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Distinct myeloid cell subsets promote meningeal remodeling and vascular repair after mild traumatic brain injury

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Abstract

Mild traumatic brain injury (mTBI) can cause meningeal vascular injury and cell death that spreads into the brain parenchyma and triggers local inflammation and recruitment of peripheral immune cells. The factors that dictate meningeal recovery after mTBI are unknown at present. Here we demonstrated that most patients who had experienced mTBI resolved meningeal vascular damage within 2–3 weeks, although injury persisted for months in a subset of patients. To understand the recovery process, we studied a mouse model of mTBI and found extensive meningeal remodeling that was temporally reliant on infiltrating myeloid cells with divergent functions. Inflammatory myelomonocytic cells scavenged dead cells in the lesion core, whereas wound-healing macrophages proliferated along the lesion perimeter and promoted angiogenesis through the clearance of fibrin and production of the matrix metalloproteinase MMP-2. Notably, a secondary injury experienced during the acute inflammatory phase aborted this repair program and enhanced inflammation, but a secondary injury experienced during the wound-healing phase did not. Our findings demonstrate that meningeal vasculature can undergo regeneration after mTBI that is dependent on distinct myeloid cell subsets.

Mild traumatic brain injury (mTBI) is the most common form of brain injury in humans¹; however, there are currently no approved treatments for this, despite some promising candidates^{2,3}. The development of effective therapies for mTBI is dependent on more-complete understanding of the injury and repair mechanisms. These mechanisms can vary

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Author contributions

M.V.R. designed and executed the animal studies, analyzed data and wrote the manuscript; L.L.L. designed the human studies and analyzed the resultant data; and D.B.M. conceived of the project, designed and interpreted the animal studies and wrote the manuscript.

Competing interests

The authors declare no competing interests.

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temporally and by anatomical location. For example, the central nervous system (CNS) is protected by various barrier systems, including the blood–brain and blood–cerebral spinal fluid barriers⁴, that can become damaged following TBI⁵. The precise location and duration of the barrier disruption can influence the subsequent inflammatory reaction and extent of damage within surrounding neural tissue. Rapid restoration of CNS barriers is probably an essential aspect of the process of recovery from mTBI.

Another component of the recovery process is inflammation. The immune system can promote positive and negative outcomes following CNS injury^{6–8}. Macrophages derived from the periphery that respond to CNS injury range in phenotype from inflammatory to anti-inflammatory and result in divergent effects^{9,10}. Inflammatory dysregulation can disrupt the differentiation of immune cells along this spectrum and possibly affect tissue repair. In addition, the meninges are far more permissive to peripheral immune-cell traffic than is the CNS parenchyma¹¹ and can support robust inflammatory reactions in response to injury, infection or autoimmune disease^{12–14}. It is unclear at present how microenvironmental differences between the CNS parenchyma and meninges affect the differentiation or function of myeloid cells.

A pathology often observed in patients who have experienced mTBI is disruption of meningeal vascular integrity, which induces a peripheral immune response¹⁴. Damage to meningeal vasculature can also promote secondary degeneration of the glial limitans and underlying brain parenchyma^{5,14}, yet the capacity of the meninges to repair following mTBI is not defined. In a rodent model of moderate TBI, regeneration of parenchymal vasculature was observed within 2 weeks of injury; however, newly formed blood vessels were irregular in morphology and density after severe TBI¹⁵. As mTBI-induced meningeal injury can occur in the absence of primary parenchymal damage, it is important to better understand pathogenesis and repair within this anatomically distinct compartment, which is injured in ~50% of patients after mTBI¹⁴. We therefore set out to identify mechanisms that influence the meningeal recovery trajectory following mTBI.

Results

Meningeal vascular recovery following mTBI in humans.

A published study found that ~50% of patients presenting to the emergency department with minor head injury had meningeal vascular damage, indicated by enhancement of the meninges on post-contrast images obtained by fluid attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI)¹⁴. To estimate the resolution rate of traumatic meningeal enhancement, we followed a population of patients, up to three visits, for ~3 months from the date of injury (Fig. 1a–c and Supplementary Table 1). Patients who were included met the following criteria: (i) they presented with suspected mTBI, (ii) they had computerized tomography (CT) of the head that was negative for traumatic intracranial findings and (iii) they received a research MRI with contrast within 48 h of injury and at subsequent visits. Of the 209 patients studied, 104 (50%) had evidence of enhancement at baseline (11.6 h (4.9–20.2 h) from injury) that was seen to resolve in 79 patients (76%) at a median of 22 d (7–37 d) from injury (Fig. 1b). The fraction of patients with persistent meningeal enhancement decreased rapidly after the first week (Fig. 1b). In patients who had a minimum of three

visits ($n = 99$), 59% had enhancement at baseline, 40% had enhancement at 12 d and 10% had enhancement at 70 d (Fig. 1c). We detected resolution of enhancement in 83% of patients who had enhancement at baseline, and the median time to resolution was 18.9 d (11.4–37.8 d). However, enhancement persisted in 17% of patients re-imaged 87 d (72–103 d) from injury, which suggested that the meninges did not undergo repair in these patients. These data demonstrated differential rates of meningeal vascular recovery following mTBI.

Meningeal vascular damage is repaired following mTBI in mice.

We used a recently developed mouse model of meningeal injury to define the mechanisms underlying meningeal damage and repair after mTBI in humans¹⁴. Meningeal compression in mice (called ‘mTBI’ here) induces immediate cellular and vascular damage, which results in the release of reactive oxygen species that secondarily damage the glial limitans and brain parenchyma¹⁴. Because concussed humans showed varying degrees of vascular recovery over time, we became interested in the factors that govern the resolution of meningeal injury. Using the mouse model, we performed intravital two-photon microscopy (TPM) through a thinned skull window to study meningeal vascular dynamics following mTBI (Supplementary Movies 1 and 2). Mechanical injury to the meninges resulted in vascular occlusion and leakage that caused meningeal cells, including vascular endothelium, to die instantaneously (Supplementary Movie 1). This event coincided with engagement of a reparative process that consisted of the deposition of fibrin(ogen) and platelets within damaged blood vessels as well as the meningeal space (Supplementary Movie 2).

We next assessed meningeal vascular pathology and repair in mice following mTBI to determine the kinetics of recovery. Meningeal vascular health was visualized by intravenous injection of fluorescent tomato lectin (a dye that labels vascular endothelial cells). We also examined the extravascular distribution of intravenously injected Evans blue to quantify the extent of meningeal vascular leakage. Temporal analysis of meningeal whole mounts revealed that mTBI induced severe vascular leakage into the meningeal space that subsided over the ensuing week (Fig. 2a,b). Over this same time frame, large lesioned areas of non-functioning or dead vessels were replaced by newly formed, healthy vasculature (Fig. 2a,c). The new vascular network was composed of intricately connected microvessels and could be distinguished from existing vasculature on the basis of the appearance of small vascular loops (Fig. 2a). To evaluate the function of the new vasculature, we analyzed blood-flow velocity using two-photon line-scan imaging (Supplementary Fig. 2). We found that there was no difference between newly formed vessels after injury and vessels on the opposite, uninjured hemisphere, in terms of their blood flow (Fig. 2d). These data indicated that angiogenesis and closure of the meningeal vascular barrier could occur within 1 week of injury.

mTBI induces meningeal recruitment of peripheral myeloid cells.

We next sought to understand the dynamics of immune-cell infiltration during meningeal injury and repair. Analysis of gene expression revealed a robust increase in transcripts encoding pro-inflammatory cytokines and chemokines at 6 h to 1 d that mostly subsided by day 7 (Supplementary Fig. 1). The genes with the highest expression among those were genes encoding chemoattractants for neutrophils (*Cxcl1*) and monocytes (*Ccl2* and *Ccl12*).

