Cerebrospinal fluid can exit into the skull bone marrow and instruct cranial hematopoiesis in mice with bacterial meningitis

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Interactions between the immune and central nervous systems strongly influence brain health. Although the blood–brain barrier restricts this crosstalk, we now know that meningeal gateways through brain border tissues facilitate intersystem communication. Cerebrospinal fluid (CSF), which interfaces with the glymphatic system and thereby drains the brain’s interstitial and perivascular spaces, facilitates outward signaling beyond the blood–brain barrier. In the present study, we report that CSF can exit into the skull bone marrow. Fluorescent tracers injected into the cisterna magna of mice migrate along perivascular spaces of dural blood vessels and then travel through hundreds of sub-millimeter skull channels into the calvarial marrow. During meningitis, bacteria hijack this route to invade the skull’s hematopoietic niches and initiate cranial hematopoiesis ahead of remote tibial sites. As skull channels also directly provide leukocytes to meninges, the privileged sampling of brain-derived danger signals in CSF by regional marrow may have broad implications for inflammatory neurological disorders.
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100 mm², we estimate that two-photon intravital microscopy (IVM) and an ex vivo imaging pipeline to visualize CSF distribution after fluorescent tracer injection revealed that 67% of them showed perivascular signal after intracisternal tracer injection (Extended Data Fig. 1). To directly test this hypothesis, we labeled cranial vasculature with intravenously injected, fluorescently conjugated CD31/Scale antibodies and CSF with intracisternally (IC) injected, fluorescently labeled ovalbumin, followed by intravitral imaging of the skull marrow cavity. We labeled cranial vasculature with intravenously injected, fluorescently conjugated CD31/Scale antibodies and CSF with intracisternally (IC) injected, fluorescently labeled ovalbumin, followed by intravitral imaging of the skull marrow cavity. In the skull marrow, we detected perivascular spaces and cells labeled with the IC injected CSF tracer (Fig. 2b).

As we anticipated, high-magnification transmission electron microscopy (TEM) analysis of skull channels revealed a perivascular space that may accommodate CSF transport beginning at the dural channel opening (Fig. 2c). To directly test this hypothesis, we labeled cranial vasculature with intravenously Texas Red Dextran and the CSF with an intracisternal FITC–Dextran injection 1 h before ex vivo microscopy of the inner skull cortex. Channel cross-sections were clearly demarcated by dextran-labeled vessels surrounded by bone visualized with second harmonic generation (Fig. 2d). The z-stacks that began on the dural surface and moved deep into the marrow cavity visualized IC injected dextran in numerous skull channels (Fig. 2d and Supplementary Video 2). This signal surrounded the blood vessel and was present from channels’ dural openings all the way into the marrow cavity. Counting CSF-tracer–containing channels revealed that 67% of them showed perivascular signal after intracisternal dextran injection (Fig. 2c, f). Frontal, parietal and occipital bone, most skull channels exhibited perivascular signal after intracisternal tracer injection (Extended Data Fig. 1). It remains to be determined whether all skull channels can accommodate CSF flow, and whether there are functional or anatomical reasons for the observed heterogeneity, for example, the direction of blood flow through the channel’s vessels or absence of a perivascular space.

Bone marrow imaging after intracisternal injection revealed a similar perivascular appearance for tracers with molecular masses from 66 kDa to 2,000 kDa along a subset of skull marrow vessels (Fig. 2g). Labeled CSF was detected in the skull marrow as early as 15–30 min after injection but was largely absent from the tibia’s marrow vasculature (Fig. 2b and Extended Data Fig. 2); this indicates that CSF was excluded from systemic circulation at early timepoints. Together, these data demonstrate that CSF can exit the subarachnoid space via perivascular flux along a subset of dural vessels that connect into the bone marrow cavity through an extensive skull channel network. This finding implicates the skull marrow as a CSF-sensing hematopoietic compartment. We next sought to examine these observations’ relevance in a mouse model of acute CNS inflammation, bacterial meningitis.

**Results**

**Perivascular CSF transits through skull channels into marrow cavities.** To understand the spatial organization of the skull channels, we first performed high-resolution, ex vivo X-ray computed tomography (CT) of the skull (Fig. 1a) and characterized regional channel networks overlying frontal, parietal and occipital brain lobes (Fig. 1b). Channels traversed the inner compact bone into the marrow-containing cavities. We observed the highest density of skull channels in the frontal and occipital regions (Fig. 1c). Given a CT-derived mean channel density >10 per mm² and an inner skull surface area >100 mm², we estimate that >1,000 channels reach into the cranial vault of an adult mouse. Frontal and parietal skull channels formed the shortest connections to the dura, ranging from 83 μm to 90 μm, whereas occipital skull channels were approximately 25% longer (Fig. 1d). Frontal and parietal skull channels were 20% narrower than their occipital counterparts (Fig. 1c), pointing to regional channel heterogeneity.

We hypothesized that these numerous links between the skull marrow and the dura enable not only cell traffic toward the meninges but also bidirectional crosstalk. We therefore implemented a two-photon intravitral microscopy (IVM) and an ex vivo imaging pipeline to visualize CSF distribution after fluorescent tracer injection into the cisterna magna. Intracisternal injection of FITC-labeled ovalbumin was combined with intravenous labeling of the blood pool using Texas Red-labeled Dextran. We observed a striking perivascular appearance of the intracisternal tracer along a subset of dural vessels (Fig. 2a and Supplementary Video 1), indicating that CSF travels perivascularly from the subarachnoid space into the dura. This led us to wonder whether we could track this CSF outflow into the skull marrow cavity. We labeled cranial vasculature with intravenously injected, fluorescently conjugated CD31/Scale antibodies and CSF with intracisternally (IC) injected, fluorescently labeled ovalbumin, followed by intravitral imaging of the skull marrow cavity. In the skull marrow, we detected perivascular spaces and cells labeled with the IC injected CSF tracer (Fig. 2b). This critical observation in the dura and marrow suggested that perivascular CSF flow into the marrow may occur through skull channels.

