-day-old C57/BL6J mice as previously described (5). Briefly, brain membrane was removed and cerebral cortex was gently dissociated in 0.25% tripsin EDTA for 10 min. The digestion was terminated by adding equal volume of DMEM medium containing 10% FBS. The cells were centrifuged at 800 rpm at 37 °C for 10 min. The supernatant was aspirated and cells were resuspended in 75 cm^2 flasks. After 10-12 days, the microglia were separated from mixed primary glia by shaking the flasks and the floating microglia were replanted into 12- or 24- well plates for approximately 48 h. The purity of the microglial cells was greater than 95%, which was examined by immunocytochemistry analysis using Iba-1 antibody. The cells were maintained in DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C under a humidified atmosphere of 5% CO_2.

DNTs-microglia coculture assay. For T cell and microglia coculture, primary microglia were seeded in 24-well plates (5×10^5 cells) for 48 h. Magentic-activated cells sorting (Mitenyi Biotech, Inc., Auburn, CA) were used to isolate DNTs from mice splenocytes using CD3, CD4 and CD8 beads. Purity of DNTs was confirmed by flow cytometry and was general more than 95%. Isolated DNTs (5×10^5 cells) were then seeded and co-cultured with microglia for 24 h in 24-well plates. After 24 h, the plates were shaken for 5 min, and then washed with PBS for three times. After that, DNTs were found to be barely visible in a microscope and microglia were left in the plates. Cells then were harvested for flow cytometry and Real-Time PCR analysis.

Condition medium (CM) experiments. To generate DNT-CM, DNTs isolated from the spleen were cultured *in vitro* in RPMI 1640 (HyClone) supplemented with 10% FBS and 1 μg/ml recombinant IL-2 (Peprotech) for 24 h. The supernatants were collected and mixed with DMEM (4:1) to prepare the DNT-CM. In the inhibition study, DNTs were pretreated with a TNF-α inhibitor (lenalidomide, 5 μM, Selleck Chemicals) or a pharmacological inhibitor of PTPN2 (ethyl-3,4-

dephostatin, 0.01 or 0.05 μM) for 24 h. In IL-10 treatment groups, we added exogenous IL-10 (30 pg/ml to 30 ng/ml) to DNT-conditioned media for stimulating microglia.

Real-Time PCR. Total RNA was extracted from primary microglia or DNTs using TRIzol (Invitrogen) and reverse-transcribed into cDNA using a PrimeScript RT Reagent kit (Takara). Quantitative Real-Time PCR was performed on an ABI 7500 PCR instrument (Applied Biosystems) using a SYBR Green kit (Takara). The relative gene expression levels were normalized to the expression of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Corresponding primers (Invitrogen) were shown as follows:

Primers for Real-Time Polymerase Chain Reaction

Gene	Primer
CD16	SENS: TTT GGA CAC CCA GAT GTT TCA G
	REVS: GTC TTC CTT GAG CAC CTG GAT C
CD86	SENS: GAC CGT TGT GTG TGT TCT GG
	REVS: GAT GAG CAG CATCAC AAG GA
CD206	SENS: TTC GGT GGA CTG TGG ACG AGC A
	REVS: ATA AGC CAC CTG CCA CTC CGG T
Arg-1	SENS: GGT TGC CAA GCC TTA TCG GA
	REVS: ACC TGC TCC ACT GCC TTG CT
S100A8	SENS: TGA CAA TGC CGT CTG AAC TG
	REVS: TAT CAC CAT CGC AAG GAA CTC
S100A9	SENS: TGT GAC TCT TTA GCC TTG AGC A
	REVS: GCT GAT TGT CCT GGT TTG TG
TIAL1	SENS: TGA TAA CAG AGC AAC CCG ATA GC
	REVS: TTC CAC AAA GCA ATA TGG GTC AT
RFTN1	SENS: TCA GGT GGA GAC CAA AGT AGA TG
	REVS: ATG GCC CGA AAG ATG TGT TCC
GPS1	SENS: GAT CCA TGT CAA GTC TCC TCC T
	REVS: CTG TTG GCT GGA GTC AGC TC
SH3KBP1	SENS: TTA CCT CCA GCT ACA TCA ACT CC
	REVS: TTG CAG TAA TCC TTG GTC TTT GT
PTPN2	SENS: ATG TCG GCA ACC ATC GAG C
	REVS: TGC AGT TTA ACA CGA CTG TGA T
C1QBP	SENS: ACG GCA CGG AGG CTA AAT TAT
	REVS: TCT TCG TGT CCA ATC TCA TCC T
LPXN	SENS: GGA GAG CAA GAT TCC CCA AAC
	REVS: GAT CCT GGA TAT TGG TTG CAT
TNF-α	SENS: TCC TGG CCA ACG GCA TGG AT
	REVS: AAT CGG CTG ACG GTG TGG GT

Western Blot. Cells were washed three times with phosphate-buffered saline (PBS), and lysed in a lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 120 mM KCl, 320 mM sucrose) on ice for 30 min, and then cell lysates were centrifuged at 12,500 rpm for 30 min. The supernatants were concentrated by an Amicon Ultra-4 Centrifugal Filter (Millipore) to a volume of 20 μL for western blot analysis. Equal amounts of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocked in 5% skim milk for 2 h at RT, the membranes were incubated with primary antibodies against PTPN2 (1:1000), TIAL1 (1:500) or β tubulin (1:5000) at 4°C overnight. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) were used to label the primary antibodies. The membranes were developed with an ECL kit (Bioworld) and visualized using a Gel-Pro system (Tanon Technologies). The intensities of the blots were quantified via densitometry.

