Brain Ischemia Suppresses Immunity in the Periphery and Brain via Different Neurogenic Innervations

**Highlights**
- Brain ischemia causes transient but severe suppression of cellular immunity
- Natural killer (NK) cells display different profiles in the CNS versus periphery after stroke
- NK cell response is shaped by organ-specific neurogenic innervation after stroke
- Modulation of neurogenic innervation limits post-stroke infection

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**In Brief**
Liu and colleagues demonstrate that brain ischemia shapes innate cellular immune responses in the periphery and the brain through different neurogenic and intracellular pathways. Targeted modulation of neurogenic innervation is capable of inhibiting post-stroke infection.

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Brain Ischemia Suppresses Immunity in the Periphery and Brain via Different Neurogenic Innervations

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SUMMARY

Brain ischemia inhibits immune function systemically, with resulting infectious complications. Whether in stroke different immune alterations occur in brain and periphery and whether analogous mechanisms operate in these compartments remains unclear. Here we show that in patients with ischemic stroke and in mice subjected to middle cerebral artery occlusion, natural killer (NK) cells display remarkably distinct temporal and transcriptome profiles in the brain as compared to the periphery. The activation of catecholaminergic and hypothalamic-pituitary-adrenal axis leads to splenic atrophy and contraction of NK cell numbers in the periphery through a modulated expression of SOCS3, whereas cholinergic innervation-mediated suppression of NK cell responses in the brain involves RUNX3. Importantly, pharmacological or genetic ablation of innervation preserved NK cell function and restrained post-stroke infection. Thus, brain ischemia compromises NK cell-mediated immune defenses through mechanisms that differ in the brain versus the periphery, and targeted inhibition of neurogenic innervation limits post-stroke infection.

INTRODUCTION

Infectious complications, predominantly pneumonia and urinary tract infection, are a leading cause of death in ischemic stroke patients (Finlayson et al., 2011; Koennecke et al., 2011; Meisel and Meisel, 2011; Westendorp et al., 2011). The impairment of immune responses after brain ischemia increases the susceptibility to infections (Hug et al., 2009; Prass et al., 2003; Wong et al., 2011). The need to better understand these events has become obvious after two large international clinical trials demonstrated that prophylactic antibiotics did not prevent lung infection or improve neurological outcomes in patients with acute ischemic stroke (Kalra et al., 2015; Westendorp et al., 2015). To identify interventional modalities for the reduction of the incidence of post-stroke infection, the mechanisms of stroke-induced immune alterations need to be clarified.

The central nervous system (CNS) has been classically considered an immunologically privileged site because the blood-brain barrier physically shields it from immune cells. However, as it has now become clear, immune surveillance plays a non-redundant role in brain homeostasis, under physiological conditions (Ousman and Kubies, 2012; Waisman et al., 2015). In the presence of a neurological insult— including brain ischemia—the CNS allows large numbers of peripheral immune cells to infiltrate its parenchyma. Depending on the timing, those infiltrates can either damage neural structures or promote tissue repair (Chamorro et al., 2012; Fu et al., 2015; Gadani et al., 2015; Iadecola and An rather, 2011; Ransohoff and Brown, 2012). During the transition from acute to subacute stage of brain ischemia, the peripheral immune system also changes from a status of activation to hypofunction (Anderson and Tracey, 2012; Fu et al., 2015; Iadecola and Anrather, 2011; Meisel et al., 2005). A prevailing notion is that brain-derived neurogenic signals modulate immune functions (Irwin and Cole, 2011; Kipnis, 2016; Sternberg, 2006; Tracey, 2009). Brain ischemia induced activation of these neurogenic pathways including the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system, and the parasympathetic nervous system, which influence the magnitude of the immune response (Fu et al., 2015; Meisel et al., 2005). However, whether brain ischemia impacts the cellular immunity within the brain is not known; and if so, whether the temporal immune alterations and underlying mechanisms are similar to those occurring in the periphery is also unknown. Also, considering that prophylactic antibiotics fail to benefit stroke patients with infectious complications, what could be effective to inhibit the prevalent and grave consequence of infection in stroke?

To address the above questions, we examined the cellular and transcriptome profiles of natural killer (NK) cells in the CNS and periphery after brain ischemia. While studies of other lymphocyte subpopulations, such as T and NKT cells, are under
Figure 1. Spleen Atrophy and Contraction of the Peripheral NK Cell Compartment in Patients with Ischemic Stroke

(A) Automated 3D reconstruction of MRI shows serial spleen images in healthy subjects (controls) and patients during early phase (<24 hr) of ischemic stroke. Scale bars represent 2 cm.

