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Invited review article

In vivo imaging in autoimmune diseases in the central nervous system

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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

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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

CO_2 concentration in the inspiratory and expiratory gas. The machine can also monitor airway pressure and isoflurane concentration. Because an animal's body temperature decreases during anesthesia, we install a heat pad under the animal. The heat pad is connected to a temperature sensor, and it keeps the body temperature stable during intravital imaging. An electrocardiogram is monitored continuously. For intravenous injection during imaging, an intravenous cannula is inserted into the tail vein and saline solution is injected slowly to prevent blood clotting. These machines are monitored and controlled by custom-made software with adjustable alarms. All of this equipment is not strictly necessary, but it helps for stable imaging.

To acquire stable images, it is very important to stabilize the animal mechanically. To accomplish this, we used custom-made devices. For spinal cord imaging, we used a forceps-like device and clamp the spinal cord from both sides of the imaging area (similar to[14]). For spleen imaging, the spleen is isolated from the body without damaging the blood vessels and placed onto a heated stage.[15] Other researchers have published schemes for stabilization of lymph nodes,[16] ear skin,[17] and liver.[18]

How to label target cells

To identify target cells in vivo, it is necessary to stably label them. Commonly used labeling methods are listed in Table 1. Early studies used synthesized dyes, such as 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR).[19] In addition to these simple dyes, functional dyes that can monitor cellular function, such as intracellular calcium levels, are widely used. Although these dyes stain cells very strongly, cells lose fluorescence and become undetectable if they are proliferating.

Genetic modification induces expression of fluorescent proteins. In addition to global expression of green fluorescent protein (GFP),[20] a variety of transgenic mice has become available as listed in Table 2. By using knock-in technology, a fluorescent protein can be inserted under the control of a specific promoter. For example, yellow fluorescent protein (YFP) was inserted under control of the CD11c promoter to cause YFP expression only in dendritic cells.[21] Alternatively, a fluorescent protein can be fused with another protein that is expressed only in special cell lineage. For example, transgenic mice expressing the FoxP3-GFP fusion protein can be useful for the study of regulatory T cells.[22] These gene-modified animals continuously produce fluorescent protein within the cells, even in proliferating cells. The gene that encodes the fluorescent protein can be delivered by a viral vector, such as a retro-, lenti- or adeno-virus. Usually viral transduction can be quickly done to compare the generation of gene-modified animals. In general, protein labeling is weaker than chemical labeling, which influences penetration depth.

We primarily used retroviral gene transfer to label our derived encephalitogenic T cells.[23] To achieve retroviral transduction, the target cells must be proliferating. Therefore, we stimulate T cells in vitro with a specific antigen; in our case, CNS-specific autoantigen together with APCs. After inducing proliferation of autoreactive T cells, they are co-cultured with retroviral vector-producing cells.[23] Transduced T cells can be selected by using antibiotics because the retroviral vector contains an antibiotic resistance gene.

Table 1
Commonly used labeling dyes for intravital imaging.

Category	Name	EX2P	EX1P	EM	Ref
Synthesized dyes	CFSE	780–890	492	517	57,58
	CMAC	800	353	466	59
	CMFDA	800	492	517	59,60
	CMTMR	800–960	541	565	59,61
	Hoechst33342	890–960	350	461	61,62
	SNARF	780–900	488–530	580/640	43,63,64
	Texas-Red	890–930	595	615	27,65
Fluorescent protein	CFP	870–910	433	475	66,67
	DsRed	935-	558	583	27
	GFP	880–960	488	509	61,68
	Kaede before conversion	1014	508	518	56
	Kaede after conversion	1014	572	582	
	TdTomato	980	554	581	69
	YFP	870–910	513	527	66,67
Calcium sensing	Cameleon	850	440	475/530	70
	Fluo4	840	494	506	71
	FuraRed	860–920	457/488	660	72
	GCaMP3	860–920	496	513	72
	Indo-PE3/AM	740	346	405/475	73
	R-CaMP2	1020	565	583	74
	TNXXL	850	433	475/527	75
	Twitch 1	835	433	475/527	41
	Twitch 2b	835	433	475/527	76
Functional sensor	ERK FRET sensor	840	433	475/527	77
	NFAT-GFP	880	488	509	43,44
	PKA FRET sensor	840	433	475/527	77

Encephalitogenic T cells in peripheral organs

When EAE is induced by adoptive transfer of encephalitogenic T cells, these T cells do not infiltrate into the CNS directly, but spend some days in peripheral organs. Odoardi *et al.* showed that T cells accumulate in the lung immediately after transfer.[24] Intravital imaging showed that encephalitogenic T cells actively migrate within the airways. Interestingly, encephalitogenic T cells acquired a migratory phenotype in the lung and penetrated into the CNS more

Table 2
List of transgenic mice which are often used for intravital imaging.