**S. pneumoniae expand near dural skull channel openings.** We adapted a model of pneumococcal meningitis to test the functional significance of skull channel connections in neuroinflammation. *S. pneumoniae* is the clinically dominant cause of bacterial meningitis. To establish a disease timeline in mice, we injected 5×10⁸ bioluminescent *S. pneumoniae* Xen10 bacteria into the cisterna magna and analyzed bacterial propagation over time, alongside control mice that received an equal volume of artificial CSF (aCSF; Fig. 3a). Whole-body bioluminescence (BLI) imaging revealed a time-dependent signal increase reporting bacterial growth (Fig. 3b). The BLI signal was observed predominantly in the skull 36 h after injection and by 48 h had spread to the spine. Bacterial burden increased exponentially by 36 h, with a peak at 48 h after injection (Fig. 3c). To assess meningeal inflammation, we measured the canonical inflammatory cytokines IL1β, IL6 and TNFα in...
We injected 5 x 10^4 GFP+ S. pneumoniae, or an equal volume of aCSF in controls, and sacrificed mice 48 h later (Fig. 3f). Confocal microscopy of cleared tissue revealed abundant GFP+ bacterial growth in the subarachnoid space of mice with S. pneumoniae meningitis, but not controls (Fig. 3e-g and Supplementary Video 3). Three-dimensional (3D) reconstructions document the proximity of GFP+ S. pneumoniae to skull channels (Fig. 3h and Supplementary Video 4). In addition to subarachnoidal colonies directly adjacent to dural channel openings, bacteria were also present in the skull marrow’s extravascular space (Fig. 3h). Taken together, these findings gave rise to the hypothesis that, in meningitis, bacteria may enter the skull bone marrow.

**Pneumococcal meningitis propagates to the skull.** To test the hypothesis that S. pneumoniae entered the skull marrow cavity, we performed in vivo confocal microscopy of the intact skull in mice with meningitis. Mice were imaged 48 h after intracisternal injection with GFP+ S. pneumoniae or aCSF in controls. We observed GFP+ bacterial colonies in the skull marrow extravascular spaces of mice with meningitis whereas no such signal was detectable in controls (Fig. 4a and Supplementary Videos 5...
As this, to our knowledge, could possibly be the first observation of *S. pneumoniae* entering the skull cavity during meningitis, we sought to corroborate these imaging data with orthogonal assays, including bacterial cultures, qPCR for bacterial genes, flow cytometry to detect GFP expressed by bacteria and electron microscopy.

Using an experimental timeline (Fig. 4b) comparable to the imaging experiments described above, we first employed a bacterial colony-forming unit assay to analyze bacterial growth 48 h after intracisternal injection of *S. pneumoniae*. Tibia and skull bone marrow was plated on blood agar plates to accommodate bacterial colony growth, similar to clinical blood cultures. Although bacteria...
Fig. 4 | Intra- and extracellular bacterial localization in the cranial marrow. a, IVM of skull marrow from sham controls or mice 48 h after intracisternal injection of GFP+ S. pneumoniae JWV500 (n = 3 for sham and 4 for meningitis from 2 independent experiments). b, Experimental scheme for d–j. c, Bacterial culture of pneumococcal growth in tibia versus skull. Skull sample contains pooled frontal, parietal and occipital bone. d, Quantification of bacterial colony-forming units (mean ± s.d.; n = 10; P value represents a Mann–Whitney two-sided rank test). e, S. pneumoniae surface adhesion gene (psaA) expression in tibia versus skull normalized to sham (mean ± s.d.; n = 9; P value represents a Mann–Whitney two-sided rank test). f, Gating strategy for GFP+ CD45+ leukocytes. g, Histogram of GFP signal in CD45+ leukocytes obtained from mice with meningitis compared with CD45+ cells from sham controls. h, Quantification of GFP+CD45+ cells (mean ± s.d.; n = 5; P value represents an unpaired, two-tailed Student’s t-test). i, TEM of S. pneumoniae in the skull marrow. Left: low-magnification view of calvarial marrow depicting a sinusoidal vessel lumen adjacent to a skull channel, the inner skull bone cortex and leukocytes. Right: insets illustrate multiple leukocytes containing S. pneumoniae (n = 2 mice). j, Flow cytometric analysis of extracellular GFP+ S. pneumoniae in supernatant fraction of tibia and skull (n = 2 for sham and 4 for meningitis).
Fig. 5 | Skull channels are conduits for pneumococcal migration into the cranial marrow. 

**a.** Whole-mount ex vivo imaging of skull channels in sham controls (122-μm stack, 0.75 μm per step) and mice after intracisternal injection of GFP+ S. pneumoniae JWV500 (43-μm stack, 0.75 μm per step; 102-μm stack, 0.75 μm per step). The images depict bacteria (green arrow) in skull channels (white arrow). Osteosense was used to label bone and a CD31/Sca1 cocktail for vasculature (n=7 for sham and 12 for meningitis mice). 

**b.** Quantification of S. pneumoniae GFP signal in skull channels in sham controls and mice with meningitis (n=7 for sham and 12 for mice with meningitis). 

**c.** Tissue clearing preceded ex vivo imaging of skull channels in sham control or after intracisternal injection of GFP+ S. pneumoniae JWV500. Skull channels are visualized using osteosense to label bone and marrow vasculature using a CD31/Sca1 cocktail. The 3D reconstructions show intrachannel S. pneumoniae location in meningitis whereas bacteria are absent in sham controls (representative data from three independent experiments). 

