






RESEARCH ARTICLE

Neutrophil β_1 adrenoceptor blockade blunts stroke-associated neuroinflammation

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Background and Purpose: Reperfusion therapy is the standard of care for ischaemic stroke; however, there is a need to identify new therapeutic targets able to ameliorate cerebral damage. Neutrophil β_1 adrenoceptors (β_1 AR) have been linked to neutrophil migration during exacerbated inflammation. Given the central role of

Abbreviations: AAR, area-at-risk; ADC, apparent diffusion coefficient; AMI, acute myocardial infarction; CSPGs, chondroitin sulfate proteoglycans; DSC, dynamic susceptibility contrast; HT, haemorrhagic transformation; I/R injury, ischaemia/reperfusion injury; Iba1, ionized calcium binding adaptor molecule 1; IS, infarct size; MBP, myelin basic protein; MVO, microvascular obstruction; NG2, neuronal/glial antigen; OGD, oxygen-glucose deprivation; PDM, perfusion-diffusion mismatch; RGMa, repulsive guidance molecule a; rt-PA, recombinant tissue-plasminogen activator; T2W, T2 weighted; YM1/Chil3, chitinase 3-like 3.

Agustín Clemente-Moragón and Eduardo Oliver contributed equally to this work.

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neutrophils in cerebral damage during stroke, we hypothesize that β 1AR blockade will improve stroke outcomes.

Experimental Approach: Rats were subjected to middle cerebral artery occlusion–reperfusion to evaluate the effect on stroke of the selective β 1AR blocker metoprolol ($12.5 \text{ mg} \cdot \text{kg}^{-1}$) when injected i.v. 10 min before reperfusion.

Key Results: Magnetic resonance imaging and histopathology analysis showed that pre-reperfusion i.v. metoprolol reduced infarct size. This effect was accompanied by reduced cytotoxic oedema at 24 h and vasogenic oedema at 7 days. Metoprolol-treated rats showed reduced brain neutrophil infiltration and those which infiltrated displayed a high proportion of anti-inflammatory phenotype (N2, YM1⁺). Additional inflammatory models demonstrated that metoprolol specifically blocked neutrophil migration via β 1AR and excluded a significant effect on the glia compartment. Consistently, metoprolol did not protect the brain in neutrophil-depleted rats upon stroke. In patients suffering an ischaemic stroke, β 1AR blockade by metoprolol reduced circulating neutrophil–platelet co-aggregates.

Conclusions and Implications: Our findings describe that β 1AR blockade ameliorates cerebral damage by targeting neutrophils, identifying a novel therapeutic target to improve outcomes in patients with stroke. This therapeutic strategy is in the earliest stages of the translational pathway and should be further explored.

KEYWORDS

Ischemic Stroke, Neutrophils, I/R, β 1AR, Neuroinflammation

1 | INTRODUCTION

Despite improved diagnostic imaging techniques and numerous attempts to establish neuroprotective strategies, stroke treatment is currently restricted to reperfusion without coadjuvant therapy (Powers et al., 2019). The only clinically available interventions are intravenous (i.v.) administration of the fibrinolytic agent recombinant **tissue-plasminogen activator** (rt-PA) and, more recently, endovascular thrombectomy, alone or in combination with rt-PA (Powers et al., 2019).

Experimental evidence shows that reperfusion after prolonged brain ischaemia can trigger a set of potentially deleterious events, known as reperfusion injury. Tissue reperfusion was recently shown to exacerbate the inflammatory response, resulting in massive leukocyte infiltration (Lin et al., 2016). Among the first cells infiltrating the brain are neutrophils (Jickling et al., 2015; Strecker et al., 2017), and neutrophil pro-inflammatory activation after stroke has been linked to increased infarct size (IS), blood–brain barrier (BBB) disruption, haemorrhagic transformation (HT), and worse neurological outcomes (Jickling et al., 2015; Strecker et al., 2017). Moreover, neutrophils form aggregates with erythrocytes and platelets, and after reperfusion, these aggregates form embolisms that impair tissue perfusion, despite the restoration of blood flow in the large cerebral arteries. This phenomenon is known as microvascular obstruction (MVO) and

What is already known

- Cerebral ischaemia/reperfusion injury is a clinical challenge because few therapeutic interventions are available.

What does this study add

- β 1AR blockade during ongoing stroke reduces brain injury and neuroinflammation, and improves neurological outcomes.
- β 1AR blockade decreases neutrophil migration and boosts pro-resolving circuits which favour tissue homeostasis.

What is the clinical significance

- Pharmacological β 1AR blockade may represent a potential candidate in clinical trials for ischaemic stroke.

is a major contributor to ischaemia–reperfusion (I/R) injury and final IS (Ibanez et al., 2015).

Neutrophils have an extensive repertoire of biological functions and remain essential contributors for the resolution of inflammation and tissue repair (Horckmans et al., 2017; Yang et al., 2019). Cell plasticity allows neutrophils to take on different characteristics (Ballesteros et al., 2020; Mantovani et al., 2011; Silvestre-Roig et al., 2016) under certain tissue environments or stimuli (Chakravarti et al., 2009; Fridlender et al., 2009; Ohms et al., 2020; Puellmann et al., 2006). Different neutrophil populations with disparate effects have been recently described in brain after stroke (Cuartero et al., 2013; Garcia-Culebras et al., 2019), and thus it is vital to evaluate their phenotype in the context of therapies.

We recently showed that in the context of myocardial infarction, acute blockade of neutrophil β_1 -adrenoceptors (β_1 AR) by metoprolol—but not by other β -blockers—ameliorates myocardial I/R injury (Clemente-Moragon et al., 2020; Garcia-Prieto et al., 2017). These experimental studies were followed by a successful randomized clinical trial showing that injection of the selective β_1 AR blocker metoprolol before reperfusion resulted in clinical benefits in patients suffering an acute myocardial infarction (AMI) (Garcia-Ruiz et al., 2016; Pizarro et al., 2014). These data prompted us to test this pharmacological strategy in ischaemic stroke. In the present study, we tested the effects of pre-reperfusion intravenous (i.v.) administration of metoprolol in a rat model of brain I/R. We show that a single injection of metoprolol before reperfusion dampened oedema formation and reduced neuronal loss. This protective effect was associated with significantly reduced neuroinflammation, better preservation of BBB (blood-brain barrier) integrity and lessened glial scar formation. Moreover, by attenuating the inflammatory environment, metoprolol reduced circulating neutrophil activation and boosted pro-resolving circuits which favour the re-establishment of tissue homeostasis. Consistent with this, the benefits of metoprolol were lost when the ischaemic stroke was induced in rats previously depleted of neutrophils.

2 | METHODS

Full details of materials and experimental procedures are available in Supporting Information. The data that support the findings of this study are available from the corresponding author on reasonable request.

All experimental and other scientific procedures with animals conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013. Animal protocols were approved by the local ethics committees and the Animal Protection Area of the Comunidad Autónoma de Madrid (PROEX 056_17 and PROEX 176.3/20). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

In summary, 8- to 10-week-old young male Wistar rats (200–250 g) were subjected to middle cerebral artery occlusion–reperfusion (MCAO/R) for 35 min and randomized to receive either a 12.5 mg·kg⁻¹ i.v. bolus of metoprolol-tartrate or vehicle 10 min before reperfusion. IS and brain oedema were evaluated by MRI at 24 h and 7 days post-reperfusion and by histopathology at 7 days post-reperfusion. Neutrophil infiltration and phenotype were assessed by immunohistochemistry (IHC) at 24 h post-reperfusion, and the effect of metoprolol was also evaluated at 24 h and 7 days in rats depleted of circulating neutrophils. Specific neutrophil *Adrb1* knock-out mice were used for mechanistic experiments exploring the effect of metoprolol on neutrophil biology.

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.1 | Statistics

Data are presented as mean \pm standard error of the mean (SEM). Differences were deemed statistically significant at *P* values below 0.05. Further details are provided in Supporting Information.

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.2 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | Selective β_1 AR blockade during ongoing stroke reduces brain injury, prevents neuronal loss and improves neurological outcomes

Rats subjected to 35 min of middle cerebral artery occlusion (MCAO) received a single i.v. injection of the selective β_1 AR blocker metoprolol (12.5 mg·kg⁻¹) (Garcia-Prieto et al., 2017) or saline (0.9% NaCl, vehicle) 10 min before reperfusion (Figure 1a). The selected i.v. metoprolol dose was based on a dose–response study showing a moderate haemodynamic effect on arterial pressure and heart rate (<20%) (Figure S1A). Rats underwent three MRI studies: during ongoing cerebral ischaemia and 24 h and 7 days after reperfusion. The extent of cerebral ischaemia provoked by MCAO (area at risk, AAR) was determined from MRI dynamic susceptibility contrast (DSC) images obtained during occlusion, and did not differ between groups