Mouse line	Express in	Ref
CCR2-RFP	Macrophages	78
c-CSF1R-GFP	Neutrophils Monocytes Macrophages	79
CD11c-YFP	DC	80
CD2-RFP	T cells	79
CFP	global	66
CX3CR1-GFP	Microglia	81
CXCR6-GFP	NK T cells	82
DsRed	global	83
FoxP3-GFP	Treg	84
GFP	Global	85
IFNγ-YFP	IFNg producing cells	86
IL17f-RFP	Th17 cells	87
Langerin-GFP	Langerhans cells	88
LysM-GFP	Neutrophils	89
Lyz2-GFP	Neutrophils	30
NG2-RFP	Pericytes	31
Thy1-TNXXL	Neuron	75
Thy1-YFP	Neuron	14

efficiently than did in vitro activated T cells. This result indicates that encephalitogenic T cells become mature in the lung. It is not yet clear whether this maturation reflects intrinsic T cell changes or influences from external factors. Because lung airways are exposed to the external environment and stimulation can be delivered from numerous sources, it may well be possible that some endogenous factors induce T cell maturation. Indeed, it has been shown that gut microbiota, which are similarly a mixture of many antigens, activate encephalitogenic T cells in the spontaneous EAE model.[25]

The lungs are not the only organ where T cells maturate. Flügel *et al.* showed that encephalitogenic T cells accumulated in the spleen and acquired a migratory phenotype there.[24,26] Gene profiling demonstrated that migratory T cells showed phenotypic changes, especially up-regulated cell adhesion molecules and chemokines that are important for migration.[24] The up-regulation of chemokine receptors is confirmed by cell surface staining of T cells prepared from the spleen.[26] More directly, retransfer of encephalitogenic T cells prepared from the spleen showed faster infiltration into the CNS.

Intravital imaging of encephalitogenic T cells in the spleen showed that T cells are continuously moving within the organ.[15] In our set-up, penetration depth was limited to approximately 100 μm from the surface, which is less than in lymph nodes or spinal cord. According to our analysis, encephalitogenic T cells did not show any directed movement. We sought to arrest T cells in an antigen-dependent manner by applying soluble antigen intravenously. Our analysis showed that T cells became slower immediately after soluble antigen injection and were arrested 20 min after antigen injection. This was surprisingly fast, yet the following results suggested that this is within reason and that the entire process happens in 20 min. First, injection of peptide, which does not need to be processed to be presented to T cells, arrested the T cells more rapidly than did whole protein. Second, MHC class II blocking diminished the soluble antigen effect dramatically. More directly, DQ-OVA, which is non-fluorescent but becomes fluorescent after protein digestion, caused fluorescent signals within 15 min after intravenous injection. Importantly, this quick effect was further supported by conventional analysis. Both mRNA and protein level data show that inflammatory cytokines are produced as early as 30 min after soluble antigen treatment. Because soluble antigen trapped encephalitogenic T cells in the spleen and prevented CNS infiltration, clinical EAE was ameliorated. This experiment elegantly showed the benefit of intravital imaging. Intravital imaging clearly showed the behavioral changes of T cells before and after injection of soluble antigen in the same animal, which was difficult to analyze using conventional methods.

T cell infiltration into the CNS: perivascular phagocytes, fibrinogen, and pericytes

After encephalitogenic T cells acquire a migratory phenotype, they leave the peripheral organs and arrive at the CNS. We asked the question, how do T cells enter the CNS? Because conventional histological studies suggested that “early bird” T cells are detected in the spinal cord leptomeninges, we performed intravital imaging there. We used myelin basic protein (MBP)-specific GFP-labeled T cells ($T_{MBP\text{-}GFP}$ cells) to induce clinical EAE. Animals showed the first clinical sign of disease on day 3 post transfer (p.t.). Therefore, we performed imaging at the spinal cord leptomeninges between day 1 and day 3 p.t.[27]

Intravital imaging showed that a few $T_{MBP\text{-}GFP}$ cells arrived at the leptomeninges on day 1 p.t., which is long before disease onset. Those pioneer cells adhered to the intraluminal surface and moved along the vessels. Similar intraluminal crawling was reported for other cell types, such as monocytes[28] and neutrophils.[29] Within the next 24 h, the number of intraluminal cells increased. Because two-photon microscopy detects signals by scanning, it is hard to detect flowing cells and rolling cells, indicating that the cells that we detected were crawling. According to our analysis, intraluminally crawling T cells prefer to migrate against the direction of flow in blood vessels. However, velocities are similar regardless of the direction of movement. The infusion of anti-integrin α4 antibody diminished intraluminal crawling, indicating that intraluminal crawling is VLA-4-dependent. Although the precise significance of intraluminal crawling is still unknown, we can speculate that those cells are looking for extravasation sites. Interestingly, $T_{MBP\text{-}GFP}$ cell crawling was observed only in the leptomeningeal vessels, but not in other blood vessels, such as those in ear connective tissues and near peripheral nerves.

Intraluminal crawling was followed by extravasation. Intravital imaging recorded that crawling $T_{MBP\text{-}GFP}$ cells arrested and then extravasated.[27] During extravasation, we often observed leakage of fluorescent dextran, which was injected intravenously to fill the blood plasma. This leakage indicates that the blood–brain barrier (BBB) had opened. However, the leakage was observed for only a short time, indicating that the BBB closed again after T cells crossed it. According to our observations, multiple $T_{MBP\text{-}GFP}$ cells extravasated, one after the other, at the same place. This suggests that there are special locations where lymphocytes prefer to extravasate. In accordance with our observations, Abtin *et al.* showed that neutrophils extravasated adjacent to perivascular macrophages in inflamed skin.[30] In addition, they showed that this localization is due to chemokines produced by perivascular macrophages. Another group suggested that there was influence from pericytes.[31] They showed that pericytes attracted myeloid leukocytes by producing macrophage migration inhibitory factor (MIF). Although these studies focused on innate immune cells in peripheral organs, similar mechanisms may exist in the spinal cord leptomeninges.

