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REVIEW ARTICLE

Immune cell gateways in the central nervous system regulated by regional neural stimulations

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Abstract

The central nervous system (CNS) is an immune-privileged tissue due to a specialized blood vessel structure, the blood–brain barrier. Indeed, the blood–brain barrier tightly limits cell migrations into the CNS. However, several immune cells, including T cells, can be found there. It is hypothesized that these cells have both beneficial and detrimental roles in immune surveillance and inflammation development, and that they can lead to multiple diseases, such as multiple sclerosis, Alzheimer's disease and Parkinson's disease. The presence of immune cells within the CNS suggests a gateway that allows access from the peripheral blood stream. We recently identified dorsal vessels of the fifth lumbar spinal cord as a gateway for autoreactive T cells that opened with excessive chemokine expression. A chemokine inducer, the inflammation amplifier, which is hyperactivated by regional neural activations such as gravity, appears critical for formation of this fifth lumbar gateway. The gating of immune cells to the CNS can be artificially controlled by electric stimulations, at least in mice. We named this neural stimulation-dependent gating the "gateway reflex." Physical and/or chemical manipulations of the gateway reflex through the inflammation amplifier hold promising therapeutic value for neuroimmunological disorders.

Introduction

The immune system is a complex and sophisticated system that orchestrates various types of hematopoietic cells, such as T cells, B cells, dendritic cells and macrophages. This system is evolutionally invaluable for combating infectious microorganisms (e.g. virus, parasites and bacteria) by inducing inflammation. Classically, inflammation has been defined as five symptoms: heat, redness, pain, swelling and the resulting tissue dysfunction. These clinical signs can be induced by a local accumulation and activation of immune cells that produce inflammatory cytokines and tissue-damaging mediators to attack infectious microorganisms. Thus, inflammation can be recognized as a local accumulation of immune cells at an affected region. In this respect, chemokine expressions, which recruit immune cells, are key for local inflammation, and the molecular mechanism that induces chemokines could be a good target for many disorders involving inflammatory cells. We have defined one such molecular mechanism, the inflammation amplifier, which operates in non-immune cells, such as endothelial cells and fibroblasts. The inflammation amplifier induces chemokines in non-immune cells, and is itself induced by the simultaneous activation of the transcription factors nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT).

The blood–brain barrier (BBB) tightly regulates the entry of immune cells to the central nervous system (CNS). However, the CNS is known to experience neuroinflammation as a result of an excessive accumulation of deregulated immune cells. Such an occurrence is common in many diseases, such as multiple sclerosis (MS), amyotrophic lateral sclerosis and ischemia. Recent studies suggest that neuroinflammation also contributes to the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease.[1] Indeed, numerous upregulated genes in hippocampus samples from Alzheimer's disease patients are related to myeloid

immune cells including monocytes, infiltrating macrophages and microglia.[2] Considering the protection of the CNS by the BBB, these immune cells might exploit a gateway to circumvent the barrier. We have identified a novel neuroimmune interaction that forms the entry site for immune cells to enter the CNS, termed the "gateway reflex." In the present review article, we discuss the gateway reflex and its underlying molecular mechanism, the inflammation amplifier, with a focus on an animal model of MS, experimental autoimmune encephalomyelitis (EAE).

Inflammation and the BBB

The BBB is known to strictly limit the inflow of substances, including proteins and cells, into the CNS to maintain a specific environment for neurons and glia cells. The BBB is formed and maintained by several cell types, including endothelial cells, pericytes and astrocytic end-feet.[3] Tight junctions play a central role in sealing the blood vessels by interacting claudins and occludins.[4] In addition, astrocytes and BBB endothelial cells promote BBB integrity by the Hedgehog pathway.[5] Dysfunction of the BBB is associated with chronic neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease.[6] Furthermore, leakage of the BBB is related to autoimmune disease in the CNS.[7] In healthy conditions, the BBB provides a tight barrier between circulating immune cells and the CNS by dense tight junction proteins, which seal the space between adjacent blood endothelial cells, whereas in inflammatory conditions, the integrity of the BBB is impaired by various cytokines. For instance, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-17A are reported to compromise the BBB. In particular, IL-17A seems critical for distributing the BBB *in vitro* and *in vivo*. Autoreactive CD4 helper T cells that secrete IL-17A (Th17 cells) play an important role in the induction of CNS autoimmunity including MS.[8] EAE mice genetically lacking IL-17A are protected from the disease development, and an anti-IL-17 neutralizing antibody is now under clinical trials for MS treatment.[9,10] The BBB dysfunction induced by IL-17A involves the formation of reactive oxygen species by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and xanthine oxidase, followed by the downregulation of tight junction molecules and the activation of the endothelial contractile machinery *in vitro*.[11] Human brain endothelial cells treated with IL-17A have increased protein permeability, suggesting reduced tight junction molecules, occludin and ZO-1.[12] Thus, it is generally accepted that the BBB is significantly affected by inflammation. However, whether the entire CNS is equally targeted by inflammation or there exist certain vulnerable sites was not known until recently.

