Supporting Information

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SI Materials and Methods

Generation of NK1.1-tdTomato Mice. The NK1.1-tdTomato knockin mice were produced by homologous recombination into the NK1.1 (Nkrp1c) locus directly in C57BL/6Tac-derived embryonic stem cells. The NK1.1 targeting construct was designed to replace exon 1 of the gene encoding Nkrp1c with the sequence coding for tdTomato (a gift from Roger Tsien, Howard Hughes Medical Institute, University of California, San Diego) and a selection cassette flanked by FLP recombination target (FRT) sites (Fig. S2*A*). Homologous arms for recombination were generated from bacterial artificial chromosome clone RP24-127M20 (Children's Hospital Oakland BACPAC Resources). The B6 (Taconic) line WY-ES7 was generated with assistance from the Rheumatic Diseases Core Center Transgenic and Knockout Mouse Facility (Department of Pathology and Immunology, Washington University), was found to have a normal male karyotype, demonstrating the capacity to produce highly chimeric mice when microinjected into blastocysts, and was used for gene targeting. Eleven clones were identified with homologous recombination by Southern analysis. Clones were microinjected into Albino B6 blastocysts to generate chimeric animals. Chimeras were bred to C57BL/6 (National Cancer Institute) to generate the colony; three clones ultimately showed germline transmission (Fig. S2*B*). Animals containing the targeted allele were born at the expected frequency and were phenotypically normal. Analysis of the immune system demonstrated that tdTomato is faithfully expressed in all cell lineages that normally express NK1.1 [i.e., natural killer (NK) and NKT cells] (Fig. S2*C*).

Animal Studies. Mice were housed in pathogen-free conditions at the animal facilities of the Barrow Neurological Institute (Phoenix, AZ), the Tianjin Neurological Institute (Tianjin, China), and Washington University (St. Louis, MO). All animal studies were approved by the Animal Care and Use Committees of the Barrow-St. Joseph's Hospital, Tianjin Neurological Institute, and Washington University.

Middle Cerebral Artery Occlusion Procedure. Middle cerebral artery occlusion (MCAO) was performed in mice under anesthesia by inhalation of 3.5% isoflurane and maintained by inhalation of 1.0–2.0% isoflurane in 70% N_2O and 30% O_2 by a face mask. Body temperature was monitored throughout surgery (via a rectal probe) and maintained at 37.0 ± 0.5 °C using a heating blanket (Sunbeam). Cerebral blood flow was monitored for 5 min both before and after MCAO, and immediately before and after reperfusion. For this procedure a small incision was made in the skin overlying the temporalis muscle, and a 0.7-mm flexible laser Doppler probe (model P10) was positioned on the superior portion of the temporal bone (6 mm lateral and 2 mm posterior from the bregma). Focal cerebral ischemia was induced for 90 min by occlusion of the right middle cerebral artery with a 6-0 surgical nylon monofilament with rounded tip, and cerebral blood flow was monitored as described previously (1). After 90 min of MCAO the mice were reanesthetized, and the occluding filament was withdrawn gently back into the common carotid artery to allow reperfusion. Relative cerebral blood flow had to rise to at least 50% of preischemic levels for the mice to be included in the study and subjected to further analyses. Relative cerebral blood flow was monitored for a further 5 min before the wound being sutured, and mice were allowed to recover from the anesthesia. Sham-operated mice underwent the same surgical procedure, except that the filament was not advanced far enough to occlude the middle cerebral artery.

Neuroimaging. To detect reactive oxygen species generation in the brain, live bioluminescence images in mice were captured using the Xenogen IVIS200 imager (Caliper LifeSciences) after i.p. injection of 200 mg/kg Luminol (Invitrogen) (2–4). A region of interest tool was used to measure chemiluminescent intensity. Data were collected as photons per second per cm^2 using Living Image software (Caliper Life Sciences).