Genes encoding inflammatory cytokines (*Il1a* and *Il1b*) were also upregulated early (6 h and 1 d) and returned to baseline expression by day 7. Neutrophils are known to infiltrate the meningeal space ~2–3 h after injury¹⁴, but the kinetics and localization of the recruitment of peripheral monocytes-macrophages in this model were undefined. Using histocytometry to analyze meningeal whole mounts¹⁶, we temporally mapped the myeloid response to mTBI over the course of 1 week using dual reporter mice expressing a green fluorescent protein (GFP) reporter for the chemokine receptor CX3CR1 and a red fluorescent protein (RFP) reporter for the chemokine receptor CCR2 (*Cx3cr1^{gfp/+} Ccr2^{rfp/+}* mice) (Fig. 3a). Loss of CX3CR1^{hi}CCR2^{lo-neg} myeloid cells was noted at 1 d after mTBI in these mice (Fig. 3c), a finding best explained by the death of meningeal macrophages observed immediately after mTBI¹⁴. That loss coincided with invasion of the lesion core by classical CX3CR1^{lo-neg}CCR2^{hi} monocytes (Fig. 3b). The number of these cells subsided over time, and they were replaced by CX3CR1^{hi}CCR2^{lo-neg} myeloid cells that were present both within the lesion and around it (Fig. 3a–c and Supplementary Movie 3), a result that was especially evident at day 4. We next analyzed the phenotype of these macrophages at day 4 after injury by staining for the mannose receptor CD206 in *Cx3cr1^{gfp/+} Ccr2^{rfp/+}* mice (Fig. 3d). This marker was shown to be expressed differentially by inflammatory (CD11b⁺CD206[−]) macrophages versus wound-healing (CD11b⁺CD206⁺) macrophages⁹. We found that most macrophages surrounding the lesion at day 4 were CD206⁺ and CCR2^{lo-neg} (Fig. 3e), suggestive of a non-inflammatory, wound-healing macrophage phenotype. These data demonstrated that the peripheral myeloid cell response to meningeal injury was temporally regulated.

Wound-healing macrophages localize to the lesion perimeter.

To further investigate post-mTBI macrophages during lesion repair, we again employed histocytometry and quantified CD206-expressing macrophages (Fig. 4a–d and Supplementary Fig. 3). At 4 d after injury, we observed that CD206⁺ wound-healing macrophages vastly outnumbered inflammatory macrophages in the mTBI lesion (Fig. 4a,b). We next mapped the anatomical positions of these macrophage subsets within the lesion (Fig. 4c and Supplementary Fig. 3). This analysis revealed that wound-healing macrophages localized mainly to the peri-lesion area, whereas inflammatory macrophages were present mostly within the lesion core (Fig. 4d). More than half of the CD206⁺ macrophages also expressed Lyve-1, a hyaluronan receptor expressed by angiogenic and wound-localizing macrophages^{17,18} (Supplementary Fig. 4a,b). These Lyve-1⁺CD206⁺ macrophages ‘preferentially’ localized to the peri-lesion area (Supplementary Fig. 4c). Intravital imaging at day 4 revealed that these lesion-perimeter macrophages were highly dynamic and juxtaposed to areas where new blood vessels would be expected to emerge (Supplementary Movie 4). We also observed a striking degree of cell proliferation in the lesion at day 4 after injury (Fig. 4e,f), with ~25% of *Cx3cr1^{gfp/+}* macrophages incorporating the thymidine analog EdU injected 1 h earlier (Fig. 4g). Proliferating cells localized mainly to the peri-lesional area where angiogenesis was probably occurring (Fig. 4h). These data indicated that CD206⁺Lyve-1⁺ wound-healing macrophages localized and proliferated around the lesion perimeter prior to the initiation of meningeal angiogenesis.

Non-classical monocytes promote meningeal angiogenesis.

The localization of CD206⁺ macrophages around the lesion perimeter led us to postulate that macrophages might participate in meningeal repair and re-vascularization following mTBI. We employed different depletion strategies to evaluate the involvement of circulating myelomonocytic cells (monocytes and neutrophils) in meningeal wound healing (Supplementary Fig. 5). Intravenous injection of antibody to the myelomonocytic cell marker Gr1 (anti-Gr1) resulted in the depletion of neutrophils and classical Ly6C^{hi} monocytes but not of non-classical Ly6C^{lo} monocytes from the circulation. Daily injection of clodronate liposomes, on the other hand, resulted in the depletion of classical and non-classical monocytes but not of neutrophils. Depletion of both monocyte subsets with clodronate markedly impaired meningeal angiogenesis, with large areas of lesioned meninges remaining non-vascularized at day 7 after injury (Fig. 5a,b). Depletion of only classical monocytes and neutrophils with anti-Gr-1 had no effect on meningeal angiogenesis (Fig. 5c), suggestive of an important role for non-classical Ly6C^{lo} monocytes in the process. To test that hypothesis, we administered a single dose of clodronate liposomes, which induced lasting depletion of non-classical monocytes but did not induce depletion of classical monocytes or neutrophils (Supplementary Fig. 5b-d). This depletion strategy significantly impeded meningeal angiogenesis (Fig. 5c). Notably, the inefficiency of meningeal vascular repair after treatment with the various depletion strategies was associated with the total number of CD206⁺ macrophages found in the lesion at day 7 after injury (Fig. 5d). Further anatomical analyses at day 4 revealed that depletion achieved through the use of anti-Gr-1 or single-clodronate treatment did not affect the proportion of macrophage subsets within the lesion core (Fig. 5e). However, a significant decrease in CD206⁺ macrophages was observed around the lesion perimeter only after a single injection of clodronate liposomes (Fig. 5f), which indicated a role for circulating non-classical Ly6C^{lo} monocytes in the derivation of wound-healing macrophages that promoted meningeal angiogenesis.

Classical monocytes scavenge dead cells in the meninges.

Because neutrophils and classical monocytes did not participate in meningeal angiogenesis, we theorized that myeloid subsets might have divergent functions following mTBI. By TPM we observed inflammatory myelomonocytic cells expressing a GFP reporter for the antibacterial enzyme lysozyme M (*Lysm*^{gfp/+}) swarming the damaged meninges and interacting with dead cells at 1 d after injury (Supplementary Movie 5). We therefore developed a novel dead-cell-clearance assay to evaluate whether those innate immune cells contributed to the uptake of dead cells instead of angiogenesis. Quantification of dead-cell clearance revealed that ~58.2% ± 3.8% (mean ± s.e.m.) of the dead cells were removed from the meninges between 3 h and 48 h after injury, a finding that was supported by TPM data showing *Cx3cr1*^{gfp/+} macrophages extending processes toward and then engulfing nucleated dead cells in the damaged meninges (Supplementary Movie 6). Depletion of neutrophils and classical monocytes with anti-Gr-1 significantly impeded the clearance of dead cells, whereas no impairment was observed following depletion of non-classical monocytes (Fig. 5g,h). These data suggested a divergent innate immune wound-healing response to mTBI, with inflammatory myelomonocytic cells participating in dead-cell removal and non-classical monocytes contributing to meningeal angiogenesis.

Macrophages clear extravascular fibrin after mTBI.

We next sought to further define the mechanisms underlying the pro-angio-genic properties of macrophages following mTBI. We initially used clearance of extravascular fibrin(ogen) as a surrogate for tissue remodeling in our mTBI lesion. Deposition of fibrinogen can form a provisional fibrin matrix on which endothelial cells migrate during neo-vascularization. This subsequently degrades as the wound-healing process ensues¹⁹. Inefficient removal of fibrin deposits can have an inhibitory effect on wound healing and promote inflammation^{20,21}. Following mTBI, fluorescence-labeled fibrin(ogen) rapidly leaked from damaged meningeal vessels into the extravascular space (Supplementary Movie 2), and a significant amount of this material remained in and around the lesion 4 d later (Fig. 6a,b). By day 7 after injury, most of the extravascular fibrin deposits were cleared, and this clearance was significantly impeded after depletion of monocytes-macrophages (Fig. 6c). Intravital imaging and immunohistochemistry revealed that myeloid cells contained fibrin within their cell bodies (Supplementary Movie 6 and Supplementary Fig. 6). These data suggested that the mechanism of clearance was direct phagocytosis, consistent with published data showing an endocytic pathway for fibrin in macrophages²².

