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INVITED REVIEW

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The emergence of the calvarial hematopoietic niche in health and disease

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Summary

The diploë region of skull has recently been discovered to act as a myeloid cell reservoir to the underlying meninges. The presence of ossified vascular channels traversing the inner skull of cortex provides a passageway for the cells to traffic from the niche, and CNS-derived antigens traveling through cerebrospinal fluid in a perivascular manner reaches the niche to signal myeloid cell egress. This review will highlight the recent findings establishing this burgeoning field along with the known role this niche plays in CNS aging and disease. It will further highlight the anatomical routes and physiological properties of the vascular structures these cells use for trafficking, spanning from skull to brain parenchyma.

KEYWORDS
aquaporin-4, arteries, arterioles, astrocytes, B cells, BBB, blood–brain barrier, bone marrow, calvaria, capillaries, cerebrospinal fluid, CSF, glymphatic, hematopoietic, hematopoietic stem cells, monocytes, neutrophils, pericytes, sinusoids, skull bone marrow, smooth muscle cells, T cells, venules

1 | INTRODUCTION

A decade ago, Ransohoff and Engelhardt[1] first offered the idea that memory T cells monitor the CNS through interactions with antigen-presenting cells in the cerebrospinal fluid (CSF) of the subarachnoid space. Since that time, numerous studies have shed light not only on the rich diversity of myeloid populations within the meningeal compartment, but also on the calvarial bone marrow niche from which they derive as well as the vascular routes by which they traffic. In light of these findings in this newly burgeoning field, this review will thoroughly discuss key findings from recent papers that allow us to definitively follow a monocyte from the calvarial bone marrow niche to brain parenchyma. We will also discuss the properties of vascular structures used for passage and the molecular cues known to induce myeloid cell egress from this bone marrow niche. We will discuss the role of glymphatics in signaling to immune cells of the meningeal compartment and how cerebral circulation contributes to glymphatic function. We will then end by highlighting the role of meningeal immune cells in central nervous system (CNS) disease. Thus, this review will shed light on the anatomy as well as cell and molecular mechanisms regulating skull bone marrow-derived myeloid cell entry into the brain and the role of skull marrow-derived myeloid cells in CNS disease.

2 | THE CALVARIAL BONE MARROW NICHE

The brain is surrounded by a case known as the cranial vault, or calvarium. This encasing is composed of 22 bones that are joined

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together through cranial sutures.[2] The cranial base possesses various foramina that serves as exit points for cranial nerves and blood vessels, and though there are many, we will only highlight the cribriform plate through which the olfactory nerve exits.[2] The calvarium is classified as a diploic bone, consisting of the external and internal laminae (also known as inner and outer tables). In between these two laminae, lies a layer of trabecular bone known as the diploë.[3] This layer houses the hematopoietic stem cell niche, which is perivascular and created by both mesenchymal stromal stem cells (MSSC) and endothelial cells.[4]

Bone marrow endothelial cells (BMECs) comprise the vascular network supporting and forming this hematopoietic niche, and a zonation is present whereby BMECs are either arterial BMECs (aBMECs) or sinusoidal BMECs (sBMECs).[5–7] These cells form a mechanical barrier that denies passage to circulating red blood cells and platelets into bone marrow and they further regulate cellular trafficking, hematopoiesis, and osteogenesis.[4,5,8] Itkin et al.[9] provided a thorough classification of the calvarial vascular tree in order to determine the mechanisms by which BMECs execute their dual regulatory roles in stem cell maintenance and cellular trafficking, and if such roles are associated with specific vascular zones and/or anatomical regions.

Anatomically speaking, aBMECs are proximally closer to ossified bone at the metaphysis or in the diaphysis, lying at a distance of <40 μm from the endosteum, with ~50% being <20 μm. Morphologically speaking, aBMECs possessed an average diameter around 10μms and were enwrapped by alpha-smooth muscle actin (SMA)+ cells, which the authors identified as pericytes. As the same vessels approached the endosteum, they narrowed to ~5 μms and were enwrapped not by pericytes but were surrounded by $Sca1^+$ mesenchymal and clusters of $Sca1^+$ hematopoietic cells. Given the size of these vessels, the mural cell classification would make sense if following criteria used for the vasculature in brain parenchyma,[10] or perhaps the pericytes here would be best classified as an ensheathing pericyte.[10] If following parenchymal vasculature criteria, however, these vessels would best be classified as capillaries based on their size rather than arterioles. In contrast to these vessels, the downstream sinusoids are much larger, having an average diameter of ~25 μms.

Physiologically speaking, aBMECs have lower permeability and significantly higher blood flow and shear rates. This is due to their preferential expression of vascular-endothelial cadherin and the tight junction protein Zonula Occludens-1. aBMECs also express slightly higher levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin, and junctional adhesion molecule-A (JAM-A), whereas sinusoids have higher expression of E-selectin, which is known to regulate hematopoietic stem and progenitor cell (HSPC) homing.[11] In addition to their role in leukocyte trafficking, these adhesion molecules also control HSPC retention by VCAM-1 or negative regulation of hematopoietic stem cell (HSC) quiescence by E-selectin. Along those lines, this study demonstrated that HSPCs localize to arterioles or sinusoids based on reactive oxygen species (ROS) levels. Specifically, HSPCs associated with arterioles were consistently ROS negative, whereas HSPCs associated with sinusoids possessed variable levels. Relative to circulating HSPCs in peripheral blood, however, ROS levels are much lower in bone marrow HSPCs. Exposure to peripheral blood plasma in vitro changed ROS levels in bone marrow HSPCs such that migratory capacity was elevated. This was in line with previous observations that enhanced reactive oxygen species levels promotes HSPC mobilization by activating motility machinery.[12,13]

Interestingly, genetic deletion of endothelial *Cxcr4* promoted HSPC trafficking while also simultaneously increasing endothelial permeability. Perturbations to permeability alone was sufficient to alter HSC fate which resulted in HSPC and mesenchymal stem and progenitor cell (MSPC) expansion, reduced bidirectional trafficking, reduced MSPC differentiation, and shifts in HSPC metabolism. Endothelial disruption further reduced HSPCs, and long-term repopulating hematopoietic stem cells and ROS levels were increased in HSPCS and MSPCs such that the frequency of ROS^{high} cells surrounding blood vessels was increased.[9]

Taken together, loss of endothelial integrity and exposure to blood plasma increased ROS levels, which increases migratory capacity. In their original mechanistic investigations,[9] the authors noted that perturbed barrier integrity was localized primarily to sinusoids. To that end, the authors observed hematopoietic cell rolling and adhesion events as well as transendothelial migration of mature leukocytes and immature HSPCs occurring exclusively in sinusoids, thus showing that sinusoids are distinct sites for leukocyte trafficking. Despite this finding, it was still unknown if the diploë hematopoietic stem cell niche supplied cells to the underlying meninges.