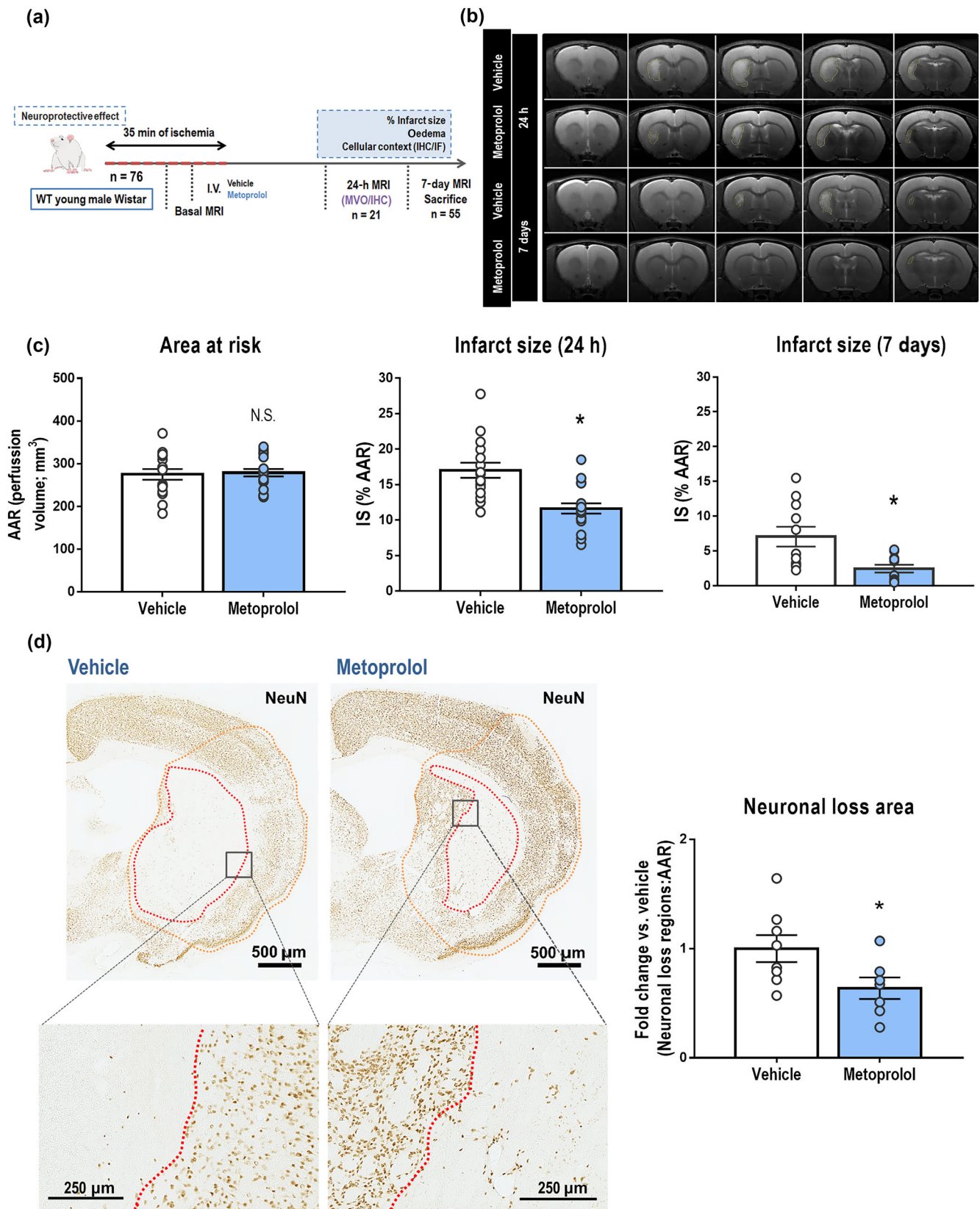


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FIGURE 1 β 1AR blockade by metoprolol reduces infarct size and neuronal loss. (a) Rat model of middle cerebral artery occlusion–reperfusion (MCAO/R), with euthanasia at 7 days post-reperfusion. WT, wild-type, MRI, magnetic resonance imaging, IF, immunofluorescence; IHC, immunohistochemistry; MVO, microvascular obstruction. (b) Comparative coronal T2-weighted (T2W) MRI at 24 h (top rows) and 7 days (bottom rows) post-reperfusion. Infarcted regions correspond to hyperintense areas. (c) Infarct size (IS) evaluated by coronal T2W MRI at 24 h (vehicle, $n = 16$; metoprolol, $n = 18$) and 7 days post-reperfusion (vehicle, $n = 11$; metoprolol, $n = 11$). Final IS was calculated as the ratio of infarct volume to the area-at-risk (AAR) and expressed as %. (d) IHC of coronal sections with anti-NeuN antibody. Higher magnifications of infarcted areas show territories of complete neuronal loss (delineated in red). Neuronal loss analysis in the middle cerebral artery territory (MCA, delineated in orange) of infarcted hemispheres shows neuronal preservation in metoprolol-treated rats at 7 days post-reperfusion (vehicle, $n = 8$; metoprolol, $n = 7$). Graphs show mean \pm S.E.M. * $P < 0.05$

($275.0 \pm 12.5 \text{ mm}^3$ and $279.1 \pm 8.85 \text{ mm}^3$ for vehicle- and metoprolol-injected rats, respectively, Figure 1c). Brain IS was measured as the AAR-normalized extent of oedema on T2-weighted (T2W) MRI images at 24 h post-reperfusion (pre-specified primary outcome) and at 7 days post-reperfusion (Figure 1b). MRI-quantified IS was significantly smaller in metoprolol-treated rats both at 24 h ($17.0 \pm 1.06\%$ of the AAR in vehicle-treated rats vs. $11.6 \pm 0.72\%$ in metoprolol-treated rats, $P < 0.05$, Figure 1c) and on day 7 (vehicle, $7.07 \pm 1.42\%$; metoprolol, $2.46 \pm 0.56\%$; $P < 0.05$, Figure 1c). A tendency towards IS reduction was observed when using a lower dose of metoprolol ($6.25 \text{ mg}\cdot\text{kg}^{-1}$) (Figure S1B).

The exclusion of rats with hemispheric infarcts (IS $> 40\%$) was an a priori exclusion criterion defined before experimentation. To rule out unintentional selection bias, we performed a post hoc analysis of all rats, including those meeting the exclusion criterion, at 24 h post-reperfusion and metoprolol maintained its association with significantly smaller IS (Figure S2).

IS was also quantified as a percentage of perfusion-diffusion (PDM), another validated normalization method. The results were consistent with IS/AAR data, with metoprolol-treated rats having significantly smaller infarcts at 24 h and 7 days post-reperfusion (Figure S3A). Furthermore, comparison of infarct volumes on T2-weighted (T2W) MRI without normalization also showed that metoprolol-infused rats had significantly smaller infarcts at 24 h and 7 days post-reperfusion (Figure S3B).

Immunohistochemical analysis of the neuronal marker NeuN at 7 days post-reperfusion showed that early i.v. metoprolol administration was associated with significant smaller regions of neuronal loss than observed in the vehicle-treated group (vehicle 1.00 ± 0.11 ; metoprolol, 0.65 ± 0.09 ; $P < 0.05$, Figure 1d).

To study whether the neuroprotective effect of metoprolol was consistent with better neurological outcomes, a longitudinal study was performed to evaluate long-term effects of brain injury on sensory-motor behaviours. The results obtained with the motor and behavioural scales (Figure S4) first showed a slow recovery of these neurological functions over the time in the groups subjected to ischaemia (metoprolol and vehicle groups), with no recovery in the sham group. With both scales, despite a better neurological function observed in the group treated with the drug in all time points, it was not until 21 days after the surgery when significant differences were observed, in particular, in the behavioural test. Our evaluation of the sensorimotor functions using the adhesive removal test again revealed an improvement in these neurological deficits in rats receiving

i.v. metoprolol, with significant differences at 24 h and 7 days between these groups in the parameter of time for the first attempt to remove the adhesive.

3.2 | β 1AR blockade reduces brain cytotoxic and vasogenic oedema and preserves the BBB

The extent of cytotoxic and vasogenic oedema was detected as areas of reduced or increased diffusion, respectively, on MRI apparent diffusion coefficient (ADC) maps (Birenbaum et al., 2011). MRI during index ischaemia (before metoprolol or vehicle injection) showed no differences in brain cytotoxic oedema (vehicle, $98.1 \pm 8.8 \text{ mm}^3$; metoprolol, $105.2 \pm 11.7 \text{ mm}^3$). At 24 h after reperfusion, metoprolol-treated rats had significantly less cytotoxic oedema than their vehicle-treated counterparts (vehicle, $31.0 \pm 2.54 \text{ mm}^3$; metoprolol, $20.6 \pm 1.62 \text{ mm}^3$; $P < 0.05$) (Figure 2a). Besides, metoprolol-treated rats had a significantly smaller extent of vasogenic oedema at 7 days post-reperfusion (vehicle, $13.8 \pm 1.87 \text{ mm}^3$; metoprolol, $7.86 \pm 1.97 \text{ mm}^3$; $P < 0.05$) (Figure 2a). Vasogenic oedema was further assessed by comparing ipsilateral and contralateral tissue water content at 7 days post-reperfusion (Figure 2b). MRI ADC maps revealed lower water content (ipsilateral:contralateral intensity ratio) in the brain tissue of metoprolol-injected rats (vehicle, 1.18 ± 0.03 ; metoprolol, 1.07 ± 0.02 ; $P < 0.05$; Figure 2c). Similar results were obtained by T2W MRI analysis (vehicle, 1.24 ± 0.04 ; metoprolol, 1.08 ± 0.04 ; $P < 0.05$; Figure 2c).

We assessed BBB integrity by immunohistochemical analysis of aquaporin-4 (AQP4), whose expression on the end-feet of astrocytes in the BBB prevents post-stroke neuroinflammation-related oedema (Fukuda & Badaut, 2012). AQP4 expression at day 7 post-reperfusion was twofold higher in metoprolol-treated rats (vehicle, 1.00 ± 0.24 ; metoprolol, 2.00 ± 0.41 ; $P < 0.05$; Figure 2d,f, Video S1). These results were consistent with an increase of IgG extravasation in the absence of AQP4 staining (Figures 2e,f and S5).

