T lymphocyte function in the delayed phase of ischemic brain injury

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Lymphocyte recruitment and activation have been implicated in the progression of cerebral ischemia-reperfusion (I/R) injury, yet the roles of specific lymphocyte subpopulations and cytokines in stroke remain to be clarified. We demonstrated that IL-23 and IL-17, rather than IFN-γ, play pivotal roles in the evolution of brain infarction. IL-23 was produced from infiltrated macrophages in the immediate phase of brain ischemia; thereafter, IL-17-producing T lymphocytes were infiltrated into the ischemic brain tissue in the delayed phase. IL-17 was mostly produced in γδT lymphocytes, and was dependent on IL-23. We discovered that not only IL-23 but also IL-17 deficiency prevented neural cell death in the delayed phase of ischemic brain injury. We also demonstrated that FTY720 administration, which blocked T cell infiltration into the brain, suppressed ischemic brain injury. Furthermore, the depletion of γδT cells also attenuated ischemic brain damages. Therefore, we propose that cerebral T lymphocytes, including γδT lymphocytes, be considered as new therapeutic targets in a novel neuroprotective strategy for ischemic brain injury during the delayed phase.
Introduction

Stroke is a leading cause of death and disability worldwide and approximately 80% of strokes in Japan are caused by brain infarction. So far, no successful therapy has been established that can benefit patients beyond the narrow time window of thrombolysis\(^1,2\). Brain infarction is the death of brain tissue caused by brain ischemia, which is the loss of cerebral blood flow mainly caused by the stenosis or occlusion of cerebral arteries. In the immediate phase of brain infarction, ischemia causes the necrotic cell death of brain tissue, and moreover, macrophages begin to infiltrate into the ischemic brain tissue and promote rapid inflammatory responses\(^3\) (Fig. 1).

These inflammatory responses continue in the delayed phase of brain infarction and cause the evolution of infarct volume and neurological worsening. In this phase, various types of inflammatory cells including macrophages, neutrophils and lymphocytes infiltrate into the ischemic brain tissue and exacerbate ischemic injury, now recognized as a secondary ischemic injury. Brain edema and apoptotic neuronal death have been suggested to contribute to the evolution of infarct volume in the delayed phase\(^4-6\), however, both the mechanism and the regulation of such events have not been fully understood.

**Fig. 1 The inflammation and brain infarction.**

Many inflammatory factors play a major role in the inflammation during the immediate phase. In this phase, macrophages begin to infiltrate the ischemic brain tissue and mediate ischemic injury. It has not, however, been clarified which cells exacerbate the ischemic damage in the delayed phase of brain ischemia. Because lymphocytes infiltrate the ischemic brain tissue in the delayed phase, we hypothesized that these infiltrating lymphocytes caused secondary inflammatory responses. If this is true, we can develop a new neuroprotective strategy which can be initiated even at the delayed phase of brain infarction.

T lymphocytes in ischemic brain injury

Recently, T lymphocytes have been suggested to play a role as mediators in the delayed phase of brain ischemia. In fact, as early as 24 hours after reperfusion, T lymphocytes are present in the ischemic brain tissue and appear to be localized to the infarction boundary zones, typically close to blood vessels\(^7,8\). The number of infiltrated T lymphocytes was reported to reach its peak in the delayed phase (day 3 from the onset) of brain I/R injury\(^9\). Furthermore, recent studies revealed that the depletion of these T lymphocytes attenuated cerebral ischemic damage. A significant reduction of infarct volume was observed in severe combined immunodeficient (SCID) mice and recombination activating gene (RAG)-deficient mice both of which are lacking T and B lymphocytes\(^10,11\). A previous study revealed that CD4-positive and CD8-positive T lymphocytes, but not B lymphocytes, exacerbate in-
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It has not been clarified whether a specific antigen of the brain is involved in the activation of these infiltrated T cells. Because the inflammatory responses induced by ischemic injury have been shown to be driven by the innate immune system, it is highly possible that these T lymphocytes mediate antigen-independent, innate inflammatory responses. However, some reports suggested the role of antigen recognition by the T lymphocytes in ischemic brain injury. Treatment with T cell receptor ligands, major histocompatibility complex class II molecules bound to myelin peptides, attenuated ischemic brain injury. In addition, myelin basic protein-tolerized animals have been shown to develop reduced infarction compared to control animals.

Fig. 2 FTY720 administration attenuated ischemic brain injury.

