

Research paper

Harnessing anti-TNFR1 therapy has a limited impact on inflammation and motor function after stroke in obese male mice

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ARTICLE INFO

Edited by Cesar V. Borlongan.

Keywords:

Stroke
Obesity
Tumor necrosis factor
Neuroinflammation
Functional outcomes

ABSTRACT

Obesity is a significant global health concern that exacerbates the risk and severity of ischemic stroke through chronic low-grade systemic inflammation and metabolic dysregulation. Tumor necrosis factor (TNF) signaling, particularly through its TNF receptor 1 (TNFR1), is involved in obesity-driven inflammation and adverse stroke outcomes. To assess the potential of TNFR1 blockade as a treatment strategy, we employed a diet-induced obesity model of ischemic stroke. Male C57BL/6 mice were fed a regular diet or high-fat diet for 6 weeks prior to stroke induction via photothrombosis and treated with either a TNFR1-neutralizing antibody or isotype control antibody. Outcomes were evaluated using motor function assessments, systemic inflammatory biomarker measurements, and brain tissue analysis, including evaluation of microglia, astrocytes, oligodendrocytes, myelin integrity, and stroke size. Results showed that obese mice exhibited worsened motor deficits and heightened systemic inflammation following stroke compared to non-obese controls. Treatment with anti-TNFR1 antibody did not ameliorate the increased functional deficits but did improve interleukin-6 cytokines levels after stroke in obese mice, while it had no effects on non-obese controls. Moreover, anti-TNFR1 therapy did not impact infarct size, microglial and astrocyte reactivity, or myelin integrity in both dietary groups. Overall, TNFR1-targeted therapy had a limited impact on obesity-exacerbated stroke outcomes, suggesting the need to explore broader or combination immunomodulatory approaches. These findings also emphasize the importance of considering comorbidities such as obesity in stroke, as they can influence both disease progression and treatment effectiveness.

1. Introduction

Obesity represents a critical health and social problem worldwide linked to increased risk of ischemic stroke (Tsao et al., 2023). Weight gain though chronic consumption of diets high in saturated fat and sugars (Western style diet) is associated to a metabolic syndrome

(MetSy) characterized by insulin resistance, hyperglycemia, hyperinsulinemia, and chronic low-grade systemic inflammation (Weisberg et al., 2003; Wisse, 2004), resulting in priming of circulating monocytes which display a heightened and dysregulated basal inflammatory state (Friedrich et al., 2019). Following ischemic stroke, this chronic low-grade systemic inflammation is believed to increase blood-brain

Abbreviations: CXCL1, chemokine (C-X-C motif) ligand 1; DAB, 3,3'-Diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; FBS, fetal bovine serum; FDR, false discovery rate; GFAP, glial fibrillary acidic protein; HRP, horse radish peroxidase; IFN- γ , interferon-gamma; IL, Interleukin; LOD, lower level of detection; LPS, lipopolysaccharide; MBP, myelin basic protein; MetSy, metabolic syndrome; NeuN, neuronal nuclei; NF-L, neurofilament light-chain; NG2, Neural/glia antigen 2; i.p., intraperitoneally; PBS, phosphate buffered saline; PFA, paraformaldehyde; RM, repeated measures; SEM, standard error of mean; SD, standard deviation; TBS, tris buffered saline; TBS-T, tris buffered saline + triton; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2.

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<https://doi.org/10.1016/j.brainres.2025.149888>

Received 4 June 2025; Received in revised form 30 July 2025; Accepted 15 August 2025

Available online 18 August 2025

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barrier permeability, which then contributes to enhanced post-stroke neuroinflammation (Maysami et al., 2015). Post-stroke neuroinflammation is characterized by increased intraparenchymal pro-inflammatory cytokine levels, glial cell reactivity, and neuronal dysfunction, as well as impaired functional recovery (Buckman et al., 2013; Lainez et al., 2018; Noronha et al., 2019; Rahman et al., 2018; Thompson et al., 2013; Valdearcos et al., 2017). We and others have previously demonstrated that when stroke occurs in the context of obesity, infarct size is typically larger, microgliosis and astrogliosis enhanced, and functional outcomes impaired (Cao et al., 2015; Deutsch et al., 2009; Haley et al., 2019; Peterson et al., 2021). We recently demonstrated that the expression of tumor necrosis factor (TNF), a key immunomodulatory cytokine known to be involved in post-stroke inflammation (Lambertsen et al., 2018), and its receptors TNFR1 and TNFR2 is significantly elevated in the brain of obese mice subjected to experimental stroke compared to lean mice (Peterson, Lechtenberg, 2021). Peripheral TNF levels are also found to be elevated in obesity, where it is a critical mediator of inflammation as well as glucose intolerance and insulin resistance (Liang et al., 2008; Tzanavari et al., 2010). Blocking TNFR1-mediated TNF signaling during the pathogenesis of obesity using a TNFR1 blocking antibody resulted in reduced body weight, reduced plasma triglycerides, along with an improvement in obesity-induced insulin resistance and hyperglycemia in obese rats (Liang et al., 2008). In the context of experimental ischemic stroke, blocking TNF-TNFR1 signaling using either a selective soluble TNF inhibitor or a TNFR1 blocking antibody in lean animals significantly improved neurological deficits and functional outcomes (Clausen et al., 2014; Lin et al., 2021).

These studies raised the possibility that blocking TNF-TNFR1 signaling in obese mice subjected to ischemic stroke may alleviate inflammation and improve functional outcomes. To address this, we used our standard paradigm to produce diet-induced obesity and glucose intolerance (Peterson et al., 2021) and compared to lean mice subjected to stroke. We fed wild-type C57BL/6 mice high-fat diet or regular diet for 6 weeks prior to stroke and treated mice with a TNFR1 blocking antibody or an isotype control antibody. We then assessed peripheral inflammation, infarct size, and glial cell reactivity at 7 days post-stroke. Finally, we assessed the effect of high-fat diet and treatment on oligodendrocyte responses and the effect on motor recovery.

2. Methods

2.1. Experimental animals

A total of 60 male, 18 weeks old C57BL/6J mice were purchased from the Jackson Laboratories (Sacramento, CA, USA). The mice were fed either a 18 % kcal fat regular diet (no. 2018SX; Harlan Laboratories, Indianapolis, IN) or a 60 % kcal high-fat diet (no. D12492; Research Diets, Inc., New Brunswick, NJ) for 6 weeks prior to induction of ischemic stroke and 1 week after stroke. Animals were housed on a 12:12 h light–dark cycle and given free access to food and water. The Stanford Institutional Animal Care and Use Committee and the NIH Guide for Care and Use of Animals approved the protocols for using animals in this study, and all experiments were conducted following the ARRIVE 2.0 guidelines for reporting animal experiments.

2.2. Photothrombotic stroke model

To induce photothrombotic stroke (Labat-gest and Tomasi, 2013), the mice were anesthetized with isoflurane and placed in a stereotaxic frame (Digital Mouse Stereotaxic Instrument, Stoelting, Wood Dale, IL) resting on a heating pad. Rose Bengal dye (no. 330,000 5G, Sigma-Aldrich, St. Louis, MO, USA), dissolved 10 mg/ml in sterile saline, was injected intraperitoneally (i.p.) at a dose of 10 mg/kg body weight. At least 10 min after injection, a midline scalp incision was made to expose the skull, and a cold light source was applied to the skull

overlying the right motor cortex (coordinates: 0.2 mm posterior and 1.75 mm laterally to the right of Bregma) for 15 min. This photo-activation of the Rose Bengal dye induced a clotting cascade and thrombus, leading to ischemic damage to the brain tissue in the motor cortex (Labat-gest and Tomasi, 2013). The scalp incision was closed by applying Surgi-lock tissue adhesive. For post-surgical antibiotics and analgesia, mice received subcutaneous injections with cefazolin (25 mg/kg, VWR) and buprenorphine SR (1 mg/kg; ZooPharm). After surgery, the mice recovered for approximately 20 min under a heated lamp to prevent hypothermia and were then returned to home cage. By the end of surgeries all mice were returned to the conventional facility. Animals were monitored daily for signs of pain or distress.

2.3. Pharmacological treatment

We administered 100 µg of a TNFR1 neutralizing antibody (T950, mAB 55R-170, Leinco, St. Louis, MO, USA) or Armenian hamster IgG isotype control (P376, Isotype control, Leinco, St. Louis, MO, USA) twice, *retro-orbitally* at 4 h post stroke and again i.p. 72 h post-stroke (Fig. 1).

2.4. Behavioral assessments

Functional outcomes were assessed 1, 3, and 7 days after stroke using behavioral tests specifically designed to detect motor deficits (Cheng et al., 2014; Schaar et al., 2010), performed as described by Peterson and colleagues (Peterson et al., 2021) (Fig. 1). Briefly, prior to stroke, mice were acclimatized for at least one hour in the behavior room to adapt to the new environment. After this, they underwent 6 days of handling and one day of habituation to each apparatus. Finally, two days of training were performed on each apparatus.

Tapered Beam: The tapered beam is 100 cm long, angled 16 degrees, one end starts as 3.5 cm wide and tapers down to 0.5 cm wide at the other end where a box is placed. Animals were placed on the tapered beam and the time to travel the beam, as well as the number of contralateral forelimb and hindlimb errors, were recorded as they progressed down the beam during three trials each testing day. An error was defined as the whole foot missing and sliding on the side of the beam (Schaar et al., 2010). At the end of training, all mice had < 2.5 total errors.

