Astrocyte-derived interleukin-15 exacerbates ischemic brain injury via propagation of cellular immunity

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Astrocytes are believed to bridge interactions between infiltrating lymphocytes and neurons during brain ischemia, but the mechanisms for this action are poorly understood. Here we found that interleukin-15 (IL-15) is dramatically up-regulated in astrocytes of postmortem brain tissues from patients with ischemic stroke and in a mouse model of transient focal brain ischemia. We generated a glial fibrillary acidic protein (GFAP) promoter-controlled IL-15–expressing transgenic mouse (GFAP–IL-15^{tg}) line and found enlarged brain infarcts, exacerbated neurodeficits after the induction of brain ischemia. In addition, knockdown of IL-15 in astrocytes attenuated ischemic brain injury. Interestingly, the accumulation of CD8$^+$ T and natural killer (NK) cells was augmented in these GFAP–IL-15^{tg} mice after brain ischemia. Of note, depletion of CD8$^+$ T or NK cells attenuated ischemic brain injury in GFAP–IL-15^{tg} mice. Furthermore, knockdown of the IL-15 receptor α or blockade of cell-to-cell contact diminished the activation and effector function of CD8$^+$ T and NK cells in GFAP–IL-15^{tg} mice, suggesting that astrocytic IL-15 is delivered *in trans* to target cells. Collectively, these findings indicate that astrocytic IL-15 could aggravate postischemic brain damage via propagation of CD8$^+$ T and NK cell-mediated immunity.

astrocyte | interleukin-15 | lymphocytes | inflammation | ischemic brain injury

Infiltrating leukocytes such as lymphocytes are major effectors of postischemic brain inflammation (1–6). The phenotype and function of infiltrating lymphocytes are largely dictated by organ-specific intrinsic factors during inflammatory responses (7–9), and such factors in the brain are unique in terms of cellular constituents, blood–brain barrier (10–12), and microenvironment (1–3, 7). As the most abundant cell type in the CNS, astrocytes constitute nearly 50% of the human brain's volume. Astrocytes contribute to the regulation of neural transmission, survival of neurons and other glia cells, and integrity of the blood–brain barrier. In the inflamed CNS, astrocytes engage in significant cross-talk with CNS-infiltrating immune cells by providing a major source of the proinflammatory cytokines and chemokines, thereby activating infiltrating lymphocytes. Evidence has shown that astrocytes can exert potent proinflammatory functions by producing factors including monocyte chemotactic protein-1 (MCP-1/CCL2), interleukin 1 beta (IL-1β), interleukin-6 (IL-6), etc., as their primary mode of action after CNS injury. In addition, astrocytes are considered as important nonprofessional antigen-presenting cells. Depending on the stage of brain pathology, astrocytes also possess antiinflammatory properties such as scar formation and restriction of inflammation by producing transforming growth factor-β (13, 14). Recent studies have shown that the inhibition of astrocytes correlates with decreased infarct size (15, 16) and that treatments capable of decreasing infarct size are often accompanied by attenuated astrocyte responses. These findings suggest a detrimental role for astrocytes after brain ischemia (15–18). However, still unknown are whether and how astrocytes shape acute CNS immune responses in the context of a postischemic brain and whether this process has any clinical significance.

IL-15 belongs to a family of cytokines using the common γ-chain as a component of their receptors (19, 20). IL-15 interacts specifically with the high-affinity IL-15 receptor α (IL-15Rα) and binds to IL-2/IL-15Rβ and a common γ-chain expressed by target cells (21–23). In the periphery, monocytes and dendritic cells are the main sources of IL-15 (24, 25). IL-15 maintains homeostasis and cytotoxic activities of lymphocytes that bear its receptor [i.e., natural killer (NK) and CD8$^+$ T cells] (19, 20). Some studies have demonstrated that IL-15 contributes to the immunopathology of several inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease (26, 27). Despite recent studies suggesting astrocytes as a major source of IL-15 in the inflamed CNS (28–30), the potential role of astrocytic IL-15 in ischemic brain injury remains elusive.

Our observations of dramatically elevated IL-15 expression in astrocytes after ischemia and reperfusion established a rationale for further investigation of astrocytes and their derived IL-15 in brain ischemia. To this end, we have generated a transgenic mouse line with efficient expression of glial fibrillary acidic protein (GFAP) promoter-controlled IL-15 (GFAP–IL-15^{tg}), characterized their lesions, and defined the mechanisms of IL-15 action. Our findings reveal that IL-15 is a key factor of astrocytes in controlling the magnitude of CNS inflammation and brain injury after ischemia.

Results

Expression of IL-15 in Astrocyte Is Highly Up-Regulated After Brain Ischemia. To gain a comprehensive view of astrocyte-derived factors after brain ischemia, we isolated glutamate/aspartate transporter-positive (GLAST$^+$) astrocytes (purity > 99%;

Significance

Ischemic stroke is a leading cause of death and disability worldwide. Evidence indicates the detrimental effects of lymphocyte infiltration into the ischemic brain. However, a knowledge gap exists relating to the brain-specific cellular constituents and environmental factors that dictate the phenotype and function of infiltrating lymphocytes. Astrocytes bridge interactions between ischemic neurons and lymphocytes. We show that brain ischemia induces robust up-regulation of astrocytic interleukin-15 (IL-15). The present study was directed toward understanding the role of astrocyte-derived factors such as IL-15 in stroke. We discovered that astrocytic IL-15 is necessary and sufficient to amplify cell-mediated immune responses that promote ischemic brain injury. These results provide definitive evidence on the role of astrocyte-derived IL-15 in ischemic brain injury.

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Fig. S1 *A* and *B*) and adopted a proteome profiler array analysis that measured 110 proteins from lysates of these purified astrocytes at 24 h after middle cerebral artery occlusion (MCAO) in mice. Among 70 factors that were detectable after brain ischemia, chemokine (C-X-C motif) ligand 1 (CXCL1), MCP-1/CCL2, intercellular adhesion molecule (ICAM-1), and IL-15 were produced by astrocytes in relatively large amounts (Fig. 1*A*). Of note, these proteins play a role in lymphocytes' migration, adhesion, and propagation and, therefore, could serve as an integral process that drives the emergence of inflammation after brain ischemia. In this regard, we paid special attention to IL-15, given its potential action on early brain-infiltrating cells. The increase in IL-15 protein expression was confirmed by ELISA, which detected a significant increase of IL-15 in cell lysates of astrocytes obtained from post-ischemic brains (Fig. 1*B*). Meanwhile, alterations of other cytokines in astrocytes were also verified by ELISA (Fig. S1*C*). In addition, flow cytometry analysis showed significantly increased counts of IL-15^{+} astrocytes at 24 h after MCAO (Fig. 1 *C* and *D*). To further assess whether astrocytes are a major source of IL-15 in the post-ischemic brain, we compared IL-15 levels in cell lysates of astrocytes (GFAP^{+}), neurons (NeuN^{+}), and microglia (CD11b^{+}CD45^{int}) isolated at 24 h after MCAO. We found that astrocytes produced more IL-15 relative to microglia or other CNS cells after brain ischemia (Fig. 1*E*). Together, these results showed that astrocyte-derived IL-15 is a specific factor up-regulated after brain ischemia and that astrocytes are a major source of IL-15 in the CNS.

Next, we stained groups of postmortem brain sections from patients with acute ischemic stroke. IL-15 expression was readily detected from astrocytes (IL-15^{+}GFAP^{+}) in the periinfarct areas, but less was present in the microglia and neurons (Fig. 1 *F* and *G*). In contrast, IL-15–expressing astrocytes were not noticeable in control sections. The finding that IL-15 is consistently up-regulated in astrocytes of MCAO mice and patients with ischemic stroke justifies further investigation into its role in brain ischemia.