**d.** Whole-mount ex vivo imaging after CUBIC tissue processing for bacterial GFP detection after intracisternal injection of GFP+ S. pneumoniae JWV500. CUBIC protocol (described in Methods) was followed by immunostaining for bacterial GFP. Bone marrow vasculature was labeled in vivo with CD31/Sca1 (n=2 mice). The green arrows indicate bacteria and the black skull channels. EM, Electron microscopy; IgG, immunoglobulin G; PV, perivascular.
were almost undetectable in tibial marrow, bacterial colonies grew from the skull marrow preparations (Fig. 4c,d). As only viable bacteria can divide, this finding documents live bacteria being present in the skulls of mice with meningitis. We next compared skull and tibial marrow using qPCR analysis for the bacterial gene *psaA*, which is not expressed in mice. Skull samples from mice with meningitis contained markedly higher levels of *psaA* transcript compared with the tibia (Fig. 4e), confirming the presence of *S. pneumoniae* within the skull marrow. Furthermore, we performed flow cytometric analysis on skull marrow isolated from mice after intracisternal injection of GFP+ *S. pneumoniae*. In addition to detecting bacterial presence, flow cytometry also determined whether bacteria are located inside cells. The skulls of mice with meningitis showed substantial numbers of CD45+ GFP+ leukocytes, which were largely absent in the skulls of control mice (Fig. 4f). We document the observed fraction of bacteria-containing leukocytes in a right-shifted GFP histogram in CD45+ leukocytes obtained from the skull marrow of mice with meningitis, compared with controls injected with aCSF (Fig. 4g,h). The presence of intracellular bacteria in the skull bone marrow was confirmed by electron microscopy (Fig. 4i). We also analyzed the...
cell-free supernatant of bone marrow suspensions after high-speed centrifugation by flow cytometry. In the skull marrow supernatant obtained from mice that received an intracisternal GFP\(^+\) S. pneumoniae injection, we noted abundant bacteria that were absent in controls (Fig. 3i). Taken together, the intravital imaging observation of bacteria in the skull marrow of mice with meningitis was confirmed by four independent assays, all supporting the notion that bacteria can propagate from the meninges to the skull marrow.

**Skull channels are conduits for S. pneumoniae into the marrow.** To directly evaluate whether S. pneumoniae reaches the skull marrow by transiting skull channels from the dura, we performed ex vivo confocal microscopy of tissue-cleared skull preparations containing intact brain tissue 48 h after intracisternal injection of either GFP\(^+\) S. pneumoniae or aCSF in controls. The z-stack projections of skull channels revealed the presence of GFP\(^+\) S. pneumoniae inside skull channels (Fig. 5a). We found that 75% of mice with meningitis showed bacterial GFP signal in their skull channels, whereas control animals without meningitis lacked any sign of S. pneumoniae (Fig. 5b). The 3D reconstructions of skull channels revealed extravascular GFP localization within the channels of mice with meningitis, but not in controls (Fig. 5c and Supplementary Videos 7 and 8). As a complementary approach to imaging bacterial GFP directly, we adapted optical clearing methods to search for GFP\(^+\) S. pneumoniae in deeper tissue areas\(^25\). Whole-mount confocal microscopy of CUBIC-processed meningitis specimens revealed a striking pattern of anti-GFP staining within skull channels (Fig. 5d). Sku...
As previously reported, this procedure led to preferential seeding of transplanted CD45.1 marrow in the skull whereas the nonirradiated marrow remained CD45.2. Two days after induction of meningitis, we detected CD45.1+ leukocytes in infected meninges at a chimerism that exceeded the chimerism in blood (Extended Data Fig. 7d,e), supporting that skull-derived leukocytes migrate to the meninges.

Finally, as bacterial sensing occurs via toll-like receptors and the adaptor molecule Myd88 (ref. 8), we sought to test whether such sensing is involved in skull LSK expansion observed in mice with meningitis. Thus, we isolated 40,000 LSKs from wild-type and Myd88−/− donor mice, labeled them with spectrally resolved membrane dyes and co-transferred this dual color mix into wild-type recipients, which were then infected with an intracranial injection of bacteria (Fig. 6). Myd88−/− hematopoietic progenitors exhibit normal frequencies and bone marrow homing if transferred into nonirradiated recipients8, which we confirmed by flow cytometry (Extended Data Fig. 8a–f). Two days after infection, using IVM enumeration of wild-type and knockout progenitors in the skull, we detected approximately twofold fewer Myd88−/− LSKs than wild-type LSKs (Fig. 6j,k). Corroborating these data with flow cytometry as an alternative method, we did not detect increased HSPC proliferation in the skull of Myd88−/− mice with meningitis (Extended Data Fig. 8g–j). We analyzed progenitor proliferation in Myd88−/− mice at 12 h, an intermediate timepoint between the observed increased LSK proliferation in wild-type mice at 6 and 24 h. These data suggest that toll-like receptor signaling in skull HSPCs is required for their expansion during meningitis, and we speculate that hematopoietic progenitor cells directly sense bacteria that have migrated to the skull marrow. However, other mechanisms, perhaps including cytokine signaling of meningeal leukocytes, may also alert hematopoietic cells in the skull. In sum, our data point to a process by which IC injected bacteria co-opt a perivascular CSF passage into the skull marrow, inciting a skull-specific increase in LSK proliferation that precedes changes in distal tibial bone marrow (Fig. 7).

Discussion

Every day, the human ventricular choroid plexus produces most of the 500 mL of CSF that provides a protective environment for the brain and receives waste from brain interstitial fluid through exchange with the glymphatic system. CSF outflow occurs via several routes: (1) through arachnoidal villi described >100 years ago, (2) along spinal and cranial nerves and (3) through dural lymphatics. We speculate that the skull marrow warrants closer scrutiny due to its proximity to and crosstalk with the meninges and the CNS. Constant sampling of CSF outflow suggests that the skull marrow state may reflect brain health and that the skull marrow has a prominent role in regulating CNS inflammation.

Online content

Any methods, additional references, Nature Research reporting summaries, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-022-01060-2.

Received: 19 October 2021; Accepted: 23 March 2022; Published online: 2 May 2022

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Methods

Mice. Mice were housed under certificated pathogen-free conditions at Massachusetts General Hospital's Center for Comparative Medicine. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee's approved protocols (Protocol Nos. 2003-00039, 2003-00030, 2009N000085, 1949-001062, and 2007N000148). Male and female C57BL/6 (all wild-type animals are derived from a C57D.2 background; Jackson Lab, catalog no. JAX 006664) aged 10–16 weeks were used for all experiments. Male Mdsd88−/− (Jackson Lab, catalog no. 009888) and CD45.1 (Jackson Lab, catalog no. 020014) ordered at age 8 weeks were also used for transplantation experiments. Mice were provided rodent chow and water and allowed to acclimate for 1 week before experimentation. All mice were group housed on a 12:12 h light:dark cycle at 22 °C with unlimited access to food and water. Animals were assigned to experimental groups randomly and experiments were performed in a blinded fashion.

S. pneumoniae. S. pneumoniae strain INVV500 (D39V hlp A-gfp; Cam−; serotype 2) was generously provided by J.-W. Veening and prepared as previously described19. S. pneumoniae Xen10 (serotype 3) was purchased from Caliper LifeSciences.

