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Invited review article

In vivo imaging in autoimmune diseases in the central nervous system

Naoto Kawakami [a, b, *]

[a] Institute of Clinical Neuroimmunology, Biomedical Center and University Hospital, Ludwig-Maximilians Universitaet Muenchen, Munich, Germany
[b] Neuroimmunology Group, Max-Planck Institute of Neurobiology, Martinsried, Germany

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Abbreviations:
APCs, antigen presenting cells; BBB, blood–brain barrier; CFP, cyan fluorescent protein; CFSE, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; CMTMR, 5-(and-6)-(((4-chloromethyl) benzoyl)amino)tetramethylrhodamine; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GRIN, gradient index; MBP, myelin basic protein; MIP, macrophage migration inhibitory factor; MRI, magnetic resonance imaging; N.A., numerical aperture; NFAT, nuclear factor of activated T cells; OVA, ovalbumin; p.t., post transfer; YFP, yellow fluorescent protein

ABSTRACT

Intravital imaging is becoming more popular and is being used to visualize cellular motility and functions. In contrast to in vitro analysis, which resembles in vivo analysis, intravital imaging can be used to observe and analyze cells directly in vivo. In this review, I will summarize recent imaging studies of autoreactive T cell infiltration into the central nervous system (CNS) and provide technical background. During their in vivo journey, autoreactive T cells interact with many different cells. At first, autoreactive T cells interact with endothelial cells in the airways of the lung or with splenocytes, where they acquire a migratory phenotype to infiltrate into the CNS. After arriving at the CNS, they interact with endothelial cells of the leptomeningeal vessels or the choroid plexus before passing through the blood–brain barrier. CNS-infiltrating T cells become activated by recognizing endogenous autoantigens presented by local antigen-presenting cells (APCs). This activation was visualized in vivo by using protein-based sensors. One such sensor detects changes in intracellular calcium concentration as an early marker of T cell activation. Another sensor detects translocation of Nuclear factor of activated T-cells (NFAT) from cytosol to nucleus as a definitive sign of T cell activation. Importantly, intravital imaging is not just used to visualize cellular behavior. Together with precise analysis, intravital imaging deepens our knowledge of cellular functions in living organs and also provides a platform for developing therapeutic treatments.

Introduction

During inflammation, many different types of immune cells migrate to and accumulate in the lesion. Those cells interact with each other and work together. The functions of each cell population have often been studied in vitro after isolation from animals, which has provided valuable information. Under these experimental conditions, cells are cultured and/or stored. However, an in vitro system lacks blood flow and often lacks three-dimensional structure, which resembles environment in the organ. In addition, purified cytokines and growth factors are often added to the culture, which might create different conditions from in vivo, where cells are exposed to a mixture of those factors. Therefore, to understand cellular functions, in vivo analysis should be considered.

In vivo experiments have certain disadvantages, as it is much more complicated and difficult to perform than in vitro experiments. The experimental animals must be kept under physiological conditions in order for proper experiments to be performed. If this is not the case, the results obtained are not accurate. In addition, researchers need to consider how to identify and analyze cells in

* Corresponding author. Institute of Clinical Neuroimmunology, Biomedical Center and University Hospital, Ludwig-Maximilians Universitaet Muenchen, Munich, Germany.
E-mail address: Naoto.Kawakami@med.uni-muenchen.de.
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living animals. In contrast to in vitro experiments in which antibody staining and cell labeling can be easily done, it is more challenging to mark target cells in vivo. Furthermore, the number of cells that can be analyzed in vivo, especially by intravital imaging, is usually much fewer than that in vitro. This means that there is always a risk that intravital imaging detect only special event at special location. Therefore, the best approach is to combine intravital imaging and conventional methods. Intravital imaging provides information regarding cellular functions under physiological conditions, whereas conventional methods provide larger quantities of data regarding cellular status.

Our group is focusing on the infiltration of encephalitogenic T cells into the central nervous system (CNS). We use experimental autoimmune encephalomyelitis (EAE), a widely used animal model for multiple sclerosis,[1] which is considered an autoimmune disease in humans. EAE can be induced by active immunization of CNS-specific antigens emulsified in complete Freund's adjuvant (active EAE). Immunized antigen is taken up by dendritic cells and macrophages and presented to CD4+ helper T cells. Thereafter, CD4+ T cells migrate through the body. Alternatively, EAE can be induced by adoptive transfer of myelin antigen-specific T cells (transfer EAE)[2] or by using transgenic mice that harbor myelin antigen-specific T cells in high numbers (spontaneous EAE).[3] In any case, CNS-infiltrating CD4+ T cells recognize specific antigens presented by local antigen-presenting cells (APCs), and they become activated, produce inflammatory cytokines, and initiate the inflammatory reaction. During inflammation, both innate immune cells (such as macrophages) and adoptive immune cells (such as T and B cells) infiltrate into the CNS and contribute to CNS inflammation. Macrophages have both pro- and anti-inflammatory roles during inflammation.[4,5] Infiltrated B cells produce antibodies in the cerebrospinal fluid,[6] which may either enhance or control inflammation. It was shown that regulatory T cells (Treg) infiltrate into the CNS, although their function there is still largely unknown. Our ultimate goal is to illustrate the functions of and interactions among infiltrating immune cells during CNS inflammation. In this review, we will focus on cellular interactions in EAE, especially by using intravital imaging.