Immunofluorescence. Human brain sections were deparaffined in xylene, and rehydrated through a graded series of ethanol baths. The sections were then placed into sodium citrate buffer (pH 6.0) and heated in a microwave oven for 10 min. After cooling, the sections were washed with PBS for 5 min twice, and then treated with 3% H_2O_2 for 10 min at RT to quench endogenous peroxidase and washed three times with PBS. Mouse brain slices were prepared as previously described (6). Non-specific antibody binding was blocked by incubation of the sections with 3% BSA in PBS for 60 min at RT. After incubating with primary antibodies [CD3 antibody (1:200), CD4 antibody (1:200), CD8 antibody (1:200) and Iba-1 antibody (1:500), CD86 antibody (1:500) or CD206 antibody (1:500) plus Iba-1 antibody (1:500)] overnight at 4 °C, the sections were washed with PBS for three times. Then incubated with fluorescent secondary antibodies (Invitrogen, 1:500) at RT for 2 h. The slices were washed with PBS and cover slipped with fluoroshield mounting medium containing DAPI (5 mg/ml in PBS). Images were taken using a confocal microscope (Olympus, FV1200, Olympus Corporation) and analyzed with ImageJ.

Cytometric Bead Array (CBA) and Enzyme-linked Immunosorbent Assay (ELISA). Cytometric Bead Array Th1/Th17 kits (mouse inflammation kit, Bioscience, San Diego, CA, USA) were used to measure the concentrations of IL-2, IL-4, IL-6, IL-17, IL-10, TNF-α and IFN-γ in each sample. The experiment was conducted following the manufacturer's instructions. Briefly, 25 μl cultures medium was incubated with coating antibody for 2 h at RT. After washing away unbound substances, 25 μl capture beads and 25 μl of PE detection reagent were added. After incubation for 1 h, the samples were washed with washing buffer. The supernatants were discarded and the plate were resuspended in 300 μl washing buffer for analysis. The absorbance was detected at 450 nm with an iMark microplate reader (Bio-Rad). The concentrations of the cytokines were calculated and evaluated based on the standard curves using FCAP Array™ software (BD Bioscience).

Proteomic Analysis. We used mass spectrometry to analyze protein alterations in DNTs from B6 and gld mice with or without MCAO induction. Proteomic analysis was performed using an AB SCIEX TripleTOF 5600 mass spectrometer (AB SCIEX) equipped with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. Proteins (200 μg) from the splenic DNTs of B6 or gld mice with or without MCAO induction were resolved via 10% SDS-PAGE and stained with Coomassie Blue G-250 for 1 h. After destaining in ultrapure H_2O, the gels were cut into blocks and digested using trypsin. The extracted peptides were dried, redissolved and analysed. Gene ontology analysis, KEGG pathway analysis and protein-protein interactions were assessed using DAVID software coupled with STRING (version 10.0).

Statistics. The data are expressed as the mean±SD of three independent experiments. The statistical significance of the differences was evaluated using SPSS software version 18.0; Student's *t*-test was used for two groups, and one-way ANOVA followed by Bonferroni's post hoc test was used for three groups. All tests were two-tailed. $P<0.05$ was considered significant.