Infarct areas were assessed using a 7T small-animal MRI, 30-cm horizontal-bore magnet, and BioSpec Avance III spectrometer (Bruker Daltonics Inc.) with a 116-mm high-power gradient set (600 mT/m) and a 72-mm whole-body mouse transmit/surface receive coil. Scan parameters and T2-weighted acquisition have been described previously (4). To identify the ischemic penumbra, diffusion and perfusion were determined on the basis of apparent diffusion coefficient and cerebral blood flow maps. The diffusion/perfusion mismatched area shows the penumbra in the ipsilateral hemisphere. MRI data were analyzed using MEDx3.4.3 software (Medical Numerics) on a LINUX workstation (4, 5).

2,3,5-Triphenyltetrazolium Chloride Staining. For 2,3,5-triphenyltetrazolium chloride (TTC) staining, MCAO brains were removed rapidly and frozen at −20 °C for 5 min. Coronal slices were made at 1–2 mm from the frontal tips, and sections were immersed in 2% TTC (Sigma-Aldrich) at 37 °C for 20 min. The presence of infarction was determined by areas on the sides that were not stained with TTC. Infarct volume was quantified by ImageJ image analysis (6).

Neurological Deficit Assessment. Neurological deficit assessment was performed by investigators blinded to the control and MCAO groups, as described elsewhere (7, 8). The rating scale was as follows: 0 = no deficit, 1 = failure to extend left forepaw, 2 = decreased grip strength of left forepaw, 3 = circling to left by pulling the tail, and 4 = spontaneous circling.

Immunohistochemistry. The sections were incubated with primary antibodies against human NKp46 (C-20; Santa Cruz Biotechnology) and βIII-tubulin (pAb; Abcam), or against mouse NKp46 (29A1.4; BD Bioscience), Qa1b (6A8.6F10; Abcam), NeuN (A60; Abcam), CX3CL1 (126315; R&D Systems), IL-1β (pAb; R&D Systems), and IL-6 (pAb; R&D Systems), respectively, at 4 °C overnight and then incubated with avidin–biotin–horseradish peroxidase complex and developed in 3,3' diaminobenzidine tetrahydrochloride, or incubated with appropriate fluorochrome conjugated secondary antibodies. Images were captured by a fluorescence microscopy (Olympus, model BX-61) (4, 5).

In Vivo Antibody Administration. mAbs directed against mouse NK1.1 (PK136 clone) were produced from hybridomas (American Type Culture Collection). Mouse IgG2a (Sigma-Aldrich) was used as the isotype control antibody. For depletion of $NK1.1^+$ cells in vivo, 200 μg of anti-NK1.1 mAb was injected i.p. into each mouse at the various time points indicated. Depletion of $NK1.1^+$ cells was confirmed by flow cytometry and was constantly >90%, as previously described (4, 9).

Cell Isolation and Passive Transfer. NK cells were sorted from pooled splenocytes of several mutant strains. All mice from which NK cells were purified were back-crossed to mice of C57BL/6

background for at least 10 generations in this study. NK cells were purified via magnetic beads selection (NK cell isolation kit; Miltenyi Biotech) coupled with two rounds of cell sorting selection with the high-speed sort of FACSAria (BD Biosciences). The purity of NK cells was confirmed by flow cytometry before transfer. Highly purified (>99%) NK cells (5×10^5) were injected i.v via the tail veil into recipient mice (4).

Cell Migration Assay. NK cells' chemotaxis toward CX3CL1 was measured using a transmigration chamber assay with 8-μm pore size inserts (Neuro Probe) according to the manufacturer's instructions. Briefly, the inserts were placed into 24-well plates containing culture medium in the presence or absence of CX3CL1 (10 nM), control neurons, ischemic neurons, or ischemic neurons plus anti-CX3CL1 antibody. *Cx3cr1*$^{+/+}$ or *Cx3cr1*$^{-/-}$ NK cells (2×10^5) were then loaded onto the upper chambers and incubated at 37 °C for 4 h. The number of cells that migrated through the insert (at least 10 different randomly selected fields in each well) was counted under a phase contrast microscope. Migration index (MI) was calculated as follows: MI = (number of cells migrating toward chemoattractants)/(number of cells migrating toward medium in the absence of any stimulant) (4).