Intravital imaging: microscopy

Many different methods of intravital imaging are available. For example, magnetic resonance imaging (MRI) is used for diagnosis of MS patients to detect inflammation. MRI is non-invasive and provides valuable information. However, a conventional MRI machine with a 3T magnetic field does not provide sufficient resolution for single cell imaging.[7] Recently, a higher-powered MRI with a 7T magnetic field was introduced that can visualize CNS inflammation with surprisingly high resolution.[7] Still, it is not sufficient to see single cells in the CNS. The same holds true for computed tomography. The above methods are very useful for diagnostic use, but not for single-cell imaging.

To achieve single-cell imaging, microscopic techniques are commonly used. In the earliest phase of intravital imaging, leukocytes were imaged in the blood vessels of frogs by using bright-field microscopy (reviewed in[8]). This opened up new methodologies for allowing the observation of cellular motility directly in vivo. However, the target tissue must be thin and relatively transparent because bright-field techniques are used. Furthermore, cell types are hard to distinguish. The use of fluorescent microscopy allows one to focus on specific cell types after proper labeling (for discussion of labeling, please refer to the next section.). Now researchers can analyze the cells of interest in the living animal. However, fluorescent microscopy can only achieve a relatively small penetration depth. Imaging is thus possible only close to the surface.

It is possible to increase the penetration depth of fluorescent imaging, either by using stronger labeling, objectives with higher numerical aperture (N.A.), or stronger excitation power. The development of confocal microscopy equipped with stronger lasers increased the penetration depth dramatically. Confocal microscopy has better spatial resolution and provides clearer images than does fluorescent microscopy. One disadvantage of confocal microscopy is slow image acquisition because of the need to do line scanning. This can be improved by using spinning disk confocal[9] or light sheet microscopy,[10] which can perform faster acquisition. Another disadvantage of confocal microscopy is phototoxicity, which is difficult to prevent because fluorochromes are excited by strong laser light. Excitation laser power can be reduced, but the emitted signal becomes weaker.

To increase the penetration depth and reduce phototoxicity, two-photon microscopy was developed.[11] Two-photon microscopy can share most equipment parts with confocal microscopy, except the excitation laser. The difference between one-photon microscopy (confocal microscopy) and two-photon microscopy is the mechanism of excitation. One photon excites one fluorescent molecule in confocal microscopy, whereas two photons excite one fluorescent molecule in two-photon microscopy. To achieve this two-photon excitation, high photon density is absolutely necessary.[12] Therefore, instead of a continuous confocal laser that emits photons spontaneously, a pulsed two-photon laser can accumulate generated photons and emit them in time intervals.[13] As a result, without changing the average laser power, a two-photon laser increases the peak power dramatically. Commonly used commercial two-photon lasers pulse at the frequency of 80 MHz (80 million pulses per second), which can provide a sufficient pulse even during very fast scanning. Two-photon excitation occurs only at the focal point due to the requirement of high photon density. To some extent, excitation of fluorochromes produces oxygen radicals, which induce cellular toxicity. Since fluorochromes are excited only at the focal point in two-photon excitation, two-photon microscopy minimizes phototoxicity. Another advantage of two-photon microscopy is penetration depth. Because two photons excite one fluorescent molecule, each photon contributes only half the amount of energy compared with conventional one-photon excitation. This indicates that two-photon microscopy is equipped with a laser of twice the wavelength than that of confocal microscopy. Because longer-wavelength light has less of a scattering effect in tissues, two-photon microscopy shows higher penetration depth. All of these factors result in two-photon microscopy being an indispensable method for intravital imaging.

Intravital imaging set-up

It is necessary to use anesthesia to stabilize animal movement. At the same time, animal conditions must be kept as close to physiological as possible during intravital imaging. We use a fentanyl mixture for induction and isoflurane during intravital imaging. Animals are intubated via tracheostomy and connected to a small animal ventilation machine. Then, isoflurane is continuously delivered during intravital imaging. As an alternative, anesthesia injection of a ketamine/xylazine mixture can be used. Injection anesthesia is relatively easy to perform because no additional equipment is necessary. However, additional injections to keep animals anesthetized are absolutely required for longer imaging times, which might be not be feasible without stopping image acquisition.

We use additional equipment as follows to monitor and control animal conditions. An anesthesia monitor is used to monitor O_2 and