Macrophages do not induce angiogenic gene expression after mTBI.

To investigate the role of macrophages in angiogenic programming, we quantified meningeal expression of 90 genes encoding molecules related to angiogenesis at day 4 after injury to identify those that were upregulated as CD206⁺ macrophages localized to the lesion perimeter (Fig. 6d,e). Of the 90 genes analyzed, we observed significant upregulation of those encoding alanine aminopeptidase (*Anpep*), annexin A2 (*Anxa2*), fibronectin (*Fnl*), the vascular endothelial growth factor receptor VEGFR2 (*Kdr*), the matrix metalloproteinase MMP-2 (*Mmp2*) and thrombospondin 1 (*Thbsp1*) (Fig. 6d). Some macrophage subsets have been shown to produce factors that could enhance or promote angiogenesis^{23–25}. To investigate this possibility, we quantified expression of the same genes encoding angiogenesis-related molecules (at day 4 after injury) following depletion of monocytes and macrophages with clodronate liposomes. Unexpectedly, depletion of monocytes-macrophages resulted in significant upregulation of several genes encoding molecules related to angiogenesis, including *Anxa2*, *Fnl* and *Kdr*, whereas others (*Anpep*, *Mmp2* and *Thbsp1*) remained unchanged relative to their expression in the control group that did not undergo depletion (Fig. 6e). These data suggested that the macrophages localizing to the day 4 lesion were not necessarily providing pro-angiogenic signals to stimulate new blood-vessel growth.

MMP-2 localizes to macrophages and promotes angiogenesis.

We next considered the possibility that the pro-angiogenic macrophages were involved mainly in matrix remodeling, a theory supported by the fibrin-clearance data (Fig. 6a–c). MMP-2 is a secreted protease involved in the breakdown of extracellular matrix proteins. MMP-2 can also serve a role in angiogenesis by promoting the migration of endothelial cells and activation of VEGF²⁶. We observed that *Mmp2* expression was significantly increased at day 4 after injury (Fig. 6d). Because quantification of *Mmp2* mRNA can be unreliable, we next evaluated MMP-2 at the protein level. Immunohistochemistry revealed that MMP-2

localized ‘preferentially’ to the lesion perimeter at day 4 (Fig. 7a,b). Notably, depletion of peripheral monocytes-macrophages with clodronate liposomes significantly decreased MMP-2 expression in the peri-lesion area but not in the lesion core (Fig. 7c). The majority of MMP-2-expressing macrophages were found to be CD206⁺ (Fig. 7d,e), which would explain the specific loss of MMP-2 expression at the lesion perimeter after depletion via clodronate. To assess the function of MMP-2 in meningeal re-vascularization following mTBI, we administered SB-3CT, a potent inhibitor of MMP-2 and MMP-9. Blockade of MMP markedly impeded meningeal re-vascularization at day 7 after injury (Fig. 7f) comparable to that observed following depletion of non-classical monocytes. We used in situ gelatinase zymography to investigate the local effects of SB-3CT at the mTBI lesion (Fig. 7g). Quantification of gelatinase activity (dye-quenched fluorescent gelatin cleaved by MMPs to produce fluorescent signal) confirmed that SB-3CT was inhibiting MMP activity at the injury site (Fig. 7h). The MMP inhibitor did not affect the total number of CD206⁺ macrophages in the mTBI lesion (Supplementary Fig. 7), which suggested that the failure to re-vascularize was not linked to altered accumulation of wound-healing macrophages in the lesion. These data suggested that CD206⁺ wound-healing macrophages promoted meningeal angiogenesis in part through the production of MMP-2.

Secondary injury influences inflammation and repair after mTBI.

Our data supported a model in which the myeloid response to the damaged meninges is temporally regulated. The first 24–48 h is characterized by the influx of inflammatory myelomonocytic cells, followed by the differentiation and expansion of tissue remodeling macrophages by day 4 after injury. Because re-injury to the brain is common within the human population, we finally evaluated whether a secondary injury encountered during these two distinct phases of inflammation affected the vascular repair process. A secondary injury experienced at 1 d after the initial injury exacerbated the lesion and significantly impeded meningeal re-vascularization, whereas re-injury at 4 d after the initial injury had no effect on vascular remodeling and lesion repair (Fig. 8a,b). This notable disparity was probably due to the inflammatory state of the lesion when the secondary injury occurred. The sensitivity of the lesion at 1 d after injury was evident by TPM. Re-injury at 1 d resulted in the rapid recruitment of intravascularly labeled neutrophils within minutes (Fig. 8c,d and Supplementary Movie 7). In contrast, neutrophils normally require ~2–3 h to fully infiltrate a primary mTBI lesion or a lesion re-injured at day 4. Neutrophils were abundant in the lesion within the first 30 min after re-injury at day 1, while they were barely detectable after primary mTBI or re-injury at day 4 (Fig. 8c). The rate at which neutrophils entered the lesion was also significantly higher following re-injury at day 1 (Fig. 8d), which indicated that lesion sensitivity influenced recruitment kinetics.

Examination of the lesion 24 h following re-injury at day 1 revealed that CCR2^{hi} inflammatory monocytes were recruited to the meninges in greater numbers than their recruitment in mice that experienced only a single injury (Fig. 8e,f). Depletion of inflammatory monocytes and neutrophils with anti-Gr-1 at 3 h prior to re-injury at day 1 modestly improved vascular repair, although this result did not reach statistical significance (Supplementary Fig. 8a,b). The structural environment of the lesion at day 4 was resistant to further damage, which was demonstrated by the complete preservation of lesion

macrophages at 3 h following re-injury (Fig. 8g,h). Not only were these macrophages resistant to mechanical injury but they also maintained MMP-2 expression at the lesion perimeter (Fig. 8i). In fact, MMP-2 expression was elevated in the lesion following a re-injury at day 4 (Fig. 8j). Collectively, these data indicated that mTBI lesions were temporally sensitive to secondary injuries, which resulted in drastically different immediate inflammatory responses and recovery trajectories.

Discussion

Our study has revealed several important observations that shed new light on the process of recovery after mTBI. First, the meninges, which are damaged in ~50% of patients after mTBI¹⁴, had a remarkable capacity to recruit peripheral immune cells and repair. Quantification of meningeal enhancement as a surrogate for vascular damage demonstrated that most patients who had experienced mTBI recovered within 1–3 weeks, although enhancement persisted in a subset of patients (17%) for several months. Using our animal model, we found that meningeal vascular repair and angiogenesis occurred during the week following a single mTBI and was temporally orchestrated by distinct myeloid cell subsets. The macrophages most supportive of angiogenesis were probably derivatives of circulating non-classical monocytes²⁷ that localized to the lesion perimeter, proliferated and provided tissue-remodeling enzymes, such as MMP-2. In contrast, inflammatory myelomonocytic cells arrived several days earlier and contributed instead to the removal of dead cells from the lesion core, which had little effect on angiogenesis. This bifurcated and temporally regulated myeloid cell response has important implications for the reparative process following mTBI. Deviation from this wound-healing program could explain the failure of some patients to recover promptly after mTBI. In addition, our data demonstrated that meningeal lesions were sensitive to reinjuries experienced during the acute inflammatory phase before the wound-healing program was established.

Meningeal vascular damage has been found in ~50% of patients who experienced minor head trauma¹⁴. Our current findings support that finding and indicate that patients also have differential rates of resolution. Failure to restore meningeal vascular integrity could explain the post-concussive syndrome that develops in ~15% of patients after mTBI²⁸. Prolonged CNS vascular leakage probably induces a chronic state of secondary cell death and inflammation⁵. To mitigate secondary neurodegeneration, it is necessary to understand and foster programs that mediate meningeal repair. The blood–brain barrier is often disrupted by trauma-induced changes in endothelial tight-junction proteins²⁹ and can remain open for months following TBI, depending on injury severity^{30,31}. Meningeal blood vessels, however, lack an ensheathing glial barrier and differ from parenchymal vessels that are wrapped by astrocytes. Thus, meningeal and parenchymal vessels should have different kinetics for leakage and recovery. We observed, in mice, that damaged meningeal blood vessels stopped leaking and initiated angiogenesis within 4 d of injury. Parenchymal angiogenesis has been reported in animal models of moderate to severe TBI, but the process is less efficient (or is possibly aborted) following severe injury^{15,32}. It is important to consider both injury location and injury severity in patients after TBI, as the anatomy of the meningeal compartment differs from that in the parenchymal compartment, which can influence symptoms, pathogenesis and the ability to recover.

