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Delayed administration of interleukin-1 receptor antagonist reduces ischemic brain damage and inflammation in comorbid rats

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Many neuroprotective agents have been effective in experimental stroke, yet few have translated into clinical application. One reason for this may be failure to consider clinical comorbidities/risk factors in experimental models. We have shown that a naturally occurring interleukin-1 receptor antagonist (IL-1Ra) is protective against ischemic brain damage in healthy animals. However, protective effects of IL-1Ra have not been determined in comorbid animals. Thus, we tested whether IL-1Ra protects against brain injury induced by experimental ischemia in aged JCR-LA (corpulent) rats, which have clinically relevant risk factors. Male, aged, lean, and corpulent rats exposed to transient (90 minutes) occlusion of the middle cerebral artery (tMCAO) were administered two doses of IL-1Ra (25 mg/kg, subcutaneously) during reperfusion. Brain injury and neuroinflammatory changes were assessed 24 hours after tMCAO. Our results show that IL-1Ra administered at reperfusion significantly reduced infarct volume measured by magnetic resonance imaging (50%, primary outcome) and blood–brain barrier disruption in these comorbid animals. Interleukin-1Ra also reduced microglial activation, neutrophil infiltration, and cytokines levels in the brain. These data are the first to indicate that IL-1Ra protects against ischemic brain injury when administered via a clinically relevant route and time window in animals with multiple risk factors for stroke.
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Introduction

Stroke is a leading cause of death and disability in adults worldwide. Fibrinolysis with recombinant tissue plasminogen activator is the only licensed therapy for stroke, but is available to only a minority of patients due to the risk of hemorrhage. Numerous agents have shown efficacy in animal models of stroke, yet have failed to show benefit in clinical trials (Paul *et al*, 2007). Recent clinical failures have led the Stroke Therapy Academics Industry Roundtable (STAIR; Fisher *et al*, 2009; STAIR, 1999) to update their guidelines in order that better translation from bench-to-bedside might be made. These updated guidelines include the need to show effectiveness of any potential stroke drug in aged and comorbid animals.

Inflammation contributes to poor outcome after stroke (Denes *et al*, 2010*a*, 2010*b*; McColl *et al*, 2009), and the proinflammatory cytokine interleukin-1 (IL-1) is a key mediator of ischemic brain injury (Allan *et al*, 2005; Simi *et al*, 2007). The IL-1 receptor antagonist (IL-1Ra) is a naturally occurring, competitive inhibitor of IL-1 action, which is highly selective. There is considerable preclinical and clinical evidence showing that IL-1Ra is a promising candidate for the treatment of stroke and related disorders (Banwell *et al*, 2009; Emsley *et al*, 2005; Hannum *et al*, 1990; Rothwell, 2003). In experimental stroke, endogenous IL-1Ra is upregulated and protects the brain (Denes *et al*, 2008; Pinteaux *et al*, 2006), while administration of exogenous IL-1Ra reduces ischemic injury, even when administered 3 hours after the insult (Garcia *et al*, 1995; Loddick

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and Rothwell, 1996; Mulcahy *et al*, 2003; Relton *et al*, 1996; Stroemer and Rothwell, 1997). Subcutaneous IL-1Ra, administered at the time of occlusion, is neuroprotective in the rat and localizes to salvageable brain tissue (Greenhalgh *et al*, 2010), and IL-1Ra showed some beneficial effects in a small phase II clinical study in acute stroke (Emsley *et al*, 2005).

However, IL-1Ra has not yet been tested in comorbid animals after stroke, a key recommendation of the recent STAIR report. We have previously shown an elevated inflammatory burden in the brain of corpulent (Cp) rats in the absence of any acute injury, and activated microglia in patients at risk of stroke (Drake *et al*, 2011). Therefore, due to the neuroprotective effects of IL-1Ra demonstrated previously, we determined the effects of subcutaneous administration of IL-1Ra after experimental stroke in aged, Cp (Mangat *et al*, 2007) and lean rats.