Regardless of antigen specificity, these data indicate that T lymphocytes play a progressive role in the delayed phase of ischemic brain injury by enhancing secondary ischemic inflammatory responses. However, it remains unclear whether these T lymphocytes act centrally or peripherally. To attempt to answer this question, we evaluated the therapeutic effect of FTY720, an immunomodulatory prodrug which is well known to prevent T lymphocyte infiltration into inflammatory tissues. We discovered that FTY720 suppressed T lymphocyte infiltration into the ischemic brain tissue without influencing macrophage infiltration. Additionally, FTY720 administration significantly reduced the infarct volume, compared to control mice (Fig. 2). These data support the contention that T lymphocytes act centrally and mediate ischemic injury. However, T lymphocytes infiltration begins 24 hours after the onset of brain ischemia. In order to develop a new neuroprotective therapy, the dose and therapeutic time window of FTY720 administration should be established in future study.

T lymphocyte cytokines in ischemic brain injury

Although recent studies clearly demonstrate the relationship between ischemic stroke and T lymphocytes, the specific function of these T lymphocytes has not been sufficiently clarified. IFN-γ from activated T cells has been proposed to promote infarction. However, the role of IFN-γ in ischemic brain injury is the subject of controversy. IL-10 is well-known to function as a neuroprotective mediator derived from T lymphocytes, especially from regulatory T cells.

We have been interested in the newly identified cytokines, IL-23 and IL-17, because they play a pivotal role in the onset and the progression of experimental autoimmune encephalomyelitis (EAE). EAE is a well-established T cell-mediated brain and spinal cord inflammation model. It has been demonstrated that IL-23 is a critical cytokine for the onset of experimental autoimmune encephalomyelitis (EAE). IL-23 is a heterodimer of IL-23p19 and IL-12p40 and has been shown to be essential for the induction
of IL-17 production from helper T cells (Th17)\(^{25}\) and \(\gamma\delta\) T lymphocytes\(^{26,27}\). IL-23 is mostly produced from antigen-presenting cells, such as dendritic cells.

On the other hand, IL-17 is produced not only from CD4-positive helper T cells (Th17) but also from \(\gamma\delta\) T lymphocytes. In EAE, IL-17 and other inflammatory mediators from Th17 cells have been implicated in the onset as well as the progression of the disease\(^{28-30}\). IL-17 has been reported to modify many inflammatory responses in the central nervous system by promoting the production of neurotoxic cytokine\(^{33}\) and blood brain barrier disruption\(^{34,35}\).

Therefore, we hypothesized that IL-23 and IL-17 were implicated in cerebral ischemic injury. Actually, elevated levels of IL-17 were found in the ischemic hemispheres of human brains, and the levels peaked 3-5 days after brain ischemia\(^{36}\). It remains to be determined which T lymphocyte subsets and inflammatory mediators participate in the evolution of brain infarction. If we can modulate these inflammatory T lymphocyte functions, a new neuroprotective therapy could be developed (Fig. 1).

**Fig. 3 Pivotal roles of IL-23 and IL-17 in the ischemic brain.**

(a) The source of IL-17 in the ischemic brain. IL-17-positive T cell populations were further analyzed with respect to the expression levels of TCR\(\gamma\delta\) or CD4.

(b) The time course of infarct volume measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining on days 1 and 4. The number of mice is shown on each bar. *\(P < 0.05\), **\(P < 0.01\) vs. WT mice (one-way ANOVA with Dunnett’s correction).

**IL-23 and IL-17 in ischemic brain injury**

Then, we established transient cerebral ischemia/reperfusion model mice by suture occlusion method and investigated the infiltrating inflammatory cells. We found that IL-23p19, IL-12p35, and IL-12p40 mRNA levels in infiltrating inflammatory cells were high on day 1 and decreased thereafter. To determine the source of IL-23, the pool of infiltrating inflammatory cells was sorted to separate the macrophages, microglia, and other cells. IL-23p19 mRNA was detected...
only in the macrophage fraction (CD45 high, CD11b high population). By using bone marrow-transferred chimeric mice, we also showed that infiltrated macrophages, but not brain cells, were the major source of IL-23, which was essential for the induction of IL-17 producing cells in the ischemic brain.