In 2018 and 2019, however, a series of studies reported the presence of ossified CD31+ vascular channels traversing the inner skull cortex,[14–16] thereby granting access to the meninges. These vessels were 21.6 ± 0.9 μm in diameter,[14] indicating that they are continuations of the sinusoids in the diploë region from which monocytes can traffic. Furthermore, these vessels were confirmed to exist in human samples too.[14] The authors, however, did not determine if the skull bone marrow is a source for meningeal myeloid cells.

To address this, a follow-up paper[17] pioneered a calvaria bone-flap transplantation method where mice expressing green fluorescent protein under human ubiquitin C promoter (UBC-GFP mice) had a rectangular portion of their skull transplanted onto wildtype (WT) mice with a matching skull vacancy, with underlying dura left intact. 30 days following transplantation, results revealed the formation of ossified channels traversing inner skull cortex from calvarial bone marrow, from which a small amount of donor GFP+ cells were found to give rise to dural $Ly6C^+$ monocytes and neutrophils. Lethally irradiating the head while shielding the body resulted in a significantly higher percentage of GFP+ cells in skull bone marrow and cranial dura. This was in contrast to the chimerism achieved when the body was irradiated, and head shielded. The authors further only found monocyte-dendritic cell progenitors (MDPs) and monocyte-committed progenitors (cMoPs) in the skull bone marrow but not cranial dura. Finally, upon characterizing these ossified vascular channels that formed in transplanted skull flaps, size revealed these vessels to be the larger sinusoids as indicated in the previous study first characterizing these vessels.

Beyond reactive oxygen species level in endothelial cells regulating HSPC quiescence or trafficking, their production of colony-stimulating factor-1 (in combination with mesenchymal stromal cells) was recently shown to be a crucial factor in supporting Ly6C$^-$ monocytes.[18] In bisected femurs, cMoPs, Ly6C$^+$, and Ly6C$^-$ monocytes were found to reside within 5 μms of sinusoidal endothelial cells, and these endothelial cells were further confirmed to the specific source of colony-stimulating factor-1 that maintains Ly6C$^-$ monocyte abundance in circulation under homeostatic conditions. Under conditions of polymicrobial sepsis, endothelial-derived colony-stimulating factor-1 promoted the recovery of Ly6C$^-$ monocytes. Additionally, other factors such as stem cell factor, CXC motif chemokine ligand 12 (CXCL12), and thrombopoietin are known to regulate HSPC maintenance within the perivascular bone marrow niche.[4,19–22]

Taken together, the aforementioned studies reveal HSPC retention and migration exist along a vascular zonation. Arteriole vessels in the diploë region are responsible for retention of HSPCs, whereas larger sinusoids in the diploë are responsible for HSPC trafficking. Ossified, sinusoidal vessels continue in the inner cortex of skull, ultimately providing anatomical routes for skull bone marrow to serve a source of myeloid cells to the underlying dura (Figure 1a–b), the properties and cellular constituents of which, will be discussed next.

3 | THE MENINGES AND ITS ASSOCIATED MYELOID LANDSCAPE

The meninges are a three-layered membrane lying just below the skull. This structure adds an additional protective covering for the brain while also sealing the CSF bathing it.[23–25] The outermost layer, known as the dura, is a thick membrane comprised of two layers. This includes the periosteal layer, which sits in close proximity to the inner skull, and the meningeal layer encased by flattened cells and sitting closer to the brain. Outside of locations where the meningeal layer enwraps blood-filled cavities known as dural sinuses, the periosteal and meningeal layers fuse together.[26]

The middle meningeal layer possesses a spiderweb-like appearance, from which its name-arachnoid mater-derives. It is composed of an outer layer of cells that form a barrier due to the expression of transmembrane tight junction proteins. This barrier separates the CSF in the subarachnoid space from the interstitial fluid (ISF) in the dura. Major arteries penetrate the brain from this CSF-filled cavity.[23]

The third meningeal layer is known as the pia ("gentle" in Latin). This thin membrane is composed for flat cells adhering firmly to the surface of the CNS parenchyma, dipping down into the brain sulci or folds in the cerebral cortex. This layer is highly vascularized and is semipermeable to the CSF present within the Virchow–Robin spaces surrounding brain-penetrating arteries. Together, the arachnoid and pial membranes form the leptomeninges.[23]

3.1 | The meningeal immune cell landscape

The diversity of myeloid inhabitants occupying the meningeal space has recently begun to be elucidated. Meningeal occupants were long thought to derive solely from the systemic circulation, but the aforementioned studies[14–16] describing vascular channels in the inner cortex of skull prompted further investigation, and as already mentioned, the calvarial niche was indeed identified as a major contributor.[17] It is known that ontogeny of and local niche surrounding myeloid cells is a major determinant in establishing their heterogeneity and physiological repertoire.[27–29] To begin identifying specific inhabitants, these authors[17] utilized parabiotic pairing between UBC-GFP and WT mice to detect GFP+ cells arising from systemic circulation and GFP−, non-blood-derived myeloid cells. Analysis via flow cytometry revealed that, relative to blood, spleen, and liver, Ly6C+ monocytes and neutrophils in the cranial dura were GFP−, suggesting a non-blood origin. In contrast, a relatively equal number of GFP+ CD4 T cells were found in cranial dura, which suggests a blood origin.