Materials and methods

Animals

Studies were conducted on 15- and 16-month-old male, lean (JCR:LA-lean (*cp/−*); 400–500 g) and Cp (JCR:LA-cp (*cp/cp*); 900–1000 g) rats (Charles River, Wilmington, MA, USA). Cp rats are homozygous for the autosomal recessive cp gene (*cp/cp*), and spontaneously develop obesity, hyperlipidemia, insulin resistance, glomerular sclerosis, and atherosclerosis with enhanced vascular contractility and reduced vascular relaxation (Mangat *et al*, 2007). Animals were allowed free access to food and water and were maintained under temperature, humidity, and light-controlled conditions. All animal procedures were performed under the University of Manchester project license number (40/3076) and adhered to the UK Animals (Scientific Procedures) Act (1986).

Treatment Groups

A pilot study was undertaken to analyze the pharmacokinetic profile of subcutaneous human-IL-1Ra (r-met-huIL-1Ra: Kineret; Amgen, Thousand Oaks, CA, USA) or placebo (Amgen). Owing to the highly lipophobic formulation of the drugs, obese Cp rats received half the dose of IL-1Ra (50%) per body weight compared with lean rats. Plasma concentrations of IL-1Ra (measured by enzyme-linked immunosorbent assay) after subcutaneous administration reached levels that we believe are clinically therapeutic concentrations ($\sim$8000 ng/ml; Emsley *et al*, 2005) and no differences in plasma concentrations were observed between lean or Cp rats (data not shown).

Animals were randomized for all the experiments, assessments were performed in a blinded manner and analysis was confirmed by two independent researchers. Lean and Cp rats received two doses (subcutaneous) of placebo or IL-1Ra (25 mg/kg and 12.5 mg/kg, respectively) at reperfusion and 6 hours later, and were allocated randomly to the following experimental groups: lean + tMCAO + placebo; lean + tMCAO + IL-1Ra; Cp + tMCAO + placebo; and Cp + tMCAO + IL-1Ra ($n = 6$ per group). For the delayed administration study, animals were injected (subcutaneous, doses as above) with placebo or IL-1Ra at 3 hours of reperfusion ($n = 8$ per group), and again, 3 hours later.

Group sizes were determined by power calculation ($\alpha = 0.05$, $\beta = 0.2$) at http://www.stattools.net/SSizAOV_Pgm.php.

Focal Cerebral Ischemia

Focal cerebral ischemia was induced by 90 minutes transient occlusion of the middle cerebral artery (tMCAO) as described previously (Chen *et al*, 1986; Liu *et al*, 1989). Isofluorane (5% for induction and 1.5% during surgery) was used as anesthetic in a mixture of 70% N_2O and 30% O_2. Core body temperature was maintained at 37.0°C ± 0.5°C throughout the surgery by a heating blanket (Homeothermic Blanket Control Unit; Harvard Apparatus, Kent, UK) and monitored after recovery. Ischemia was induced by a transient ligature (90 minutes) of the left MCA trunk with a 10-0 suture (Prolene, Ethicon, Somerville, NJ, USA). Occlusion and reperfusion were confirmed visually under the surgical microscope. After surgery, animals were returned to their cages and allowed free access to water and food. It was decided, *a priori*, to exclude from the study those animals that showed brain hemorrhage at any time of the surgery or with no reperfusion (8% in total, corresponding to lean and Cp treated with IL-1Ra). The survival rate was 100%.

Magnetic Resonance Imaging

The primary outcome was infarct volume measured by magnetic resonance imaging (MRI) 23 hours after tMCAO. Animals anesthetized with isofluorane (5% for induction and 1.5% during scanning) were scanned using a Magnex 7-Tesla, horizontal-bore magnet (Agilent Technologies, Oxford Industrial Park, Yarnton, Oxford, UK) connected to a Bruker Biospec Avance III console (Bruker Biospin, Banner Lane, Coventry, UK) with a transmit/receive 2.5-cm surface coil. Rectal temperature and respiration rate were monitored. A T_2-weighted fast spin-echo sequence based on RARE (Hennig *et al*, 1986) was used (repetition time = 4.8 seconds, base echo time = 20 ms, effective echo time = 60 ms, number of echos = 8, number of samples = 256, number of views = 128, number of averages = 2 with 25 1 mm slices), which results in sufficient anatomical information to measure brain damage (Calamante *et al*, 1999; Wegener *et al*, 2006). Lesion volume was determined using Anatomist software (http://brainvisa.info/).

