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BASIC SCIENCE
Disease management

Post-ischaemic administration of the murine Canakinumab-surrogate antibody improves outcome in experimental stroke

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Aims The CANTOS trial underscored the efficacy of selective antibody-based interleukin (IL)-1β inhibition with Canakinumab in secondary prevention of cardiovascular events. Despite the success of the trial, incidence of stroke was not reduced likely due to the low number of events and the relatively young age of patients enrolled. Given the established role of IL-1β in stroke, we tested the efficacy of the murine Canakinumab-equivalent antibody in a mouse model of ischaemic stroke. To mimic the clinical scenario of modern stroke management, IL-1β inhibition was performed post-ischaemically upon reperfusion as it would be the case in patients presenting to the emergency room and eligible for thrombolytic therapy.

Methods and results Transient middle cerebral artery occlusion (tMCAO) was performed in wild type mice; upon reperfusion, mice were randomly allocated to anti-IL-1β antibody or vehicle treatment. Following tMCAO, cerebral IL-1β levels, unlike tumour necrosis factor-α, were increased underscoring a role for this cytokine. Post-ischaemic treatment with IL-1β antibody reduced infarct size, cerebral oedema and improved neurological performance as assessed by 2,3,5-triphenyltetrazolium chloride staining, Bederson and RotaRod tests. Antibody-treated animals also exhibited a reduced neutrophil and matrix metalloproteinase (MMP)-2 but not MMP-9, activity in ipsilateral hemispheres as compared to vehicle-treated mice. Noteworthy, tMCAO associated vascular endothelial-cadherin reduction was blunted in IL-1β antibody-treated mice compared to vehicle-treated, likely providing the mechanistic explanation for the improved outcome.

Conclusion Our data for the first time demonstrate the efficacy of selective post-ischaemic IL-1β blockade in improving outcome following experimental ischaemia/reperfusion brain injury in the mouse and encourage further focused clinical studies assessing the potential of the approved IL-1β antibody Canakinumab, as an adjuvant therapy to thrombolysis in acute ischaemic stroke patients.

Keywords IL-1β • Monoclonal antibody • Canakinumab • Middle cerebral artery occlusion • Stroke

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Translational perspective

The CANTOS trial recently demonstrated the efficacy of Canakinumab, a monoclonal anti-interleukin (IL)-1β antibody, in cardiovascular secondary prevention. In spite of this success, the incidence of stroke was not significantly reduced by treatment with Canakinumab likely due to the low number of events and the relatively young age of patients enrolled.

In view of the previously demonstrated link between IL-1β pathway and stroke and the limited therapeutic options for its treatment, this study was initiated to consider the therapeutic potential of Canakinumab in this setting. We here demonstrated that antibody-based inhibition of IL-1β by the murine Canakinumab-equivalent antibody is an effective approach to blunt cerebral damage and improve neurological performance after brain ischaemia/reperfusion in mice. Importantly, the antibody was administered only after ischaemia upon reperfusion thus mirroring the clinical situation of a patient undergoing thrombolytic therapy after symptoms onset. Our data encourage further clinical studies aimed at assessing potential of the approved IL-1β antibody Canakinumab as an adjuvant therapy for thrombolysis in the setting of acute ischaemic stroke.

Introduction

The inflammation theory of atherosclerosis[1] has recently been supported by the results of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial underscoring the clinical efficacy of selective interleukin (IL)-1β inhibition in secondary prevention of cardiovascular adverse events after an acute coronary syndrome.[2]

Despite the strong reduction in the primary composite endpoint of non-fatal myocardial infarction, non-fatal stroke, or cardiovascular death, the incidence of stroke was not reduced likely due to the low number of events with an incidence rate of 0.74/100 person-years and the relatively young age of patients enrolled (median age 61.1 years).[3]

Stroke represents a longstanding unmet clinical challenge as highlighted by the poor effectiveness of current therapies to reduce its incidence and improve outcome.[4] Although thrombolysis has revolutionized the management of acute stroke, reperfusion injury with oedema formation, and secondary bleeding is an unresolved issue. This is even more important as the incidence of stroke is expected to rise given the increasing global life expectancy.[5] An involvement of inflammation and specifically of IL-1β in stroke outcome is established by numerous pre-clinical studies which however, failed to translate into approved medications.[4,6]

Thus, it was the aim of this study to test the efficacy of the murine Canakinumab-equivalent IL-1β antibody in a mouse model of ischaemic stroke employing a clinically relevant experimental set-up. To this end, IL-1β inhibition was performed after the ischaemic event upon reperfusion as it would be the case in patients presenting to the emergency care unit and eligible for thrombolytic therapy.