Myeloid cells are important contributors to wound repair in all injured tissues³³. Following tissue injury, the early arrival of neutrophils is usually followed within 1–2 d by the arrival of peripheral monocytes-macrophages³⁴. During meningeal repair, macrophages were found within and surrounding the mTBI lesion. Microglia and macrophages can adopt different inflammatory phenotypes following TBI^{10,35}, yet their specific roles during repair were unclear. A key finding of our study was that the origin (classical monocytes versus non-classical monocytes) of injury-responding macrophages dictated the anatomical position and phenotype of these cells within the meningeal lesion. This finding prompts further consideration of the relationship between immune cells and the wound microenvironment.

Published TBI studies have demonstrated that infiltration of the CNS by CCR2^{hi} inflammatory monocytes is detrimental and results in larger lesions and impaired recovery^{36,37}; however, our data showed that the recruitment of classical and non-classical monocytes derived from the periphery to the meninges could be beneficial after mTBI. Under steady-state conditions, classical monocytes can give rise to non-classical monocytes in the blood, and each subset has a distinct differentiation potential^{38,39}. In response to a sterile injury, classical monocytes can also convert to non-classical monocytes in situ⁴⁰. Although we did not observe conversion in our mTBI model, this could explain the residual CD206⁺ macrophages we found in the perimeter of meningeal lesions following the depletion of non-classical monocytes. Nevertheless, our data clearly showed that one monocyte subset was unable to compensate for the other in our mTBI model. We observed that non-classical monocytes promoted meningeal angiogenesis but did not scavenge dead cells, whereas the opposite was true for classical monocytes. The finding of divergent functions for classical monocytes versus non-classical monocytes following tissue injury is supported by their response to myocardial damage⁴¹. Collectively, these data were consistent with the notion that non-classical monocytes are predetermined to differentiate into wound-healing macrophages⁴². It is possible that injury severity and location can also influence the immune response in the CNS. The controlled cortical-impact and fluid-percussion models of TBI generate substantially more parenchymal damage than does our mTBI model⁴³. As we observed no peripheral-immune-cell infiltration of the brain parenchyma after a single injury, it is possible that more substantial disruption of CNS barriers (e.g., destruction of the glial limitans) must occur before neutrophils and monocytes will infiltrate the parenchyma. Understanding how localization of peripheral immune cells to the meninges versus the parenchyma influences the neurological outcome following TBI will be instrumental in guiding the development of therapeutics for patients.

Wound-healing macrophages can promote angiogenesis through the production of soluble pro-angiogenic factors^{23–25}. For example, macrophages have been shown to produce VEGF and fibroblast growth factor in the brain after TBI^{44,45}. However, our data revealed that depletion of macrophages had no effect on angiogenic programming in the damaged meninges. Instead, myeloid cells seemed to have a more important role in meningeal tissue remodeling. Extravascular fibrin is involved in angiogenesis as a provisional matrix component¹⁹ and is also an inflammatory modulator²¹. We observed that depletion of monocytes significantly increased the amount of fibrin remaining in meningeal mTBI lesions, which was associated with impaired re-vascularization. Because the glial limitans is damaged following mTBI¹⁴, these excessive fibrin deposits could potentially enter the

parenchyma and exacerbate pathology and inflammation⁴⁶. Macrophages can also produce extracellular matrix–remodeling enzymes^{47,48}. We evaluated this function in situ and observed expression and enzymatic activity of MMP-2 in peri-lesional macrophages that was significantly reduced following depletion of macrophages. In addition, administration of an inhibitor of MMP-2 impeded meningeal revascularization after mTBI. The proximity of MMP-2 to repairing meningeal blood vessels is probably critical for angiogenesis, as MMP-2 is known to activate VEGF²⁶. It is also likely that other MMPs produced by macrophages⁴⁹ have a role in meningeal repair following mTBI.

Repetitive brain trauma in humans can lead to long-term neurodegeneration and serious neurological complications^{50,51}. Rodent models of repetitive TBI have focused on histopathological and behavioral changes associated with repetitive injuries^{52,53}. Notably, our data demonstrated that the state of the mTBI lesion influenced the degree of secondary pathology and inflammation following re-injury. Acute inflammatory mTBI lesions were highly sensitive to recruitment of additional myelomonocytic cells after a second injury, and this disrupted meningeal re-vascularization. In contrast, mTBI lesions that had entered the wound-healing phase at day 4 became refractory to secondary damage and inflammation. This disparity could be explained by changes at the cellular level. A published study has demonstrated that immune cells in *Drosophila* become desensitized to damage-associated molecular patterns and exhibit attenuated responses to sequential injuries⁵⁴, which might also happen to immune cells in the mTBI lesion. Another possibility is that the meninges become structurally more resistant to secondary damage over time. It will be important in future studies to identify the mechanisms underlying this differential susceptibility to repeat CNS injuries.

In conclusion, our findings have provided important insights into CNS repair following mTBI. Meningeal tissue can undergo efficient vascular repair with aid from myeloid cells derived from the periphery that exhibit bifurcated functions. Deviation from this beneficial immune response might explain why a fraction of patients still exhibit meningeal vascular damage months after mTBI. It will be crucial to identify fluid biomarkers in patients who have experienced mTBI⁵⁵ that define different stages of repair. This should result in more-informed decision-making about when to resume potentially injurious activities. It will also be important in future studies to identify factors that shift the balance toward wound-healing programs in patients who fail to resolve their lesions after mTBI.

Methods

Human subjects.

This study was reviewed and approved by the appropriate human patient protection authorities at the National Institutes of Health, Johns Hopkins Suburban Hospital, and MedStar Washington Hospital Center. All patients or surrogates provided informed consent before any study procedure. We conducted a retrospective analysis of patients with known and suspected non-penetrating acute TBI who enrolled in an IRB-approved Traumatic Head Injury Neuroimaging Classification Study (THINC) NCT01132937 and who completed study procedures between Oct 2010 and Feb 2017 (Supplementary Table 1). Patients were enrolled and received a research MRI within 48 h of head injury at Johns Hopkins Suburban

Hospital (Bethesda, MD) or MedStar Washington Hospital Center (Washington, DC). Cases were included that had suspected mild TBI with (i) a Glasgow coma score > 12 on presentation, (ii) a loss of consciousness < 30 min, (iii) post-traumatic amnesia < 24 h, (iv) a negative non-contrast head CAT scan (CT), and (v) at least one follow-up MRI with contrast at a nominal 5, 30 or 90 d from injury. Imaging was performed on 1.5 T and 3.0 T MRI machines from three commercial vendors. The MRI research protocol included a post-contrast T2-FLAIR (fluid attenuated inversion recovery) series of ~2.5 min duration acquired ~5 min after contrast administration. Traumatic meningeal enhancement was noted as a hyperintensity on post-contrast FLAIR (not seen pre-contrast) of the presumed interface between the dura and arachnoid membrane along the convexity or of dural reflections in the falx cerebri, tentorium cerebri or diaphragm sellae. The duration of traumatic meningeal injury was estimated as time first seen without enhancement minus time last seen with meningeal enhancement. Loss to follow-up was left censored as time last seen with enhancement.

Mice.

C57BL/6J (B6), B6.129P-CX3CR1^{tm1Litt}/J (*Cx3cr1*^{gfp/gfp}), and B6.129(Cg)-CCR2^{tm2.1Ifc}/J (*Ccr2*^{gfp/rfp}) mice were purchased from Jackson Laboratories. *Cx3cr1*^{gfp/+} *Ccr2*^{gfp/+} double reporter mice were generated by breeding *Cx3cr1*^{gfp/gfp} and *Ccr2*^{gfp/rfp} together. *Lysm*^{gfp/+} were provided by T. Graf⁵⁶ and maintained in closed breeding colony at the NIH. All mice in this study were handled in accordance with the guidelines set forth by the NIH Animal Care and Use Committee.