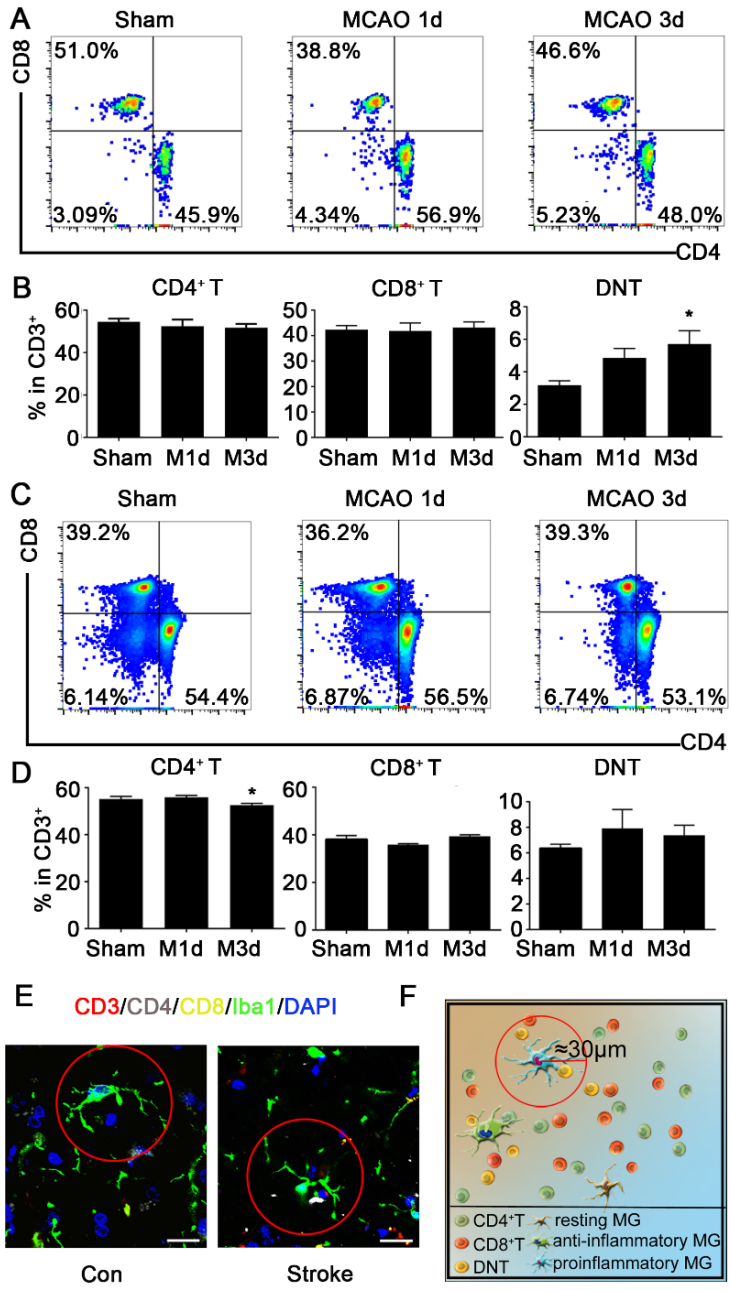

Fig. S1. T cells in the mouse periphery. (A) Representative FACS plots of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T cells in the blood 1 d and 3 d after MCAO. (B) The percentages of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells in the blood. (C) Representative FACS plots of the $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells in the spleen 1 d and 3 d after MCAO (n=10 per group). (D) The percentages of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells in the spleen (n=10 per group). *P<0.05 vs. the sham group. (E) Representative confocal images of T cells and microglia in brain sections from stroke patients. (F) Statistical schematic diagram showed the statistic process of the percentages of different T cells around microglia.

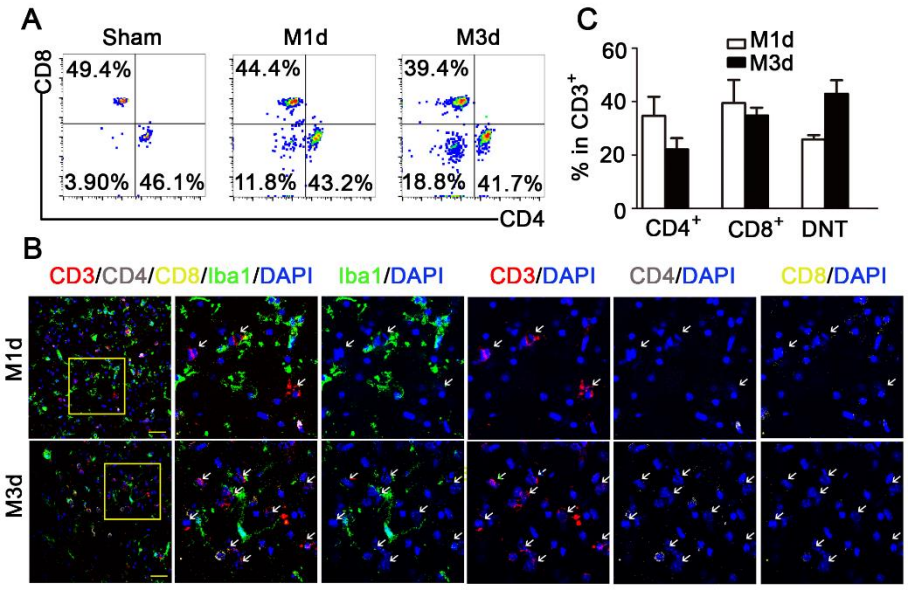

Fig. S2. T cells infiltrated in the mouse brain. (A) Representative FACS plots of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T cells in the brain 1 d and 3 d after MCAO. (B and C) Representative images and quantification of infiltrated DNTs (red), $CD4^+$ T cells (grey), $CD8^+$ T cells (yellow) and activated microglia (green) in mouse ischemic hemisphere. Scale bar: 100 μm. n=4 mice per group.

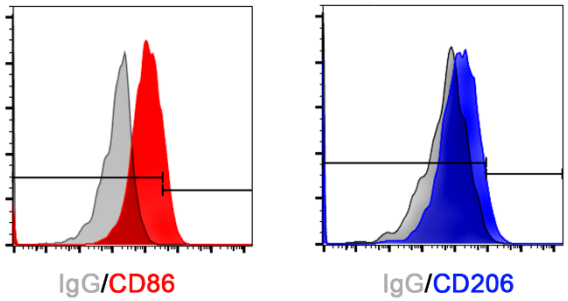

Fig. S3. Grey indicates isotype lgG; Red indicates CD86; Blue indicates CD206.

