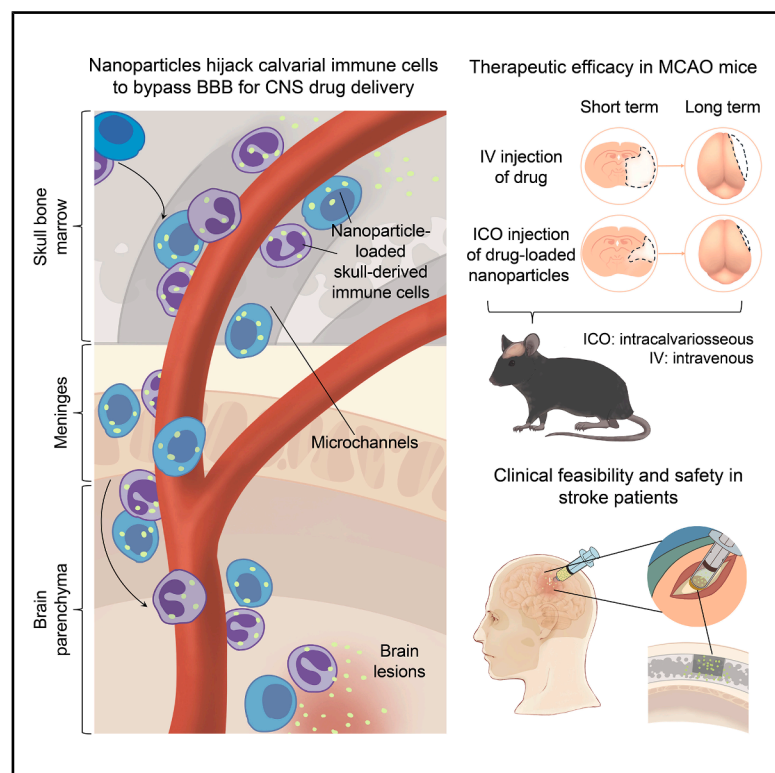


Nanoparticles hijack calvarial immune cells for CNS drug delivery and stroke therapy

Graphical abstract



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In brief

Drug-loaded albumin NPs hijack calvarial immune cells to bypass the BBB via skull-meninges microchannels for CNS drug delivery and stroke therapy, establishing skull-based delivery and immune-assisted transport as a clinically translatable strategy for neurological therapeutics.

Highlights

- Drug-loaded albumin NPs hijack calvarial immune cells *in vivo* via ICO injection
- The NP-loaded cells migrate toward the CNS via skull microchannels, bypassing the BBB
- The NP-loaded cells promote targeted drug delivery to CNS lesions
- The NP-loaded cells relieve stroke injury, showing clinical feasibility and safety



Article

Nanoparticles hijack calvarial immune cells for CNS drug delivery and stroke therapy

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<https://doi.org/10.1016/j.cell.2025.12.008>

SUMMARY

The rapid accessibility of calvarial immune cells to the brain, in principle, may offer transformative opportunities for overcoming drug delivery barriers in central nervous system (CNS) disorders. Here, we hijacked calvarial immune cells using drug-loaded nanoparticles (NPs) and leveraged their unique migration mechanism through skull-meninges microchannels to bypass the blood-brain barrier (BBB) for CNS drug delivery. We constructed NP-loaded immune cells *in situ* via intracalvariososseous (ICO) injection, validated their prompt migration in response to CNS perturbation, and targeted therapeutic delivery to CNS lesions. Compared with conventional delivery approaches, this strategy achieved promising therapeutic efficacy in improving both short- and long-term outcomes in preclinical stroke models. Our prospective clinical trial further supports the translational feasibility of ICO immune access in treating malignant stroke. These findings establish skull-based delivery as a promising, clinically translatable route for CNS drug delivery and highlight immune-assisted transport as a potentially transformative strategy for improving therapeutic outcomes in neurological disorders.

INTRODUCTION

Development of effective medications for central nervous system (CNS) disorders has long been plagued by a high failure rate in clinical trials.^{1–3} One major obstacle is the restrictive permeability of the blood-brain barrier (BBB) to most drugs, thereby leading to insufficient brain accumulation and unsatisfactory therapeutic outcomes.^{1–3} At the same time, the lack of specificity in drug targeting often leads to adverse effects, which are particularly problematic in the CNS due to the region-specific vulnerabilities in most neurological disorders.^{1–3} Despite recent advancements in multiple drug delivery platforms proposed in the open literature, efficacious drug delivery to the CNS remains a daunting challenge.

Immune cells, which are actively involved in the pathogenesis of most CNS disorders, have emerged as promising vectors for CNS drug delivery due to their innate ability to traverse the BBB and target CNS lesions.^{4–6} Nevertheless, clinical translation of immune cell-based drug delivery strategies still faces several challenges. One primary limitation is that current strategies rely on systemic administration of drug-loaded cells, which are prepared *ex vivo* and typically isolated from either actual patients or healthy donors.^{7–10} Off-target effects during systemic circulation, along with the low expression of leukocyte adhesion molecules on the BBB, often result in limited infiltration of drug-loaded immune cells into the brain parenchyma.^{7–10} Additionally, the infusion of large numbers of immune cells may raise safety concerns, including the risk of graft-versus-host diseases and graft



immune rejection.^{11–13} The possibility of immune rejection may also diminish the persistence of infused cells and undermine their therapeutic benefits.^{12,13} Finally, the complexity and expertise required for *ex vivo* manipulation procedures render the accessibility and scalability of these therapies barely possible.^{6,8} These hurdles underscore the need to develop alternative cell-based delivery strategies that can achieve efficient CNS delivery while minimizing the associated risks.

More recently, the skull bone marrow and the meninges encasing the brain have been recognized as critical immune niches for CNS surveillance.^{14–19} These niches continuously monitor changes in the brain's immune environment and dynamically adjust immune responses to maintain CNS homeostasis. Under homeostasis, the skull bone marrow replenishes the meninges with a repertoire of immune cells through skull-meninges channels (SMCs).^{18,20} In CNS disorders, cerebrospinal fluid (CSF), laden with disease-specific biomarkers, engages directly with these cells through SMCs and prompts their chemotactic infiltration into the brain parenchyma.^{19,21–23} Distinct from peripheral circulating immune cells, the calvaria-derived immune cells are in immediate proximity to the brain and further exhibit a unique migration mechanism that can bypass the BBB through the brain-bordering immune gateway (i.e., SMCs), thus enabling rapid targeting of CNS lesions.^{14,19} Additionally, populated immune cells and relatively low liquidity in the skull bone marrow provide a favorable setting to facilitate drug-loaded cells *in vivo*, thereby circumventing the introduction of high numbers of cells or the inefficiencies associated with intravascular *in vivo* loading methods.^{4–6,24} Therefore, an alternative approach that can construct drug-loaded immune cells in these brain-adjacent immune niches, exploit their immune specificity, and use the brain-targeted immune gateway to bypass the BBB for enhanced CNS drug delivery, has recently attracted considerable attention in research communities.^{1,14}

Here, we propose to hijack the calvarial immune cells with drug-loaded nanoparticles (NPs), leveraging their unique brain-targeted migration through SMCs for CNS drug delivery (Figure 1A). We employed albumin NPs because of their tropism for immune cells via complement opsonization.^{25–27} We observed rapid internalization of the albumin NPs by the immune cells following intracalvariosseous (ICO) injection. Using a lipopolysaccharide (LPS)-induced neuroinflammation model and an experimental stroke model induced by middle cerebral artery occlusion (MCAO), we discerned a substantial migration of NP-loaded cells toward the brain via the SMCs, with a subsequently markable accumulation of albumin NPs in the target neurons at CNS lesions rather than the unlesioned tissues. To validate the therapeutic potentials of this strategy, we loaded nerinetide (Tat-NR2B9c and NA1), a clinically validated neuroprotective eicosapeptide, onto albumin NPs via electrostatic interaction and evaluated their efficacy in treating acute ischemic stroke using the MCAO mouse model.²⁸ Comparative evaluation revealed that ICO administration of NA1-loaded NPs not only significantly alleviated ischemic infarction and cerebral edema during the acute phase but also mitigated brain atrophy and neurological deficit in long term. Remarkably, these effects, achieved with only 1/15 of the conventional dosage, greatly exceeded those from clinical intravenous (i.v.) administration, direct ICO injection of free NA1,²⁹ or i.v. injection of the NA1-

loaded NPs that may have peripheral immune cell-mediated brain targeting as reported.²⁴ Moreover, to assess clinical translation, we conducted a prospective, randomized, open-label clinical trial, SOLUTION (ClinicalTrials.gov identifier: NCT05849805), to treat patients with malignant middle cerebral artery infarction (mMCAI). Real-world data from this trial demonstrates the feasibility and safety of our approach, underscoring its translational potential. Together, these findings establish a clinically translatable platform for CNS drug delivery, harnessing unique brain-bordering immune mechanisms to bypass the BBB and achieve lesion-specific targeting, thereby unlocking considerable potential for CNS disorder treatments.

RESULTS

NPs target calvarial myeloid cells *in vivo* through ICO injection

Calvarial immune cells, particularly those of the myeloid lineage, possess a distinct transcriptomic and proteomic profile for CNS immune surveillance.³⁰ Compared with the peripheral immune cells, the calvarial myeloid cells (monocytes and neutrophils) exhibit a mature phenotype with upregulated genes related to migration and phagocytosis.³⁰ We therefore hypothesized that injecting NPs into the skull bone marrow could directly load them into calvarial myeloid cells *in vivo*. High-resolution *ex vivo* X-ray computed tomography (CT) revealed highly interconnected marrow cavities in the mouse interparietal bone and numerous microchannels extending to the meninges, identifying this site as optimal for ICO injection (Figures S1A and S1B).²³ Following injection, radiographic data of the harvested mice skulls confirmed both the accessibility of the bone marrow and the integrity of the compact inner table (Figure S1C). The data indicate that NPs firstly distribute within the marrow cavity, interact with resident myeloid cells, and then enter the CNS via microchannels, rather than direct meningeal diffusion. Complete skull healing was observed within 5 weeks (Figure S1C).

To target calvarial myeloid cells *in vivo*, we developed albumin NPs, a clinically approved material with known myeloid tropism via complement-mediated opsonization.^{25–27} Dynamic light scattering (DLS) and scanning electronic microscopy (SEM) show uniform diameters and good stability of the albumin NPs (Figures 1B, 1C, and S1D–S1F). To confirm phagocytosis of the albumin NPs by calvarial myeloid cells, we grafted the NPs with fluorescein isothiocyanate (FITC) and used a combination of confocal microscopy, flow cytometry, and transmission electron microscopy (TEM) for characterizations. *In vitro* co-incubation with skull and tibial bone marrow cells shows up to 90% uptake efficiency (Figures S2A–S2C). *In vivo*, 2 h after ICO injection, confocal imaging reveals clear colocalization between FITC-NPs and granulocyte receptor-1 (Gr1+) cells (Figure 1D), confirming phagocytosis by calvarial myeloid cells (Figure 1D). Flow cytometry experiments further quantified the *in vivo* phagocytosis by myeloid cell subsets, with the proportion of FITC+ neutrophils and Ly6Chi monocytes at 29.7% and 49.4%, respectively (Figures 1E and 1F). By contrast, i.v. administration resulted in 12.6% of peripheral neutrophils and 8.3% of peripheral Ly6Chi monocytes being FITC-labeled (Figure S2D), further supporting the enhanced targeting efficiency of the ICO route.

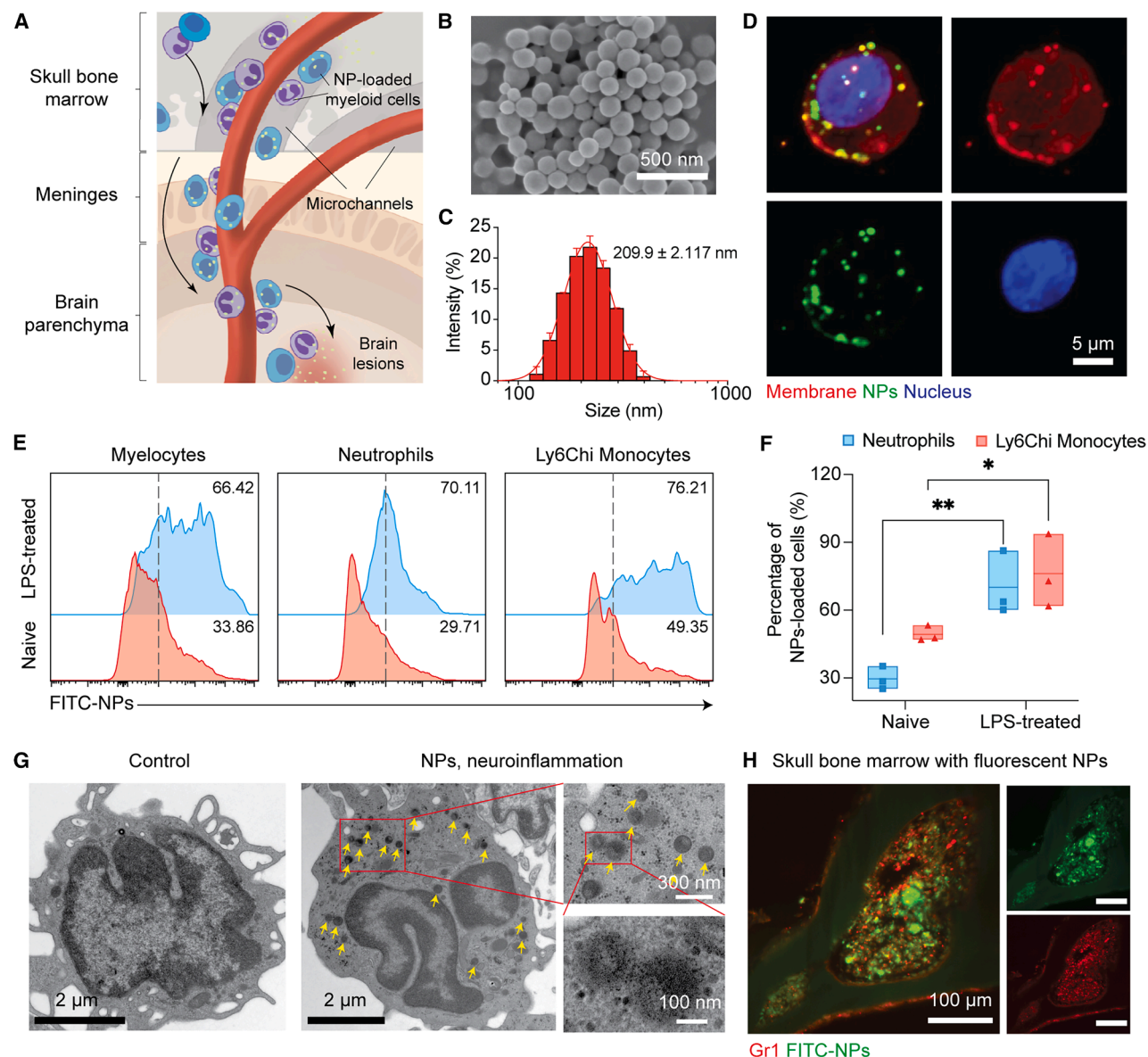


Figure 1. NPs target calvarial myeloid cells *in vivo*

(A) Schematic of the NPs administered into skull bone marrow targeting calvarial myeloid cells *in vivo*.

(B and C) SEM (B) and DLS (C) characterization of the albumin NPs.

(D) Confocal images show the phagocytosis of albumin NPs by Gr1+ cells isolated from skull bone marrow.

