

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



The Gateway Reflex, a Novel Neuro-immune Interaction, is Critical for the Development of Mouse Multiple Sclerosis (MS) Models

Daisuke Kamimura, Yasunobu Arima,
Andrea Stofkova, Naoki Nishikawa, Kotaro Higuchi,
Takuto Ohki and Masaaki Murakami

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/62938>

Abstract

The central nervous system (CNS) is an immune-privileged tissue protected by the brain–blood barrier (BBB), which limits the absorption of substances and cells from blood flow. In the case of inflammatory diseases in the CNS, such as multiple sclerosis (MS), however, autoreactive T cells that attack brain autoantigens, including myelin proteins, circumvent the BBB. Despite the wide distribution of brain autoantigens, demyelination often occurs as discrete foci. This fact suggests that there might be a certain cue that guides autoreactive T cells to particular site(s) in the CNS. In other words, there exists a mechanism that facilitates a site-specific accumulation of autoreactive T cells in the CNS. Using a murine model of MS, experimental autoimmune encephalomyelitis (EAE), we identified dorsal vessels of the fifth lumbar (L5) spinal cord as the initial entry site of immune cells. The formation of a gateway for immune cells is defined by local neural stimulations. For example, neural stimulation by gravity creates this gateway by increasing the expression of chemokines that attract autoreactive T cells. Regional neural activation by the other stimuli, such as electric pulses or pain sensation, also induces gateway formation, but at different blood vessels via chemokine expression. These neuro-immune interactions are examples of the gateway reflex and are extensively reviewed in this chapter.

Keywords: gateway reflex, inflammation amplifier, NF- κ B, STAT, multiple sclerosis

1. Introduction

Multiple sclerosis (MS) is a common neurological disease that is estimated to inflict more than 2.5 million patients worldwide [1–4]. MS is associated with chronic inflammation of the central nervous system (CNS) with myelin antigens as the immune target during the inflammation processes. The clinical manifestations of MS are variable and often include both symptoms of upper motor neurons, such as hyperreflexia, ataxia, spasticity, and visual defects, and lower motor neuron symptoms, such as sensory neuron defects and paralysis [5]. Given the uniform presence of myelin antigens in the CNS, however, variable local, rather than systemic clinical symptoms raise the possibility that there exists specific sites in the CNS that are vulnerable to immune cells thus triggering auto-reactivity. As discussed below, we observed using a murine model of MS, experimental autoimmune encephalomyelitis (EAE), that regional neural stimulations permit entry of immune cells into the CNS, which may explain the variable MS symptoms among patients.

Genetic susceptibility of MS is well studied, and major histocompatibility complex (MHC) genes and genes associated with CD4⁺ helper T-cell activation and homeostasis are identified as susceptible genes. The strongest genetic linkage was found at certain alleles of MHC class II, which suggests a relationship between autoreactive CD4⁺ T cells and MS development in humans [6]. Consistently, autoreactive CD4⁺ T cells have important roles in the development and relapse of animal models of MS [4, 7–13]. In addition to MHC genes, several genome-wide association studies (GWAS) of MS patients have identified additional genetic loci including interleukin (IL)-17, IL-2 receptor (CD25), and IL-7 receptor (CD127), which are important for CD4⁺ T-cell effector function, activation, and survival [14–16]. These lines of genetic evidence suggest that blocking CD4⁺ T-cell entry into the CNS, thereby blocking subsequent inflammation in the CNS, would be an effective way for the treatment of MS. Indeed, drugs that target T-cell migration such as fingolimod (FTY720) and natalizumab (anti-VLA4 antibody) have shown clinical success in the treatment of MS. Fingolimod is an orally available sphingosine-1-phosphate receptor modulator, which reduces CD4⁺ T-cell invasion to the CNS due to the inhibition of lymphocyte egress from lymph nodes [17]. Natalizumab, on the other hand, targets alpha4 integrin (a subunit of VLA4), which is required for the migration of CD4⁺ T cells to inflamed CNS [4, 18]. The prominent clinical effects of these drugs provide a proof of concept for therapeutic strategies to suppress the invasion of autoreactive CD4⁺ T cells into the CNS. Because side effects such as progressive multifocal leukoencephalopathy warrant caution for the use of fingolimod and natalizumab [19, 20], a novel strategy including a blockade of the entry site, or gateway, of immune cells to the CNS is desired.

It is widely known that the CNS is an immune-privileged site, protected by the blood–brain barrier (BBB), which restricts the exchange of substances and cell migration into and out of the organ. However, CNS invasion by immune cells occurs in not only neuropathologic conditions including MS, but also normal healthy conditions. We have been studying where and how immune cells can enter the CNS using EAE models. In this chapter, our

recent findings about these gateways, a mechanism to operate them, and perspectives are discussed.