Why do T cells infiltrate into the immune-privileged CNS? The CNS is protected by the BBB, and the infiltration of immune cells is tightly controlled but not prohibited.[32] After ovalbumin (OVA)-specific GFP-labeled T cells ($T_{OVA\text{-}GFP}$ cells) were transferred into naïve animals, a very small number of cells were found in the CNS,[27] supporting the idea of immune surveillance in the CNS. However, Davalos *et al.* used intravital imaging to suggest that fibrinogen leakage from blood vessels induced clustering of microglia, which further induced neuronal damage.[33] This small amount of damage may change the permeability of the BBB and recruit immune cells to the CNS.

The spinal cord leptomeninges is not the only location where encephalitogenic T cells begin infiltration. It was shown that small numbers of T cells enter the CNS and are distributed in the parenchyma within 3 h after adoptive transfer, suggesting direct infiltration into the CNS parenchyma.[34] In addition, it was shown that T cells enter the CNS via dorsal blood vessels at the 5th lumbar spinal cord.[35] This is due to CCL20 production caused by activation of sensory neurons by the soleus muscles. T cells also seem to infiltrate via the cerebrospinal fluid (CSF). Reboldi *et al.* showed that CCR6-deficient mice are resistant to EAE and, interestingly, T cells were stacked at the choroid plexus, where CCL20 is constitutively expressed.

T cell activation in the CNS

Once encephalitogenic T cells enter the CNS, it was shown that T cells recognized antigen presented by bone marrow-derived perivascular macrophages.[36] To visualize the interaction between encephalitogenic $T_{MBP\text{-}GFP}$ cells and APCs, we performed intravital imaging at the spinal cord leptomeninges. We visualized APCs by intrathecal injection of fluorescent dextran (size: 70 kDa) into the

cisterna magna. Intravital imaging found that $T_{MBP\text{-}GFP}$ cells interact with APCs for a relatively long time, whereas $T_{OVA\text{-}GFP}$ cells showed only a short period of contact. This result suggests that $T_{MBP\text{-}GFP}$ cells recognize endogenous antigen presented by local APCs. Indeed, $T_{MBP\text{-}GFP}$ cells in the spinal cord meninges and parenchyma, but not in the spleen, produced inflammatory cytokines, indicating activation. In addition, $T_{OVA\text{-}GFP}$ cells in the spinal cord leptomeninges became arrested after administering OVA-pulsed APCs intrathecally, which again suggests antigen-dependent interaction and subsequent activation. However, due to lack of proper methods, it was not possible to visualize T cell activation in vivo.

There were several remarkable attempts to detect cellular activation in vivo. One of them involved detecting an immunological synapse, which is the special structure formed when TCR recognizes its specific antigen in the context of MHC.[37] For this purpose, lck or CD3ζ is fused to GFP and expressed in cell lines using retroviral gene transfer.[38] After TCR-mediated stimulation, the fusion protein was recruited into an immunological synapse, and it was imaged in vitro. However, it is not easy to apply these fusion proteins to in vivo experiments. In in vitro experiments, one can predict where immunological synapses will appear by using a monolayer of APCs. In contrast, an immunological synapse can appear at any place on the cell surface in vivo, which indicates that one must scan entire cells with precise z-stacks to detect it. Such precise scanning requires more time and loses temporal resolution. Another attempt to detect cellular activation in vivo involved using GFP knock-in mice under the control of the immediate early gene Nr4a1 (Nur77) was performed.[39] By quantifying GFP expression, activation status and signal strength can be analyzed. However, this was not suitable for intravital imaging because there is an unavoidable time gap between T cell stimulation and GFP expression. A simpler approach is to use calcium sensing dyes to analyze neuronal activities.[40] Unfortunately, this approach cannot be used for T cells because T cells lose their staining within a short time due to proliferation and actively pumping out the dyes.[41] To overcome these problems, we decided to use protein-based sensors that detect T cell activation immediately after TCR stimulation. More specifically, we attempted to detect increasing intracellular calcium and translocation of nuclear factor of activated T cells (NFAT) from cytosol to nucleus (Fig. 1). These sensors are functionally distinguishable. Calcium signaling can be induced by relatively weak stimulation, whereas NFAT translocation can occur only after absolute T cell activation. It makes sense to use two sensors to obtain a more precise picture of the status of T cell activation.