Rotating Beam: A rotating beam was resting on two columns, with one end having the motor (6 rpm) and the other end having a box, and with 150 cm between the two (Cheng et al., 2014). Animals were placed on the rotating beam and the distance covered and time spent on the beam prior to falling or entering the box was recorded for four trials each testing day. At the end of training, all mice had < 3 total errors.

2.5. Tissue processing

Seven days after stroke, the animals were deeply anesthetized and approximately 0.5 mL whole blood was obtained by cardiac puncture. The animals were then perfused transcardially with heparinized 0.9 % saline solution. The blood samples were kept on ice for no longer than 30 min before being centrifuged at 4 °C for 15 min at 5,000 rpm. Plasma was carefully isolated to avoid disrupting the blood layers, and flash-frozen in liquid nitrogen for storage. Brains were immersion fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight. The brains were then sunk through 30 % sucrose in 1X PBS and stored at 4 °C. A freezing sliding microtome (Microm HM430) was used to collect 40-µm-thick coronal brain sections in 12 parallel series. The sections were stored at −20 °C in a cryoprotective medium (30 % glycerol, 30 % ethylene glycol, and 40 % 0.5 M sodium phosphate buffer) until further processing.

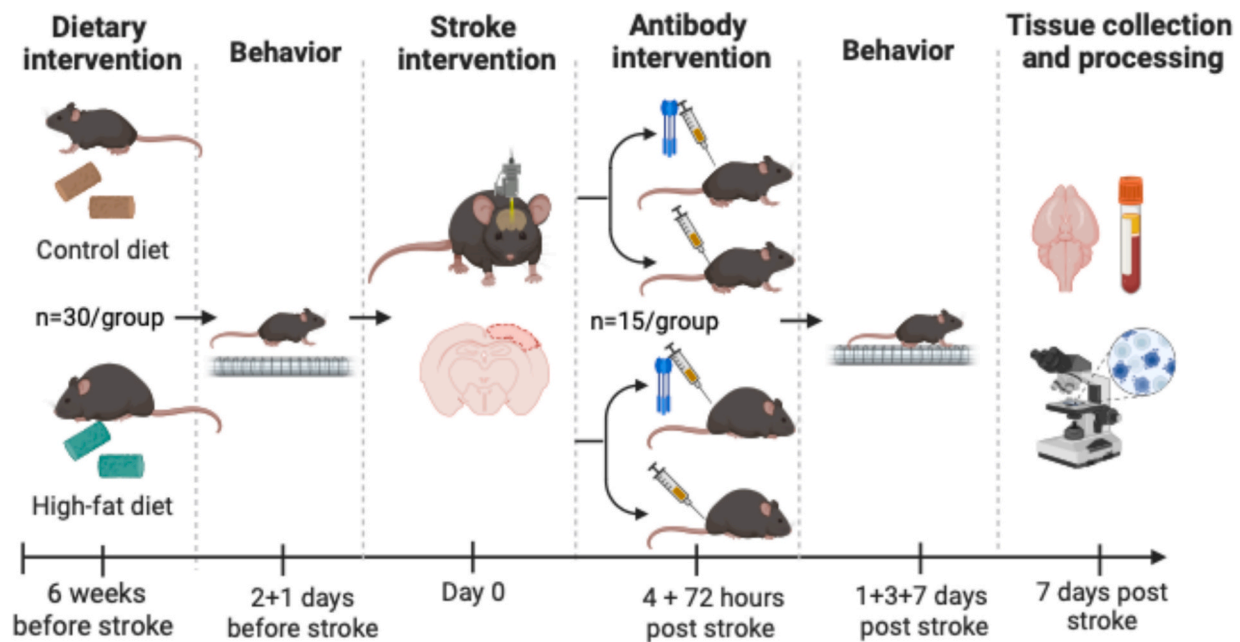


Fig. 1. Experimental design.

2.6. Immunohistochemistry

For standard immunohistochemistry procedures every sixth section was stained with specific antibodies against neuronal nuclear antigen (NeuN), CD68, and glial fibrillary acidic protein (GFAP). Sections were rinsed 3x 15 min in Tris-buffered saline (0.05 M TBS, pH 7.4) and placed on a shaker at 4 °C overnight. Next day, sections were rinsed 3x 5 min in TBS, 3x 10 min in TBS containing 0.5 % Triton (TBS-T), and blocked with 10 % fetal bovine serum (FBS; ThermoFisher Scientific) for 1 h at room temperature. Tissue was placed on a shaker and incubated at 4 °C for two days in primary antibody: biotinylated anti-NeuN (mouse, 1:500, Millipore, MAB377B), anti-GFAP (rabbit, 1:1200, DakoCytomation, Z0334), or anti-CD68 (rat, 1:400, BioRad, MCA1957S). After two days of incubation, tissue sections were adjusted to room temperature, rinsed 3x 15 min in TBS-T, followed by blocking for endogenous peroxidase activity for 10 min using 1:1:8 H₂O₂, methanol and TBS. For CD68 staining, tissue sections were rinsed 3x 5 min in TBS-T, incubated for 2 h in biotinylated anti-rat IgG (1:200, Vector Laboratories, BA-4001) diluted in TBS-T with 10 % FBS, followed by 3x 15 min rinses in TBS-T before finally being incubated with HRP-conjugated streptavidin (1:200, DakoCytomation, PO3971) diluted in TBS-T with 10 % FBS at 4 °C overnight gently on a shaker. For NeuN staining, sections were rinsed 3x 15 min in TBS-T and incubated with horse radish peroxidase (HRP)-conjugated streptavidin diluted in TBS-T with 10 % FBS at 4 °C overnight gently on a shaker. For GFAP staining, tissue sections were incubated with EnVision+ (HRP, rabbit, Dako) diluted 1:10 in TBS-T with 10 % FBS at 4 °C overnight gently on a shaker. The following day, sections were rinsed 3x 15 min in TBS and treated with 3,3'-Diaminobenzidine (DAB) solution (50 mg DAB (Sigma) per 100 mL of TBS with 0.33 % H₂O₂ added) for development of the immunohistochemical signal. Sections were then mounted on glass slides and allowed to air-dry. Finally, they were dehydrated in increasing concentrations of ethanol, cleared in xylene, and coverslipped with Depex mounting medium (VWR International, Søborg, Denmark). The images of brain sections were acquired with a NanoZoomer slide scanner (Hamamatsu).

2.7. Immunofluorescence

For immunofluorescent staining for neuron-glial antigen 2 (NG2) and myelin basic protein (MBP), sections were rinsed in PBS with 0.3 %

Triton. Non-specific binding was blocked by incubating sections in 10 % normal goat serum diluted in PBS with 0.3 % Triton, whereafter sections were incubated overnight at 4 °C with the following primary antibodies: anti-NG2 (rabbit, 1:1000, Sigma Aldrich, ZRB5320) and anti-MBP (rat, 1:100, ThermoFisher Scientific, MAB386) diluted in PBS with 0.3 % Triton and 5 % goat serum. The following day, sections were incubated with goat anti-rabbit Alexa 594 (1:600, Thermo Fisher Scientific, A-11012) and goat anti-rat Alexa 488 (1:600, Thermo Fisher Scientific, A-11006) diluted in PBS with 0.3 % Triton and 1 % goat serum for 2 h at room temperature. Nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, 1:000, Thermo Fisher Scientific, D1306) before mounting the slides with Aquatec (Abcam, Cambridge, UK). Images have been acquired at 40 × magnification using a Nikon ECLIPSE Ti2 confocal microscope.

2.8. ELISA

Plasma levels of lipopolysaccharides (LPS) 7 days post-stroke in regular diet and high-fat diet mice treated with vehicle or anti-TNFR1 antibody were measured with a mouse LPS ELISA kit (My Biosource, MBS261904) using a 1:10 dilution, according to the manufacturer's instructions.

2.9. Electrochemiluminescence analysis

Electrochemiluminescence assays were used to quantify the protein levels of TNF receptors, cytokines, neurofilament-light chain (NF-L), GFAP, and Tau as previously described (Madsen et al., 2016). TNFR1 was measured using a U-Plex Mouse TNF-R1 assay (K1520VK), TNFR2 using a R-Plex Mouse/Rat TNF-R2 assay (K150ZSR), cytokines (TNF, interferon (IFN)γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70) using a V-Plex Proinflammatory Panel1 (mouse) assay (K15048D), and NF-L, GFAP and Tau using a S-Plex Neurology Panel assay (K15639S-1), all from Mesoscale Discovery. All analyses were performed on a SECTOR Imager 6000 Plate based on a signal 2.5 standard deviations (SD) above the blank (zero) calibrator. For protein levels below LLOD, a value of 0.5 LLOD was used for statistical analysis. LLODs were: GFAP = 28,030 fg/mL, NF-L = 76,039 fg/mL, Tau = 766 fg/mL, TNF = 0.45 pg/mL, CXCL1 = 0.40 pg/mL, IL-6 = 12.3 pg/mL, IL-5 = 0.43 pg/mL, IL-4 = 0.45 pg/mL, IL-2 = 0.78 pg/mL, IL-1β = 0.25 pg/mL,

IL-12p70 = 4.39 pg/mL, IL-10 = 0.58 pg/mL, IFN- γ = 0.26 pg/mL, TNFR1 = 1.88 pg/mL, and TNFR2 = 1.19 pg/mL. IL-4 was below detection levels.