Methods

Animals

Experiments were performed on 12-week-old male C57BL/6 wild type mice (Charles-River Lab, Freiburg im Breisgau, Germany); all rodents were kept in a temperature-controlled animal facility under normal light/dark cycle with free access to food and water. All procedures were approved by the local animal committee. Animal experiments were performed conform to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Transient middle cerebral artery occlusion

To induce ischaemia/reperfusion (I/R) brain injury, transient middle cerebral artery occlusion (tMCAO) was performed as described previously.[7] Briefly, mice were anaesthetized using isoflurane 3% and 1.5% for induction and maintenance, respectively, while body temperature was tidily controlled and kept at 37°C. For analgesia, buprenorphine HCl was infiltrated at the incision side (0.1 mg/kg). Ischaemia was induced by inserting a 6-0 silicone-coated filament (Doccol Corporation, Sharon, MA, USA) into the common carotid artery until the origin of the left middle cerebral artery after the dissection of common, internal and external carotid arteries. After 45 min of ischaemia, the filament was retracted and reperfusion allowed for 48 h before animal euthanasia with carbon dioxide.

Monoclonal anti-interleukin-1β antibody treatment

Monoclonal anti-IL-1β antibody was kindly provided by Novartis (Basel, Switzerland). Since the human monoclonal anti-IL-1β antibody (i.e. Canakinumab) does not react with rodent antigen, a surrogate antibody (i.e. 01BSUR) with a murine IgG2a/k isotype was used. This antibody was used by Novartis in all parallel pre-clinical studies performed for the development of Canakinumab.[8,9]

Upon retraction of the filament (beginning of reperfusion), animals were randomly allocated to receive either IL-1β antibody or vehicle (i.e. NaCl 0.9%).01BSUR was administered intravenously via tail vein injection, the unique dose was 10 μg/g diluted in saline so as to reproduce serum Canakinumab concentration in patients enrolled in the clinical trial CACZ885A2102 (NCT00487708), as described previously.[9–11]

Stroke volume

For determination of stroke volumes, murine brains were cut using a brain slicer matrix (Zivic instruments, Pittsburgh, PA, USA) into 5 (2 mm thick) coronal sections and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, Chemie GmbH, Buchs, Switzerland) at 37°C for 20 min. Stroke areas, were quantified using ImageJ software. The following formula was applied in order to compensate for cerebral swelling (oedema) and subsequent overestimation of the infarct volume as described previously[7]: corrected infarct volume = contralateral hemisphere volume - (ipsilateral hemisphere volume - infarct volume). Accordingly, oedema size was calculated by subtracting ipsilateral hemisphere volume to the contralateral ones.

Neurological deficit assessment

Neurological status was assessed 2, 24, and 48 h after tMCAO by a four-point scale neurological score based on Bederson *et al.*[12] as follows: grade 0, normal neurological function; grade 1, forelimb and torso flexion to the

contralateral side upon lifting the animal by the tail; grade 2, circling to the contralateral side; grade 3, leaning to the contralateral side at rest; grade 4, no spontaneous motor activity, as described previously.[13] Neurological performance was determined by the RotaRod test: animals were placed on a rotating rod at increasing speeds (4–44 revolutions/min) and latency to fall was recorded.[13] An experimenter blinded to the group allocation evaluated the neurological deficit at 2 h, 24 h, and 48 h after tMCAO.