(B) Left: spleen volume in healthy controls and patients with ischemic stroke during early (<24 hr) and later (day 7–10) stages. Control: n = 40; day 1: n = 40; day 7–10: n = 30. Right: paired comparison shows change of spleen volume in the individual stroke patients. Day 1: n = 30; Day 7–10: n = 30. *p < 0.05 by one-way ANOVA.

(C) Counts of circulating lymphocytes in patients after onset of ischemic stroke.

(D) Dot plots show gated peripheral NK cells (CD3−CD56+) in a healthy control and an ischemic stroke subject. Bar graph shows NK cell counts in healthy controls and patients with ischemic stroke during early (<24 hr) and later (day 3 and day 7–10) phases.

(legend continued on next page)
way to establish their potential roles in host susceptibility during post-stroke infection (Prass et al., 2003; Hug et al., 2009; Wong et al., 2011), we chose to focus on NK cells because of these cells’ capacity to produce multiple cytokines that enable orchestration of the first steps and tuning of the intensity of both innate and adaptive immune responses (Long et al., 2013; Poli et al., 2013; Vivier et al., 2011). Additionally, NK cells operate as very early responders to pathogen invasion through their cytolytic activity and, importantly, NK cells are among the first cell subsets to home to the brain promptly after brain ischemia, partly driven by neuron-derived fractalkine (Chu et al., 2014; Gan et al., 2014; Mracsko et al., 2014; Zhang et al., 2014). NK cells were abundant in post-mortem tissues from stroke patients (Gan et al., 2014). In mice with large infarcts induced by middle cerebral artery occlusion (MCAO), NK cells promoted local inflammation and neuronal hyperactivity, lost tolerance to neurons, and exacerbated brain infarction (Gan et al., 2014; Zhang et al., 2014). Because of these reasons, by determining the compartmentalized immune alterations on the ischemic brain and the role of neurogenic innervation, we demonstrate that the modulation of neurogenic pathways can restore NK cell-mediated immune defense against post-stroke infection.

RESULTS

Spleen Atrophy and Peripheral NK Cell Contraction in Patients with Ischemic Stroke

The impact of brain ischemia on the immune system has been documented mostly in peripheral lymphocytes (Hug et al., 2009; Liesz et al., 2009; Prass et al., 2003). Here, we examined both lymphoid organs and lymphocytes. Patients undergoing acute phase of ischemic stroke (≤24 hr) exhibited marked atrophy of the spleen, as measured by magnetic resonance imaging (MRI). Seven to ten days after onset of brain ischemia, the spleen size returned to its initial volume (Figures 1A and 1B). Those patients also exhibited lymphopenia (Figure 1C).

In parallel with the reduced spleen size, circulating NK cell numbers were significantly reduced, and this deficit was accompanied by a decrease in the expression of activating receptor (CD69) at day 1 after brain ischemia that normalized with a return to basal values at days 7–10 (Figures 1D and 1E).

NK cell numbers in the ischemic brain peaked during early phase of stroke onset (<24 hr), then declined by 3 to 10 days later (Figure 1F), as indicated by the NKp46 marker that is specific for NK cells in humans and in mice (Walzer et al., 2007). Expression of the CD69 on NK cells was also higher in the ischemic brain during acute phase and declined thereafter (Figure 1G). In all, NK cells underwent compartment contraction in the periphery and CNS of patients with ischemic stroke.

Effects of Brain Ischemia on NK Cells in the Periphery versus the CNS in Mice

To confirm the findings in patients with ischemic stroke, we induced transient ischemia-reperfusion in mice via transient 60 min MCAO. Seven-Tesla (7T) rodent magnetic resonance imaging (MRI) was used to measure spleen volume in these mice. At 24 hr after onset of brain ischemia, spleen volumes were significantly reduced (Figure 2A) as compared to sham-operated controls. Resolution of splenic atrophy followed at day 7 after brain ischemia (Figure 2A).

Administration of fingolimod (FTY720), which sequesters sphingosine 1-phosphate receptor (S1PR)-expressing immune cells within the spleen prior to MCAO (Aktas et al., 2010; Brinkmann et al., 2010), failed to prevent the decrease in spleen volume after MCAO (Figure 2A). This suggests that S1PR-dependent egress of immune cells from the spleen into the circulation did not contribute to the rapid reduction in spleen size after brain ischemia.