3.3 | β 1AR blockade during brain ischaemia prevents microglia/macrophage response and reduces subacute glial scar formation

In vehicle-injected rats, reactive and hypertrophic astrocytes, detected by up-regulated expression of glial fibrillary acidic protein

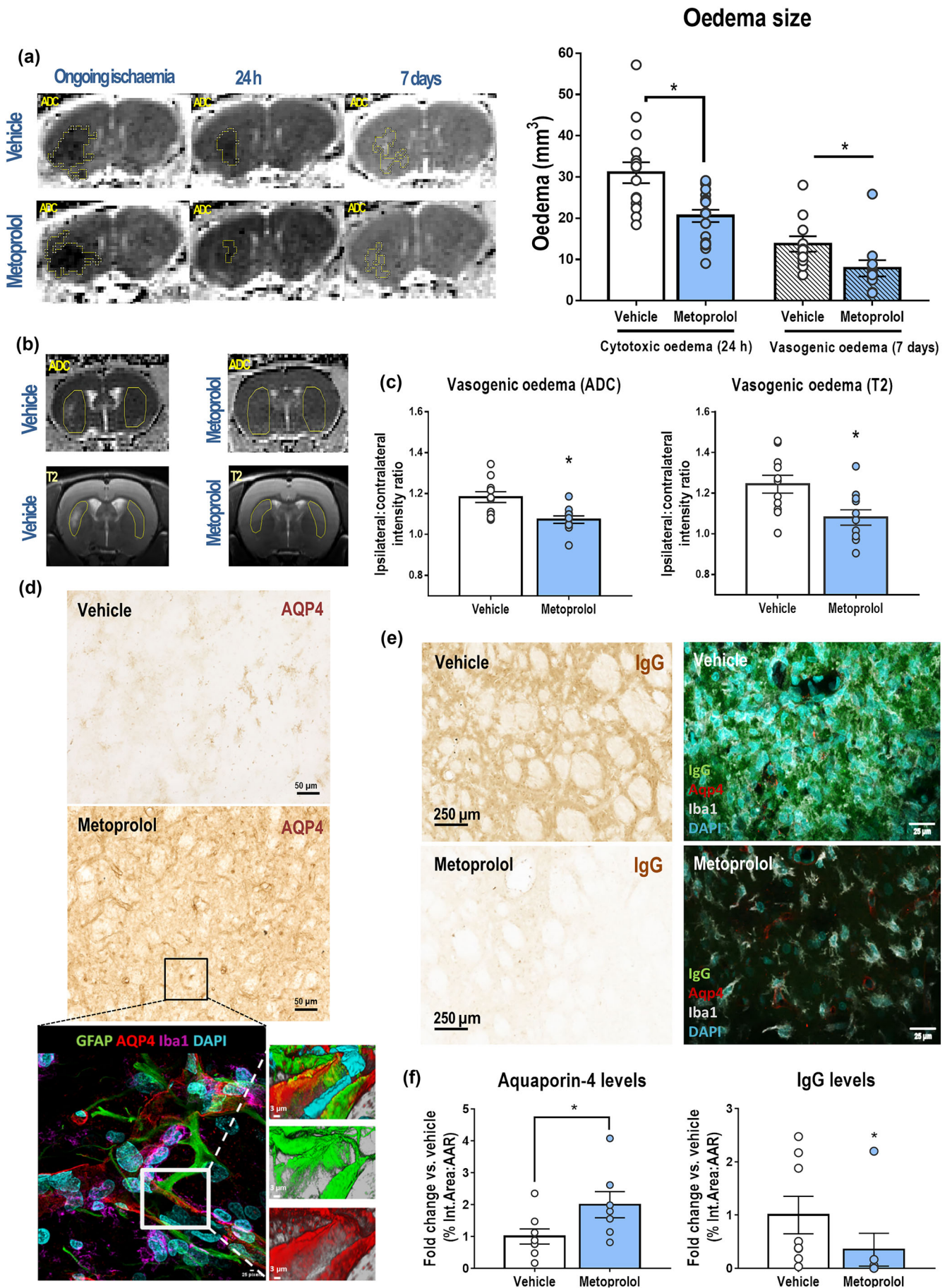


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FIGURE 2 β 1AR blockade by metoprolol reduces cerebral oedema and preserves the blood–brain barrier (BBB). (a) Coronal parametric MRI apparent diffusion coefficient (ADC) maps comparing rats receiving i.v. vehicle (top row) or metoprolol (bottom row) during ongoing ischaemia and 24 h and 7 days post-reperfusion. Restricted diffusion was identified at 24 h as hypointense areas and increased diffusion at 7 days as hyperintense areas (outlined in yellow). Metoprolol reduces cytotoxic oedema at 24 h post-reperfusion (vehicle, $n = 16$; metoprolol, $n = 18$) and vasogenic oedema at 7 days post-reperfusion (vehicle, $n = 11$; metoprolol, $n = 11$). (b) Tissue water content analysed by comparison of ipsilateral (with infarct) and contralateral (without infarct) intensities in similar-sized areas (yellow outlines), in a single slice per rat on ADC maps and T2W MRI. (c) Quantification of ipsilateral-to-contralateral intensity ratios from ADC maps and T2W images, showing reduced brain water content in metoprolol-treated rats. (d) IHC of coronal sections at 7 days post-reperfusion with anti-aquaporin 4 (AQP4) antibody. The bottom panel shows representative triple GFAP⁺AQ4⁺Iba1⁺ immunofluorescence, illustrating AQP4 preservation (red) in astrocyte (GFAP; green) end-feet and consequent preservation of BBB integrity. Microglia/macrophages (Iba1; purple) were found around vessels. Nuclei were revealed with DAPI (blue). (e) IHC of coronal sections at 7 days post-reperfusion with anti-immunoglobulin G (IgG) antibody (left panels). The right panels shows representative triple IgG⁺AQ4⁺Iba1⁺ immunofluorescence, illustrating IgG extravasation (green) in the absence of AQP4 (red). Microglia/macrophages (Iba1; grey) were found more reactive in the core lesion of vehicle-treated rats and nuclei were revealed with DAPI (blue). (f) Quantification of AQP4 and IgG IHC in the MCA of infarcted hemispheres shows preservation of BBB integrity at 7 days post-reperfusion in rats receiving i.v. metoprolol (vehicle, $n = 8$; metoprolol, $n = 7$). Graphs show mean \pm S.E.M. * $P < 0.05$. Other abbreviations as in Figure 1

(GFAP), accumulated in a barrier pattern surrounding the core lesion and expressed large amounts of chondroitin sulfate proteoglycans (CSPGs). Metoprolol injection reduced glial scar formation by fivefold (vehicle, 1.00 ± 0.18 ; metoprolol, 0.19 ± 0.07 ; $P < 0.05$; Figures 3a–c and S6A). In line with this result, proteoglycan production in the core lesion identified by CSPG deposition was reduced by 10-fold in rats receiving i.v. metoprolol (vehicle, 1.00 ± 0.33 ; metoprolol, 0.09 ± 0.04 ; $P < 0.05$; Figures 3b,d and S6A,B), as well as microglia/macrophage response (vehicle, 1.00 ± 0.17 ; metoprolol, 0.52 ± 0.09 ; $P < 0.05$; Figure 3a,b,e). Confirming these results, double-immunohistochemistry (IHC) and immunofluorescence (IF) for CSPG and GFAP in the vehicle group revealed astrocytes surrounding a scar containing a matrix of proteoglycans, whereas this phenomenon was not seen in metoprolol-treated rats (Figure S6D). Moreover, quadruple IF showed that the absence of a glial scar and proteoglycan matrix in the core lesion of metoprolol-treated rats was accompanied by a reduced microglia/macrophage response in the same area (Figure 3a), as observed with Iba1 immunohistochemistry (Figure 3b,e). IHC results at 24 h post-reperfusion indicated that glial scarring was not present in any of the treatment groups. The latter suggests that metoprolol did not have a direct effect on glia (Figure S7).

Increased inflammation leads to secondary damage to other cell populations, such as neurons and oligodendrocytes (Hackett & Lee, 2016). Myelin basic protein (MBP) IHC, a marker of the state of axonal myelination, showed good preservation of myelin sheaths in metoprolol treated rats (vehicle, 1.00 ± 0.16 ; metoprolol, 2.02 ± 0.36 ; $P < 0.05$; Figures 3a,b,f and S6C). Myelin sheaths in the vehicle group showed evidence of damage, and there were large amounts of debris accumulated in the core lesion, coinciding with CSPG deposition (Figure S6E). Triple IF confirmed these results, illustrating how metoprolol injection was associated with better preserved myelin sheaths that resembled those on the contralateral side (Figure 3a). Moreover, the core lesions of vehicle-treated rats were positive for release of **repulsive guidance molecule** a (RGMa), indicating a context inhibitory to axonal and neural regeneration, whereas RGMa was absent from the core lesions of metoprolol-infused rats (Figure S8).

3.4 | β 1AR blockade reduces brain microvascular obstruction (MVO) and blunts neutrophil infiltration into brain parenchyma

Upon reperfusion, MVO induced by circulating cells contributes significantly to the final extent of injury (Ibanez et al., 2015). We quantified trapped erythrocytes as a surrogate for MVO by treating brain tissues from rats killed 24 h post-reperfusion (Figure 4a) with NaBH₄, which renders haemoglobin fluorescent. Pre-reperfusion metoprolol injection was associated with a twofold reduction in the density of erythrocytes trapped in capillary networks in penumbra areas (vehicle, 1.00 ± 0.16 ; metoprolol, 0.50 ± 0.11 ; $P < 0.05$) (Figure 4b). Neutrophil pro-inflammatory activation after stroke is associated with increased IS and BBB breakdown (Strecker et al., 2017). Brain neutrophil infiltration at 24 h (Figure 4a) was significantly twofold less in metoprolol-treated rats 24 h after reperfusion (vehicle, 1.00 ± 0.25 ; metoprolol, 0.46 ± 0.10 ; $P < 0.05$, Figure 4c).