ELISA. The brain homogenates were prepared from MCAO brains, and the total protein concentration was adjusted to 1 mg/mL protein extract. CX3CL levels in homogenates were quantified by ELISA (R&D Systems) and converted into pg/mg protein extract. A Multi-Analyte ELISArray kit (SABioscience) was used to measure cytokines in brain homogenates according to the manufacturer's instructions, as previously described (4).

Flow Cytometry. Single-cell suspensions were prepared from spleens or central nervous system and stained with fluorochrome-conjugated antibodies. All antibodies were purchased from BD or eBioscience, Inc. unless otherwise indicated. Antibodies were directly labeled with one of the following fluorescent tags: fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerCP-Cy5.5, or allophycocyanin (APC). The following antibodies to mouse Ags were used: CD3 (145-2C11), NK1.1 (PK136), NKp46 (29A1.4), NKG2A (20d5), and NKG2D (CX5). Cell surface phenotype and sorting were performed on a FACSAria flow cytometer, and data were analyzed with FACSDiva and FCS Express 4 software.

Primary Cultures of Cortical Neurons and Oxygen Glucose Deprivation. We previously described primary cultures of cortical neurons (10). Briefly, 1- to 3-d-old, C57BL/6 mice were killed, and their cortexes were dissected under a stereological microscope. Tissue was minced with scissors in ice-cold Neurobasal medium (Invitrogen) and then digested with papain (20 U/mg; Worthington) at 37 °C for 20 min in tubes shaken at 120 rpm in a water bath shaker. After enzyme digestion the reaction was stopped by adding inactivated FBS to the medium. After filtering and centrifugation three times, the supernatant was replaced with Neurobasal medium supplemented with 0.5% (wt/vol) L-glutamine and 2% B27 serum-free supplement. Cells were plated at a density of $4.5–5.0 \times 10^4$ cells per cm^2 in 35-mm culture dishes.

To model ischemic-like conditions in vitro, primary cultured neurons were exposed to oxygen glucose deprivation (OGD) as previously described with modifications (11). In brief, two-thirds of the culture medium was replaced four times with serum- and glucose-free medium, resulting in a final glucose concentration of less than 1 mM. The serum-glucose deprived cultures were then placed in a Billups-Rothenberg modular incubator chamber according to the manufacturer's instructions, followed by flushing with 95% nitrogen and 5% CO_2 for 5 min and then sealing. The chamber was moved to a water-jacketed incubator at 37 °C/5% CO_2 for 15 or 60 min and then returned to 95% air, 5% CO_2, and glucose-containing medium for 12 h. The PO_2 of the serum and glucose-free media in the hypoxic chamber were repeatedly measured using a Clark-type electrode (Hansatech Instruments) at several OGD time points. Control glucose-containing cultures were incubated for the same periods of time at 37 °C/5% CO_2.

Qa1 Overexpression with Lentivirus. Qa1 expression with lentivirus in cultured cortical neurons was performed as previously described by us, with modification (4). Briefly, full-length Qa1 cDNA was amplified by RT-PCR from RNA isolated from splenocytes of C57BL/6 mice. After verification of the sequence, cDNA was ligated into the pLVX-IRES-mCherry vector (Takara Bio Inc.). The recombinant lentivirus was produced by Lenti-X 293T cells (Takara Bio Inc.) with a Lenti-X HTX Packaging System (Takara Bio Inc.). Virus was collected 72 h after transfection, and titers were up to $5–8 \times 10^8$ infectious U/mL. Cortical neuron cultures were infected with virus in 12-well plates precoated with 20 μg/mL poly-L-lysine. The virus-containing transduction medium was then replaced with fresh growth medium; the cells' incubation then continued for 48 h at 37 °C and 5% CO_2.