Lymphopenia occurred in mice subjected to MCAO and subsequently recovered to normal lymphocyte counts at day 7 after MCAO (Figure 2B). Next, we compared the NK cell phenotypes in the spleens and brains of MCAO mice. Molloy RNIDahamine B (MIRB) is an ultra-small, super-paramagnetic iron oxide particle (USPIO) of 35 nm that is not toxic for NK cells and can be visualized by MRI (Jin et al., 2016). NK cells were obtained from wild-type mice, labeled with MIRB in vitro, and then passively transferred into 

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Lymphopenia occurred in mice subjected to MCAO and subsequently recovered to normal lymphocyte counts at day 7 after MCAO (Figure 2B). Next, we compared the NK cell phenotypes in the spleens and brains of MCAO mice. Traditionally, NK cells are expressed by the spleen prior to MCAO (Aktas et al., 2010; Brinkmann et al., 2010), failed to prevent the decrease in spleen volume after MCAO (Figure 2A). This suggests that S1PR-dependent egress of immune cells from the spleen into the circulation did not contribute to the rapid reduction in spleen size after brain ischemia.

In (C)–(E), dot plots represent the results from three independent experiments. n = 6 patients per group. *p < 0.05, **p < 0.01 by one-way ANOVA.
highest number of NK cells appeared during early stages of ischemia, then contracted afterward, suggesting a contraction of NK cells in the brain after early stages of ischemia.

**Brain Ischemia Induces Different Transcriptome of NK Cells in the Spleen and Brain**

To evaluate whether the above differences between NK cells from spleen and brain after brain ischemia are associated with gene alterations, we performed nCounter digital multiplexed gene expression analysis (Kulkarni, 2011; Yamasaki et al., 2014). Using flow cytometry-sorted NK cells from spleen and brain at different time points after sham or MCAO procedures (Figure 3A), we observed alterations of inflammatory transcriptomes in brain-derived NK cells after ischemia (Figures 3B and 3C). In NK cells isolated from brains 1 day after MCAO, the expression of 120 genes was altered as compared to sham controls, whereas in splenic NK cells, 55 genes were altered (Figure 3C). At day 3 after MCAO, changes of 127 genes were seen in NK cells isolated from brains of MCAO mice versus sham controls, whereas in splenic NK cells 80 genes were altered (Figure 3C). These findings suggest that NK cells in the ischemic brain undergo different changes than those in the periphery.

In the analysis of the effects of brain ischemia on canonical pathways in brain-infiltrating and splenic NK cells at day 3 after MCAO (Figures 3D and 3E), alterations were found for molecules related to cytokine-mediated signaling, cellular defense responses, and cytotoxicity including the JAK-STAT pathway, Eomes, Soxs3, Runx3, and downstream molecules including Ifng, Cd69, Prf1, Gzm, Cd266, and Tnfaip3 (Figure 3E). Ifng and Cd69 were among the most markedly decreased factors, and notably dysregulated factors were associated with NK cell activation or inhibition (Figures 3E and 3F).

Together, these data indicate differences between NK cells in the brain and in the periphery after brain ischemia, including an altered expression of molecules involved in cell signaling, cell activation, and inhibition.

