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The mouse cortical meninges are the site of immune responses to many different pathogens, and are accessible to intravital imaging

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ABSTRACT

A wide range of viral and microbial infections are known to cause meningitis, and there is evidence that the meninges are the gateway to pathogenic invasion of the brain parenchyma. Hence observation of these regions has wide application to understanding host-pathogen interactions. Interactions between pathogens and cells of the immune response can be modified by changes in their environment, such as suppression of the flow of blood and lymph, and, particularly in the case of the meninges, with their unsupported membranes, invasive dissection can alter the tissue architecture. For these reasons, intravital imaging through the unperforated skull is the method of choice. We give a protocol for a simple method of two-photon microscopy through the thinned cortical skull of the anesthetized mouse to enable real-time imaging with sub-micron resolution through the meninges and into the superficial brain parenchyma. In reporter mice in which selected cell types express fluorescent proteins, imaging after infection with fluorescent pathogens (lymphocytic choriomeningitis virus, *Trypanosoma brucei* or *Plasmodium berghei*) has shown strong recruitment to the cortical meninges of immune cells, including neutrophils, T cells, and putative dendritic cells and macrophages. Without special labeling, the boundaries between the dura mater, the leptomeninx, and the parenchyma are not directly visualized in intravital two-photon microscopy, but other landmarks and characteristics, which we illustrate, allow the researcher to identify the compartment being imaged. While most infectious meningitides are localized mainly in the dura mater, others involve recruitment of immune cells to the leptomeninx.

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1. Investigating immune responses in the cranial meninges

The very wide range of pathogens that can cause meningitis includes viruses [1–4], bacteria [5–7], protozoa [8–11], nematodes [12], and fungi (notably *Cryptococcus* [13,14]). A common symptom in humans is headache, which implies nervous activity in the meninges, and activation, at the very least, of resident mast cells [12,15,16]. However, knowledge of meningeal immune responses to microbial infection is meagre, so the great convenience of working with mouse models, notably the availability of reporter mice in which selected classes of cells express fluorescent proteins (e.g., [17,18]), outweighs the disadvantage that pathological changes in the transcriptome may not perfectly mimic those in humans [19,20]. To date, the few reports of immune responses to pathogens in compartments of the cranial meninges have been based on studies on mice; these have described recruitment of neutrophils [21], and T lymphocytes and monocytes [10,11,21].

In vivo imaging, by recording movements and interactions of pathogens and different classes of immune cells, can give information, not only of numbers and location, but also about the activation states of immune cells. User-friendly programs, such as Volocity or Imaris, can extract the position co-ordinates of cells at each time-point in a video, draw the tracks, and calculate the speed and the x,y,z, components of the velocity of each cell for each time interval, or the angle turned between two time intervals [22,23]. From these values, statistics can be calculated such as the mean speed along a track [21], the displacement rate (the rate at which a cell moves further from its starting point), the mean x,y and z components of velocity [10], the fraction of time intervals during which the cell is stationary (the 'arrest coefficient' [11,21]), the direction of movement relative to blood vessels [24] or the duration of interactions between two cells [25]. These parameters can change markedly during infection.

The architecture of the meninges, with its large spaces separated by flimsy membranes, is difficult to preserve if the overlying skull is removed, and an important part of an immune response is extravasation of cells (or pathogens) from flowing blood. It is therefore desirable to observe meningeal pathophysiology in vivo and through the skull. The most informative technique to date is two-photon microscopy, which is based on the theoretical prediction of Maria Göppert-Mayer [26] [27]. Perhaps surprisingly, the skull bone overlying the cortical meninges is an asset for imaging, rather than a handicap. When an area of the bone is mechanically thinned, it provides a transparent window to the meninges, while the surrounding bone provides a solid anchorage for immobilizing the imaging field. Other laboratories have described methods for making a window that remains transparent over weeks or months and allows repeated imaging sessions [28,29], the video by Marker et al. [30] being particularly recommended. Our priorities are slightly different. We have chosen not to allow mice to recover from anesthesia, for the scientific reason that the stress of the imaging session would modify the disease progression, for the practical reasons that a quick and reliable preparation is convenient and it can be interesting to image other parts of the mouse after dissection, and for the ethical reason that disease progression should be terminated as soon as clinical symptoms develop. Statistically significant data on the movements of immune cells require videos lasting several tens of minutes at several sites per mouse, with an accurately controlled temperature. We have therefore chosen a fairly quick, non-sterile preparation, adjustable gas anesthesia, and perfusion of the chamber over the thinned area. A perfusion system had initially been set up for ex-vivo experiments [25,31]. It is useful during thinning (for removing debris and for cooling), and, while the mouse is under the two-photon microscope, to control the temperature above the thinned skull and to maintain the water contact with the objective during video imaging lasting tens of minutes, and for subsequent ex vivo imaging at a controlled temperature.