(E and F) Representative flow cytometry data and statistics show *in vivo* targeting efficiency of the albumin NPs to myeloid cells and subpopulations, including neutrophils and Ly6Chi monocytes in naive and LPS-treated mice. $n = 3$ biological replicates for each group. Data are mean $\pm$ SD. Two-way ANOVA with Šidák's multiple comparisons test. * $p < 0.05$ and ** $p < 0.01$.

(G) Representative TEM images of the mouse skull bone marrow cells after ICO injection of saline control (left) and the albumin NPs (right).

(H) Fluorescent images of optically cleared calvaria show *in vivo* targeting of the Gr1+ cells by FITC-labeled albumin NPs.

See also Figures S1 and S3.

To investigate whether the CNS perturbation enhances NP uptake, we employed an LPS-induced neuroinflammation model for verification.^{31,32} Compared with the naive control, flow cytometry analysis reveals a significant increment of calvarial myelocyte subsets co-stained with the NPs in the neuroinflammatory group, with FITC+ neutrophils and Ly6Chi monocytes

rising to 70.1% and 76.1%, respectively (Figures 1E and 1F). It is noteworthy that the proportion of FITC+ neutrophils demonstrated a more pronounced increase than that of Ly6Chi monocytes, which may be attributed to their mature late-stage phenotype and rapid immune responses.^{30,33} Moreover, TEM images clearly demonstrated the presence of a large number

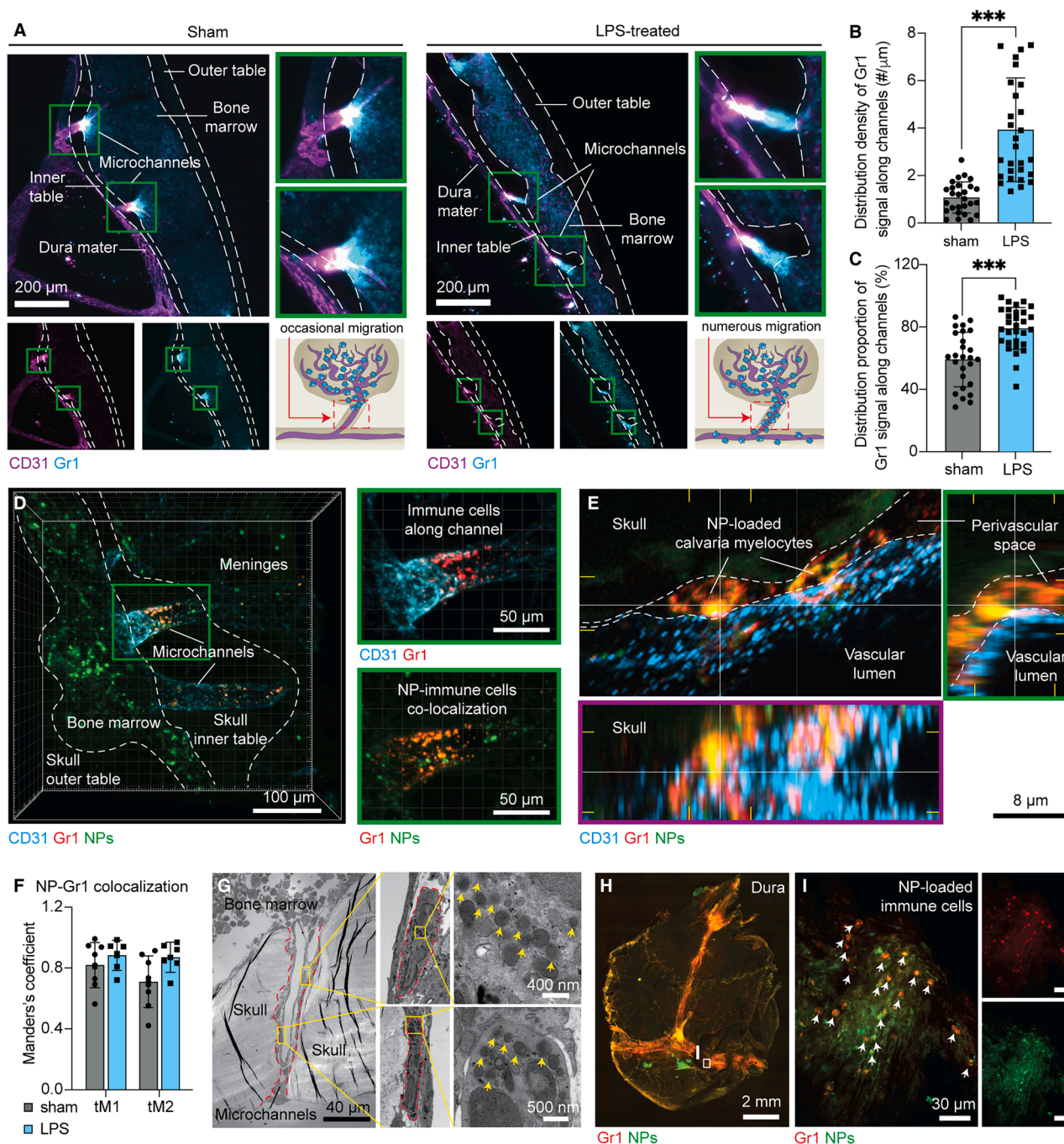


Figure 2. Calvarial myeloid cells mediate NP delivery to CNS border via skull-meninges immune gateway

(A) Lightsheet images of the optically cleared calvaria show different responses of the calvarial myeloid cells in LPS-treated mice or sham control. In LPS-treated mice, the calvarial myeloid cells demonstrate massive migration through the microchannels. In sham control, the migration is occasional.

(B and C) Statistics of the distribution density (B) and distribution proportion (C) of Gr1+ signals along the microchannels. $n = 6-8$ technical replicates from 4 biological replicates for each group. Data are mean $\pm$ SD. Unpaired t test. $***p < 0.001$.

(D) Representative image of the optically cleared calvaria shows Gr1+ cells delivered NPs toward CNS through the microchannels.

(E) Extended orthogonal views of the NP-loaded Gr1+ cells migrating through the perivascular space of skull-meninges microchannels.

(F) Colocalization analysis between NPs and Gr1+ cells along the channels. Thresholded Manders' correlation coefficients are shown among groups. $n = 7-9$ technical replicates for each group. Data are mean $\pm$ SD.

(legend continued on next page)

of NP-loaded cells inside the bone marrow (Figure 1G).³⁴ After *in vitro* co-incubation or *in vivo* injections of the albumin NPs, no decrease in the viability of the myeloid cells was observed, indicating good biocompatibility of the NPs (Figure S2E).²⁵ Systemic leakage following the ICO administration was also assessed. Flow cytometry of peripheral blood at multiple time points revealed minimal presence of FITC+ myeloid cells (Figure S2G). *Ex vivo* imaging at 24 h further demonstrated negligible NP accumulation in peripheral organs, in sharp contrast to the widespread distribution observed with i.v. delivery, highlighting low systemic exposure via the ICO route (Figure S2F).

Collectively, these results establish ICO injection as a feasible strategy to construct NP-loaded calvarial myeloid cells at brain boundaries, offering high efficiency and minimal systemic leakage under both homeostatic and pathological conditions.

NP-loaded calvarial myeloid cells migrate toward CNS border through the skull-meninges immune gateway

To determine whether the NP-loaded calvarial myeloid cells would subsequently migrate toward the CNS through the skull-meninges immune gateway,^{18,19} we performed *ex vivo* immunofluorescence (IF) imaging on mouse interparietal skulls after ICO injection. Consistent with the TEM findings (Figure 1G), optically cleared skulls showed successful *in vivo* labeling of myeloid cells, evidenced by colocalization of Gr1+ cells and FITC-NPs in the bone marrow of both sham and LPS-treated mice (Figure 1H). Based on the optically cleared skulls, the skull-meninges microchannels could be distinctly identified as the discontinuities of the inner table of the skull created by CD31-labeled blood vessels (Figures 2A, S3A, and S3B). In the sham control, occasional Gr1+ cells were seen traversing microchannels (Figure 2A, left). By marked contrast, however, LPS-treated mice showed pronounced accumulations along these pathways (Figure 2A, right). Quantification analysis further revealed a significant increase in both density and proportional distribution of Gr1+ signals along microchannels of the LPS-treated mice, as compared with the sham control (Figures 2B and 2C). These findings align with the early studies that show the distinct supply of the immune cells under homeostatic and pathological conditions by the adjacent skull bone marrow niches,¹⁸ suggesting feasibility to utilize these cells for responsive drug delivery to the CNS in disease settings.

Furthermore, we confirmed that the Gr1+ cells distributed along the microchannels were extensively colocalized with the NPs in both the LPS-treated mice and the sham control (Figures 2D–2F and S3C; Video S1). TEM further revealed the widespread presence of NPs inside these cells (Figure 2G), consistent with the high cell loading efficiency observed after ICO NP injections (Figure 1F). Although some NPs were observed dissociating outside cells (Figure S3D), the finding suggests that chemotactic cell migration represents an alternative naturally evolved pathway for delivery of the NPs to the CNS, complementing their simple diffusion to the brain surface via the physical microchannels.^{18,19}

In the majority of cases, these NP-loaded cells were observed to migrate toward the CNS through the perivascular space (Figures 2E and 2G).^{19,23} Occasionally, these cells were also observed entering the intravascular space (Figure S3E), which would get access to the systemic circulation.³⁵ Whole-mount dura imaging further confirmed the wide presence of NP-loaded Gr1+ cells within the dura mater following LPS-induced inflammation (Figures 2H, 2I, and S3F), providing direct evidence that calvarial immune cells can deliver NPs to CNS borders when responding to CNS perturbations.

Collectively, these findings reveal a CNS-targeting paradigm in which NPs leverage calvarial myeloid cells and skull-meninges trafficking pathways to reach CNS borders, representing an alternative to conventional systemic strategies limited by the BBB.

Calvarial myeloid cells promote targeted delivery to CNS lesions

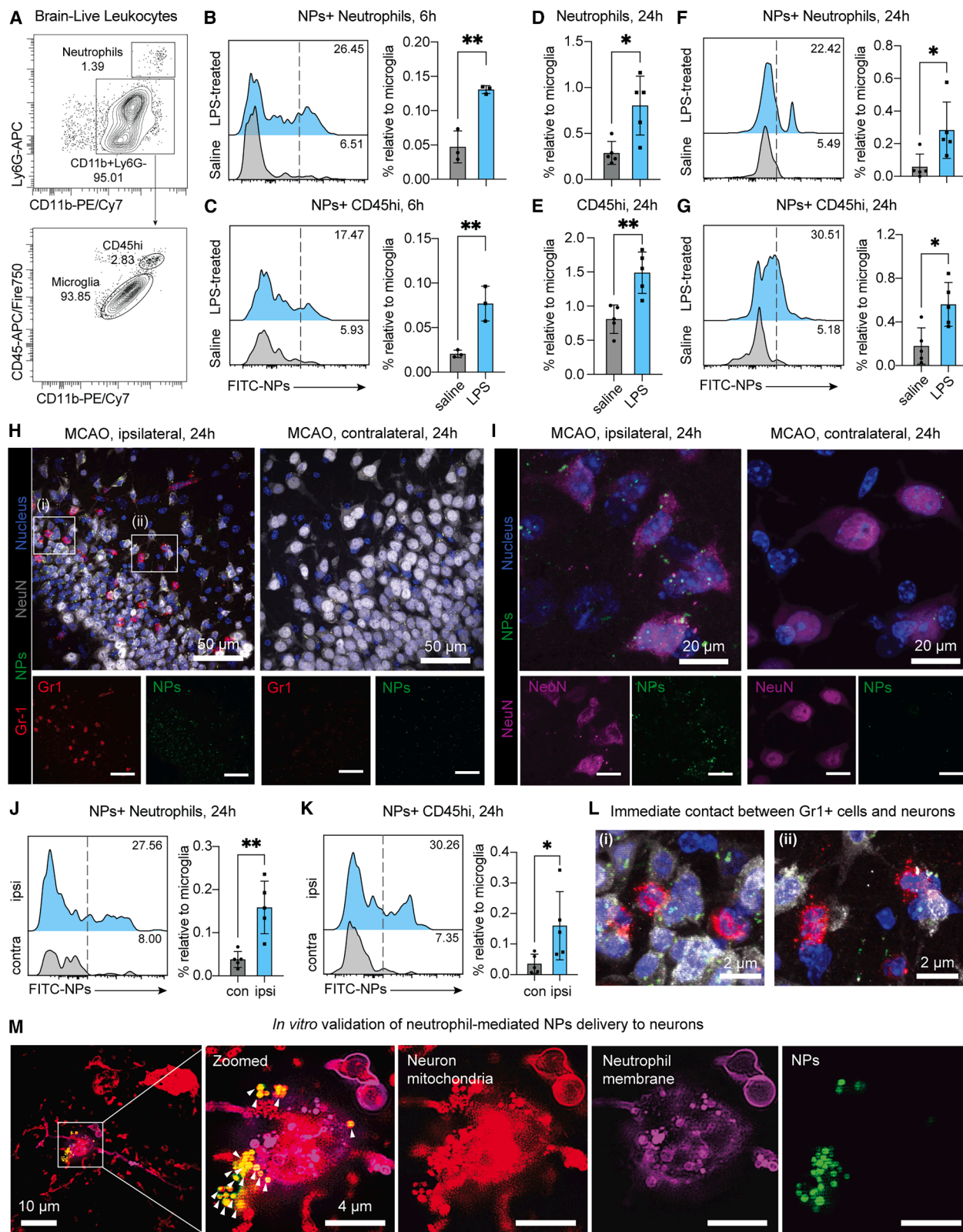
Having confirmed that the NP-loaded myeloid cells could migrate to CNS borders through the skull-meninges microchannels in response to CNS perturbations, we then ask whether they would deliver the NPs into the deep brain parenchyma. Flow cytometry of brains collected 6 h after ICO NP injection in LPS-induced neuroinflammatory mice revealed robust NP loading in brain-infiltrating myeloid cells, with a markedly higher proportion of NP-carrying cells compared with sham controls (Figures 3A–3C). By 24 h after LPS injection, this response became more pronounced. The numbers of neutrophils and CD45hi leukocytes were markedly elevated compared with sham mice (Figures 3D and 3E), resulting in a substantially increased ratio of NP-loaded myeloid cells to resident microglia (Figures 3F and 3G). These results together support the feasibility of leveraging calvarial myeloid cells to transport therapeutic cargos into the inflamed brain parenchyma.

To investigate whether these myeloid cells may achieve targeted delivery to CNS lesions, we employed an experimental MCAO model to simulate acute ischemic stroke and performed a combination of IF analysis and flow cytometry for validation. 24 h after ischemia onset, the decreased fluorescent signals of neuron-specific nuclear protein (NeuN) or microtubule-associated protein 2 (MAP-2) clearly delineated the ischemic region in the ipsilateral hemibrain (Figure S4A). Within these lesions, a substantial accumulation of the Gr1+ cells was observed, markedly higher than that of the contralateral side (Figures 3H and S4A). Similarly, FITC-NPs were observed more frequently concentrated in the ipsilateral cerebral cortex instead of the contralateral side (Figures 3H and 3I). By marked contrast, in sham-operated mice, both the Gr1+ cells and FITC-NPs exhibited minimal infiltration into the brain parenchyma, with no significant difference in their distributions between the two hemibrains (Figures S4B and S4C). Flow cytometry further confirmed these observations, revealing a significantly higher accumulation of the NP-loaded Ly6G+ neutrophils and CD45hi cells in the

(G) Representative TEM images of the NP-loaded bone marrow cells migrating through the perivascular space of skull-meninges microchannels.

(H and I) Representative image (H) and magnified view (I) showing the presence of NP-loaded Gr1+ cells in the dura. The white arrows indicate the Gr1+ cells co-stained with FITC-labeled albumin NPs.