**Catecholaminergic and HPA Axis Innervations Suppress Peripheral NK Cells after Brain Ischemia**

We hypothesized that neurogenic signals received by NK cells contributed to splenic atrophy and impaired function in stroke. To study this possibility, we used the β-adrenergic receptor blocker propranolol for adrenergic blockade, RU486 for glucocorticoid receptor blockade, and nicotinic acetylcholine receptor Chrb2−/− mice for the genetic deletion of cholinergic innervation for NK cells. We found that treatment with propranolol or RU486 reduced splenic atrophy after MCAO (Figures 4A–4C). Treatment with propranolol together with RU486 further precluded splenic atrophy (Figures 4A–4C). In contrast, perturbation of cholinergic innervations via genetic deletion of Chrb2 failed to prevent splenic atrophy (Figure S1). Next, we examined the effects of propranolol, RU486, or propranolol + RU486 on NK cell phenotype after brain ischemia in mice. Treatment with either propranolol or RU486 significantly but only partially prevented the decline of NK cell counts, preserved expression of CD69 and IFN-γ in NK cells, and limited NK cell apoptosis after MCAO (Figures 4D and 4E). Treatment with propranolol + RU486 preserved at a greater extent the number of NK cells and their expression of CD69 and IFN-γ (Figures 4D and 4E). Conversely, perturbation of cholinergic innervations via genetic deletion of cholinergic receptors failed to prevent stroke-induced NK cell deficiency in the periphery (Figure S2). Of note, silencing the β2-adrenergic receptor and glucocorticoid receptor using siRNA preserved peripheral NK cell competence after MCAO (Figure S3). In addition to NK cells, a systematic assessment of numbers of other immune cell types in spleen of wild-type mice subjected to MCAO also revealed some effect of RU486 + propranolol on T cells, but no significant effect on monocytes, neutrophils, and dendritic cells (Figure S4). However, the effect of RU486 and propranolol on NK cells was not affected by absence of T cells in Rag2−/−/Il2rg−/− mice subjected to NK cell transfer and followed by MCAO (Figure S4). These results also suggest that the effect of RU486 and propranolol on NK cells may not result from their impact on other immune cell types.

To determine the specific changes in NK cell genes in response to propranolol and RU486 treatment, we compared the NK cell signatures in MCAO mice with or without propranolol and RU486 treatment. Among the genes whose expression was altered by propranolol and RU486 treatment, the suppressor of cytokine signaling 3 (SOCS3) was affected, together with genes from STAT family members and downstream targets (Figures 5A–5D). Considering that SOCS3 functions as an inhibitor of NK cell activity (Braunschweig et al., 2011;
Toka et al., 2009; Xu et al., 2012), we hypothesize that SOCS3 may contribute to the contraction of the NK cell response in the periphery caused by adrenergic and HPA axis input. Indeed, silencing SOCS3 with siRNA rescued to a large extent the number and activity of NK cells in the spleen (Figures 5E–5G), suggesting that SOCS3 can represent an integral mediator of neural regulation for NK cells in the periphery after brain ischemia.

Cholinergic Innervation Inhibits Brain-Infiltrating NK Cells after Brain Ischemia
In the ischemic brain, NK cells localize in close proximity to ischemic neurons (Gan et al., 2014; Zhang et al., 2014). This physical proximity may allow NK cells to receive neurogenic signals that enable the decline of NK cells after the acute stage of brain ischemia. One well-recognized neurotransmitter that influences lymphocyte functions is ACh, which binds
We previously demonstrated that the mRNA expression of \( \beta^2 \)-nAChR in splenic NK cells is stronger than in other nicotinic receptor subtypes (Hao et al., 2013). The expression of \( \beta^2 \)-nAChR protein in NK cells was confirmed at the protein level (Figures 6A and 6B), suggesting that \( \beta^2 \)-nAChR engagement on NK cells could influence their activity. To test this hypothesis, we examined the contribution of \( \beta^2 \)-nAChR-mediated signaling to the alteration of the NK cell response in the ischemic brain. Genetic deletion of *Chrnb2* prevented the decline of the NK cell response in the ischemic brain at day 3 after MCAO, as indicated by increased numbers of hypointensive dots (MIRB-labeled NK cells) (Figures 6C and 6D). This finding was consistent with the results of flow cytometry (Figures 6E and 6F). Also, genetic deletion of *Chrnb2* preserved NK cell expression of CD69 and IFN-\( \gamma \) (Figures 6G and 6H) but had no significant impact on infarct volume (Figure S2).
To determine the direct effect of ACh on NK cells, we exposed wild-type and Chrnb2−/− NK cells to ACh in vitro. Among the genes altered in ACh-treated wild-type NK cells but not in ACh-treated Chrnb2−/− NK cells, we identified a decrease in the runt-related transcription factor 3 (RUNX3) gene, along with a dysregulation of RUNX3-related transcription factors and downstream target genes (Figures 6I–6K). RUNX3 is a key transcription factor for proper NK cell function. A deficiency of RUNX3 results in the contraction of the NK cell compartment and the reduced production of effector molecules including IFN-γ (Ebihara et al., 2015; Lai and Marger, 2012; Levanon et al., 2014; Lotem et al., 2013; Ohno et al., 2008). To understand the potential contribution of RUNX3 downregulation to NK cell alteration in the ischemic brain, we transferred NK cells with transgenic expression of RUNX3 into Rag2−/− Il2rg−/− recipients prior to MCAO (Figure 6L). Rescuing RUNX3 downregulation preserved NK cell responses in the brain at day 3 after MCAO (Figure 6M), suggesting that RUNX3 silencing on splenic NK cell counts and expression of IFN-γ or CD69 at day 1 after MCAO. Plots represent the results from three independent experiments. n = 15 mice per group. *p < 0.05, **p < 0.01 by two-tailed unpaired Student’s t test. Error bars represent mean ± SEM.