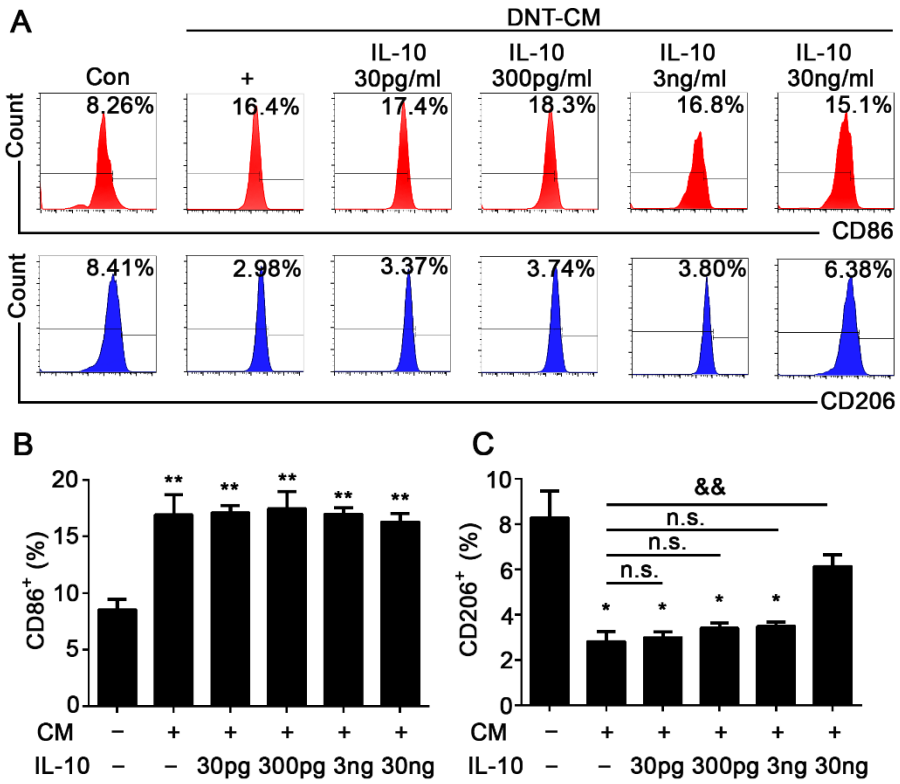

Fig. S4. Low levels of IL-10 as detected in DNTs-CM failed to impact microglia polarization. DNTs isolated from the spleens of C57BL/6J mice 3 d after treatment with or without MCAO were cultured in vitro for 24 h. The CM from the cultured DNTs was collected and supplied with IL-10 to generate different concentration (30 pg/ml to 30 ng/ml) before used to stimulate microglia for 24 h The percentage of $CD86^+$ pro-inflammatory and $CD206^+$ anti-inflammatory microglia were determined using FACS, n=9 per group. **P*<0.05 and ***P*<0.01 compared with the control group; $^{\&\&}$ *P*<0.01 compared with the CM only group.

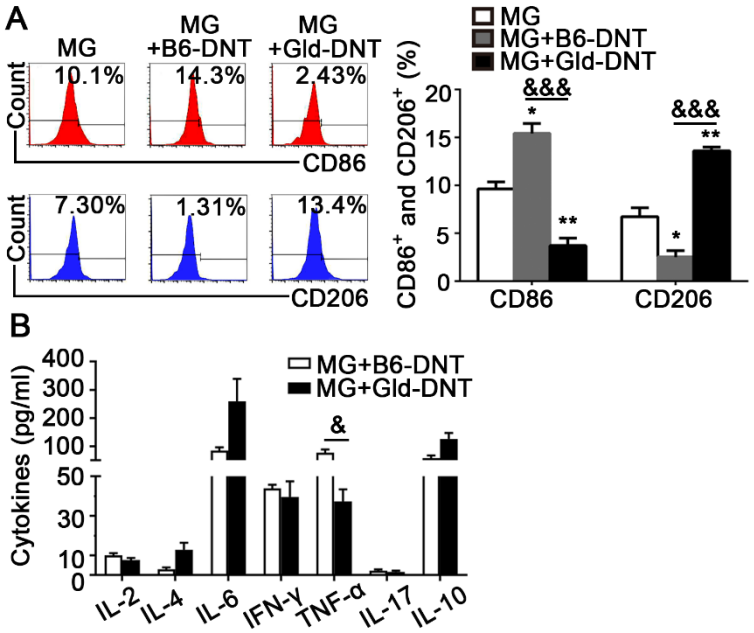

Fig. S5. FasL deficiency inhibits TNF-α secretion from DNTs and attenuates pro-inflammatory microglia activation after stroke. (A) DNTs (5×10^5 cells) sorted from the spleens of C57BL/6J and gld mice 3 d after MCAO were co-cultured with microglia (5×10^5 cells) for 24 h. The percentages of $CD86^+$ microglia and $CD206^+$ microglia were determined using FACS. (B) The cytokine profiles in the supernatants were evaluated using CBA. n=12 samples per group. **P*<0.05 and ***P*<0.01 compared with the control group (microglia only); $^{\&}P$<0.05, $^{\&\&}P$<0.01 and $^{\&\&\&}P$<0.001 compared with the B6 group.