See also Figure S2.



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ipsilateral side (Figures 3J and 3K). The data indicate a strong correlation between the targeted delivery of NPs and the tropism of myeloid cells for the CNS lesion.

In these brain lesions, we further confirmed the effective delivery of the NPs into the affected neurons, as evidenced by the presence of abundant NPs within the NeuN-labeled cells (Figure 3I). Concurrently, Gr1⁺ cells were observed in immediate contact with neurons (Figure 3L). To further explore whether the immune cells may facilitate this neuronal uptake, we co-cultured NP-loaded neutrophils with mouse primary neurons *in vitro*. These experiments recapitulated the *in vivo* phenomena, showing close contact between neutrophils and neurons and the presence of the NPs within neurons (Figures 3M and S4D). A time course analysis further shows that co-culturing neurons with NP-loaded neutrophils could lead to neuronal internalization of NPs within a few hours (Figure S4D), comparable to that observed under conditions of direct exposure to higher NP concentrations (Figure S4E). Together, these observations suggest a potential role of neutrophils in facilitating NP transfer to neurons; however, the underlying mechanisms remain uncertain and require more investigation.

Collectively, these findings demonstrate the important role of calvarial myeloid cells in the targeted delivery to neurons in the CNS lesions.

Therapeutic calvarial myeloid cells alleviate acute ischemic stroke

We next sought to investigate whether the BBB-bypassing drug delivery strategy, which leverages the skull-meninges immune gateway and the chemotactic migration of calvarial myeloid cells, could be utilized to alleviate CNS disorders. We selected eicosapeptide nerinetide (Tat-NR2B9c or NA1), a promising i.v.-administered neuroprotectant in a phase 3 clinical trial, which may reduce infarct volume and preserve neurological function after stroke, as a test drug to verify the delivery and therapeutic efficacy in MCAO mice.^{28,36} NA1 was surface-loaded onto albumin NPs via electrostatic interaction between its positive charge and the negatively charged NP surface (Figure S1G). High-performance liquid chromatography showed encapsulation efficiencies above 85% across doses (Figure S1I), and DLS confirmed minimal size change and stable surface potential

(>−20 mV) after loading, indicating good colloidal stability (Figure S1H).

To evaluate both the therapeutic efficacy and dose-response relationship of NA1 NPs delivered via ICO injection, we administered the formulations immediately after ischemia-reperfusion (Figure 4A). As a positive control, NA1 was given i.v. at a validated effective dose of 10 nmol/g in mice.³⁷ Additionally, to compare the cell-mediated delivery strategy with direct ICO injection (diffusion-based delivery), free NA1 was administered at an optimized dose equivalent to one-half of the i.v. dose.²⁹ For the NA1 NPs group, the administered doses were adjusted to 1/30 to 1/3.75 of the i.v. dosage.

Before stroke induction, physiological parameters did not differ among the groups. At 24 h after the ischemia onset, 2,3,5-triphenyltetrazolium chloride (TTC) staining revealed a marked reduction in infarct volume in mice treated with either NA1 or NA1 NPs compared with the placebo, with decreases of at least 25.6% (Figures 4B, 4C, S5A, and S5B). Notably, all cohorts administered with the NA1 NPs via ICO injection showed greater reductions in infarct volume compared with those receiving i.v. NA1, with the 1/15 dosage group achieving the highest efficacy, exhibiting an additional 26.7% decrease in infarct size and improved neurological functions (Figures 4B, 4C, and S5A–S5C). Thus, the 1/15-dose regimen was selected for subsequent studies.

To evaluate the therapeutic time window, we administered NA1 NPs at delayed time points of 4.5 and 6 h after the stroke onsets. Strikingly, while ICO delivery of NA1 NPs at 6 h conferred no therapeutic benefit, administration at 4.5 h still yielded significant neuroprotection (Figures S5D and S5E). By contrast, i.v. administration of NA1 did not achieve significant efficacy at either delayed time point, likely due to limited drug penetration across the BBB and insufficient accumulation at the ischemic site.²⁹ These findings highlight the advantage of the ICO route in maintaining efficacy within a clinically relevant therapeutic window (Figures S5D and S5E).

For long-term evaluation, we performed continuous magnetic resonance imaging (MRI) analysis and behavioral observations over a period of up to 28 days. Notably, TTC staining at 24 h showed comparable infarct volumes between ICO-NA1 NPs and free NA1 administered via ICO injection, consistent with the prior reports highlighting the hyperacute neuroprotective

Figure 3. Calvarial myeloid cells promote targeted delivery to CNS lesions

(A) Gating strategy of skull-derived neutrophils and CD45^{hi} cells in brain parenchyma.

(B and C) Representative flow data (left) and the statistic (right) of the percentage of NP-loaded neutrophils (B) and CD45^{hi} cells (C) in the brain of LPS-treated mice and sham mice relative to native microglia 6 h after LPS injection. *n* = 3 biological replicates for each group.

(D and E) Statistics of percentage of neutrophils (B) and CD45^{hi} cells (C) in the brain relative to native microglia. *n* = 5 biological replicates for each group.

(F and G) Representative flow data (left) and the statistic (right) of the percentage of NP-loaded neutrophils (F) and CD45^{hi} cells (G) in the brain of LPS-treated mice and sham mice relative to native microglia 24 h after LPS injection. *n* = 5 biological replicates for each group.

(H and I) Representative IF images of the ipsilateral (left) and contralateral (right) hemibrain of MCAO mice 24 h after stroke onset showing the targeted migration of Gr1⁺ cells and targeted accumulation of NPs.

(J and K) Representative flow data (left) and the statistic (right) of the percentage of NP-loaded neutrophils (J) and CD45^{hi} cells (K) in the contralateral hemibrain and ipsilateral hemibrain relative to native microglia. *n* = 5 biological replicates for each group.

(L) Enlarged images in (H, left) show the immediate contact between Gr1⁺ cells and neurons.

(M) Representative IF images of primary mouse neurons 12 h after co-incubation with NP-loaded neutrophils showing the neutrophil-mediated NPs delivery to neurons. The white triangles indicate the co-staining of NPs and neurons.

Data are mean ± SD. Unpaired *t* test. **p* < 0.05 and ***p* < 0.01.

See also Figures S2 and S4.

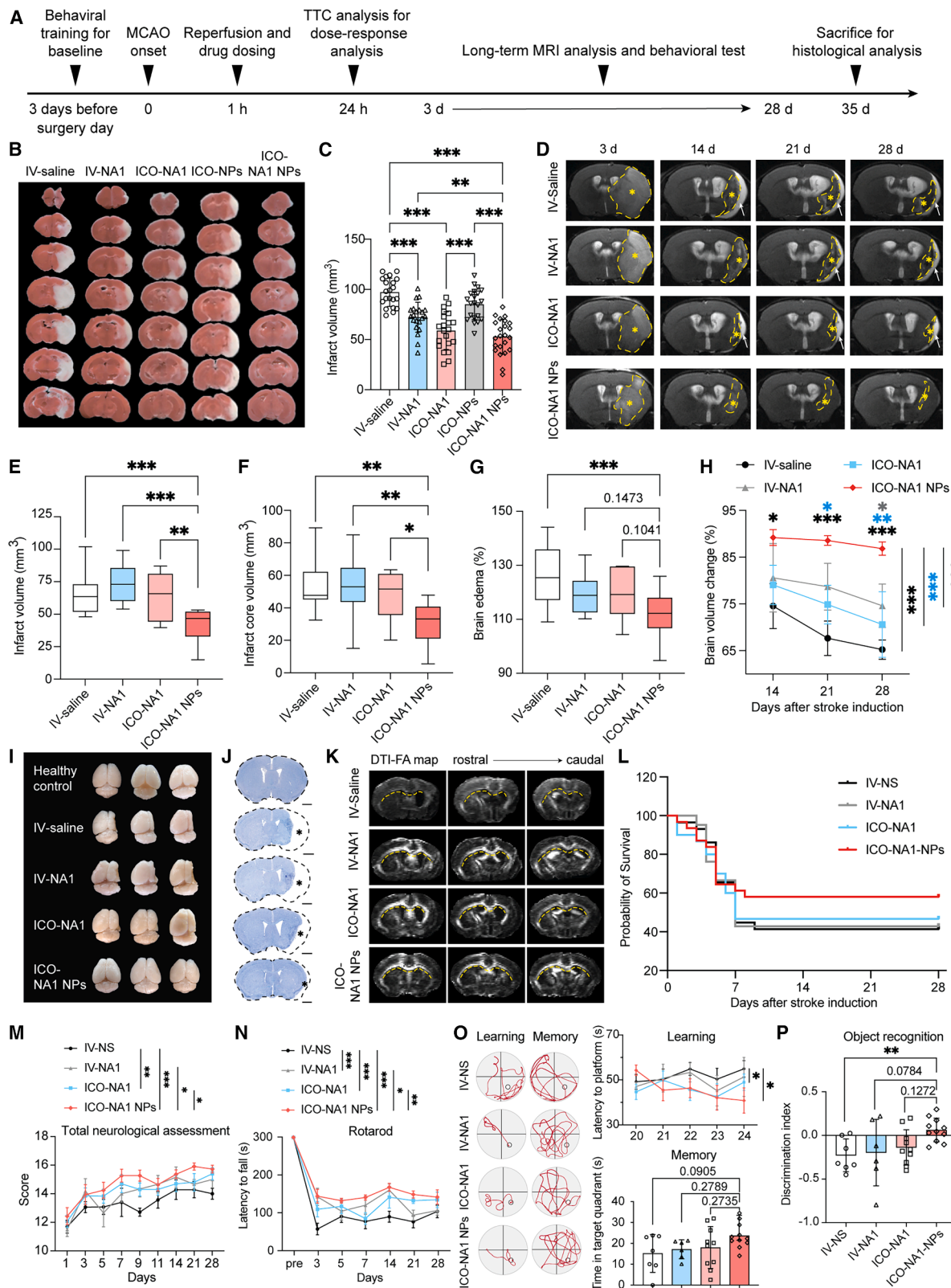


Figure 4. Therapeutic calvarial myeloid cells alleviate acute ischemic stroke

(A) A time scheme of the therapeutic evaluation study.

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effect of NA1.^{29,36,37} By 72 h, however, T2-weighted MRI and TTC staining revealed markedly smaller ischemic regions in the ICO-NA1 NP group compared with all other treatments (Figure S5F). Apparent diffusion coefficient (ADC) imaging further demonstrated the improved therapeutic efficacy of the ICO-administered NA1 NPs in relieving infarct cores over free NA1, regardless of the delivery route (Figure 4F). Moreover, ICO-NA1 NPs significantly alleviated stroke-induced cerebral edema compared with other groups (Figures 4D and 4G), underscoring their multifaceted neuroprotective capabilities during the acute phase. By contrast, i.v.-NA1 and ICO-NA1, while effective at 24 h, failed to confer these protections at 72 h, likely reflecting the transient nature of free NA1 due to rapid clearance rather than sustained release from NPs.^{29,38,39} To assess this, we quantified the NA1 concentration in the brain with liquid chromatography tandem mass spectrometry (LC-MS/MS) 24 h after stroke induction. The results show that the NA1 NPs delivered by ICO route achieved the highest NA1 concentrations in the brain even at substantially lower dosages, along with markedly higher accumulation in the ipsilateral hemibrain compared with the contralateral side (Figure S4F), confirming both the efficiency and targetability of ICO-delivered NA1 NPs. In addition, brain-to-organ ratios, particularly brain-to-spleen, brain-to-lung, and brain-to-kidney, were markedly higher with ICO-NA1 NPs than with other treatments (Figures S4G–S4J), further indicating enhanced CNS selectivity and reduced systemic exposure. In the follow-up imaging observations up to 28 days, these beneficial effects persisted, collectively highlighting their durable therapeutic efficacy in ameliorating ischemic stroke (Figure 4D).

To estimate the long-term neuroprotective effect on tissue damage, we assessed brain atrophy and white matter integrity up to 28 days post-stroke (Figures 4H–4K). We quantified brain atrophy by measuring volumetric reduction of tissues on the T2-weighted images on 14, 21, and 28 days after stroke. The data indicate that ICO administration of NA1 NPs alleviates brain atrophy more effectively than free NA1 administered intravenously or intracalvariosesously, suggesting its long-term histological neuroprotective effects (Figure 4H). More impressively, 35 days after the stroke induction, the mouse brains in the NA1

group administered either intravenously or intracalvariosesously showed severe brain atrophy on the lesional side, akin to those in the placebo group. By marked contrast, ICO delivery of the NA1 NPs significantly reduced stroke-induced brain atrophy, with some cases even demonstrating brain integrity indiscernible from that of the healthy control (Figures 4I and 4J). Diffusion tensor imaging (DTI) further showed that ICO-NA1 NPs preserved corpus callosum integrity, maintaining continuous fraction anisotropy (FA) signals and fiber tracts, in contrast to the disrupted architecture seen in saline or free NA1 groups (Figure 4K). Ultimately, 35 days after stroke induction, NA1 NP-treated MCAO mice demonstrated an improved survival rate compared with the MCAO mice treated with either i.v. or direct ICO injection of free NA1 (Figure 4L).

Throughout the 28-day observation period, we also conducted neurological assessments using neurological severity scores (NSSs) and behavioral evaluations, including the rotarod test, water maze test, and object recognition test. Animals treated with ICO-NA1 NPs demonstrated improved NSS scores from the initial 24 h after ischemia onset and throughout the 28-day period (Figure 4M). This group also presents significantly better performance in the rotarod task than other groups during the continuous observations (Figure 4N). We also confirmed that NA1 NPs administered through skulls significantly improved the performance of mice in the water maze and object recognition tasks (Figures 4O and 4P). Improvements in learning, memory, and object discrimination indicated an evident overall enhancement in cognitive ability (Figures 4O and 4P).

Given that peripheral circulating myeloid cells can infiltrate the brain after stroke and serve as potential carriers for cell-mediated drug delivery,^{5,6,40} we also evaluated the therapeutic efficacy of i.v. administered NA1 NPs. Notably, the ICO group received only 1/15 of the NA1 dose used in the i.v. group. Despite the lower dosage, animals in the ICO-NA1 NPs group exhibited markedly smaller infarct volumes compared with the i.v.-NA1 NPs group, as shown by TTC staining (Figure S5F). MRI analyses corroborated these findings, revealing significantly smaller infarct volumes and reduced perilesional edema as early as day 3, confirming a more effective neuroprotective response (Figures S5G and S5H). These advantages persisted up to

(B and C) Representative TTC-stained sections (B) and statistics of the infarct volume (C) 24 h after stroke induction. $n = 21$ –23 biological replicates for each group.

(D) Representative T2-weighted images at different time points after stroke induction. The yellow dotted circles indicate the lesioned area. The white arrows indicate the lost brain tissue.

(E–G) Statistics of the infarct volume (E), infarct core (F), and brain edema (G) 3 days after stroke induction. $n = 11$ –13 biological replicates for each group.

(H) Brain volume change after stroke induction. $n = 3$ –7 biological replicates for each group.

(I and J) Representative images of dissected mouse brain (I) with different treatments 35 days after stroke induction and their Nissl's stained sections (J). The blank area in (J) indicated by the asterisk represents the lost brain tissue based on mirroring the contralateral side. Scale bar, 1 mm.

(K) Representative DTI axial views of FA map acquired 28 days after stroke induction. The yellow dotted lines indicate the corpus callosum.

(L) Survival curve of MCAO mice with different treatments.

(M and N) Sensorimotor functions assessed by the neurological deficit assessment (M) and the rotarod test (N). $n = 14$ –16 biological replicates for each group.