MS and EAE

MS is the most common neurological disease of young adults, and there are more than 2.5 million patients worldwide.[13–16] A genome-wide association study has clearly shown the involvement of immune cells, in particular autoreactive CD4 T cells and their cytokines, in the disease.[17–20] EAE is an animal model to study the pathogenesis of MS, and can be induced by immunizing animals with myelin antigens, such as myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein and glial fibrillary acidic protein.[21,22] The importance of the BBB integrity is shown to prevent CNS disease in EAE models. For example, inhibition of the Hedgehog pathway, which promotes BBB integrity, increases disease severity.[5] Endothelial specific leptin receptor knockout mice show ameliorated EAE symptoms by reducing the BBB dysfunction associated with cytokine and chemokine expressions in spinal cord microvessels.[23] In addition, EAE can also be induced by the adoptive transfer of autoreactive CD4 T cells into naïve animals. This transfer EAE model is particularly useful to study the behavior of the disease-causing pathogenic CD4 T cells, because unusual systemic inflammation, and continuous production and activation of autoreactive CD4 T cells caused by adjuvant treatment in the immunization step do not occur. CNS-specific autoreactive CD4 T cells, especially IL-17-producing Th17 cells, are critical for EAE development.[8,9]

The clinical symptom of typical EAE begins with a loss of tonicity in the tail tip. Paralysis progresses to the hind limbs, and finally the forelimbs are affected. This upward progression of the disease symptom suggests that immune attacks might begin at a particular site despite the wide distribution of autoantigens within the CNS. Using intravital imaging, the infiltration of pathogenic CD4 T cell blasts into the CNS can be observed in the lumbar region of the spinal cord. After the intravenous transfer of pathogenic CD4 T cells into naïve mice, these cells transiently reside in the lung, where they gain the capacity to enter the CNS. Transcriptional profiles of CD4 T cell blasts in the lung were reprogrammed to more migratory phenotypes by increased expressions

of cellular locomotion molecules, chemokine and adhesion receptors. Although the precise mechanism remains undefined, the lung potentially represents a niche for reactivation of autoreactive CD4 T cells, such that the cells become pathogenic before invading the target organ.[24,25] However, the precise location of the entry site of the pathogenic T cells and the molecular mechanism governing the location specificity were unclear until recently.

Where is the entry site for immune cells into the CNS?

To find the entry point into the CNS, we examined the localization of disease-causing, IL-17-producing CD4 T cells (hereinafter referred to as pathogenic Th17 cells) using a macrotome. Whole-mount sagittal sections of adult mice at a preclinical stage of EAE induced by a single adoptive transfer of pathogenic Th17 cells showed that these cells preferentially localized in the lumbar spinal cord, but not in the upper region of the spinal cord or brain. Closer examination of each lumbar cord by immunohistochemistry and flow cytometry showed that pathogenic Th17 cells are mainly accumulated around the dorsal vessels of the fifth lumbar (L5) cord at the early stage of EAE (Fig. 1).[26] The cellular accumulation in the L5 cord is dependent on IL-17A secreted from pathogenic Th17 cells and CCL20, a chemokine that potently recruits Th17 cells. Indeed, CCL20 expression was highest in the L5 dorsal vessels among spinal cord segments. In addition, various other chemokines were also upregulated at L5 dorsal vessels even in steady state compared with the dorsal vessels of the other spinal levels. These results identify the L5 dorsal vessels as a possible gateway for pathogenic Th17 cells into the CNS at the initial stage of EAE.[26] Consistent with these results, magnetic resonance imaging of EAE mice showed that the L5 spinal cord exhibits edema just before the onset of EAE clinical manifestation.[27] Intriguingly, the selective increase of chemokine expressions at the L5 dorsal vessels was observed even under steady state, albeit to a lesser degree than expressions in EAE.[26] As a detectable number of immune cells reside within the CNS under steady state, the L5 gateway might also function for CNS resident immune cells, although further studies are required to confirm this theory.