Generation and Characterization of GFAP–IL-15^{tg} Mice. To test the potential participation of astrocyte-derived IL-15 in ischemic brain injury, we generated a transgenic mouse line in which the expression of IL-15 was targeted to astrocytes by the 2.2-kb human GFAP promoter (31). The transcription construct of IL-15 is shown in Fig. 2*A*. Genotyping of the animals was accomplished by PCR analysis of genomic tail DNA targeting the 2.9-kb sequence included in the transgene construct (Fig. 2*B*). In GFAP–IL-15^{tg} mice, the mRNA and protein levels of IL-15 were significantly increased in the brain compared with those from wild-type (WT) littermates, but not in the peripheral organs such as spleen, liver, or kidney (Fig. 2 *C* and *D*). Flow cytometry analysis confirmed a robust increase of IL-15 expression in astrocytes, but not in microglia, in the brains of GFAP–IL-15^{tg} mice as compared to their WT littermates (Fig. 2 *E*–*H*).

Importantly, GFAP–IL-15^{tg} mice developed normally without showing any clinical sign of neurological disease, infertilities, or behavioral abnormalities. In addition, flow cytometry analysis showed that astrocytic overproduction of IL-15 did not affect the number of peripheral immune cell subsets (Fig. 2 *I* and *J*), suggesting that the overproduction of IL-15 was restricted to the CNS. Upon neuroimaging of brain vasculature, no difference in cerebral arteries was apparent (Fig. 2*K*). Notably, although IL-15 is considered to be a proinflammatory cytokine, no inflammatory infiltrates were seen in brain tissues of normal GFAP–IL-15^{tg} mice (Fig. 2*L*), suggesting that the overproduction of IL-15 by astrocytes may not alter brain inflammation under physiological conditions.

Astrocyte-Targeted Expression of IL-15 Exacerbates Ischemic Brain Injury. Upon induction of MCAO, GFAP–IL-15^{tg} mice exhibited more severe neurological deficits than WT mice (Fig. 3*A*). The 7T-MRI, in conjunction with 2,3,5-triphenyltetrazolium chloride (TTC) staining, revealed significantly enlarged infarcts in GFAP–IL-15^{tg} mice compared with WT mice after reperfusion (Fig. 3 *B* and *C* and Fig. S2). Aggravated neurologic deficits and enlarged brain infarcts were noticed as early as at day 1 after MCAO and persisted to day 7 (Fig. 3 *A*–*C*). GFAP–IL-15 transgenic mice also exhibited more severe impairment in motor, sensory, and balance than WT mice at day 14 after MCAO (Fig. S3).

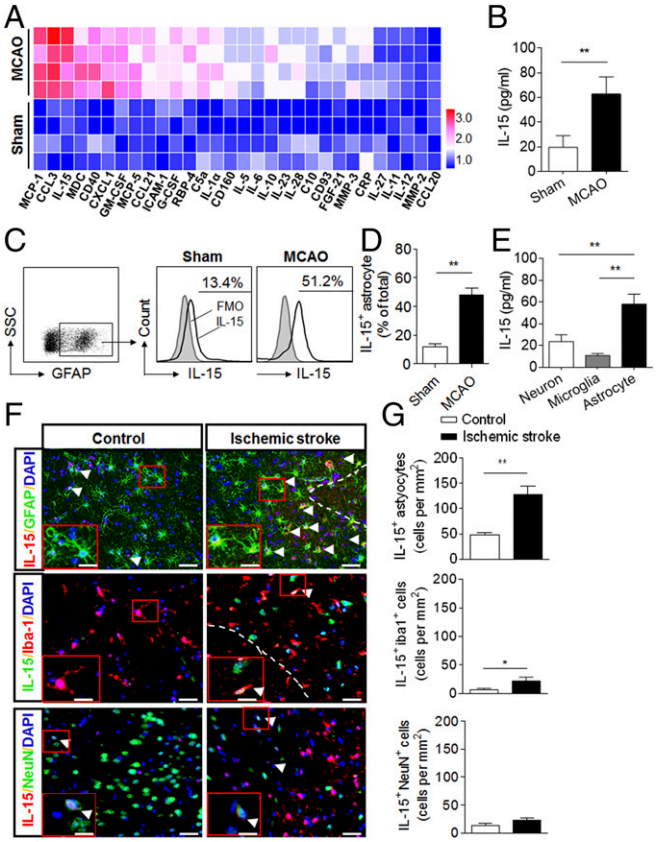

Fig. 1. Astrocytic IL-15 is robustly up-regulated after cerebral ischemia in mouse and human brains. (*A*) Heat map shows the cytokine/chemokine expression profiles of the lysates from fluorescence-activated cell sorted (FACS)-astrocytes (GLAST^{+}, >99%) from the mouse brain at 24 h after 60 min MCAO or sham controls. Procedures for the isolation and purification of astrocytes from the mouse brain are shown in Fig. S1. All gates were set by using fluorescence minus one (FMO) controls. Results were generated based on clustering of proteome profiler array measurements of the listed proteins normalized to individual reference controls. Red shades represent increased expression of proteins relative to other cell types. $n = 9$ mice per group. Data are from four individual experiments. (*B*) Quantification of ELISA shows highly up-regulated IL-15 protein expression level in lysates of astrocytes obtained from the mouse brain at 24 h after MCAO compared with sham controls. $n = 9$ mice per group. Data represent three individual experiments. (*C*) Representative flow cytometry plots show increase of IL-15^{+} astrocytes in MCAO mice at 24 h after reperfusion relative to sham controls. All gates were set by using FMO controls. (*D*) Summarized flow cytometry data show significant increase of IL-15^{+} astrocytes in MCAO mice at 24 h after reperfusion compared with sham controls. $n = 9$ mice per group. Results are from three individual experiments. (*E*) Quantification of ELISA results shows measurements of IL-15 protein in cell lysates of FACS-sorted astrocytes (GLAST^{+}), neurons (NeuN^{+}), and microglia (CD11b^{+}CD45^{int}) obtained from the mouse brain at 24 h after MCAO. All gates were set by using FMO controls. $n = 9$ mice per group. (*F* and *G*) Brain sections from patients with stroke and nonneurological disease control show that IL-15 is up-regulated after stroke and that IL-15–expressing cells in the brain are predominantly astrocytes (IL-15^{+}GFAP^{+}cell), located in the periinfarct areas. Fewer microglia (Iba-1^{+}) and neurons (NeuN^{+}) express IL-15 in the periinfarct areas of patient with stroke. [Scale bars, 40 μm (*F*) and 20 μm (*F*, *Insets*).] For IL-15 staining in astrocytes, microglia, or neurons, $n = 12$ sections from 6 patients with ischemic stroke; $n = 12$ sections from 4 nonneurological disease controls. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