1.1. The functional anatomy of the meninges

Popular views of the structure of the cortical meninges have been influenced by text-book images based on an artistically very attractive drawing published by Weed in 1923 [32]. This shows the dura as a thin membrane overlying a capacious subarachnoid space (SAS) in the leptomeninx. While this is true over the basal brain, over the convexities of the cortex, as Weed [33] himself stated, "the subarachnoid space is only of capillary thickness". In primates, the SAS extends into sulci, which are bridged by the dura, but mice and other rodents have no sulci, and over the dorsal brain the SAS is deep only where is penetrates the medial longitudinal fissure. In contrast, the dura is thick enough to accommodate bundles of collagen, a rich vasculature, and extracellular lacunae [34,35]. It is extensively innervated [36] and activity in trigeminal neurons with endings in the dura is perceived as headache (see [37]). The dura also contains lymph vessels, which were described in man by Mascagni in 1787 (reproduced in [38]), and have been shown in rat [36] and in mouse [39,40].

The functional compartmentation of the meninges is still not clear. Electron microscopy shows that the cells of the outer layer of the leptomeninx, the arachnoid membrane, are connected by tight junctions [41] and it can be assumed that it is this membrane that separates the cerebrospinal fluid (CSF) in the SAS from the extracellular fluid of the dura [42]. The nature of the barrier, as well as its location, is of interest as there is some evidence that particulate matter and T-cells can pass from the leptomeninx into lymph vessels within the dura [40,43] and, in the opposite direction, T-cells may pass from the dura to the perivascular spaces of vessels penetrating the parenchyma [10,44]. The CSF present in the SAS can contain pro-inflammatory cytokines secreted by the choroid plexus [45,46] so the paths of flow of CSF are relevant to the immunopathology of the meninges and the brain parenchyma. There is agreement that CSF reaches the dorsal SAS via channels adjacent to cerebral arteries (e.g. [43,47]) but uncertainty about whether or not there is flow of CSF down the paravascular spaces of arteries penetrating the parenchyma, and up the paravascular spaces of emerging veins [37,48–52].

In vivo two-photon microscopy, which allows simultaneous imaging of several fluorophores (Fig. 1A), in Z-stacks that can be reconstructed as 3D images (Fig. 1B), is expected to contribute to resolving these questions. Here, we focus on whether a given immune response is in the dura or in the leptomeninx.

1.2. Locating cells and structures in the mouse meninges

Blood vessels can be labeled by i.v. injection of fluorescently labeled dextran, or inert quantum dots (see Section 2.2. for more details); in Fig. 1B they are shown in green. Bone, and also collagen, are readily imaged with a two-photon microscope using the phenomenon of second harmonic generation (SHG, [54]). A very intense pulse of light from a near-infrared laser generates the second harmonic, so that, for example, an exciting wavelength of 900 nm produces emission at 450 nm, which can be used to construct an image as if from a conventional fluorophore (blue in Fig. 1B). Blood vessels in the dura are relatively leaky, and fluorescent nuclear dyes injected i.v. rapidly label nuclei in the dura, as well as nuclei of vascular endothelial cells throughout the mouse [9,10]. This is seen in the vertical sections shown in Fig. 1D,E and also in Fig. 2A. In Fig. 1D we suggest where the arachnoid membrane might be located, below the labeled nuclei and above