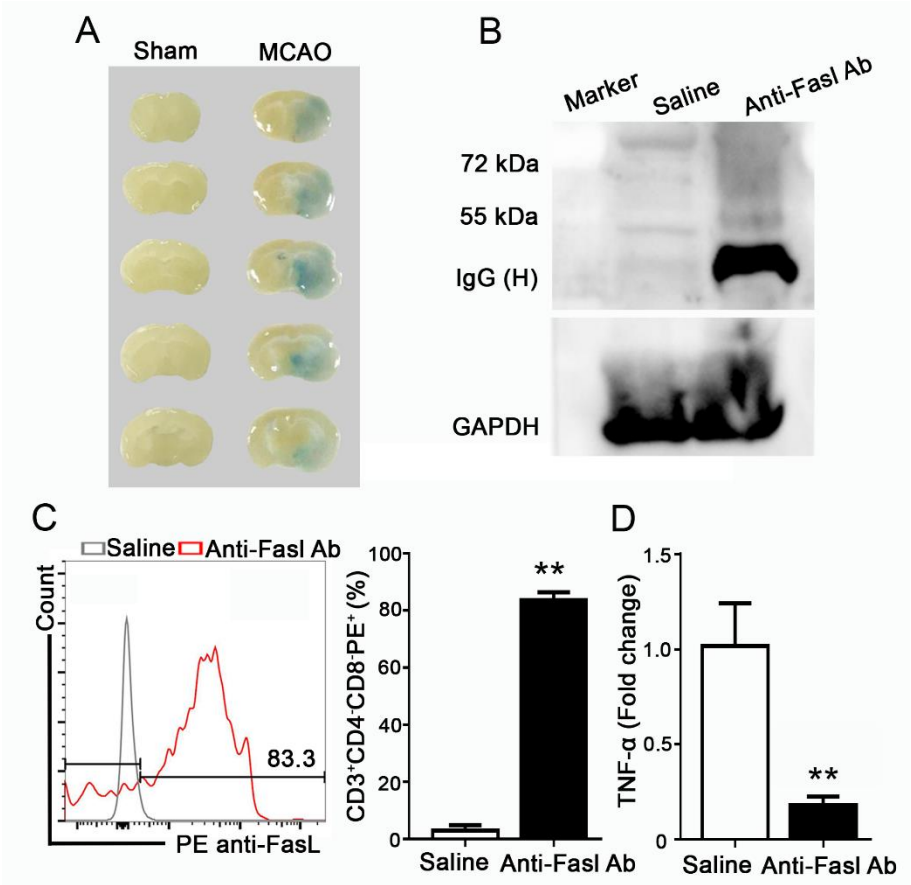

Fig. S6. Anti-FasL antibody massively penetrated to ischemic mouse brain, effectively bound to DNTs and significantly blocked FasL signaling. Purified Hamster Anti-mouse FasL antibody (0.002 g/kg mouse weight) was injected three times by i.p. at 30 min, 24 h and 48 h after MCAO in wild-type B6 mice. In parallel experiments, mice were i.p. injected with saline. BBB permeability was determined by the i.v. injection of Evans Blue (EB) 3 d after MCAO. (A) Representative pictures of Evans Blue leakage in brain sections from sham controls and MCAO mice. (B) Western blots analysis of the heavy chain of anti-FasL antibody in MCAO brain. (C) DNTs isolated from MCAO mice brain were stained with PE-conjugated secondary anti-Armenian Hamster IgG antibody followed by flow cytometry analysis. PE positive population indicated the DNTs with anti-FasL binding. (D) RT-PCR results showing the mRNA levels of TNF-α in DNTs sorted from the neutralizing antibody treated group and saline control group.

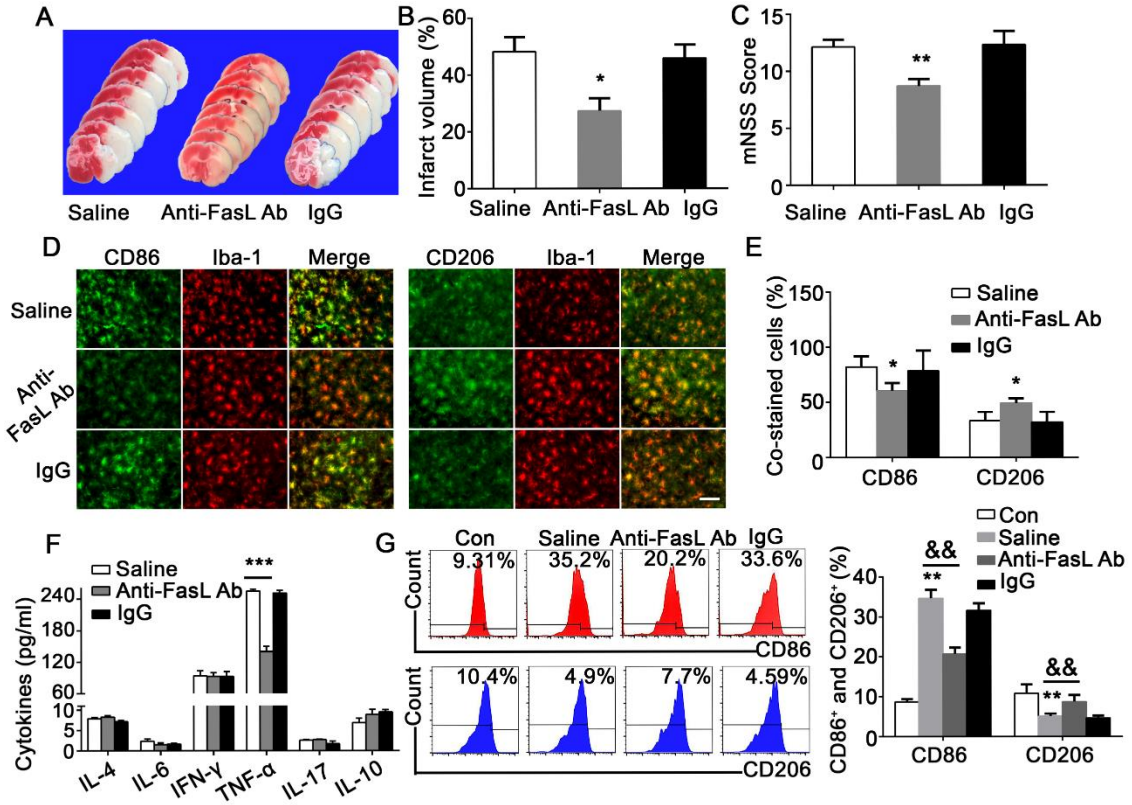

Fig. S7. Therapeutic blockade of FasL ameliorates brain injury and DNT-mediated inflammation in ischemic stroke. Anti-FasL antibody (0.002 g/kg mouse weight) was injected three times by i.p. at 30 min, 24 h and 48 h after MCAO in wild-type B6 mice. In parallel experiments, mice were i.p. injected with control isotype IgG. First, TTC staining images (A) depict the size of the brain infarction (B) from each group. The quantification of neurological deficits was assessed using the mNSS score (C). (D and E) Cortex co-stained for CD86 (green), CD206 (green) and Iba-1 (red). Scale bar: 50 μm. n=9 animals per group. **P*<0.05 and ***P*<0.01 vs. the saline control group. In the following experiments, splenic DNTs were isolated and cultured for 24 h, and their conditioned medium were applied to microglia for 24 h. (F) The cytokine profiles in the supernatant were evaluated by Cytometric Bead Array. (G) The percentage of $CD86^+$/$Iba\text{-}1^+$ and $CD206^+$/$Iba\text{-}1^+$ cells were analyzed by FACS. ***P*<0.01 vs. the control group (microglia only); $^{\&}P$<0.05, $^{\&\&}P$<0.01 vs. the saline control group.