FPG S. pneumoniae and S. pneumoniae Xen10 were cultured in brain–heart infusion broth (BHI) containing 4.5 μg/ml of chloramphenicol or 200 μg/ml of kanamycin, respectively. S. pneumoniae strains were cryopreserved in BHI with 30% glycerol, thawed the day before the experiment in a 37 °C water bath and diluted in fresh BHI with the antibiotic. Doses between 1000 and 100 colony-forming units (cfu) were used, depending on experimental design.

Optical densities (at 600 nm) were used for all bacterial experiments to approximate colony-forming units, but serial plating dilutions confirmed actual colony-forming units on BHI agar.

Micro-CT protocol and channel analysis. Samples were imaged using a high-resolution CT scanner (μCT40, Scanco Medical AG). Data were acquired using a 6-μm isotropic voxel size, 70-Kp peak X-ray tube intensity, 114-μA X-ray tube current and 200-ms integration time, and were subjected to Gaussian transport were obtained using a customized two-photon excitation microscope.

Two-photon IVM and ex vivo imaging. Mice were anesthetized and placed on a customized stereotactic frame. During imaging, anesthesia was maintained with 1.5–1.3% isoflurane in 100% oxygen, with small adjustments to maintain the respiratory rate at ~1 Hz. To fluorescently label the microvasculature, Texas Red Dextran (40 μl, 2.5%, molecular mass (Mw) = 70,000 kDa, Thermo Fisher Scientific) in saline was injected retro-orbitally immediately before imaging. The 3D datasets of the calvarial bone marrow, meninges, meningeal vasculature and CSF transport were obtained using a customized two-photon excitation microscope. Imaging was done using 830-μm, 120-fs pulses from a Ti:Sapphire laser oscillator (Spectra-Physics InSight X3). The laser beam was scanned by polygon scanners (30 frames s−1) and focused into the sample using a x80 water-immersion objective lens for high-resolution imaging (numerical aperture of 1.1, Olympus). The emitted fluorescence was detected on photomultiplier tubes through the following emission filters: 400/60 nm for second harmonic generation, 525/50 nm for Alexa-488/FTTC and 605/50 nm for Texas Red. Laser scanning and data acquisition were controlled by custom-made software. Stacks of images were spaced at 1 μm axially.

Cisterna magna injections. Mice were anesthetized by isoflurane inhalation (3–4% induction, 1–2% maintenance), injected with buprenorphine (0.1 mg/kg−1 before surgery and every 12 h until sacrifice), followed by hair removal from the back of the neck (Nair). We used a thermometer and feedback-controlled heating blanket (Harvard Apparatus) to maintain body temperature at 37 °C. The head and neck were shaved and washed with 70% (v/v) ethanol solution (AgriLabs). An intracranial injection was performed as described above. After the injections, the exposed tissue was sealed with cyanoacrylate adhesive (Loctite) and dental cement (Co-Oral-Ile Dental).

Skull marrow preparation and confocal microscopy. For in vivo imaging, the mouse head was shaved and held in a stereotactic skull holder (Harvard Apparatus). Calvarial bone marrow was exposed, as previously described16, by incising a skin flap and then applying glycerol (Sigma–Aldrich) to prevent tissue drying. Skull marrow microscopy was performed with a single-photon confocal microscope (IV 100, Olympus) equipped with IV10-ASW 01.00.05.05 software (Olympus). A Zeiss Axio Imager M1 equipped with a ×4 magnification objective (corresponding to a 2.390 × 2.390 μm2 magnification covers a 458 × 458 μm2 area consisting of 512 × 512 pixels for ex vivo skull preparation and imaging after tissue clearing, mice were humanely sacrificed. Then, the head with brain intact was split longitudinally along the sagittal sinus and fixed for 1 h in 4% paraformaldehyde (PFA), after which tissue was washed and subjected to clearing in RapiClear 1.49 (SunJinLab) for 1–2 h, depending on sample size. Specimens were then mounted on a customized tissue holder with a hanging, covered coverslip (Harvard Apparatus). As indicated in specific figure panels with regard to the intravenous injection timing (retro-orbital intravenous injection, 100-μl total volume in phosphate-buffered saline (PBS)), mice received the following reagents for IV and ex vivo imaging: 30 μl of CD31-AF647 (BioLegend, MECD13-3), 30 μl of DAPI (BioLegend, D35307) and 30 μl of 1:106 colony-forming units (c.f.u.) were used, depending on experimental design. Optical densities (at 600 nm) were used for all bacterial experiments to approximate colony-forming units, but serial plating dilutions confirmed actual colony-forming units on BHI agar.

TEM. Skull samples were collected for TEM as described above with the following modifications. Cardiac perfusion with PBS was followed by 20 ml of Karnovsky’s fixative (0.1 M sodium cacodylate, 2.5% glutaraldehyde and 2% PFA). The skull was excised and trimmed to contain the frontal, parietal and occipital bones, and fixed for 3 h in Karnovsky’s fixative followed by 48-h fixation at 4 °C. Marrow-containing 2 × 4-mm pieces were decalcified over 2 weeks in 140 mM EDTA, pH 7.4 (Boston BioProducts). Specimens were fixed in 1% glutaraldehyde in cacodylate buffer overnight at 4 °C. Specimens were infiltrated in 1% osmium tetroxide, rinsed in cacodylate buffer and then dehydrated. Specimens were then incubated in a 1:1 mix of propylene oxide and Eponate resin (Ted Pella). The following day, specimens were incubated for at least 3 h in 100% Eponate resin, then placed into molds with 100% Eponate resin and allowed to polymerize in a 60 °C oven. Semi-thin sections (1 μm) were collected on to slides and stained with 0.1% Toluidine Blue to preview channels. Then, 70-μm sections were cut using a Leica EM UC7 ultramicrotome,
collected on to formvar-coated grids, stained with 2% uranyl acetate and Reynold's lead citrate, and examined in a JEOL JEM 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital imaging system with proprietary image capture software (Advanced Microscopy Techniques).

**Tissue clearing.** This protocol was adapted from Tainaka and colleagues to define a region of interest across the head, neck and spine. This region of interest was then uniformly fitted to each individual mouse.