2.10. Quantification procedures

2.10.1. Infarct volumetric analysis

Estimation of the infarct volume was performed on NeuN-stained sections using the QuPath software as previously described (Lechtenberg et al., 2019), using the volume of the contralateral cortex for comparison to control for edema formation. The following formula was used:

Infarct volume (%) = volume of infarct / volume of contralateral hemisphere x 100.

2.10.2. Analysis of CD68 and GFAP positive area

To quantify CD68-positive and GFAP-positive cells, images of the infarct core and *peri*-infarct cortex were exported from total section scans at 20x magnification using the NDP.view2 software (Hamamatsu). These images were then converted to 8-bit grayscale using ImageJ, and a consistent threshold was applied to quantify the percentage of positive area for each staining (Raffaele et al., 2021, Raffaele et al., 2023).

2.10.3. Analysis of NG2-positive cell density

Images of NG2 staining in the *peri*-infarct cortex were analyzed using ImageJ using the “cell count” tool. All NG2 positive cells were manually counted; the tools “unsharp mask”, “enhance local contrast” and “color balance” were used to highlight the NG2⁺ cells and remove the background noise. The count values obtained for each image were normalized on the analyzed area so to obtain the cell density expressed as number of positive cells per mm² (Raffaele et al., 2021, Raffaele et al., 2023).

2.10.4. Analysis of myelin integrity

Images of MBP staining in the *peri*-infarct cortex were opened in ImageJ and the tools “unsharp mask” and “color balance” were used to maximize the contrast. Images were then converted to 8-bit grayscale using ImageJ, and a consistent threshold was applied to quantify the percentage of MBP-positive area (Raffaele et al., 2021, Raffaele et al., 2023).

2.11. Statistical analyses

Sample sizes were chosen based on previous studies using similar outcome measures and expected effect sizes, with consideration of statistical power. Animals were randomly assigned to experimental groups using alternating allocation, and when done on separate days all experiments included animals in all groups. The experimenter was blinded for all experiments and analysis. Animals that met euthanizing criteria or did not survive until the final endpoint were excluded from all immunohistochemical and behavioral analysis. Data distributions were assessed for normality using the Shapiro-Wilk test prior to statistical analyses. A Kaplan-Meier analysis was used to assess the significance of the survival curve. Histological and blood biomarker data were analyzed using a two-way ANOVA followed by Tukey’s multiple comparison test. Weight analyses were performed using a mixed-effects analysis followed by Tukey’s multiple comparison test. For behavior analyses we used a two-way repeated measures (RM) ANOVA and Fisher’s Least Significant Difference test using a 1 % false discovery rate (FDR). All statistical analyses and graphing were done using GraphPad Prism 9 Software and

considered significant at $P \leq 0.05$. Quantitative data are presented as means $\pm$ standard error of mean (SEM).

3. Results

3.1. High-fat diet causes weight gain and increased mortality rate post-stroke

First, we evaluated the mortality rate and body weight progression of non-obese and obese mice treated with anti-TNFR1 antibody or isotype control antibody after stroke. Obese mice showed a significantly higher mortality rate of 27 %, compared to non-obese mice with a mortality rate of 0 % ($p = 0.03$, Fig. 2a). No difference in mortality was observed between isotype- and anti-TNFR1-treated mice in any of the diet groups.

After 6 weeks, mice on high-fat diet exhibited a significant weight gain and became obese compared to non-obese mice (Fig. 2b). After stroke the mice lost a substantial amount of weight with the biggest weight loss being in the obese mice. Weight loss after stroke was similar between mice receiving the anti-TNFR1 antibody and the isotype antibody (Fig. 2b).

3.2. Anti-TNFR1 treatment did not affect stroke-related motor deficits exacerbated by obesity

The effect of diet on functional outcomes after photothrombotic stroke, as well as the impact of anti-TNFR1 treatment, were evaluated using the rotating beam and the tapered beam tests to assess motor functions on day 1, 3 and 7.

After stroke, obese mice displayed a significant reduction in the distance travelled and speed on the rotating beam compared to non-obese controls (Fig. 2c-d). Moreover, high-fat diet exposure induced a significantly increased latency on the tapered beam compared to regular diet (Fig. 2e). The total number of errors and the number of forelimb or hindlimb errors on the tapered beam (Fig. 2f-h) remained unchanged between groups. These data indicate impaired motor recovery after stroke in obese mice compared to non-obese controls.

When comparing the effect of the anti-TNFR1 treatment, we found no significant differences in non-obese mice between the isotype and the anti-TNFR1-treated group in terms of motor behavior outcomes (Fig. 2c-h). Conversely, in obese mice treated with anti-TNFR1 therapy, we did observe a trend toward reduction in the number of hindlimb errors compared to the isotype control antibody (Fig. 2h), indicating a potential mild improvement in motor function after anti-TNFR1 administration in the obese mice, which however did not reach statistical significance. No overall effect was observed in the other motor parameters evaluated in obese mice receiving the anti-TNFR1 antibody or isotype antibody (Fig. 2c-g).

3.3. Anti-TNFR1 therapy had a limited impact on circulating biomarkers of inflammation after stroke in obese mice

Given the established association between obesity and low-grade systemic inflammation, we wondered whether exposure to high-fat diet may result in heightened inflammation after stroke and if this could be dampened by anti-TNFR1 therapy. We therefore analyzed plasma levels of TNF receptors (TNFR1 and TNFR2) and a panel of cytokines to assess systemic inflammation 7 days after stroke in non-obese and obese mice treated with isotype antibody or anti-TNFR1 antibody.

Anti-TNFR1 treatment did lead to elevated TNFR1 levels, indicating target engagement and a likely positive feedback loop (Fig. 3a).

High-fat diet alone significantly increased TNFR2, TNF, IL-6, IL-1 β , IL-10, and IL-2 levels, compared to regular diet, indicating an elevated

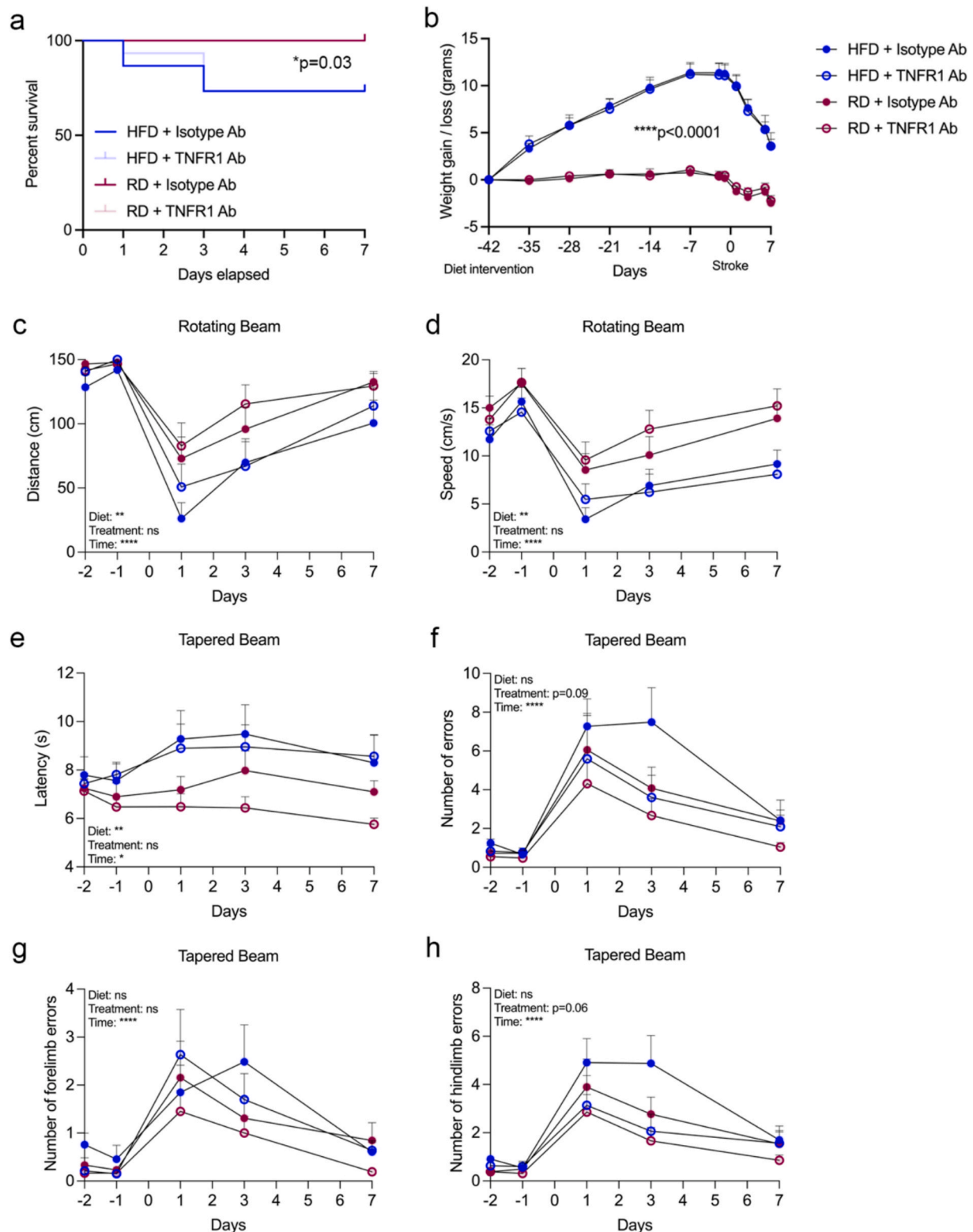
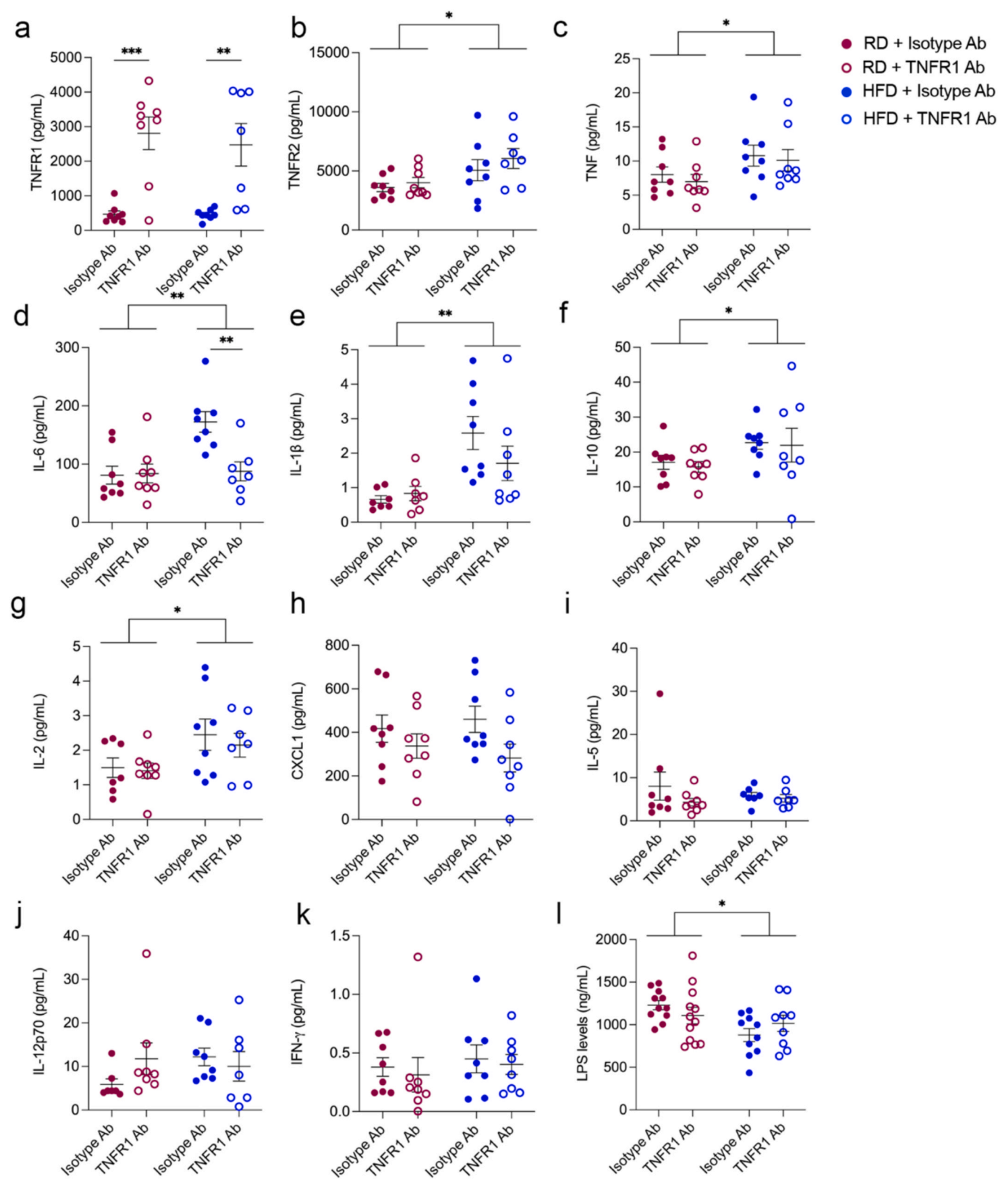