2. Blood–brain barrier and inflammation

The BBB is a specialized blood vessel formed by several cell types including vascular endothelial cells, pericytes, and astrocyte endfeet. In addition, tight junctions are critical for barrier development and are established through interactions of tight junction molecules, such as ZO-1, claudins, and occludins, between the vascular endothelial cells to cause a size exclusion of about 500 daltons [21, 22]. Despite this rigid barrier system, autoreactive T cells can enter to the CNS and cause autoimmune diseases such as MS. The breakdown of the BBB is also observed in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [23]. Because neuroinflammation is associated with almost all diseases in the CNS [24], the relationship between a dysfunctional BBB and inflammatory cytokines has been well studied. For example, TNF α increases BBB permeability during sepsis, and IL-1 β regulates the BBB via reactivation of the HIF-VEGF axis in MS [25, 26]. IFN γ and TNF α decrease the expression of TWIK-related potassium channel-1 in human brain microvascular endothelial cells, which leads to increase the transmigration of various immune cells across the endothelial cells in vitro [27]. IL-17A, which is a signature cytokine of Th17 cells, also contributes to BBB leakage in vitro and in vivo. EAE was significantly suppressed in IL-17A-deficient mice [28], and IL-17A-deficient CD4⁺ T cells hardly invade the CNS through the fifth lumbar (L5) spinal cord unlike normal ones [12]. IL-17A-mediated BBB dysfunction involves the formation of reactive oxygen species by cellular oxidases, which down-regulate tight junction molecules and the activation of the endothelial contractile machinery in vitro [29, 30]. Seeing that GWAS and animal model data have revealed that autoreactive CD4⁺ T cells producing many cytokines play a central role in MS, understanding the in vivo behavior of disease-causing CD4⁺ T cells offers crucial insights about the pathophysiology of MS. Intravital two-photon imaging using a rat transfer EAE model showed that there are at least three stages for CNS invasion by autoreactive CD4⁺ T cells: intraluminal scanning, perivascular scanning, and CNS-surface scanning [31]. This study also suggested that the reactivation of CNS-invading CD4⁺ T cells by perivascular/ meningeal antigen-presenting cells is followed by parenchymal infiltration of pathogenic CD4⁺ T cells [31]. Chemokine guidance and cellular adhesion events of pathogenic CD4⁺ T cells are also critical for the CNS invasion [12, 31]. Myelin autoantigen candidates associated with MS including Myelin Basic Protein (MBP), myelin-oligodendrocyte protein (MOG) and proteolipid proteins (PLP) [32–35] are present throughout CNS white matter, while MS lesions are often observed by MRI as focal plaques [36]. These facts suggest that there exists an additional signal that directs pathogenic CD4⁺ T cells to initiate inflammatory reactions at particular region(s) of the CNS. We recently demonstrated that this signal is provided by several types of neural activations. We also defined a molecular mechanism of the resulting neuro-immune interactions for the development and relapse of murine models of MS, as discussed in the following sections.

3. Gravity-mediated gateway reflex

We first considered the initial site where the autoreactive CD4⁺ T cells invade the CNS. In EAE models, mice or rats are generally immunized with various CNS antigens such as myelin-oligodendrocyte protein (MOG) emulsified with complete Freund's adjuvant (CFA) in the presence of pertussis toxin administration, which are critical for the generation of autoreactive CD4⁺ T cells and the induction of MS-like symptoms, including a progressive paralysis that usually begins at the tip of the tail. EAE can also be induced in wild-type animals by the transfer of autoreactive CD4⁺ T cells isolated from other EAE mice. This transfer EAE model is induced without antigens, CFA, or pertussis toxin treatment, making this model suitable for investigating the behavior of the disease-causing CD4⁺ T cells in detail. Whole-mount sagittal sections of adult mice at a preclinical stage of the transfer EAE model (day 4 or 5 after MOG-reactive CD4⁺ T-cell transfer) revealed that MOG-reactive pathogenic CD4⁺ T cells preferentially accumulated at the L5 spinal cord, but not in the brain at this early stage. This observation suitably explains the first clinical symptom of typical EAE, a weakness of the tail tip. A closer analysis of the L5 spinal cord revealed that the accumulation of MOG-reactive CD4⁺ T cells occurred around the dorsal vessels. Various chemokines including Th17-attracting CCL20 were up-regulated at the L5 dorsal vessels during EAE compared with dorsal vessels from other spinal cords. Indeed, anti-CCL20 antibody treatment or transfer of MOG-reactive CD4⁺ T cells lacking its receptor, CCR6, compromised the L5 accumulation of these CD4⁺ T cells and subsequent development of EAE, which is consistent with a published study [37]. Interestingly, the selective up-regulation of chemokines at the L5 dorsal vessels was observed even at steady state, albeit to a lesser degree than in the EAE condition. These results suggested that the L5 dorsal vessels are the first gateway for pathogenic CD4⁺ T cells in this transfer EAE system (**Figure 1**), and some physiological effects under steady state that modulate chemokine levels at the L5 dorsal vessels are enhanced during EAE.

Further investigation revealed that the selective up-regulation of chemokines at the L5 dorsal vessels at steady state were the result of neuro-immune interactions. It is known that neurons in the dorsal root ganglion (DRG) beside the L5 spinal cord are connected to the soleus muscles, the main anti-gravity muscles [38]. Consistent with a constant stimulation of the soleus muscles by Earth's gravity, the L5 DRG is the largest among all DRGs in mice and humans [39]. We hypothesized that constant stimulation by gravity might mediate the uniqueness of the L5 spinal cord in establishing the gateway of immune cells including autoreactive CD4⁺ T cells. Consistent with this hypothesis, we found two peaks of CCL20 expression at the dorsal vessels of the cervical and lumbar cords, although CCL20 expression was higher at the latter. To examine the effect of gravity, mice were suspended by their tails so that their hind legs were released from gravity. This treatment resulted in a reduced accumulation of MOG-specific CD4⁺ T cells at the L5 cord. Instead, these CD4⁺ T cells invaded the cervical cords as if a new gateway was created by the additional gravitational burden on the forefeet muscles due to the tail suspension [12]. Consistently, the tail suspension downregulated CCL20 levels in the L5 dorsal blood vessels, but up-regulated CCL20 in the cervical cords. Moreover, electric stimulations to the soleus muscles in tail-suspended mice restored CCL20 levels and CD4⁺ T-cell

accumulation at the L5 spinal cord [12]. These results suggest that a certain neural activation triggered by gravity contributes to the gateway at the L5 dorsal blood vessels for autoreactive CD4⁺ T cells to infiltrate the CNS (**Figure 1**).

IntechOpen

Figure 1. Gravity-mediated gateway reflex. Constant gravity-mediated stimulation of the soleus muscles induces the activation of sensory nerves, whose cell bodies are located at the dorsal root ganglion (DRG) of the fifth lumbar (L5) spinal cord. Signals via L5 DRG neurons travel to sympathetic neurons located nearby, resulting in norepinephrine (NE) secretion at the dorsal vessels there. NE enhances the inflammation amplifier (Amp) in the L5 dorsal vessel endothelium, causing an up-regulation of CCL20 and subsequent accumulation of pathogenic CD4⁺ T cells.