^{51}Cr Release Assay. NK cell cytotoxicity was evaluated with a standard ^{51}Cr release assay. Normal neurons or neurons transfected with lentiviral vector encoding Qa1 exposed to OGD were used as target cells and labeled with ^{51}Cr. Various effector:target cell (E:T) ratios were adopted, as we previously described (4).

Neuronal Electrophysiology. The standard external solution contained 140 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM D-glucose, and 10 mM Hepes, adjusted to pH 7.4 with Tris base. The internal solution for current-clamp recordings to measure intrinsic excitability contained 130 mM $KMeSO_4$, 10 mM KCl, 10 mM Hepes/K-Hepes, 2 mM $MgSO_4$, 0.5 mM EGTA, and 3 mM ATP; the pH was adjusted to 7.3 with KOH. For miniature excitatory postsynaptic currents (mEPSCs) recordings, 0.3 μM TTX, 50 μM picrotoxin, and 50 μM (2R)-amino-5-phosphonopentanoate (APV) were added into external solution. For whole-cell recordings of ligand-gated ion channels and mEPSCs recordings, the internal solution contained the following: 140 mM potassium gluconate, 5 mM KCl, 10 mM Hepes, 0.2 mM EGTA, 2 mM $MgCl_2$, 4 mM MgATP, 0.3 mM Na2GTP, and 10 mM Na2-phosphocreatine (pH 7.3 with KOH). Current-clamp recordings to measure neuronal excitability were performed with a modified protocol as previously described (12, 13). Neuronal intrinsic excitability was measured as the number of spikes in response to 500-ms current injection (0.09 nA). Data were acquired by Axonpatch 200B amplifier at 2 kHz with pClamp 9.2 software (Molecular Devices) and analyzed with clampfit 9.2 software (Molecular Devices) and Mini Analysis software (Synaptosoft).

Statistics. Statistical analyses were performed using GraphPad Prism software. Two-tailed unpaired Student *t* test was used to determine the significance of differences between two groups. One-way ANOVA followed by Tukey post hoc test were used for three or more groups. Two-way ANOVA with Bonferroni post hoc test were performed for multiple comparisons. $P < 0.05$ was considered significant.

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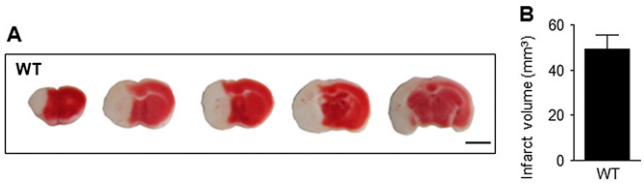

Fig. S1. Infarct volume determined by TTC staining. (*A*) Representative coronal sections from WT C57BL/6 mice after 24 h of reperfusion following 90 min MCAO were stained with TTC (1). The red regions show intact areas; pale regions show infarct areas. (Scale bar, 1 mm.) (*B*) Infarct volume was quantified from eight WT mice 24 h after MCAO.