Brain sampling for cytokine and neutrophil elastase assessment

Brains were collected after euthanasia and snap frozen in liquid nitrogen. Later, they were homogenized in the lysis buffer (Tris 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, Na3VO4 0.1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, and NP-40 0.5%) and total protein concentration was determined by Bradford protein assay according to the manufacturer's recommendations (VWR Life Science AMRESCO, Solon, OH, USA). Levels of IL-1beta, tumour necrosis factor (TNF)-α and neutrophil elastase were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) following the manufacturer instruction (Thermo Fischer Scientific, Waltham, MA, USA for TNF-α; R&D Systems, Minneapolis, MN, USA for IL-1β and neutrophil elastase). Mean intra- and inter-assay coefficients of variation were <10% for all markers measured by ELISA methods. Protein concentration as detected by ELISA was then normalized according to the total protein content of the sample, cytokines and neutrophil elastase brain content was expressed as pg per mg of total protein.

Western blotting

Brains were lysed in lysis buffer as described above and proteins were separated using SDS-PAGE before being transferred to a polyvinylidene fluoride membrane by wet transfer (Bio-Rad Laboratores AG, Fribourg, Switzerland). Membranes were incubated with primary antibodies: anti-matrix-metalloproteinases (MMP)-2 (ab37150; 1:1000; Abcam, Cambridge, UK), MMP-9 (ab38898; 1:1000; Abcam), vascular endothelial (VE)-cadherin (PA5-19612, 1:1000, Thermo Fischer Scientific) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (MAB374; 1:40 000; Merck Millipore, Billerica, MA, USA) over night at 4°C on a shaker. Secondary antibodies anti-mouse (1031-05) and anti-rabbit (4050-05) were obtained from Southern Biotechnology (Birmingham, AL, USA) and applied for 1 h at room temperature. Densitometric analyses were performed (Amersham Imager 600, General Electric; Healthcare Europe GmbH, Glattbrugg, Switzerland) and protein expression was normalized to GAPDH.

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, CA, USA). Data were analysed by using one-way analysis of variance (ANOVA) with Tukey *post hoc* test for multiple comparisons or unpaired two-tailed Student's *t*-test as appropriate. For repeated measurements, two-way ANOVA with Sidak *post hoc* test was used. A probability value (*P*) below 0.05 was considered as statistically significant.

Results

Brain ischaemia/reperfusion specifically increases interleukin-1β brain levels

To test the specific pathophysiological relevance of IL-1β during I/R brain injury, tMCAO was performed for 45 min followed by 48 h of reperfusion. Concentrations of different pro-inflammatory cytokines were determined in the affected (ipsilateral) hemisphere and compared to the contralateral side. I/R strongly induced the expression of IL-1β in the ipsilateral brain hemisphere, as compared to contralateral side (3.40 ± 0.92 vs. 2.40 ± 0.68 pg/mg of protein, $P = 0.027$, *Figure 1A*). In contrast, tissue levels of the pro-inflammatory cytokine