Sympathetic nerve activation was involved in the gravity-mediated chemokine expressions at the L5 dorsal vessels. c-Fos expression, which is a marker of neural activation, was higher in L5 sympathetic ganglions than in L1 sympathetic ganglions. Blood flow speeds at the L5 dorsal vessels, but not at other blood vessels including L1 dorsal vessels, femoral artery, and portal vein, became slower when mice were tail suspended, while electronic stimulation of the soleus muscles increased the flow speed, suggesting an involvement of autonomic nerves including sympathetic ones in this response [12]. Furthermore, pharmacological blockade of adrenergic receptors significantly inhibited CCL20 levels, NF- κ B activation and MOG-reactive pathogenic CD4⁺ T-cell accumulation at the L5 dorsal vessels, and also suppressed clinical signs of EAE [12]. Thus, sensory nerve activation due to anti-gravity responses leads to a sympathetic nerve stimulation that creates a gateway for immune cells to pass through the CNS via the L5 dorsal vessels [12]. This neuro-immune response, which leads to change in the status of the vascular endothelium, is named the “gateway reflex” [40–42]. Other examples of the gateway reflex are described below.

4. Electric stimulation-mediated gateway reflex

Neural activations can be artificially induced by various methods including electric stimulations and treatment with reagents. We wondered if electric stimulation of different muscles could create gateways in blood vessels at distinct positions via regional neural activations. As discussed earlier, weak electric stimulation to the soleus muscles restored chemokine expression at the L5 dorsal vessels of tail-suspended mice, because the cell body of sensory neurons in the soleus muscles is mainly located in the L5 DRG. We applied this methodology to other muscles. As expected, electric stimulations to thigh muscles including the quadriceps, which are known to be regulated by L3 DRG neurons, led to an increased expression of chemokines in L3 dorsal vessels in mice, while electric stimulations of upper regions, such as forefoot muscles including the epitrochlearis and triceps brachii, resulted in an up-regulation of chemokines in the fifth cervical to fifth thoracic cord dorsal vessels, which is where the DRG

IntechOpen

Figure 2. Electric stimulation-mediated gateway reflex. Artificially induced neural activation by weak electric stimulation followed by sensory-sympathetic cross talk can trigger the gateway reflex. Electric pulses to the triceps induce chemokine up-regulation at the dorsal vessels of the fifth cervical (C5) to fifth thoracic (T5) spinal cord through activation of the inflammation amplifier (Amp). Similarly, stimulation of the quadriceps induces a gateway at the dorsal vessels of the third lumbar (L3) cord, whereas the gateway dorsal vessels of the L5 cord are created by electric pulses to the soleus muscles.

neurons of the C5-T5 spinal cords project to/from these muscles. These results established the electric stimulation-mediated gateway as another example of the gateway reflex (**Figure 2**). In addition, they offer an important implication that the gateway reflex can be controlled by artificially stimulating specific neurons, providing a promising opportunity for a novel therapeutic strategy against inflammatory diseases in the CNS.

5. Pain-mediated gateway reflex

It is reported that many MS patients experience pain, and some patients take pain relief medication to improve their quality of life [43]. Pain is a common symptom in many diseases and disorders, but it is often considered a symptomatic effect that arises from tissue damage caused by the disease. However, it is known that pain triggers neural inputs via sensory neurons expressing nociceptors such as TRPV1 [44, 45]. Therefore, we assumed that a pain-mediated gateway reflex might exist, which would affect the disease status of MS and EAE. In EAE, modulations of nociception (pain sensation) are reported during the disease development [46]. Adoptive transfer of MOG reactive, pathogenic CD4⁺ T cells induces a transient EAE within a week after transfer, followed by a remission phase. Mice that experienced the first episode of EAE and are in the remission phase, which we termed EAE-recovered mice, never develop EAE symptoms again under normal conditions. We experimentally induced pain sensation in these EAE-recovered mice by ligating the middle branch of the trigeminal nerves, which has been reported as being composed of only sensory neurons [47]. Pain induction at the time of pathogenic T-cell transfer resulted in a persistence of EAE symptoms and caused considerable delay of the remission phase. On the contrary, suppressing pain by medication inhibited EAE development in several mouse models including an active immunization model in SJL mice. Because a majority of MS progresses with relapse and it is reported that pain is more intense in MS patients with higher disease scores [48], we examined whether pain induction triggers EAE relapse in mice. EAE-recovered mice were subjected to trigeminal nerve ligation to induce pain sensation. In separate experiments, pain-processing nerves were also chemically activated by the injection of capsaicin and substance P at the cheek or forefeet of EAE-recovered mice. All these treatments induced relapse of EAE, indicating that pain is not a simple by-product of the disease, but significantly contributes to the development and EAE relapse [13]. Similar to the gravity-mediated gateway reflex, a sensory-sympathetic pathway is involved in the pain-mediated EAE relapse, because pharmacological or genetic inhibition of pain-processing molecules such as TRPV1 and Nav1.8, and chemical ablation of sympathetic neurons by 6-hydroxydopamine (6-OHDA) suppressed EAE relapse induced by trigeminal nerve ligation. Interestingly, stress-mediated events, including immobilization stress and forced swimming stress (about 20 minutes/day), did not trigger EAE relapse although serum corticosterone, norepinephrine, and epinephrine were induced at a similar level to pain induction, suggesting that specific sensory-sympathetic nerve pathways, rather than systemic hormonal stress responses, mediate the relapse of EAE (**Figure 3**).