Stroke Outcome

Motor and behavioral outcomes and the adhesive removal test were assessed 24 hours before and 23 hours after tMCAO, as described previously (Hunter *et al*, 2000; Madrigal *et al*, 2003; Denes *et al*, 2008). Functional assessments were all performed by an observer blinded for treatments. Each test was conducted three times per trial, and the average taken to determine outcome.

Histology/Immunohistochemistry

Blood samples for pharmacokinetic analysis and cytokine assays were obtained from the tail vein before stroke (time 0), at reperfusion (time 1.5 hours), and at 6 hours, and from the right heart ventricle at 24 hours. Using isofluorane as anesthetic and after the final blood sample, animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (pH = 7.4). Brains were removed, post-fixed, cryoprotected in 30% sucrose and frozen at −20°C. Sections (30 μm) were cut on a sledge microtome (Bright series 8000; Bright Instruments, Huntingdon, UK) and stored at −20°C in antifreeze solution (30% ethylene glycol and 20% glycerol in phosphate-buffered saline) until processing.

Nissl staining was used to quantify the infarct volume on brain sections. Blood–brain barrier (BBB) damage was assessed by immunoglobin G immunostaining, as described previously (Greenhalgh *et al*, 2010). Double or triple immunofluorescence was performed on free-floating brain sections as previously described (Drake *et al*, 2011). The primary antibodies used were as follows: goat anti-rat metalloproteinase-9 (MMP-9; 1:100; R&D Systems, UK), rabbit anti-neutrophil serum (1:100; SJC; Anthony *et al*, 1998), rabbit anti-Iba1 (1:1000; Wako Chemicals, Germany), goat anti-Iba1 (1:1000; Abcam, Cambridge, UK), goat anti-chemokine (C-X-C motif) ligand (CXCL1; 1:100; R&D Systems, Abingdon, UK), rabbit polyclonal anti-IL-6 (1:100; Abcam). Biotinylated tomato lectin (1:100; Sigma-Aldrich, Poole, UK) was used to stain cerebral blood vessels. Antigens were visualized by using anti-goat or anti-rabbit fluorochrome-conjugated (Alexa Fluor 594 or Alexa Fluor 488, 1:500; Molecular Probes, Invitrogen, Paisley, UK) secondary antibodies and to visualize the lectin, streptavidin Alexa Fluor 350 conjugate, 1:500) was used. After mounting and applying coverslips, images from the sections were collected on an Olympus BX51 upright microscope and captured using a Coolsnap ES camera (Photometrics, Tucson, AZ, USA) through MetaVue Software (Molecular Devices, UK). Plasma cytokine levels were measured by cytometric bead array (R&D Systems; Denes *et al*, 2010*a*, 2010*b*).

Metalloproteinase-9/lectin-positive blood vessels, granulocytes, and Iba1/CXCL1-double-positive cells were counted in two and three random fields of view, respectively, inside the cortex, covering the infarct and penumbra, per section over three sections per animal (2 mm apart) covering the whole infarct in a rostro-caudal manner. Activated microglia were identified as showing: (1) increased Iba1 immunopositivity, (2) an enlarged and/or amenorrhea cell body, (3) complete or partial loss of thin, elongated processes. For Iba1/IL6-double-positive cells, all the sections per animal (14 sections) were analyzed.

Statistical Analysis

Data are expressed as mean ± s.d. Statistical analysis was performed as follows: for two groups, Student's *t*-test (two-tailed), and for three or more groups, one-way or two-way analysis of variance followed by Bonferroni's *post hoc* multiple-comparison or paired comparison ($n = 6$–8/experimental group).