We used the calcium sensor Twitch, which consists of cyan- and yellow-fluorescent protein (CFP and YFP, respectively) connected with a troponin C domain. In a low calcium environment, excitation of CFP induces emission from CFP. In contrast, in a higher calcium environment, calcium binding to the troponin C domain changes the protein conformation.[42] Subsequently, excitation of CFP results in emission from YFP due to a fluorescence resonance energy transfer (FRET) effect. To improve expression levels in mouse T cells, we developed a codon-diversified Twitch and used it for intravital imaging in the mouse EAE model.[41] Twitch was expressed in MOG-specific T cells ($T_{MOG\text{-}Twitch}$ cells) by using retroviral gene transfer and imaged in peripheral lymph nodes and spinal cord leptomeninges. In peripheral lymph nodes, $T_{MOG\text{-}Twitch}$ cells showed occasional short-duration calcium spikes, often coincident with lower motility. Because it is not likely that MOG antigen is presented in the peripheral lymph node, we considered those short-duration calcium spikes to be antigen-independent. The application of antigen stimulation arrested T cells within a short time, as we observed in the spleen.[15] At the same time, $T_{MOG\text{-}Twitch}$ cells showed saturated long-duration calcium elevation, indicating that the Twitch sensor detected T cell activation. $T_{MOG\text{-}Twitch}$ cells were also imaged in the spinal cord leptomeninges at the time of EAE onset. Substantial numbers of $T_{MOG\text{-}Twitch}$ cells showed elevated intracellular calcium, and the duration of calcium spikes

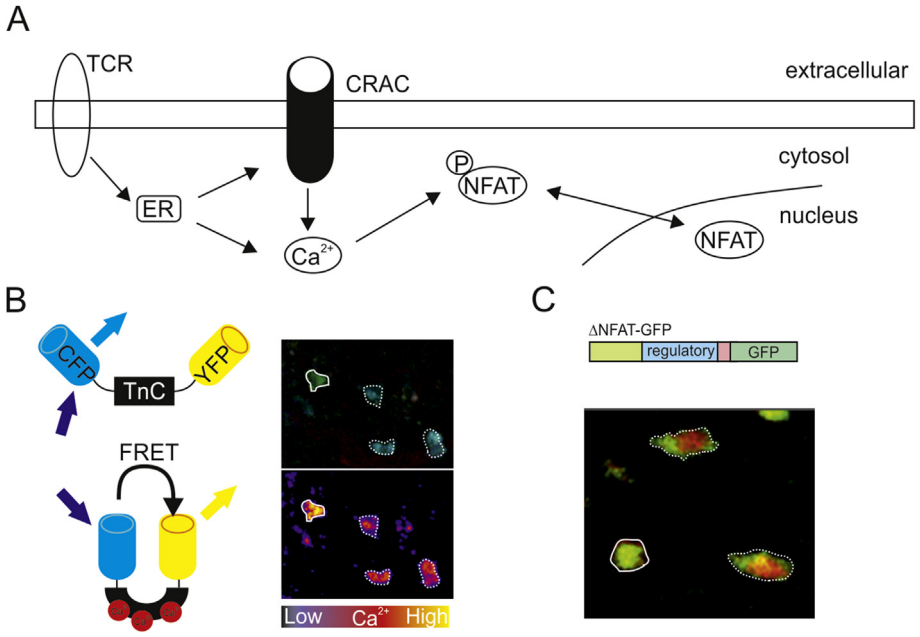

Fig. 1. Scheme of activation sensors. (**A**) TCR stimulation induces calcium release from endoplasmic reticulum (ER). Emptying calcium in the ER opens Calcium Release-Activated Channels (CRAC) on cell surface, which induce influx of extracellular calcium. Increased intracellular calcium induced DE phosphorylation of NFAT, followed by relocation of NFAT from cytosol to nucleus. (**B**) Structure of Twitch calcium sensing protein. CFP and YFP are connected with troponin C calcium sensing protein. Twitch changes its confirmation according to calcium concentration. Excitation of CFP results emission of blue and yellow at low and high calcium environment, respectively. Right pictures show representative cells of both activated (line) and not activated (dotted lines). (**C**) Protein structure of NFAT-based activation sensor Picture shows representative cells of both activated (line) and not activated (dotted lines).

was approximately 6 min, which is considerably longer than those observed in lymph nodes. Importantly, those activations were often observed in perivascular areas or near APCs. Because Twitch-labeled OVA-specific T cells rarely showed long-duration calcium spikes, we concluded that $T_{MOG\text{-}Twitch}$ cells are activated by endogenous autoantigens.

To detect the subcellular location of NFAT, truncated NFAT was fused to GFP and expressed in MBP-specific T cells ($T_{MBP\text{-}NFAT\text{-}GFP}$ cells).[43] $T_{MBP\text{-}NFAT\text{-}GFP}$ cells were first imaged when T cells were within the leptomeningeal vessels. Intravital imaging showed that both rolling and crawling cells had cytosolic NFAT, indicating that they were not activated. In contrast, substantial numbers of extravasated $T_{MBP\text{-}NFAT\text{-}GFP}$ cells showed NFAT-GFP in their nuclei. Intravital imaging clearly showed that a non-activated T cell, which has cytosolic NFAT, interacted with local APC and the interaction quickly induced translocation of NFAT to the nucleus. Interestingly, some, but not all, APCs stimulated T cells efficiently. Similar observations were also reported by another group.[44]

In summary, by using protein-based activation sensors, we could visualize T cell activation in the CNS after contact with local APCs. Of course, these activation sensors can be applied in other cells, as shown.[45]