IntechOpen

Figure 3. Pain-mediated gateway reflex. Pain-induced sensory neuron activation results in the activation of specific sympathetic neurons that control norepinephrine (NE) expression around the ventral vessels of every level of the spinal cords. This system is regulated by the anterior cingulate cortex (ACC) in the brain. Because the fifth lumbar (L5) spinal cord abundantly contains MHC class II high activated monocytes, this region is affected significantly during pain sensation. NE from pain-specific sympathetic neurons at the ventral vessels induces the production of CX3CL1 from the activated monocytes, further recruiting these cells in an auto/paracrine manner. The MHC class II high activated monocytes are able to present MOG antigens to pathogenic CD4⁺ T cells, which in turn activate the inflammation amplifier (Amp) in regional endothelial cells and subsequently cause a relapse in the disease.

During the first episode of EAE, MOG-reactive CD4⁺ T cells enter the CNS from the dorsal vessels of the L5 spinal cord [12]. The transferred CD4⁺ T cells are then found at the upper levels of the spinal cord and brain, which matches typical clinical manifestations of EAE including the initial tail tip weakness and subsequent ascending paralysis. Intriguingly, after pain induction in apparently normal EAE-recovered mice, the relapse also starts from the loss of tonicity of the tail tip, suggesting that the L5 cord could again be a gateway for relapse. However, an immunohistochemical examination of the L5 spinal cord from EAE-recovered mice showed differences with naïve mice, with many MHC class II high monocytes around the meningeal region. After pain induction, these cells accumulated at the L5 ventral vessels, but not dorsal vessels within a day. This accumulation is dependent on a chemokine CX3CL1, which is secreted from the MHC class II high monocytes themselves and astrocytes after norepinephrine stimulation. Therefore, the pain-mediated gateway reflex induces norepinephrine secretion from sympathetic neurons around the L5 ventral vessels and subsequent auto/paracrine induction of CX3CL1 followed by MHC class II high monocyte accumulations. Because these monocytes are able to present MOG peptides, circulating MOG-reactive pathogenic CD4⁺ T cells can recognize the L5 ventral vessels as an entry site to the CNS. Indeed, a depletion of CD4⁺ T cells including pathogenic ones from EAE-recovered mice abrogated the clinical symptoms of EAE relapse (i.e. paralysis), but the accumulation of MHC class II high monocytes around the L5 ventral vessels remained intact. These results suggested that the activated monocyte accumulation is an upstream event relative to pathogenic CD4⁺ T-cell invasion and required for EAE relapse induced by pain sensation [13].

The sympathetic nerve activation caused by pain appeared to be relatively systemic compared with that via the sensory pathway, since the expression levels of neural activation marker c-Fos increased in certain nerve cells in sympathetic ganglions at all spinal levels investigated, which is in contrast to the gravity-mediated gateway reflex in which the L5 sympathetic ganglion alone showed the highest expression of c-Fos. However, the pain-mediated gateway reflex affects blood vessels at the ventral sides of the L5 spinal cord. Why does the pain-mediated gateway reflex influence the L5 level alone, similar to the gravity-mediated gateway reflex? We assume that this property is related to the fact that the L5 cord is the first lesion in the transfer EAE model. Even at the remission phase of transfer EAE, MHC class II high monocytes that have infiltrated in the CNS during the initial episode remained mostly in the L5 spinal cord despite the disappearance of clinical symptoms [13]. Otherwise, gravity-mediated neural inputs might make the L5 environment different from other CNS regions. Whichever the explanation, we found that pain induction similarly triggers sympathetic neural activation to some neuron cells of every spinal level, but the L5 spinal cord responded strongest, most likely due to an abundance of activated monocytes.

It is known that pain-sensing neurons activate sympathetic neurons at least partially via the anterior cingulate cortex (ACC) in the somatosensory area of the brain [49, 50]. Blockade of this sensory-sympathetic connection by the injection of an N-methyl-D-aspartate (NMDA) receptor antagonist at the ACC suppressed the accumulation of MHC class II high monocytes at the L5 ventral vessels even after pain induction, and conversely, injection of an NMDA agonist at the ACC induced the accumulation of these cells without pain stimulation in EAE-recovered mice. These results clearly suggest that the sensory-sympathetic connection at the ACC is involved in pain-mediated EAE relapse. Thus, we showed a third pain-mediated gateway reflex where neural signals travel from a TRPV1 and Nav1.8-dependent sensory circuit to sympathetic neurons via the ACC, reaching the dorsal vessels of the spinal cords [13] (**Figure 3**).

The study of the pain-mediated gateway reflex also highlights the importance of MHC class II high monocytes for EAE relapse. Parabiosis experiments, in which two different congenically marked mice are joined together surgically to share blood circulation, revealed that MHC class II high monocytes that accumulated at the L5 ventral vessels were derived from the peripheral blood stream during the first EAE development and stayed in the CNS, but not from resident microglia in the CNS [13]. These infiltrated MHC class II high monocytes survive in the CNS during the remission phase and play a pathogenic role upon relapse when the mice have a pain sensation. Therefore, in addition to controlling neural pathways and/or molecular machinery of the gateway reflexes, MHC class II high monocytes could also be a potential cellular target for treatment of relapse-remitting MS and progressive-relapsing MS.

6. Other gateway reflexes

In addition to the sensory perceptions described above, including gravity-, electric stimulation-, and pain-mediated stimuli, neural excitations occur in response to emotional alterations

and physical/mental stresses. These physiological events too are often associated with MS symptoms [51]. Worsening of the clinical symptoms of neurological diseases including MS when the body is exposed to high ambient temperatures, called Uhthoff's phenomenon, is another well-known example [52]. It is also known that stress burdens are associated with activation of the sympathetic nervous system, such as increased noradrenaline levels in the peripheral blood. Despite these correlations, a mechanical link between stress and disease development remains elusive. Neuronal activations, noradrenaline surge, and disease development are fundamental of the gateway reflex. Stress-induced neural signals traveling to the CNS might modulate specific blood vessels depending on stress type, opening or closing a gateway for immune cells in the CNS. To examine this possibility, we have been testing the effects of various stresses on the pathogenesis of EAE. Although restraint stress and forced swim stress did not provoke EAE relapse [13], some stresses were found to worsen the clinical symptoms, whereas another stress prevented EAE development. These phenomena represent the fourth and fifth examples of the gateway reflex. The effects of good stress, or eustress, have also been reported in cancer models, in which tumor growth is delayed when mice are reared under an enriched environment with running wheels, tunnels, etc. in a larger cage [53, 54]. We suggest it may be possible to prevent diseases if stimulating the appropriate neurons can trigger a good gateway reflex.