**Bacterial colony-forming unit assay.** Colony-forming unit assays from blood and CSF of sham and meningitis mice were performed 48 h after injection. Mice were fixed on to a stereotactic frame in a manner similar to the orientation used for intracisternal injections. To sample the CSF, the injection site was re-opened and the dura mater punctured with a glass micropipette to aspirate 5–10 μl of CSE. Mice were then removed from the frame and 100 μl of blood was collected by cardiac puncture. For colony-forming units of skull and tibia, bones were aseptically harvested from the skull and meninges groups, and the meninges were dissected from the skull in sterile PBS containing 5% bovine serum albumin (BSA) and 2 mM EDTA. Pilot experiments were performed to titer dilutions of CSF, blood, skull or tibia homogenate necessary to visualize bacterial growth on blood agar plates containing 10 μl of kanamycin (TEKnova, catalog nol. T0194). A cell spreader was used to evenly distribute homogenates across the plate and the spread was stored at 37°C for 24 h before analysis. Plates were photographed and colonies quantified for relative comparisons.

**S. pneumoniae detection by qPCR.** We analyzed relative amounts of the S. pneumoniae gene psaA from tibias and skulls excised from the sham and meningitis cohorts of adult mice 48 h after intracisternal injection of aCSF or S. pneumoniae Xen10. Injections were performed as described above and bones were aseptically excised from mice. All mice were perfused with 20 ml of PBS before removal of the tibia and skull bones. After the bones had been excised, the meninges were dissected from the skull bone, after which the tibia and skull bones were snap-frozen in liquid nitrogen and stored at −80°C overnight before subsequent analysis.

**Flow cytometric detection of bacteria.** To assess meningee, blood, skull and tibia bone marrow hematopoietic cells, mice were anesthetized, sacrificed and perfused with 20 ml of PBS to remove blood. Tibia and skull were excised, and then the meninges were removed from the skull and mechanically homogenized in buffer (PBS with 5% BSA and 2 mM EDTA). Homogenate was filtered through a 40-μm strainer, centrifuged for 5 min at 3400 rpm and resuspended in FACS buffer (PBS with 0.5% BSA) for antibody staining. To analyze HSCPs, we followed previously established gating strategies. Cells were first stained with biotin-conjugated, anti-mouse antibodies (all used at 1:100 dilution) against CD3 (BioLegend, clone 145-2C11), CD4 (BioLegend, clone GK1.5), CD8a (BioLegend, clone 53-6.7), CD49b (BioLegend, clone DX5), CD90.2 (BioLegend, clone 30-H12), CD19 (BioLegend, clone 6D5), B220 (BioLegend, clone RA3-6B2), NK1.1 (BioLegend, clone PKP36), TER119 (BioLegend, clone TER-119), CD11b (BioLegend, clone M1/70), CD11c (BioLegend, clone N418) and Gr1 (BioLegend, clone RB6-8C5, all diluted 1:500), washed twice in and left in LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies, 1:100). Staining was done for 30 min on ice followed by a wash/spin and resuspension in FACS buffer for secondary staining (all at 1:100). LSK analysis was performed by staining cells with ekit-PE-Cy7 (BioLegend, clone 2B8), Sca1-BV605 (BioLegend, clone D7) and Streptavidin-APC-Cy7 (BioLegend, 1:100). LSKs were identified as Lin−c-kit+CD45+LSK. For tissue clearing, cells were centrifuged stained with CD34 (vendor, clone, dilution, catalog number; BD, RAM34, 1:100, 553733) and CD16/32 (BioLegend, 93, 1:100, 101337). Antibodies listed in the secondary staining panel were used at 1:100 dilution in a 500-μl single-cell suspension buffer for smoking 30 min on ice. Cells were further stained with the APC BioLegend (clone G01, no. 555298) following the manufacturer's guidelines for analysis. BeD was administered via intrapetinal injection 3 h before sacrifice to analyze LS cell proliferation 6 h after intracisternal bacterial injection. Cells were then washed with FACS buffer, spun down at 3400 rpm for 5 min and resuspended in 400 μl of buffer for analysis. Events were recorded on an LSRII flow cytometer and accompanying FACS DIVA 6.1 software (BD Biosciences). Data were analyzed flow with FlowJo software (Becton Dickinson) and the CellQuest software was used to define a region of interest across the head, neck and spine. This region of interest was then uniformly fitted to each individual mouse. Bacterial colony-forming unit assay. Colony-forming unit assays from blood and CSF of sham and meningitis mice were performed 48 h after injection. Mice were fixed on to a stereotactic frame in a manner similar to the orientation used for intracisternal injections. To sample the CSF, the injection site was re-opened and the dura mater punctured with a glass micropipette to aspirate 5–10 μl of CSE. Mice were then removed from the frame and 100 μl of blood was collected by cardiac puncture. For colony-forming units of skull and tibia, bones were aseptically harvested from the skull and meninges groups, and the meninges were dissected from the skull in sterile PBS containing 5% bovine serum albumin (BSA) and 2 mM EDTA. Pilot experiments were performed to titer dilutions of CSF, blood, skull or tibia homogenate necessary to visualize bacterial growth on blood agar plates containing 10 μl of kanamycin (TEKnova, catalog nol. T0194). A cell spreader was used to evenly distribute homogenates across the plate and the spread was stored at 37°C for 24 h before analysis. Plates were photographed and colonies quantified for relative comparisons.

