Supplementary File 2: Leukocyte coverage post micro-embolism surgery.

Introduction

Tween20 (or its closely related compound, sorbitan Tween80) is commonly used at low concentrations as a dispersant for arterial injections *in vivo*, with no reported blood–brain barrier (BBB) disruptions or local toxicity[1-3]. The low concentration, injection volume, and injection speed of Tween20 used in this study are not expected to cause adverse effects on the BBB or induce inflammation. To verify this, CD45 staining was performed to assess immune cell coverage in the brain and confirm that Tween20 does not exert negative effects.

Methods

Tie2–GFP transgenic mice (Jackson Laboratory, Stock No. 003658) were used. Male (n = 7) and female (n = 8) mice aged 2–6 months were housed under controlled environmental conditions (12-hour light/dark cycle, 21 °C), with access to food and water *ad libitum*. To model microvascular occlusions and study the capillary response to embolic obstruction, 10 µm fluorescent polystyrene microspheres (Invitrogen, F8829) were injected into the left internal carotid artery under anesthesia (1.5% isoflurane). Body temperature was maintained at 37 °C using a heating pad. Mice were euthanized at 1, 7, or 28 days post-surgery by cardiac perfusion with 4% paraformaldehyde (PFA) (**Supplementary Table 1**). Brains were isolated and fixed for 24 hours at 4°C in 4% PFA, followed by 48 hours in 30% sucrose. Prior to sectioning, whole brains were stored at –80°C. Coronal sections were cut at 50 µm (Cryomicrotome Cryostat NX70) and maintained in a cryoprotectant solution containing 30% sucrose and 30% ethylene glycol. Sections were stored at -20°C until further use.

Time point *(day(s) post-surgery)*	**Number of mice *(F/M)***
1	5 (3F / 2M)
7	5 (3F / 2M)
28	5 (2F / 3M)
Total	**15 (8F / 7M)**

Supplementary Table 1. **Overview of experimental time points and gender distribution of mice.** *F = female; M = male.*

Immunohistochemistry

Tissues were washed three times for 15 minutes each in phosphate-buffered saline (PBS). Subsequently, sections were incubated for 1 hour in a blocking buffer containing PBS, 5% goat serum, and 2% Tween-20. After blocking, tissues were incubated overnight at 4°C in blocking buffer with rabbit anti-CD45 (1:50, Abcam, ab10558) antibody to label leukocytes. The next day, sections were washed three times for 15 minutes each in PBS and then incubated overnight at 4 °C with secondary antibody, goat anti-rabbit IgG conjugated to Cy5 (1:400, Invitrogen, A10523), diluted in blocking buffer. Finally, tissues were washed twice for 15 minutes each in PBS, followed by a 10-minute incubation with bis-benzimide (ThermoFisher; 1:100 dilution in PBS). After two final PBS washes, tissues were mounted onto SuperFrost microscope slides using a fluorescence-compatible mounting medium (DAKO, S3023), covered with glass coverslips, and stored at 4°C until imaging.