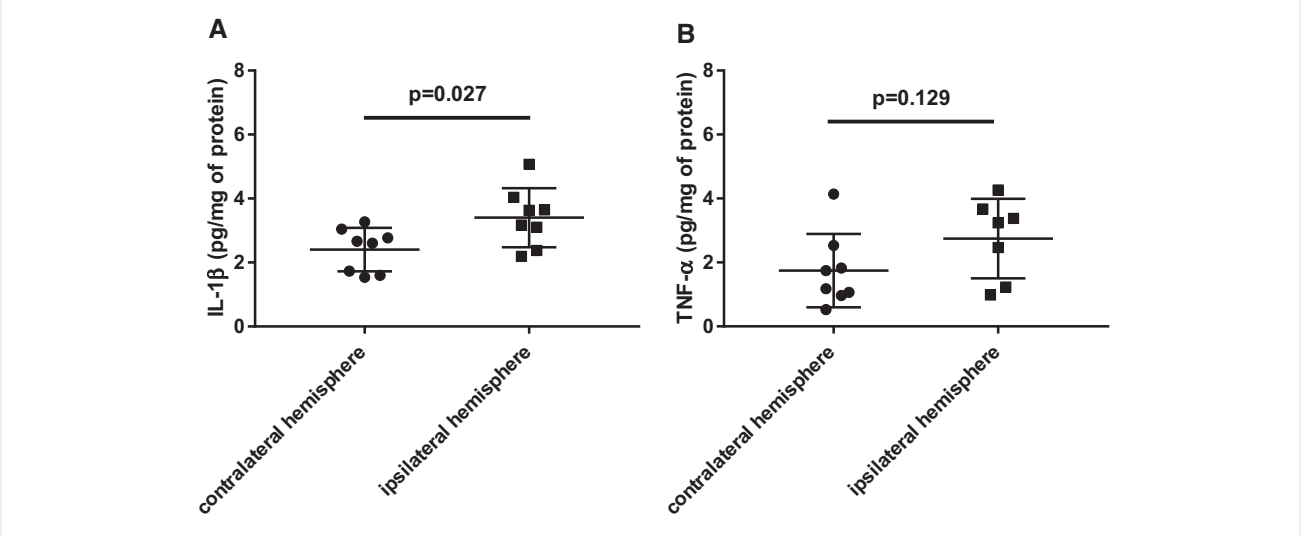

Figure 1 Interleukin-1β and tumour necrosis factor-α cerebral levels after transient middle cerebral artery occlusion. (*A*) Cerebral interleukin-1β concentration increased in ipsilateral hemisphere of mice undergoing transient middle cerebral artery occlusion for 45 min followed by 48 h reperfusion, as compared to contralateral side ($n = 8$, unadjusted $P = 0.027$). (*B*) Tumour necrosis factor-α levels in the ipsilateral side remained comparable to contralateral hemisphere ones after transient middle cerebral artery occlusion ($n = 7$–8, unadjusted $P = 0.029$).

TNF-α did not differ statistically between the ipsilateral and unaffected side 48 h after I/R injury (2.75 ± 1.24 vs. 1.75 ± 1.15 pg/mg of protein, $P = 0.129$, *Figure 1B*).

Post-ischaemic interleukin-1β inhibition by monoclonal antibody reduces infarct size and preserves post-stroke neurological function

To assess the therapeutic potential of post-ischaemic IL-1β inactivation by a monoclonal antibody on stroke outcome, 01BSUR (10 μg/g i.v.) was administered after tMCAO upon reperfusion (*Figure 2*). Stroke size, oedema and neurological deficits were assessed and compared to vehicle-treated animals. Following 48 h of reperfusion, mice treated with IL-1β antibody showed a 50% reduction in infarct volume, as assessed by TTC staining (36.99 ± 6.18 vs. 71.04 ± 12.74 mm^3, $P < 0.0001$, *Figure 3A* and *B*). Accordingly, IL-1β inhibition also reduced the calculated oedema volume (14.44 ± 13.28 vs. 34.04 ± 14.69 mm^3, $P = 0.018$, *Figure 3C*). Neurological deficits at different time points after tMCAO were quantified by two tests: 2 h after stroke, a tendency towards an improved neurological performance as assessed by Bederson score was already observed in IL-1β-blocked animals despite not being statistically significant (1.60 ± 0.51 vs. 2.08 ± 0.28, $P = 0.111$, *Figure 3D*). 24 h and 48 h after stroke however, anti-IL-1β-treated mice displayed a significantly improved Bederson score compared to vehicle-treated ones, indicating a blunted post-stroke impairment of neurological function in these rodents (1.07 ± 0.70 vs. 1.85 ± 0.56 and 0.60 ± 0.73 and 1.62 ± 0.65, $P = 0.003$ and $P < 0.0001$, respectively, *Figure 3D*). Accordingly, at the same time points, control animals showed worse neuromotor function than 01BSUR-treated ones thus falling markedly faster from the rotating rod as assessed by RotaRod test (42 ± 14 vs. 29 ± 17 and 49 ± 13 vs. 28 ± 14 s, $P = 0.049$ and $P = 0.0009$, respectively, *Figure 3E*).