Kevin Tracey and his colleagues have reported that activation of the vagus nerves, which mainly consist of parasympathetic nerves, leads to the suppression of systemic inflammation during septic shock in mice. This neural reflex is specifically called the "inflammatory reflex." In this context, lipopolysaccharide treatment in mice leads to norepinephrine release in the spleen via vagus and splenic nerves. A subset of T cells that receive norepinephrine signaling produces acetylcholine, which acts on macrophages to suppress the lipopolysaccharide-induced expression of inflammatory mediators such as $\text{TNF}\alpha$ [55], thus acting as a negative feedback system for excessive inflammatory reactions such as septic shock. It is also reported that electro-acupuncture in mice at the ST36 Zusanli acupoint, which is located close to the common peroneal and tibial branches of the sciatic nerve, or directly to the sciatic nerve prevents a sepsis model through vagal activation and dopamine production [56]. A similar strategy may generate the gateway reflex in humans.

7. Inflammation amplifier

As described above, regional sensory neural activations transmit neural signals to sympathetic neurons, which then secrete norepinephrine at the target blood vessels to establish a gateway and recruit immune cells. Because chemokines have a critical role in immune cell recruitment, we hypothesized that machinery responsible for the production of chemokines could act as an interface between neural signals and immune reactions. Indeed, we discovered the inflammation amplifier, which is a local chemokine inducer. In this section, we describe the inflammation amplifier as a molecular mechanism for neuro-immune interactions in blood vessels.

IntechOpen

Figure 4. Inflammation amplifier. Simultaneous activation of the transcription factors NF- κ B and STATs creates a synergistic effect on the production of inflammation mediators, such as chemokines, growth factors, and IL-6, in nonimmune cells, such as vascular endothelial cells and fibroblasts. Factors activating NF- κ B and STATs are various and include IL-6, TNF α , and IL-17. IL-6 is expected to act on nonimmune cells to form a positive feedback loop that amplifies this synergistic effect. Massive chemokine and growth factor production by this mechanism, called the inflammation amplifier, plays a central role in the pathogenesis of many inflammatory diseases and disorders.

To establish an IL-6-dependent autoimmune mouse model, we previously generated a knock-in mouse strain called F759 mice, which lacks the SOCS3 binding site of the IL-6 signal transducer, gp130, thereby inhibiting SOCS3-dependent negative signaling and excessive STAT3 activation [57]. Accordingly, F759 mice developed spontaneous rheumatoid arthritis-like joint disease and autoantibody production [58], which is consistent with the high levels of IL-6 and clinical success of its signaling blockade in rheumatoid arthritis patients [59, 60]. Using these autoimmune-prone F759 mice, we performed mechanistic analyses for the pathogenesis of their autoimmunity and found that IL-6 signaling in nonimmune cells, such as vascular endothelial cells and fibroblasts, rather than immune cells, is important for the exaggerated inflammation [61, 62]. Mechanistically, coactivation of the transcription factors NF- κ B and STATs in nonimmune cells led to a synergistic production of inflammatory agents, including chemokines and growth factors, compared with NF- κ B or STAT activation alone (**Figure 4**). Factors that activate NF- κ B and STATs and therefore trigger the inflammation amplifier include IL-17A, TNF α , IL-22, IL-6, and IFN γ . Furthermore, neurotransmitters, including

norepinephrine and several growth factors, augment the amplifier by enhancing NF- κ B activation [12, 63]. Since chemokines can recruit immune cells and promote inflammation, we named this synergistic mechanism in nonimmune cells the “inflammation amplifier” [64, 65]. Mice lacking gp130 or STAT3 in type 1 collagen+ cells including various nonimmune cells were highly resistant to animal models of rheumatoid arthritis, MS, and chronic graft rejection via suppression of the regional accumulation of immune cells [12, 13, 62, 63, 66, 67]. The inflammation amplifier is also seen in astrocytes, resulting in the development of EAE [68]. In addition, we found evidence of the inflammation amplifier activation in human clinical specimens, and human disease-associated genes are highly enriched in inflammation amplifier-related genes according to genome-wide RNA functional screening [67]. These data suggested that the inflammation amplifier is a critical mechanism for the development of various inflammatory diseases via excessive expression of inflammatory chemokines and growth factors from nonimmune cells (**Figure 4**).

Subsequent studies about the gateway reflex demonstrated that neural signals translate into inflammatory signals by the inflammation amplifier in target vascular endothelial cells. In the case of the gravity-mediated gateway reflex, neural signals from the soleus muscles reached the L5 dorsal vessel endothelium via sensory-sympathetic cross talk, where norepinephrine from the activated sympathetic neurons excessively stimulated the inflammation amplifier by increasing NF- κ B activity to secrete various NF- κ B-targets including chemokines. Moreover, the L5 but not L1 dorsal vessel endothelium showed activation of STAT3. Pharmacological inhibition of beta-adrenergic receptors suppressed NF- κ B activation, chemokine production, and pathogenic CD4+ T-cell accumulation in the L5 dorsal vessels and EAE development [12]. Similarly, during the pain-mediated gateway reflex, treatment with a beta-adrenergic receptor antagonist resulted in no accumulation of activated monocytes or pathogenic CD4+ T cells around the target dorsal vessels of the L5 spinal cord. Neutralization of cytokines that activate the inflammation amplifier such as IL-6 and IL-17A also suppressed the pain-induced EAE relapse and pathogenic CD4+ T-cell accumulation [13]. These results indicate that the inflammation amplifier is a foundation of the immunological response induced by the gateway reflex.