Neural stimulations operate the L5 gateway

It is known that sensory neurons in the dorsal root ganglion (DRG) located at the L5 spinal level are connected to the soleus muscles, the main antigravity muscles in humans and mice.[28] The L5 DRG is the largest DRG in both mice and humans, possibly because of constant stimulation of the soleus muscles by Earth's gravity.[29] Interestingly, when mice were suspended by their tails so that the hind limbs were released from gravitational force, pathogenic Th17 cells did not accumulate at the L5 dorsal vessels. Instead, they were detected at the cervical spinal cords as if a new gateway was formed by the increased gravitational burden on the forearm muscles by the tail suspension.[26] Consistent with this

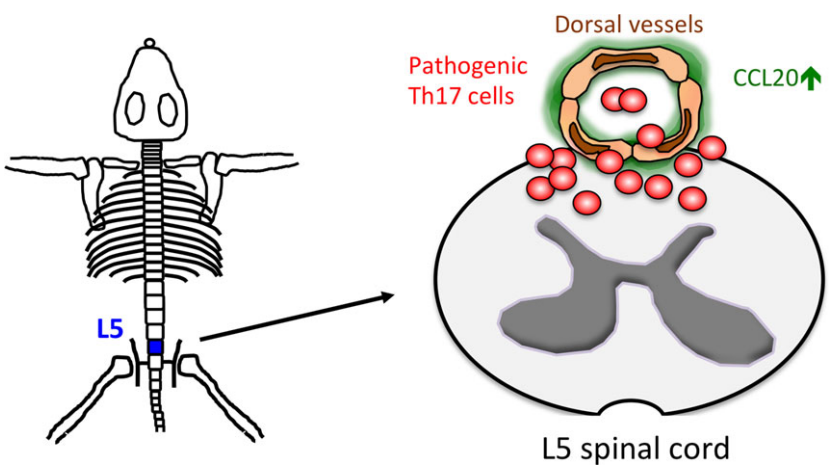

Figure 1 The entry of pathogenic Th17 cells is the fifth lumbar (L5) spinal cord. Interleukin-17-producing CD4 T cells specific for the central nervous system antigens (pathogenic Th17 cells) preferentially invade the central nervous system at the dorsal vessels of the L5 cord at an early, preclinical stage of experimental autoimmune encephalomyelitis through upregulation of CCL20, a chemokine that potently recruits these cells. This observation is consistent with the typical clinical symptom of experimental autoimmune encephalomyelitis beginning with the loss of tonicity at the tail tip.

observation, tail suspension inhibited the expression of CCL20 at the L5 dorsal vessels, and reciprocally increased it at the dorsal vessels of the cervical cord. In addition, electric stimulations to the soleus muscles of tail-suspended mice dose-dependently restored CCL20 expression and pathogenic Th17 cell accumulation at the L5 spinal cord in a manner dependent on regional sensory activation.[26] These results suggest that sensory neural signals from the Earth's gravity can be converted into inflammatory signals in the L5 dorsal blood vessels with the elevated expression of various chemokines including Th17-recruiting CCL20.

Subsequent experiments showed that a pathway between the activation of sensory neurons and the upregulation of chemokines at the L5 dorsal vessels involves sympathetic nerves. Blood flow speeds at the L5 dorsal vessels, but not other regions, became slower in tail-suspended mice, while electronic stimulations to the soleus muscles increased the blood flow, suggesting that autonomic nerves, which control blood flow, were involved in this response.[26] Furthermore, the pharmacological inhibition of adrenergic receptor signaling, which blocks noradrenaline signaling, suppressed CCL20 expression and pathogenic Th17 cell accumulation at the L5 dorsal vessels, and also inhibited the development of EAE.[26] Thus, continuous stimulation of sensory neurons in the soleus muscles by gravity leads to activation of local sympathetic nerves, and creates a gateway for immune cells to enter the CNS through the L5 dorsal vessels, although the precise neuronal connection is not yet elucidated (Fig. 2).[26] Our findings imply that various types of neural activities, including mental depression and pain, which are often associated with MS, might affect the MS