S. pneumoniae detection by qPCR. We analyzed relative amounts of the S. pneumoniae gene psaA from tibias and skulls excised from the sham and meningitis cohorts of adult mice 48 h after intracisternal injection of aCSF or S. pneumoniae Xen10. Injections were performed as described above and bones were aseptically excised from mice. All mice were perfused with 20 ml of PBS before removal of the tibia and skull bones. After the bones had been excised, the meninges were dissected from the skull bone, after which the tibia and skull bones were snap-frozen in liquid nitrogen and stored at −80°C overnight before subsequent analysis. DNA isolation protocol and primers used to detect S. pneumoniae psaA were adapted from an established protocol. Briefly, three primers were custom synthesized (IDT): psaA forward (5′-GCCTTAAATAATGTGGGACATGAA-3′), psaA reverse (5′-GAGCAGGTAGCTATTTCTTTCGCC-3′) and psaA probe (5′-HEX-CTTAGACTCTCTACCTAAGAATTCGGAGAAGAA-3′-phosphate) for qPCR-based relative expression analysis. DNA and tibia bones were trimmed to 50 mg and homogenized in 180 μl of ALT buffer containing 0.04 g ml−1 of lysozyme (Sigma-Aldrich) and 75 μl m−1 of mutanolysin (Sigma-Aldrich). Digestion was performed for 1 h at 37°C in a shaking water bath. DNA isolation and purification were performed following the manufacturer’s guidelines from the QIAamp DNA Mini-Kit manual. The genomic DNA for each sample was sequenced photometrically measured (Thermo Fisher Scientific, NanoDrop 2000) and equivalent DNA amounts were loaded for subsequent qPCR. Reactions were allowed to run for 4 thermal cycles for amplification in duplicate. Cycle threshold values >40 were considered negative.

Flow cytometric detection of bacteria. Mice were IC injected with GFP + S. pneumoniae as described above and analyzed 48 h after injection. Skulls were aseptically excised followed by meningeal dissection under a light microscope. Samples were mechanically homogenized in buffer (PBS with 5% BSA and 2 mM EDTA), filtered through a 40-μm strainer and spun down for 5 min at 3400 rpm. Supernatant containing bacteria was isolated and further spun at 5,000g.
for 10 min and resuspended in 400 μl to analyze free-floating bacteria. Cell pellets were stained with CD45-AF700 (BioLegend, clone 30F11, 1:100) in FACS buffer on ice for 30 min. Samples were washed with 2 ml of FACS buffer, spun down at 340g for 5 min and resuspended in FACS buffer for analysis. Events were recorded on an LSRII flow cytometer and accompanying FACS DIVA 6.1 software (BD Biosciences). Data were analyzed with FlowJo 10 software (Becton Dickinson). Cells were gated for single cells by forward scatter and side scatter parameters, as previously described, and leukocytes were defined as CD45+ cells.

RNA extraction and qPCR. RNA was isolated from the meninges using the RNeasy Micro kit (QiAGEN). High-Capacity RNA to cDNA kit (Applied Biosystems) was used for first-strand complementary DNA synthesis from meningeval RNA. TaqMan gene expression kits were utilized to measure target genes of interest: Il-1β (catalog no. Mm00265778_m1, Thermo Fisher Scientific), Il-12p70 (catalog no. Mm00446190_m1, Thermo Fisher Scientific) and Tf-a (catalog no. Mm00432358_m1, Thermo Fisher Scientific). Target gene primers were all FAM-MGB and all target gene relative expression analyses were normalized to a housekeeping gene, Gapdh (VIC-MGB, Mm99999915_g1, Thermo Fisher Scientific). Samples were run on a 7900 Real-Time PCR machine (Applied Biosystems).