While this study also found that GFP+ B cells were less prevalent at brain borders, another study published at a similar time thoroughly characterized the meningeal B cell phenotype.[30] Prior studies had characterized an immature B cell cluster in the CNS,[31,32] yet they did not exclusively focus on the meningeal compartment. Through the utilization of multiple experimental techniques—including flow cytometry, confocal microscopy, and scRNAseq—Brioschi et al.[30] defined the meningeal B cell constituency as that spanning multiple stages of B cell development, including pro-B to mature B cells. This is in alignment with other bone marrow signatures yet in contrast to peripheral B cells, which exhibit a mature B cell phenotype.[30]

To confirm calvarial bone marrow contribution of B cells, just as the previously mentioned study did,[17] the authors performed an irradiation experiment where only the body was shielded. These head irradiated *Cd45.2* mice then received bone marrow cells from *Cd45.1* mice, with assessment of chimerism in different tissues assessed four weeks following this. The reverse experiment was performed where *Cd45.1* mice were irradiated and received BM cells from *Cd45.2* mice. Both experiments demonstrated a large population of donor-derived B cells in the calvarial bone marrow and dura. To determine what degree circulating B cells may contribute to the meningeal B cell population, a parabiosis experiment was performed between WT and CD19-Tomato mice. Four weeks following surgery, blood and spleen possessed a large population of parabiont-derived B cells, whereas only a minor constituency could be found in brain and dura. These results were replicated upon adoptive transfer of CD19-Tomato splenocytes into WT-recipient mice. Interestingly, just as was found in 17, CD4 T cells were found to largely derive from circulation rather than calvarial bone marrow. Finally, through the use of X-ray tomography and confocal imaging, the authors demonstrated IgM- B cell trafficking through the aforementioned calvarial inner cortical channels downstream of trabecular sinusoids (Figure 1b).

In light of the major calvarial bone marrow contribution to meningeal myeloid populations, what dura-derived factors might be responsible for promoting myeloid egress from skull bone marrow? To answer this question, Cugurra et al.[17] identified ligand expression for monocyte and neutrophil chemokine receptors using RNA-magnet algorithms from whole dura single-cell RNA sequencing