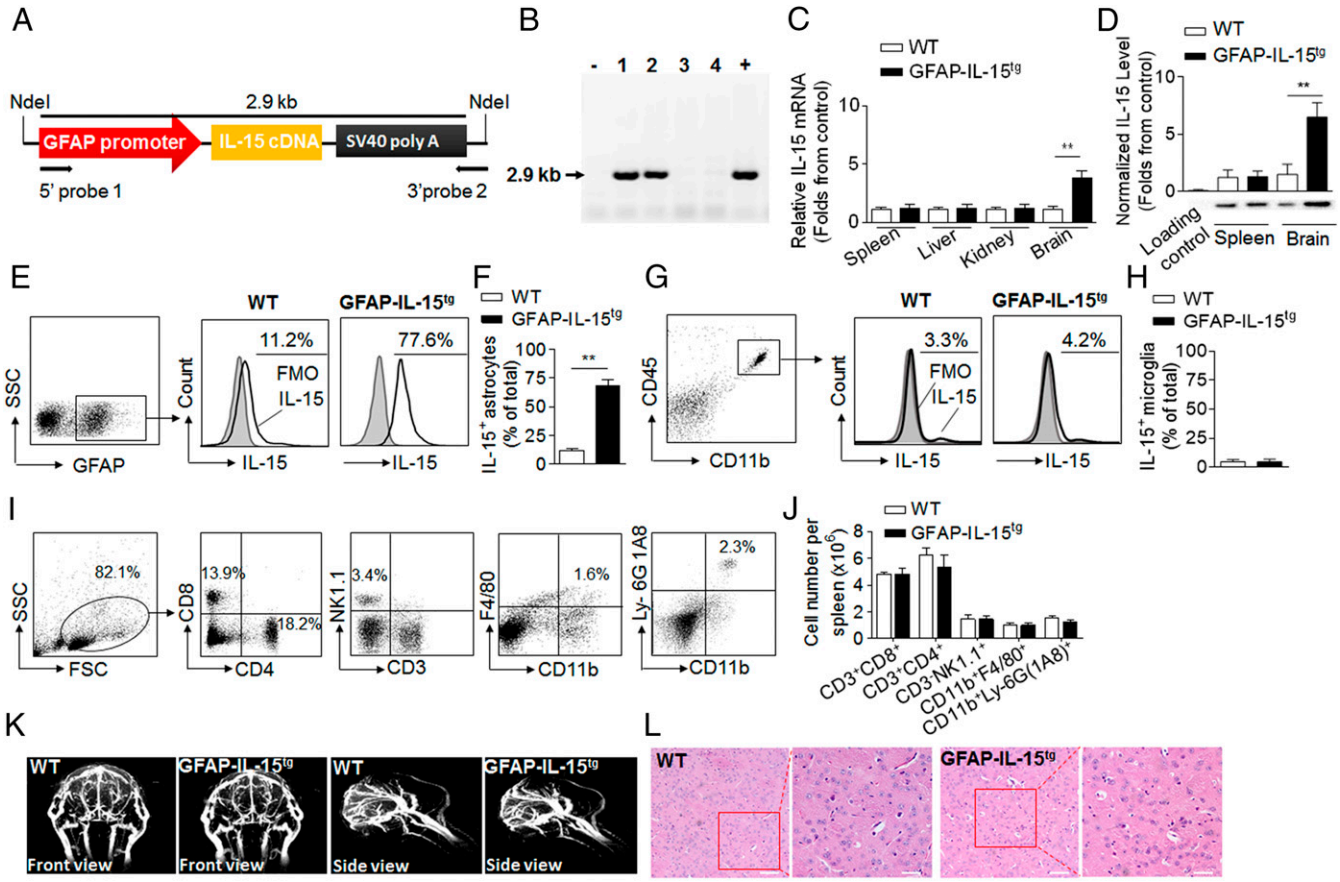

Fig. 2. Generation and characterization of GFAP–IL-15^{tg} mice. (*A*) Design of the GFAP–IL-15–SV40 construct used to generate the GFAP–IL-15^{tg} mouse. The construct incorporates mouse IL-15 cDNA under a GFAP promoter followed by the addition of a SV40 polyA signal. Construction primers are indicated by arrows under the diagram. The 5′ and 3′ terminal restriction enzymes, NdeI, enables the cleavage and subcloning of GFAP–IL-15–SV40. (*B*) Genotyping of chromosomal DNA isolated from the tails of targeted mice. Nos. 1 and 2 indicate positive germ-line transmission of the targeted allele identified by PCR; 3 and 4 indicate negative germ lines. –, negative control; +, positive control. (*C*) Results from RT-PCR demonstrate the increased expression of IL-15 mRNA in the brain tissues of GFAP–IL-15^{tg} mice. $n = 9$ mice per group. (*D*) Western blot analysis confirms the enhanced expression of IL-15 protein in the brains of GFAP–IL-15^{tg} mice. $n = 9$ mice per group. In *C* and *D*, results are from four individual experiments. (*E–H*) Flow cytometry plots show that astrocytes (GFAP$^+$), but not microglia (CD45^{int}CD11b$^+$), exhibit increased IL-15 expression in GFAP–IL-15^{tg} mice compared with WT littermates under physiological conditions. All gates were set by using FMO controls. $n = 9$ mice per group. (*I*) Representative flow cytometry plots show gating strategy of immune cell subsets in the spleen. (*J*) Quantification shows that astrocytic overproduction of IL-15 does not affect the numbers of immune cell subsets in the spleen. $n = 9$ mice per group. Results are from four individual experiments. (*K*) MRI of brain vasculature shows no alterations of cerebral vasculatures in GFAP–IL-15^{tg} mice. (*L*) After H&E staining, no inflammatory infiltrates are noticeable in brain sections of GFAP–IL-15^{tg} mice. [Scale bars, 50 μm (*L*) and 20 μm (*L*, *Insets*).] Data are expressed as mean ± SEM. $^{**}P < 0.01$.

Reactive oxygen species (ROS) production was also significantly increased in GFAP–IL-15^{tg} mice vs. WT controls at 24 h after MCAO (Fig. S4). An additional comparison then verified that astrocytes' IL-15 expression was much greater in GFAP–IL-15^{tg} mice than in WT mice at 24 h after MCAO (Fig. 3 *D–G*). Of note, knockdown of astrocytic IL-15 by using shIL-15–expressing lentivirus reduced neurodeficits and infarct volume in WT mice subjected to MCAO and reperfusion (Fig. S5). These results demonstrate that astrocyte-derived IL-15 exacerbates cerebral infarction during the acute and delay phase after brain ischemia.

Augmented Accumulation of CD8$^+$ T and NK Cells in GFAP–IL-15^{tg} Mice After Cerebral Ischemia. Reportedly, IL-15 is essential for the maintenance of lymphocytes, such as NK and CD8$^+$ T cells, in the periphery. To determine the potential impact of IL-15 overexpression on the brain's cellular infiltrates after ischemia, we measured the accumulation of infiltrating leukocytes and resident microglia after MCAO in brains of GFAP–IL-15^{tg} and WT mice by using flow cytometry (gating strategy in Fig. 4*A*). At 24 h after reperfusion, we did not observe any significant difference in the numbers of macrophages (CD11b$^+$CD45^{high}F4/80$^+$), neutrophils (CD11b$^+$CD45^{high}Ly-6G$^+$), microglia (CD11b$^+$CD45^{int}), or CD4$^+$ T cells (CD3$^+$CD4$^+$) in the ischemic brains of WT compared with GFAP–IL-15^{tg} mice (Fig. 4 *B* and *C*). In addition, the expression of proinflammatory (CD86 and CD68) or antiinflammatory (CD206) markers in microglia was not significantly different in the ischemic brains of WT and GFAP–IL-15^{tg} mice (Fig. 4*C*). In contrast, GFAP–IL-15^{tg} mice had significantly increased cerebral accumulations of CD8$^+$ T (CD3$^+$CD8$^+$) and NK (CD3$^-$NK1.1$^+$) cells after MCAO compared with their WT littermates (Fig. 4 *D–G*), and these significant differences persisted at day 7 (Fig. 4 *D* and *E*). Of interest, significantly reduced numbers of CD8$^+$ T and NK cells, but not other cell subsets, were also present in the spleen after MCAO (Fig. 4 *H–J*).