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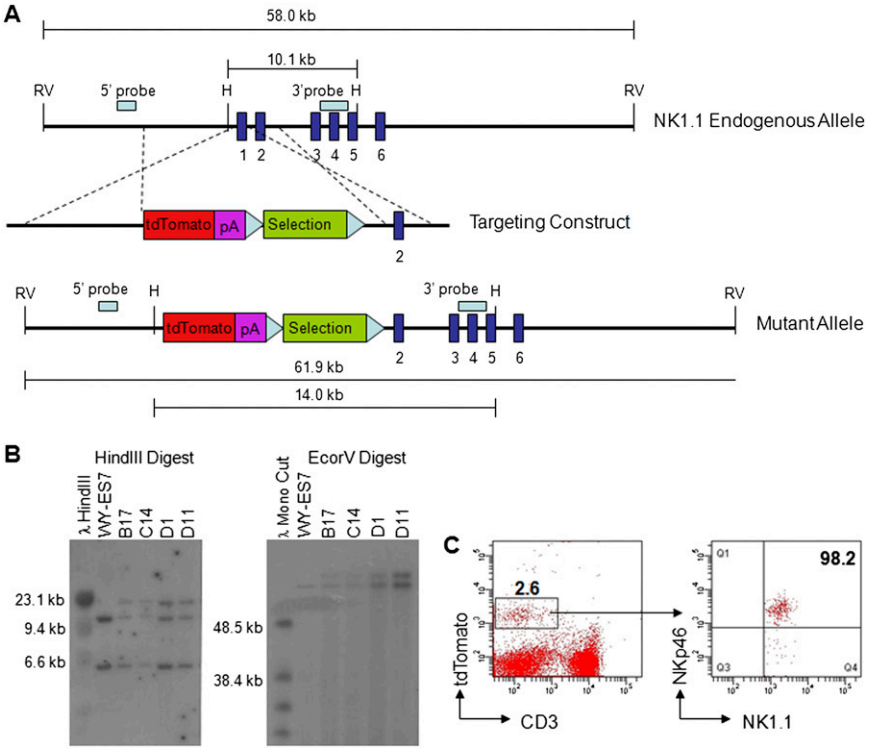

Fig. S2. Generation and identification of tdTomato transgenic mouse. (*A*) Structure of the endogenous NK1.1 allele, targeting construct, and mutant allele after recombination. The 5′ and 3′ external probes are indicated by the light blue bars. The enzymes critical for analysis are EcoRV and HindIII. The 5′ arm is 10,104 bp in length, and the 3′ arm is 3,566 bp in length. The selection cassette contains Em7-Zeo as well as PGK-Neo, flanked by FRT sites. A Sv40 polyA signal was added after the stop codon of tdTomato (1), which completely replaces exon 1 of NK1.1. (*B*) Southern blot analysis of ES DNA isolated from targeted clones. DNA was digested with EcoRV or HindIII and hybridized with the 5′ or 3′ external probe, respectively. Expected band sizes are as follows: EcoRV digest with the 5′ probe: WT is 58.0 kb and the targeted allele is 61.9 kb; HindIII digest with the 3′ probe: WT is 10.1 kb and targeted allele is 14.0 kb. Clones B17, C14, D1, and D11 are positive for homologous recombination compared with the parental ES control. Clones B17, C14, and D11 resulted in germ-line transmission of the targeted allele. (*C*) Identification of NK cells from NK1.1-tdTomato mouse. Single suspension of splenocytes was isolated from NK1.1-tdTomato mouse and stained with NKp46 FITC and NK1.1 PerCP-Cy5.5 antibodies. FACS showed that the majority (more than 98%) of tdTomato-positive cells were also NKp46- and NK1.1- positive. n =10.

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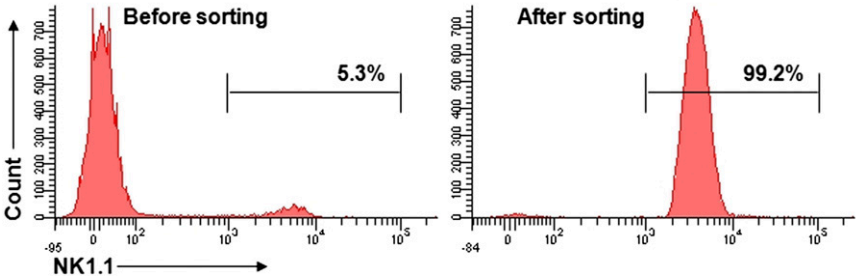

Fig. S3. Sorted NK cell purity >99% was validated by FACS. NK cells were isolated from pooled splenocytes of WT mice. NK cells were then purified via magnetic beads selection (NK cell isolation kit; Miltenyi Biotech) coupled with two rounds of cell sorting selection with the high-speed sort of FACSAria flow cytometer (BD Biosciences). The purity of NK cells was analyzed by flow cytometry. Representative plots show the density of NK cells from splenocytes of WT mice (*Left*) and the purity (99.2%) after sorting (*Right*).