3.5 | β 1AR blockade specifically blunts pro-inflammatory neutrophils, which results in a shift towards an anti-inflammatory environment in brain

Neutrophils have been shown to be plastic cells with distinct modifiable phenotypes and specific functional-derived properties (Ballesteros et al., 2020; Mantovani et al., 2011; Silvestre-Roig et al., 2016). Because neutrophil plasticity makes them sensitive to changeable environmental stimuli (Chakravarti et al., 2009; Fridlender et al., 2009; Ohms et al., 2020; Puellmann et al., 2006), a heterogeneous population of these cells could be found in brain after stroke (Cuartero et al., 2013; Garcia-Culebras et al., 2019). After documenting that metoprolol reduced neutrophil presence in the brain 24 h after reperfusion stroke, we phenotypically characterized the infiltrated neutrophils by IF on the basis of Chitinase 3-like 3 (YM1/Chil3) staining, which is as a marker of alternative anti-inflammatory N2 neutrophils. Surprisingly, whereas the number of YM1⁺ neutrophils was not significantly different between metoprolol-treated and control rats (vehicle, 85.6 ± 8.85 neutrophils mm⁻²; metoprolol, 59.2 ± 18.4

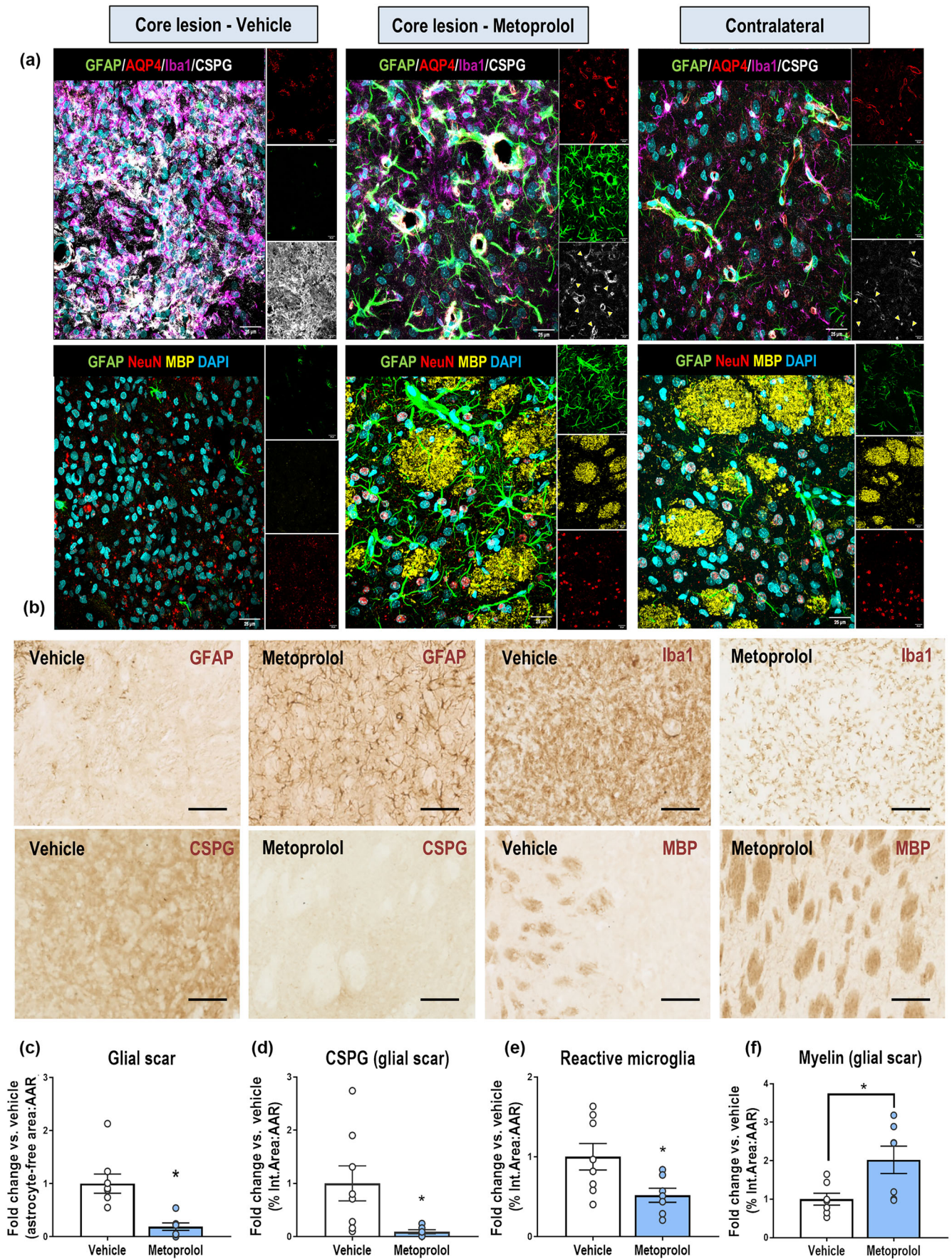


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FIGURE 3 β 1AR blockade by metoprolol prevents microglia/macrophage response and reduces subacute glial scar formation. (a) Single and merged channels of quadruple GFAP⁺AQP4⁺Iba1⁺CSPG⁺ (top row) and triple GFAP⁺MBP⁺NeuN (bottom row) IF. The quadruple IF shows glial scar formation (GFAP; green), proteoglycan deposition (CSPG; grey), microglia/macrophage response (Iba1; purple), and BBB disruption (AQP4; red) in a vehicle-treated rat (left panels), contrasting the absence of glial scar in a metoprolol-treated rat (central panels). Yellow arrowheads show a suggesting neural/glial antigen 2 expression by CSPG⁺ perivascular pericytes. The triple IF shows representative degeneration of neurons (NeuN; red) and the myelin sheath (MBP; yellow) in a vehicle-treated rat (left panels) and their preservation in a metoprolol-treated rat (central panels), where the lesion resembles the contralateral hemisphere (right panels) except for the astrocyte hypertrophy (GFAP; green). Nuclei were revealed with DAPI (blue). (b) Representative IHC (vehicle, left panels; metoprolol, right panels) of GFAP (glial fibrillary acidic protein), CSPGs (chondroitin sulfate proteoglycans), Iba1 (ionized calcium-binding adapter molecule 1) and myelin basic protein (MBP) on coronal sections at 7 days post-reperfusion, showing a reduction in glial scar formation (c), proteoglycans deposition (d), reactive microglia response and myelin sheath deterioration in metoprolol-treated rats. Graphs show mean \pm S.E.M. * $P < 0.05$. $n = 6-8$ rats per group. Other abbreviations as in Figure 1

neutrophils mm^{-2}), there was a strong reduction in YM1⁺ neutrophil population (vehicle, 153 ± 8.42 neutrophils mm^{-2} ; metoprolol, 50.7 ± 9.62 neutrophils mm^{-2} ; $P < 0.05$). Consequently, the relative density of N2 anti-inflammatory neutrophil subset was overtly higher in metoprolol-injected rats (vehicle, $36.2 \pm 2.55\%$; metoprolol, $53.9 \pm 5.13\%$; $P < 0.05$). These results suggest that metoprolol selectively inhibit pro-inflammatory neutrophil extravasation without affecting the dynamics of anti-inflammatory neutrophils (Figure 4d,e).

Since N2 polarization has been shown to be associated with an increased neutrophil clearance and inflammation resolution (Cuartero et al., 2013), we performed a TUNEL assay on brain sections that allows us to assess neutrophil apoptosis by immunofluorescence. The density of apoptotic neutrophils was significantly higher in metoprolol-treated rats (vehicle, $60.7 \pm 1.11\%$; metoprolol, $76.9 \pm 4.51\%$; $P < 0.05$). Apoptotic neutrophils clearance in brain is mainly carried out by phagocytic microglia via efferocytosis, in which therefore an immunosuppressive, pro-resolving and tissue-repair response is activated (Doran et al., 2020; Jones et al., 2016). We observed microglia preferential engulfment of TUNEL⁺ neutrophils (vehicle, $49.4 \pm 3.06\%$; metoprolol, $61.7 \pm 3.15\%$; $P < 0.05$) (Figure 4f-i, Video S2).

Overall, these data show that β 1AR blockade results in a selective inhibition of pro-inflammatory N1 neutrophils infiltration and a faster resolution of inflammation in the post-stroke brain.

3.6 | Metoprolol exerts a specific disruptive effect on neutrophils in a β 1AR-dependent manner

To evaluate whether metoprolol blunts neutrophil migratory capacities through β 1AR, we exposed isolated neutrophils from WT and *Adrb1* KO mice across the transwell filter to the chemoattractant CXCL1 in the presence or absence of metoprolol (10 μM). The number of cells migrating across the transwell membrane was quantified by flow cytometry after 90 min. Metoprolol inhibited baseline WT neutrophil migration across the CXCL1 gradient (vehicle, $1.83 \pm 0.41 \times 10^3$ neutrophils; metoprolol, $1.04 \pm 0.23 \times 10^3$ neutrophils; $P < 0.05$), whereas no effect was seen in the absence of neutrophil β 1AR expression (vehicle, $1.23 \pm 0.32 \times 10^3$ neutrophils; metoprolol, $1.20 \pm 0.23 \times 10^3$ neutrophils) (Figure 5a-c). Next, we explored whether the effect of metoprolol on neutrophil crawling dynamics

was abolished in neutrophil specific *Adrb1* KO mice. To this end, we used 2D intravital microscopy (IVM) to image migration in the cremaster muscle vessels of mice injected with TNF α , which triggers massive neutrophil recruitment (Figure 5d) (Sreeramkumar et al., 2014). Consistent with the in vitro results, metoprolol disrupted in vivo neutrophil kinetics (velocity, accumulated and Euclidean distance, and directionality), as well as neutrophil-platelet interactions only in WT but not in neutrophil specific *Adrb1* KO mice (Figure 5e-h, Video S3A-D). Altogether, these data demonstrate that metoprolol regulates neutrophil dynamics through β 1AR modulation.