Image analysis. Images were processed and analyzed using Fiji v2.1.0, MATLAB R2015b (Mathworks) or Volocity 3D imaging software v6.3 (Perkin Elmer). To complete ex vivo 2-photon microscopy channel analysis for CSF tracer presence, z-stacks were untitled using MATLAB (2020b) such that the skull surface spanned the least number of z depths, that is, it lay approximately flat. Afterwards, enhanced contrast was performed on each channel (R, G, B) to correct for intensity attenuation due to light absorption in the tissue. For confocal images, z-stacks of 0.75, 1.0 or 2.0 μm per slice were taken depending on the imaging objective (x4, x10 or x20). These conditions were consistent across all single-photon confocal microscopy images in both ex vivo imaging and IVM experiments. Images were 3D max-intensity or sum-intensity projected using Fiji and background subtracted, followed by an automated protocol to remove speckle-noise based. Manual thresholding and contrast adjustment were applied uniformly across all samples. To ensure processing step uniformity, Fiji macros were recorded for the first image processed and then applied to all images from the same cohort. Where indicated, certain images were further analyzed using Volocity software for preparing 3D surface reconstructions. The 3D rendering was automatically generated and the image contrast, density and brightness were uniformly set across all images analyzed. Movies were generated in Volocity using snapshots of post-processed datasets and then incorporated into a single video in AVI format.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (GraphPad Software v10). All quantitative results are reported as the mean ± s.e.m. For single variable comparisons of parametric datasets for two groups, an unpaired Student’s t-test was performed. For nonparametric datasets of unpaired data, a Mann–Whitney test was performed. For parametric datasets for multiple groups, a one-way analysis of variance (ANOVA) was performed with a post-hoc correction for multiple comparisons as indicated in individual legends. For nonparametric datasets for multiple groups, a Kruskal–Wallis test was performed, followed by post-hoc correction for multiple comparisons where indicated. For all statistical tests, a P value <0.05 was considered significant. No statistical methods were used to predetermine sample sizes but our sample sizes are in agreement with those reported in previous publications. Animals were not excluded from experimental data analysis, with the exception of two mice that died before the experimental timepoint (6h and 24h), caused by a failed intracranial magna injection. Due to the preparation of groups necessary for intracranial injections and subsequent nature of the data acquisition of these experiments, it was possible to perform these experiments only in a semi-blinded fashion. As tissue harvesting and preparation required two to three people, only one person was made aware of the control versus experimental groupings, with the remaining experiment members blinded. Cell sorting of LSKs was also done in a blinded fashion (for example, wild-type versus Myd88 knockouts) by staff scientists at our flow cytometry core. Animals were randomly assigned to control or experimental groups.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Extended Data Fig. 1 | CSF tracer outflow in occipital, parietal and frontal skull bones. a, Experimental outline. Ex-vivo z-stack (54 μm stack at 1 μm/step) of occipital, parietal and frontal skull cortex after IC and IV injection of fluorescently labeled dextran. Bone is visualized by second harmonic generation around channels b, Imaging of CSF tracer outflow through channels in different skull bones, assessed in n=2 mice. Bar graphs depict the proportion of skull channels that were positive for CSF tracer.
Extended Data Fig. 2 | Dynamics of CSF outflow into bone marrow. a, Ex vivo imaging of whole-mount skull 10 min after intracisternal (IC) injection of ovalbumin. Intravenous (IV) injection of CD31/Sca1 labeled vasculature and IV osteosense the bone. b, Ex vivo imaging of tibia 10 minutes after intracisternal injection of ovalbumin. c, Imaging 30 minutes after intracisternal injection of ovalbumin. Data is representative of 2 independent experiments.
Extended Data Fig. 3 | Inflammation in the meninges driven by bacterial meningitis. qPCR analysis of meninges isolated from either sham controls that were intracisternally injected with artificial CSF or mice 48 hours after intracisternal infection for relative expression analysis of a, Il1β, b, Il6 and c, Tnfa (mean ± SD; n=6 mice per group; P values represent an unpaired two-tailed t-test from a single experiment). d, Raw images obtained by whole mount ex vivo imaging of the skull 48 hours after intracranial sham or S. pneumoniae injection. First representative image is the original data from Fig. 5b while the second set represents additional examples of channel morphology and bacterial propagation. Green arrow highlights bacteria (scale: 50 and 25 μm).
Extended Data Fig. 4 | Analysis of skull hematopoietic progenitors during meningitis. a, Experimental outline for calvarial hematopoietic progenitor analysis. b, Flow cytometry gating. c, Quantitation of calvarial BrdU+ common myeloid progenitors (CMP) 6 hours after intracisternal sham or S. pneumococci injection. (n=11 sham and 12 meningitis, P value represents an unpaired, two-tailed t-test). d, Quantitation of calvarial BrdU+ CMP 24 hours after intracisternal sham or S. pneumococci injection. (n=6 mice per group, P value represents an unpaired, two-tailed t-test). e, Quantitation of calvarial BrdU+ LSK 24 hours after intracisternal sham or S. pneumococci injection. (n=6 mice per group, P value represents an unpaired, two-tailed t-test).
Extended Data Fig. 5 | Analysis of calvarial leukocytes during meningitis. a. Experimental outline of calvarial leukocyte analysis. b. Flow cytometry gating. c-f. Quantitation of calvarial leukocytes 6 hours after intracisternal S. pneumococci injection shows neutrophils, monocyte subsets and total lymphocytes (n=5 mice per group). g-j. Quantitation of calvarial leukocytes 12 hours after S. pneumococci injection including neutrophils (g), Ly6C<sup>hi</sup> monocytes (h), Ly6C<sup>lo</sup> monocytes (i) and total lymphocytes (j) (n=9 sham and 8 meningitis). k-n. Quantitation of calvarial leukocytes 24 hours after S. pneumococci injection including neutrophils (k), Ly6C<sup>hi</sup> monocytes (l), Ly6C<sup>lo</sup> monocytes (m) and total lymphocytes (n) (n=6 mice per group). (P values represent unpaired, two-tailed t-tests, data are mean values ± SD).
Extended Data Fig. 6 | Meningeal leukocytes expand in bacterial meningitis. **a**, Experimental outline. **b**, flow cytometry plots of control meninges (upper panel) and meninges 48 hours after infection. **c**, quantification of CD11b+ myeloid cells and **d**, Ly6G+ neutrophils in meninges (n=4 sham and 7 meningitis mice, P values represent unpaired, two-sided t tests, data are mean values ± SD).
Extended Data Fig. 7 | Tracking of skull leukocytes to infected meninges. a, Experimental outline indicating skull marrow transplantation, followed by induction of meningitis 4 weeks later. b, flow plots and (c) quantitation of myeloid cell chimerism in irradiated skull versus lead-shielded tibia 4 weeks after transplantation (n=7 recipient mice, P value represents unpaired, two-tailed t test, data are mean values ± SD). d, flow plots and (e) quantification of myeloid cell chimerism in the meninges and in blood (n=7 recipient mice, P value represents unpaired, two-tailed t tests, data are mean values ± SD).
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Myd88-related sensing in the skull marrow. a, Experimental groups include wild type and Myd88−/− mice. The skull marrow was assessed by flow cytometric staining for lineage markers Sca-1 and c-kit. b, Flow cytometry plots and c, quantitation of LSK % as a total of all live lineage negative single cells in the calvarial marrow of steady-state Myd88−/− or wild-type C57/BL6 mice (n=9 sham and 6 meningitis mice, P value represents an unpaired, two-tailed t-test, data are mean values ± SD). d, Experimental outline. Non-irradiated wild type recipient mice received a mix of 40,000 LSK from wild type donors (labeled with the membrane dye DiD) and from Myd88−/− donors (labeled with Dil). e, Flow cytometry gating and f, analysis of the skull bone marrow 3 days later showed a similar seeding of LSK irrespective of phenotype (n=4 mice per group, unpaired, two-tailed t-test). g, Experimental outline of calvarial progenitor analysis in Myd88−/− mice with and without meningitis. h, Flow cytometry gating. i, Quantitation of calvarial BrdU+ CMP 12 hours after intracisternal sham or S. pneumococci injection. (n=11 sham and 12 meningitis mice per group, unpaired two-tailed t-test, data are mean values ± SD). j, Quantitation of calvarial BrdU+ LSK 24 hours after intracisternal sham or S. pneumococci injection. (n=6 mice group, unpaired two-tailed t-test, data are mean values ± SD).
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- Confirmed

☐ X The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ X The statistical test(s) used AND whether they are one- or two-sided
  *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐ ☐ A description of all covariates tested
☐ X A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐ ☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ ☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  *Give P values as exact values whenever suitable.*
☐ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ ☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Flow cytometry data were acquired on an LSRII flow cytometer running FACS DIVA 6.1 software for event acquisition. For LSK cell-sorting, a BD FACs Aria running FACS DIVA 6.1 (BD Biosciences) was used by a staff scientist at the Harvard Stem Cell Institute and Center for Regenerative Medicine Flow Core. qPCR was performed on a Bio-Rad CFX96 Real Time PCR Machine. Single photon confocal microscopy was performed on an Olympus IX100 microscope equipped with IX10-ASW 01.01.00.05 software (Olympus). 2-photon microscopy was performed on a custom-built two-photon excitation microscope equipped with a Ti: Sapphire laser oscillator (Spectra-Physics InSight X3) and coupled with Olympus 60x water-immersion objective lens. Laser scanning and acquisition were controlled by custom-built software.