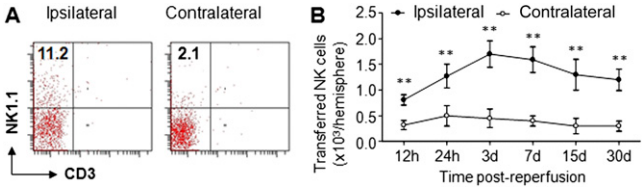

Fig. S4. NK cells survive in vivo after transfer during the course of 30 d. (*A*) Representative dot plots show that transferred NK cells survived in the ischemic brains of recipient Rag2$^{-/-}\gamma c^{-/-}$ mice 3 d after MCAO. (*B*) Adoptively transferred NK cells can survive in *Rag2*$^{-/-}\gamma c^{-/-}$ recipient mouse brains until day 30 after MCAO. Quantification of NK cells infiltrating the ipsilateral and contralateral hemispheres of *Rag2*$^{-/-}\gamma c^{-/-}$ MCAO brains are shown in the graph. Data were obtained from eight mice. $^{**}P < 0.01$.

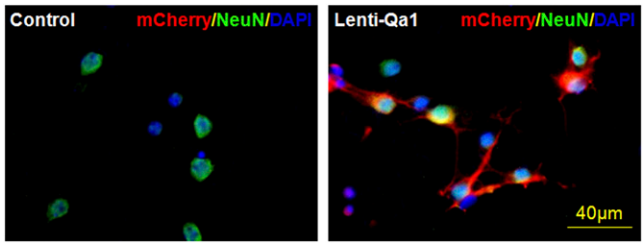

Fig. S5. Overexpression of Qa1 in neurons using lentiviral transfection. Representative images show Qa1 expression in neurons using lentiviral transfection (*Right*). Qa1 expression with lentivirus in cultured cortical neurons was performed as we previously described, with modification (1). Immunofluorescence staining revealed stably expressed Qa1 (mCherry, red) in NeuN^{+} (a neuronal maker, green) cells after pLVX-IRES-mCherry-Qa1 lentivirus transfection. The nuclei were stained with DAPI (blue).

1. Hao J, et al. (2010) Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *J Exp Med* 207(9):1907–1921.

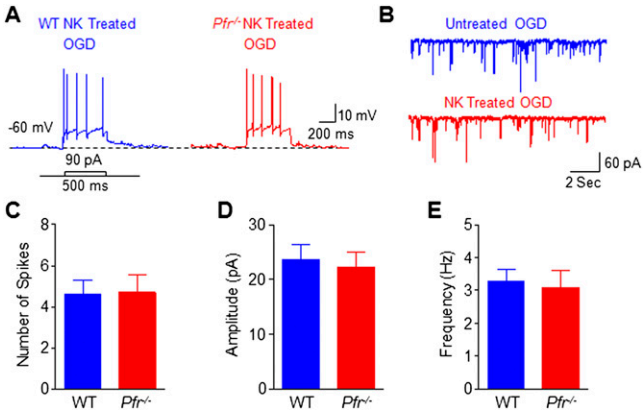

Fig. S6. NK cells' effects on ischemic neuronal excitability and synaptic excitatory transmission are independent of perforin. (*A*) Typical traces of action potential spikes in response to 90-pA current injection show that OGD neurons cocultured with WT or *Pfr*$^{-/-}$ NK cells for 24 h have similar neuronal excitability. (*B*) OGD neurons cocultured with WT or *Pfr*$^{-/-}$ NK cells have similar mEPSCs. (*C*) Bar graph summarizes action potential numbers in OGD neurons elicited by injection of 90-pA currents after coculture with WT or *Pfr*$^{-/-}$ NK cells for 24 h. (*D* and *E*) Frequency and amplitude of mEPSCs in OGD neurons were comparable when cocultured with WT or *Pfr*$^{-/-}$ NK cells for 24 h. Electrophysiology data were collected from 11 cells in each group. $P > 0.05$ in *C*–*E*.