Fig. 2. Effects of high-fat diet and anti-TNFR1 treatment on mortality, weight change, and motor functions after stroke. (a) Survival curve showing mortality rates after photothrombotic stroke in mice fed a regular diet or high-fat diet and receiving an anti-TNFR1 or isotype antibody treatment. There was a significant difference between obese and non-obese mice. Log-Rank (Mantel-Cox) test ($X^2 = 9.08$, $*p = 0.03$, $n = 11-15$ /group). (b) Weights of non-obese and obese mice treated with either anti-TNFR1 antibody or isotype antibody shown as weight gain/loss compared to starting weight. Timepoints shown are the weekly weight assessments during the 6 weeks feeding period, before stroke surgeries, after behavioral testing, and before and after the 24-hour fasting period prior to the glucose tolerance test and sacrifice. (c) Distance travelled on the rotating beam. (d) Speed on the rotating beam. (e) Latency to transverse the tapered beam. (f-h) Number of errors on the tapered beam presented as total errors (forelimb and hindlimb) (f), forelimb errors (g), and hindlimb errors (h). Data are expressed as mean $\pm$ SEM. RM three-way ANOVA, $n = 11-14$ /group. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, ns: not significant. ANOVA summary results are included in each panel to highlight the effects of diet, treatment, and time. Ab, antibody; HFD, high-fat diet; RD, regular diet.



(caption on next page)

Fig. 3. Effects of high-fat diet and anti-TNFR1 treatment on blood levels of LPS, TNF receptors and selected cytokines after stroke. (a-k) Analysis of blood levels of TNFR1 (Treatment: $F_{1, 27} = 34.15$, **** $p < 0.0001$; Diet: $F_{1, 27} = 0.21$, $p = 0.65$; Interaction: $F_{1, 27} = 0.19$, $p = 0.67$) (a), TNFR2 (Treatment: $F_{1, 27} = 1.13$, $p = 0.30$; Diet: $F_{1, 27} = 6.99$, * $p = 0.01$; Interaction: $F_{1, 27} = 0.1$, $p = 0.66$) (b), TNF (Treatment: $F_{1, 28} = 0.41$, $p = 0.53$; Diet: $F_{1, 28} = 4.86$, * $p = 0.04$; Interaction: $F_{1, 28} = 0.02$, $p = 0.9$) (c), IL-6 (Treatment: $F_{1, 27} = 6.23$, * $p = 0.02$; Diet: $F_{1, 27} = 8.43$, ** $p = 0.007$; Interaction: $F_{1, 27} = 7.18$, * $p = 0.01$) (d), IL-1 β (Treatment: $F_{1, 26} = 0.84$, $p = 0.37$; Diet: $F_{1, 26} = 13.09$, *** $p < 0.001$; Interaction: $F_{1, 26} = 1.83$, $p = 0.19$) (e), IL-10 (Treatment: $F_{1, 28} = 0.13$, $p = 0.73$; Diet: $F_{1, 28} = 4.22$, * $p < 0.05$; Interaction: $F_{1, 28} = 0.01$, $p = 0.92$) (f), IL-2 (Treatment: $F_{1, 26} = 0.33$, $p = 0.57$; Diet: $F_{1, 26} = 6.16$, * $p = 0.02$; Interaction: $F_{1, 26} = 0.1$, $p = 0.75$) (g), CXCL1 (Treatment: $F_{1, 28} = 4.46$, * $p = 0.043$; Diet: $F_{1, 28} = 0.01$, $p = 0.92$; Interaction: $F_{1, 28} = 0.65$, $p = 0.43$) (h), IL-5 (Treatment: $F_{1, 26} = 1.3$, $p = 0.26$; Diet: $F_{1, 26} = 0.12$, $p = 0.73$; Interaction: $F_{1, 26} = 0.67$, $p = 0.42$) (i), IL-12p70 (Treatment: $F_{1, 26} = 0.44$, $p = 0.51$; Diet: $F_{1, 26} = 0.66$, $p = 0.42$; Interaction: $F_{1, 26} = 2.09$, $p = 0.16$) (j), and IFN γ (Treatment: $F_{1, 28} = 0.27$, $p = 0.61$; Diet: $F_{1, 28} = 0.51$, $p = 0.48$; Interaction: $F_{1, 28} = 0.007$, $p = 0.93$) (k) 7 days post-stroke in isotype antibody- or TNFR1 antibody-treated non-obese and obese mice ($n = 8$ /group). (l) Analysis of blood LPS levels 7 days post-stroke in isotype antibody- or TNFR1 antibody-treated non-obese and obese mice (Treatment: $F_{1, 38} = 0.009$, $p = 0.93$; Diet: $F_{1, 38} = 7.1$, ** $p < 0.01$; Interaction: $F_{1, 38} = 2.45$, $p = 0.13$, $n = 10$ – 12 /group). Two-Way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean $\pm$ SEM. Ab, antibody.

inflammatory profile after stroke associated with obesity (Fig. 3b-g). Anti-TNFR1 treatment was able to significantly reduce IL-6 levels in obese mice (Fig. 3d), while it did not affect other cytokines elevated in obesity (Fig. 3b-g). Neither diet nor treatment affected peripheral IL-5, IL-12p70, or IFN- γ levels (Fig. 3h-k).

Next, we assessed circulating LPS levels to further evaluate the

effects of diet and treatment on gut barrier permeability and systemic inflammation. To our surprise LPS levels were reduced in isotype antibody-treated obese mice compared to non-obese mice, with no evident effect of anti-TNFR1 therapy (Fig. 3l).