The role of β AR in astrocytes and microglia in the context of stroke has been a matter of controversy (Follesa & Mocchetti, 1993; Goyagi et al., 2006; Han et al., 2009; Junker et al., 2002; Lechtenberg et al., 2019; Monai et al., 2019; Semkova et al., 1996). We wanted to explore whether β 1AR blocked in these cells had an impact on neutrophil recruitment. Astrocyte/microglia neonatal co-cultures (Figure S8A) were subjected to 1 h oxygen-glucose deprivation (OGD) followed by 24 h of reperfusion (Figure 5i). Neutrophils isolated from WT mice were then placed on a transwell filter having the post-OGD astrocyte/microglia as the attractant stimuli. WT astrocyte/microglia subjected to 1 h OGD triggered a significant increase in neutrophil migration across the transwell compared to astrocyte/microglia not undergoing OGD (control) (control, $0.69 \pm 0.16 \times 10^3$ neutrophils; vehicle, $1.14 \pm 0.18 \times 10^3$ neutrophils; $P < 0.05$). This effect was consistent with an increase in CXCL1 levels in the supernatants of the co-culture (control, 2.40 ± 0.56 ng·ml⁻¹; vehicle, 5.38 ± 0.73 ng·ml⁻¹; $P < 0.05$, Figure S6B) and disruption of neutrophil migration after CXCL1 antibody-mediated neutralization ($0.86 \pm 0.13 \times 10^3$ neutrophils; $P < 0.05$, Figure 5j). When metoprolol (10 μM) was added on neutrophil ($0.78 \pm 0.15 \times 10^3$ neutrophils; $P < 0.05$) chamber migration was significantly disrupted; however, this effect was not seen when added on astrocyte/microglia chamber (before reperfusion). Consistent with this, CXCL1 levels in supernatant of metoprolol-treated co-cultures were not altered. Moreover, CXCL1 levels in supernatants of co-cultures treated with metoprolol after reperfusion indicate that metoprolol does not have any effect at that point (Figure S8B). These results were replicated in astrocyte/microglia co-cultures from whole body *Adrb1* KO littermate mice, but no additional effects were observed in the absence of astrocyte/microglia β 1AR expression (Figures 5i,k and S8C).

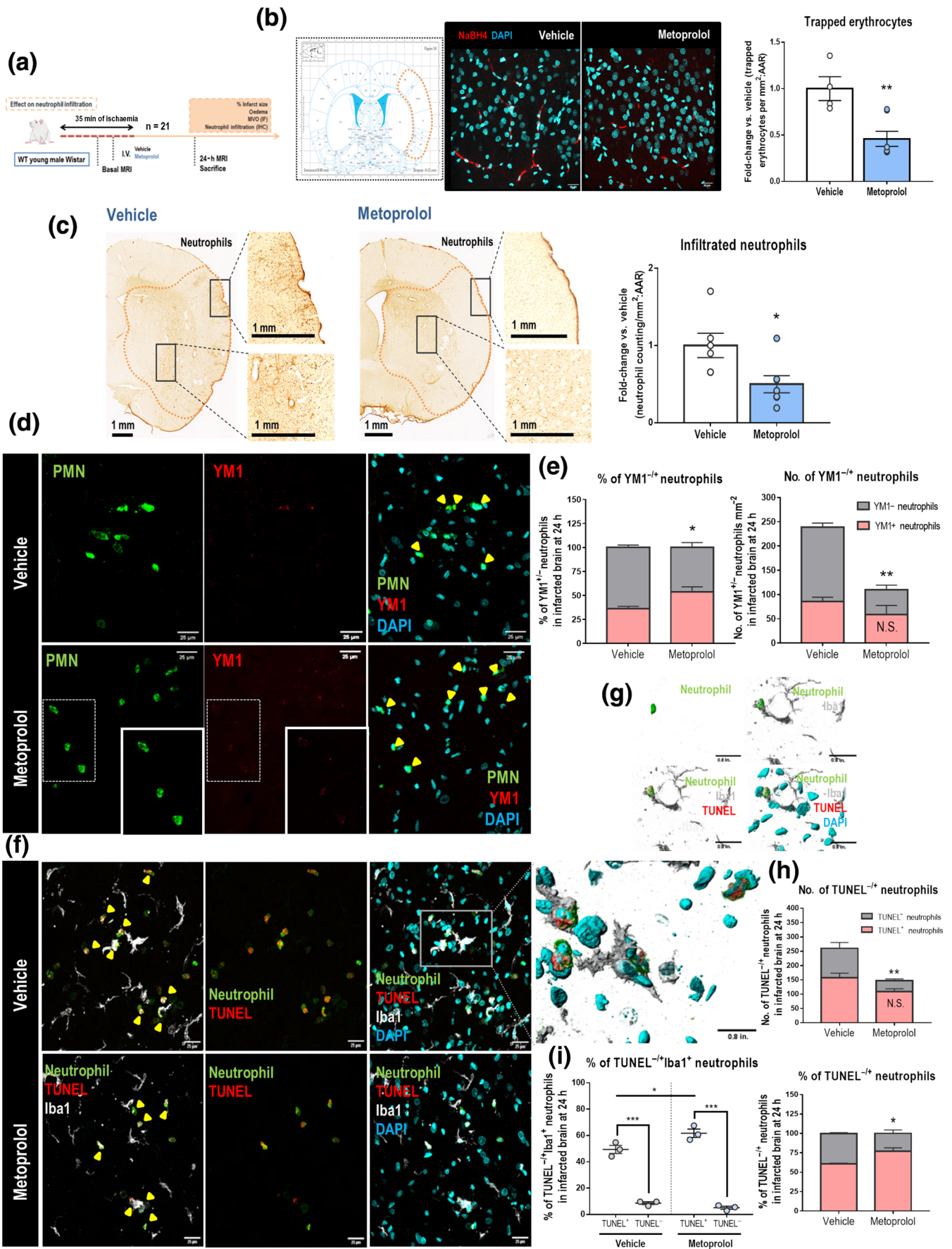


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FIGURE 4 Metoprolol reduces MVO and neutrophil infiltration into brain parenchyma via β 1AR. (a) Rat model of MCAO/R. Rats were killed at 24 h post-reperfusion. (b) Representative confocal imaging of NaBH_4 -treated tissues, showing trapped erythrocytes (red) in penumbra areas of rats receiving pre-reperfusion vehicle (left) or metoprolol (right). Penumbra areas correspond to the orange-bordered brain cortex region in the rat brain atlas. Nuclei were revealed with DAPI (blue). The relative numbers of trapped erythrocytes mm^{-2} in penumbra areas show reduced MVO in metoprolol-treated rats. (c) Representative anti-neutrophil IHC on coronal sections at 24 h post-reperfusion. Quantification of neutrophil infiltration shows lower numbers of neutrophils mm^{-2} in brain parenchyma of metoprolol-treated rats at 24 h post-reperfusion (vehicle, $n = 5$; metoprolol, $n = 7$). (d) Evaluation of infiltrated neutrophils in brain (green) expressing YM1 (Chitinase 3-like 3 protein, red) by IF at 24 h post-reperfusion in vehicle- (top row) and metoprolol-treated rats (bottom row). Nuclei were revealed with DAPI (blue). (e) The number of YM1^- neutrophils mm^{-2} , but not of YM1^+ , is significantly reduced by metoprolol and, as a consequence, the % of alternative neutrophils ($\text{N2}, \text{YM1}^+$) was then higher in brain parenchyma. (f) Evaluation by IF of apoptotic (TUNEL⁺, red) neutrophils (green) in brain being engulfed by microglia (Iba1, grey) at 24 h post-reperfusion in vehicle- (top row) and metoprolol-treated rats (bottom row). Nuclei were revealed with DAPI (blue). (g) 3D reconstructions of confocal images show how neutrophils undergoing apoptosis are preferably phagocytized by microglia. (h) % of apoptotic neutrophils was increased in brain parenchyma, as the number of TUNEL⁻ neutrophils mm^{-2} , but not of TUNEL⁺, was significantly reduced by metoprolol. (i) Apoptotic neutrophils were preferentially engulfed by microglia. $n = 3$ rats per group. Graphs show mean \pm S.E.M. * $P < 0.05$. Other abbreviations as in Figure 1

Additionally, proliferation assays based on EdU staining were performed to study astrocyte behaviour in response to ischaemia, as an indirect marker of reactivity and glial scar formation (Li et al., 2015; Ou-Yang et al., 2018; Wang et al., 2012). EdU⁺/DAPI⁺ nuclei were quantified and related to proliferative astrocytes due to their large numbers in co-cultures (~85%) compared to microglia. Astrocytes subjected to OGD prior to reperfusion exhibit a greater proliferation compared to controls; however, metoprolol treatment did not rescue significantly astrocytes from proliferation. Neither GFAP intensity nor GFAP area/nuclei significantly changes among conditions (Figure S8D,E). Immunoblot analyses of M2 markers (YM1, ARG1, and CD243) were performed to assess whether metoprolol exerts an effect on glia polarization; nevertheless, no differences were observed among conditions (Figure S8F,G).

3.7 | The neuroprotective effect of β 1AR blockade by metoprolol is abolished in the absence of circulating neutrophils

To further explore whether the benefits of metoprolol in stroke are mediated by an effect on neutrophils, as it has been previously described for myocardial infarction (Garcia-Prieto et al., 2017), we evaluated the impact of β 1AR blockade in the absence of circulating neutrophils (Figure 6a). Neutrophils were selectively depleted (~75%) by i.p. administration of anti-polymorphonuclear (PMN) cell serum 24 h and 48 h before stroke induction (Figure 6a,b). Reduction in lymphocytes and platelets counts was much lower in comparison (~20%) (Figure S7A). Metoprolol injection before reperfusion produced no additional benefit in rats previously depleted of neutrophils at 24 h post-reperfusion (vehicle, $12.1 \pm 0.73\%$; metoprolol, $13.9 \pm 2.87\%$, Figures 6c,d and S10B). Consistent with previous literature (Frieler et al., 2017; Herz et al., 2015; Wang et al., 2019), pre-stroke neutrophil depletion was associated with a significant reduction in IS in rats subjected to MCAO/R (non-depleted vehicle, $17.0 \pm 1.06\%$; depleted vehicle, $12.1 \pm 0.73\%$; $P < 0.05$, Figures 6e and S10C), as well as in cytotoxic oedema (non-depleted vehicle, $31.0 \pm 2.54 \text{ mm}^3$; depleted

vehicle, $12.4 \pm 1.16\%$; $P < 0.05$, Figure 6g), at 24 h post-reperfusion. As expected, in the case of metoprolol treatment, there were not differences in IS or cytotoxic oedema at 24 h post-reperfusion (Figures 6f,g and S10C).