8. Future directions

The CNS is an immune-privileged site protected by the BBB, but the gateway reflex, which can be triggered by various neural stimulations, can induce gateways for immune cells to circumvent the BBB. So far, three kinds of gateway reflex have been identified: gravity, electric stimulation, and pain-induced, all of which involve sensory-sympathetic communication. Further study of the mechanisms driving the gateway reflex should consider the neural network involved and whether it is present in other organs and tissues. Newly developed imaging techniques and tools including a tissue decolorization reagent, CUBIC [69, 70], will help elucidate the former. For the latter, it is recently reported there exists barrier architecture with similar components to the BBB in the gut endothelium, the so-called gut-vascular barrier [22]. A similar system may explain how immune cells breach this barrier. Because neuronal circuits run throughout the body, the gateway reflex could have a tremendous

clinical benefit, as closing a specific gateway would dampen autoimmune inflammation in a target organ without massive systemic immune suppression, while opening it in tumors could enhance cancer immunotherapy.

Acknowledgements

We thank the excellent assistance by Ms. Chiemi Nakayama, Ms. Mitsue Ezawa and Ms. Satomi Fukumoto (Hokkaido University, Sapporo, Japan), and Dr. Peter Karagiannis for reading the manuscript. This work was supported by KAKENHI (D. K., Y. A., T. A., and M. M.), Takeda Science Foundation (M. M. and D. K.), Institute for Fermentation Osaka (M. M.), Mitsubishi Foundation (M. M.), Uehara Memorial Foundation (M. M.), Mochida Memorial Foundation for Medical and Pharmaceutical Research (D. K.), Suzuken Memorial Foundation (D. K. and Y. A.), Japan Prize Foundation (Y. A.), Ono Medical Research Foundation (Y. A.), Kanzawa Medical Research Foundation (Y. A.), Kishimoto Foundation (Y. A.), Nagao Takeshi Research Foundation (Y. A.), Tokyo Medical Research Foundation (M. M. and Y. A.), JSPS Postdoctoral Fellowship for Foreign Researchers (A.S.), JST-CREST program (M. M.), and the Osaka Foundation for the Promotion of Clinical Immunology (M. M.). The authors declare they have no conflicting financial interests.

Author details

Daisuke Kamimura*, Yasunobu Arima, Andrea Stofkova, Naoki Nishikawa, Kotaro Higuchi, Takuto Ohki and Masaaki Murakami

*Address all correspondence to: murakami@igm.hokudai.ac.jp

Molecular Neuroimmunology, Graduate School of Medicine, Institute for Genetic Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

References

- [1] Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. *N Engl J Med* 2000;343(13):938–952. DOI: 10.1056/NEJM200009283431307
- [2] Hafler DA, Slavik JM, Anderson DE, O'Connor KC, De Jager P, Baecher-Allan C. Multiple sclerosis. *Immunol Rev* 2005;204:208–231. DOI: 10.1111/j.0105-2896.2005.00240.x
- [3] McFarland HF, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 2007;8(9):913–919. DOI: 10.1038/ni1507

- [4] Steinman L. Immunology of relapse and remission in multiple sclerosis. *Annu Rev Immunol* 2014;32:257–281. DOI: 10.1146/annurev-immunol-032713-120227
- [5] Keegan BM, Noseworthy JH. Multiple sclerosis. *Ann Rev Med* 2002;53:285–302. DOI: 10.1146/annurev.med.53.082901.103909
- [6] International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, et al.: Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011;476(7359):214–219. DOI: 10.1038/nature10251
- [7] Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 2002;20:101–123. DOI: 10.1146/annurev.immunol.20.081701.141316
- [8] Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* 2005;23:683–747. DOI: 10.1146/annurev.immunol.23.021704.115707
- [9] Steinman L, Zamvil SS. Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. *Trends Immunol* 2005;26(11):565–571. DOI: 10.1016/j.it.2005.08.014
- [10] Goverman J. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* 2009;9(6):393–407. DOI: 10.1038/nri2550
- [11] Rangachari M, Kuchroo VK. Using EAE to better understand principles of immune function and autoimmune pathology. *J Autoimmun* 2013;45:31–39. DOI: 10.1016/j.jaut.2013.06.008
- [12] Arima Y, Harada M, Kamimura D, Park JH, Kawano F, Yull FE, et al.: Regional neural activation defines a gateway for autoreactive T cells to cross the blood–brain barrier. *Cell* 2012;148(3):447–457. DOI: 10.1016/j.cell.2012.01.022
- [13] Arima Y, Kamimura D, Atsumi T, Harada M, Kawamoto T, Nishikawa N, et al.: A pain-mediated neural signal induces relapse in murine autoimmune encephalomyelitis, a multiple sclerosis model. *eLife* 2015;4. DOI: 10.7554/eLife.08733
- [14] Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A, et al.: Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet* 2007;39(9):1083–1091. DOI: 10.1038/ng2103
- [15] Lundmark F, Duvefelt K, Iacobaeus E, Kockum I, Wallstrom E, Khademi M, et al.: Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. *Nat Genet* 2007;39(9):1108–1113. DOI: 10.1038/ng2106