To identify the IL-17-producing cells in ischemic brain tissue, we performed intracellular FACS analysis on the infiltrating inflammatory cells. The number of IL-17-positive cells as well as IFN-γ-positive cells in ischemic brain tissue was first measurable on day 3 and decreased on day 6. Importantly, we barely detected IL-17-producing cells in infiltrating mononuclear cells from IL-23 knockout (KO) mice. Therefore, IL-23 is a key factor for the induction of IL-17 producing cells in the ischemic brain.

All of the IL-17-positive and IFN-γ-positive cells were also CD45-positive. Approximately 90% of the IL-17-positive cells and IFN-γ-positive cells were CD3-positive T lymphocytes. Surprisingly, most of the IL-17-producing T lymphocytes were CD4-negative but γδ TCR-positive (Fig. 3a). These data indicate that γδ T lymphocytes, rather than Th17 cells, are the source of IL-17.

Furthermore, to investigate the functional significance of IL-23 and IL-17 in brain infarction, we used an experimental brain ischemia model in gene-disrupted mice. IL-23p19 KO mice exhibited a significant reduction in infarct volume at day 1 and 4, compared to wild-type (WT) mice (Fig. 3b). IL-17 KO mice also exhibited a significant reduction of infarct volume on day 4, but no drastic difference was observed on day 1 (Fig. 3b). However, there was no statistical difference between IFN-γ KO and WT mice at day 1 and 4. We also revealed the importance of IL-17-producing T lymphocytes rather than brain cells for the progression of ischemic damages by an adoptive transfer experiment. These data indicate that IL-23p19 functions in the immediate phase of ischemic brain injury, while IL-17 plays a role in the delayed phase (after day 3). Additionally, our results suggest that IL-23 promoted ischemic brain injury at the immediate phase by IL-17-independent mechanism.

γδ T lymphocytes in ischemic brain injury

Infiltrating γδ T lymphocytes were found in the ischemic brain tissue and their presence peaked at day 3. These γδ T lymphocytes appeared to be localized to the infarct boundary zones (Fig. 4a), suggesting a role in neural cell death in the penumbra region. We confirmed that the administration of anti-TCRγδ antibody almost completely depleted cerebral γδ T lymphocytes and IL-17-positive T lymphocytes in ischemic brain tissue. Furthermore, anti-TCRγδ antibody treatment exhibited a neuroprotective effect even 24 hours after the induction of brain ischemia (Fig. 4b). These data suggest that IL-17 is the most potent effector of γδ T lymphocytes.

Discussion

In the immediate phase of brain ischemia, infiltrated macrophages produce IL-23. Thereafter, in the delayed phase of brain ischemia, T lymphocytes, including γδ T lymphocytes, infiltrate the ischemic brain tissue, and IL-23 induces IL-17 production by γδ T lymphocytes. IL-17 exacerbates ischemic brain injury by promoting blood brain barrier disruption and the production of neurotoxic factors.

Recent studies have revealed that γδ T lymphocytes can more rapidly produce IL-17 than can Th17, and they exacerbate autoimmune disease such as EAE. IL-17-producing γδ T lymphocytes have also been shown to be involved in the development of EAE. These γδ T lymphocytes, which produce IL-17 but not IFN-γ, were reported to be antigen-naïve and a naturally-occurring type of γδ T lymphocytes. Therefore, it is reasonable to conclude that IL-23-induced IL-17-producing γδ T lymphocytes play a role in ischemic brain injury.

Our findings raise the intriguing possibility that T lymphocytes, including γδ T lymphocytes which produce IL-17, could be a new therapeutic target for stroke, widening the therapeutic time window for neuroprotection against brain ischemia. A recent study identified the IL-17-producing cells in the ischemic brain of stroke patients, and that their presence peaked 3-5 days after injury. In addition, it has been reported that the infarct volume evolved until 3-5 days after the onset. Thus, it is highly likely that infiltration of IL-17-producing T lymphocytes plays a pivotal role not only in mouse but also in human brains. We propose that suppres-
sion of a specific pathogenic T lymphocyte subset, such as IL-17 producing γδT lymphocytes, is useful for neuroprotection against brain ischemia.

Fig. 4 The effects of γδT lymphocyte infiltration in ischemic brain tissue.
(a) Anti-mouse TCRγδ immunostaining of ischemic brain tissue at day 4.
(b) The infarct volume of mice which were PBS- or antibody-treated 24 hours after the induction of brain ischemia. Mice were sacrificed at day 7 and the infarct volumes were evaluated by anti-MAP2 immunostaining. **P = 0.028, vs. control mice [two-sided Student’s t-test]. The number of mice is shown on each bar.

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