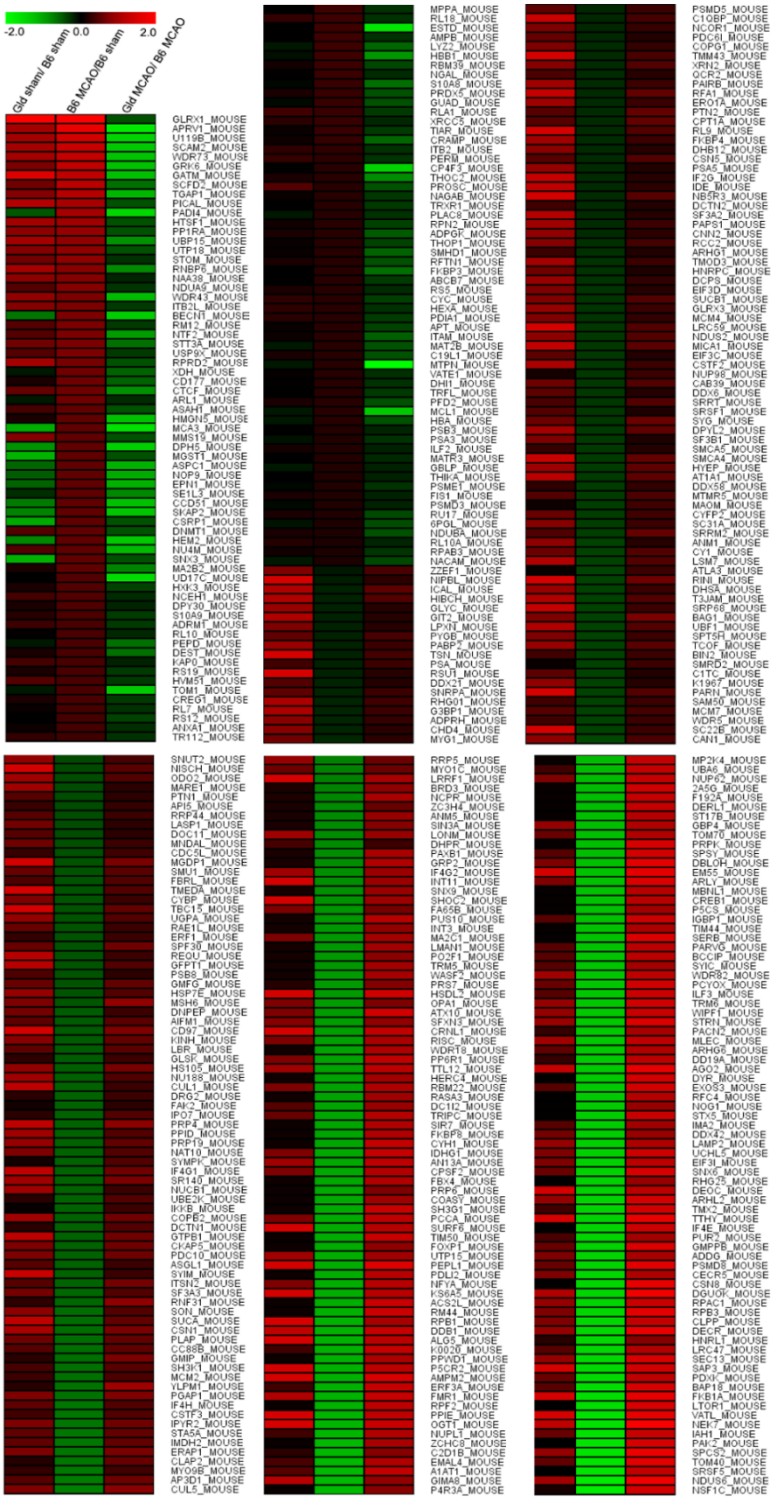

Fig. S8. Proteomic analysis of the DNTs isolated from B6 and gld mice after ischemic brain injury. A heat map generated based on the MeV of the differentially expressed proteins between B6 MCAO group and Gld MCAO group. The experimental groups are presented on the horizontal axis, and the proteins are on the vertical axis. The colours are consistent with the protein expression levels; red indicates upregulation, and green indicates downregulation.