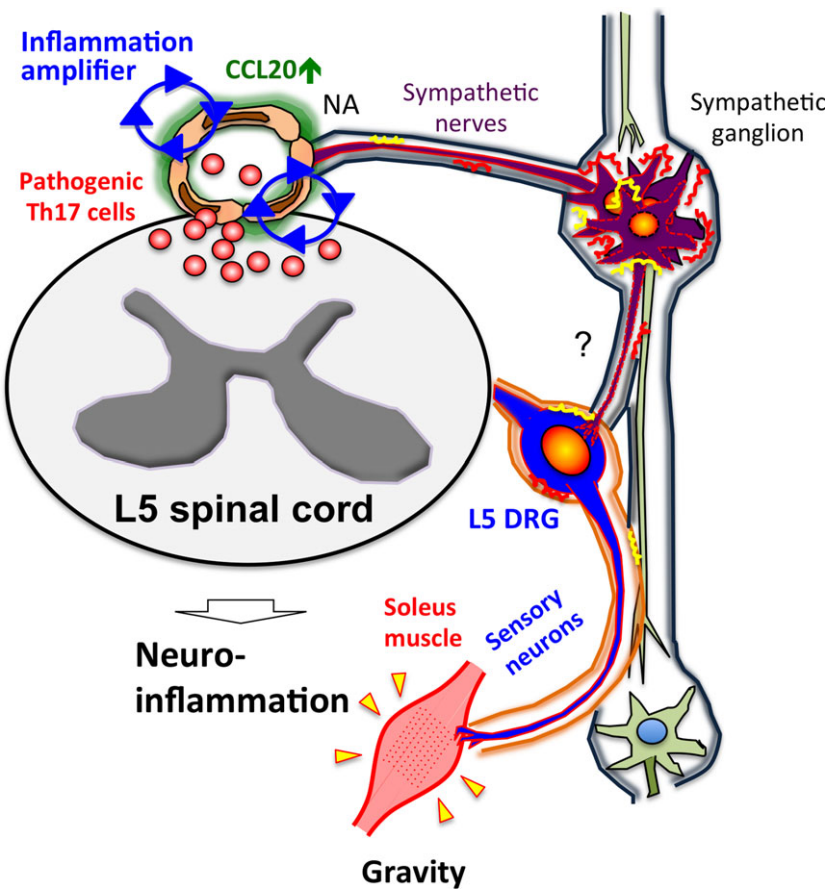

Figure 2 Regional neural activation directs Th17 cells into the fifth lumbar (L5) gateway. Continuous stimulation of the soleus muscles by Earth's gravity leads to the constant activation of dorsal root ganglion (DRG) sensory neurons in the L5 spinal cord followed by activation of sympathetic neurons at the L5 sympathetic ganglia through unidentified neural connections. Noradrenaline (NA), a neurotransmitter from sympathetic neurons, augments the inflammation amplifier at the dorsal vessels of the L5 spinal cord, leading to further activation of the inflammation amplifier (i.e. the induction of chemokines including CCL20) and accumulation of Th17 cells around the dorsal vessels of the L5 spinal cord.

disease status differently.[30–32] Indeed, increasing evidence suggests an association between stressful life events; for example, war or the death of a child, with relapse in MS patients.[33] Similarly, we found that some stresses cause a deterioration or relapse of EAE in mice (unpublished observations), which is a useful model system to study the molecular mechanisms of MS relapses. Taken together, regional neural activations can impact neuroinflammation.

Gateway reflex

As discussed earlier, regional neural activations can modify the status of local blood vessels by upregulating the expression of various chemokines, leading to the formation of the L5 gateway for immune cells. Neurons exist throughout the body and transmit information to exert organ control. We speculated that if the proper neurons connecting target organs are artificially stimulated, local inflammation could be controlled by modulating chemokine expression at the target site. To examine this possibility, we stimulated various muscles with electric pulses and examined local chemokine expressions in mice. Thigh muscles are regulated by L3 DRG neurons. As expected, electronic stimulations of these muscles increased CCL20 expression in the L3 spinal cord. Likewise, chemokine levels in the fifth cervical to fifth thoracic spinal cord vessels were elevated by stimulation of upper arm muscles.[26] Based on these results, we proposed the gateway reflex, which explains how blood vessels act as a gateway for immune cells to target organs depending on the regional neural stimulation.[34–37] Whether the gateway reflex can be applied to tissues beyond the CNS is currently under investigation. The gateway reflex could potentially be a novel therapeutic target, as closing a gateway should dampen autoimmune inflammation in the target organ without systemic suppression of the immune system, whereas opening it in tumors could enhance cancer immunotherapy by inducing the recruitment of immune cells.