(O and P) Cognitive functions assessed by the Morris water maze test (O) and the object recognition test (P). $n = 6$ –11 biological replicates for each group.

Data are presented as mean $\pm$ SD (C, O [bottom], and P), mean $\pm$ SEM (H, M, N, and O [top]), or median (minimum, maximum) (E–G). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by one-way ANOVA with Tukey's (C) or Dunnett's multiple comparisons test (E–G, O [bottom], and P) or by two-way ANOVA with Sidak's multiple comparisons test (H, M, N, and O [top]).

See also Figures S4 and S5.

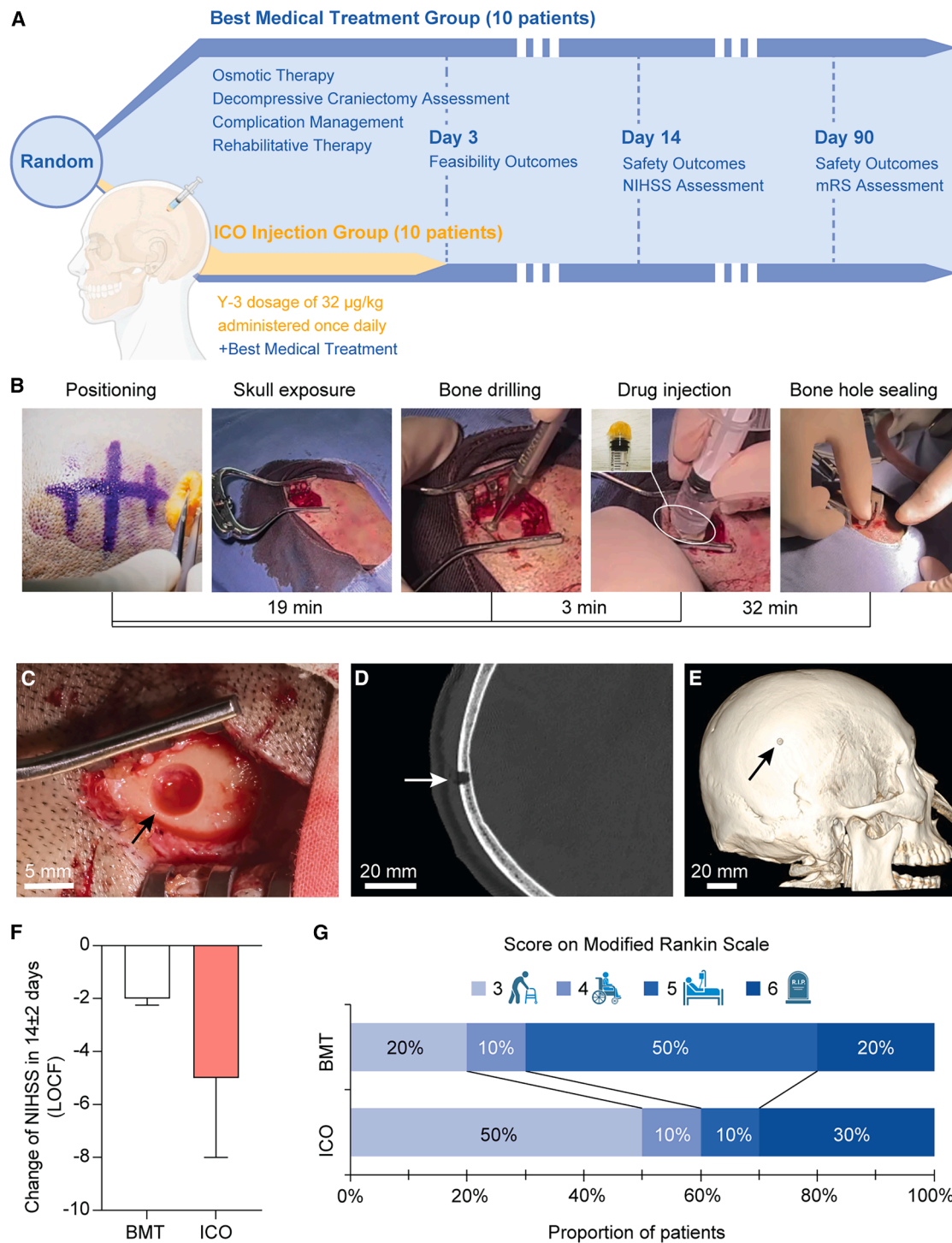


Figure 5. Clinical feasibility, safety, and exploratory efficacy of ICO drug administration in mMCAI patients

(A) Schematic of study design and treatment allocation.

(B) Major steps and duration of the ICO protocol. The head cone of the syringe was covered by three layers of foam dressing, indicated by the white circle.

(C) The drilled hole (black arrow) on the surgical field of the skull.

(D) CT image showing the drilled hole (white arrow) on the skull. The drilling only penetrated the outer plate of the skull without affecting the inner plate.

(legend continued on next page)

28 days, with the ICO group showing consistently reduced secondary tissue loss and improved survival rates compared with the i.v.-treated animals (Figures S5I and S5J), likely owing to the high and sustained drug accumulation in the ischemic brain during the early acute phase (Figure S4F).

Taken together, these data demonstrate the promising potential of the ICO administration of drug-loaded NPs for CNS drug delivery and disease alleviation.

Clinical feasibility and safety of ICO drug administration in mMCAI patients

To assess the translational potential of ICO delivery, we conducted the first-in-human SOLUTION trial (ClinicalTrials.gov number, NCT05849805), a prospective, randomized, investigator-initiated pilot study designed to assess the feasibility, safety, and exploratory efficacy of ICO administration in patients with mMCAI. Between April 14, 2023, and November 6, 2023, 20 patients with mMCAI were enrolled and randomized 1:1 to receive either best medical treatment (BMT) alone or BMT plus ICO injection of Y-3, a neuroprotective agent approved by the China National Medical Products Administration, for 3 days (Figure 5A). The demographic characteristics of the two groups were mostly the same (Table S1), and both groups received similar treatments aside from ICO injection. The primary outcome was set to evaluate the feasibility and safety of ICO injection. Secondary outcomes encompassed exploratory efficacy outcomes such as the score on the modified Rankin scale (mRS) at 90 days or the National Institutes of Health Stroke Scale (NIHSS) score at multiple time points.

The procedural workflow for ICO injection is illustrated in Figure 5B. The operation consists of five sequential steps: (1) positioning and preoperative marking, (2) skin incision and skull exposure, (3) drilling through the outer table to access the diploic space, (4) drug administration using a syringe, and (5) sealing of the cranial opening with bone wax. The second- and third-day injections utilized the pre-drilled holes. The median duration of the entire procedure with drilling was 32 min, while follow-up injections required only 22 min, indicating its acceptable procedure duration for time-sensitive treatment (Table S2). Representative intraoperative and radiographic images confirmed precise drilling limited to the outer table, with an intact inner plate verified by CT and 3D reconstruction (Figures 5C–5E). The procedure was well-tolerated, with no intraoperative complications such as inner plate penetration or drug leakage throughout the study (Table S2).

Safety outcomes over the 90-day follow-up period were comparable between groups. One ICO-treated patient was diagnosed with meningitis at day 75, considered secondary to decompressive craniectomy by the clinical event committee. Symptomatic and non-symptomatic intracranial hemorrhages were reported in 7 cases in the BMT group and 4 in the ICO group (relative risk, 0.57; 95% confidence interval [CI], 0.24–1.35; $p = 0.37$) (Table S3). All patients completed follow-up, while two fatal

cases were reported in the BMT group and three fatal cases were reported in the ICO group due to stroke-related or other causes. No statistically significant differences in safety events and serious adverse events of various organ systems were observed between the two groups (Tables S3, S4, and S5).

In terms of exploratory efficacy outcomes, the ICO group demonstrates a reduction in NIHSS score at day 14 compared with the BMT group under the last observation carried forward (LOCF) analysis, which includes all patients (median -5 versus -2 , $p = 0.65$) (Figure 5F; Table S6). Only one patient in the BMT group shows a reduction in NIHSS score greater than 4 points, whereas this proportion reached 60% in the ICO group (relative risk, 6.0; 95% CI, 0.87–41.21; $p = 0.057$). The mRS scores at 90 days are presented in Figure 5G and Table S6, showing no statistically significant difference but providing exploratory data for evaluating potential efficacy.

These findings provide evidence regarding the feasibility and safety profile of ICO administration, thereby establishing a foundation for its further clinical translation.

DISCUSSION

Efficacious drug delivery to the CNS remains a daunting challenge due to the restriction of the BBB. This study tackled the challenge by hijacking calvarial immune cells and leveraging their unique migration mechanism to bypass BBB for enhanced and targeted drug delivery. We identified the feasibility and efficacy of the *in situ* construction of NP-loaded calvarial immune cells and their unique brain-targeting delivery mechanism through the skull-meninges microchannels. This mechanism enables direct delivery to brain lesions without crossing BBB. The model payloads, delivered using this approach, were confirmed to be able to target the lesion sites and led to clear improvement in both short- and long-term outcomes after acute ischemic stroke. Furthermore, the clinical feasibility and safety of the ICO procedure were validated in a pilot clinical trial, supporting its translational potential. Together, these findings establish a clinically translatable platform that harnesses brain-bordering immune pathways to bypass the BBB and achieve enhanced CNS therapy.

A clear advantage of this drug delivery strategy is the complete circumvention of the need to cross the BBB, combined with the rapid responsiveness and active tropism for brain lesions of calvaria-derived immune cells. Compared with conventional cell-mediated delivery methods reported in the open literature,^{4–6} the proposed approach can reduce off-target effects and safety concerns associated with systemic administration through a minimally invasive, local delivery strategy and does not require complex *ex vivo* cell preparation.^{10–12} While i.v. infusion of the cell-targeted NPs can construct drug-loaded cells *in vivo*,⁴⁰ the fluid dynamics and dilution effect during systemic circulation significantly limit the delivery efficiency (Figures S4F–S4J) and therapeutic efficacy (Figures S5F–S5J). By contrast, our

(E) Three-dimensional reconstruction of the skull CT scan showing the drilled hole (black arrow, patient 7 in the ICO group).

(F) Median and interquartile range of change of NIHSS score in 14 ± 2 days. Mortality cases' 14-day NIHSS score was assigned by LOCF. $n = 10$ patients for each group.

(G) Score on the mRS at 90 ± 7 days after onset of the symptoms among patients. BMT, best medical treatment; ICO, intracalvariosseous.

See also Tables S1–S3, S4, S5, and S6.

approach constructs drug-loaded cells *in situ* by directly injecting drug-loaded albumin NPs into the skull bone marrow, a low-liquidity environment enriched with immune cells in the brain borders.^{14,30} These immune cells, which are entrusted for CNS surveillance, respond specifically to the CNS perturbation and deliver the drugs to the lesion site. Importantly, this method avoids the introduction of exogenous immune cells, potentially reducing the risk of immune-related complications.^{11–13} Although the current therapeutic validation focuses on acute ischemic stroke and primarily engages myeloid cells, the underlying delivery strategy—based on the BBB-bypassing and lesion-targeted mobilization of skull-derived, brain-bordering immune cells—is mechanistically conserved and flexibly extensible. Whether similar immune-cell-mediated delivery dynamics occur in other neurological conditions remains to be determined.^{14,18,30,41}

Additionally, our strategy lies in utilizing NPs for cell hijacking, which may enhance the applicability of this method for various therapeutic agents. While direct ICO drug injection can enhance cerebral drug accumulation when compared with systemic administration,^{29,38,39} its therapeutic efficacy remains limited by two major factors: meningeal barriers that restrict drug entry into the brain parenchyma^{29,38,39} and the reliance on glymphatic diffusion, which suffers from limited penetration depth and poor lesion specificity.^{15,17,42} By contrast, our approach utilizes NPs not only as carriers of therapeutic payloads but also as key mediators to guide the phagocytic process by calvarial immune cells in order to leverage their active tropism for brain lesions to ensure effective drug delivery into the deep brain and reduce off-target effects on healthy tissues. In the case of acute ischemic stroke, we demonstrated that the ICO route enabled targeted accumulation of NPs and therapeutic payloads within ischemic lesions, achieving robust therapeutic efficacy with only 1/15 of the i.v. dose (Figures 5 and S5). This underscores the efficiency of the approach and suggests its potential for other drugs with limited BBB permeability. In addition, the sustained-release properties of NA1 NPs enable prolonged drug exposure in the brain throughout the acute phase of ischemic stroke (Figure S4F), which may mitigate reperfusion-induced secondary injury and ongoing neuroinflammatory damage. This extended CNS bioavailability, together with targeted delivery mediated by skull marrow-derived immune cells, likely contributes to enhanced and more durable neuroprotection compared with free NA1, underscoring the advantage of ICO-NA1 NPs in maintaining therapeutic efficacy during the critical early post-stroke period (Figures S4E and S4F). This NP-guided technique may also offer a unique platform for a promising spectrum of pharmaceuticals, with potential for multiple drug delivery or stimulus-responsive release to cater to different clinical demands.

Several strategies could be explored in the future to improve the brain accumulation and lesion-targeting effects of drugs by these therapeutic calvarial immune cells. One approach could involve specific modifications of the NPs to optimize selective cellular loading and improve drug payload efficiency.^{4,26} In addition, these calvarial immune cells could be engineered *in situ* to enhance their specificity for pathological sites.^{43,44} External field-responsive mechanisms could also be explored to enable controlled migration or drug release, providing greater precision in therapeutic delivery.⁵

In summary, this study demonstrates the promising strategy that hijacks calvarial immune cells and the skull-meninges immune shortcut for targeted CNS drug delivery and stroke alleviation. Although we focused here on drug delivery to the brain, this approach may be applied to deliver functional nanotransducers for future-generation neuro-interfacing^{45,46} or engineered immune cells, such as chimeric antigen receptor T cell (CAR-T),¹¹ for immunotherapy. The approach may also be extended to advanced therapeutic modalities, such as closed-loop feedback regulation devices that respond to neurological or pathological signals, for timely, sustained, or controlled CNS therapy.^{47,48} These qualities prompt further exploration of hijacking calvarial immune cells to circumvent the CNS delivery barrier, potentially opening a paradigm for targeted delivery for CNS disorders.