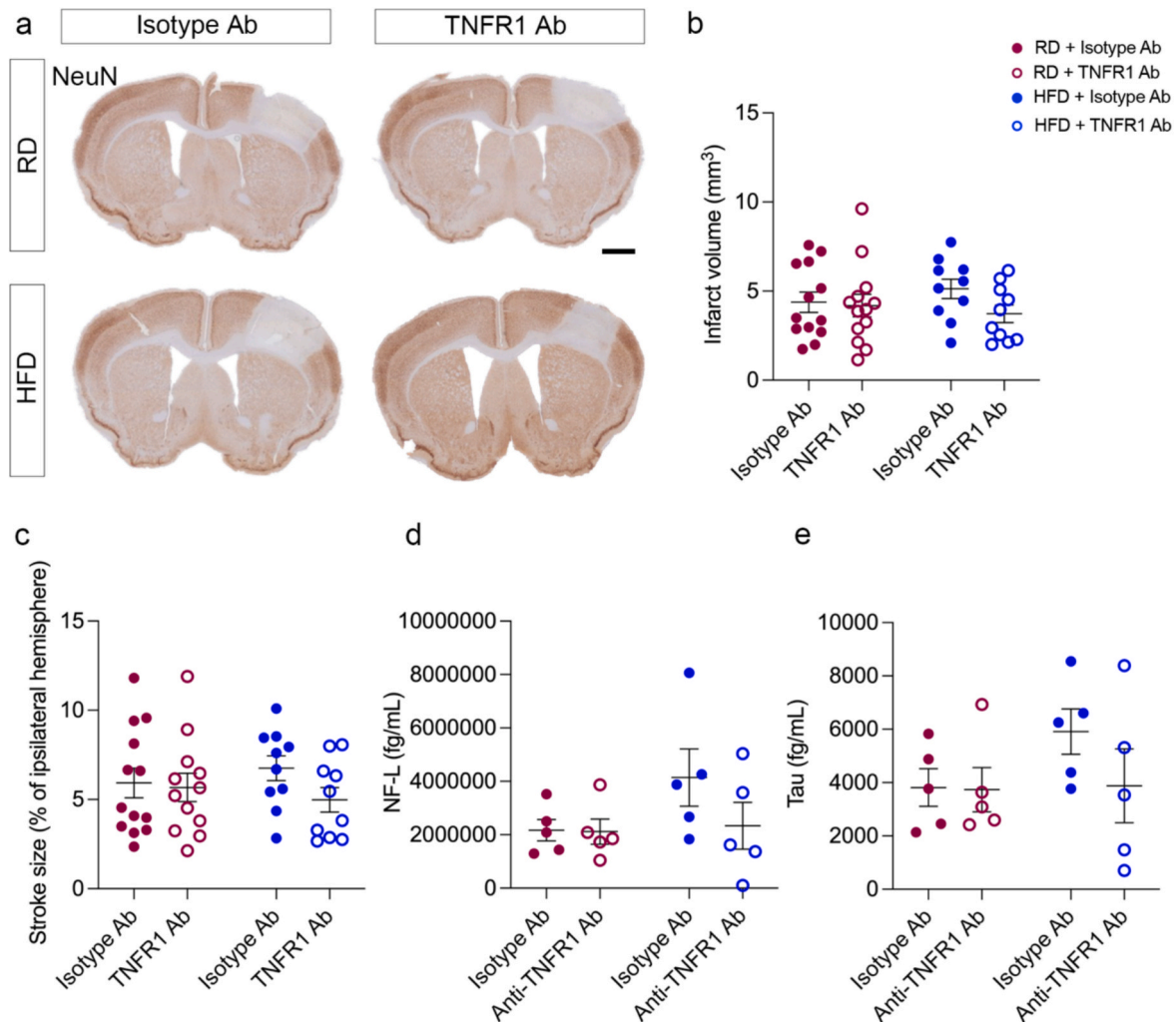


Fig. 4. Effects of high-fat diet and anti-TNFR1 treatment on infarcts size and neurodegenerative blood biomarkers post-stroke (a) NeuN staining of frontal brain sections from regular diet and high-fat diet isotype antibody- or TNFR1 antibody-treated mice treated 7 days post-stroke. Scale bar: 1 mm. (b) Estimation of cortical infarct volume in isotype antibody- or TNFR1 antibody-treated regular diet and high-fat diet mice 7 days after stroke (Treatment: $F_{1, 42} = 1.86$, $p = 0.18$; Diet: $F_{1, 42} = 0.06$, $p = 0.8$; Interaction: $F_{1, 42} = 1.06$, $p = 0.31$, $n = 10$ – 13 /group). (c) Estimation of infarcted area as percentage of the ipsilateral hemisphere in isotype antibody- or TNFR1 antibody-treated non-obese and obese mice 7 days post-stroke (Treatment: $F_{1, 41} = 1.68$, $p = 0.93$; Diet: $F_{1, 41} = 0.007$, $p = 0.93$; Interaction: $F_{1, 41} = 0.95$, $p = 0.34$, $n = 10$ – 12 /group). (d-e) Analysis of blood neurofilament light chain (NF-L) levels (Treatment: $F_{1, 16} = 1.5$, $p = 0.24$; Diet: $F_{1, 16} = 2.1$, $p = 0.17$; Interaction: $F_{1, 16} = 1.34$, $p = 0.26$) (d) and blood Tau levels (Treatment: $F_{1, 16} = 1.17$, $p = 0.3$; Diet: $F_{1, 16} = 1.32$, $p = 0.27$; Interaction: $F_{1, 16} = 0.1$, $p = 0.33$) (e) levels 7 days after stroke in isotype antibody- or TNFR1 antibody-treated non-obese and obese mice ($n = 5$ /group). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's multiple comparison test. Ab, antibody; HFD, high-fat diet; RD, regular diet.

3.4. Anti-TNFR1 treatment did not modify infarct size or blood biomarkers of neurodegeneration after stroke

To assess the effect of anti-TNFR1 treatment on infarct volume, frontal brain sections from non-obese and obese mice treated with either isotype antibody or anti-TNFR1 antibody were stained for the neuronal marker NeuN at day 7 after stroke (Fig. 4a). Quantitative analysis of direct infarct volume (Fig. 4b) and stroke size as a percentage of the ipsilateral hemisphere (Fig. 4c) revealed no significant differences between diet or treatment groups. Furthermore, analysis of blood NF-L (Fig. 4d) and Tau (Fig. 4e) levels as biomarkers of neurodegeneration showed no significant difference between diet or treatment groups.

3.5. Anti-TNFR1 treatment had no effects on microglial/macrophage and astrocyte reactivity after stroke

To evaluate microglial/macrophage and astrocyte reactivity in the *peri*-infarct region 7 days after stroke, immunostaining for CD68 (Fig. 5) and GFAP (Fig. 6), respectively, was conducted in non-obese and obese mice treated with isotype antibody or anti-TNFR1 antibody. For CD68, we estimated CD68 expression in the stroke border as well as in the *peri*-infarct area (Fig. 5a). Quantitative analysis of the percentage area covered by CD68 staining in the stroke border (Fig. 5b) and *peri*-infarct area (Fig. 5c) showed no effect of diet or treatment on microglia/macrophage reactivity 7 days post-stroke.

For astrogliosis, we estimated GFAP expression in the *peri*-infarct area (Fig. 6a) and found no significant differences between groups in the percentage area covered by GFAP staining (Fig. 6b). Estimation of blood

GFAP levels revealed increased levels in obese mice compared to non-obese mice after stroke, however, anti-TNFR1 treatment did not affect GFAP blood levels (Fig. 6c).

3.6. Anti-TNFR1 treatment did not alter NG2⁺ cell density or myelin integrity post-stroke

To evaluate the effect of high-fat diet as well as anti-TNFR1 treatment on the density of oligodendrocyte precursor cells (OPCs) and myelin integrity 7 days after stroke, we performed immunofluorescent staining for NG2 and myelin basic protein (MBP), respectively. The density of NG2⁺ OPCs in the *peri*-infarct region was comparable between diet and treatment groups (Fig. 7a,b). Moreover, we did not observe any effect of diet or treatment on MBP⁺ area fraction in the cortical *peri*-infarct area (Fig. 7c,d).

4. Discussion

In the present study, we examined for the first time the effects of anti-TNFR1 treatment on ischemic stroke outcome in mice with diet-induced obesity and compared to non-obese mice. High fat consumption has a negative effect on glucose homeostasis, causing glucose intolerance and weight gain, contributing to increased mortality and worsened outcome after ischemic stroke (Haley et al., 2019, Lin et al., 2021, Peterson et al., 2021, Williams et al., 2014a). Here, 6 weeks of high-fat diet increased body weight and mortality after stroke, and impaired functional outcomes, compared to regular diet-fed mice. Anti-TNFR1 treatment did not influence the amount of weight loss after stroke and did not lead to