Interleukin-1β targeting reduces post-stroke cerebral neutrophil infiltration

After stroke, proinflammatory cells infiltrate the brain parenchyma, and contribute to tissue damage.[4] In order to assess neutrophil infiltration, we measured neutrophil elastase tissue levels which predominantly reflect neutrophil activity although being also produced to a lesser extent by macrophages.[14] Neutrophil cerebral activity was increased compared to the contralateral side in the control group (55.29 ± 22.33 vs. 165.60 ± 68.96 pg/mg of protein, $P < 0.0001$, *Figure 4A*). Of note, treatment with anti-IL-1β antibody 01BSUR significantly reduced neutrophil infiltration in the stroke side (95.64 ± 21.48 vs. 165.6 ± 68.96 pg/mg of protein, $P = 0.016$, *Figure 4A*).

Reduced matrix metalloproteinase-2 and preserved vascular endothelial-cadherin expression in ipsilateral side of anti-interleukin-1β-treated animals

The lower oedema volume in mice treated with the IL-1β antibody 01BSUR, suggests a blunted blood brain barrier (BBB) damage following I/R. Among several mechanisms contributing to post-stroke BBB dysfunction, MMPs enzymatically digest inter-epithelial tight and adherens junctions leading to increased BBB permeability. In line with the above, in animals treated with 01BSUR active MMP-2 expression was reduced significantly more in ipsilateral hemisphere than in vehicle-treated controls, as compared to contralateral side (-0.35 ± 0.17 vs. -0.16 ± 0.07, $P = 0.047$, *Figure 4B*). Meanwhile, levels of the active form of MMP-9 were unaffected by anti-IL-1β treatment (0.08 ± 0.11 vs. -0.06 ± 0.36, $P = 0.397$, *Figure 4C*). In line with the blunted reduction in MMP2 activity, VE-cadherin protein content was similar between ipsilateral and contralateral hemispheres of antibody-treated mice (0.92 ± 0.18 vs. 0.85 ± 0.20, $P = 0.967$, *Figure 4D*) unlike vehicle-treated animals where a reduction was observed (1.01 ± 0.28 vs. 0.57 ± 0.31, $P = 0.045$, *Figure 4D*).

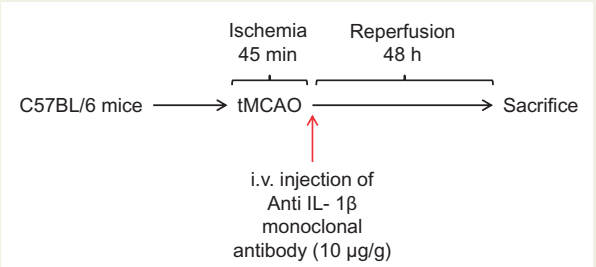

Figure 2 Schematic of clinically relevant experimental study design. In an attempt to simulate the clinical situation of a patient with stroke of new onset that, once arrived at ED, is submitted to thrombolysis, the animals were treated with murine Canakinumab-surrogate antibody only after the ischaemic period and at the beginning of reperfusion (removal of filament from middle cerebral artery origin). Interleukin-1β monoclonal antibody was administered to the animal intravenously via tail vein injection in a unique dose of 10 μg/mg, vehicle (i.e. NaCl 0.9%) was used as negative control. After 45 min of transient middle cerebral artery occlusion, 48 h of reperfusion and antibody action were allowed prior to euthanize the animal.

Discussion

Stroke is the second cause of death worldwide and the leading cause of adult neurological disability in the Western World, furthermore its incidence is set to increase given the current gradual aging of the population.[15,16] Very recently, the CANTOS study demonstrated the efficacy of a monoclonal IL-1β antibody (Canakinumab, Novartis) in cardiovascular secondary prevention.[2] In spite of this success, the incidence of stroke was not significantly reduced by treatment with Canakinumab likely due to the low number of events and the relatively young age of patients enrolled. Indeed, only 254 out of the 10 061, i.e. 2.5%, of the patients enrolled suffered a stroke with no statistical difference between the treatment groups.[2]

Given the established role of IL-1β in determining outcome after stroke,[6] we hereby assessed the efficacy of the murine Canakinumab-equivalent antibody (01BSUR, Novartis) in a mouse model of I/R brain injury. In order to augment the translational value of our results, a clinically relevant experimental setup was employed whereby anti-IL-1β antibody was administered to the animal only after the ischaemic event and upon reperfusion mirroring the situation of a patient undergoing thrombolytic therapy after symptoms onset.