Limitations of the study

While this study demonstrates a promising strategy for CNS drug delivery via skull marrow-derived immune cells, several limitations warrant further investigations. First, this study focuses on myeloid cell-mediated delivery in acute stroke and neuroinflammatory models. The involvement of other immune cell types and the generalizability of this mechanism across different CNS diseases remain to be determined. Second, the mechanism underlying the neutrophil-mediated NP transfer to neurons remains to be further defined. Our findings demonstrate intimate interactions between neutrophils and neurons and support the possibility that immune cells facilitate neuronal NP access; however, the precise route of transfer remains elusive. Future studies employing real time, high-resolution imaging and molecular tracing will be crucial to delineate these dynamic processes and clarify how the immune cells contribute to neuronal NP delivery. Third, although our histological and imaging analyses confirmed a clear discrepancy in neuroprotective outcomes between i.v. NA1 and ICO NA1 NP administration at 24 h and later time points, and our tissue drug quantification suggests that this divergence may stem from distinct acute-phase pharmacokinetic behaviors of free NA1 versus NA1 NPs in metabolically active mice, the underlying mechanisms may still require further elucidation. In addition, while ICO injection has proven safety and effectiveness in both preclinical models and clinical studies,²⁹ technical improvements are required to enhance procedural precision, reduce invasiveness, and enable repeated dosing. The current clinical approach requires drilling through the skull, which is feasible but still invasive. Future work may therefore prioritize the development of minimally invasive injection devices to support wider clinical usages. In parallel, optimizing dosing protocols and conducting comprehensive risk-benefit assessments will be essential. Finally, interspecies differences in skull marrow anatomy and immune responses may affect translation. Thus, validation in large-animal models with long-term follow-up is needed to better evaluate safety, bio-distribution, and efficacy under clinically relevant conditions.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Yilong Wang (yilong528@aliyun.com).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

Original data related to this paper were deposited at Mendeley at <http://doi.org/10.17632/zygw533zx.2>. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (62350710211), the National Key Research and Development Program of China (2023YFB4705500), the National Natural Science Foundation of China (T24B2008, 62173200, and 82425101), the Noncommunicable Chronic Diseases-National Science and Technology Major Project (2023ZD0504800, 2023ZD0504801, 2023ZD0504802, 2023ZD0504803, and 2023ZD0504804), the Beijing Municipal Science & Technology Commission (Z2311000048 23036), the Capital's Funds for Health Improvement and Research (2022-2-2045), the National Key Research and Development Program of China (2024YFC3044800, 2022YFF1501500, 2022YFF1501501, 2022YFF1501502, 2022YFF1501503, 2022YFF1501504, and 2022YFF1501505), and the Shuimu Tsinghua Scholar Program (2025SM234). We thank Dan Zhang, Jiahuan Lian, Bin Liang, and Pengcheng Jiao from the Core Facility of the Center of Biomedical Analysis of Tsinghua University for technical support with tissue immunolabeling, tissue clearing, lightsheet imaging, confocal imaging, image processing, and flow cytometry analysis. We thank Yan Yang, Chenguang Zhao, Qian Li, and Yanfei Hu from the Cell Biology Facility affiliated with the Center of Biomedical Analysis of Tsinghua University for technical assistance with TEM sample preparation. We thank Weihua Wang and Fang Wei from the Center of Pharmaceutical Technology of Tsinghua University for the technical support with high-performance liquid chromatography (HPLC) and LC-MS/MS analysis. We are grateful to Dr. Ziming Xu from the Center for Biomedical Imaging Research of Tsinghua University for the technical assistance of magnetic resonance imaging and data analysis.

AUTHOR CONTRIBUTIONS

Conceptualization, X.G., Mingjun Zhang, and Y.W.; methodology, X.G., X.L., N.W., C.C., W.L., M.Y., Mingjun Zhang, and Y.W.; investigation, X.G., X.L., N.W., C.C., W.L., M.Y., Q.L., Y.O., A.N., Meiyang Zhang, X.W., S.Q., Y.L., D.F., H.L., L.S., and M.L.; writing—original draft, X.G., N.W., and Mingjun Zhang; writing—review and editing, X.G., N.W., M.Y., and Mingjun Zhang; funding acquisition, X.G., Mingjun Zhang, and Y.W.; resources, Mingjun Zhang and Y.W.; and supervision, Mingjun Zhang and Y.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors did not use AI tools or services.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- [KEY RESOURCES TABLE](#)
- [EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS](#)
 - Animals
 - Primary cell cultures
 - Human participants
- [METHOD DETAILS](#)
 - Nanoparticle preparation and characterization
 - ICO injection in mice

- *In situ* construction of NP-loaded myeloid cells
- LPS induced neuroinflammation
- Ischemic stroke induction
- Tissue harvest and processing
- Transmission electron microscopy
- Immunofluorescence staining and imaging
- Tissue clearing and imaging
- Single cell harvest for flow cytometry
- *In vitro* co-incubation of neurons and NP-loaded neutrophils
- *In vitro* co-incubation of neurons and fluorescent NPs
- LC-MS/MS
- Histological and MRI therapeutic evaluation
- Behavioral assessments
- Randomization and Interventions of clinical trial
- ICO injection in patients
- Clinical outcomes

- [QUANTIFICATION AND STATISTICAL ANALYSIS](#)
- [ADDITIONAL RESOURCES](#)

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cell.2025.12.008>.

Received: February 1, 2025

Revised: November 16, 2025

Accepted: December 5, 2025

Published: January 16, 2026

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-mouse Ly-6G/Ly-6C (Gr1) (RB6-8C5) monoclonal antibody	Cell Signaling Technology	CAT# 31469; RRID:AB_2888647
Rat anti-mouse Ly-6G monoclonal antibody	Biolegend	CAT# 127601; RRID: AB_1089179
Goat anti-rat IgG H&L AF647 polyclonal antibody	Abcam	CAT# ab150167; RRID: AB_2864291
Rabbit anti-mouse CD31 polyclonal antibody	Abcam	CAT# ab182981; RRID: AB_2920881
Goat anti-rabbit IgG AF568 polyclonal antibody	Abcam	CAT# ab175471; RRID: AB_2576207
Donkey anti-rabbit AF647 plus polyclonal antibody	Thermo Fisher Scientific	CAT# A32795; RRID: AB_2762835
Rat anti-mouse MAP2 polyclonal antibody	Abcam	CAT# ab32454; RRID: AB_776174
Rabbit anti-mouse NeuN (D3S3I)	Cell Signaling Technology	CAT# 12943; RRID: AB_2630395
Rat anti-mouse NeuN monoclonal antibody	Abcam	CAT# ab279297; RRID: AB_3095692
APC rat anti-mouse Ly-6G antibody	Thermo Fisher Scientific	CAT# 17-9668-82; RRID: AB_2573307
APC/Fire 750 rat anti-mouse CD45 antibody	Biolegend	CAT# 103153; RRID: AB_2572115
PE/Cyanine7 rat anti mouse CD11b antibody	Biolegend	CAT# 101215; RRID: AB_312798
eFluor450 rat anti-mouse Ly-6C antibody	Thermo Fisher Scientific	CAT# 48-5932-80; RRID: AB_10805518
Chemicals, peptides, and recombinant proteins		
Fixable Viability Dye eFluor™ 506	Thermo Fisher Scientific	CAT# 65-0866-14
Zombie NIR	Biolegend	CAT# 423105
NHS-Fluorescein	Thermo Fisher Scientific	CAT# 46410
Hoechst 33342	Thermo Fisher Scientific	CAT# H3570
DiD	MCE	CAT# HY-D1028
MitoTracker	Solarbio	CAT# M9940
Multi Tissue Dissociation Kit 1	Miltenyi	CAT# 130-110-201
Debris Removal Solution	Miltenyi	CAT# 130-109-398
Bovine Serum Albumin	Macklin	CAT# B824162
2% TTC solution	Macklin	CAT# T775774
Red blood cell lysis buffer	Biolegend	CAT# 420301
Nerinetide, YGRKKRRQRRRLSSIESDV	Nanjing Aite Biotechnology Co., Ltd	N/A
Y-3	Neurodawn Pharmaceutical Co., Ltd	https://www.neurodawn.cn/sy
aniline-suc-KLVFFC	Nanjing Aite Biotechnology Co., Ltd	N/A
Lipopolysaccharides from E.coli O111:B4	Sigma-Aldrich	CAT# L4391
Experimental models: Cell lines		
Primary mouse cortical neuron	C57BL/6 mouse embryos at embryonic day 15	N/A
Deposited data		
Preclinical and clinical dataset	This paper	Mendeley Data: http://doi.org/10.17632/zyygw533zx.2
Software and algorithms		
Graphpad Prism 10	GraphPad	https://www.graphpad.com/
Imaris 10.2	Oxford Instruments	https://imaris.oxinst.com/
Fiji/ImageJ	Schindelin et al. ⁴⁹	http://imagej.net/Fiji;https://imagej.nih.gov
ITK-snap	Yushkevich et al. ⁵⁰	https://www.itksnap.org/pmwiki/pmwiki.php?n=Main.HomePage
SAS 9.4	SAS Institute	https://www.sas.com/en_us/home.html

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SCIEX OS 3.1.6	SCIEX	https://sciex.com
FlowJo 10	BD Biosciences	https://www.flowjo.com

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**Animals**

All animal procedures were approved by and performed according to the animal care and ethics committee of Tsinghua University (22-ZMJ1.G23-1) and Beijing Tiantan Hospital (202102001). All animal experiments used male C57BL/6 mice and female C57BL/6 mice at 15 days of pregnancy, which were purchased from Laboratory Animal Resources Center of Tsinghua University and Vitalriver Co., Ltd. Mice are maintained under standard housing conditions (12 light/12 dark cycle, 22–24 °C with humidity set at 40–50%).

Primary cell cultures

Primary cortical neurons were isolated from C57BL/6 mouse embryos at embryonic day 15. Pregnant mice were euthanized, and embryos were harvested under sterile conditions. The bilateral cerebral cortices were dissected, minced, and enzymatically dissociated using a standard digestion protocol. Dissociated neurons were seeded at a density of 1×10^6 cells/mL onto poly-D-lysine (PDL)-coated confocal culture dishes (coated overnight at 4°C). Four hours after plating, the medium was completely replaced to remove debris and unattached cells. Cultures were maintained with half-medium changes every three days and used for experiments on day 7 *in vitro*.

Human participants

The trial enrolled patients aged 18–75 years old without gender restriction and scored 16 to 30 on the National Institutes of Health Stroke Scale (NIHSS, ranges from 0 to 42, higher scores indicating more severe neurological deficits). Patients also met the inclusion criteria that they had a core infarction volume (defined as relative cerebral blood flow [rCBF] <30% in CT perfusion [CTP]) occupying more than a half of the MCA territory or an Alberta Stroke Program Early CT Score (ASPECTS, a scale from 0–10 to measure infarct size, with lower scores reflecting larger infarcts) of ≤ 6 . All the patients were routinely screened for eligibility of reperfusion therapy within 24 hours. Patients who underwent reperfusion therapy were still eligible if they had poor treatment outcomes (defined as decreased NIHSS score or the improvement of post-treatment NIHSS ≤ 4 , and the total NIHSS score remained between 16 to 30). Additionally, patients all shared a pre-stroke modified Rankin Scale (mRS) score ≤ 1 (The scale ranging from 0–6 assesses capacity for daily activities, with higher scores indicating greater disability. A score of 6 signifies death). A full list of clinical characteristics of patients is available in the [Table S1](#). The primary exclusion criteria included: presence of acute cerebral hemorrhage or sub-arachnoid hemorrhage; acute posterior circulation ischemia; bilateral pupil fixation or disappearance of pupillary reflex; schedule of craniectomy before randomization; contraindications for ICO drug administration, e.g. skull fracture, skull infection, subdural/external hematoma, sub-scalp hematoma.

This study was approved by the Institutional Review Board (IRB) of Beijing Tiantan Hospital (IRB approval number: KY2023-052-02), and was conducted in accordance with the principles of the Declaration of Helsinki and the International Council for Harmonization of Good Clinical Practice guidelines. A steering committee was responsible for the design and implementation of the trial and presentation of the results. An independent data- and safety-monitoring committee oversaw the trial and conducted regular safety assessments. All clinical and adverse events were confirmed by an independent clinical event adjudication committee (CEC). The data were analyzed by the Data Management Center of the Chinese Neurological Clinical Research Center. All patients or their legal representatives were informed of the study protocol and provided written informed consent prior to enrolment. Nanjing Neurodawn Pharmaceutical provided the trial drug Y-3, but did not participate in the design, implementation or data analysis of the study. No commercial support was received for this study. No confidentiality agreements were obtained between the authors and any commercial entities. All authors vouch for the accuracy and completeness of the data, and the fidelity of the trials.

METHOD DETAILS**Nanoparticle preparation and characterization**

The albumin nanoparticles (NPs) were prepared using a desolvation method as previously described.²⁵ Briefly, 7.5 ml of ethanol was continuously added into 3 ml 10 mg/ml of bovine serum albumin solution under magnetic stirring (1000 rpm). After 1 hour of stirring, 80 μ L of 2.5% glutaraldehyde was introduced to crosslink the NPs. The reaction was allowed to proceed for 10 hours under stirring, after which the NPs were washed three times with deionized water to remove excess ethanol and unreacted albumin. The purified NPs were then redispersed in saline for subsequent use. The size distribution and surface zeta potential of the NPs were determined by dynamic light scattering instrument (Malvern). The morphology was observed by scanning electron microscopy (HITACHI,

SU8600). To produce NPs for fluorescent observation, the prepared albumin NPs were grafted with fluorescein-N-hydroxysuccinimide (FITC-NHS) through NHS-amine chemistry. After incubated with FITC-NHS for 2h, the fluorescent NPs were washed three times with deionized water to remove excess free dye molecules.

To prepare NA1-loaded NPs, NA1 of different concentrations were added into the albumin solution under magnetic stirring. The electrostatic interaction between the NA1 molecules and albumin NPs enables the surface loading of drug. After 1 hours of stirring, the NPs suspension was centrifuged to remove free NA1 and resuspended in saline for further use. The concentration of free NA1 in the supernatant was tested to determine the encapsulation efficiency of NA1 by high performance liquid chromatography (Agilent).

ICO injection in mice

All procedures were conducted on mice anesthetized with tribromoethanol (Avertin, 300 mg/kg). Body temperature was maintained at 37°C throughout the surgery using a heating pad. The head was shaved, a midline skin incision was made, and the interparietal skull was exposed.

The injection procedure followed a previously described method with modifications.¹⁹ Briefly, an electric drill (RWD, 78001) was carefully used to thin the compact bone overlying the bone marrow in the interparietal bones aided by a dissecting microscope, leaving only a thin layer of periosteum intact. A Hamilton syringe mounted with a 34G blunt needle was then used to puncture the periosteum manually, creating a small hole for injection. All injections were performed manually, with each injection consisting of a maximum volume of 10 μ l and taking approximately 60 seconds. After each injection, the needle was kept in place for 15 seconds to minimize leakage of the injected solution. After sacrifice, skulls were dissected from mice for 3D X-ray characterization (Zeiss, Xradia620 Versa). The results were evaluated to determine the accessibility of the bone marrow, the integrity of the inner table of the skull, and the time required for the skull to recover after injection.

In situ construction of NP-loaded myeloid cells

To construct NP-loaded myeloid cells *in situ*, ICO microinjection of NPs was performed under anesthesia. For each ICO spot, 10 μ l of NP solution was injected using a 5 μ l Hamilton syringe mounted with a 34G blunt needle. To validate *in situ* construction of NP-loaded calvarial myeloid cells, the skull bone marrow was isolated for fluorescent imaging and flow cytometry. Briefly, mice were sacrificed by cervical dislocation 2 hours after ICO injection of NPs, and their interparietal bones were dissected and cut into small pieces. Because the bone marrow cavities of the interparietal bone are highly interconnected, a 32G needle containing RPMI 1640 medium can flush most of the bone marrow cells out of the interparietal fragments to obtain a single-cell suspension. After washing thrice with ice-cold PBS or RPMI 1640 medium, free NPs were removed by centrifugation and cells were redispersed for further characterization.

To visualize the phagocytosis of NPs, the cells were stained with Hoechst 33342 (nucleus, blue) and DiD (membrane, red), and observed by a 3D confocal laser scanning microscope (Zeiss, LSM980 Airyscan2). Flow cytometry (Cytex Biosciences, Cytex Aurora) was used to quantify the NP phagocytosis efficiency. After stained with an antibody cocktail, the myeloid cells were screened as Zombie NIR- or Fixable Viability Dye eFluor506-, CD45+ and CD11b+. Subpopulations including neutrophils and Ly6Chi monocytes were identified as Ly6G- and Ly6G-Ly6C+, respectively. Skull bone marrow cells without ICO injection of NPs or fluorescent labelling were served as blank control. The myeloid cells co-stained with FITC-NPs were identified as the NP-loaded myeloid cells. *In situ* NPs targeting efficiency of myeloid cells and each subset were calculated from the resulting fluorescence signals.