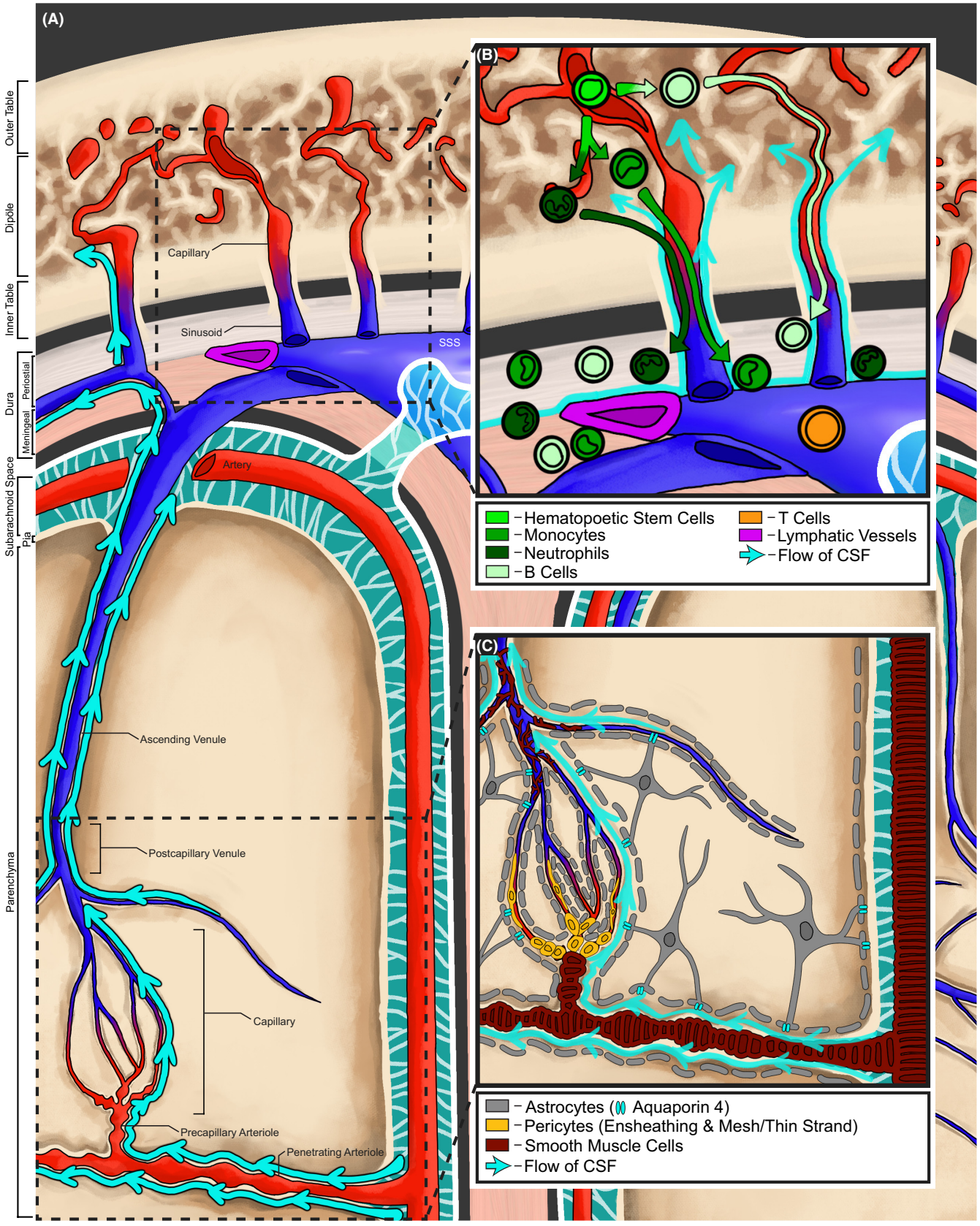

FIGURE 1 Anatomy and physiology of myeloid cell egress from the calvarial bone marrow stem cell niche. (A) Overview of anatomical structures relevant to myeloid cell egress. Starting at top, capillaries (red) and sinusoids (blue) in the diploë region form a vascular network, where sinusoids feed into ossified vascular channels traversing the inner table of skull and feed into the dural layer of the meninges. This dural layer forms the first of three layers, and it is here that sinusoidal structures, such as the superior sagittal sinus (SSS), feed into brain parenchyma. Running alongside and parallel to the SSS are meningeal lymphatic vessels (magenta). Arteries (red) begin their descent in the arachnoid (second meningeal layer), which contains the cerebrospinal fluid (cyan)-filled subarachnoid space. As they penetrate through the inner meningeal, or pial layer, they are surrounded by the Virchow–Robin space, which is a continuation of the subarachnoid space. Surrounding and meeting this space beginning at the pial layer are astrocyte endfeet, which form the glia limitans (not shown here). As arteries dive deeper into brain, they transition to smaller arterioles, precapillary arterioles, capillaries, postcapillary venules, and finally ascending venules. Note that CSF flow (indicated in cyan and cyan arrow) flow perivascularly from arteries to venules, eventually reaching the skull bone marrow niche. (B) Arterioles (red) in the diploë region maintain hematopoietic stem cells in a quiescent state. These cells can differentiate into monocytes, neutrophils, and B cells, which can traffic along sinusoids and through ossified channels in the inner table of skull to the underlying dural layer. T cells (orange) are largely localized to sinusoidal regions within the dural layer. (C) CSF flow (cyan) progresses from arteries to venules through convective flow. Arteriole pulsatility and aquaporin 4-expression (cyan) on astrocyte endfeet are vital to CSF flow. Note that vascular mural coverage varies in morphology as one progresses through various vascular zones. Ring-like and fast contractile smooth muscle cells on arteries and arterioles transition to ensheathing pericytes (yellow) on precapillary arterioles, and eventually to mesh and thin strand pericytes. Smooth muscle cells are also located on ascending venules, but their morphology differs from that of arteries and arterioles. Postcapillary ascending venules are also the site of immune cell entrance into CNS parenchyma. For a reference on mural cell morphology and vessel size along the vascular tree in brain parenchyma, see Hill et al.[125]

analysis. This unbiased approach revealed high expression of C-C motif chemokine ligand (CCL) 2, CCL12, and CCL8, which can signal through C-C motif chemokine receptor (CCR) 2 to recruit monocytes. They also found high expression of CCL6, which can signal through CCR1 to recruit neutrophils. Taken together, the dura contains monocytes, neutrophils, and B cells, which are largely derived from calvarial bone marrow, while T cells in meninges derive largely from blood circulation. The dura contains signaling factors under homeostatic conditions that promotes myeloid cell egress from calvarial bone marrow, thus pointing to this hematopoietic niche as a major contributor to neuroimmune function. What other structures further support myeloid cell trafficking from the meninges? If T cells are blood-derived and do not exist in brain parenchyma under homeostatic conditions, how do they sample CNS-derived antigens? This will be discussed next.

4 | LOCATIONS OF TRAFFICKING AND OF ANTIGEN PRESENTATION-LYMPHATIC, DURAL, AND CEREBRAL BLOOD VESSELS

Seminal experiments conducted by Peter Medawar in the early 1900s led to the concept of CNS immune privilege via brain barriers restricting the passage of antigens from brain to peripheral circulation. Specifically, it was demonstrated that skin grafts transplanted into brains of previously immunized animals undergo swifter rejection relative to non-sensitized animals. The rediscovery of a functional meningeal lymphatic system, however, later challenged the concept of CNS immune privilege.[33–36] Prior to this, it was accepted that the meningeal compartment was a space of continual immune surveillance,[1,37,38] but upon the discovery that two to three lymphatic vessels run parallel and adjacent to the dural sinuses,[34] an additional site of fluid exchange, and hence peripheral access to CNS antigen, emerged (Figure 1a).

Meningeal lymphatic vessels express canonical markers of lymphatic vessels, including lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1), the transcription factor Prox1, podoplanin, the vascular endothelial growth factor receptor 3 (VEGFR3), and CCL21.[34,36] The interaction between VEGFR3 and VEGF-C is important both for the development and physiology of meningeal lymphatic vessels. In contrast to other peripheral organs where lymphatic development commences in utero, meningeal lymphatic vessel development begins postnatally in response to arterial/venous smooth muscle cell-derived VEGF-C and blocking this ligand-receptor interaction results in deficient vessel development.[35] Furthermore, the interaction between VEGF-C and VEGFR3 modulates meningeal lymphatic vessel diameter.[34]

Lymphatic vessels possess a variety of phenotypes based off the presence of cellular junctions and variability in thickness of basement membrane coverage, both of which function to regulate vessel permeability.[39] Capillary lymphatic vessels have higher permeability due to a thin basement membrane and lack of mural cell coverage. These endothelial cells are further connected by discontinuous junctions, known as buttons, and hence, these vessels are characterized by the transmigration of immune cells and uptake of ISF. In contrast, endothelial cells of collecting lymphatic vessels are connected by continuous junctions, termed zippers.[40] Furthermore, these vessels are enwrapped by a contractile smooth muscle cells and basement membrane such that permeability is reduced. Retrograde lymph and blood reflux is prevented in collecting vessels by the presence of intraluminal valves.[39] In addition to these phenotypes, some peripheral lymphatic vessels are characterized by an intermediate precollector phenotype—possessing mixed button—and zipper-like junctional patterns along with lymphatic valves without abluminal smooth muscle cell coverage.[39] Dorsal meningeal lymphatic vessels lack lymphatic valves and possess a non-continuous basement membrane, thus making them more akin to these peripheral lymphatic capillaries.[34,36] Basal vessels, on the contrary, possess a precollector

phenotype having a button-like junctional pattern. They further possess lymphatic valves but lack smooth muscle cells.[33,35,36]

While cellular and soluble constituents of the CSF were known to induce immune responses in the cervical lymph nodes, the proposed pathway was through the cribriform plate into lymphatic vessels within the nasal mucosa.[41] Upon discovering meningeal lymphatic vessels, it was shown that these vessels do communicate with deep cervical lymph nodes (dCLN) via Evan's Blue injection. In contrast, when injecting Evan's Blue into the nasal mucosa, no Evan's Blue was detected in the deep cervical lymph nodes, suggesting that indeed meningeal lymphatic vessels and not nasal mucosal lymphatic vessels represent the primary route for CSF drainage into the dCLN. Physiologically speaking, however, one study failed to find substantive CSF reuptake and drainage by dorsal meningeal lymphatic vessels using controlled low-rate and volume stereotactic CSF tracer injection.[42] Furthermore, other studies demonstrated that injection of fluorescent or contrast tracers into brain parenchyma or lateral ventricle resulted in transport occurring primarily through the basal meningeal lymphatic vessels into the dCLNs rather than dorsal lymphatic vessels.[33,36]