Modulation of Neurogenic Pathways Preserves NK Cell Function and Improves Host Immune Defense against Post-stroke Infection

In some patients with ischemic stroke, the Gram-positive intracellular bacterium Listeria monocytogenes (LM) causes infection and/or septicemia that can be fatal (Hamon et al., 2006; Pamer, 2004). Since NK cell-derived IFN-γ confers immune protection from LM infection (Hamon et al., 2006; Pamer, 2004), we utilized a LM infection model to evaluate if and how NK cells could control LM infection after brain ischemia. Adoptive transfer of NK cells into Rag2−/− Il2rg−/− mice prior to sham operations protected them from sublethal LM infection (Figures 7A–7C). In contrast, the mortality rate from LM infection was high in Rag2−/− Il2rg−/− mice that received NK cells and underwent MCAO (Figure 7A) and associated with a heavy LM burden in the brain, liver, and spleen during early infection, together with insufficient IFN-γ amount (Figures 7B–7F). Thus, the...
otherwise effective NK cell-mediated control of LM infection was impaired after stroke both in the CNS and systemically. Of note, the administration of propranolol and RU486 reduced the mortality rate from LM infection in Rag2\(^{-/-}\)Il2rg\(^{-/-}\) mice that had received NK cells and MCAO (Figure 7A). Protection was accompanied by increased systemic amount of IFN-\(\gamma\) (Figure 7B) and a decreased LM burden in the liver and spleen (Figures 7D and 7E). Moreover, adoptive transfer of Chrnb2\(^{-/-}\)-NK cells into Rag2\(^{-/-}\)Il2rg\(^{-/-}\) recipients prior to MCAO promoted an IFN-\(\gamma\) burst, reduced LM burden in the ischemic brain, and prolonged survival after LM infection, as compared to Rag2\(^{-/-}\)Il2rg\(^{-/-}\) mice transferred with wild-type NK cells (Figures 7A and 7C–7F). Last, Rag2\(^{-/-}\)Il2rg\(^{-/-}\) mice receiving Chrnb2\(^{-/-}\)-NK cells prior to MCAO plus propranolol and RU486 before infection with LM displayed an IFN-\(\gamma\) burst, reduced LM burden, and further increased survival (Figures 7A–7F).

Pneumonia is the leading cause of death among stroke patients (Kaira et al., 2015; Westendorp et al., 2015). Consistent with previous reports (Prass et al., 2003; Wong et al., 2011), all MCAO mice spontaneously developed pneumonia. Therefore, we determined the contribution of NK cells to the immune defense against pneumonia after brain ischemia. Our results show that blockade of adrenergic and HPA axis innervation of NK cells in the periphery by administering propranolol and RU486 significantly boosted the NK cell-mediated immune defense against post-ischemic pneumonia (Figure S6).

Together, these data indicate that manipulation of neurogenic innervation with pharmacological or genetic approaches can preserve NK cell competence and boost NK cell-mediated immune defenses against post-stroke infection (Figure S7).

**DISCUSSION**

Pneumonia, urinary tract infection, and infections in other organ systems are relatively common during the subacute stage of stroke (21%–65% of patients), associated with ~20% of all deaths, and related to considerable morbidity in survivors (Westendorp et al., 2011). Recent clinical trials conducted in stroke patients haven’t demonstrated significant benefit of prophylactic antibiotics (Kaira et al., 2015; Westendorp et al., 2015), mandating the need to better understand the basis of stroke-induced immune suppression and possible identification of new modalities of intervention for the suppressed host immune defense after stroke. Here we have revealed that brain ischemia impacts immunity differentially in the periphery and brain. This is reflected by distinctive kinetics, cellular and transcriptome features of NK cells, and the immune defense orchestrated by these cells in these two compartments. Neurogenic innervations as well as cell signaling pathways that govern post-stroke immunosuppression also differ. Finally, our results demonstrated that targeting specific neurogenic pathways can combat post-stroke infection, thereby offering an alternative and potentially effective means to antibiotics.