LPS induced neuroinflammation

The delivery of NPs into the brain parenchyma was evaluated using a neuroinflammation model induced by lipopolysaccharide (LPS).³¹ In this model, animals received an intraperitoneal (i.p.) injection of LPS at a dose of 5 mg/kg body weight (Escherichia coli O111:B4, Sigma-Aldrich), prepared in sterile, nonpyrogenic 0.9% saline solution. The injection volume was standardized at 10 μ L per gram of body weight. For the control group, animals were injected intraperitoneally with an equivalent volume (10 μ L per gram of body weight) of sterile saline solution. 1 hour after LPS or saline injection, fluorescence-labelled NPs were intracalvariosseously injection, followed by the collection of calvaria bones 2 hours after NP injected. For brain tissue analysis, mice were sacrificed 24 hours after LPS or saline injection, and their brains without meninges were dissected, dissociated and stained for flow cytometry analysis.

Ischemic stroke induction

Experimental stroke was induced using the filament middle cerebral artery occlusion (MCAO) model for transient and focal brain ischemia. Under anesthesia (isoflurane), a silicon coated 6/0 filament was inserted from the external carotid artery (ECA) of mice until it obstructed the middle MCA. The ECA was then ligatured with the occluding suture for 60 minutes before reperfusion. Sham-operated mice received the same surgery procedure without filament insertion. 24 hours after onset of MCAO or sham operation, mouse brains without meninges were dissected, dissociated and stained for flow cytometry analysis.

Tissue harvest and processing

Mice were deeply anesthetized by intraperitoneal injection of tribromoethanol (Avertin, 300 mg/kg) and transcardially perfused with 1X PBS to remove the blood before fixation. Following perfusion, mice were decapitated immediately posterior to the interparietal bone, and all excess skins, muscles and mandibles were trimmed to remove from the skull. Skull was trimmed bilaterally from the interparietal bone towards the olfactory bulb to be released from the mouse head. The skull cap was then incubated in 4%

paraformaldehyde (PFA) at 4°C for 24 hours and decalcified in 10% EDTA in PBS for 10 days at room temperature. Brains were fixed in 4% PFA for additional 24 hours and placed in PBS until used. To harvest meninges, the mouse head whole mounts were fixed in 4% PFA for 8 hours, the brain tissue was removed, and the dura mater was gently dissected with fine surgical forceps from the intracranial surface and placed in 4°C PBS until further use.

Transmission electron microscopy

Tissues were fixed via cardiac perfusion with 4% PFA, followed by overnight post-fixation in a solution containing 2% PFA and 2.5% glutaraldehyde. Dissected skulls were decalcified in 10% EDTA in PBS at 4°C, with the solution changed daily for 10 days. After decalcification, the tissues were washed 4 times with PB buffer (0.1 M). The tissue underwent post-fixation with Osmium tetroxide (1%) and Tetrapotassium hexacyanoferrate trihydrate (1.5%) for 1 h at 23°C, followed by ethanol dehydration in graded solutions (50%, 70%, 80%, 90%, 100%, 100%, 100%) for 15 min each. Then, 1, 2-Epoxypropane twice for 15 min each and gradient infiltration with mixture of 1, 2-Epoxypropane and Epon 812 resin for 8 h (SPI, America). Subsequently, Pure Epon 812 twice and polymerizing in oven (60°C). Blocks of polymerized resin were sectioned using a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Ultra-thin sections (80 nm) were mounted and dried on coated copper grids. Sections were stained on-grid with 2% uranyl acetate (25 min) and lead citrate (5 min). Imaging was carried out using transmission electron microscope (Hitachi, HT7800).

Immunofluorescence staining and imaging

To obtain fixed brain tissue, mice underwent transcatheter perfusion using 0.9% normal saline followed by 4% PFA. For immunofluorescence staining, tissues were rinsed in PBS and washed by 0.1% PBST for 10 min followed by incubation in blocking buffer (Beyotime, QuickBlock) for 10 min with gentle agitation. Autofluorescence quencher was used to reduce the background signals following the manufacturer's instructions. Tissue slices were then incubated with diluted primary antibodies overnight at 4°C, washed thrice and incubated with secondary antibodies for 2 hours at room temperature. After rinsing in PBST thrice, mounted on slides, dehydrated, and immersed in antifade reagent with DAPI, the tissue slices were stored at 4°C until imaging using widefield microscopy at 10x objective (Olympus Slideview VS200) and confocal microscopy at 63x objective (Zeiss, LSM980 Airyscan2).

Tissue clearing and imaging

For cleared mouse head whole-mount or skull imaging, the fixed tissues were cleared using the iDISCO method.⁵¹ After decolorization, samples were decalcified by 20% EDTA in PBS for 4 days at room temperature and then dehydrated, permeabilized, stained and cleared using the iDISCO methods as described. Cleared samples were incubated in dibenzyl ether for optical matching and 3D imaging using light-sheet microscopy (Zeiss, Lightsheet 7) or confocal laser microscopy (Zeiss, LSM 980 airyscan2). Images were stitched and reconstructed using Imaris software.

Single cell harvest for flow cytometry

For blood analysis, blood was collected before perfusion from the retroorbital sinus. Blood was centrifuged at 350 g for 5 min. Red blood cell lysis was then performed by resuspending the cell pellet in the red blood cell lysis buffer (Biolegend) for 10 min. After centrifugation at 350 g for 5 min, the pellet was resuspended in FACS buffer (1X PBS with 2% fetal bovine serum) until use. For brain analysis, 24 hours after LPS injection or stroke induction, mice were anesthetized and perfused to remove the intravascular leukocytes. Sham-operated mice were set as control. Tissues were harvested as described above. Skull bone marrow cells were isolated by flushing using FACS buffer. Brains were triturated into single cell suspensions by a tissue dissociator (Miltenyi, gentleMACS) with tissue dissociation kits (Miltenyi) according to the provided protocols. The ischemic ipsilateral brain hemisphere and the contralateral brain hemisphere were analyzed separately. Cell debris were removed before test using a debris removal solution (Miltenyi). Single cell suspensions were obtained by filtering through a 70-μm cell strainer and kept in FACS buffer on ice until use. The antibody list is provided in [key resources table](#) and the gating strategy is provided in [Figures 3A](#) and [S2](#). Zombie NIR or Fixable Viability Dye eFluor506 was used to select living cells. Unstained controls and single-stained controls were prepared for negative/positive determination and color compensation. Cells isolated from mice that did not receive administration of FITC-NPs were used as negative controls for NP-loaded cells to eliminate potential interference of cell autofluorescence. After stained with an antibody cocktail, the calvaria-derived myeloid cells were screened as Zombie NIR- or Fixable Viability Dye eFluor506-, CD45+ and CD11b+. Subpopulations including neutrophils and CD45hi cells were identified as Ly6G+ and Ly6G-CD45hi, respectively. Microglia were identified as Ly6G-CD45mid. Samples were acquired with Aurora spectral flow cytometer (Cytek) and data were analyzed by Flowjo.

In vitro co-incubation of neurons and NP-loaded neutrophils

On the co-culture day, neutrophils are isolated from C57BL/6 mice by collecting blood through orbital puncture, followed by immunomagnetic bead sorting. Neutrophils are seeded at 1×10^6 /ml in serum-free RPMI-1640 medium and co-incubated with fluorescent NPs for two hours. After centrifugation at 1500 rpm for five minutes and removal of the supernatant, the neutrophils containing fluorescent NPs are resuspended in 1 ml of medium. Neutrophils are stained with DiD membrane dye (working concentration 1:1000) at 37°C for 20 minutes, followed by Hoechst 33342 nuclear dye (working concentration 1:2) for 10 minutes at 37°C. After staining, cells are washed twice with PBS to remove excess dyes and resuspended in 1 ml of medium. Neurons are stained with MitoTracker mitochondrial fluorescent probe (working concentration 1:2500) and Hoechst 33342 nuclear dye at 37°C for 10 minutes. After washing

twice with PBS, 500 μ L of neuronal culture medium is added. For co-culture, 500 μ L of the neutrophil suspension is evenly added to the neuron culture dish and incubated at 37°C for different times before imaging. Imaging is performed using a HIS-SIM super-resolution structured illumination microscope at 100 $\times$ magnification. Multiple fields of view are captured for ultra-high-resolution imaging. The acquired super-resolution images were sequentially processed using Wiener reconstruction and Sparse deconvolution, followed by further analysis with Fiji.

In vitro co-incubation of neurons and fluorescent NPs

To assess whether neurons can uptake fluorescent NPs directly, the primary mouse neurons were directly co-incubated with fluorescent NPs. On day 7 of primary mouse neuronal culture in confocal dishes, the neurons were first stained with MitoTracker and Hoechst 33342 to label mitochondria and nuclei, respectively, using previously optimized concentrations and incubation times. Following staining, the neurons were washed twice with PBS and maintained in 1 mL of neuronal complete medium. NPs were then introduced into the culture medium at a 1:20 dilution ratio, and the co-culture system was incubated at 37°C. To investigate the temporal dynamics of NPs internalization, neurons were co-cultured with NPs for 2, 4, 6, and 12 hours, respectively. After each incubation period, high-resolution structured illumination microscopy (HIS-SIM) was employed for super-resolution imaging, with consistent imaging parameters and post-processing methods as previously described.

LC-MS/MS

Tissue samples were accurately weighed and flash-frozen in liquid nitrogen, followed by pulverization. The homogenized tissue was resuspended in 200 μ L of 0.1% formic acid in water and vortexed thoroughly. A 100 μ L aliquot of the suspension was then mixed with 300 μ L of acetonitrile containing 0.1% formic acid and the internal standard (aniline-suc-KLVFFC, Molecular weight: 931.18 Da, 20 ng/mL). The mixture was vortexed for 3 min and centrifuged at 13,000 rpm for 10 min at 4 °C. The resulting supernatant was transferred to a clean vial for UPLC-MS/MS analysis. Calibration standards were prepared by spiking blank tissue homogenates with known concentrations of the analyte and internal standard. Chromatographic separation was performed using a Thermo Hypersil GOLD C4 column (2.1 $\times$ 150 mm, 3 μ m) on an Exion LC UPLC system. The column temperature was maintained at 40 °C and the autosampler was set at 8 °C. The mobile phases consisted of solvent A (acetonitrile containing 0.4% formic acid) and solvent B (water containing 0.4% formic acid), delivered at a flow rate of 0.3 mL/min. The injection volume was 3 μ L. The elution gradient was as follows: 0–2 min, 5% A; 2–4 min, 90% A; 4–4.1 min, 5% A; 4.1–10 min, 5% A. The mass spectrometric analysis was conducted on a SCIEX Triple Quad™ 7500 system equipped with an electrospray ionization (ESI) source operating in positive ion mode with multiple reaction monitoring (MRM). The source parameters were as follows: source temperature, 400 °C; curtain gas, 40 psi; ion spray voltage, 3000 V; nebulizer and auxiliary gases, 35 and 60 psi, respectively. The following MRM transitions were monitored: m/z 504.65 $\rightarrow$ 601.1 for the analyte (RT 1.54 min, Q1: 45 V, CE: 23 V) and m/z 931.53 $\rightarrow$ 516.23 for the internal standard (RT 2.90 min, Q1: 70 V, CE: 45 V). Quantification was performed using a calibration curve constructed with matrix-matched standards. Data acquisition and processing were carried out using SCIEX OS software (version 3.1.6).

Histological and MRI therapeutic evaluation

Mice received MCAO surgery were intravenously or intracalvariosseously administered with NA1 or NA1 NPs after 60-min occlusion. Control mice were treated with PBS. Eicosapeptide NA1, or Nerinetide (YGRKKRRQRRRLSSIESDV) with 99% purity was purchased from Nanjing Aite Biotechnology Co., Ltd. For dose-response relationship studying, mice received different dose of NA1 were assessed for neurological deficit scoring 24 hours after MCAO and then sacrificed to quantify the brain infarct volume using 2,3,5-triphenyltetrazolium chloride (TTC) staining. For long-term evaluation, *in vivo* MRI imaging was performed on a 9.4T BioSpec 94/30 USR animal scanner (Bruker BioSpin, Germany) using a quadrature transmit/receive volume coil of inside diameter of 35 mm. Animals were kept under isoflurane anesthesia (1.2–1.8% in 80% air and 20% oxygen) during the entire data acquisition period. Physiological signals included heart rate, body temperature and respiratory frequency were monitored throughout the experiment. Temperature was maintained around 37°C with a hydrothermal pad. Anatomical images were acquired with a T2 turboRARE, Diffusion-weight-EPI-sat and Diffusion-Tensor-EPI-sat sequences in a coronal plane. Analysis was finished using ITK-Snap contouring software (Pittsburgh). Stroke volumes were calculated on TTC and T2 images as

$$\text{Infarct volume} = V_{\text{contra}} - (V_{\text{ipsi}} - V_{\text{infarct}})$$

where V_{contra} , V_{ipsi} , V_{infarct} are the volume of contralateral hemisphere, ipsilateral hemisphere and infarct zone, respectively.

Infarct core volumes were calculated on ADC map with severely reduced ADC < 620. Brain edema and brain atrophy was assessed by calculating the percentage ratio of the volume of the ipsilateral hemibrain to the volume of the contralateral hemibrain. 35 days after MCAO, the mice were sacrificed and the brains were harvested for section and Nissl staining.

Behavioral assessments

A modified Garcia score test was used to evaluate sensorimotor function at 1, 3, 5, 7, 9, 11, 14, 21, and 28 days after MCAO. Seven tests were conducted, including body proprioception, vibrissae touch, limb symmetry, lateral turning, forelimb walking, motor test and spontaneous activity. The scores of each test ranged from 0 to 3, with a total maximum possible score of 21.

The rotarod test was conducted to assess motor function at 3, 5, 7, 9, 11, 14, 21, and 28 days after ischemia. Briefly, the mice were trained on a rotarod apparatus (Panlab, Barcelona, Spain) for 3 successive days (speed from 4 to 40 rpm). The time for each mouse to fall onto the platform was recorded, with a maximum time of 300 seconds per trial.

To investigate learning and memory/recognition in mice, the novel object recognition test was performed. Briefly, this two-trial test consisted of a training and a testing phase. During the training phase, the animal was allowed to explore two identical objects for 10 minutes. After a 6-hour interval, one of the training objects was replaced with a novel object, and the animal was allowed to explore the two different objects for another 10 minutes during the testing phase. Mice which did not reach a minimum exploration time (20 s) in the training phase were excluded from the analysis. The recognition index was calculated as the time spent exploring the novel object divided by the total time spent exploring the novel object and the familiar object. The objects were cleaned between mice using 75% ethanol.

The Morris water maze test is used to assess spatial learning and memory abilities in mice. The apparatus consists of a circular pool with a diameter of 109 cm, a circular platform with a diameter of 11 cm that is elevated 2 cm above the water surface, and spatial cues placed around the pool. A camera is positioned above the setup for monitoring. Prior to sample collection, mice undergo spatial learning training for an appropriate duration each day: each mouse is placed into the pool from four different locations (northwest, north, east, southwest), and their ability to locate the platform within 60 seconds is observed. The time taken for the mouse to reach the platform is recorded. After each trial, the mouse is allowed to remain on the platform for 15 seconds. Training is conducted for four consecutive days. On the day following the completion of training, a spatial memory test is performed. The circular platform is removed, and the mouse is released into the pool from the location farthest from the original platform. The time spent in the target quadrant (where the platform was previously located) within 60 seconds and the average swimming speed are recorded.