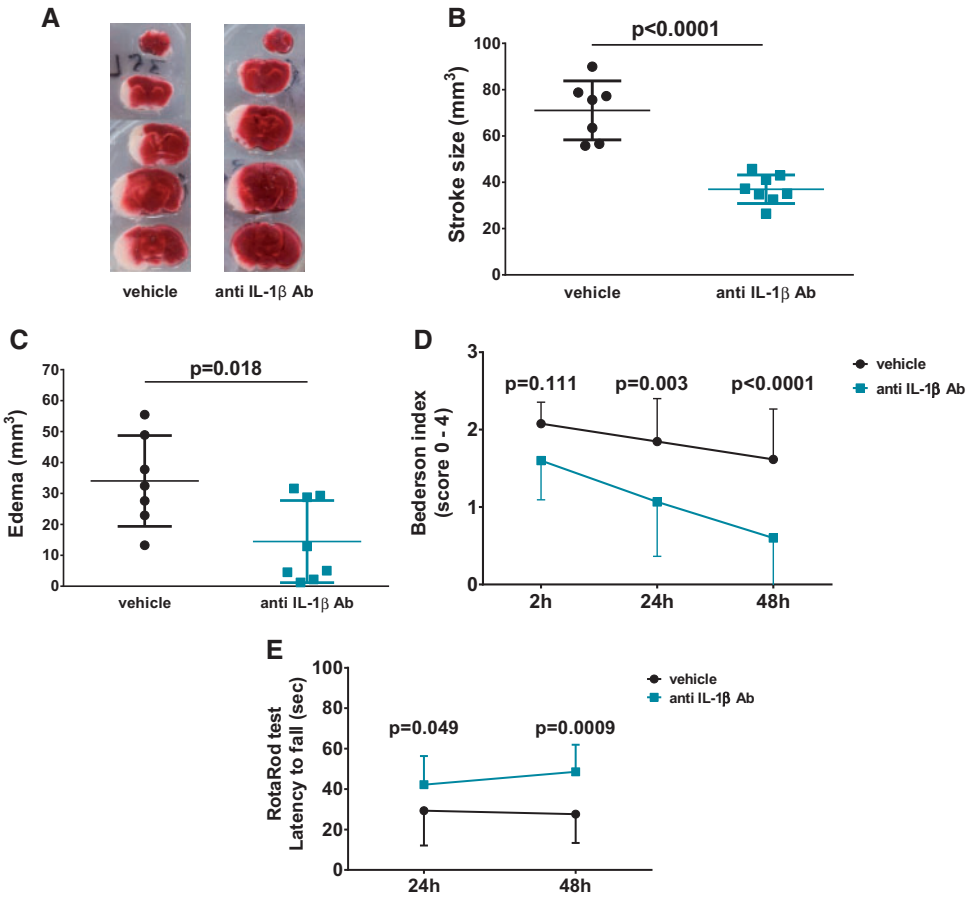

Figure 3 Impact of post-ischaemic treatment with interleukin-1β antibody on cerebral lesion and neurological deficit after transient middle cerebral artery occlusion in mice. (*A*) Representative pictures of 2,3,5-triphenyltetrazolium chloride-stained brain slices. (*B*) Animals treated with monoclonal antibody against interleukin-1β (anti-interleukin-1β Ab) showed decreased stroke volumes by 50% ($n = 7–8$, unadjusted $P < 0.0001$) and (*C*) decreased oedema ($n = 7–8$, unadjusted $P = 0.018$) as assessed by 2,3,5-triphenyltetrazolium chloride-staining 48 h after transient middle cerebral artery occlusion, compared to animals treated with vehicle alone. Accordingly, treatment with anti interleukin-1β Ab improved post-stroke neurological function as assessed by (*D*) RotaRod test or (*E*) Bederson-based neurological score, as compared to treatment with vehicle alone ($n = 13–15$ for both, adjusted $P = 0.003$ and <0.0001 for Bederson index at 24 h and 48 h, respectively; adjusted $P = 0.049$ and 0.0009 for Rotarod test at 24 h and 48 h, respectively).