Other types of immune cells in the CNS: macrophages, Treg, B cells, and microglia

Although CD4+ T cells are considered the key player in initiating CNS inflammation, other types of cells, which can be either brain-resident cells or infiltrating cells, also contribute. One of them is Treg, which can suppress the function of encephalitogenic T cells. Because depletion of Treg at the acute phase of EAE enhances clinical severity dramatically, we aimed to image Treg in the spinal cord leptomeninges to analyze interaction with encephalitogenic T cells.[46] We crossed T-Red mice, in which T cells express RFP,[47] and DEREG mice, in which Treg express GFP and diphtheria toxin receptor under FoxP3 promoter.[48] Intravital imaging at the spinal cord leptomeninges was performed at the peak of EAE with or without Treg depletion. We found that the encephalitogenic T cells moved slower and stopped more often in the absence of Treg. This suggests that Treg can influence inflammation in the CNS. During intravital imaging, we observed that Treg interacted with both effector T cells and APCs, indicating that suppression of disease can be via direct effect on effector T cells or indirect effect via APCs.

There are other players in CNS inflammation. B cells are known to produce antibodies in the CSF[6] and contribute significantly to CNS inflammation. The depletion of B cells is beneficial for both EAE[49] and MS.[50] Mononuclear phagocytes, such as microglia[33] and macrophages[51] induce neuronal damage. In addition, oligodendrocytes have a critical role in myelination and neurons are targeted to be destroyed. However, in vivo imaging to study these cells, with the exception of neurons, has rarely been performed, and their roles are largely unknown.

Platform to develop therapeutic treatment

The results obtained from intravital imaging can be used for developing therapeutic treatment. For example, we showed that the infusion of anti-integrin α4 antibody diminished intraluminal crawling within minutes. As a consequence, infiltration of encephalitogenic T cells into the CNS is also blocked, resulting in prevention of clinical EAE.[27] Indeed, anti-integrin α4 antibody is approved as an MS treatment and shows beneficial effects. Our intravital imaging clearly showed the mechanism of this antibody treatment. In addition, we have shown that the calcium inhibitor, BZ194, ameliorated clinical EAE in both preventive and therapeutic treatments.[52] Intravital imaging showed that BZ194 treatment increased T cell motility in the CNS. We speculate that BZ194 prevented T cell arrest by blocking intracellular calcium signaling; therefore, T cells do not get sufficient stimulation to induce inflammation. Furthermore, we have shown the effect of soluble antigen treatment in EAE. When soluble antigen was given before the onset of EAE, the treatment ameliorated clinical severity dramatically.[15] In contrast, soluble antigen worsened EAE when it was applied after the onset of disease.[53] In both cases, soluble antigen activates encephalitogenic T cells. The difference lies in where the T cell activation occurs. Before the onset of EAE, the majority of encephalitogenic T cells are in the periphery, and activation of them does not result in deleterious effects. However, after the onset of EAE, many encephalitogenic T cells are in the CNS, and their activation results in a lethal level of inflammation. One always needs to keep in mind that results from rodent models cannot be applied directly to humans. However, intravital imaging holds great potential for understanding the cellular mechanisms of disease pathogenesis and for developing and evaluating therapeutic treatments.

Future directions

Intravital imaging in the immunology field started in the early 2000s to study cellular motility in the explanted organ.[54] Currently, multicolor imaging and functional imaging have become popular. There are interesting, and potentially very robust, new methods that have been introduced recently. One of them involves gradient index (GRIN) lenses.[55] This method uses an endoscope that can perform imaging within the tissue. Because the penetration depth of two-photon microscopy is superior, but still limited, such an endoscope is the method of choice. Recently, an interesting study using photoconvertible dyes was published.[56] This study explored the functional difference between migratory and resident dendritic cells in the lymph nodes. This kind of study has very high potential because cells are migrating in the body, and the consequences of a particular event may not happen in the same place. For example, a cell receives stimulation in one organ and shows effector function in another organ. Lastly, it is extremely important to analyze data and obtain fruitful results. Two-photon microscopy has become user-friendly and it is now easier to acquire excellent images. However, this is only one component of intravital imaging and researchers must translate imaging data to fruitful messages.

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Conflict of interest

The author received the research funding from Genzayme.

References

1. Gold R, Linington C, Lassmann H. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 2006;**129**:1953–71.
2. Ben-Nun A, Wekerle H, Cohen IR. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* 1981;**11**:195–9.
3. Pöllinger B, Krishnamoorthy G, Berer K, Lassmann H, Bösl M, Dunn R, et al. Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. *J Exp Med* 2009;**206**:1303–16.