Inflammation amplifier, inflammation-inducing machinery

As aforementioned, gravity-mediated sensory neural signals are converted to inflammatory signals through sympathetic activation. A molecular link between regional neural activations and the subsequent chemokine expressions at local blood vessels has also been shown. We have been studying the pathogenesis of chronic inflammatory diseases using animal models of MS and rheumatoid arthritis (RA). IL-6 is one important pro-inflammatory cytokine, and is upregulated in the serum and affected areas of human diseases, such as RA and MS. In addition, there is reported clinical success of an anti-IL-6 receptor antibody for the treatment of RA.[38] However, it is unclear how IL-6 induces chronic inflammation. Thus, the establishment of an IL-6-dependent animal model has been awaited.

IL-6 signaling induces the activation of transcription factor, STAT3, which is responsible for the induction of many IL-6 target genes. *SOCS3* is one target gene, and negatively controls IL-6 signaling by binding to the IL-6 signal transducer gp130.[39,40] High levels of IL-6 in human diseases prompted us to hypothesize that uncontrolled enhanced IL-6 signaling might induce inflammatory diseases, such as RA. We generated a knock-in mouse strain called F759 mice that have a point mutation at the SOCS3 binding site of gp130, Y759F, thereby abolishing the negative regulation of IL-6 signaling *in vivo*. As expected, IL-6 signaling (i.e. activation of STAT3) is prolonged in these knock-in mice.[41] F759 mice produce autoantibodies, show splenomegaly and spontaneously develop RA-like joint diseases as they age.[42] Furthermore, they show severe EAE, suggesting enhanced inflammatory responses. To identify the cellular populations responsible for the enhanced inflammatory responses, we created bone marrow chimera mice. Interestingly, F759 host mice that received the wild-type immune cells developed the arthritis, whereas wild-type hosts with F759 immune cells did not, suggesting that the mutation in irradiation-resistant non-immune cells is responsible for the induction of F759 arthritis. Genetic studies showed that the development of arthritis in F759 mice requires CD4 T cells, IL-6 and IL-17A.[43,44] Consistently, the injection of IL-17A and IL-6 into F759 mice accelerated the onset of arthritis. The cytokine injection induced further secretion of IL-6, suggesting a positive feedback loop of IL-6 production.[44] This result was confirmed *in vitro*. Non-immune cells, such as endothelial cells and fibroblasts, stimulated with a combination of IL-17A and IL-6 produced a large amount of inflammatory chemokines and IL-6 compared with stimulation with IL-17A or IL-6 alone. The effect of these two cytokines is synergistic (Fig. 3a).[44] As local chemokine expression can recruit immune cells to cause inflammation, we named this synergistic mechanism the inflammation amplifier.[45] We also found that noradrenaline, which is involved in the gateway at L5 dorsal vessels, further increases the expression of chemokines

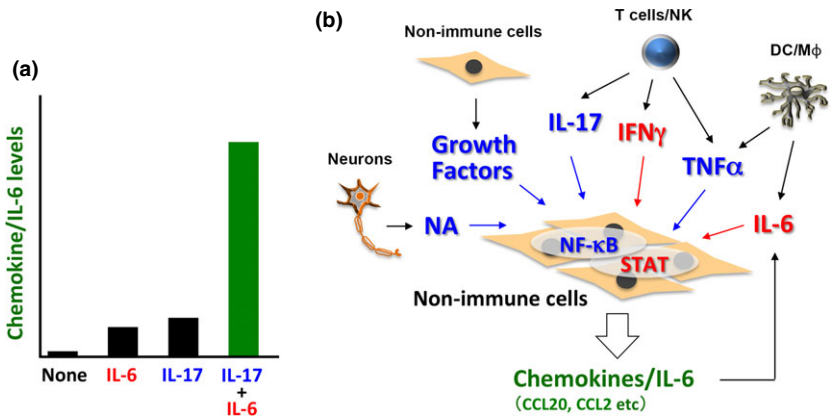

Figure 3 The inflammation amplifier. (a) Stimulation of non-immune cells including fibroblasts and endothelial cells with interleukin (IL)-17 and IL-6 induces a synergistic effect on the production of inflammatory chemokines, such as CCL20 and IL-6. An imaginary figure is shown. (b) The synergistic effect requires the simultaneous activation of two transcription factors, nuclear factor (NF)-κB and STAT, in non-immune cells. In addition to IL-17 and IL-6, various soluble factors activating or sustaining the activation of NF-κB and STAT can augment the amplifier. NF-κB-activating factors are written in blue and STAT activating molecules in red. DC, dendritic cells; IFN-γ, interferon-γ; Mϕ, macrophages; NA, noradrenaline; NK, natural killer cells; TNF-α, tumor necrosis factor-α.