To examine whether astrocyte-targeted expression of IL-15 had an impact on the functional status of CD8$^+$ T and NK cells, we measured the expression of CD69, natural-killer group 2 member D (NKG2D), interferon gamma (IFN-γ), and perforin in CD8$^+$ T and NK cells after MCAO (gating strategy in Fig. 5*A*). We found significantly increased expression of CD69 and NKG2D in CD8$^+$ T cells in the ischemic brain of GFAP–IL-15^{tg} mice (Fig. 5*B*). Similarly, increased expression of CD69, NKG2D, and IFN-γ was also

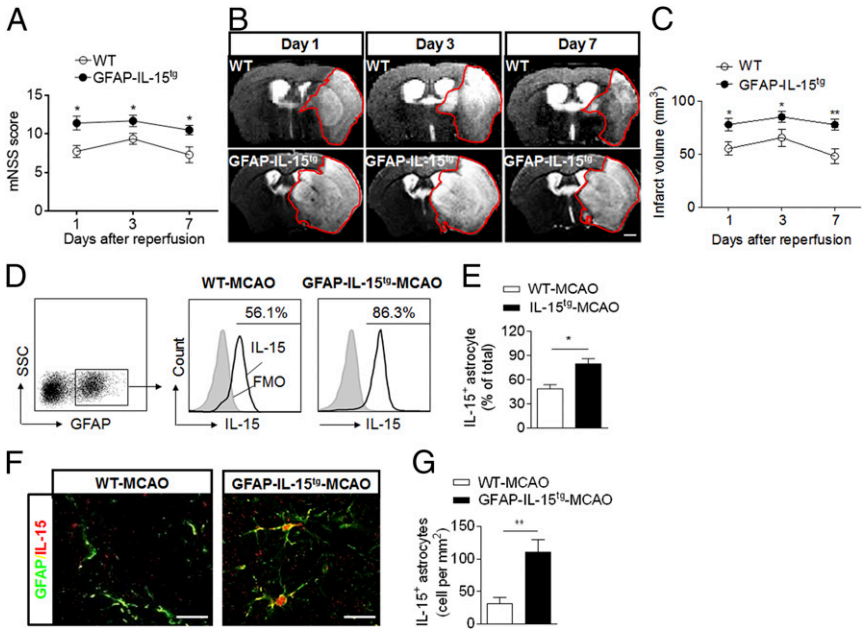

Fig. 3. Exacerbated ischemic brain injury in GFAP–IL-15^{tg} mice. (*A*) GFAP–IL-15^{tg} mice exhibited more severe neurological deficits than their WT littermates after transient focal ischemia. (*B*) Longitudinal 7T MRI images depict larger brain infarcts in GFAP–IL-15^{tg} mice than in WT littermates. (Scale bar, 1 mm.) (*C*) Quantification of 7T MRI shows significantly increased infarct volume in GFAP–IL-15^{tg} mice relative to their WT littermates. In *A* and *C*, $n = 15$ mice per group. (*D*) Flow cytometry plots show an increase of IL-15–expressing astrocytes in GFAP–IL-15^{tg} mice after brain ischemia. All gates were set by using FMO controls. (*E*) Quantification shows significant increase of IL-15–expressing astrocytes in GFAP–IL-15^{tg} mice at 24 h after MCAO. $n = 6$ per group. (*F*) Immunostaining shows IL-15 expression by astrocytes from WT and GFAP–IL-15^{tg} mice at 24 h after MCAO. The merged (yellow) images demonstrate that IL-15 (red) colocalizes with GFAP$^+$ (green) astrocytes. Astrocytes in GFAP–IL-15^{tg} mice express higher levels of IL-15. (Scale bars, 40 μm.) (*G*) Quantification shows significant increase of IL-15–expressing astrocytes in GFAP–IL-15^{tg} mice at 24 h after MCAO. $n = 15$ per group. Results are from three individual experiments. Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

seen in brain-infiltrating NK cells of GFAP–IL-15^{tg} mice after reperfusion (Fig. 5*C*). On the contrary, the expression of CD69, NKG2D, IFN-γ, and perforin was unaltered in splenic CD8$^+$ T and NK cells after MCAO (Fig. 5 *D* and *E*). These results suggest that astrocyte-derived IL-15 may contribute to the activation and accumulation of CD8$^+$ T and NK cells in the ischemic brain.

Impact of CD8$^+$ T and NK Cells on Ischemic Brain Injury in GFAP–IL-15^{tg} Mice. Recent studies have shown that CD8$^+$ T and NK cells are both prominent lymphocyte subsets in the ischemic brain and may contribute to ischemic brain injury (32–35). To determine whether the exacerbation of such injury in GFAP–IL-15^{tg} mice is mediated by the impact of astrocytic IL-15 on CD8$^+$ T and NK cells, we induced ischemia in WT and GFAP–IL-15^{tg} mice given anti-CD8 or -NK1.1 mAb 1 d before the induction of MCAO. As reported (32, 36, 37), CD8$^+$ T and NK1.1$^+$ cells (NK and NKT cells) can be efficiently depleted with anti-CD8 and -NK1.1 mAb, respectively (Fig. S6). Isotype IgG was used as a control. In MCAO mice treated with anti-CD8 mAb, anti-NK1.1 mAb, or anti-CD8 mAb + anti-NK1.1 mAb, infarct size and neurodeficits were similar in WT and GFAP–IL-15^{tg} mice (Fig. 6). In other words, the depletion of CD8$^+$ T and/or NK cells reversed the augmented brain injury in GFAP–IL-15^{tg} mice. In contrast, in MCAO mice treated with the IgG control, the neurodeficits were more severe and infarct sizes enlarged in GFAP–IL-15^{tg} mice compared with their WT littermates (Fig. 6). Because NKT cells reportedly do not significantly influence stroke (32, 33), the observed effects of anti-NK1.1 mAb treatment, if any, could be attributed to NK cell depletion. Combined, these data demonstrate the necessity of CD8$^+$ T and NK cells for astrocyte-derived IL-15 to exacerbate ischemic brain injury.

Astrocyte-Derived IL-15 Propagates CD8$^+$ T and NK Cell Responses. To further investigate the impact of astrocyte-derived IL-15 on CD8$^+$ T and NK cells, we cocultured WT or GFAP–IL-15^{tg} astrocytes exposed to oxygen glucose deprivation (OGD) with WT CD8$^+$ T or NK cells. CD8$^+$ T and NK cells were isolated from pooled WT splenocytes and purified (purity > 98%; Fig. S7). Upon coculture with GFAP–IL-15^{tg} astrocytes for 48 h, the percentages of activated CD8$^+$ T cells (CD69$^+$CD8$^+$) or NK cells (CD69$^+$CD3$^-$NK1.1$^+$) and IFN-γ–expressing CD8$^+$ T cells (IFN-γ$^+$CD3$^+$CD8$^+$) or NK cells (IFN-γ$^+$CD3$^-$NK1.1$^+$) were significantly increased compared with CD8$^+$ T or NK cells cocultured with WT astrocytes (Fig. 7). Moreover, the effects of GFAP–IL-15^{tg} astrocytes on CD8$^+$ T or NK cells were blocked by coincubation with an IL-15 mAb (Fig. 7), which selectively neutralized IL-15. These results suggest that astrocyte-derived IL-15 can directly enhance CD8$^+$ T and NK cell responses.

Astrocytes Deliver IL-15 *in Trans* to CD8$^+$ T and NK Cells. Distinguished from many other cytokines, intracellular IL-15 binds to the high-affinity IL-15Rα to form a complex, which is subsequently transported to the cell surface for an efficient cross-presentation to target cells. This process is termed "transpresentation," and membrane-bound, rather than secreted, IL-15 is crucial in mediating its effects in vivo (38–42). We showed that CD8$^+$ T or NK cells are within close proximity to astrocytes in the periinfarct area of brain sections at 24 h after MCAO (Fig. 8*A*). We also performed ELISA to detect secreted IL-15 in WT and GFAP–IL-15^{tg} astrocyte-conditioned medium, but secreted IL-15 levels were minimum and around the detection threshold of the assay (~10 pg/mL). This result suggests that IL-15 secreted by astrocytes may not have a major impact on the target cells of interest (i.e., CD8$^+$ T or NK cells).