Anatomically speaking, meningeal lymphatic vessels run alongside the major venous sinuses, arteries, and cranial nerves exiting alongside these structures at the base of the skull via the aforementioned foramina.[34,36,43] This includes the cribriform plate underneath the olfactory bulb where olfactory nerves traverse bone into nasal mucosa. Thus, previously identified periarterial and perineural locations of ISF and CSF flow are also locations of meningeal vessels.[43–47] These findings have been confirmed in human brain as well.[48] Interestingly, following experimental autoimmune encephalomyelitis (EAE), lymphatic vessels at the cribriform plate were recently demonstrated to increase in cell number,[49] with EAE driving cribriform plate lymphatic endothelial cells into a slightly elevated proliferative state indicative of lymphangiogenesis. These vessels upregulated proteins such as podoplanin and programmed death-ligand 1 in an interferon gamma-dependent manner. These proteins are known to mediate leukocyte adhesion/chemotaxis, and indeed, EAE resulted in increased leukocyte binding to cribriform plate lymphatic endothelial cells. This included dendritic cells, myeloid cells, and $CD4^+$ T cells.[49] Interestingly, these lymphatic vessels were further shown to capture and present CNS-derived antigens, with prime access to CSF provided by discontinuity of the arachnoid membrane near the cribriform plate.[49]

Beyond EAE, meningeal lymphatic vessels have been reported to play varying roles with regards to improving or exacerbating disease pathology. With viral infection,[50] Alzheimer's Disease (AD),[51] brain tumor models,[52,53] and traumatic brain injury,[54] deficits in meningeal lymphatic vessel function worsens disease outcomes. In multiple sclerosis,[55,56] however, ablation of meningeal lymphatic vessels may improve disease outcome by reducing antigen transport and T cell migration.

Taken together, lymphatic vessels are a gateway for CNS-derived antigens to access peripheral immune cells. These meningeal lymphatic vessels possess canonical markers of lymphatic vessels in the periphery, and their permeability varies based off the presence of cellular junctions and basement membrane coverage. Basal meningeal lymphatic vessels may be primary route for fluid transport into the deep cervical lymph nodes relative to their dorsal counterparts. Beyond meningeal vessels, however, where else might peripheral or calvarial immune cells access CNS-derived antigen?

4.1 | Sites of antigen presentation—the dural sinusoids

Initial studies characterizing the CNS immune cell landscape revealed the presence of border-associated macrophages that were distinct from parenchymal microglia.[57,58] These studies did not elucidate the exact anatomical locations in which immune cells reside. To clarify this, Rustenhoven et al.[59] visualized a clustering of T cells and major histocompatibility complex II ($MHCII^+$) antigen-presenting cells around dural sinuses, where these dural T cells polarized towards a Th1, Th2, Th17, and Treg subsets. Single-cell RNA sequencing of dural endothelial cells revealed a subset of von Willebrand Factor (VWF^+) cells, and immunostaining of meningeal whole mounts clarified that VFW^+ cells were indeed dural sinus endothelium, with human superior sagittal sinus also possessing VWF^+VCAM1^+ endothelial cells. Dural sinus endothelial cells further possessed higher expression of the adhesion molecules *Vcam1*, *Icam1*, and *Selp* relative to other vessel types present in dura. In vivo imaging at the confluence of sinuses in RAG2 knockout mice transplanted with CD4 T cells from UBC-GFP mice revealed the preferential occurrence of T cell adhesion events at dural sinuses relative to other dural vessels, with some events occurring at larger cerebral veins projecting into the sinus. Blocking antibodies were injected to disrupt the binding of P-selectin, VCAM1, and ICAM1 with the leukocyte integrins P-selectin glycoprotein ligand-1, alpha4beta 1 integrin, and lymphocyte function-associated antigen 1, respectively. This treatment nearly abolished adherence events completely, thus demonstrating that adhesion molecules are necessary for homeostatic trafficking to the dural sinus. Finally, CD4 T cell infiltration into the meninges across the dural sinus was examined six days following peripheral UBC-GFP CD4 T cell transfer into RAG2 knockout mice, with results revealing a nearly identical perisinusal localization of CD4 T cells as that observed in WT mice.[59]

Beyond the presence of adhesion molecules, single-cell RNAseq of the entire dural meninges revealed additional stromal-derived ligands that serve to recruit CD4 T cells to dural sinuses. The most notable was *Cxcl12*, and immunostaining in CXCL12-dsRed reporter mice further confirmed dural sinus expression to be substantially higher than other non-sinus sites. Additional physiological experiments confirmed the critical yet non-exclusive role this signaling pathway plays in T cell recruitment. Finally and critically, this study demonstrated that CSF effluxes to perisinal dura where sinus-associated macrophages and dendritic cells capture CNS-derived antigens and present them to patrolling T cells. Taken together, the dural sinuses are sites of T cell patrolling and antigen presentation,

and upon activation, T cells further transmigrate into dura at these vascular locations.[59]

In addition to the dural sinus, this study[59] further characterized other vascular locations and mural cell composition using single-cell RNA sequencing. Beyond the aforementioned $VWF^{+}VCAM1^{+}$ endothelial cells, dural endothelial cells lack tight junctions Claudin 5 and Occludin and additionally possess markers for fenestrated endothelia, such as plasmalemmal vesicle-associated protein. $NG2^{+}$ pericytes were localized to capillaries and alpha smooth muscle actin smooth muscle cells to larger caliber blood vessels (arteries, arterioles, and venous sinuses). Furthermore, a large population of platelet-derived growth factor receptor ß ($PDGFR\beta^{+}$) fibroblast-like cells occupied a distinct perivascular localization or were not embedded in the collagen-1 extracellular matrix. Beyond this larger population of fibroblast-like cells and vascular zonation, the barrier properties of dural vasculature differ from the leptomeninges and parenchymal vasculature, which will be discussed next.