Randomization and Interventions of clinical trial

Patients with potential mMCAI were continuously screened in the emergency room (ER). Those enrolled were randomly assigned to the ICO group or the best medical treatment group at a 1:1 ratio through simple random allocation. The randomization code was obtained from the Statistics and Data Centre at the China National Clinical Research Centre for Neurological Diseases. All the patients received active treatment and care according to the Evidence-Based Guidelines for the Management of Large Hemispheric Infarction, and the European Stroke Organization (ESO) guidelines for the management of space-occupying brain infarction. Additionally, the ICO group received ICO Y-3 injections, with a dosage calculated as 32 $\mu\text{g/kg}$ (The dosage is calculated from preclinical studies²⁹). All the participants were admitted to the neurointensive care unit (NICU) prior to ICO injection operation. The ICO injection procedure was performed at the bedside in the ICU and required an isolated aseptic operating space. Referring to stereotactic aspiration surgical procedures, we designed a specific operational protocol. Operations in the ICO group were carried out under the monitoring, with salvage equipment and professional medical personnel available. During hospitalization, patients routinely underwent CT scans, and laboratory tests to detect existence of cerebral edema, hemorrhagic transformation, or potential infectious events and other adverse events. Patients were transferred to general wards for rehabilitation training if out of danger.

ICO injection in patients

Patients were positioned with the shoulder on the affected side elevated and the head tilted toward the contralateral side for ease of operation. The injection site was selected at the region of the ipsilateral parietal bone where the diploe was most prominent. A 2–3 cm longitudinal scalp incision was made at this location. After standard disinfection, draping, and local lidocaine infiltration, the scalp was incised, hemostasis achieved, and the temporal muscle retracted to expose the skull. Using a surgical drill, a 2–3 mm-deep hole was created in the outer plate to expose the diploe. In most cases, slow oozing of dark red blood indicated successful access to the skull marrow cavity. If no bleeding is observed, the drill bit can be changed to grind open the barrier until bleeding occurs. A 5 mL needleless syringe was prepared by wrapping three layers of foam dressing around the syringe cone and screw thread. After pressing the syringe firmly against the bone hole, the therapeutic agent was slowly injected into the skull bone marrow. Upon completion, the bone opening was immediately sealed using bone wax. The scalp incision was then closed with sutures. On postoperative days 2 and 3, additional doses were administered through the same bone access point by repeating the injection process, followed by resealing with bone wax and scalp closure. After each procedure, the surgical site was dressed according to standard sterile technique, and the wound was regularly monitored for healing and signs of infection.

Clinical outcomes

The primary outcomes of our clinical study were set to evaluate feasibility and safety of ICO Y-3 injection for mMCAI patients. Feasibility outcomes were defined as drilling through the internal plate of skull; terminated due to drug leakage during the injection; the patient's refusal to continue; and failure for other reasons during the 3-day ICO injection treatments. Safety outcomes included infection events (skin infection, osteomyelitis, or intracranial infection), symptomatic and asymptomatic intracranial hemorrhage, moderate to severe bleeding (defined by the GUSTO), hepatic insufficiency, renal insufficiency during the treatment, severe or extremely severe anemia (hemoglobin < 60g/L), mortality, incidence of other adverse events/serious adverse events.

Secondary efficacy outcomes included: Changes in NIHSS scores from baseline values to 14 $\pm$ 2 days or at discharge; NIHSS score increase of ≥ 4 at 14 $\pm$ 2 days compared to baseline; mRS score of 0–3 points at 90 $\pm$ 7 days. NIHSS score and mRS score were evaluated by two specialized neurologists. In instances of discordant assessment outcomes, adjudication was performed

by a senior clinician to ensure the accuracy and reliability of the diagnostic evaluation. Prior to scoring, the physicians ensured that the heads of the patients in both groups were covered and appearance of medical dressings were consistent. The neurologists validating the scoring and the CEC members were blinded to the patients' group assignments.

QUANTIFICATION AND STATISTICAL ANALYSIS

All *in vitro* experiments were conducted independently at least three times. For preclinical study, sample or replicate numbers (*n*) are provided in the figure legends. Data are presented as mean values $\pm$ standard deviation (SD), mean values $\pm$ standard error (SEM) or median (minimum, maximum). For comparisons between two groups, unpaired t-test was applied. Multiple group comparisons were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test to compare each group to the ICO-NA1 NPs group, or Tukey's post hoc test for pairwise comparisons among treatment groups. For time-course data, such as lesion volume change, brain volume change, sensorimotor and cognitive functions, two-way repeated measures ANOVA was used, with subsequent Šidák test to calculate p-values, enabling comparisons between each treatment group and the control, or among all pairs of treatment groups all time points, respectively. Statistical significance was defined as $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. GraphPad Prism 10 was used for data analysis. Details of statistical analysis conducted are included in the Figure legends.

For clinical study, all numeric variables were analyzed by the Shapiro-Wilk test to determine whether they followed normal distributions. Normally distributed data are described as means $\pm$ standard deviations (SD), while non-normally distributed data were described as median (interquartile range, IQR) values. Categorical variables were expressed as counts and frequencies. Feasibility measures were only statistically described. All safety measures were compared between groups using Fisher's exact test. The primary effectiveness measures were compared using the Mann-Whitney U test. Since NIHSS score and GCS score are not applicable for mortality cases, relevant score was treated as missing values and assigned by last observation carried forward (LOCF) which meant the values were assigned by the most recent valid scores. Among the secondary effectiveness measures, rates of NIHSS improvement, NIHSS motor limbs score improvement and mRS 0-3 in 90 days were compared using Fisher's exact test. Statistical analyses were performed using SAS software, version 9.4 (SAS Institute). All statistical tests will be two-sided, with $p < 0.05$ considered statistically significant.

ADDITIONAL RESOURCES

Clinical trials related to this work: <https://clinicaltrials.gov/study/NCT05849805?cond=%20NCT05849805&rank=1>.

Supplemental figures

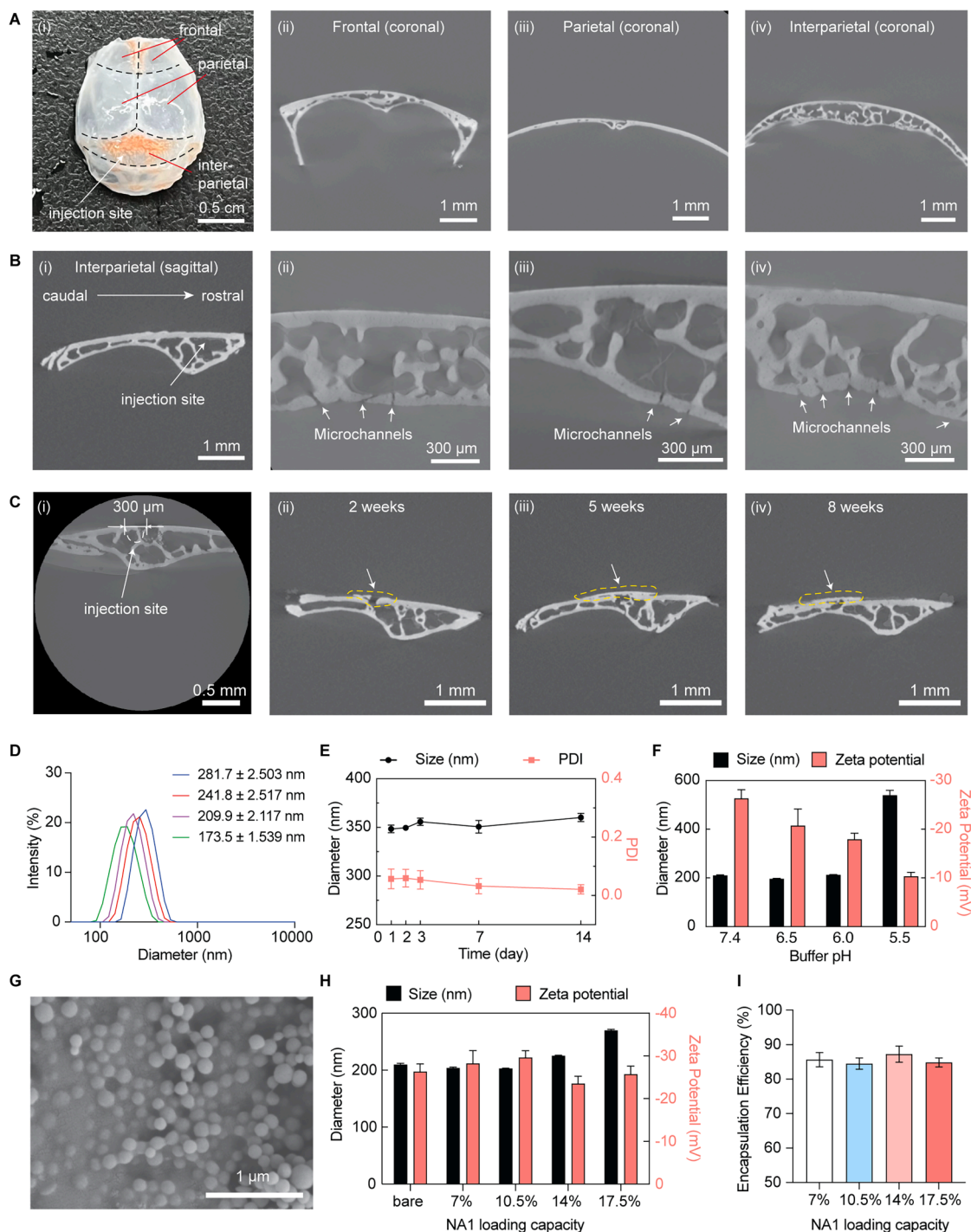


Figure S1. Characterization of mouse skulls and albumin NPs, related to Figure 1

(A) Representative photograph (Ai) and X-ray images of the dissected dorsal part of the mouse skull, including the frontal (Aii), parietal (Aiii), and interparietal (Aiv) parts.

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(B) Representative sagittal view of the interparietal skulls shows the large and highly interconnected bone marrow cavities (Bi) along with abundant microchannels (Bii–Biv).

(C) Representative X-ray images show the minimally invasive ICO injection (Ci) and fast recovery after injection (Cii–Civ).

(D) Representative size distribution of the albumin NPs with different diameters characterized by DLS.

(E) Stability analysis of the albumin NPs by DLS. Data are presented as mean $\pm$ SEM.

(F) Size and zeta potential of the albumin NPs in buffers with different pH values. Data are presented as mean $\pm$ SD. $n = 3$ technical replicates for each group.

(G) Representative SEM images of NA1-loaded albumin NPs.

(H) Size and zeta potential of albumin NPs with different loading capacities of NA1 tested by DLS. Data are presented as mean $\pm$ SD. $n = 3$ technical replicates for each group.

(I) Encapsulation efficiency of albumin NPs with different loading capacities of NA1 tested by HPLC. Data are presented as mean $\pm$ SD. $n = 3$ technical replicates for each group.

All experiments were repeated at least three times independently, with similar results.

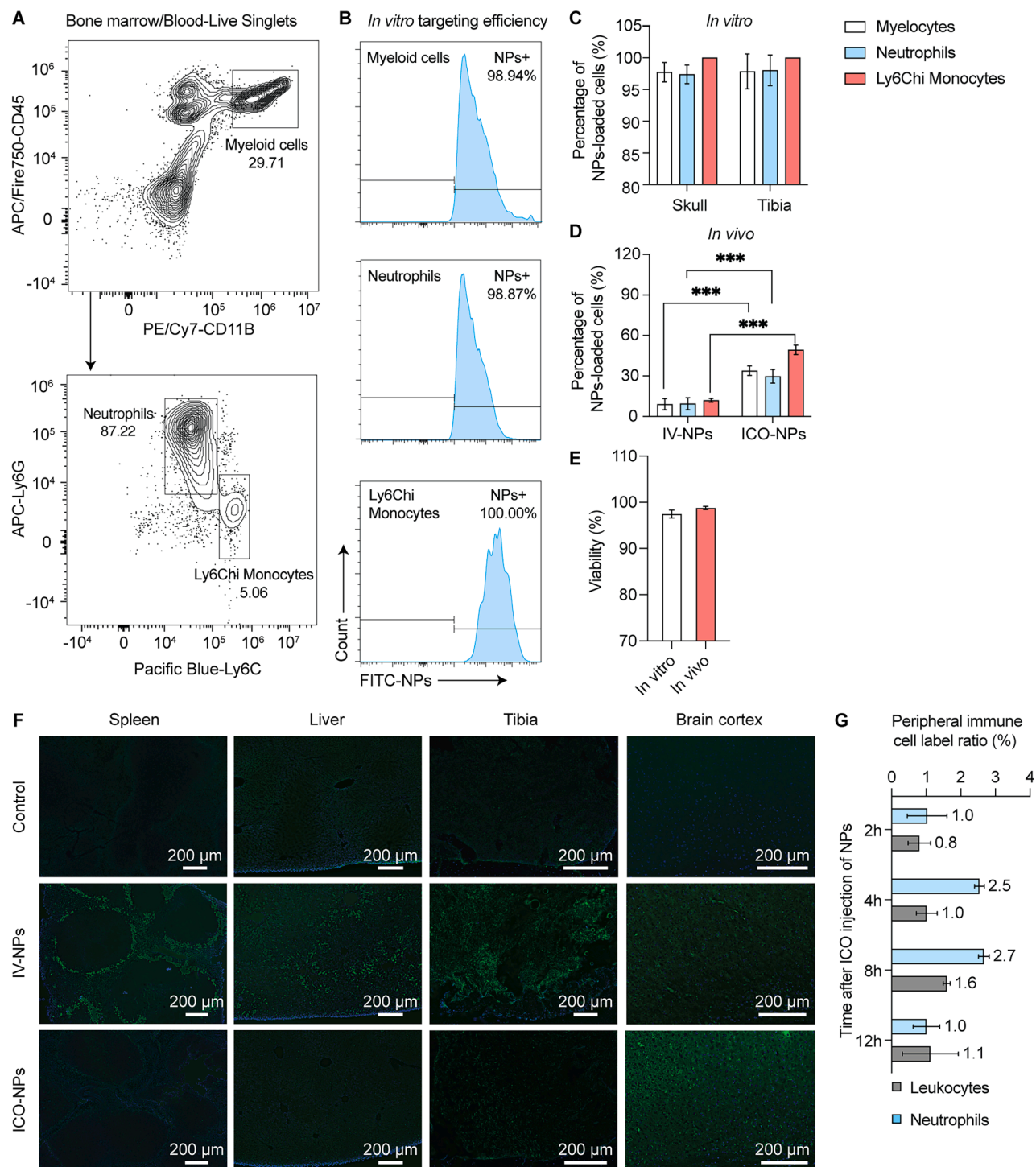


Figure S2. Immune cell targeting efficiency and biodistribution of albumin NPs administered via ICO versus i.v. routes, related to Figures 1 and 3

(A) Gating strategy of the myeloid cell subsets in bone marrow and blood.

(B and C) Representative histogram (B) and statistics (C) of *in vitro* targeting efficiency of albumin NPs for myeloid cells and their subsets 2 h after co-incubation. $n = 3$ biological replicates for each group. Data are presented as mean $\pm$ SD.

(D) Statistics of *in vivo* targeting efficiency of albumin NPs for myeloid cells and their subsets 2 h after i.v. injection and ICO injection. $n = 3$ biological replicates for each group. Data are presented as mean $\pm$ SD. *** $p < 0.001$ by two-way ANOVA with Šidák's multiple comparisons test.