Our data show that the cytokine IL-1β, unlike TNF-α, is upregulated in the murine ipsilateral hemisphere following I/R brain injury. The pathophysiological relevance of this up-regulation was underscored by the fact that post-ischaemic, antibody based, IL-1β inhibition upon reperfusion reduces infarct size and cerebral oedema in mice exposed to tMCAO. Treatment with IL-1β antibody 01BSUR also improved neurological performance as demonstrated by Bederson and RotaRod tests. Deeper investigation of the underlying protective molecular mechanisms of IL-1β inhibition, revealed a reduction of cellular inflammation as assessed by neutrophil elastase and MMP-2 (but not MMP-9) tissue levels. As a consequence, while vehicle-treated mice showed decreased VE-cadherin expression following tMCAO, IL-1β blockade prevented this reduction likely providing the explanation for the reduced oedema and lesion size in this group.

Non-selective interference with IL-1β pathway was previously shown to exert beneficial effects on I/R cerebral injury in preclinical studies.[17,18] Such studies prompted two phase 2 randomized control trials to assess safety and efficacy of an IL-1β receptor antagonist (IL-1Ra, Anakinra) in patients with stroke. Although this drug was confirmed to have good tolerability,[19] results on its efficacy (ISRCTN74236229), initially announced for May 2017, have not been published yet. Furthermore, Anakinra requires daily injections and blocks both IL-1α and IL-1β, thereby potentially impairing host defence.[3]

Canakinumab was administered every 3 months in the CANTOS trial and is highly specific for IL-1β; indeed, it has already been approved by the FDA for the treatment of rare periodic fever syndromes and active systemic juvenile idiopathic arthritis.[3] In light of the above and the recently published results from the CANTOS trial, IL-1β specific targeting via a monoclonal antibody appears to be a more promising therapeutic approach, which deserves further assessment in the setting of stroke. In this sense, data from this study showing that post-ischaemic treatment with the Canakinumab-surrogate antibody improves outcome after stroke in the mouse, support this concept.