4.2 | The blood–brain barrier

The cerebrovasculature can be categorically separated into six distinct segments, which includes pial arteries, penetrating arteries, arterioles, capillaries, postcapillary venules, and veins.[60] Endothelial cells (ECs) form the vasculature but have transcriptional identities that shift according to their location within the vascular tree (arteriole, venous, or capillary).[61] For the arterial zone, genes such as *Bmx*, *Vegfc*, *Efnb2*, *Gkn3*, and *Sema3g* shape endothelial identity, whereas the venous identify is shaped by genes *Nr2f2* and *Slc38a5*.[61] *Mfsd2a* and *Tfrc* comprise the capillary zone, and in general, transcription factors are a dominant attribute of the arterial endothelium whereas transporters shape capillary and venous endothelial transcriptional identity.[61]

The blood–brain barrier (BBB) is found in penetrating arterioles, capillaries, and ascending venules. It specifically refers to a continual structure existing at the plasma membranes of endothelial cells of lying adjacent to one another.[62] The BBB was first described by Paul Ehrlich over a century ago upon his observation of systemically injected water-soluble dye exclusion in brain and spinal cord.[63,64] Ehrlich's student, Edwin Goldmann, expanded upon these observations and found that trypan blue injection into CSF results in confinement to the CSF only and not the periphery.[65] It was not until Max Lewandowsky's experiments, however, that the term blood–brain barrier was used. He determined that the detrimental impact of exposure to neurotoxic substances only occurred when injected directly into the brain.[66] Many studies since that time have characterized the presence of both physical and chemical barriers along with a lack of fenestrations. Additionally, CNS vessels possess low expression of transcytotic vesicles, and these aforementioned properties distinguish CNS vessels from those in the periphery.[61] Due to these properties, only lipid-soluble molecules possessing fewer than 8 hydrogen bonds and being <400 Da can pass freely via lipid-mediated diffusion.[67]

The molecular constituents conferring this low permeability to CNS vessels include adherens and tight junction proteins.[68] Specifically, junctional adhesion molecules A-C (JAM A-C), platelet endothelial cell adhesion molecule (PECAM-1), and vascular endothelial cadherin (VE-cadherin) form the adherens junctions, which are located on the apical side of the endothelial cell, or that side facing brain parenchyma.[69] α-, β-, and γ-catenin proteins link these proteins to the actin/vinculin-based cytoskeleton.[70]

In the CNS, Occludin[69] and Claudins 1,[71] 3, 5, and 12[72,73] interact to form tight junctions. The BBB's exclusivity to different molecular size is differentially regulated by particular Claudins.[69] As an example, deletion of Claudin-5 leads to extravasation of molecules less than 800 Da and neonatal death.[72] In contrast, deletion of Occludin does not perturb barrier integrity,[74] yet when only the N-terminal domain is deleted, TJ strand formation is altered.[75] Adherence to the actin-based cytoskeleton holds these proteins in place, where members of the peripheral membrane-associated guanylate kinase protein (MAGUK) family, zonula occludens 1–3 (ZO1-ZO3), serve as the connecting node between these proteins.[68] Beyond the provision of cytoskeletal anchorage, zonula occludin proteins regulate the spatial distribution of Claudins through their PDZ-binding domains.[69]

4.3 | Induction of BBB properties

As mentioned, these proteins and physiological properties are distinguishing characteristics of CNS vasculature. The first clue at understanding how these properties are induced came from seminal studies conducted by Steward and Wiley.[76] They performed chick-quail chimera studies to show that the neural microenvironment is responsible for the induction of BBB properties in CNS endothelial cells. An additional study cultured isolated BBB endothelial cells outside their CNS environment and demonstrated a reduction in transendothelial electrical resistance and increased barrier permeability.[77] Together, these studies ushered in an era of determining how various CNS cell types contribute to the BBB formation in development and maintenance throughout adulthood.

Many early studies co-cultured endothelial cells with astrocytes or astrocyte-conditioned media and found that, as a result, endothelial cells possessed more complex tight junctions[78] and transendothelial electrical resistance.[77,79] One particular study performed endothelial cell implantations into the anterior chamber of the eye and found that newly formed vessels interacting with astrocytes retained Evan's blue dye. This was not the case, however, when fibroblasts were implanted.[80] A follow-up study[81] repeated this experiment but provided additional electron microscopy data to show that the endothelial cell implants in the present of astrocytes were poorly vascularized, and that of the few vascular structures formed, none possessed characteristics of CNS capillaries. In contrast to this, those implants with fibroblasts possessed high numbers of fenestrated capillaries, and the authors concluded from this that the differences in dye extravasation observed in the prior study were actually due to differences in

vascularization rather than BBB-inducing factors from astrocytes. Follow-up in vitro studies utilized astrocyte-conditioned media to show that electrical resistance and permeability to large and small molecular tracers can reinduced in CNS endothelial cells.[82–84] Further in vitro support for astrocyte induction of BBB properties was provided when a study cultured non-CNS endothelial cells in the presence of astrocytes or astrocyte-conditioned media and found that BBB-specific properties such as P-glycoprotein (p-gp) and tight junction expression was induced.[84–86] Even direct contact with astrocytes in culture was shown to be sufficient to induce formation of mature BBB properties.[87] Taken together, there was a litany of in vitro studies supporting astrocyte induction of BBB properties, but in vivo evidence was lacking.

Following these early studies on astrocytes, there was a seminal study in 2010[88] that demonstrated the necessity of pericytes in establishing mature BBB properties in development, specifically by downregulating the number of transcytotic vesicles rather than inducing tight junction protein expression for example. This BBB developmental program is completed by the time astrogliogenesis commences in the brain, which usually begins around E18.5.[89] In light of this finding, the consensus around astrocytes and the BBB shifted towards a view that astrocytes were necessary for BBB maintenance in adulthood. The first in vivo study supporting this view was published in 2003[72] that demonstrate the necessity of the Src-suppressed C Kinase substrate (SSeCKS) in regulating the expression of ZO-1, where SSeCKS is expressed in astrocytes. Other studies have further shown impacts to BBB integrity upon genetically ablating astrocytes[90] or knocking out astrocyte-specific proteins. One such study knocked-out connexin 40 and 43,[91] and another study astrocyte-specific laminin,[92] with both studies showing dye extravasation in striatum but not cortex. Additionally, one other study employing diphtheria toxin to ablate astrocytes found no BBB breakdown in spinal cord.[93] With the exception of the aforementioned study[90] observing extravasation of <1 kDa Cadaverine in cortex upon genetic ablation of astrocytes, the prevailing view of the literature points to astrocyte maintenance of the BBB in deeper brain regions rather than cortex. Pericytes, on the contrary, are necessary for BBB development.[88] Future studies should expand astrocyte regulation of BBB properties beyond the expression of tight junction proteins to transporters that may not be expressed until later developmental timepoints coinciding with astrogliogenesis.