Meningeal compression injury.

All mTBI experiments were performed as described⁵⁷. 7- to 10-week-old mice (weighing 22–25 g) were anaesthetized ketamine (85 mg/kg), xylazine (13 mg/kg) and acepromazine (2 mg/kg) in PBS and were maintained at a core temperature of 37 °C. Hair was removed from the head using hair clippers and Nair. Lidocaine was applied to the scalp, followed by cleaning of the area with chlorohexidine and ethanol. Under aseptic conditions, an incision was made in the scalp to expose the skull and a metal bracket was secured on the skull bone over the barrel cortex (2.5 mm from bregma × 2.5 mm from sagittal suture). A cranial window (1 mm × 1 mm) was quickly thinned to a thickness of ~20–30 µm. Once thinned, the blunt end of a microsurgical blade was used to lightly compress the skull bone into a concavity without cracking the skull. After compression, the incision was closed with sterile wound clips and the mice were injected subcutaneously with 0.1 mg/kg Buprenex for pain management. The mice were kept warm until they fully recovered from anesthesia. For the re-injury studies described in Fig. 8, mice were anesthetized as described above, after which the original injury was exposed and compressed again with the blunt end of a microsurgical blade.

In vivo cell depletions and MMP inhibitor treatment.

All antibodies used for cell depletion were purchased from BioXcell. B6 mice were given intraperitoneal injection of 400 µg of anti-Gr-1 (RB6-8C5) 1 d prior to mTBI, and this was repeated daily until the denoted time points. For monocyte depletion, 200 µl of clodronate liposomes (<https://clodronateliposomes.com>) were injected intravenously 3 h prior to mTBI

and this was repeated daily for 4 d or, alternatively, the clodronate liposomes were given as a single injection administered 24–48 h prior to mTBI. The MMP-2 and MMP-9 inhibitor SB-3CT was reconstituted in 25% DMSO, 65% PEG-200 and 10% H₂O at a concentration of 12.5 mg/ml as previously described²³. B6 mice were given intraperitoneal injection of 25 mg/kg SB-3CT daily beginning 2 d after mTBI.

Meningeal whole mounts and immunohistochemistry.

Vasculature was labeled by intravenous injection of fluorescence-conjugated tomato lectin (Vector Labs) 5–10 min before euthanasia. Mice were perfused with either saline or 5% neutral buffered formalin (NBF). A small area of skull with meninges attached encompassing the mTBI lesion was removed with scissors and placed in staining buffer (PBS containing 2% FBS). All meninges were stained while still attached to the skull bone. FcR block and mouse IgG (Jackson ImmunoResearch) were added to staining buffer, and the meninges were incubated for 30 min at room temperature (RT; ~26 °C). For saline-perfused meninges, primary antibodies were added directly to staining buffer and incubated for 1 h at RT. After primary staining, meninges were washed three times in staining buffer. Secondary antibodies were added and incubated for 1 h at RT. Meninges were washed three times in staining buffer and placed in 5% NBF for 30–60 min at RT. For NBF-perfused meninges, primary antibodies were added directly to staining buffer and incubated 16–18 h at 4 °C. After primary staining, meninges were washed three times in staining buffer, then secondary antibodies were added and incubated at RT for 1 h. Meninges were finally washed three times in staining buffer. Meninges were removed from the skull by careful peeling of the tissue from the bone using fine-tipped forceps. The free-floating meninges were placed in one drop of FluorSave Reagent (Calbiochem) on a slide and a coverslip was added. Meninges were stained with the following primary antibodies: anti-CD11b PE (M1/70), anti-CD206 Brilliant Violet 421 or AlexaFluor 488/647 (C068C2), anti-Lyve-1 eFluor 660 (clone ALY7; eBiosciences), polyclonal anti-laminin (catalog# ab11575; Abcam), polyclonal anti-fibrinogen (catalog# ab3426; Abcam) and polyclonal anti-MMP2 (catalog# ab3715; Abcam). All directly conjugated antibodies except anti-Lyve-1 were purchased from BioLegend. Secondary antibodies included AlexaFluor488-conjugated polyclonal anti-GFP (catalog# 600-141-215; Rockland), rhodamine red-X or Alexa Fluor 647-conjugated donkey anti-rabbit (Jackson ImmunoResearch). Tile scan images of the meninges were acquired using an Olympus FV1200 laser-scanning confocal microscope equipped with four detectors, six laser lines (405, 458, 488, 515, 559 and 635 nm) and five objectives (4 × /0.16 NA, 10 × /0.4 NA, 20 × /0.75 NA and 40 × /0.95 NA, and chromatic aberration-corrected 60 × /1.4 NA). Tile scans were also acquired using a Leica SP5 laser-scanning confocal microscope equipped with three detectors, an 8000 Hz resonant scanner, three laser lines (488, 594 and 633 nm) and two objectives (5 × /0.15 NA and 10 × /0.40 NA). All confocal images were imported to Imaris version 9.0 software (Bitplane) for further analysis.

Evans blue leakage assay.

200 µl of a 1 mg/ml solution of Evans blue (Sigma) was injected intravenously and allowed to circulate for 1 h before euthanasia. Mice were perfused with saline to wash out intravascular Evans blue. Meningeal whole mounts were prepared as described above, tile scans were captured by confocal microscopy and quantification of Evans blue fluorescence

(excited with the 633 nm laser line) was performed using Imaris as described below ('Confocal image analysis').

EdU proliferation assay.

1 mg of EdU (dissolved in water) was injected intraperitoneally into mice 1 h before euthanasia. Mice were perfused with 5% NBF and the meninges were removed as described above. EdU was detected using a Click-iT Plus EdU Alexa Fluor 647 kit (ThermoFisher) and was imaged with an Olympus FV1200 laser scanning confocal microscope. Quantification of EdU⁺ cells was performed by histo-cytometry as described below.

Intravital two-photon microscopy.

mTBI and control mice were imaged using a Leica SP8 two-photon microscope equipped with an 8,000-Hz resonant scanner, a 25 × color-corrected water-dipping objective (1.0 NA), a quad HyD external detector array, a Mai Tai HP DeepSee Laser (Spectra-Physics) tuned to 905 nm (for GFP, Alexa Fluor 488, Evans blue, propidium iodide) and an Insight DS laser (Spectra-Physics) tuned to 1,050 nm (for red fluorescent protein) or 1,200 nm (for Alexa Fluor 647). Three-dimensional time-lapse movies were captured in *z*-stacks of 15–30 planes (3-μm step size) at 1- to 2-min intervals. Signal contrast was enhanced by averaging 10–12 video frames per plane in resonance scanning mode. For blood vessel visualization, 1 mg/ml Evans blue (Sigma) or tomato lectin (Vector Labs) was injected intravenously prior to mTBI. For dead-cell visualization, propidium iodide (ThermoFisher) was applied transcranially for 30–60 min prior to imaging. Intravascular labeling of neutrophils was achieved by injection of 10 μg anti-Ly6G Alexa Fluor 647 or FITC (clone 1A8; BioLegend). Intravascular labeling of platelets was achieved by injection of 10 μg anti-CD41-PE (clone MWRReg30; BioLegend). Antibodies were injected intravenously 30 min before imaging.

Blood-flow-velocity quantification.