Acute ischemic brain injury swiftly activates the sympathetic, parasympathetic, and HPA axis pathways, leading to the release of norepinephrine (NE), ACh, and glucocorticoids (GCs) (Meisel et al., 2005; Prass et al., 2003; Rosas-Ballina and Tracey, 2009; Wong et al., 2011). Adrenergic and the HPA axis pathways act synergistically to induce splenic atrophy and NK cell deficiency in the peripheral compartment via coordinated effects of NE and GCs. The subsequent upregulation of SOCS3 induced by adrenergic and HPA axis innervation
acts as a negative feedback inhibitor to suppress NK cells in the peripheral compartment. In the CNS, we and others reported that NK cells are reduced in numbers after acute stroke, although the kinetics differs from that of periphery (Gan et al., 2014; Zhang et al., 2014). The close proximity of these NK cells to ischemic neurons (Gan et al., 2014; Zhang et al., 2014), as well as the inhibitory effect of ACh on NK cells (Hao et al., 2013), suggests that different mechanisms operate in the periphery and CNS to suppress cellular immunity. In line with those findings, we now identify cholinergic innervation through β2-nAChR as the factor responsible for altered NK cell responses in the brain after ischemia, and the involvement of RUNX3. The difference in dynamic transition of brain-infiltrating NK cells from early accumulation with effector function to a subsequent reduced number and activity as compared to the periphery suggests that intrinsic organ-specific elements can shape NK cell responses after NK cells enter the brain.

This study has clinical implications for the management of stroke patients. The identification of compartmental effects and specific neurogenic pathways suggests that immune modulation needs to take the compartmental effects, i.e., CNS versus the periphery, into consideration. Additionally, due to the high prevalence and morbidity of infection in stroke patients and the lack of benefits in the use of antibiotics in those patients (Kalra et al., 2015; Westendorp et al., 2015), the finding that pharmacological manipulation can preserve spleen size and NK cell competence offers means to improve host defenses against pneumonia or LM infection in stroke patients. This could provide an approach for the prevention of post-stroke infection. Another finding of this study is that the identified time window during which NK cells progress from competence to contraction can allow the inhibition of NK cell function for attenuation of brain damage in acute stroke, whereas a boost would be needed during subacute stroke to combat infection. Finally, this study proposes pharmacologic strategies—that need further testing—to improve host immune defense while leading to reduced ischemic damage and improved clinical outcomes.

Figure 7. Neurogenic Modulation Restores NK Cell Activity and Their Immune Defense against LM Infection after Stroke, Both at the Systemic and CNS Levels

Wild-type and Chrnb2−/− NK cells were obtained from wild-type and Chrnb2−/− mice, respectively. 3 × 10⁶ wild-type or Chrnb2−/− NK cells were injected i.v. into Rag2−/−Il2rg−/− mice followed by sham or MCAO operations with or without treatment of propranolol and RU486. Another group of Rag2−/−Il2rg−/− mice was treated with RU486 + propranolol and did not receive NK cells. Immediately after surgery, mice were inoculated i.p. with 300 CFU LM.

(A) Survival of post-ischemic Rag2−/−Il2rg−/− mice receiving the treatments indicated. n = 15 mice per group. Data are from four independent experiments.

(B and C) Blood (B) or brain homogenate (C) were collected at day 3 after surgery, and IFN-γ expression was analyzed by ELISA. n = 9 mice per group. *p < 0.05, **p < 0.01 by one-way ANOVA.

(D–F) Bacterial culture from liver (D), spleen (E), and brain (F) of controls (sham) or post-ischemic Rag2−/−Il2rg−/− mice at day 3 after surgery. n = 9 mice per group. *p < 0.05, **p < 0.01 by one-way ANOVA.

Error bars represent mean ± SEM. Please see also Figures S6 and S7.
**EXPERIMENTAL PROCEDURES**

**Human Subjects and Magnetic Resonance Imaging**

Forty patients who presented at the Tianjin Medical University General Hospital with clinically definite cerebral infarction within 24 hr of symptom onset were recruited into the study (Table S1). The inclusion criteria were a clinically definite cerebral infarction (CI) within 24 hr of symptom onset and age of at least 18 years. Details on human subjects and MRI parameters are provided in the Supplemental Experimental Procedures.