4.4 | Immune cell trafficking at postcapillary venules

Within the CNS parenchyma, immune cell extravasation occurs at postcapillary venules[94] similar to sinuses in the diploë and meningeal layer. As already mentioned, T cell migration across the glia limitans from the meninges into brain parenchyma requires antigen recognition on perivascular or dural antigen-presenting cells.[59] In brain parenchyma, however, migration across the BBB is independent of antigen recognition on endothelial cells.[95–100]

Trafficking across postcapillary venules occurs in a stepwise, progressive fashion. These include cell arrest, cell rolling, integrin activation, cell arrest and adhesion, cell polarization, crawling opposite the direction of blood flow, and finally, diapedesis or migration across the vascular wall.[94] Interactions between CD4 T cell alpha4beta1 integrin and VCAM1 is known to be necessary for capture, and VCAM1 is further involved in arrest and adhesion.[100–102] Other molecules such as E- and P-selectins are necessary for cell rolling,[103–106] and intercellular adhesion molecule 1/2 underlies cell arrest, adhesion, polarization, and crawling against the direction of blood flow.[101,107–109] Additionally, g-protein-coupled receptor signaling is necessary for T cell adhesion[100] and arrest[105] to endothelial cells. For a thorough review on immune cell trafficking across the parenchymal BBB, see Marchett & Engelhardt.[94]

5 | THE ROLE OF CEREBROSPINAL FLUID AND THE GLYMPHATIC SYSTEM IN SIGNALING

In tandem with the BBB is the blood-CSF barrier. Together, these two entities keep the blood separate from brain parenchyma and help maintain its extracellular environment through regulation of the ionic and biochemical composition of the brain's various fluid compartments.[110] There are four total compartments including the CSF, ISF, intracellular fluid, and the aforementioned blood vasculature.[111] In contrast to the BBB, the blood-CSF barrier is comprised mostly of choroid plexus epithelial cells. Unlike the cerebrovasculature spanning brain parenchyma or choroid plexus epithelial cells, plexus capillary endothelial cells lack tight junction proteins thereby increasing their permeability to macromolecules. It is the presence of epithelial transporters at the choroid plexus regulating the passage of molecules from blood to CSF.[112]

CSF is produced primarily by the choroid plexuses. These highly vascularized structures are expansions of the ependymal epithelium lining the lateral, third, and fourth ventricles. Osmotic gradients generated across epithelial cells from the transport and exchange of ions is a crucial process in CSF production. These gradients propel water from blood to the ventricle lumen.[110,112,113] The Na^+/K^+-ATPase has emerged as a crucial ionic transporter underlying CSF production and is localized to the apical membrane of the choroid plexus epithelial cell. It functions to maintain a low intracellular sodium concentration in epithelial cells due to active transport of sodium across the epithelial cell into the CSF housed within the ventricular lumen.[112–116] It is further thought that bicarbonate (HCO_3-) and its transcellular exchange with chloride is important for CSF production.[111] Beyond these transported ions, it is thought that CSF production is regulated by intracranial pressure[112,117] or through autonomic regulation,[117] but more clarification is needed regarding this. For a more detailed review regarding CSF production, see Jessen et al.[111]

The four aforementioned ventricles are linked by foramina, and CSF flows through these ventricles into the subarachnoid space of the cortex and spinal cord. From the subarachnoid space, CSF flows into the brain's Virchow–Robin periarterial spaces. Arterial pulsatility (Figure 1c), respiration, CSF pressure gradients, and the loose fibrous matrix of the perivascular space can be viewed as a low resistance highway for CSF influx. Together, this convective influx facilitates the interchange of CSF and ISF.[118]

Critical for the transport of CSF from the periarterial space to brain parenchyma are astrocytes and their expression of the water-channel Aquaporin-4 (Figure 1c). These cells extend long, flattened processes called endfeet that surround endothelial cells, separated only by the basal lamina. Endfeet cover up to 99% of the cerebrovascular surface[119] and most protoplasmic astrocytes maintain direct contact to three blood vessels.[120] On average, each astrocyte possesses 3.5 endfeet, though some can have as few as one or as many as seven.[121] Furthermore, astrocytes can dynamically maintain endfoot coverage through replacement of vascular regions stripped of endfoot coverage.[122] The aforementioned water channel Aquaporin 4 is localized to endfeet, and hence it is in prime position to help transport the periarterial CSF to brain parenchyma.

Using two-photon imaging, Iliff et al.[118] visualized the passage of tracers injected into the cisterna magna. Their observations revealed a pathway whereby CSF enters the brains on a path parallel to the cortical pial arteries. From there, tracers entered periarterial sites surrounded by astrocyte endfeet and exited the brain mostly along central deep veins and the lateral-ventral caudal rhinal veins. Interestingly, when performing the same studies in Aquaporin-4 knockout mice, there was roughly a 65% reduction in CSF passage through brain parenchyma relative to WT controls. This was also accompanied by a roughly 55% reduction in clearance of radiolabeled ß-amyloid injected into striatum.[112] The polarized nature of Aquaporin-4 expression at endfeet surrounding blood vessels enabling the convective fluid flux with CSF/ISF interchange was entitled the glymphatic system given similarities to the lymphatic system, and this process of CSF perfusion throughout the brain is known as glymphatic clearance.[118]