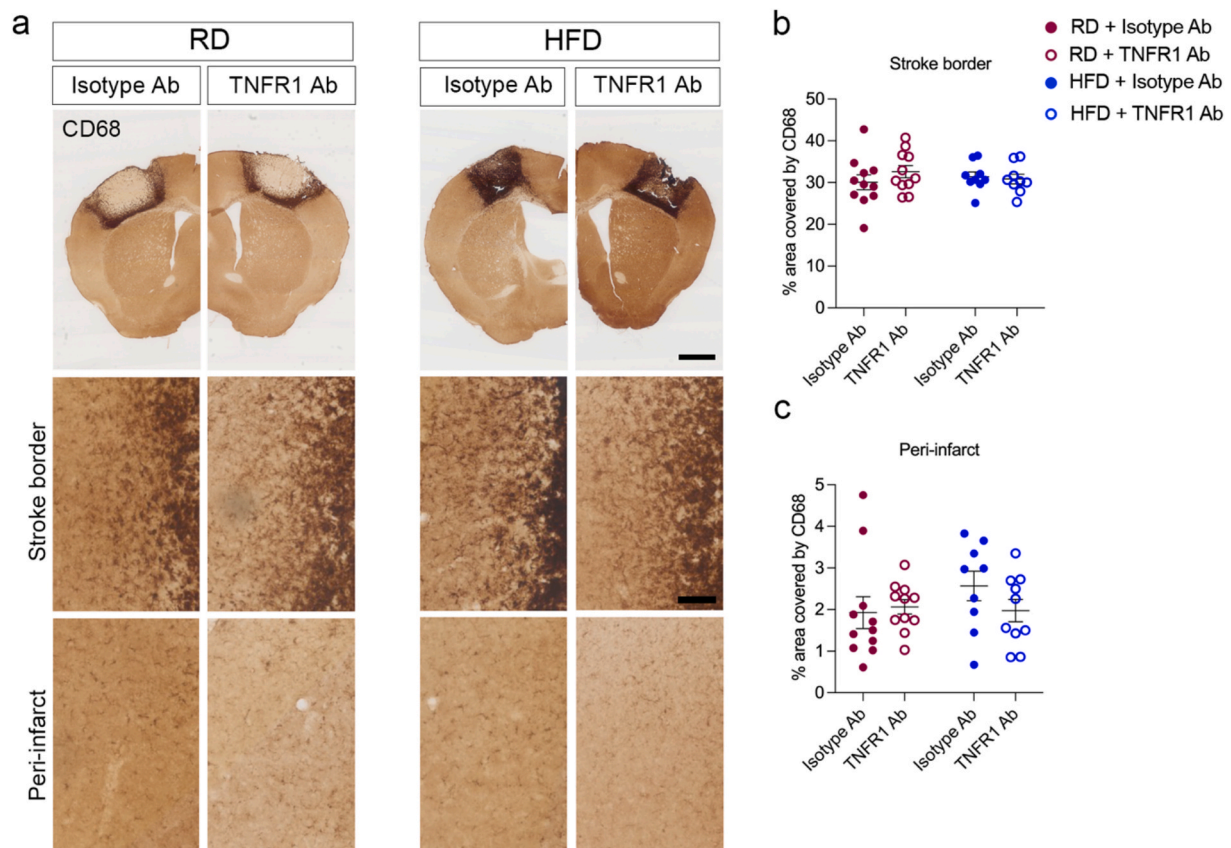


Fig. 5. Effects of high-fat diet and anti-TNFR1 treatment on microglial/macrophage responses 7 days post-stroke (a) Representative images of CD68 staining of frontal brain sections from regular diet and high-fat diet isotype antibody- or TNFR1 antibody-treated mice 7 days post-stroke. Scale bars: upper panel 1 mm, lower panel 50 μ m. (b) Percentage area covered by CD68 in the stroke border (Treatment: $F_{1,36} = 0.46$, $p = 0.50$; Diet: $F_{1,36} = 0.02$, $p = 0.89$; Interaction: $F_{1,36} = 1.12$, $p = 0.30$, $n = 9-11$ /group). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's multiple comparison test. (c) Percentage area covered by in the *peri*-infarct region (Treatment: $F_{1,37} = 0.56$, $p = 0.46$; Diet: $F_{1,37} = 0.82$, $p = 0.37$; Interaction: $F_{1,37} = 1.45$, $p = 0.24$, $n = 9-11$ /group). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's multiple comparison test. Ab, antibody; HFD, high-fat diet; RD, regular diet.

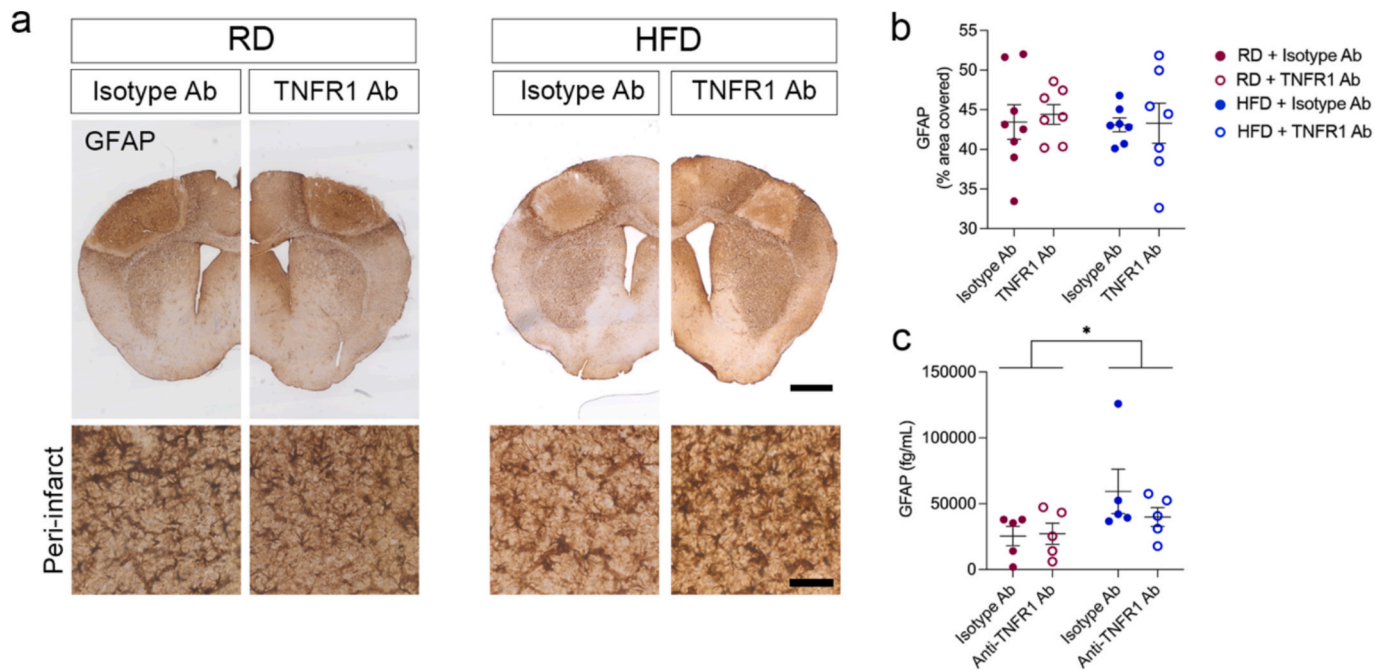


Fig. 6. Effects of high-fat diet and anti-TNFR1 treatment on astrocyte reactivity 7 days post-stroke (a) Representative images of GFAP staining of frontal brain sections from regular diet and high-fat diet isotype antibody- or TNFR1 antibody-treated mice 7 days after stroke. Scale bars: upper panel 1 mm, lower panel 50 μ m. (b) Percentage area covered by GFAP (Treatment: $F_{1, 25} = 0.09$, $p = 0.76$; Diet: $F_{1, 25} = 0.15$, $p = 0.70$; Interaction: $F_{1, 25} = 0.04$, $p = 0.84$, $n = 7-8$ /group). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's multiple comparison test. (c) Analysis of blood GFAP levels 7 days post-stroke in isotype antibody- or TNFR1 antibody-treated non-obese and obese mice (Treatment: $F_{1, 16} = 0.68$, $p = 0.42$; Diet: $F_{1, 16} = 4.76$, $*p = 0.04$; Interaction: $F_{1, 16} = 0.98$, $p = 0.34$, $n = 5$ /group). Data are expressed as mean $\pm$ SEM. Two-Way ANOVA followed by Tukey's multiple comparison test. $*p < 0.05$. Ab, antibody; HFD, high-fat diet; RD, regular diet.

an improvement in mortality, suggesting that systemic anti-TNFR1 treatment alone is not sufficient to improve survival outcomes in this context, and that additional factors contribute to the increased mortality observed in our obese mice.

For evaluation of functional outcomes after photothrombotic stroke targeting the motor cortex, we assessed motor behavior using the tapered beam task and the rotating beam task. Our results show that obese mice performed worse than non-obese mice with decreased average speed and distance on the rotating beam and increased latency on the tapered beam. Anti-TNFR1 treatment did not significantly affect functional outcome after stroke, however, there was a trend toward an effect of treatment on the total number of errors and the number of hindlimb errors on the tapered beam. Especially, we observed that anti-TNFR1 treatment seemed to have a greater positive effect on transiently improving motor deficits by reducing the number of hindlimb errors and total errors in obese mice, but not in non-obese mice, 3 days after stroke. This aligns with what a previous study found to be the timepoint with obesity exacerbated neuroinflammation, including higher levels of TNF (Peterson et al., 2021), and it supports the possibility that the altered immune response in obesity leads to a different response to treatments. In line with previous reports demonstrating improved recovery trajectories in mice that are not driven by lesion size or location (Kalantari et al., 2025; Yu et al., 2015), all groups significantly improved their behavioral performance over time after photothrombotic stroke, reflecting spontaneous recovery in both obese and non-obese mice. Not many other studies have evaluated the potential of targeting inflammation to treat obesity-exacerbated stroke outcomes. Nonetheless, a recent study investigating the effects of fingolimod, a drug with immunomodulatory properties, on stroke outcomes in obese ApoE $^{-/-}$ mice (a model of hyperlipidemia, a common stroke comorbidity) found that fingolimod reduced infarct size in these obese mice (Diaz Diaz et al., 2022). Interestingly, however, the same treatment increased infarct size in control mice, indicating that the response to immunomodulatory

treatments may differ in obese compared to non-obese mice, in line with what we found in our study. The finding that the response to the anti-TNFR1 treatment differed between obese and non-obese mice emphasizes the importance in considering common co-morbidities to stroke when investigating possible treatments.