4. Kroner A, Greenhalgh AD, Zarruk JG, Passos Dos Santos R, Gaestel M, David S. TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord. *Neuron* 2014;**83**:1098–116.
5. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci* 2013;**16**:1211–8.
6. Obermeier B, Mentele R, Malotka J, Kellermann J, Wekerle H, Lottspeich F, et al. Matching of oligoclonal Ig transcriptomes and proteomes of cerebrospinal fluid in multiple sclerosis. *Nat Med* 2008;**14**:688–93.
7. Sinnecker T, Othman J, Kuhl M, Mekle R, Selbig I, Niendorf T, et al. 7T MRI in natalizumab-associated PML and ongoing MS disease activity: a case study. *Neurol Neuroimmunol Neuroinflamm* 2015;**2**:e171.
8. Weigert R, Porat-Shliom N, Amornphimoltham P. Imaging cell biology in live animals: ready for prime time. *J Cell Biol* 2013;**201**:969–79.
9. Oreopoulos J, Berman R, Browne M. Spinning-disk confocal microscopy: present technology and future trends. *Methods Cell Biol* 2014;**123**:153–75.
10. Keller PJ, Ahrens MB. Visualizing whole-brain activity and development at the single-cell level using light-sheet microscopy. *Neuron* 2015;**85**:462–83.
11. Denk W, Strickler JH, Webb WW. 2-Photon laser scanning fluorescence microscopy. *Science* 1990;**248**:73–6.
12. Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Meth* 2005;**2**:932–40.
13. Kawakami N, Bartholomaus I, Pesic M, Mues M. An autoimmunity odyssey: how autoreactive T cells infiltrate into the CNS. *Immunol Rev* 2012;**248**:140–55.
14. Davalos D, Lee JK, Smith WB, Brinkman B, Ellisman MH, Zheng BH, et al. Stable in vivo imaging of densely populated glia, axons and blood vessels in the mouse spinal cord using two-photon microscopy. *J Neurosci Methods* 2008;**169**:1–7.
15. Odoardi F, Kawakami N, Li ZX, Cordiglieri C, Rune K, Klinkert WEF, et al. Instant effect of soluble antigen on effector T cells in peripheral immune organs during immunotherapy of autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2007;**104**:920–5.
16. Mempel TR, Scimone ML, Mora JR, von Andrian UH. In vivo imaging of leukocyte trafficking in blood vessels and tissues. *Curr Opin Immunol* 2004;**16**:406–17.
17. Sen D, Forrest L, Kepler TB, Parker I, Cahalan MD. Selective and site-specific mobilization of dermal dendritic cells and Langerhans cells by Th1- and Th2-polarizing adjuvants. *Proc Natl Acad Sci U S A* 2010;**107**:8334–9.
18. Tanaka K, Morimoto Y, Toiyama Y, Okugawa Y, Inoue Y, Uchida K, et al. Intravital dual-colored visualization of colorectal liver metastasis in living mice using two photon laser scanning microscopy. *Microsc Res Tech* 2012;**75**:307–15.
19. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002;**296**:1869–73.
20. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 1997;**407**:313–9.
21. Jung S, Unatmaz D, Wong P, Sano GI, De los Santos K, Sparwasser T, et al. In vivo depletion of CD11c + dendritic cells abrogates priming of CD8 + T cells by exogenous cell-associated antigens. *Immunity* 2002;**17**:211–20.
22. Zhou XY, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med* 2008;**205**:1983–91.
23. Flügel A, Willem M, Berkowicz T, Wekerle H. Gene transfer into CD4 + T lymphocytes: green fluorescent protein engineered, encephalitogenic T cells used to illuminate immune responses in the brain. *Nat Med* 1999;**5**:843–7.
24. Odoardi F, Sie C, Streyl K, Ulaganathan VK, Schläger C, Lodygin D, et al. T cells become licensed in the lung to enter the central nervous system. *Nature* 2012;**488**:675–9.
25. Berer K, Mues M, Koutroulos M, Al Rasbi Z, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 2011;**479**:538–41.
26. Flügel A, Berkowicz T, Ritter T, Labeur M, Jenne D, Li Z, et al. Migratory activity and functional changes of green fluorescent effector T cells before and during experimental autoimmune encephalomyelitis. *Immunity* 2001;**14**:547–60.
27. Bartholomäus I, Kawakami N, Odoardi F, Schläger C, Miljkovic D, Ellwart JW, et al. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* 2009;**462**:94–8.
28. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science* 2007;**317**:666–70.
29. Phillipson M, Heit B, Colarusso P, Liu LX, Ballantyne CM, Kubes P. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med* 2006;**203**:2569–75.
30. Abtin A, Jain R, Mitchell AJ, Roediger B, Brzoska AJ, Tikoo S, et al. Perivascular macrophages mediate neutrophil recruitment during bacterial skin infection. *Nat Immunol* 2014;**15**:45–53.
31. Stark K, Eckart A, Haidari S, Tirniceriu A, Lorenz M, von Bruhl ML, et al. Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. *Nat Immunol* 2013;**14**:41–51.
32. Brown KA. Factors modifying the migration of lymphocytes across the blood-brain barrier. *Int Immunopharmacol* 2001;**1**:2043–62.