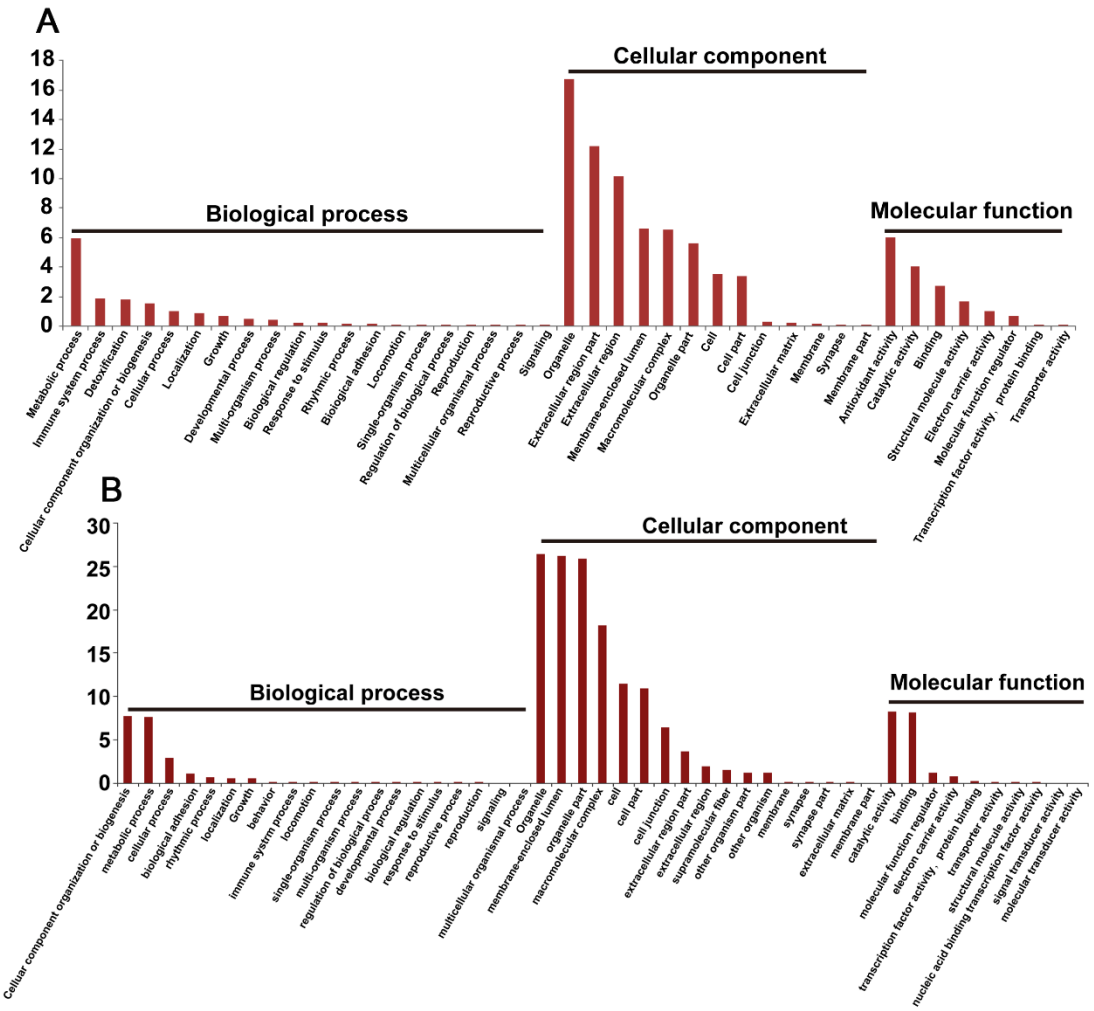

Fig. S9. Proteomic analysis of the DNTs isolated from B6 and gld mice after ischemic brain injury. (A and B) GO analysis of differentially expressed proteins that overlap in Fig.6 A and Fig.6 B and involve biological processes, cellular components and molecular functions. The vertical axis indicates –log (p).

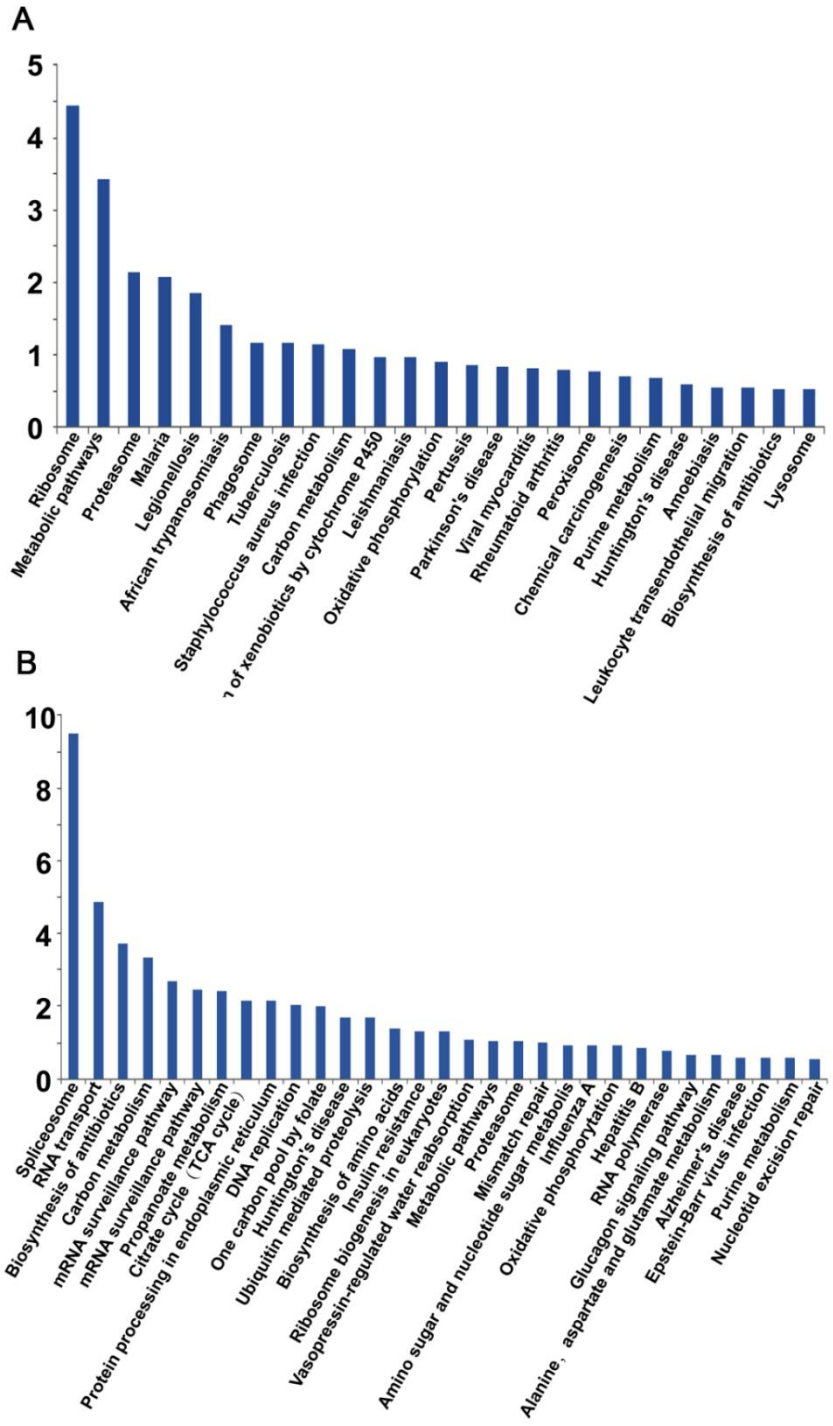

Fig. S10. Proteomic analysis of the DNTs isolated from B6 and gld mice after ischemic brain injury. (A and B) KEGG pathway analysis of differentially expressed proteins that overlap in Fig.6 A and Fig.6 B involving biological processes, cellular components and molecular functions. The vertical axis indicates –log (p).