Diet-induced obesity has been shown to shift immune cells towards a more pro-inflammatory state. Adipocytes can release chemokines and other cytokines to recruit blood and tissue monocytes and macrophages to infiltrate the adipose tissue, where they acquire a more pro-inflammatory state (Li et al., 2010; Lumeng et al., 2007; Lumeng et al., 2008). Adipose tissue then acts as a pro-inflammatory organ and drives a concomitant increase in pro-inflammatory cytokines in the blood (Galvez et al., 2019; Greenberg and Obin, 2006). This eventually leads to chronic low-grade systemic inflammation impacting systemic tissues and, as cytokines can cross the blood-brain barrier, these are likely to act directly on the brain. We investigated the dietary effect of anti-TNFR1 therapy on systemic inflammation 7 days after stroke in obese and non-obese mice. In line with the literature, we found that high-fat diet significantly increased plasma levels of IL-1 β , IL-6, IL-10, IL-2, TNF, and TNFR2 levels compared to regular diet-fed controls, indicating a prolonged worsening of systemic inflammation post-stroke. It is worth mentioning that such alterations exceed the reported time window in which pro-inflammatory cytokines peak in lean mice, which is restricted within the first 24–48 h after stroke (Lambertsen et al., 2012) and may thus be responsible for long-term detrimental effects on motor and cognitive functions associated with obesity.

Interestingly, we found that anti-TNFR1 treatment significantly increased circulating TNFR1 levels, suggesting a compensatory mechanism to cope with blocked receptor activity that might impair treatment efficacy but nevertheless confirms target engagement. However, anti-TNFR1 treatment only significantly reduced IL-6 levels in obese mice, indicating that this approach has a limited efficacy in this context and that multiple pro-inflammatory signaling pathways may contribute to

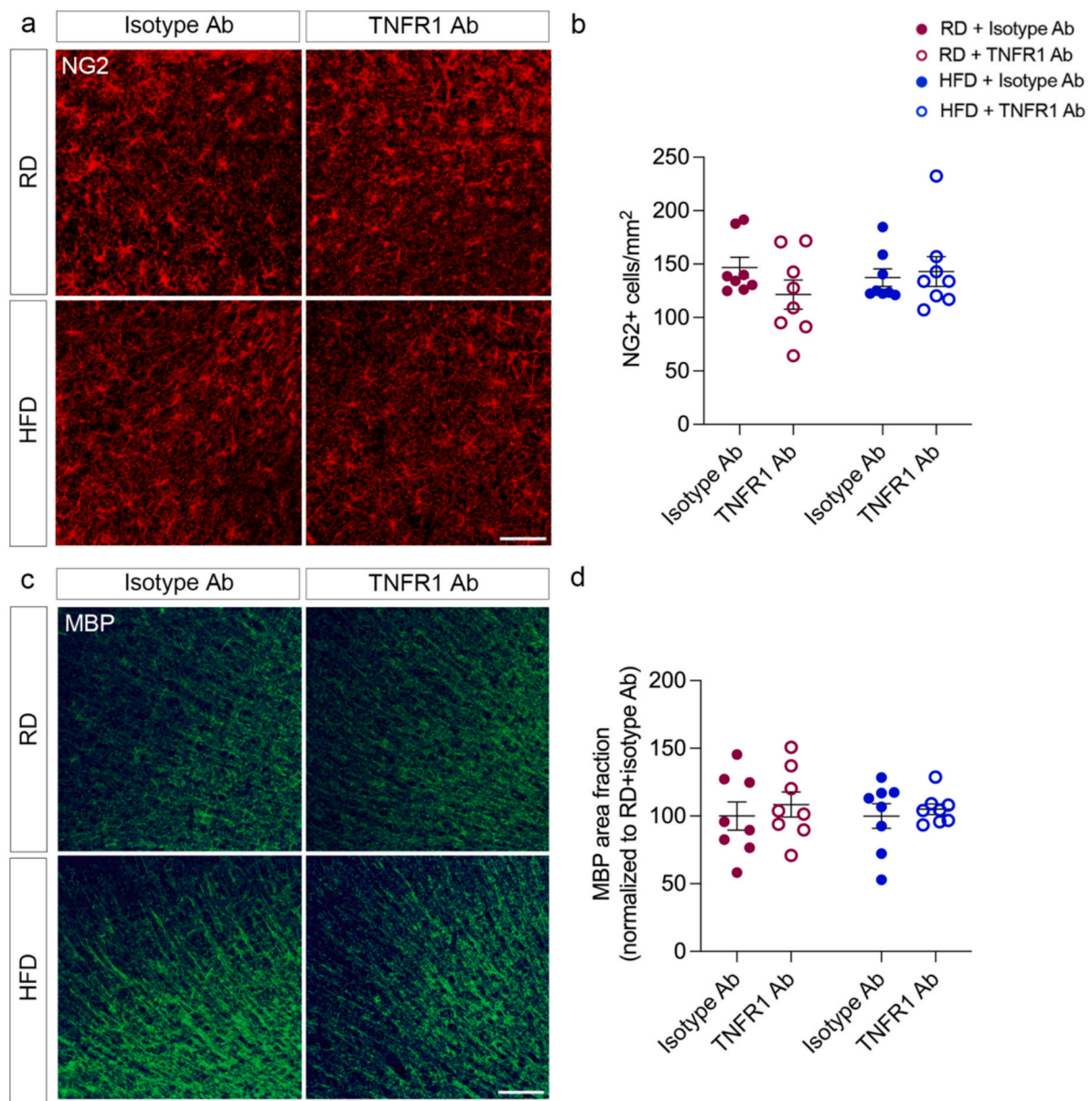


Fig. 7. Effects of high-fat diet and anti-TNFR1 treatment on oligodendrocytes and myelin integrity after stroke (a) Representative images of NG2⁺ OPCs located in the peri-infarct area (0–500 μ m) 7 days post-stroke in isotype antibody- or TNFR1 antibody-treated regular diet and high-fat diet mice. (b) Quantification of the density of NG2⁺ OPCs 7 days post-stroke in isotype antibody- or TNFR1 antibody-treated regular diet and high-fat diet mice (Treatment: $F_{1, 28} = 0.7$, $p = 0.41$; Diet: $F_{1, 28} = 0.27$, $p = 0.61$; Interaction: $F_{1, 28} = 1.78$, $p = 0.19$, $n = 8$ /group). (c) Representative images of myelin visualized using myelin basic protein (MBP) staining in the ipsilateral cortex adjacent to the boundary of the ischemic lesion at day 7 post-stroke in isotype antibody- or TNFR1 antibody-treated regular diet and high-fat diet mice. (d-e) Quantification of MBP integrated density (Treatment: $F_{1, 28} = 0.65$, $p = 0.43$; Diet: $F_{1, 28} = 0.04$, $p = 0.85$; Interaction: $F_{1, 28} = 0.04$, $p = 0.8$) (d) and MBP area fraction (Treatment: $F_{1, 28} = 0.62$, $p = 0.44$; Diet: $F_{1, 28} = 0.04$, $p = 0.84$; Interaction: $F_{1, 28} = 0.04$, $p = 0.84$) (e) in the ipsilateral cortex 7 days post-stroke in isotype antibody- or TNFR1 antibody-treated non-obese and obese mice ($n = 8$ /group). Scale bars: 100 μ m. Two-Way ANOVA followed by Tukey's multiple comparison test. Data are expressed as mean $\pm$ SEM. Ab, antibody; HFD, high-fat diet; RD, regular diet.

exacerbated inflammation in obesity. It was also interesting that there were some mice with less of a change in circulating TNFR1 levels, and we did a post-hoc analysis of our results and found no difference, including in the reduction in IL-6. We also observed an effect of the diet on circulating TNFR2 levels with significantly increased levels in obese mice, compared to non-obese mice, however, there was no effect of anti-TNFR1 treatment. In obese mice, systemic TNFR1 blockage is known to shift TNF signaling away from pro-inflammatory TNFR1 pathways (Wandrer et al., 2020) and to enhance TNFR2-mediated regulatory functions (Atrekhany et al., 2018; Chen et al., 2007), suggesting that promoting TNFR2 signaling pathways post-stroke may be protective. However, we recently demonstrated that systemic treatment with a

TNFR2 agonist in non-obese, male mice induced changes in peripheral immune cells and only transiently affected neuroinflammation, which was not enough to improve long-term functional outcome after stroke (Thougaard et al., 2023). Thus, it is important to note that there are complex regulatory mechanisms that may respond to perturbations in TNF signaling and that future therapies may need to target additional arms of the pathway to be efficacious.

The gut microbiota and their metabolites are believed to play important roles in ischemic stroke and to modulate brain functions directly or indirectly through immune, endocrine, vagal or other humoral pathways (Zhang et al., 2023). Obese mice have been shown to have increased blood levels of LPS, which are associated with impaired

humor and cognition (de Paiva et al., 2024), while in humans, fecal LPS levels were associated with an increased risk of poor functional outcomes at day 90 after stroke (Jiang et al., 2023; Kuprash et al., 2005). We therefore assessed the effect of diet and anti-TNFR1 therapy on blood LPS levels 7 days after stroke. To our surprise, we observed reduced plasma LPS levels 7 days after stroke in our obese mice, compared to non-obese mice, an effect that was independent of anti-TNFR1 treatment. This may be related to a previously unexplored combined effect of obesity and stroke on gut microbiota composition, potentially favoring bacteria that produce less LPS, or to obesity-related alterations in circulating lipoproteins and inflammation, which might paradoxically affect LPS processing and clearance (Kumar et al., 2024). Future research is warranted to further elucidate the multifaceted interplay between obesity, inflammation, and microbiota and the implications for stroke recovery.