33. Davalos D, Ryu JK, Merlini M, Baeten KM, Le Moan N, Petersen MA, et al. Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation. *Nat Commun* 2012;**3**:1227.
34. Hickey WF, Hsu BL, Kimura H. T lymphocyte entry into the central nervous system. *J Neurosci Res* 1991;**28**:254–60.
35. 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**:447.
36. Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone-marrow derived and present antigen in vivo. *Science* 1988;**239**:290–3.
37. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science* 1999;**285**:221–7.
38. Richie Ehrlich LI, Ebert PJR, Krummel MF, Weiss A, Davis MM. Dynamics of p56lck translocation to the T cell immunological synapse following agonist and antagonist stimulation. *Immunity* 2002;**17**:809–22.
39. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med* 2011;**208**:1279–89.
40. Stosiek C, Garaschuk O, Holthoff K, Konnerth A. In vivo two-photon calcium imaging of neuronal networks. *Proc Natl Acad Sci U S A* 2003;**100**:7319–24.
41. Mues M, Bartholomaus I, Thestrup T, Griesbeck O, Wekerle H, Kawakami N, et al. Real-time in vivo analysis of T cell activation in the central nervous system using a genetically encoded calcium indicator. *Nat Med* 2013;**19**:778–83.
42. Mank M, Reiff DF, Heim N, Friedrich MW, Borst A, Griesbeck O. A FRET-based calcium biosensor with fast signal kinetics and high fluorescence change. *Biophys J* 2006;**90**:1790–6.
43. Pesic M, Bartholomaus I, Kyratsous NI, Heissmeyer V, Wekerle H, Kawakami N. 2-photon imaging of phagocyte-mediated T cell activation in the CNS. *J Clin Invest* 2013;**123**:1192–201.
44. Lodygin D, Odoardi F, Schlager C, Korner H, Kitz A, Nosov M, et al. A combination of fluorescent NFAT and H2B sensors uncovers dynamics of T cell activation in real time during CNS autoimmunity. *Nat Med* 2013;**19**:784–90.
45. Marangoni F, Murooka TT, Manzo T, Kim EY, Carrizosa E, Elpek NM, et al. The transcription factor NFAT exhibits signal memory during serial T cell interactions with antigen-presenting cells. *Immunity* 2013;**38**:237–49.
46. Koutrolos M, Berer K, Kawakami N, Wekerle H, Krishnamoorthy G. Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS. *Acta Neuropathol Commun* 2014;**2**:163.
47. Pittet MJ, Mempel TR. Regulation of T-cell migration and effector functions: insights from in vivo imaging studies. *Immunol Rev* 2008;**221**:107–29.
48. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3 + regulatory T cells induces a scurfy-like disease. *J Exp Med* 2007;**204**:57–63.
49. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* 2012;**209**:1001–10.
50. Kitsos DK, Tsiodras S, Stamboulis E, Voumvourakis KI. Rituximab and multiple sclerosis. *Clin Neuropharmacol* 2012;**35**:90–6.
51. Nikic I, Merkler D, Sorbara C, Brinkoetter M, Kreutzfeldt M, Bareyre FM, et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat Med* 2011;**17**:495–9.
52. Cordiglieri C, Odoardi F, Zhang B, Nebel M, Kawakami N, Klinkert WEF, et al. Nicotinic acid adenine dinucleotide phosphate-mediated calcium signalling in effector T cells regulates autoimmunity of the central nervous system. *Brain* 2010;**133**:1930–43.
53. Odoardi F, Kawakami N, Klinkert WEF, Wekerle H, Flügel A. Blood-borne soluble protein antigen intensifies T cell activation in autoimmune CNS lesions and exacerbates clinical disease. *Proc Natl Acad Sci U S A* 2007;**104**:18625–30.
54. Stoll S, Delon J, Brotz TM, Germain RN. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 2002;**296**:1873–6.
55. Barretto RPJ, Ko TH, Jung JC, Wang TJ, Capps G, Waters AC, et al. Time-lapse imaging of disease progression in deep brain areas using fluorescence microendoscopy. *Nat Med* 2011;**17**:223–8.
56. Kitano M, Yamazaki C, Takumi A, Ikeno T, Hemmi H, Takahashi N, et al. Imaging of the cross-presenting dendritic cell subsets in the skin-draining lymph node. *Proc Natl Acad Sci U S A* 2016;**113**:1044–9.
57. Worbs T, Mempel TR, Bölter J, von Andrian UH, Förster R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Med* 2007;**204**:489–95.
58. Wei SH, Rosen H, Matheu MP, Sanna MG, Wang SK, Jo E, et al. Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nat Immunol* 2005;**6**:1228–35.
59. Henrickson SE, Mempel TR, Mazo IB, Liu B, Artymov MN, Zheng H, et al. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol* 2008;**9**:282–91.
60. Mempel TR, Henrickson SE, von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 2004;**427**:154–9.
61. Mempel TR, Pittet MJ, Khazaie K, Weninger W, Weissleder R, von Boehmer H, et al. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. *Immunity* 2006;**25**:129–41.