- [16] Australia, New Zealand Multiple Sclerosis Genetics C. Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. *Nat Genet* 2009;41(7):824–828. DOI: 10.1038/ng.396
- [17] Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, et al.: Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N Engl J Med* 2010;362(5):402–415. DOI: 10.1056/NEJMoa0907839
- [18] Steinman L. Blocking adhesion molecules as therapy for multiple sclerosis: natalizumab. *Nat Reviews Drug Discov* 2005;4(6):510–518. DOI: 10.1038/nrd1752
- [19] Bloomgren G, Richman S, Hotermans C, Subramanyam M, Goelz S, Natarajan A, et al.: Risk of natalizumab-associated progressive multifocal leukoencephalopathy. *N Engl J Med* 2012;366(20):1870–1880. DOI: 10.1056/NEJMoa1107829
- [20] Van Schependom J, Gielen J, Laton J, Nagels G. Assessing PML risk under immunotherapy: if all you have is a hammer, everything looks like a nail. *Mult Scler* 2015. DOI: 10.1177/1352458515596458
- [21] Steed E, Balda MS, Matter K. Dynamics and functions of tight junctions. *Trends Cell Biol* 2010;20(3):142–149. DOI: 10.1016/j.tcb.2009.12.002.
- [22] Spadoni I, Zagato E, Bertocchi A, Paolinelli R, Hot E, Di Sabatino A, et al.: A gut-vascular barrier controls the systemic dissemination of bacteria. *Science* 2015;350(6262):830–834. DOI: 10.1126/science.aad0135
- [23] Zlokovic BV. The blood–brain barrier in health and chronic neurodegenerative disorders. *Neuron* 2008;57(2):178–201. DOI: 10.1016/j.neuron.2008.01.003.
- [24] Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease--a double-edged sword. *Neuron* 2002;35(3):419–432.
- [25] Tsao N, Hsu HP, Wu CM, Liu CC, Lei HY. Tumour necrosis factor-alpha causes an increase in blood–brain barrier permeability during sepsis. *J Med Microbiol* 2001;50(9):812–821.
- [26] Argaw AT, Zhang Y, Snyder BJ, Zhao ML, Kopp N, Lee SC, et al.: IL-1beta regulates blood–brain barrier permeability via reactivation of the hypoxia-angiogenesis program. *J Immunol* 2006;177(8):5574–5584.
- [27] Bittner S, Ruck T, Schuhmann MK, Herrmann AM, Moha ou Maati H, Bobak N, et al.: Endothelial TWIK-related potassium channel-1 (TREK1) regulates immune-cell trafficking into the CNS. *Nat Med* 2013;19(9):1161–1165. DOI: 10.1038/nm.3303
- [28] Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al.: IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2006;177(1):566–573.

- [29] Huppert J, Closhen D, Croxford A, White R, Kulig P, Pietrowski E, et al.: Cellular mechanisms of IL-17-induced blood–brain barrier disruption. *FASEB J* 2010;24(4):1023–1034. DOI: 10.1096/fj.09-141978
- [30] Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, et al.: Human TH17 lymphocytes promote blood–brain barrier disruption and central nervous system inflammation. *Nat Med* 2007;13(10):1173–1175. nm1651 [pii] 10.1038/nm1651
- [31] Bartholomaeus I, Kawakami N, Odoardi F, Schlager C, Miljkovic D, Ellwart JW, et al.: Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* 2009;462(7269):94–98. DOI: 10.1038/nature08478
- [32] Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL, Hafler DA. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* 1990;346(6280):183–187. DOI: 10.1038/346183a0
- [33] Allegretta M, Nicklas JA, Sriram S, Albertini RJ. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science* 1990;247(4943):718–721.
- [34] Zhang J, Markovic-Plese S, Lacet B, Raus J, Weiner HL, Hafler DA. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* 1994;179(3):973–984.
- [35] Reindl M, Di Pauli F, Rostasy K, Berger T. The spectrum of MOG autoantibody-associated demyelinating diseases. *Nat Reviews Neurol* 2013;9(8):455–461. DOI: 10.1038/nrneurol.2013.118
- [36] Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al.: Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011;69(2):292–302. DOI: 10.1002/ana.22366
- [37] Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, et al.: C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 2009;10(5):514–523. DOI: 10.1038/ni.1716
- [38] Ohira Y, Kawano F, Stevens JL, Wang XD, Ishihara A. Load-dependent regulation of neuromuscular system. *J Gravit Physiol* 2004;11(2):P127-128.
- [39] Shen J, Wang HY, Chen JY, Liang BL. Morphologic analysis of normal human lumbar dorsal root ganglion by 3D MR imaging. *AJNR Am J neuroradiol* 2006;27(10):2098–2103.
- [40] Tracey KJ. Immune cells exploit a neural circuit to enter the CNS. *Cell* 2012;148(3):392–394. DOI: 10.1016/j.cell.2012.01.025

- [41] Andersson U, Tracey KJ. Neural reflexes in inflammation and immunity. *J Exp Med* 2012;209(6):1057–1068. DOI: 10.1084/jem.20120571
- [42] Sabharwal L, Kamimura D, Meng J, Bando H, Ogura H, Nakayama C, et al.: The Gateway Reflex, which is mediated by the inflammation amplifier, directs pathogenic immune cells into the CNS. *J Biochem* 2014;156(6):299–304. DOI: 10.1093/jb/mvu057
- [43] Feinstein A, Magalhaes S, Richard JF, Audet B, Moore C. The link between multiple sclerosis and depression. *Nat Rev Neurol* 2014;10(9):507–517. DOI: 10.1038/nrneurol.2014.139
- [44] Morales-Lazaro SL, Simon SA, Rosenbaum T. The role of endogenous molecules in modulating pain through transient receptor potential vanilloid 1 (TRPV1). *J Physiol* 2013;591(Pt 13):3109–3121. DOI: 10.1113/jphysiol.2013.251751
- [45] Bennett DL, Woods CG. Painful and painless channelopathies. *Lancet Neurol* 2014;13(6):587–599. DOI: 10.1016/S1474-4422(14)70024-9
- [46] Khan N, Smith MT. Multiple sclerosis-induced neuropathic pain: pharmacological management and pathophysiological insights from rodent EAE models. *Inflammopharmacology* 2014;22(1):1–22. DOI: 10.1007/s10787-013-0195-3
- [47] Thygesen TH, Baad-Hansen L, Svensson P. Sensory action potentials of the maxillary nerve: a methodologic study with clinical implications. *J Oral Maxillofac Surg : official journal of the American Association of Oral and Maxillofacial Surgeons* 2009;67(3):537–542. DOI: 10.1016/j.joms.2008.02.015
- [48] Ehde DM, Gibbons LE, Chwastiak L, Bombardier CH, Sullivan MD, Kraft GH. Chronic pain in a large community sample of persons with multiple sclerosis. *Mult Scler* 2003;9(6):605–611.
- [49] Ikemoto K, Kitahama K, Nishimura A, Jouvet A, Nishi K, Arai R, et al.: Tyrosine hydroxylase and aromatic L-amino acid decarboxylase do not coexist in neurons in the human anterior cingulate cortex. *Neurosci Letters* 1999;269(1):37–40.
- [50] Critchley HD. Neural mechanisms of autonomic, affective, and cognitive integration. *J Comp Neurol* 2005;493(1):154–166. DOI: 10.1002/cne.20749
- [51] Karagkouni A, Alevizos M, Theoharides TC. Effect of stress on brain inflammation and multiple sclerosis. *Autoimmun Rev* 2013;12(10):947–953. DOI: 10.1016/j.autrev.2013.02.006
- [52] Frohman TC, Davis SL, Beh S, Greenberg BM, Remington G, Frohman EM. Uhthoff's phenomena in MS--clinical features and pathophysiology. *Nat Rev Neurol* 2013;9(9):535–540. DOI: 10.1038/nrneurol.2013.98
- [53] Cao L, Liu X, Lin EJ, Wang C, Choi EY, Riban V, et al.: Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition. *Cell* 2010;142(1):52–64. DOI: 10.1016/j.cell.2010.05.029.