We quantified blood flow velocities using an adaptation of a previous method⁵⁸. At day 7 after injury, B6 mice were given intravenous injection of 20 μl of 0.5-μm polychromatic red fluorescent microspheres (Polysciences) and 10 μl of Qtracker 655 (ThermoFisher) diluted in 250 μl PBS ~30 min before imaging. A custom double-wide metal bracket was glued to the skull to allow access to the mTBI lesion as well as to the opposite uninjured hemisphere. Using only a microsurgical blade, the skull bone of the uninjured hemisphere was thinned down to ~50 μm to visualize uninjured meningeal vasculature. New vasculature on the mTBI hemisphere was evident under the two-photon microscope due to its unique morphology (Fig. 2a). Intravital imaging of the midline of individual blood vessels was performed in *xt* line-scan mode, which allows rapid imaging of a single line in the *x* plane over time (*t*) (Supplementary Fig. 2). Imaging was performed on randomly selected capillaries by two-photon microscopy using a resonance scanner tuned to 16,000 Hz. This resulted in images like the one in Supplementary Fig. 2c every 512 ms. Blood-flow velocity was calculated within the Leica LASX software. Individual fluorescent microspheres appeared as bright diagonal lines in *xt* images as the bead traveled a certain distance over 512 ms (Supplementary Fig. 2c,d). The actual velocity was calculated as the slope of this line ($\frac{\Delta x}{\Delta t}$) and converted to mm/s (Supplementary Fig. 2d). Four individual vessels were sample

from each mouse, and four independent microsphere-velocity measurements were calculated for each vessel and then averaged.

Dead cell–clearance assay.

Immediately following mTBI compression in B6 mice, NucGreen Dead 488 vital dye (ThermoFisher) was applied transcranially through the surgically thinned skull bone. The dye was incubated on the skull for 3 h after mTBI, labeling all cells that died in the meningeal area immediately below the thinned window within the first 3 h of injury. The dye was then removed, the incision was sealed and the mice were allowed to recover. At 48 h after injury, a 100- μ m z-stack was captured by two-photon microscopy to visualize and quantify dead cells remaining in the meninges. To quantify dead-cell clearance, a 100- μ m z-stack of the same region was captured at 3 h and 48 h after injury. Uncleared dead cells were clearly visible in the same region in both the 3 h and 48 h z-stack images, whereas cleared regions were evident by the lack of dead cells at 48 h. The number of dead cells at these two time points were quantified using Imaris as described below ('Two photon image analysis').

Fibrinogen clearance assay.

B6 mice were injected intravenously with 250 μ g of Alexa Fluor 488–conjugated human fibrinogen (ThermoFisher) ~30 min before mTBI. As shown in Supplementary Movie 2, fibrinogen leaked from injured vessels immediately following mTBI. Fluorescent fibrinogen that leaked into the extravascular spaces would remain there unless cleared or degraded. Remaining extravascular fibrinogen at 7 d after injury was quantified by collection of a 100- μ m z-stack through the thinned skull bone by two-photon microscopy. This scan was performed to visualize the un-cleared extravascular fibrinogen deposits. Fibrinogen fluorescence was quantified using Imaris as described as described below ('Two photon image analysis').

In situ gelatin zymography.

Vehicle or SB-3CT (25 mg/kg) was injected intraperitoneally into B6 mice once daily beginning 2 d after mTBI. In situ MMP activity was assayed using the EnzChek Gelatinase Assay Kit (ThermoFisher) 4 d after injury. Mice were euthanized ~15 min after final injection of vehicle or SB-3CT. Fresh meninges (attached to skull) were incubated in 1 $\times$ reaction buffer with protease inhibitors (Roche) and 10 μ g/ml DQ-gelatin for 1 h at 37 °C. Meninges were then washed in deionized water three times before incubation in 5% NBF for 1 h at RT. Meninges were then removed, whole-mounted on slides and imaged by confocal microscopy as described above. MMP activity was quantified as the sum of gelatin fluorescence within a 2 mm $\times$ 2 mm area centered on the mTBI lesion as described below ('Confocal image analysis').

Histocytometry.

Confocal images were imported into Imaris version 9.0 software (Bitplane). Using the channel arithmetic MatLab function, all cell-surface stains for analysis were combined into a new single channel (pan channel). Using the surfaces tool, individual cell surfaces were generated based on the new pan channel. Statistics for mean fluorescent intensities of each

individual stain were exported for every surface generated. For anatomical regional distinctions, a surface was manually drawn either within the lesion core (as defined by absence of lectin-positive vessels) or around the cluster of cells surrounding the lesion. Using the surface mask function, a new channel was generated that contained only signal from the pan channel within each designated surface area. The intensity values of the new region-specific channels served as arbitrary values in determining if a cell surface object was in the lesion core or peri-lesion areas. After surface statistics were exported, an Excel table was generated with each row corresponding to a cell surface and each column corresponding to the fluorescent intensity of individual channels. The Excel file was imported into FlowJo software (TreeStar). Data were analyzed using standard flow-cytometry gating strategies.

Confocal image analysis.

All confocal images were imported into Imaris version 9.0 software (Bitplane). mTBI lesions were quantified by manual drawing of areas using the surfaces tool that did not contain functioning lectin-positive vessels but did contain laminin-positive structures. The area in μm^2 was extracted from the statistics of the surface. Cell number and fluorescence quantification were obtained by generating surfaces for all cells within a $2\text{ mm} \times 2\text{ mm}$ region of interest centered on the epicenter of the lesion. For further phenotyping of the cells, the statistics for these surfaces were imported into FlowJo (TreeStar) and analyzed by histocytometry as described above. Fibrinogen staining, MMP-2 staining, Evans blue fluorescence and gelatin fluorescence were quantified by measurement of the sum fluorescence intensity within a $2\text{ mm} \times 2\text{ mm}$ area centered on the lesion. Fibrinogen, MMP-2, Evans blue fluorescence and gelatin fluorescence within the injured meninges were compared with values obtained in areas of equal size from uninjured meninges.

Two-photon image analysis.

Three-dimensional z-stacks were imported for analysis into Imaris software. To quantify dead cells, a surface object was created based on the second harmonic signal produced by the skull bone. A masked channel was created for dead cells that excluded any fluorescence within the skull. Dead cells were quantified by generating surfaces for each NucGreen⁺ nuclei below the surface of the skull, greater than $30\text{ }\mu\text{m}^3$ in volume. For extravascular fibrinogen quantification, a surface object for second harmonic signal was generated and a masked channel was made excluding fibrinogen signal within the skull. The total fluorescence sum of fibrinogen below the skull was then quantified. For neutrophil quantification following re-injury, anti-Ly6G labeled cells were counted manually at single time frames corresponding to 0 min (first frame of imaging) and 30 min after injury.

Quantitative PCR.

Mice were euthanized and perfused with saline. Superficial cortical tissue and meninges were removed from a small area surrounding the mTBI lesion and placed in RPMI. Tissue was mechanically homogenized using sterile zirconia/silica beads (Biospec). Homogenized tissue was spun down at $12,000\text{ g}$ at $4\text{ }^\circ\text{C}$ and resuspended in Trizol (ThermoFisher). Chloroform was added to the mixture and spun down ($12,000\text{ g}$ at $4\text{ }^\circ\text{C}$) and the supernatant was isolated. RNA was extracted using a PureLink RNA Mini kit (ThermoFisher). RNA concentrations were quantified using a Nanodrop, and gDNA was digested using a DNase I

kit (ThermoFisher). cDNA was generated using an iScript cDNA Synthesis kit (Bio-Rad). 96-well custom or pre-made PrimePCR plates (Bio-Rad) were used for qPCR experiments. 10–20 ng of cDNA and SYBR green reagent (Bio-Rad) were added to lyophilized primers in each well and the plate was read on a Bio-Rad CFX96 Real-Time with C1000 Thermal Cycler system. On each PrimePCR plate there were control wells for RNA quality and gDNA contamination. qPCR data was analyzed using Bio-Rad Gene Study software, with expression values relative to those of the control genes *Gapdh* and *Tbp*. The original data and genes analyzed on the PrimePCR plates are provided in Supplementary Tables 2–4.

Flow cytometry.

Blood was collected from B6 mice in heparinized capillary tubes and placed into FACS buffer (PBS plus 2% FBS). Red blood cells were lysed by addition of ACK lysis buffer and were incubated for 5–10 min at RT. Cells were pelleted and stained in FACS buffer with CD11b PE/Cy7 (M1/70), Ly6C Alexa Fluor 488 or Brilliant Violet 570 (HK1.4), Ly6G Pacific Blue (1A8), CD115 APC (AFS98). All directly conjugated antibodies purchased from BioLegend. Samples were acquired using an LSR II digital flow cytometer (BD), and data were analyzed using FlowJo software version 10.0.7 (Tree Star).