Data analysis

Flow data was analyzed with FlowJo 10 software (BD Biosciences). Quantitative analysis of flow data and qPCR data was organized on Microsoft Excel and exported onto GraphPad Prism 10 software (GraphPad). Confocal and 2-photon microscopy data was analyzed on FIJI open-source software v2.1. 3-D reconstructions were generated using Volocity software version 6.3 (PerkinElmer). 2-PM image data was analyzed with MATLAB v2020b code to construct z-stacks and Amira v5.3.2 software was used for microCT data analysis.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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All raw data can be made available upon reasonable request and a statement in the manuscript reflects the availability status of our collective data. The authors will make any source data within the manuscript available upon reasonable request. The large file sizes accompanying the extensive imaging data used can be provided along with relevant accessibility information for software packages associated with each file. Requests may be sent to any of the corresponding authors.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Sample size was approximated and derived from extensive publications within the hematopoiesis field which are based on previous a priori power testing. No statistical methods were used to pre-determine sample sizes but our sample sizes are in agreement with those reported in previous publications 8,19, 43.

Data exclusions
No data points were excluded.

Replication
Experiments were reproduced at least once to confirm the obtained results. For flow cytometric analyses of the bone marrow, experiments were split into groups of 3-4 control and 3-4 experimental sizes and then repeated at least once in identical fashion to allow for adequate sample sizes as well as independent, replication of cumulative results that were separately obtained.

Randomization
Animals were randomly assigned to control or experimental groups.

Blinding
Experiments were performed in blinded or semi-blinded fashion for experiments involving ICM injections. For the latter setting, the investigator performing the injection of sham or experimental biologic/reagent was not blinded while the 2 collaborators harvesting the data were blinded to the groups. Data acquisition during cell sorting of LSKs was also done in blinded fashion (eg WT vs Myd88 KO) by staff scientists at our flow cytometry core along with all other data acquisition for EM and flow cytometry when possible.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| ☑ Antibodies                    | ☑ ChIP-seq |
| ☑ Eukaryotic cell lines         | ☑ Flow cytometry |
| ☑ Palaeontology and archaeology | ☑ MRI-based neuroimaging |
| ☑ Animals and other organisms   |         |
| ☑ Human research participants   |         |
| ☑ Clinical data                 |         |
| ☑ Dual use research of concern  |         |

Antibodies

Antibodies used
Flow cytometry antibodies:
- Biotin ABs and Live/Dead (all at 1:100): CD3 (BioLegend, clone 145-2C11, 100304), CD4 (BioLegend, clone GK1.5, 100404), CD8a (BioLegend, clone S3-6.7, 100704), CD49b (BioLegend, clone DX5, 108904), CD90.2 (BioLegend, clone 30-H12, 105304), CD19
To assess skull and tibia bone marrow hematopoietic cells, mice were anesthetized, sacrificed and perfused with 20 mL PBS to remove blood cells. Tibia and skull were excised, and then meninges were removed from the skull and mechanically homogenized in homogenization buffer (PBS with 5% BSA and 2 mM EDTA). Homogenate was filtered through a 40 μm strainer, centrifuged for 5' at 340g and resuspended in FACS buffer (PBS with 0.5% BSA) for surface antibody staining.

Antibody panels used are described above in greater detail in the "Antibody" section. Briefly, cells were first stained with APC/Fire750 fluorophores which were not commercially available until the last year. These antibodies were tested alongside their respective PE and AF647 conjugated counterparts to ensure specific and comparable labeling. Finally, identification of LSKs was performed as recently demonstrated by our lab and others in Frodermann, V., Rohde, D., Courties, G. et al. 2007N000148.

Fluorescent AB used at 1:100 dilution: ckit-PE-Cy7 (BioLegend, clone 6D5, 115503), B220 (BioLegend, clone RA3-6B2, 103204), NK1.1 (BioLegend, clone PK136, 108704), TER119 (BioLegend, clone TER-119, 116204), CD11b (BioLegend, clone M1/70, 101204), CD11c (BioLegend, clone N418, 117304) and Gr1 (BioLegend, clone RB6-8C5, 108404), LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies).

For imaging: CD31 (Biolegend, clone MEC13.3, 25 uL IV, catalog #s: 102516, 102528, 102508 ), Sca1 (Biolegend, clone D7, 25 uL IV catalog #s: 108146, 108118, 108108), Chicken anti-GFP (Abcam, 1:50, ab13790), Goat anti-chicken AF555 (Thermofisher, 1:200, A-21437), CD115 (Biolegend, AFS98 , 1:100 135517), Ly6G (Biolegend, 1A8, 1:100 127617). Exercise reduces inflammatory cell production and cardiovascular inflammation via instruction of hematopoietic progenitor cells. Nat Med 25, 1761–1771 (2019).

Validation

All antibodies used for flow cytometry and microscopy were previously validated for the respective application by the vendor. Individual listings of citations (>5 for all listed antibodies) describe the use of these reagents in the imaging or cytometric application in which they were used on each antibody’s manufacturer page which can be accessed using the catalog numbers provided above. Additionally, our laboratory has utilized this panel of intravital microscopy and flow cytometry antibodies in recent publications including (Vandoorne, Rohde D et al Circ Res 2018; 123 (4): 415-427) with the only exception being CD31 and Sca1 antibodies which were conjugated with APC/Fire750 fluorophores which were not commercially available until the last year. These antibodies were tested alongside their respective PE and AF647 conjugated counterparts to ensure specific and comparable labeling. Finally, identification of LSKs was performed as recently demonstrated by our lab and others in Frodermann, V., Rohde, D., Courties, G. et al. Exercise reduces inflammatory cell production and cardiovascular inflammation via instruction of hematopoietic progenitor cells. Nat Med 25, 1761–1771 (2019).
For sorting of LSKs from WT and Myd88 KO mice, purity was confirmed to be greater than 95% during the duration of all Flow data was analyzed with FlowJo 10 software (BD Biosciences). Quantitative analysis of flow data and qPCR data was Flow cytometry data were acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). For cell-sorting, a BD

For gating of DiL or DiD LSKs, single cell suspension of skull were stained for live cells. FMO controls for cells lacking DiD or DiL were used to set gates for their respective channels i.e. cells stained with live/dead and DiD were used to set the gate for DiL-cells and vice-versa for DiD. All gating strategies are thoroughly described and shown in extended figures.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.