Interestingly, the of CSF has proven to be a critical signaling conduit for CNS-derived signal to access and promote myeloid cell egress from the skull bone marrow niche. It was recently demonstrated[123] that CSF accesses the calvarial bone marrow niche through the aforementioned channels traversing the inner table of skull from upstream sinusoids.[14–16] Not only was this the case for dorsal skull where ossified channels are known to exist, but also at the base of the skull through similar channels. CSF was shown to interact with both macrophages and hematopoietic stem cells, and accessibility to the niche did not change with aging. Injection of the CXCR4 antagonist, AMD3100, into the CSF promoted myeloid cell egress from the bone marrow niche. Additionally, intracerebral injection of fluorescent ovalbumin also labeled cells in the bone marrow niche, and given that access does not changing with aging, this suggests that both parenchymal and CSF-derived factors can uniquely shape the nice throughout the lifespan. To that end, the transcriptional identity of cells within this compartment was unique from that of the periphery, further supporting this conclusion. Spinal cord crush, an injury that leaves both meninges and CSF access to bone marrow niche intact, resulted in myeloid cell egress from the calvarial bone marrow niche. Finally, one other recent study[124] also confirmed that CSF reaches skull bone marrow through the ossified vascular channels in the inner table of skull cortex, and taken together, these results point to CSF as the etiological source of molecular cues signaling myeloid cell egress from local calvarial bone marrow niches.

6 | CONCLUSION

This review has highlighted the recent emergence of the calvarial hematopoietic niche and the properties comprising the vasculature structures by which they traffic. Taken together, a framework emerges whereby sinusoids form the locations of hematopoietic trafficking. This is afforded by their combined expression of E-selectin, colony-stimulating factor-1, and increased permeability due to absence or lower expression of tight junction proteins. The discovery of ossified vascular channels traversing the inner table of skull provided a physical route by which hematopoietic stem cells can traffic through calvarial sinusoids to the underlying meninges.

Within the meninges, the dural sinuses and lymphatic vasculature lie alongside each other and provide a site by which peripheral immune cells can access CNS-derived antigens, where an emphasis has been placed primarily on T cells. CSF carries these antigens as it is propelled by glymphatic clearance, where astrocytes are a crucial arbiter to this process. Important, however, is to understand how the calvarial bone marrow niche and associated vascular structures change with normal biological aging and disease.

6.1 | The role of skull bone marrow-derived immune cells in aging and CNS disease

Ossified vascular channels lying in the inner skull cortex[14] are particularly relevant to acute inflammatory conditions of the brain. For example, ischemic stroke and intracisternal injection of carrageenan resulted in higher contributions of skull-derived rather than tibial-derived neutrophils. In contrast, acute myocardial infarction resulted in equivalent contributions from skull and tibia, indicating that the skull's proximity to brain allows it to serve as a primary donor of myeloid cells during cerebral insult. How might aging might impact these calvarial-derived immune cells?

As previously mentioned, B cells in the meninges and dura of young mice have minimal contribution from peripheral circulation.[30] Interestingly, this study also found not only a marked increase in total dural B cell number during aging, but also that clonal overlap with blood B cells increased, suggesting peripheral cell infiltration. Single-cell RNA sequencing of 8–12 week and 20–25-month-old mice allowing clustering of cells into subsets, where one unique subset from aged mice was found. These cells have been named age-associated

B cells (ABCs), and they were found to substantially increase in aged dura. Single-cell RNA sequencing further revealed a transcriptional identity suggestive that ABCs were antigen-experienced B cells.

This study also characterized dural plasma cells in aging. Not only did they increase in number, but differential gene expression analysis revealed that, while dural plasma cell in young mice were predominantly IgA$^+$, they became predominantly IgM+ in aged mice. In contrast to B cells, plasma cells in young mice show peripheral overlap with blood, indicating that mostly IgA+ plasma cells in young dura infiltrate from the periphery. This changes with aging, where mostly IgM+ plasma cells show minimal overlap with the blood repertoire, indicative that they are not from the periphery. Finally, dural plasma cells in aging exhibit a clonal overlap with the aforementioned ABCs, suggestive that aging may induce some dural ABCs to undergo local terminal differentiation into IgM-secreting plasma cells.

Aging also elevates CD4 and CD8 T cells within the dura.[59] As discussed above, in young mice, these cells are localized to the sinuses. In aging, however, these cells localize to non-sinus sites, and this was due elevated VCAM-1 expression on non-sinus vasculature. Further single-cell RNA sequencing studies revealed that aging does not change CD4 T cell phenotypes when compared to young mice.

Do blood and bone marrow-derived monocytes have differential roles in diverse CNS injury and inflammatory conditions? Cugurra et al.[17] performed three injury paradigms to address this including experimental autoimmune encephalomyelitis (EAE), spinal cord injury, and optic nerve crush injury. In the context of EAE and spinal cord injury, this study found through parabiosis experiments that a substantial pool of dural and CNS-infiltrating Ly6C$^+$ monocytes did not originate from the blood, whereas differing observations were made for neutrophils, CD4 T cells, and Ly6C$^-$ monocytes. This suggests that these cells are blood-derived, at least at the time point for which the experiment was conducted. The only change observed in the optic nerve crush injury was that both infiltrating Ly6C$^+$ monocytes and neutrophils did not appear to be blood-derived. Single-cell RNA sequencing revealed that blood-derived cells possess a pro-inflammatory transcriptional profile relative to calvarial bone marrow-derived cells, which possess a largely anti-inflammatory transcriptional profile. Together, these results suggests that myeloid cells may play varying roles in CNS disease dependent upon their origin, though future studies will be needed to clarify this.

Given that the CSF can access the bone marrow niche across the lifespan, future studies should characterize how CNS antigens trafficking through CSF change across the lifespan and in various disease contexts. Additionally, is preferential trafficking from calvarial or peripheral stem cell niches just a matter of spatial localization to site of insult or injury? While the simplest explanation, might there be molecular mechanisms regulating this? It seems more likely that the periphery and CNS will share common mechanisms to promote myeloid cell egress from stem cell niches, but perhaps each compartment possesses varying mechanisms to differentially promote physiological aspects of immune cells. For example, perhaps the calvarial niche's exposure to CSF results in differential education of B cells. Future studies should aim to clarify this.

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The authors declare no competing financial interests.

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Data sharing not applicable to this article as no datasets were generated or analysed during the current study

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