To determine whether astrocytes provide IL-15 *in trans* to target cells, we cultured CD8$^+$ T or NK cells in cell culture reservoirs with or without culture-insert separation from cocultured WT or GFAP–IL-15^{tg} astrocytes. Consistent with previous studies showing that surface-bound IL-15 has a major effect on

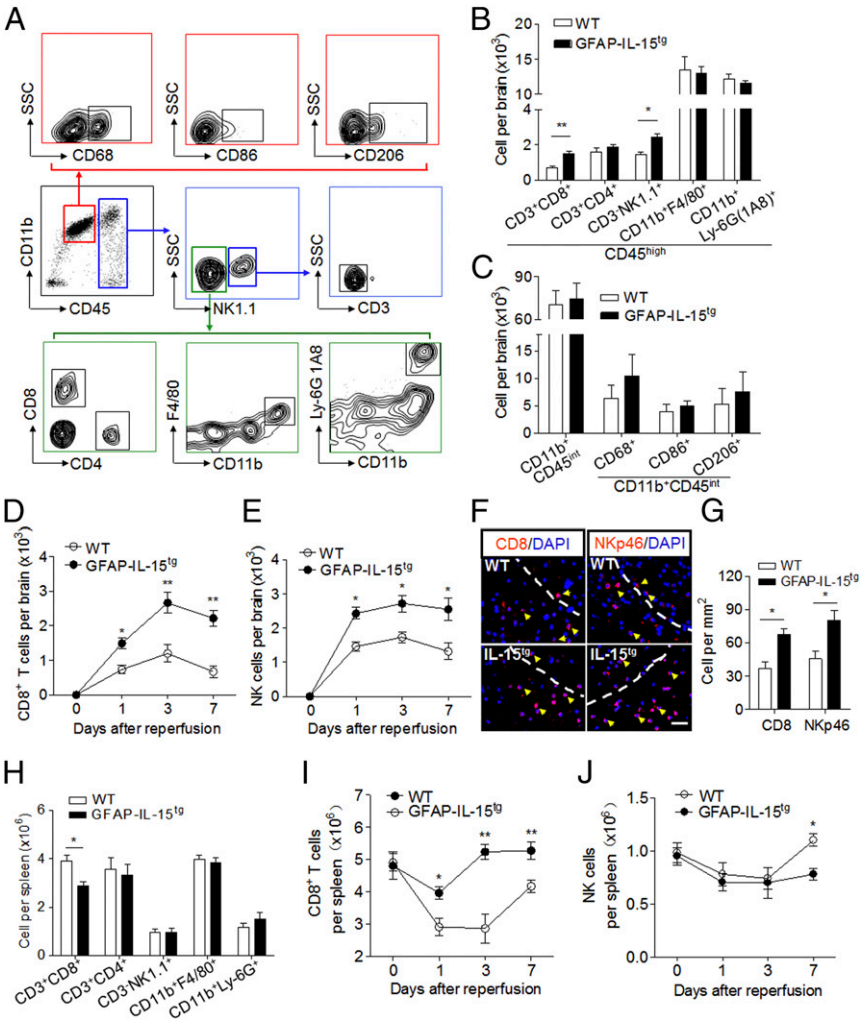

Fig. 4. Enhanced accumulation of lymphocytes in brains of GFAP–IL-15^{tg} mice after MCAO. (*A*) Gating strategy for leukocytes and microglia isolated from the brain after MCAO. All gates were set by using FMO controls. (*B*) Counts of macrophages (CD11b$^+$CD45^{high}F4/80$^+$), neutrophils (CD11b$^+$CD45^{high}Ly-6G$^+$), CD4$^+$ T cells (CD3$^+$CD4$^+$), CD8$^+$ T cells (CD3$^+$CD8$^+$), and NK cells (CD3$^-$NK1.1$^+$) in the ischemic brains of WT and GFAP–IL-15^{tg} mice at 24 h after MCAO. Data represent absolute cell numbers per brain. $n = 8$ per group. (*C*) Numbers of microglia (CD11b$^+$CD45^{int}), CD86-, CD68-, or CD206-expressing microglia in brain of WT and GFAP–IL-15^{tg} mice at 24 h after MCAO. $n = 8$ per group. (*D* and *E*) Quantification shows significantly increased counts of CD8$^+$ T and NK cells in brains of GFAP–IL-15^{tg} mice after MCAO. $n = 8$ per group. (*F* and *G*) Immunostaining of brain sections shows increased counts of CD8$^+$ T and NK cells in periinfarct areas of GFAP–IL-15^{tg} mice relative to their WT littermates at 24 h after MCAO. $n = 8$ per group. (Scale bar, 50 μm.) (*H*) Counts of splenocytes in WT and GFAP–IL-15^{tg} mice at 24 h after reperfusion. $n = 8$ per group. (*I* and *J*) Counts of CD8$^+$ T cells (*I*) and NK cells (*J*) in spleens of WT and GFAP–IL-15^{tg} mice at days 1, 3, and 7 after reperfusion. $n = 8$ per group. Results are from four individual experiments. Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

target cells (38–40, 42, 43), we found that GFAP–IL-15^{tg} astrocyte-mediated enhancement of CD8$^+$ T and NK cell responses, as manifested by their expression of CD69 or IFN-γ, was cell–cell contact-dependent (Fig. 8 *B*–*F*). Furthermore, to investigate the involvement of IL-15Rα, we knocked down IL-15Rα expression in GFAP–IL-15^{tg} astrocytes using IL-15Rα siRNA, which efficiently reduces the expression of IL-15Rα in GFAP–IL-15^{tg} astrocytes (Fig. S8). After coculture, a significantly reduced expression of CD69 or IFN-γ in CD8$^+$ T cells and CD69 in NK cells was seen after silencing of IL-15Rα in GFAP–IL-15^{tg} astrocytes (Fig. 8 *D* and *F*). These findings demonstrate that membrane-bound IL-15 transpresented by IL-15Rα is essential for the activation and effector function of CD8$^+$ T and NK cells.

To determine whether the impact of astrocytic IL-15 on CD8$^+$ T/NK cells requires transpresentation in vivo, we constructed a lentivirus vector to specifically knock down IL-15Rα in astrocytes. At day 5 after vector injection, down-regulated IL-15Rα was seen in ∼90% of astrocytes in the injected hemisphere of GFAP–IL-15^{tg} mice (Fig. S9 *A* and *B*). We also found that the percentages of CD8$^+$ T and NK cells expressing CD69, NKG2D, or IFN-γ were decreased after knockdown of IL-15Rα in GFAP–IL-15^{tg} mice compared with GFAP–IL-15^{tg} mice receiving a control vector at 24 h after MCAO (Fig. S9 *C*–*F*). These results suggest that expression of IL-15Rα is necessary for astrocytic IL-15 to boost CD8$^+$ T and NK cell responses.

Additionally, we noticed that human CD8$^+$ T (CD8$^+$) or NK cells (NKp46$^+$) resided in close proximity to astrocytes (GFAP$^+$) in the periinfarct area (Fig. 8 *G* and *H*). These findings show an astrocytic up-regulation of IL-15 and physical closeness between IL-15–expressing astrocytes and CD8$^+$ T or NK cells.