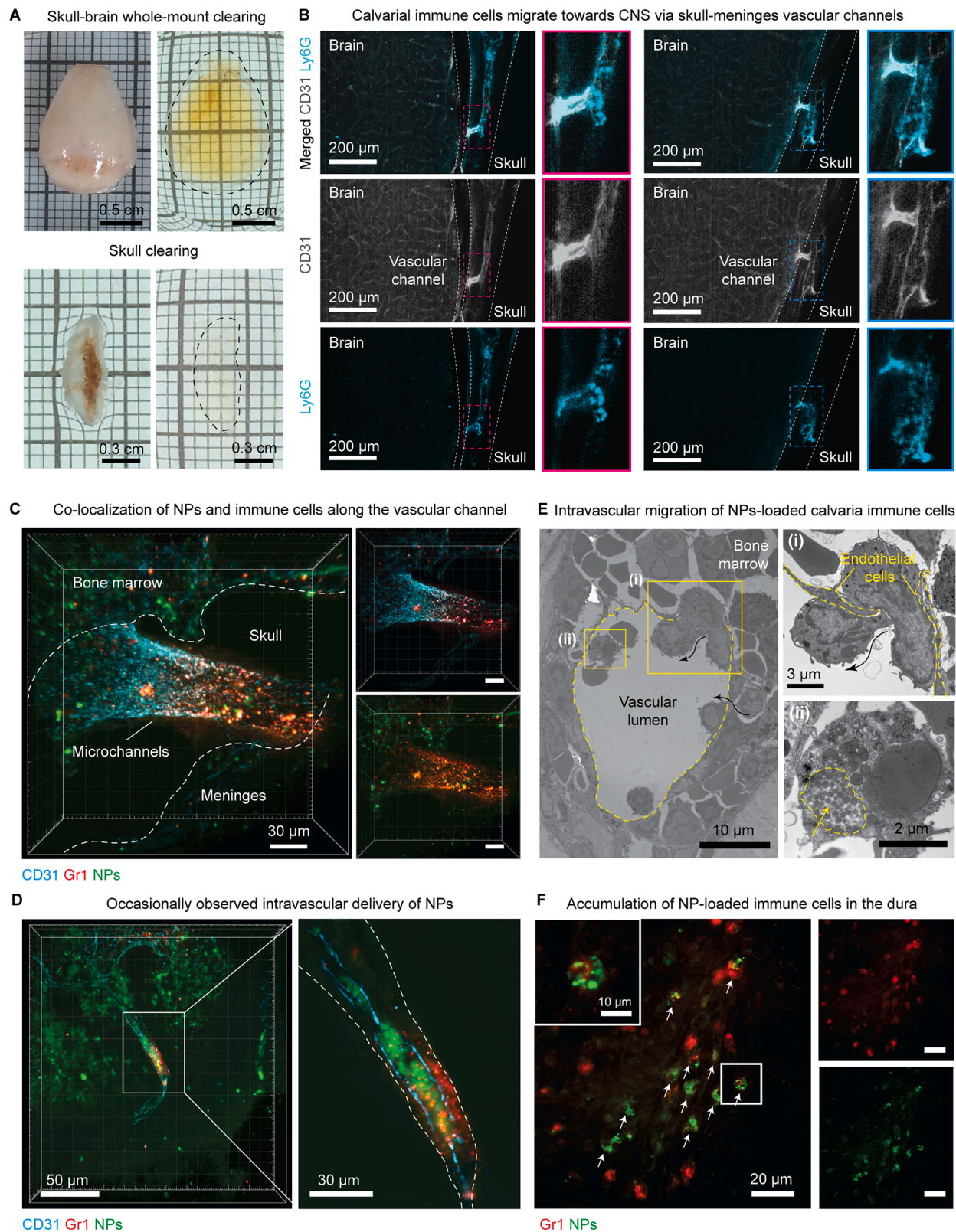
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(E) Viability of bone marrow cells 2 h after co-incubation with albumin NPs *in vitro* or ICO injection *in vivo*. Data are presented as mean $\pm$ SD. $n = 3$ biological replicates for each group.

(F) Representative IF images of the spleen, liver, tibia, and brain cortex of LPS-treated mice 24 h after administration of FITC-labeled albumin NPs via i.v. or ICO route. Blue, nucleus; green, NPs.

(G) Statistics of FITC-labeled peripheral blood immune cells at different time points after ICO injection of FITC-NPs. Data are presented as mean $\pm$ SD. $n = 3$ biological replicates for each group.

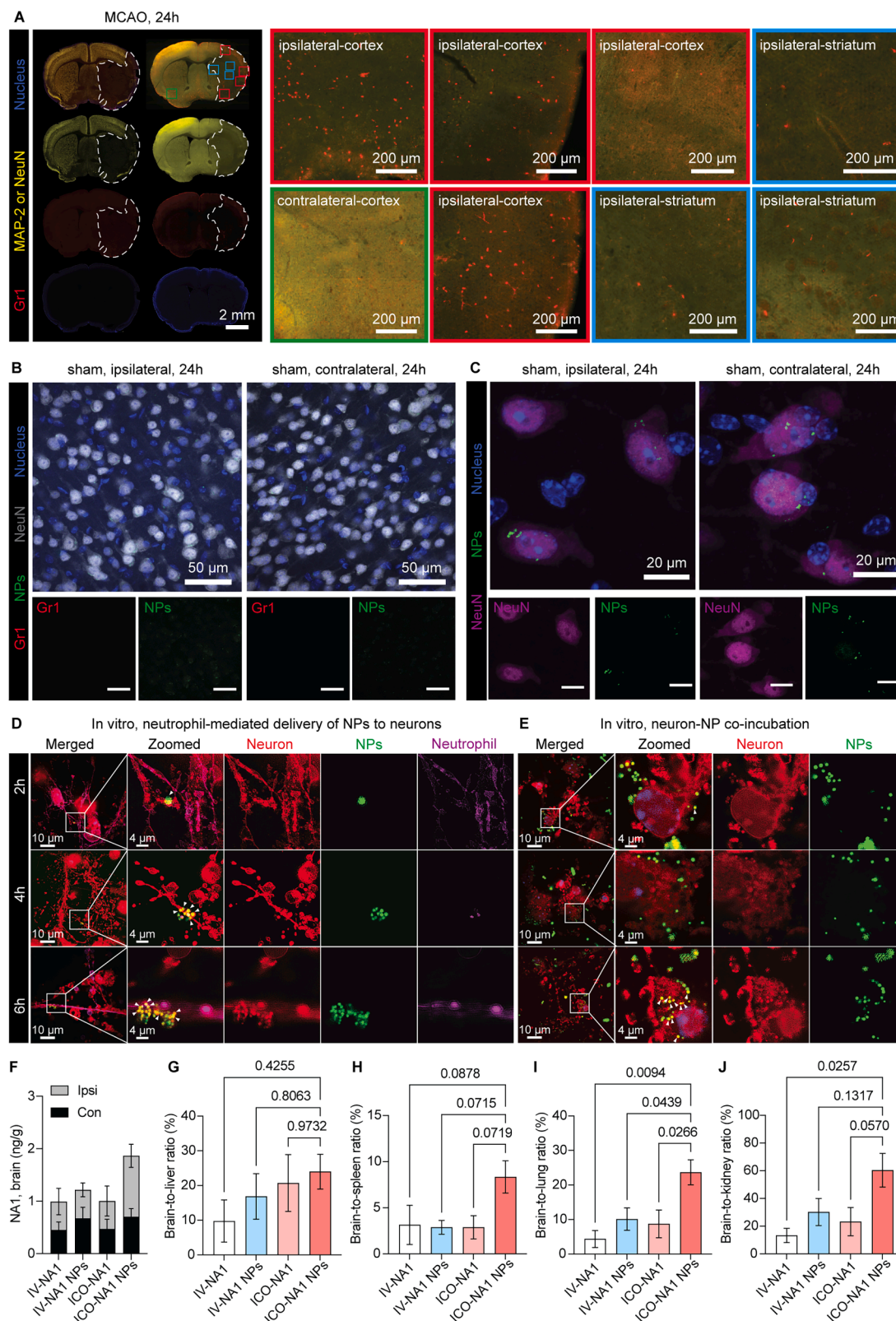
All experiments were repeated at least three times independently, with similar results.



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Figure S3. Migration of NP-loaded calvaria immune cells toward CNS through the skull-meninge vascular microchannel, related to Figure 2

- (A) Photographs of an optically cleared mouse skull-brain whole-mount (top) and mouse skull (bottom).
 (B) Representative image of the calvarial neutrophils migrating toward the CNS along the vascular channel.
 (C) Representative image of the colocalization of NPs and calvarial immune cells along the vascular channel.
 (D) Representative image of the occasionally observed intravascular delivery of NPs.
 (E) Representative TEM image of the intravascular migration of NP-loaded calvarial immune cells.
 (F) Representative image showing the accumulation of NP-loaded immune cells in the dura. The white arrows indicate the Gr1+ cells co-stained with FITC-NPs.
 All experiments were repeated at least three times independently, with similar results.



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Figure S4. Enrichment of immune cells, NPs, and therapeutics in ischemic brain lesions, related to Figures 3 and 4

(A) Representative IF images of brain sections from MCAO mice collected 24 h after stroke onset. Neurons were stained with NeuN MAP-2 to delineate low-intensity infarct regions, highlighted by white-dotted circles. The right panels show enlarged views of (A), illustrating the distribution of Gr1+ cells in the contralateral and ipsilateral hemibrains.

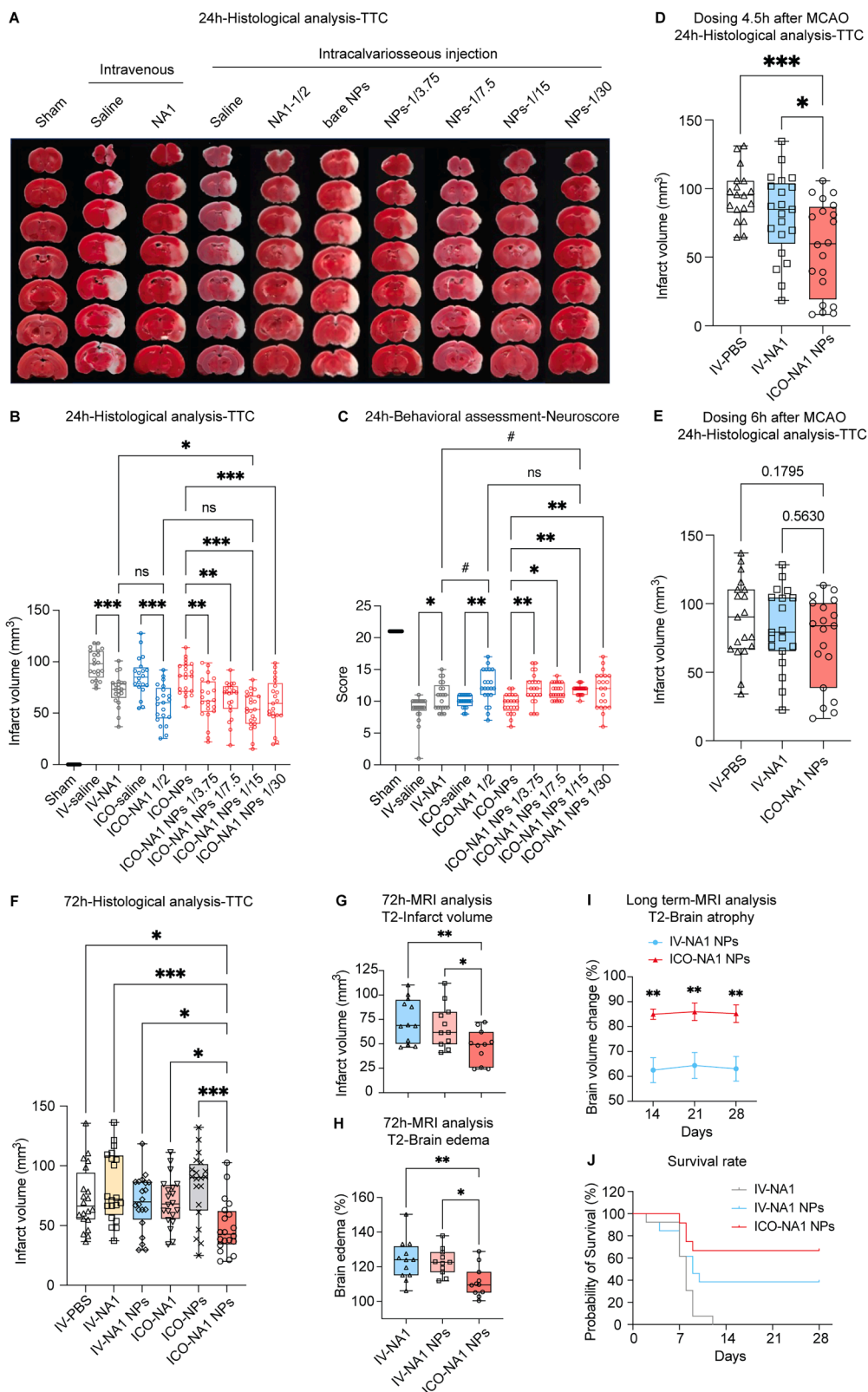
(B and C) Representative IF images of the ipsilateral and contralateral hemibrain of sham-operated mice 24 h after stroke onset.

(D) Representative IF images of primary mouse neurons co-incubated with NP-loaded neutrophils for different durations. The white triangles indicate the co-staining of neurons and NPs, confirming the neutrophil-mediated NPs delivery to neurons.

(E) Representative IF images of primary mouse neurons co-incubated with fluorescent NPs for different durations. The white triangles indicate the co-staining of neurons and NPs, showing the active uptake of NPs by neurons. The purple coloration observed in the images results from the overlap of DAPI-stained nuclei (blue) and neuron staining (red).

(F) Statistics of drug weight ratio in the ipsilateral and contralateral brain hemispheres following different delivery routes 24 h after stroke onset. $n = 4-5$ biological replicates for each group. Data are presented as mean $\pm$ SD.

(G-J) Statistics of brain-to-peripheral organ ratios 24 h after stroke onset, including liver (G), spleen (H), lung (I), and kidney (J). $n = 4-5$ biological replicates for each group. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test.



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Figure S5. Dose dependence, timing, and efficacy of ICO-NA1 NPs versus other delivery routes and formulations, related to Figure 4

(A) Representative images of mouse brain sections after TTC staining showing the infarct area induced by stroke.

(B and C) Statistics of brain infarct volume (B) and neurological scores (C) 24 h after stroke. $n = 20$ – 23 biological replicates for each group. Data are presented as median (min, max). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by one-way ANOVA with Tukey's multiple comparisons test. $^{\#}p < 0.05$ by unpaired t test.

(D and E) Time window studies in MCAO mice were administered 4.5 (D) and 6 h (E) after the onset of 60-min MCAO. $n = 18$ – 21 biological replicates for each group. Data are presented as median (min, max). $^*p < 0.05$ and $^{***}p < 0.001$ by one-way ANOVA with Dunnett's multiple comparisons test.

(F) Statistics of brain infarct volume 72 h after stroke demonstrated by TTC staining. $n = 20$ biological replicates for each group. Data are presented as median (min, max). $^*p < 0.05$ and $^{***}p < 0.001$ by one-way ANOVA with Dunnett's multiple comparisons test.

(G and H) Statistics of the infarct volume (G) and brain edema (H) 72 h after stroke induction demonstrated by T2 images. $n = 11$ – 12 biological replicates for each group. Data are presented as median (min, max). $^*p < 0.05$ and $^{**}p < 0.01$ by one-way ANOVA with Dunnett's multiple comparisons test.

(I) Brain volume change after stroke induction demonstrated by T2 images. $n = 3$ – 6 biological replicates for each group. Data are presented as mean $\pm$ SEM. $^{**}p < 0.01$ by two-way ANOVA with Šídák's multiple comparisons test.

(J) Survival curve of MCAO mice with different treatments. The 100% mortality rate of i.v.-NA1 in this batch of animals may be attributed to the additional anesthesia burden during MRI scans in the acute and high-risk post-MCAO period.