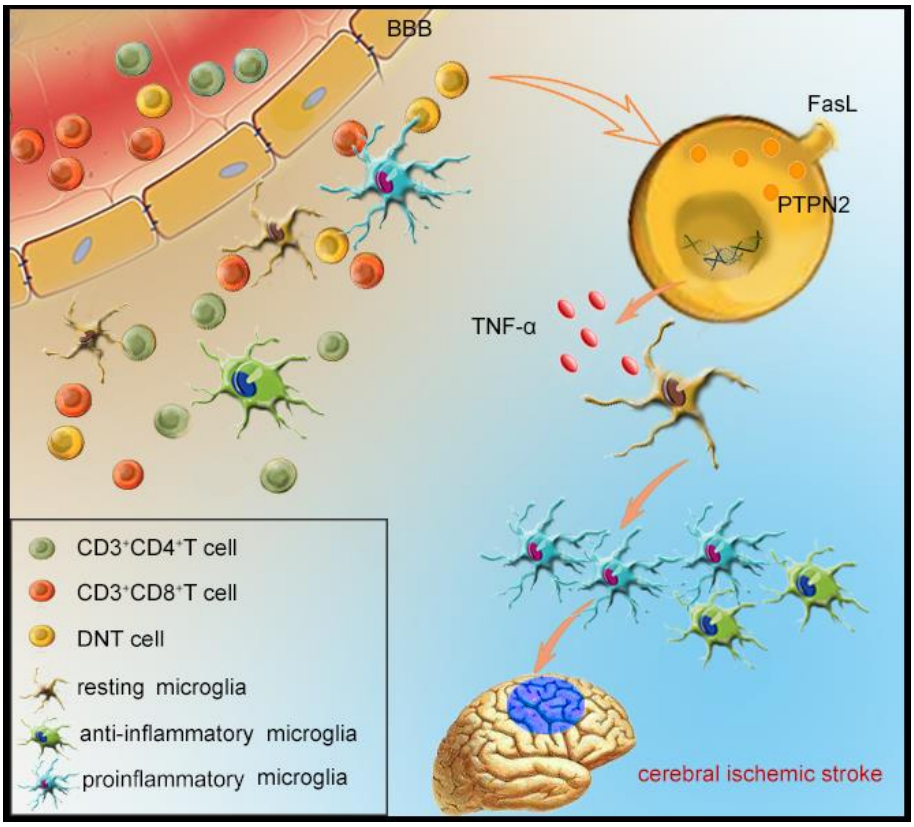

Fig. S11. Schematic illustrating the mechanisms underlying DNT-mediated ischemic brain injury. DNTs infiltrate into the brain after ischemic stroke and located closely to microglia. DNTs enhance microglial inflammation and brain injury via the FasL-PTPN2-TNF-α axis.

Table S1. Clinical characteristics of control group, and Stroke

	Control (n=33)	Stroke (n=47)
Gender(F/M)	24/9	29/18
Age (year)	69.73±6.296	72.77±11.25
Hypertension	0/33 (0%)	30/47 (64%)
Diabetes Mellitus	0/33 (0%)	13/47 (28%)
Hyperlipidemia	0/33 (0%)	4/47 (8.5%)

Table S2. Patients clinical characteristics of atherothrombotic stroke

	Stroke (n=47)
Gender (F/M)	29/18
Age (year)	72.77±11.25
Hypertention	30/47 (64%)
Diabetes Mellitus	13/47 (28%)
hyperlipidemia	4/47 (8.5%)
Admission status	
SBP (mmHg)	148.72±22.90
DBP (mmHg)	81.89±12.39
Glouse (mmol/L)	5.56±1.39
HbA1c (%)	6.14±1.36
Cholesterol (mmol/L)	4.07±1.02
Triglycerides (mmol/L)	1.22±0.67
LDL (mmol/L)	2.37±0.85
HDL (mmol/L)	1.06±0.34
Apolipoprotein A (g/L)	1.05±0.32
Apolipoprotein B (g/L)	0.82±0.24
Urea nitrogen (mmol/L)	6.7±3.54
Creatinine (umol/L)	92.96±112.47
Uric acid (umol/L)	334.51±114.50
eGFR ml/min/1.73m^2	83.36±37.31
Homocysteine (umol/L)	17.69±4.87
Cerebral infarction volume (mm^3)	35839.82±72977.51
NIHSS	7.89±4.96

Table S3. postmortem information of control group, and Stroke

	Control (n=3)	**Stroke (n=6)**
Gender(F/M)	1/2	4/2
Age (year)	82±4	88±4
Cause of death	non-neurological disease	ischemic stroke
Time of death after stroke (day)	-	3~7
Location of stroke lesion	-	cortical areas supplied by the middle cerebral artery
Collected time after death (h)	＜4	＜4
Autoimmune disease	0/3 (0%)	0/6 (0%)
Heart failure	0/3 (0%)	0/6 (0%)
Acute myocardial infarction	0/3 (0%)	0/6 (0%)
Hematological system disease	0/3 (0%)	0/6 (0%)
Infection before stroke	0/3 (0%)	0/6 (0%)

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