Despite previous studies showing that conventional TNFR1 knock out (KO) mice develop larger infarct sizes after ischemic stroke, compared to controls (Lambertsen et al., 2009; Taoufik et al., 2007), inhibiting TNFR1 using a neutralizing antibody, similar to the one used in this study, resulted in reduced infarct size and improved metabolic changes after stroke in non-obese mice (Lin et al., 2021), indicating a protective effect of TNFR1 inhibition. In the present study, we therefore investigated the effect of systemically inhibiting TNFR1 in obese and non-obese mice subjected to photothrombotic stroke and allowed 7 days survival. We did, however, not observe any effect of diet or treatment on infarct size or blood biomarker levels of brain injury (NF-L and Tau) 7 days after photothrombotic stroke. Given that anti-TNFR1 treatment has been effective in improving post-ischemic hyperglycemia (Lin et al., 2021), it is plausible that the transient therapeutic effects observed on functional outcome in our study may be partially mediated through the modulation of glucose metabolism alongside the anti-inflammatory properties of TNFR1 blockade.

Transient or permanent occlusion of the middle cerebral artery has been shown to increase astro- and microgliosis in obese mice, compared to non-obese mice (Clain et al., 2025; Peterson et al., 2021), suggesting that diet-induced obesity enhances stroke-induced neuroinflammation. In the present study, we did, however, not observe a dietary effect on CD68 microglial/macrophagic reactivity at the stroke border or in the *peri*-infarct area. We also did not observe a dietary effect on GFAP astroglial reactivity in the *peri*-infarct area, despite our findings of increased peripheral blood GFAP levels in obese mice, compared to non-obese mice. Moreover, systemic TNFR1 inhibition did not affect CD68 and GFAP quantifications in either obese and control groups. These findings might suggest that the glial responses visible at the histological level in our experimental timeframe are not dramatically altered by high fat diet or anti-TNFR1 treatment. However, glial reactivity, particularly in microglia and astrocytes, is increasingly understood to be multifaceted as both cell types exhibit highly dynamic changes in morphology, function, and gene expression in response to injury and metabolic status (Escartin et al., 2021; Paolicelli et al., 2022). For instance, microglia may adopt diverse reactive states ranging from pro-inflammatory to neuroprotective or reparative phenotypes, which can differ in subtle morphological features or in the expression of specific transcripts and metabolic profiles (Cutugno et al., 2024). Similarly, astrocytes can develop into functionally distinct subtypes that contribute variably to inflammation, synaptic remodeling, or metabolic support of neurons, many of which are not well reflected by GFAP immunoreactivity alone (Obara-Michlewska, 2022). Therefore, while our immunohistochemical analysis did not detect gross differences in glial reactivity across diet and treatment groups, this does not rule out more subtle or regionally restricted effects on glial state. In future studies, techniques such as single-cell RNA sequencing, high-resolution morphometric analysis, or metabolic profiling would be better suited to capture these nuanced changes.

Selective inhibition of soluble TNF using dominant negative soluble TNF inhibitors, such as XPro1595 (Steed et al., 2003), has been shown to

block detrimental soluble TNF-TNFR1 signaling and improve remyelination in animal models of multiple sclerosis (Brambilla et al., 2011; Karamita et al., 2017) and spinal cord injury (Novrup et al., 2014), a process believed to be regulated via TNFR1-dependent microglial phenotypic polarization (Boutou et al., 2024). Therefore, we investigated the effect of anti-TNFR1 treatment on OPC density and myelin integrity in the cerebral cortex in areas ipsilateral to the infarct in non-obese and obese mice 7 days after stroke. We did, however, not observe an effect of anti-TNFR1 treatment on the density of NG2⁺ OPCs or in MBP immunoreactivity of obese or non-obese mice 7 days after stroke. This lack of observable effect may, however, reflect temporal limitations of our experimental design rather than an absence of therapeutic potential. The process of remyelination is inherently delayed following ischemic injury, particularly in models such as photothrombotic stroke where demyelination is often partial and localized. In such models, the initial phase post-injury is typically dominated by inflammation and tissue remodeling, with robust oligodendrocyte differentiation and myelin repair occurring predominantly in subacute to chronic phases, often beyond 14 days after stroke (Han et al., 2024). Moreover, OPC proliferation and migration typically precede differentiation, and early-stage assessments may not adequately reflect downstream effects on myelin regeneration (Raffaele and Fumagalli, 2022). Future studies extending the observation period beyond 7 days will be essential to determine whether anti-TNFR1 treatment exerts delayed promyelinating effects after stroke, and whether these effects are modulated by metabolic status.

A limitation of the present study is that we only utilized male mice. We initially chose male mice because they exhibit more harmful responses than female mice in cardiac ischemia models (Wang et al., 2006). We thus reasoned that if we were to see an effect of the antibody therapy on behavior, it would be seen more prominently in males. However, we cannot rule out from this work that females might have a different response. Indeed, obese female mice develop larger infarcts compared to non-obese female mice (Peterson et al., 2021), while central blockage of TNFR1 using anti-TNFR1 therapy improved functional cortical plasticity in non-obese female mice early after stroke (Liguz-Leczna et al., 2015). And female mice do have a therapeutic response to anti-TNFR1 therapy in a mouse model of multiple sclerosis, where treatment reduced central immune cell infiltration through downregulation of TNF-induced adhesion molecules (Williams et al., 2018; Williams et al. (2014b)).

We also acknowledge that this study is primarily descriptive, however we did test the effect of anti-TNFR1 therapy in acute stroke in male mice. Because there were only modest effects we did not pursue additional studies to investigate mechanisms further. However, we feel it is important to report this kind of data in order to help others in the field move forward. We also think that a mechanism can be inferred from our results that could be tested in the future by others interested in this pathway. The changes in IL-6 levels in obese mice with anti-TNFR1 therapy, compared to isotype treatment, especially point towards a change in downstream TNFR signaling cascades shifting TNFR1 signaling towards TNFR2 signaling. It is possible that anti-TNFR1 treatment modified the systemic immune response by leading to a shift in downstream signaling pathways in adipocytes and immune cells. TNFR1 blockade will likely result in changes in TNFR1-dependent RIP1 ubiquitination and decreased activation of the canonical nuclear factor-kappa B (NF- κ B) pathway and altered apoptosis signal-regulating kinase 1 (ASK1) and Janus kinase (JNK) activity, while the preservation of TNF signaling through TNFR2 in contrast may potentially lead to an increase in PI3K-Akt (Fischer et al., 2020; Siegmund and Wajant, 2023). This may ultimately result in an ameliorated immune response in obese mice; however, this remains to be elucidated.

Taken together, our data demonstrate that obesity exacerbates stroke outcome through increased mortality, impaired functional recovery, and elevated systemic inflammation. Although TNFR1 blockade showed modest effects on circulating cytokine levels and motor performance, it

did not confer substantial neuroprotection or modulate key cellular responses in the brain. These findings suggest that targeting TNFR1 alone may be insufficient as a therapeutic strategy for improving stroke recovery in the context of obesity and highlight the need to explore broader or combination immunomodulatory approaches.

5. Consent for publication

Not applicable.

6. Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

7. Authors' contributions

C.M.H. designed and performed the research, analyzed the data, and wrote the paper together with K.L.L., S.R., and M.S.B. K.B. helped design the research, assisted with surgeries and interpretation of the data and statistical analyses. S.C. performed immunohistochemistry and immunofluorescence staining and analyses. S.R. helped design the research and assisted with analysis together with M.F. K.L.L., and M.S.B. helped design the research and assisted with interpretation of the data and statistical analyses. All authors read and approved the final manuscript.

CRedit authorship contribution statement

Christine Haarslev: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Karen Bradshaw:** Writing – review & editing, Supervision, Methodology, Investigation. **Silvia Corradini:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Stefano Raffaele:** Writing – original draft, Validation, Supervision, Methodology, Formal analysis. **Marta Fumagalli:** Writing – review & editing, Supervision, Resources. **Marion S. Buckwalter:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Kate Lykke Lambertsen:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

8. Ethics approval and consent to participate

The Stanford Institutional Animal Care and Use Committee and the NIH Guide for Care and Use of Animals approved the protocols for using animals in this study, and all experiments were conducted following the ARRIVE 2.0 guidelines for reporting animal experiments.

9. Funding

This work was supported by the Lundbeck Foundation's DARE Programme (C.M.H.), the A.P. Møller Foundation – Fonden for Lægevidenskabens Fremme (C.M.H.), Fondazione Umberto Veronesi (S.R.), the Novo Nordic Foundation (NNF22OC0079804) (K.L.L.), the Independent Research Fund Denmark (0134-00084B) (K.L.L.), American Heart Association/Allen Frontiers Group Brain Health Award (19PABHI34580007) (M.S.B.), and Leducq Foundation Transatlantic Network of Excellence (19CVD01) (M.S.B.).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We acknowledge skilled technical help from Li Zhu, Stanford University and Ulla Munk, University of Southern Denmark.

Data availability

Data will be made available on request.

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