Microscopy

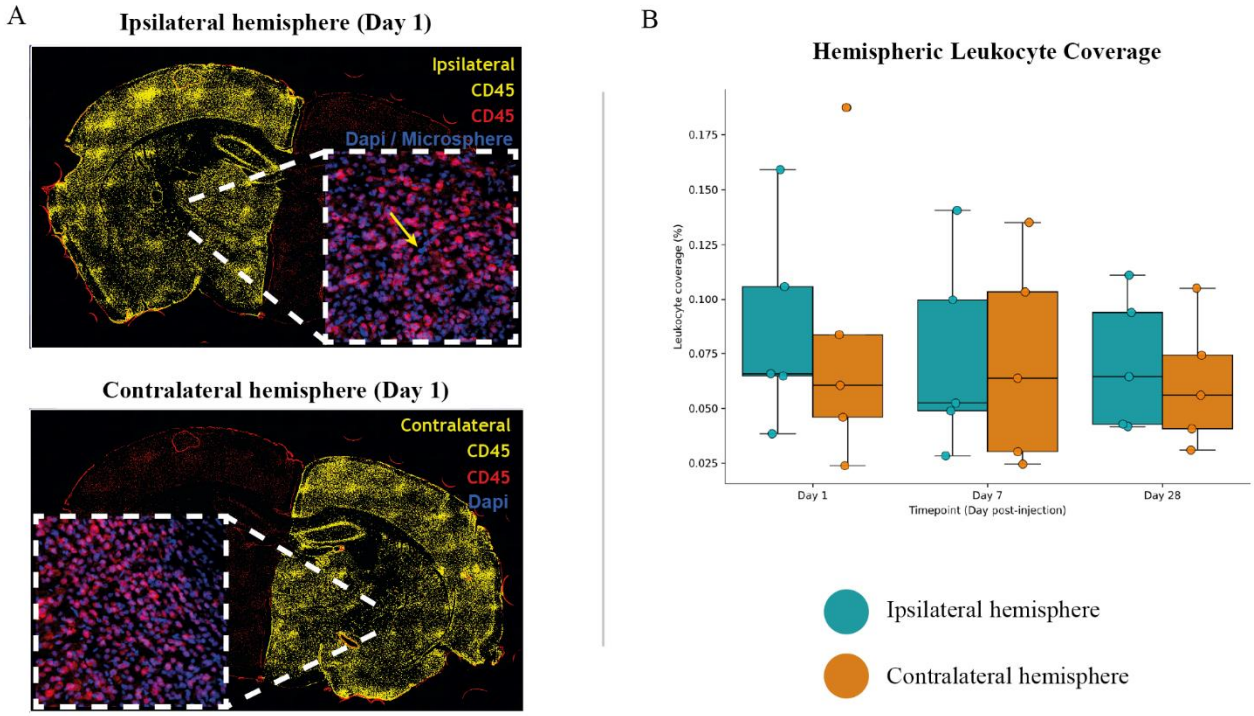
Widefield fluorescence imaging was performed using the MICA (Thunder, Leica LAS-X) system to capture entire brain sections and assess hemispheric inflammation. Images were acquired with a HC Plan Apo CS2 10x/0.40 dry objective (NA 0.32, WD 11.2 mm) in widefield fluorescence mode, using both transmitted light (brightfield) and fluorescent illumination. Thunder image enhancement was enabled via the Large Volume Computational Clearing (LVCC) algorithm, which provides real-time background suppression and deconvolution. For volumetric imaging, Z-stacks were captured by activating 3D-imaging and setting automatic focal limits with the Z-Stack Finder tool. Since MICA's adaptive acquisition algorithm optimizes exposure time, illumination intensity, and detector gain for each specimen, scan settings varied slightly between tissue sections. Total immune cell coverage per hemisphere was analyzed using ImageJ.

Results

Supplementary Figure 2 shows immunohistochemical analysis of CD45 coverage in brain sections, revealing no differences between the ipsi- and contralateral hemispheres at multiple time points following micro-embolism surgery.

Conclusion

Our findings indicate that Tween20, used as a dispersant in arterial microsphere injections, does not induce detectable immune cell infiltration or alter CD45 coverage in the brain. The lack of differences between the ipsilateral and contralateral hemispheres at multiple time points post micro-embolism surgery supports the safety of Tween20 at the concentrations, volumes, and injection parameters applied in this study for *in vivo* cerebral circulation models.



Supplementary Figure 2: Immunohistochemical assessment of CD45 coverage in brain sections at 1, 7, and 28 days following micro-embolism surgery, comparing the ipsilateral

and contralateral hemispheres. A) Representative images of immunohistochemical CD45 staining. B) Quantification of leukocyte coverage (CD45) at days 1, 7, and 28, comparing the ipsilateral and contralateral hemispheres.

References

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