As our previous results pointed, neutrophil stunning by metoprolol creates thereafter a distinct tissue environment, which follows a positive feedback in favour of resolution in which neutrophils feature a key role. Because of that, neutrophil-depleted rats were followed-up until 7 days in order to assess whether the absence of neutrophil determines the final stroke outcome. At 7 days there was not any difference between treatment conditions (vehicle, $5.44 \pm 0.93\%$; metoprolol, $8.88 \pm 1.80\%$, Figures 6c,d and S10B); however, the absence of neutrophils did not protect for long (Figures 6e and S10D–F). The neuroprotection exerted by metoprolol at 7 days post-reperfusion was surprisingly eradicated in the absence of neutrophils (non-depleted metoprolol, $2.46 \pm 0.56\%$; depleted metoprolol, $8.88 \pm 1.80\%$; $P < 0.05$; Figure 6f). Moreover, vasogenic oedema also was increased compared to non-depleted rats under metoprolol treatment (Figure 6h,i). These results highlight the relevance of neutrophil contribution to counteract and balance the inflammatory response and promote tissue recovery.

3.8 | β 1AR blockade by metoprolol inhibits neutrophil–platelet interactions in stroke patients

To investigate whether β 1AR blockade alters neutrophil dynamics and inhibits neutrophil–platelet interactions in humans, whole blood drawn from patients with reperfused acute ischaemic stroke was incubated ex-vivo with metoprolol (10 μM) or vehicle. Platelet (CD61⁺ cells) and neutrophils (CD45⁺/CD66⁺ cells) were detected by flow cytometry, and neutrophils positive for CD61 staining were identified as neutrophil–platelet co-aggregates. Metoprolol significantly inhibited neutrophil–platelet co-aggregates in ischaemic stroke patients receiving reperfusion therapy (vehicle, $14.0 \pm 3.90\%$; metoprolol, $12.3 \pm 3.46\%$; $P < 0.05$), whereas it did not exert any effect in healthy volunteers (Figure 7a,b).

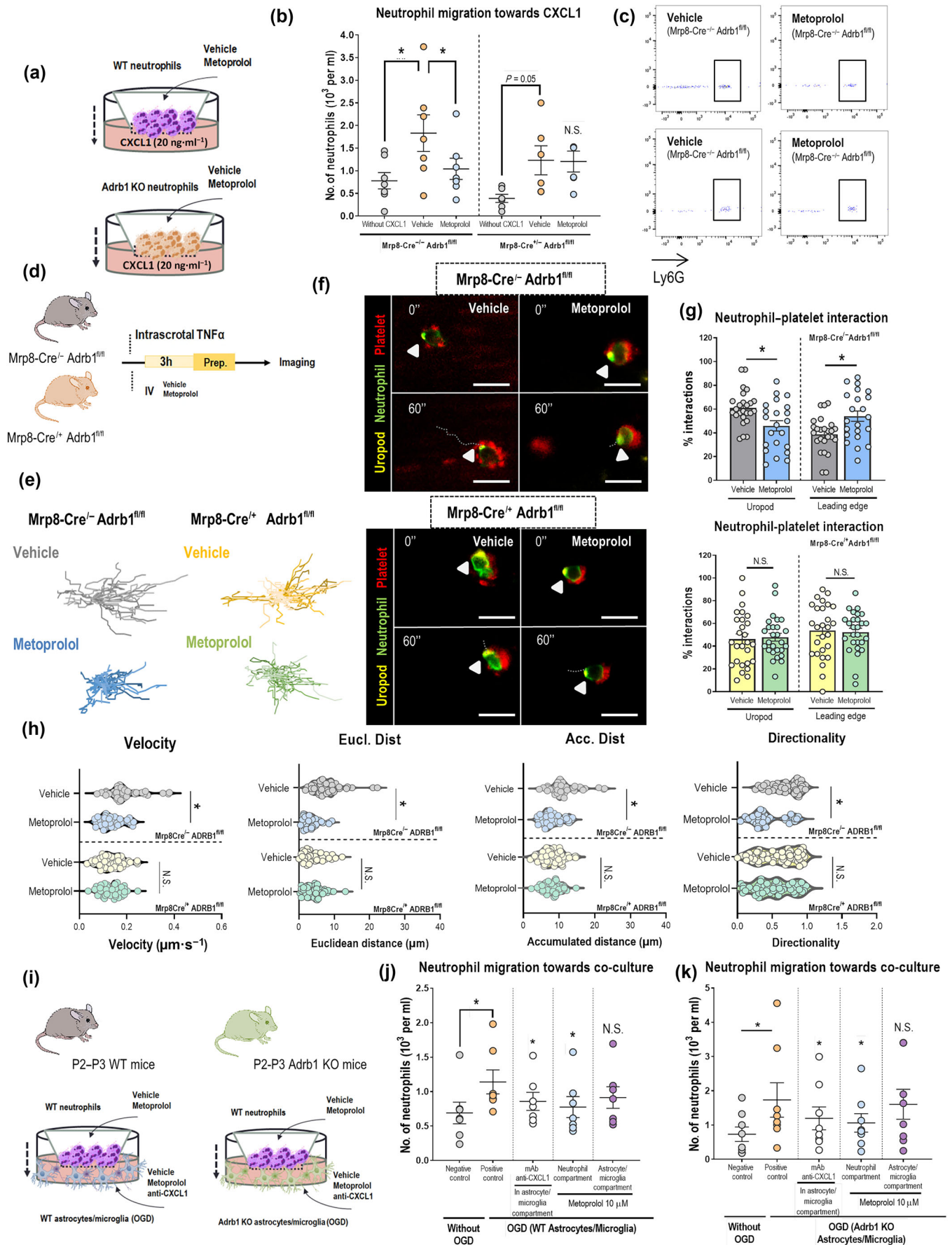


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FIGURE 5 β 1AR blockade by metoprolol blunts pro-inflammatory neutrophils and favours an anti-inflammatory environment in brain. (a) Experimental scheme for CXCL1-induced transwell migration analysis. (b) Limiting effect of metoprolol on chemokine-induced neutrophil migration in WT but not in conditional *Adrb1* KO mice. (c) Flow cytometry plots illustrating reduced migration of neutrophils (Ly6G^+ cells) upon treatment with metoprolol only in the presence of β 1AR. Each independent experiment was conducted with leukocytes pooled from five to six animals, and each condition was run with two technical replicates ($n = 8$ for WT; $n = 7$ for *Adrb1* KO). (d) Experimental scheme for 2D intravital microscopy. (e) Representative tracks of crawling neutrophils within inflamed vessels. (f) Representative time-lapse images of platelets (CD41^+ cells, red) with the polarized neutrophil uropod (CD62L^+ domain, yellow) or leading edge (Ly6G^+ domain, green). Arrowheads indicate interactions with the uropod domain, and dotted lines indicate displacement of the neutrophil over 60 s. (g) Percentage of platelet interactions with the neutrophil uropod or leading edge; $n = 22$ –25 cells from three mice per condition. (h) 2D intravascular motility parameters: velocity ($\mu\text{m}\cdot\text{s}^{-1}$), accumulated and Euclidean distance (μm), and directionality; $n = 35$ –54 cells from three mice per condition. WT mice: *Mrp8-Cre*^{-/-} *Adrb1*^{fl/fl}; neutrophil specific *Adrb1* KO mice: *Mrp8-Cre*^{+/+} *Adrb1*^{fl/fl}. (i) Experimental schemes of neutrophil migration towards WT ($n = 7$ per condition) or *Adrb1* KO ($n = 8$ per condition) glia-based co-cultures. (j, k) The limiting effect of metoprolol on neutrophil migration was directly exerted on these cells, as it did not alter astrocyte/microglia chemoattractant properties. Graphs show mean \pm S.E.M. * $P < 0.05$. Other abbreviations as in Figure 1

A sub-analysis was performed based on the reperfusion therapy. Likewise, metoprolol limited neutrophil–platelet interactions in all groups of reperfusion treatment: thrombectomy plus thrombolysis (vehicle, $14.6 \pm 5.41\%$; metoprolol, 12.5 ± 4.92 ; $P < 0.05$), thrombectomy (vehicle, $18.7 \pm 5.57\%$; metoprolol, $17.2 \pm 4.96\%$; $P = 0.05$) and thrombolysis (vehicle, $22.3 \pm 9.20\%$; metoprolol, $17.9 \pm 8.64\%$; $P < 0.05$) (Figure 7c–e).

4 | DISCUSSION

Timely reperfusion is the standard of care for ischaemic stroke; however, increasing evidence supports the notion that reperfusion of an ischaemic arterial bed induces a secondary form of brain damage, known as reperfusion injury, which leads to BBB dysfunction and HT of the ischaemic tissue. Moreover, with the recent advent of endovascular therapy (extending the time window for stroke treatment; Nogueira et al., 2018), I/R injury has become an increasing clinical challenge because there are few interventions able to limit the damage. Neutrophils feature as key protagonists in the proposed mechanisms of cerebral I/R injury, related to their capacity to exacerbate local vascular and tissue injury (Jickling et al., 2015; Kolaczowska & Kubes, 2013; Lin et al., 2016; Strecker et al., 2017).