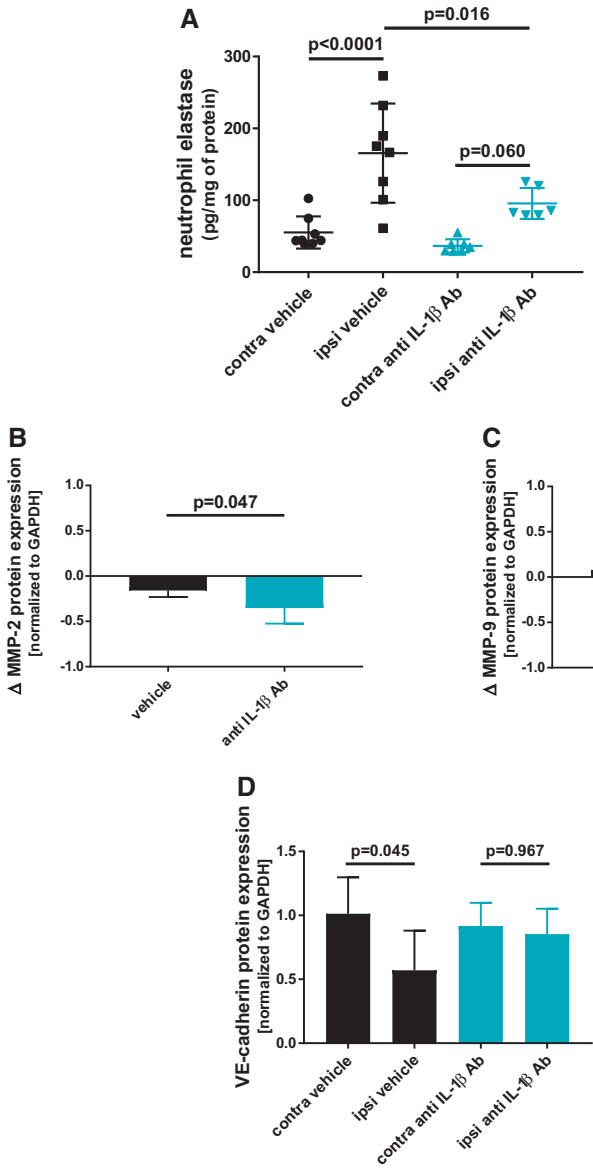

Figure 4 Effects of interleukin-1β blockade on post-stroke cerebral neutrophil infiltration, matrix metalloproteinases and vascular endothelial-cadherin expressions. (*A*) Control animals showed an increased expression of neutrophil elastase in the ipsilateral hemisphere (ipsi) as compared to the contralateral (contra) ones ($n = 8$, adjusted $P < 0.0001$). In the stroke side, animals treated with interleukin-1β monoclonal antibody (anti-interleukin-1β Ab) showed a statistical significant lower concentration of this enzyme with respect to vehicle-treated rodents ($n = 6$–8, adjusted $P = 0.016$). (*B*) A lower reduction in matrix metalloproteinase-2 levels was observed in the stroke side from interleukin-1β-inhibited animal with respect to contralateral hemisphere (ΔMMP-2), as compared to rodents treated with vehicle alone ($n = 5$–6, unadjusted $P = 0.047$), (*C*) the same effect was not observed for matrix metalloproteinase-9 ($n = 6$, unadjusted $P = 0.397$). (*D*) vascular endothelial-cadherin protein expression was significantly reduced in the ipsilateral (ipsi) hemisphere of vehicle-treated animals, as compared to contralateral (contra) one ($n = 5$, adjusted $P = 0.045$). Of interest, the levels of this adherens junction protein were not affected in the ipsilateral side of animal treated with interleukin-1β monoclonal antibody ($n = 6$, adjusted $P = 0.967$).

BBB permeability plays a pivotal role in determining stroke outcome.[20] During I/R brain injury, this tightly regulated structure is disrupted thus promoting vascular leakage and oedema formation.[21] IL-1β is known to negatively affect the layer of microvascular endothelial cells connected via tight and adherens junctional proteins that make up the BBB through different mechanism including increased adhesion molecules expression which mediate the parenchymal infiltration of circulating white blood cells.[22] In line with this, we showed that neutrophil elastase tissue levels—a marker of neutrophil activity—are lower in ipsilateral hemispheres of anti-IL-1β treated mice as compared to vehicle treated ones.

Neutrophil infiltration enhances the local inflammatory response by increasing cytokine and chemokine production (the same

neutrophil elastase can cleave pro-IL1β to its active form)[23] eventually causing an over activation of microglia and endothelial cells. In turn, released MMPs lead to junctional proteins cleavage and loss of BBB integrity, thus feeding the deleterious inflammatory loop.[24] In particular, IL-1β was previously shown to modulate astrocytic MMP-2 expression[25] which in turn directly cleaves the adherens junction protein VE-cadherin.[26–28] In line with this notion, our data show that treatment with IL-1β antibody preserves BBB function by specifically decreasing MMP-2 cleavage and preserving VE-cadherin.

In conclusion, we show that IL-1β is predominantly upregulated in brains of mice undergoing tMCAO. Furthermore, post-ischaemic treatment with the murine Canakinumab-surrogate IL-1β antibody improved morphological and functional outcome after tMCAO in mice. The cerebral anti-inflammatory effect of IL-1β blockade was mediated by a reduction in neutrophilic infiltration, MMP-2 tissue levels in the infarcted parenchyma, thus blunting the degradation of VE-cadherin, a protective adherens junction protein involved in maintaining BBB functionality.

Our data encourage further clinical studies aimed at assessing the potential of the approved IL-1β antibody Canakinumab, as an adjuvant therapy for thrombolysis in the setting of acute ischaemic stroke.

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Conflict of interest: T.F.L. has been member of the Canakinumab advisory board of Novartis and has received honoraria as well as educational grants for the *Postgraduate Course on Heart Failure* to the institution. All other authors declare no conflict of interest.

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