induced by the inflammation amplifier. During EAE, mice lacking gp130 or STAT3 in endothelial cells (i.e. defective activation of the inflammation amplifier in blood vessels) showed significantly reduced invasion of immune cells at the L5 cord and disease development. Thus, the inflammation amplifier is the molecular basis of the gateway reflex. Furthermore, not only the combination of IL-17A and IL-6, but also many stimuli inducing or supporting the activation of NF-κB and STAT, including TNF-α, growth factors and interferon-γ, drive the inflammation amplifier (Fig. 3b).[46,47] In addition, mice devoid of amplifier activation in non-immune cells are highly resistant to animal models of not only MS, but also RA and allogeneic graft rejection, showing that the inflammation amplifier is fundamental for many chronic inflammatory diseases *in vivo*.[26,44,46,48]

The aforementioned observations suggested that genes involved in the regulation of the inflammation amplifier could make potential drug targets for chronic inflammatory diseases. Therefore, we carried out genome-wide functional screening and DNA microarray, and identified approximately 1700 genes related to the activation of the inflammation amplifier.[49] The Genetic Association Database at the National Institute of Health compiles information about genes genetically associated with many types of diseases and disorders. Searching for amplifier-related genes in the database showed that genes known to be associated with autoimmunity, including MS and cancer, were significantly enriched in inflammation amplifier-related genes.[49,50] In addition, genes genetically linked with neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, were also significantly enriched in the candidate genes, suggesting that the inflammation amplifier contributes to the pathogenesis of these neuroinflammatory diseases.[49] The activation of the inflammation amplifier was evidenced by the phosphorylations of NF-κB and STAT3 in human clinical samples.[51] Furthermore, soluble factors that are induced by the amplifier, such as epiregulin and other growth factors, are elevated in the serum of patients with MS and RA, suggesting activation of the inflammation amplifier in humans.[47,49] We propose that *de novo* mutations and/or epigenetic changes induced in these amplifier-related genes in non-immune cells during acute inflammation could cause dysregulated enhancement of the amplifier and cause pathogenic chronic inflammation.

Beneficial aspects of resident immune cells in the CNS under steady state

The presence of immune cells in non-lymphoid organs under steady state is often regarded as immune surveillance, and contributes to the prevention of tumor development and infection. In the brain, however, it seems that resident immune cells play other roles. Mice lacking T cells showed dramatic impairments in cognitive functions, such as hippocampal-dependent spatial learning and memory in a water maze. In addition, transgenic mice

that had T cells specific for a brain antigen showed better learning and memory than those having T cells recognizing an irrelevant antigen.[52–54] In contrast, B-cell deficiency or CD8 T cell depletion did not have such effects, suggesting the central role of CD4 T cells in cognitive function.[53] After learning tasks, CD4 T cells increased in the meninges of the brain, where they produced IL-4. Indeed, IL-4-deficient mice showed an impairment of cognitive abilities, and adoptive transfer of CD4 T cells from wild-type, but not IL-4-deficient, mice restored the learning defects.[54] As aforementioned, the expression of many chemokines are selectively elevated in L5 dorsal vessels under steady state, which suggests the gateway reflex caused by gravity might regulate the migration of CNS resident CD4 T cells, leading to beneficial roles for cognition. It might be possible then that the manipulation of the gateway reflex can also be applied to improve cognitive functions in the elderly.

Conclusions

The BBB is a barrier system that limits immune cell migration into the CNS. The presence of immune cells in the CNS has both beneficial and harmful aspects. We identified the dorsal vessels of the L5 spinal cord as an entry site for immune cells, particularly autoreactive pathogenic CD4 T cells, into the CNS, and defined the gateway reflex as the operator of this entry site. A series of mouse studies further suggests that the inflammation amplifier, which drives hyperinduction of chemokines by simultaneous activation of NF-κB and STAT in non-immune cells, is a molecular mechanism that underlies the gateway reflex. We have observed the co-localization of activated NF-κB and STAT in human clinical specimens, and an elevation of inflammation amplifier target molecules in serum from patients, suggesting pathophysiological operations of the inflammation amplifier in humans as well.[47,49,51] Additional studies suggest that weak electric pulses or acupuncture might control the gateway reflex in humans. Therefore, elucidating the precise molecular mechanisms and neural networks for the gateway reflex and inflammation amplifier will have huge benefit on the inflammatory responses seen in chronic inflammatory diseases.

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Conflict of interest

None declared.

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