Discussion

This study identifies IL-15 as a key factor produced by astrocytes in control of brain inflammation and neural injury after ischemia and reperfusion. As documented here, astrocyte-derived inflammatory mediators such as IL-15 are necessary and sufficient to promote cell-mediated immune responses to brain ischemia. These amplified immune responses, as reflected by CD8$^+$ T and NK cells, in

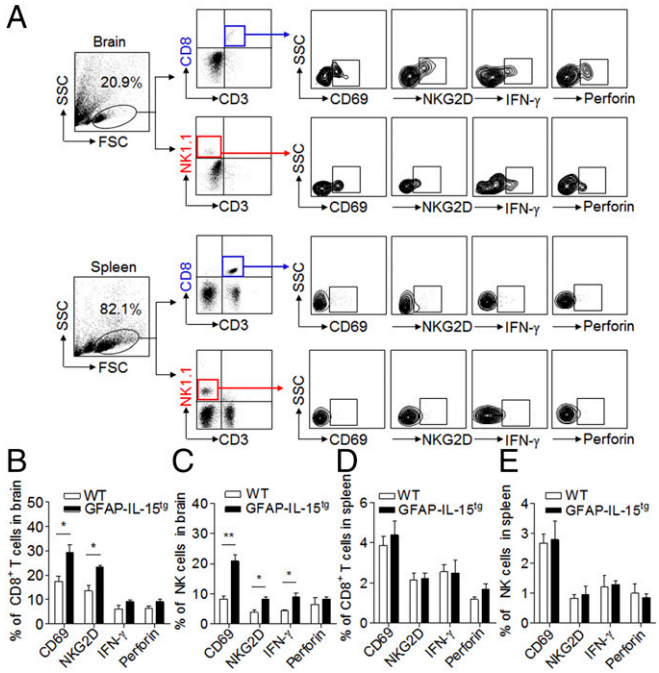

Fig. 5. CNS-restricted enhancement of $CD8^+$ T and NK cell responses in GFAP–IL-15^{tg} mice after brain ischemia. At 24 h after MCAO, the brains and spleens from GFAP–IL-15^{tg} mice and WT littermates were obtained to isolate single cells for flow cytometry analysis. (*A*) Gating strategy for the expression of CD69, NKG2D, IFN-γ, and perforin in $CD8^+$ T and NK cells in brains and spleens after MCAO. All gates were set by using FMO controls. (*B* and *C*) Quantification shows expression of CD69, NKG2D, IFN-γ, and perforin in $CD8^+$ T cells (*B*) or NK cells (*C*) isolated from brains of WT and GFAP–IL-15^{tg} mice at 24 h after reperfusion. $n = 8$ per group. (*D* and *E*) Quantification shows expression of CD69, NKG2D, IFN-γ, and perforin in $CD8^+$ T cells (*D*) or NK cells (*E*) isolated from spleens of WT and GFAP–IL-15^{tg} mice at 24 h after reperfusion. $n = 8$ per group. Results are from four individual experiments. Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

turn, contribute to ischemic neural injury. Our findings provide a better understanding of how astrocyte response to ischemia promotes brain injury.

Brain-homing immune cells participate in the onset and progression of inflammatory responses in the ischemic brain (32–34), where they become receptive to CNS cells that they had not encountered in the periphery. Among an array of factors derived by CNS cells, astrocytic IL-15 can impact the accumulation, activation, and effector function of brain-infiltrating immune cells, such as $CD8^+$ T and NK cells, both of which are prominent lymphocyte subsets considered detrimental in ischemic brain injury (32, 34, 35). Consistent with these findings, we found that both $CD8^+$ T and NK cells are preferential targets for astrocytic overproduction of IL-15 to exacerbate ischemic brain damage. These findings are supported by previous results showing that IL-15 promotes $CD8^+$ T and NK cell responses in other diseases such as rheumatoid arthritis (44–46) and multiple sclerosis (47), suggesting that the enlarged infarct size seen in GFAP–IL-15^{tg} mice may result from the enhanced $CD8^+$ T and NK responses. This notion is supported by the observation of an astrocytic IL-15–induced increase of activated $CD8^+$ T and NK cells, which suggests the involvement of direct cytotoxicity by $CD8^+$ T and NK cells in postischemic lesion enlargement. In addition to direct cytotoxicity, the production of IFN-γ by $CD8^+$ T and NK cells might activate other brain-homing immune cells, such as proinflammatory macrophages, and favor CNS inflammation. Together, our results show that brain cells such as astrocytes can shape the phenotype and function of immune cells.

Different from many other cytokines, intracellular IL-15 binds to the high-affinity IL-15Rα to form a complex, which is subsequently transported to the cell surface and delivered to target cells, a process called transpresentation. In the periphery, monocytes and dendritic cells are the main source of IL-15 (24, 25) and deliver IL-15 *in trans* to target cells. Despite a lack of direct evidence, recent studies suggest that astrocytes may serve as a potential source to deliver IL-15 *in trans* to target cells in the inflamed CNS (47, 48). Here, we show that the impact of astrocyte-derived IL-15 on $CD8^+$ and NK cells depends on both cell-to-cell contact and the expression of IL-15Rα in astrocytes, similar to the process of IL-15 transpresentation seen in peripheral organs (20, 21, 49). In addition, we found that the amount of secreted IL-15 derived by astrocytes is just above the detectable threshold and much lower than that seen in astrocyte lysates, suggesting that the impact of secreted IL-15 on target cells such as $CD8^+$ T and NK cells is minimal. Overall, our data provide evidence that astrocytes are a major source of IL-15 in the ischemic brain. Furthermore, transpresentation may serve as a crucial mechanism responsible for the detrimental effect of astrocytic IL-15 on ischemic brain injury.

Our data assign a role to astrocytes as stimuli of infiltrating $CD8^+$ T and NK cells in the ischemic brain. We noticed that overproduction of astrocytic IL-15 had no significant impact on the numbers of other immune subsets, such as monocytes and neutrophils; however, we could not exclude the potential impact of these cells on astrocytic IL-15–induced exacerbation of ischemic brain injury. Although not addressed in this study, IL-15 could participate in other aspects of neuroinflammation, given its chemotactic

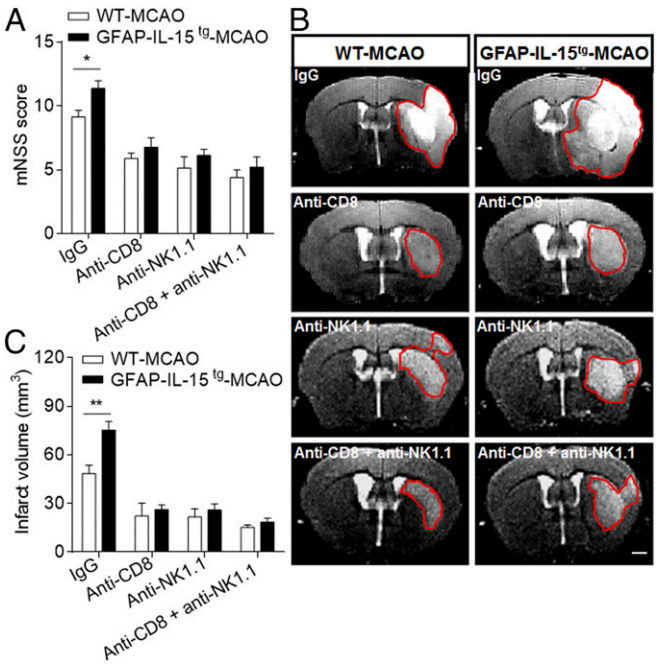

Fig. 6. $CD8^+$ T and NK cells are required for astrocyte-targeted expression of IL-15 to exacerbate ischemic brain injury. At 24 h before MCAO, GFAP–IL-15^{tg} mice and their WT littermates received i.p. injection of 200 μg of anti-NK1.1 or -$CD8^+$ mAb. (*A*) Depletion of $CD8^+$ T cells, NK cells, or $CD8^+$ T cells plus NK cells abolished the exacerbation of neurological deficits in GFAP–IL-15^{tg} mice relative to their WT littermates after brain ischemia. $n = 15$ mice per group. (*B*) The 7T MRI images show brain infarcts in WT and GFAP–IL-15^{tg} mice receiving IgG, anti-CD8, -NK1.1, or -CD8 + anti-NK1.1 mAbs at 24 h after reperfusion. (Scale bar, 1 mm.) (*C*) Quantification of brain infarct volume shows that depletion of $CD8^+$ T cells, NK cells, or $CD8^+$ T cells plus NK cells abolished the enlargement of infarct in GFAP–IL-15^{tg} mice relative to their WT littermates at 24 h after reperfusion. $n = 18$ mice per group. Results are from four individual experiments. Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