Recently, we have reported that selective blockade of β 1AR in neutrophils by metoprolol has a unique and pronounced effect on their activity that results in an amelioration of I/R injury and, consequently, smaller myocardial infarctions (Clemente-Moragon et al., 2020; Garcia-Prieto et al., 2017). We then speculated that a single injection of the selective β 1AR blocker metoprolol might protect the brain during stroke. Even though some studies attributed neuroprotective properties to β -blocker drugs in stroke (Ajmo et al., 2009; Goyagi et al., 2006; Goyagi et al., 2010, 2011; Gui et al., 2013; Hertz et al., 2014; Iwata et al., 2010; Little et al., 1982; Savitz et al., 2000), their role has always been controversial and raised debatable discussion. The diversity of β -blocker profiles, routes, and timings of drug administration, as well as the different stroke models and methods for IS and oedema evaluation, has made it difficult to come to a conclusion regarding the use of these agents in stroke

outcome. In addition, few mechanisms have been ascribed to this neuroprotection.

Herein, our findings provide strong evidence that the modulating effect of metoprolol on neutrophil dynamics (Garcia-Prieto et al., 2017) significantly reduces brain injury and promotes inflammatory resolution and tissue repair in a validated model of MCAO/R (Cai et al., 2016) (Graphical Abstract, in Supporting Information). By attenuating acute neutrophil infiltration, metoprolol reduced brain injury and prevented neuronal loss and cerebral oedema progression. This outcome resulted in better preservation of BBB integrity and reduced subacute neuroinflammation. Owing to the initial neutrophil blockade, metoprolol disrupted the self-amplifying pro-inflammatory loop and created a pro-resolving environment that led to a tissue condition favourable to recovery. Furthermore, the ability of metoprolol to decrease IS was abolished in the absence of neutrophils, strongly suggesting that β 1AR modulation by metoprolol in these cells is mechanistically involved in these benefits.

Since reperfusion therapy is clearly associated with clinical benefits, and given that endovascular thrombectomy has recently extended the reperfusion time window (Nogueira et al., 2018), we decided to test a therapy with the potential to reduce reperfusion-related injury in a rodent model of MCAO/R. The non-invasive nature of MRI allowed us to perform a longitudinal analysis of IS at early (day 1) and late (day 7) after reperfusion. This was important because there is uncertainty about the optimal time for IS quantification (Neumann-Haefelin et al., 2000). Additionally, MRI performed during brain ischaemia allowed us to accurately estimate the size of the hypoperfused brain region (AAR) and exclude any influence of anatomical variation on recorded differences between groups (Figures 1 and S2).

Cerebral oedema is a severe complication of ischaemic stroke, and medical strategies to reduce its impact are limited (Thoren et al., 2017). Our results indicate that systemic administration of the selective β 1AR blocker metoprolol before reperfusion reduces acute cytotoxic and subacute vasogenic oedema (Figure 2). Acute excessive neutrophil infiltration is directly linked to neuronal damage and BBB breakdown. Preservation of BBB integrity is associated with better clearance of toxic cellular by-products, less subsequent permeability and vasogenic oedema, and less secondary neuroinflammation and

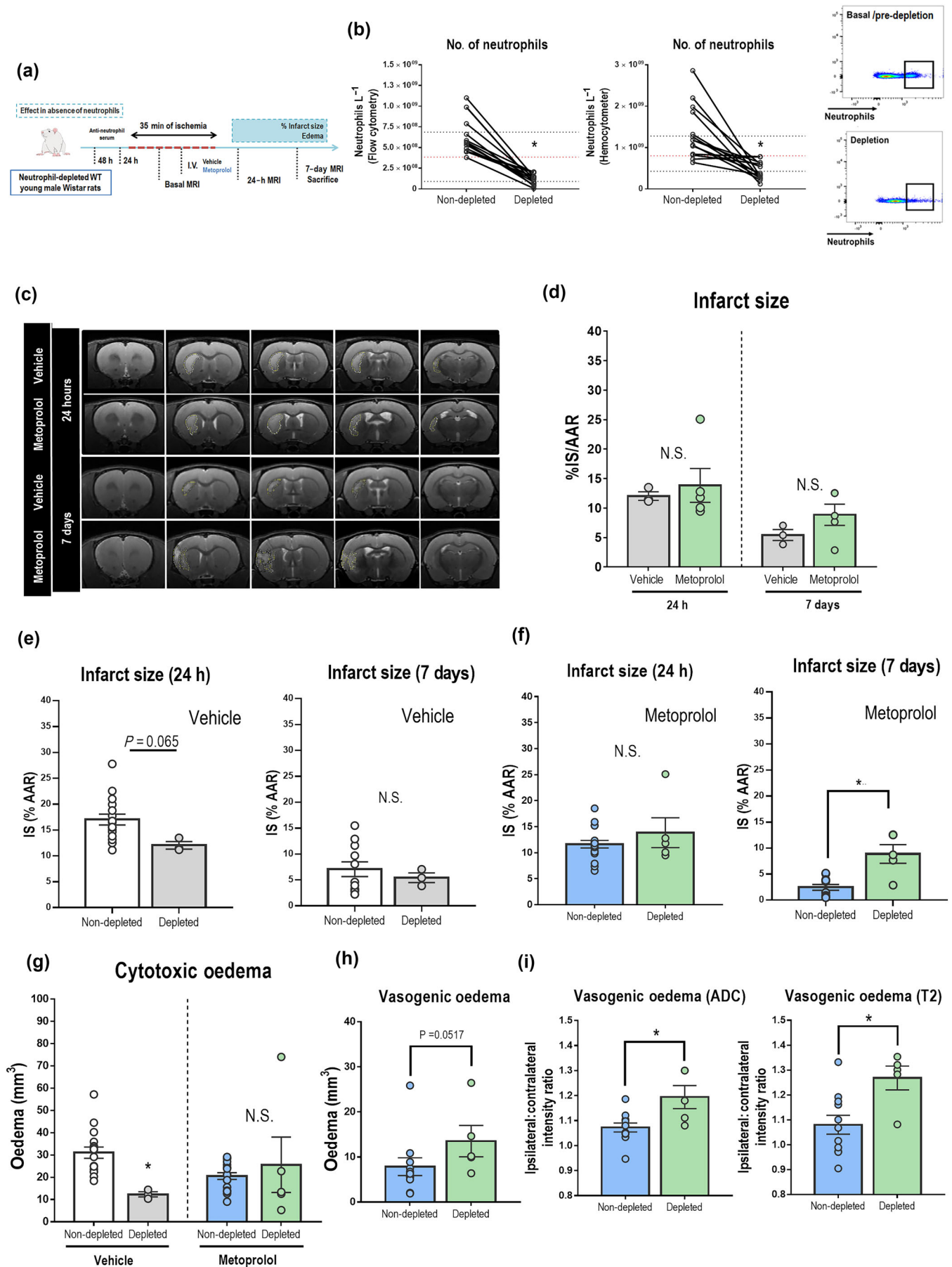


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FIGURE 6 The neuroprotective effect of β 1AR blockade by metoprolol is abolished in neutrophil-depleted rats. (a) Rat model of neutrophil depletion before stroke induction by MCAO/R. Rats were killed at 7 days post-reperfusion. (b) Depletion of circulating neutrophils with anti-PMN serum assessed by flow cytometry and haemocytometry. Upper and lower dotted black lines represent peripheral blood neutrophil counting means (baseline and after depletion). Red dotted lines represent minimal neutrophil threshold of depletion. Flow cytometry plots illustrate the reduction in peripheral blood neutrophils after depletion. The black square outlines the anti-neutrophil⁺ population. (c) Comparative coronal T2W MRI at 24 h (top rows) and 7 days (bottom rows) post-reperfusion. Infarcted regions correspond to hyperintense areas. (d) IS evaluated by coronal T2W MRI at 24 h and 7 days post-reperfusion. Final IS was calculated as the ratio of infarct volume to the AAR and expressed as %. (e) Neutrophil-depletion was associated with smaller IS at 24 h but not at 7 days post-reperfusion. (f) Metoprolol treatment in the absence of neutrophils did not provide any additional benefit at 24 h (f, g); however, its effect is completely abolished at 7 days post-reperfusion, as determined by IS and oedema quantification (h, i). Non-depleted vehicle, $n = 16$; non-depleted metoprolol, $n = 18$; depleted vehicle, $n = 3$; depleted metoprolol, $n = 5$. Graphs show mean \pm S.E.M. * $P < 0.05$. Abbreviations as in Figure 1.

neuronal death (Abdullahi et al., 2018). Our results show that early pre-reperfusion i.v. metoprolol prevents MVO and neutrophil infiltration, stemming progression of the infarct to distant areas and preserving BBB structural integrity, evaluated by the presence of AQP4 water channels at astrocyte end feet and a suggesting neural/glial antigen 2 expression by CSPG⁺ perivascular pericytes (Sharif et al., 2018). BBB integrity preservation by metoprolol was also confirmed by the absence of IgG extravasation (Figures 2–4).

Neutrophils' capacities expand further from first-line defence functions, and their plastic nature makes them able to reprogram and adapt their functions in different inflammatory contexts (Ballesteros et al., 2020; Mantovani et al., 2011; Silvestre-Roig et al., 2016). In the case of stroke, an excessive neutrophil accumulation is deleterious, while some degree of neutrophil infiltration into brain might be needed to promote infarct resolution. In line with this, metoprolol first protects the brain from an excessive and harmful neutrophil migration by disrupting their dynamics via β 1AR, as demonstrated by 2D IVM when exposed to TNF α . This β 1AR-mediated modulatory action results highly specific for neutrophils as metoprolol did not show any significant effect on astrocyte and microglia functionality (Figure 5). Besides, the lack of benefit from β 1AR blockade in the absence of neutrophils strongly confirms that the mechanism responsible for this protection is by modulating the deleterious effect of neutrophil accumulation in brain (Figure 6).