**Human Post-mortem Brain Tissue**

Human brain sections were provided by the Department of Pathology of the Ohio State University and Banner Boswell Medical Center. Among the 12 cases studied, 4 were from patients with ischemic stroke who died within 1 day after stroke onset, 4 were from patients with ischemic stroke who died within 3–10 days after stroke onset, and another 4 control subjects were from individuals with no history of neurological or neuropsychiatric diseases who died from non-neurological disease. Brain tissues were collected within 4 hr after death. Postmortem histopathological examination of brain sections by a neuropathologist confirmed that no pathological alterations were related to neurological or neuropsychiatric diseases. Stroke patients and control subjects did not differ significantly in terms of age at death (day 1: 78.3 ± 6.6 years of age; day 3–10: 72.5 ± 8.3 years of age; controls: 68.8 ± 6.2 years of age; p > 0.05, one-way ANOVA; mean ± SEM).

**Mice**

Male C57BL/6 (B6, H2b) and Rag2−/−T2rg−/− (H2b) mice were purchased from Taconic. nAChR β2-subunit genetically ablated (Chrnb2−/−) mice were kindly provided by Dr. M. Picciotto (Yale University). To specifically track NK cells in the absence of NK1.1-expressing, CD1d-dependent NKT cells, Cd1d−/−NK1.1−tdTomato mice were generated by crossing Cd1d−/− and NK1.1−tdTomato mice provided by Dr. W. Yokoyama (Washington University). All mutant mice were backcrossed to the B6 background for at least 12 generations. Details of mice used in this study are given in the Supplemental Experimental Procedures.

**MCAO Procedure, Neuroimaging, and Immunosuppressing**

Adult male 10- to 12-week-old mice were subjected to 60 min focal cerebral ischemia produced by transient intraluminal occlusion of the middle cerebral artery (MCA) by using a filament, as described previously (Gan et al., 2014; Jin et al., 2016). Details of the MCAO procedures, MRI scan, and immunosuppressing are provided in the Supplemental Experimental Procedures.

**Drug Administration**

All drugs were injected i.p. into the animals. RU486 (Sigma-Aldrich) was dissolved in an ethanol/sesame oil solution (1:10, 6 mg/mL) and given at 30 mg/kg body weight immediately after MCAO. Propranolol (Sigma-Aldrich) was dissolved in saline at 6 mg/mL and given at 30 mg/kg immediately after MCAO. FTY720 (Sigma-Aldrich) was dissolved in saline at 1 mg/mL and given at 1 mg/kg immediately after MCAO.

**Cell Isolation, Cell Labeling, Cell Passive Transfer, RNA Interference, and Plasmid cDNA Transfection**

NK cells were sorted from pooled splenocytes of wild-type or mutant mouse strains. Details on NK cell isolation, cell labeling, cell passive transfer, RNA interference, and plasmid cDNA transfection are given in the Supplemental Experimental Procedures.

**Flow Cytometry**

Quantitative analysis of immune cell subsets prepared from spleen or brain tissue were stained with fluochrome-conjugated antibodies as described (Gan et al., 2014; Hao et al., 2010; Liu et al., 2016). Details are given in the Supplemental Experimental Procedures.

**Gene Expression Profiling and Real-Time PCR**

NK cells were isolated from pooled splenocytes and brain tissues of B6 mice at days 1 or 3 after MCAO or sham operations (Gan et al., 2014; Hao et al., 2010; Liu et al., 2016). Details on gene expression profiling and real-time PCR are given in the Supplemental Experimental Procedures.

**In Vivo Infection, Microbiological Analyses, and ELISA**

Listeria monocytogenes (LM) strain EGD was cultured in Brain Heart Infusion broth as previously described (Liu et al., 2012) and stored in 30% glycerol at −80°C until use. Details on in vivo infection, microbiological analyses, and ELISA are given in the Supplemental Experimental Procedures.

**Statistical Analyses**

Details on statistical analyses are given in the Supplemental Experimental Procedures.

**ACCESSION NUMBERS**

The accession number for the Nanostring data reported in this paper is GEO: GSE95100.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes several figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2017.02.015.

**AUTHOR CONTRIBUTIONS**

Q.L. and F.D.-S. designed the studies; Q.L., W.-N.J., Y.L., K.S., H.S., F.Z., and C.Z. performed the studies; R.J.G., K.N.S., and A.L.C. advised on design, execution of experiments, and interpretation of results; and Q.L., W.-N.J., A.L.C., and F.D.-S. wrote and edited the manuscript.

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