Statistical analysis.

Statistical significance ($P < 0.05$) was determined using Student's *t*-test (two groups) or one-way analysis of variance (ANOVA) (more than two groups). ANOVA on ranks was used for datasets with a non-Gaussian distribution and more than two groups. A two-tailed Fisher exact test was used to analyze the data in Fig. 1c. All statistical analyses were performed using GraphPad Prism 7.0 or SigmaPlot 11.0.

Reporting Summary.

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability statement.

The data that support the findings of this study are available from the corresponding author upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1 |. Kinetics of meningeal vascular damage and repair following mTBI in humans.
a, Post-contrast FLAIR MRI of a patient with traumatic meningeal enhancement (white arrowheads), imaged over the first 3 months after mTBI (time below image); asterisks indicate a superficial scalp contusion. **b**, Fraction of patients with persistent meningeal enhancement after mTBI (among a cohort of 209 patients), plotted against time between injury and imaging; cross-hatches indicate patient lost to follow-up (time last seen with enhancement). **c**, Prevalence of enhancement at various time points (horizontal axis) in the

99 patients seen for at least three study visits; numbers above bars indicate precise values.

* $P = 0.009$ and ** $P < 0.001$ (two-tailed Fisher exact test).

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Fig. 2 |. Meningeal vascular leakage and re-vascularization in a mouse model of mTBI.

a, Microscopy of meningeal whole mounts, showing meningeal vascular damage and repair in mice after mTBI: green, functional vessels (visualized by intravenous injection of tomato lectin); blue, leakage of Evans blue into core of lesion after vessel destruction; red, laminin staining; white dotted line (right) delimits the area of complete vascular repair. Scale bar, 200 μm . **b,c**, Quantification of the leakage of Evans blue (**b**) and the lesion area (**c**) in uninjured (naive) mice and mice at 1 d (24 h), 4 d and 7 d after mTBI (horizontal axis). **d**, Quantification of blood-flow velocity by two-photon intravital imaging of meningeal vessels in either the uninjured hemisphere (left) or the injured hemisphere of mice at 7 d after mTBI (right), assessed as the average velocity for a blood vessel calculated by tracking of fluorescent microspheres in 512-ms *xt* line scans (additional information on blood-flow quantification, Supplementary Fig. 2). Each symbol represents an individual mouse (**b,c**) or blood vessel (**d**). NS, not significant ($P>0.05$); * P 0.05, ** P 0.01 and *** P 0.001 (one-

way analysis of variance (ANOVA) with Tukey's multiple-comparisons test (**b–c**) or two-tailed Student's *t*-test (**d**). Data are representative of two independent experiments with three to four mice per group (**a–c**; mean $\pm$ s.e.m. in **b,c**) or two independent experiments with four mice per group (**d**; mean $\pm$ s.e.m.).

Fig. 3 | Dynamics and phenotype of peripheral myeloid cells in the meninges following mTBI.
a, Microscopy of resident CX3CR1^{hi} meningeal macrophages (green) and peripheral CCR2⁺ monocytes (red) in *Cx3cr1^{gfp/+} Ccr2^{rfp/+}* reporter mice without injury (Naive) and at 1 d, 4 d and 7 d (above images) after mTBI; white dotted lines delimit the mTBI lesion. **b,c**, Quantification of CX3CR1^{hi}CCR2^{hi} monocytes (**b**) and CX3CR1^{hi}CCR2^{lo-neg} monocytes-macrophages (**c**) derived from the periphery, assessed in mice as in **a** (horizontal axis). **d**, Microscopy of a meningeal whole mount (left) and enlargements showing macrophages in the mTBI lesion (right) at 4 d after injury in *Cx3cr1^{gfp/+} Ccr2^{rfp/+}* mice, with myeloid cells

expressing CX3CR1 (green) and/or CCR2 (red) stained for CD206 (blue). **e**, Quantification of CCR2 expression (low to negative (CCR2^{lo-neg}) or high (CCR2^{hi})) on CD206⁺ macrophages at 4 d after injury as in **d**. Scale bars (**a,d**), 100 μ m (**a**), 200 μ m (**d** (left)) or 50 μ m (**d** (right)). Each symbol (**b,c,e**) represents an individual mouse. * P 0.05, ** P 0.01, *** P 0.001 and **** P 0.0001 (one-way ANOVA with Tukey's multiple-comparisons test (**b-c**) or two-tailed Student's t -test (**e**)). Data are representative of two independent experiments with three mice per group (**a-c**; mean $\pm$ s.e.m. in **b,c**) or three independent experiments with four mice per group (**d,e**; mean $\pm$ s.e.m. in **e**).

Fig. 4 |. Anatomical position of myeloid cells and cellular proliferation at mTBI lesion 4 d after injury.

a, Microscopy of myeloid populations within and surrounding the mTBI lesion at 4 d after injury in mice, with meninges stained for CD206 (green), CD11b (red) and tomato lectin (via intravenous injection; white). **b**, Quantification of myeloid cells (gated on CD11b⁺ cells by histocytometry), wound-healing (CD206⁺) macrophages and inflammatory (CD206⁻) macrophages (horizontal axis) in the uninjured hemisphere or the injured hemisphere (key) of mice as in **a** at 4 d after mTBI. **c**, Histocytometry of the meninges (as in **a**) showing cells within the lesion core or the peri-lesion area (key, bottom left), with those areas first drawn as surface objects (with Imaris software) and cells within these surfaces then assigned

coordinates to designate their position. **d**, Frequency of wound-healing (CD11b⁺CD206⁺) or inflammatory (CD11b⁺CD206⁻) myeloid cells (horizontal axis) in the lesion core or perilesion area (key) of mice as in **a**. **e**, Microscopy of proliferating CX3CR1^{hi} macrophages (green) in the injured or uninjured meninges (above images) at 4d after mTBI in mice given injection of EdU (white) 1 h before euthanasia. **f,g**, Quantification of total EdU⁺ cells (**f**) and frequency of EdU⁺CX3CR1^{hi} macrophages (**g**) in mice at 4 d after mTBI, presented relative to that of the uninjured hemisphere. (**h**) Quantification of the anatomical position (horizontal axis) of proliferating EdU⁺ cells in mice at 4 d after mTBI. Scale bars (**a,e**), 200 μ m. Each symbol (**b,d,f-h**) represents an individual mouse; small horizontal lines (**b,d**) indicate the mean ($\pm$ s.e.m.). * P 0.05, ** P 0.01, *** P 0.001 and **** P 0.0001 (two-tailed Student's t -test). Data are representative of two independent experiments with four mice per group (mean $\pm$ s.e.m. in **f-h**).

Fig. 5 | Distinct myeloid cell subsets promote vascular regeneration or clearance of dead cells following mTBI.

a, Microscopy of meningeal whole mounts of mice treated with vehicle (control (Ctrl)) or clodronate liposomes (Clod) (above images) before mTBI, assessed 7 d after mTBI, with meninges stained for laminin (red) and CD206 (blue) and functional blood vessels visualized by intravenous injection of fluorescent tomato lectin (green). **b**, Quantification of the lesion area in mice given injection of vehicle or clodronate liposomes (horizontal axis) 3 h before mTBI and then daily for the first 4 d after mTBI, assessed at 7 d after mTBI. **c**,