- [54] Li G, Gan Y, Fan Y, Wu Y, Lin H, Song Y, et al.: Enriched environment inhibits mouse pancreatic cancer growth and down-regulates the expression of mitochondria-related genes in cancer cells. *Sci Rep* 2015;5:7856. DOI: 10.1038/srep07856
- [55] Andersson U, Tracey KJ. Reflex principles of immunological homeostasis. *Annu Rev Immunol* 2012;30:313–335. DOI: 10.1146/annurev-immunol-020711-075015
- [56] Torres-Rosas R, Yehia G, Pena G, Mishra P, del Rocio Thompson-Bonilla M, Moreno-Eutimio MA, et al.: Dopamine mediates vagal modulation of the immune system by electroacupuncture. *Nat Med* 2014;20(3):291–295. DOI: 10.1038/nm.3479
- [57] Ohtani T, Ishihara K, Atsumi T, Nishida K, Kaneko Y, Miyata T, et al.: Dissection of signaling cascades through gp130 in vivo: reciprocal roles for STAT3- and SHP2-mediated signals in immune responses. *Immunity* 2000;12(1):95–105.
- [58] Atsumi T, Ishihara K, Kamimura D, Ikushima H, Ohtani T, Hirota S, et al.: A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis. *J Exp Med* 2002;196(7):979–990.
- [59] Nishimoto N, Kishimoto T, Yoshizaki K. Anti-interleukin 6 receptor antibody treatment in rheumatic disease. *Ann Rheum Dis* 2000;59 Suppl 1:i21-27.
- [60] Nishimoto N, Kishimoto T. Inhibition of IL-6 for the treatment of inflammatory diseases. *Curr Opin Pharmacol* 2004;4(4):386–391. DOI: 10.1016/j.coph.2004.03.005
- [61] Sawa S, Kamimura D, Jin GH, Morikawa H, Kamon H, Nishihara M, et al.: Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4+ T cells. *J Exp Med* 2006;203(6):1459–1470. DOI: 10.1084/jem.20052187
- [62] Ogura H, Murakami M, Okuyama Y, Tsuruoka M, Kitabayashi C, Kanamoto M, et al.: Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. *Immunity* 2008;29(4):628–636. DOI: 10.1016/j.immuni.2008.07.018
- [63] Harada M, Kamimura D, Arima Y, Kohsaka H, Nakatsuji Y, Nishida M, et al.: Temporal expression of growth factors triggered by epiregulin regulates inflammation development. *J Immunol* 2015;194(3):1039–1046. DOI: 10.4049/jimmunol.1400562
- [64] Murakami M, Hirano T. The pathological and physiological roles of IL-6 amplifier activation. *Int J Biol Sci* 2012;8(9):1267–1280. DOI: 10.7150/ijbs.4828
- [65] Atsumi T, Singh R, Sabharwal L, Bando H, Meng J, Arima Y, et al.: Inflammation amplifier, a new paradigm in cancer biology. *Cancer Res* 2014;74(1):8–14. DOI: 10.1158/0008-5472.CAN-13-2322
- [66] Lee J, Nakagiri T, Oto T, Harada M, Morii E, Shintani Y, et al.: IL-6 amplifier, NF-kappaB-triggered positive feedback for IL-6 signaling, in grafts is involved in allogeneic rejection responses. *J Immunol* 2012;189(4):1928–1936. DOI: 10.4049/jimmunol.1103613

- [67] Murakami M, Harada M, Kamimura D, Ogura H, Okuyama Y, Kumai N, et al.: Disease-association analysis of an inflammation-related feedback loop. *Cell Rep* 2013;3(3):946–959. DOI: 10.1016/j.celrep.2013.01.028
- [68] Ma X, Reynolds SL, Baker BJ, Li X, Benveniste EN, Qin H. IL-17 enhancement of the IL-6 signaling cascade in astrocytes. *J Immunol* 2010;184(9):4898–4906. DOI: 10.4049/jimmunol.1000142
- [69] Susaki EA, Tainaka K, Perrin D, Kishino F, Tawara T, Watanabe TM, et al.: Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. *Cell* 2014;157(3):726–739. DOI: 10.1016/j.cell.2014.03.042
- [70] Tainaka K, Kubota SI, Suyama TQ, Susaki EA, Perrin D, Ukai-Tadenuma M, et al.: Whole-body imaging with single-cell resolution by tissue decolorization. *Cell* 2014;159(4):911–924. DOI: 10.1016/j.cell.2014.10.034