62. Hauser AE, Junt T, Mempel TR, Sneddon MW, Kleinstein SH, Henrickson SE, et al. Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. *Immunity* 2007;**26**:655–67.
63. Celli S, Lemaître F, Bousso P. Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4 + T cell activation. *Immunity* 2007;**27**:625–34.
64. Celli S, Garcia Z, Bousso P. CD4 T cells integrate signals delivered during successive DC encounters in vivo. *J Exp Med* 2005;**202**:1271–8.
65. Matheu MP, Beeton C, Garcia A, Chi V, Rangaraju S, Safrina O, et al. Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel block. *Immunity* 2008;**29**:602–14.
66. Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks in vivo. *Nat Immunol* 2004;**5**:1243–50.
67. Schwickert TA, Lindquist RL, Shakhar G, Skokos D, Kosco-Vilbois MH, Dustin ML, et al. In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* 2007;**446**:83–7.
68. Kawakami N, Nägerl UV, Odoardi F, Bonhoeffer T, Wekerle H, Flügel A. Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J Exp Med* 2005;**201**:1805–14.
69. Bauer CA, Kim EY, Marangoni F, Carrizosa E, Claudio NM, Mempel TR. Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. *J Clin Invest* 2014;**124**:2425–40.
70. Nishida K, Matsumura S, Taniguchi W, Uta D, Furue H, Ito S. Three-dimensional distribution of sensory stimulation-evoked neuronal activity of spinal dorsal horn neurons analyzed by in vivo calcium imaging. *PLoS One* 2014;**9**:e103321.
71. Nitsch R, Pohl EE, Smorodchenko A, Infante-Duarte C, Aktas O, Zipp F. Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue. *J Neurosci* 2004;**24**:2458–64.
72. Burford JL, Villanueva K, Lam L, Riquier-Brison A, Hackl MJ, Pippin J, et al. Intravital imaging of podocyte calcium in glomerular injury and disease. *J Clin Invest* 2014;**124**:2050–8.
73. Bhakta NR, Oh DY, Lewis RS. Calcium oscillations regulate thymocyte motility during positive selection in the three-dimensional thymic environment. *Nat Immunol* 2005;**6**:143–51.
74. Inoue M, Takeuchi A, Horigane S, Ohkura M, Gengyo-Ando K, Fujii H, et al. Rational design of a high-affinity, fast, red calcium indicator R-CaMP2. *Nat Methods* 2015;**12**:64–70.
75. Radbruch H, Bremer D, Mothes R, Gunther R, Rinnenthal JL, Pohlan J, et al. Intravital FRET: probing cellular and tissue function in vivo. *Int J Mol Sci* 2015;**16**:11713–27.
76. Thestrup T, Litzlbauer J, Bartholomaus I, Mues M, Russo L, Dana H, et al. Optimized ratiometric calcium sensors for functional in vivo imaging of neurons and T lymphocytes. *Nat Methods* 2014;**11**:175–82.
77. Mizuno R, Kamioka Y, Kabashima K, Imajo M, Sumiyama K, Nakasho E, et al. In vivo imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines. *J Exp Med* 2014;**211**:1123–36.
78. Yamasaki R, Lu H, Butovsky O, Ohno N, Rietsch AM, Cialic R, et al. Differential roles of microglia and monocytes in the inflamed central nervous system. *J Exp Med* 2014;**211**:1533–49.
79. Looney MR, Thornton EE, Sen D, Lamm WJ, Glenny RW, Krummel MF. Stabilized imaging of immune surveillance in the mouse lung. *Nat Meth* 2011;**8**:91–6.
80. Shakhar G, Lindquist RL, Skokos D, Dudziak D, Huang JH, Nussenzweig MC, et al. Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity in vivo. *Nat Immunol* 2005;**6**:707–14.
81. Fuhrmann M, Bittner T, Jung CKE, Burgold S, Page RM, Mitteregger G, et al. Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease. *Nat Neurosci* 2010;**13**:411–3.
82. Lee WY, Moriarty TJ, Wong CH, Zhou H, Strieter RM, van Rooijen N, et al. An intravascular immune response to Borrelia burgdorferi involves Kupffer cells and iNKT cells. *Nat Immunol* 2010;**11**:295–302.
83. Coppieters K, Martinic MM, Kiosses WB, Amirian N, von Herrath M. A novel technique for the in vivo imaging of autoimmune diabetes development in the pancreas by two-photon microscopy. *PLoS One* 2010;**5**:e15732.
84. Deane JA, Abeynaike LD, Norman MU, Wee JL, Kitching AR, Kubes P, et al. Endogenous regulatory T cells adhere in inflamed dermal vessels via ICAM-1: association with regulation of effector leukocyte adhesion. *J Immunol* 2012;**188**:2179–88.
85. Kim JV, Kang SS, Dustin ML, McGavern DB. Myelomonocytic cell recruitment causes fatal CNS vascular injury during acute viral meningitis. *Nature* 2009;**457**:191–5.
86. Overstreet MG, Gaylo A, Angermann BR, Hughson A, Hyun YM, Lambert K, et al. Inflammation-induced interstitial migration of effector CD4(+) T cells is dependent on integrin alphaV. *Nat Immunol* 2013;**14**:949–58.
87. Xu C, Shen Y, Littman DR, Dustin ML, Velazquez P. Visualization of mucosal homeostasis via single- and multiphoton intravital fluorescence microscopy. *J Leukoc Biol* 2012;**92**:413–9.
88. Zaid A, Mackay LK, Rahimpour A, Braun A, Veldhoen M, Carbone FR, et al. Persistence of skin-resident memory T cells within an epidermal niche. *Proc Natl Acad Sci U S A* 2014;**111**:5307–12.
89. Li W, Nava RG, Bribriesco AC, Zinselmeyer BH, Spahn JH, Gelman AE, et al. Intravital 2-photon imaging of leukocyte trafficking in beating heart. *J Clin Invest* 2012;**122**:2499–508.