Quantification of the lesion area in mice treated with vehicle, with anti-Gr-1 daily starting 1 d before mTBI (Gr-1 Ab) or with a single injection of clodronate liposomes 1 d before mTBI (Single clod), assessed at 7 d after injury; results were normalized to the mean for the control group. **d**, Quantification of CD206⁺ macrophages around the meningeal lesion in mice as in **c** (left and middle) or **b** (right) at 7 d after injury; results normalized as in **c**. **e,f**, Quantification of wound-healing (CD11b⁺CD206⁺) or inflammatory (CD11b⁺CD206⁻) myeloid cells (horizontal axis; gated by histocytometry as CD11b⁺) in the lesion core (**e**) or peri-lesion area (**f**) of mice as in **c** (key) at 4 d after injury. **g**, Maximal projections of three-dimensional two-photon z-stacks, showing uncleared dead cells in the meninges of mice as in **c** (above images) at 48 h after injury, with dead cells labeled by transcranial application of a vital dye at 3h after mTBI, and z-stacks captured through a surgically thinned skull at 48 h after injury by two-photon microscopy; results normalized as in **c**. **h**, Quantification of dead cells remaining in the meninges of mice as in **c** (horizontal axis) at 48 h after injury; results normalized as in **c**. Scale bars (**a,g**), 150 μ m (**a**) or 50 μ m (**g**). Each symbol (**b–f,h**) represents an individual mouse. * P 0.05, ** P 0.01, *** P 0.001 and **** P 0.0001 (two-tailed Student's t -test (**b,c,h**) or one-way (**d**) or two-way (**e,f**) ANOVA with Tukey's multiple-comparisons test). Data are representative of three independent experiments with three to four mice per group (**a,b**), are pooled from two experiments (anti-Gr-1) or three experiments (clodronate) with three to six mice per group (**c**), are pooled from the experiments in **b,c** with three to six mice per group (**d**), are representative of two independent experiments with four mice per group (**e–f**) or are representative of three experiments (anti-Gr-1) or two experiments (clodronate) with four to six mice per group (**g,h**) (mean $\pm$ s.e.m. in **b–f,h**).

Fig. 6 |. Macrophages clear extravascular fibrinogen during wound repair but do not participate in the angiogenesis program.

a, Microscopy of meningeal whole mounts of injured or uninjured meninges (above images) of mice at 4 d after mTBI, showing the distribution of CD206⁺ macrophages (green), fibrin(ogen) (red) and functional blood vessels (visualized by intravenous injection of fluorescent tomato lectin; white). Scale bar, 100 μ m. **b**, Quantification of the fibrin(ogen) signal as in **a** in the injured and uninjured meninges (horizontal axis) at 4 d after mTBI. **c**, Quantification of uncleared fibrin(ogen) in the meninges in mice given injection of vehicle or clodronate liposomes 3 h before mTBI and daily for the first 4 d after mTBI, plus intravenous injection of fluorescence-conjugated fibrin(ogen) 30 min before mTBI, followed by imaging of fibrin(ogen) through a thinned skull window by two-photon microscopy at 7 d after injury. **d**, qPCR analysis of genes encoding angiogenesis-related molecules (horizontal axis) in naive mice and mice 4 d after mTBI (key); results were normalized to those of the control genes *Gapdh* and *Tbp* and are presented relative to the mean of the naive group. **e**, qPCR analysis of genes encoding angiogenesis-related molecules (horizontal axis) in mice

given injection of vehicle or clodronate as in **c**; results normalized as in **d** and presented relative to the mean of the control group. Each symbol (**b–e**) represents an individual mouse. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (two-tailed Student's t -test). Data are representative of three independent experiments with three mice per group (**a,b**), two independent experiments with four mice per group (**c,d**) or three independent experiments with four to six mice per group (**e**) (mean $\pm$ s.e.m. in **b–e**).

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Fig. 7 |. Macrophages mediate angiogenesis via local MMP-2 production by wound healing macrophages.

a, Microscopy of meningeal whole mounts of the injured and uninjured meninges (above images) of *Cx3cr1*^{tgfp/+} mice (green) 4 d after mTBI, showing the expression of MMP-2 (white). **b**, Quantification of the MMP-2 signal as in **a** from the injured and uninjured meninges (horizontal axis) in mice at 4 d after injury. **c**, Quantification of the MMP-2 signal within the lesion core and peri-lesion area (horizontal axis) in mice given injection of vehicle or clodronate liposomes 3 h before mTBI and daily for the first 4 d after mTBI (key),

assessed at 4 d after injury. **d**, Microscopy of a meningeal whole mount (left) and magnified image of macrophages and MMP-2 expression (right) in mice at 4 d after mTBI, with meninges stained for CD11b (red), CD206 (green) and MMP-2 (white). **e**, Quantification of CD206 expression (horizontal axis) on MMP-2 expressing myeloid cells in mice at 4 d after mTBI. **f**, Quantification of the lesion area in mice subjected to mTBI and treated with vehicle or given daily intraperitoneal injection of the MMP-2 inhibitor SB-3CT beginning 2 d after injury (horizontal axis), assessed at 7 d after mTBI. **g**, In situ gelatin zymography of meningeal whole mounts of mice as in **f** (above images) at 4 d after injury, with blood vessels stained with tomato lectin (green) and MMP activity visualized by cleaved gelatin (pink). **h**, Quantification of MMP activity (cleaved gelatin signal) in mice as in **g** (horizontal axis). Scale bars (**a,d,g**), 200 μ m (**a, d** (left), **g**) or 50 μ m (**d** (right)). Each symbol (**b,c,e,f,h**) represents an individual mouse; small horizontal lines (**c**) indicate the mean ($\pm$ s.e.m.). * P 0.05 and ** P 0.01 (two-tailed Student's t -test). Data are representative of three independent experiments with three mice per group (**a,b**), two independent experiments with six mice per group (**c**), two independent experiments with four mice per group (**d,e**), three independent experiments with six mice per group (**f**) or three independent experiments with four to six mice per group (**g,h**) (mean $\pm$ s.e.m. in **b,e,f,h**).

Fig. 8 | Re-injury during the inflammatory phase enhances inflammation and impedes angiogenesis, but injury during the wound healing phase does not.

a, Microscopy of meningeal whole mounts of mice given a single mTBI or re-injured at day 1 or day 4 after the initial mTBI (above images), assessed at 7 d after the initial injury, showing meninges stained with laminin (red) and functional blood vessels visualized by intravenous injection of fluorescent tomato lectin (green). **b**, Quantification of the lesion area in mice as in **a** (horizontal axis) at 7 d after the initial mTBI, normalized to the mean of the control group. **c**, Quantification of neutrophils in mice as in **a** (key) assessed at 0 min and 30 min after injury (mice were immediately brought to the microscope after mTBI or re-injury and imaging was initiated within a few minutes); lines connect results for the same mouse. **d**, Quantification of neutrophil influx rate (cells per minute) in mice as in **a** (horizontal axis). **e**, Microscopy of meningeal whole mounts of *Cx3cr1^{gfp/+} Ccr2^{flp/+}* mice

given a single mTBI or re-injured at day 1 after the initial mTBI (above images), assessed at 2 d after the initial injury, showing blood vessels stained with tomato lectin (via intravenous injection; white) and myeloid cells expressing CX3CR1 (green) and CCR2^{hi} (red). **f**, Quantification of inflammatory CCR2^{hi} monocytes in the mTBI lesion of mice as in **a**, at 2 d after the initial injury. **g**, Microscopy of meningeal whole mounts of mice given a single mTBI or re-injured at day 4 after the initial mTBI (left margin), assessed at 3h after-re-injury, with meninges stained for CD206 (green) and MMP-2 (white) and with tomato lectin (by intravenous injection; red). **h,i**, Quantification of CD206⁺ macrophages (h) and frequency of CD206⁺ macrophages expressing MMP-2 (i) in mice as in **g** (horizontal axis), assessed 3 h after re-injury. **j**, Quantification of the MMP-2 signal as in **g** in mice as in **g** (horizontal axis) at 3h after re-injury. Scale bars (**a,e,g**), 150 μ m (**a**) or 200 μ m (**e,g**). Each symbol (**b,d,f,h-j**) represents an individual mouse; small horizontal lines (**d**) indicate the mean ($\pm$ s.e.m.). * P 0.05, ** P 0.01, *** P 0.001 and **** P 0.0001 (two-tailed Student's t -test). Data are pooled from three independent experiments with three to six mice per group (**a,b**; mean $\pm$ s.e.m. in **b**) or two independent experiments with five to seven mice per group (**c,d**) or are representative of two independent experiments with four mice per group (**e-j**; mean $\pm$ s.e.m. in **f,h-j**).