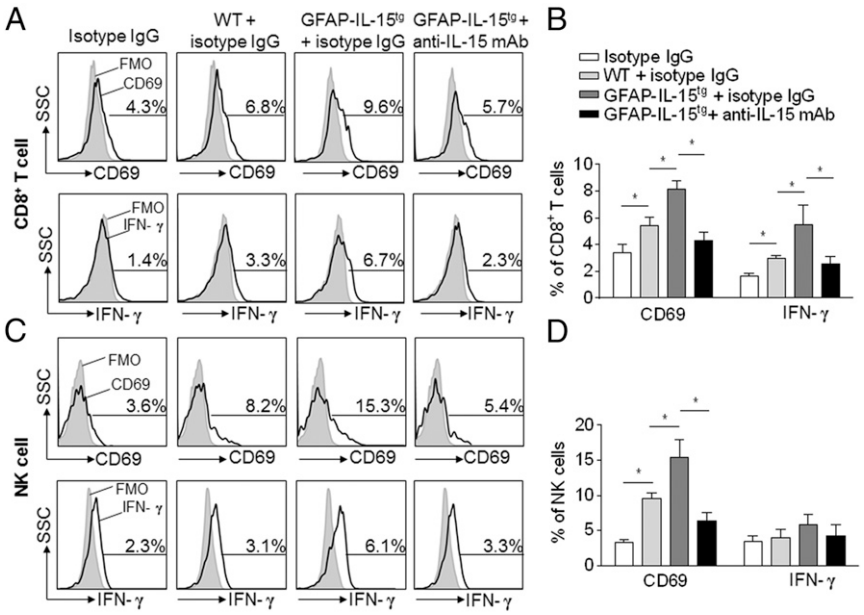

Fig. 7. Astrocyte-derived IL-15 promotes $CD8^+$ T and NK cell responses. WT $CD8^+$ T and NK cells were sorted from pooled splenocytes and cultured. Primary cultured WT and GFAP–IL-15^{tg} astrocytes were subjected to OGD and subsequently cocultured with $CD8^+$ T or NK cells for 48 h. (*A*) Flow cytometry plots show expression of CD69 and IFN-γ in $CD8^+$ T cells cultured alone, cocultured with WT astrocytes, cocultured with GFAP–IL-15^{tg} astrocytes, or cocultured with GFAP–IL-15^{tg} astrocytes plus anti–IL-15 mAb. All gates were set by using FMO controls. (*B*) Bar graph shows expression of CD69 and IFN-γ in $CD8^+$ T cells that were cultured alone, cocultured with WT astrocytes, cocultured with GFAP–IL-15^{tg} astrocytes, or cocultured with GFAP–IL-15^{tg} astrocytes plus anti–IL-15 mAb. $n = 9$ per group. (*C*) Flow cytometry plots show expression of CD69 and IFN-γ in NK cells that were cultured alone, cocultured with WT astrocytes, cocultured with GFAP–IL-15^{tg} astrocytes, or cocultured with GFAP–IL-15^{tg} astrocytes plus anti–IL-15 mAb. All gates were set by using FMO controls. (*D*) Bar graph shows expression of CD69 and IFN-γ in NK cells cultured alone, cocultured with WT astrocytes, cocultured with GFAP–IL-15^{tg} astrocytes, or cocultured with GFAP–IL-15^{tg} astrocytes plus anti–IL-15 mAb. Results are from three individual experiments, $n = 9$ per group. Data are expressed as mean ± SEM. $^*P < 0.05$.

and prosurvival functions (50, 51). For instance, IL-15 can serve as a potent T-cell chemotactic agent and enhance T-cell migration to inflamed tissue (50). Its prosurvival properties could also contribute to aspects of neuroinflammation.

Brain ischemia activates microglia that can damage neural structure or facilitate neural repair, depending on timing and context (52–55). Microglia express IL-15 receptors and thus could be receptive to astrocytic IL-15. In determining the number of microglia and factors derived from them, we did not observe significant alteration of microglia in IL-15^{tg} mice after MCAO. Nevertheless, the potential contribution of microglia to the observed exacerbation of stroke's severity cannot be ruled out in GFAP–IL-15^{tg} mice. Microglia possess a variety of capacities (i.e., phagocytic activity, secretion of proinflammatory and antiinflammatory factors, antigen presentation, etc.). These capabilities of microglia may enable them to directly or indirectly impact ischemic brain injury or tissue repair. It is important to note that there are also other brain ischemia-induced factors other than IL-15 that can activate microglia, such as danger signals or damage-associated molecular pattern molecules (HMGB1, CX3CL1, SPARC, galectin, notch, etc.) released within hours after ischemia (56). In all, whether and to what extent astrocytic IL-15 affects microglia and immune cells other than $CD8^+$ or NK cells to impact infarct development is of interest and warrants further investigation.

Evidence suggests that aging impacts the baseline brain inflammatory profile (57–60) and outcome of ischemic brain injury (61). In an effort to determine whether astrocytic IL-15 operates in the aged brain, we also compared stroke severity in aged GFAP–IL-15^{tg} and WT mice. We noticed larger brain infarcts and more severe neurodeficits in aged GFAP–IL-15^{tg} vs. WT mice (Fig. S10). Together with the finding of increased IL-15–expressing astrocytes in aged human brain samples after stroke onset, these results suggest that astrocytic IL-15 also operates in the aged brain.

Our findings have important clinical implications for the operating mechanisms of astrocytes as a prominent contributor to CNS immune and inflammatory responses after brain ischemia. Studies in humans support a role for IL-15 in the target tissues of patients with rheumatoid arthritis (62, 63), autoimmune myositis (64), obesity (65), celiac disease (66), and multiple sclerosis (47, 48). Exposure of lymphocytes such as $CD8^+$ T cells to IL-15 greatly enhances their cytotoxicity toward target cells, which has been considered as a mechanism at the basis of the tissue destruction. Recent studies have demonstrated immune interventions targeting key inflammatory mediators as a viable approach to restrict brain inflammation and tissue damage after brain ischemia. Immune therapies targeting IL-1 are ongoing in clinical trial with promising initial results (67). Therefore, the identification of astrocyte-derived IL-15 as a prominent contributor to ischemic brain injury may provide a useful target for the future design of selective treatment for patients with ischemic stroke.

In conclusion, our findings implicate IL-15 as an astrocyte-derived factor with a pronounced effect on ischemic brain injury in mice. We also demonstrate that IL-15–producing astrocytes are present in the brain of patients with acute ischemic stroke. These data warrant further investigation of the therapeutic potential of IL-15–modifying treatments in isolation and combination with current antithrombotic treatments for ischemic stroke.