Even though neutrophil infiltration is acutely blocked by metoprolol, there still remain a number of neutrophils which do infiltrate, and could regulate the inflammatory reaction and play a key role in the eventual outcome of brain infarct. Under pro-inflammatory stimuli neutrophils become activated and prolong their lifespan by delaying apoptosis, which favour their accumulation at sites of inflammation (Brostjan & Oehler, 2020). However, by attenuating the acute neutrophil inflow, metoprolol completely counteracts the pro-inflammatory state, while conserving a population mostly of anti-inflammatory neutrophils (N2, YM1 or CD206⁺), more prone to apoptosis. The latter favours neutrophil clearance by microglia and triggers anti-inflammatory and pro-resolving responses (Figure 4) (Doran et al., 2020; Jones et al., 2016).

Consistent with all these data, the fact that the rats receiving pre-reperfusion i.v. metoprolol had better long-term behavioural and motor capacities, through a wide range of neurological tests, reinforce and consolidate the translational potential of this therapy as a neuroprotective strategy (Figure S4). Although there is abundant

experimental evidence that neutrophils play a central role in initiating brain I/R injury, translation to the clinic has so far not yielded clear benefits. The ROS scavenger **edaravone** was found to improve functional outcomes in several clinical trials when administered within 24 h of stroke onset (Enomoto et al., 2019). However, trials testing the ability of agents to block neutrophil–endothelium adhesion failed to improve outcomes (e.g., **natalizumab** in the ACTION trial (Elkins et al., 2017) and the recombinant protein UK-279,276 in the ASTIN trial (Krams et al., 2003). The strategy used in the present study differs from those tested in these earlier trials. β 1AR blockade by metoprolol has an anti-migratory effect in neutrophils and significantly reduces neutrophil–erythrocyte and neutrophil–platelet (Garcia-Prieto et al., 2017) interactions, as illustrated here in our cohort of ischaemic stroke patients undergoing reperfusion therapy (Figure 7). Moreover, the anti-neutrophil–endothelium agents in the ACTION and ASTIN trials were initiated after reperfusion, whereas in our study metoprolol was injected during ongoing cerebral ischaemia. The timing of administration seems critical, since MVO occurs immediately upon reperfusion. Intravenous administration of the selective β 1AR blocker used in this study has been shown to be safe in several clinical trials enrolling acutely ill patients (Chatterjee et al., 2013), including a recent study showing that its use in COVID-19 patients with acute respiratory distress results in a massive reduction of lung neutrophil infiltration and this translates into less days on mechanical ventilation (Clemente-Moragon et al., 2021). In addition, metoprolol is easy to administer and inexpensive. Therefore, this study constitutes a largely proof of principle which proposes metoprolol as a strong and promising candidate to be further explored for ischaemic stroke treatment.

5 | STUDY LIMITATIONS

This study is at the very earliest stages of the translational pathway and therefore presents some limitations that should be addressed in order to translate the potential impact of our findings to the clinics. One of the main limitations is that the study was carried out in young male rats, without including females or any comorbidity such as ageing, hypertension, or diabetes. Moreover, although the MCAO/R model in rodents is one of the most aggressive of ischaemic stroke, 35 min of ischaemia is known to cause mild but not severe strokes. To better establish the therapeutic time window of metoprolol, the

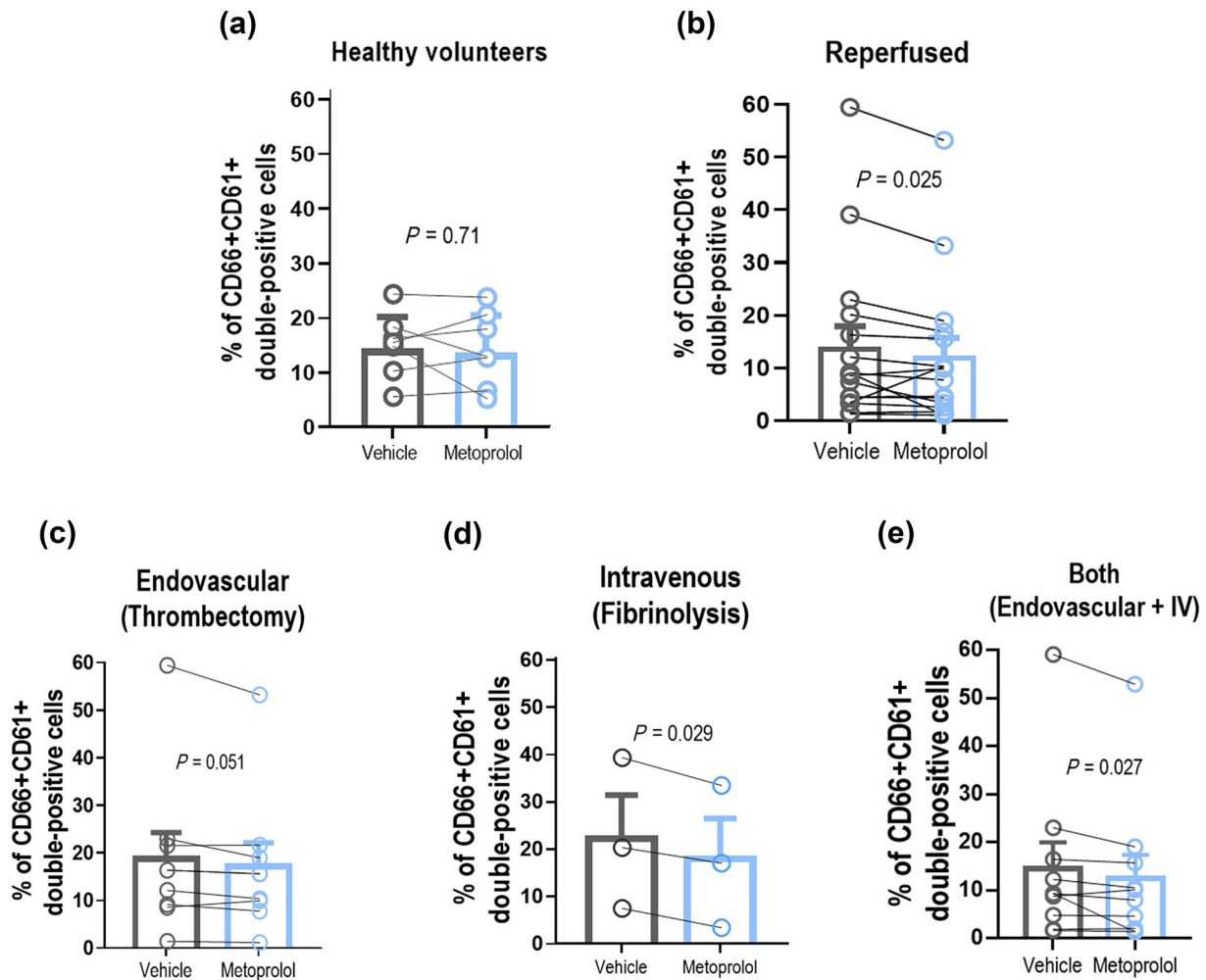


FIGURE 7 β 1AR blockade blocks neutrophil–platelet aggregates in ischaemic stroke patients. (a, b) Platelet (CD61⁺ cells) and neutrophils (CD45⁺/CD66⁺ cells) were detected by flow cytometry, and neutrophils positive for CD61 staining were identified as neutrophil–platelet co-aggregates. Neutrophil–platelet interactions were inhibited by metoprolol in ischaemic stroke patients who received reperfusion therapy (therapy, $n = 16$; non-therapy, $n = 8$). These interactions were also determined in healthy volunteers ($n = 7$), who did not respond to metoprolol treatment. A subanalysis was performed based on reperfusion therapy (endovascular thrombectomy, $n = 9$; fibrinolysis, $n = 3$; or both, $n = 10$). Likewise, metoprolol limited co-aggregates in all groups of treatment (c–e). Graphs show mean \pm S.E.M. * $P < 0.05$

neuroprotective effect of its administration should be explored at different times of cerebral ischaemia and upon (or early after) reperfusion. In addition, testing pre-reperfusion i.v. metoprolol in a stroke model of longer time of ischaemia (≈ 90 min) could be also interesting to be considered before translation to clinics.

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CONFLICT OF INTEREST

Javier Sanchez-Gonzalez is an employee of Philips Healthcare (Madrid, Spain). All other authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

B.I., E.O., M.D. and A.C-M. are responsible for the design of the entire study. Animal maintenance, MCAO/R surgery and followed-up by MRI was performed by D.C. and L.C. Haemodynamic dose-response study was carried out by M.G., A.C-M. and E.O. Brain Magnetic Resonance Imaging Protocol was designed by J.G-S., L. C. and M.D. Quantification of IS and oedema by MRI was done by A.C-M. Analyses of MRI results were carried out by A.C-M., E.O. and J.S-G. Sensory-motor assessment was performed by J.M-P. and M.A.M. Neutrophil depletion and evaluation of cell population levels was carried out by A.C-M. Tissue processing and immunostaining experiments were done by A.C-M. Immunohistochemical analyses were done by A. C-M., E.O. and M.C-C. Inflammatory in vitro and in vivo models in mice were carried out by A.C-M. Evaluation of human neutrophil-platelet co-aggregates was done by R.C., N.R. and J.M.B. Analyses of neutrophil-platelet interactions were done by R.C., N.R., J.M.B., J.C. F-F, J.C-R, A.C-M. and E.O. Statistical analyses were done by A.C-M. and E.O. All results were interpreted by A.C-M, M.C-C., J.S-G., E.O. and B.I. Manuscript was drafted by A.C-M., and critically revised by E.O., M.C-C, J.S-G., V.F. and B.I. B.I., M.D., E.O. and A.C-M are responsible for the final version of the manuscript, which was approved by all authors.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design and Analysis](#), [Immunoblotting and Immunochemistry](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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