Materials and Methods

Human Brain Tissue. Formalin-fixed paraffin-embedded brain tissue cross-sections were obtained from the Sun Health Research Institute (Sun City, AZ) and Department of Pathology, Ohio State University (Columbus, OH). Patients or their caregivers provided informed consent for brain donation, as well as for the purpose of research analysis at the Sun Health Research Institute and Ohio State University. The protocols and informed consent were

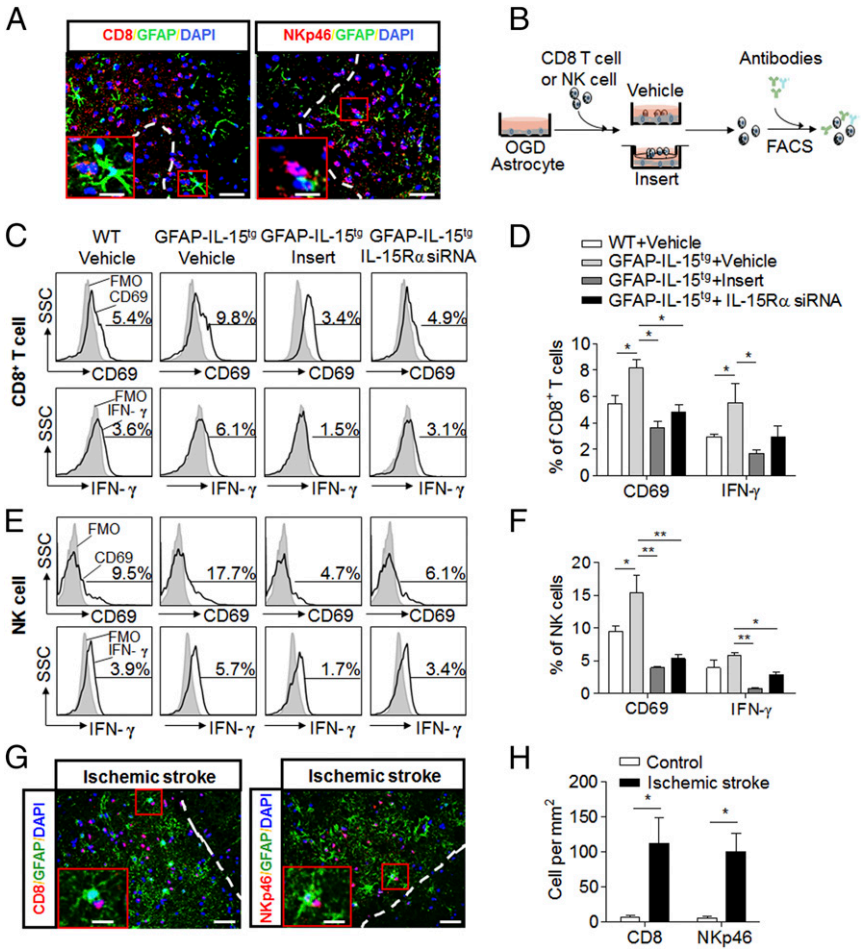

Fig. 8. Astrocyte-derived IL-15 is transported to $CD8^+$ T and NK cells via transpresentation. WT $CD8^+$ T and NK cells were sorted from pooled splenocytes and cultured. Primary cultured WT and GFAP–IL-15^{tg} astrocytes were subjected to OGD before being subsequently cocultured with $CD8^+$ T or NK cells. (*A*) Immunostaining brain sections shows $CD8^+$ T and NK cells located predominantly in proximity to astrocytes in periinfarct areas of GFAP–IL-15^{tg} mice at 24 h after MCAO. $n = 6$ per group. [Scale bars, 40 μm (*A*) and 20 μm (*A*, *Insets*).] (*B*) Scheme depicted shows procedures for cell isolation, coculture, culture-insert, and FACS analysis. (*C* and *D*) Flow cytometry plots (*C*) and summarized bar graph (*D*) show expression of CD69 and IFN-γ in $CD8^+$ T cells cocultured with WT astrocytes, cocultured with GFAP–IL-15^{tg} astrocytes (with cell–cell contact), cocultured with GFAP–IL-15^{tg} astrocytes plus culture insert (no cell–cell contact), or cocultured with GFAP–IL-15^{tg} astrocytes with IL-15Rα knockdown. All gates were set by using FMO controls. $n = 12$ per group. (*E* and *F*) Flow cytometry plots (*E*) and summarized bar graph (*F*) show expression of CD69 and IFN-γ in NK cells cocultured with WT astrocytes, cocultured with GFAP–IL-15^{tg} astrocytes (with cell–cell contact), cocultured with GFAP–IL-15^{tg} astrocytes plus culture insert (no cell–cell contact), or cocultured with GFAP–IL-15^{tg} astrocytes with IL-15Rα knockdown. All gates were set by using FMO controls. $n = 12$ per group. (*G*) Immunostaining shows $CD8^+$ T cells ($CD8^+$; red) and NK cells (NKp46; red) located in proximity to astrocytes ($GFAP^+$; green) in periinfarct areas of brain sections from patients with middle cerebral artery ischemic stroke. [Scale bars, 50 μm (*G*) and 25 μm (*G*, *Insets*).] (*H*) Quantification shows numbers of $CD8^+$ T and NK cells in the periinfarct areas of brain slices from patients with middle cerebral artery ischemic stroke (<7 d after onset) compared with nonneurological disease controls. $n = 4$ in nonneurological disease control group; $n = 6$ in ischemic stroke group. Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

approved by the Institutional Review Board of the Banner Sun Health Institute and Ohio State University. Among the 10 human samples used in this study, 6 stroke samples were obtained from patients who died 3–7 d after onset (male, 2; female, 4). The locations of stroke lesions were within the cortical areas supplied by the middle cerebral artery. Four control samples were from patients who died from nonneurological diseases (male, three; female, one). Control samples with nonneurological disease had no past history of neurological or neuropsychiatric diseases. In addition, postmortem pathological examination confirmed no pathological changes in brain sections beyond those expected in "control" nonneurological disease. Patients with ischemic stroke and controls did not differ significantly in terms of their mean age at death (stroke patients, 82 ± 9 y; controls, 87 ± 6 y, mean ± SEM; $P > 0.05$, Student's *t* test). Brain tissues were collected within 4 h after death.

Mice. WT C57BL/6 (B6, H-2^b) mice were purchased from Taconic. GFAP–IL-15^{tg} mice were produced by using a GFAP promoter to direct astrocyte-specific transcription (31, 68). GFAP–IL-15^{tg} mice were back-crossed to the B6 background for 12 generations. All animal experiments were reported according to the Animal Research: Reporting in Vivo Experiments guidelines (69, 70). All animal experimental protocols were approved by the Animal Care and Use Committees of Barrow-St. Joseph's Hospital or Tianjin Neurological Institute. Details of mice used and GFAP–IL-15^{tg} mouse creation are provided in *SI Materials and Methods*.

MCAO Surgery, Neuroimaging, Neurological Function, and Neuropathological Assessment. Adult male mice (8–10 wk of age, GFAP-IL-15^{tg} mice and WT littermates) were subjected to transient MCAO for 60 min by using the filament method, as we described (32). Details of MCAO surgery, 7T MRI scan, neurological function assessment, TTC staining, and immunohistochemistry are given in *SI Materials and Methods*.

In Vivo Cell Depletion and the Construction and Administration of shIL-15 and shIL-15 Rα Lentivirus Vectors Specifically in Astrocyte in Vivo. $CD8^+$ T cells and NK cell depletions were performed in vivo (32, 36, 71, 72). The construction and administration of shIL-15 and shIL-15Rα lentivirus vectors in vivo are provided in *SI Materials and Methods*.

Cells Cocultured in Vitro, Knockdown IL-15Rα in Astrocytes, OGD Treatment, Proteome Profiler Mouse Cytokine Array, ELISA, and Flow Cytometry. $CD8^+$ T cells or NK cells cocultured with OGD-treated astrocytes. Details of protocols

for isolation of $CD8^+$ T cells, NK cells, and astrocytes (73), OGD treatment (32, 74, 75), IL-15Rα siRNA-infected astrocyte, proteome profiler mouse cytokine array, ELISA, and flow cytometry are provided in *SI Materials and Methods*.

Statistics. Details for statistical analyses are provided in *SI Materials and Methods*. Data are expressed as mean ± SEM. $P < 0.05$ was considered significant.

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