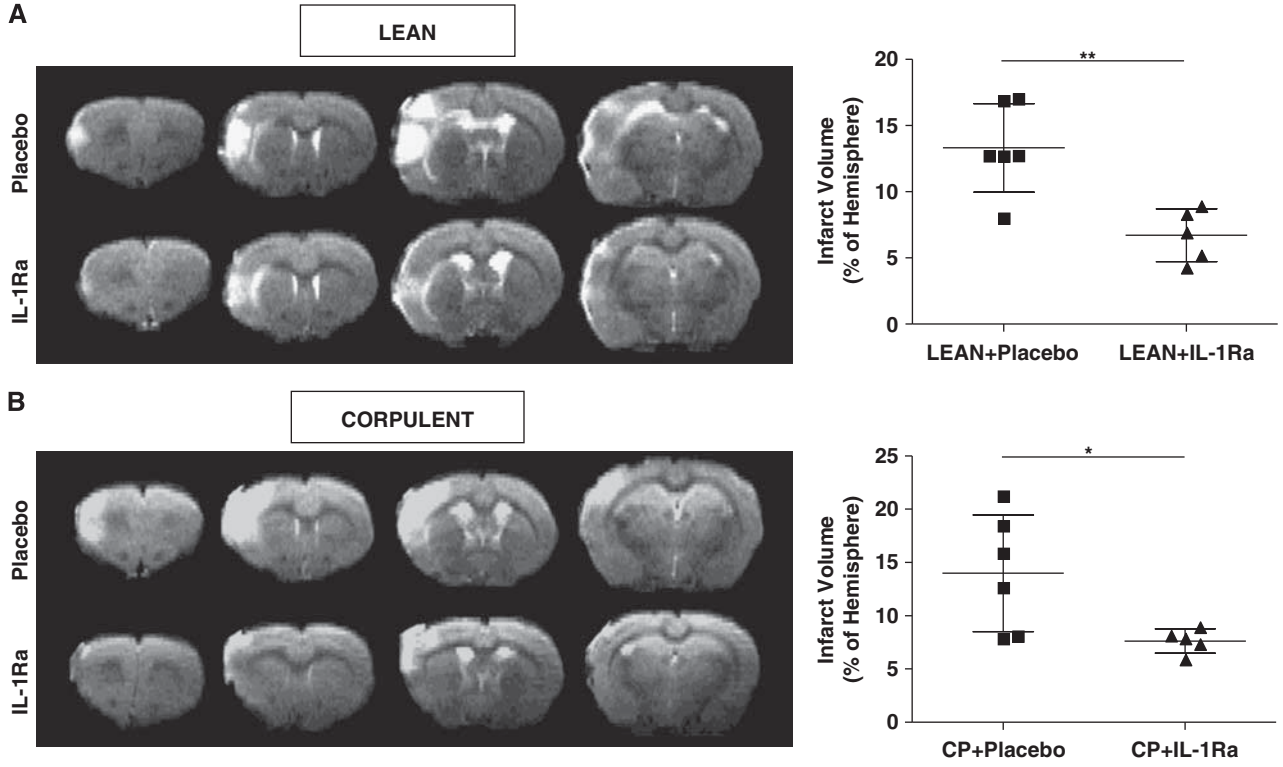

Figure 1 Effect of interleukin-1 receptor antagonist (IL-1Ra)/placebo on brain injury after transient occlusion of the middle cerebral artery (tMCAO), administered at reperfusion. Total infarct volume, expressed as percentage of ipsilateral hemisphere, was measured 23 hours after tMCAO on T_2W images in lean (**A**) and in corpulent rats (Cp, **B**; $n = 6$). $^*P < 0.05$; $^{**}P < 0.01$, Student's *t*-test.

Results

IL-1Ra Reduces Infarct Volume and BBB Breakdown in Aged Lean and Corpulent Rats

Interleukin-1Ra administration at reperfusion resulted in a significant (50%) reduction of infarct volume as measured by T_2W images in lean and Cp when compared with placebo-treated animals (lean + placebo: 13.3% ± 3.3% versus lean + IL-1Ra: 6.7% ± 2% and Cp + placebo: 14% ± 5.5% versus Cp + IL-1Ra: 7.6% ± 1.1%; $P < 0.05$; Figures 1A and 1B) 23 hours after tMCAO. When measured by Nissl staining on post-mortem tissue at 24 hours after stroke, IL-1Ra significantly reduced infarct volume by 65% and 53% in leans and Cp, respectively (lean + placebo:11% ± 3.4% versus lean + IL-1Ra:3.7% ± 1.4% and Cp + placebo:12.3% ± 5.8% versus Cp + IL-1Ra:5.7% ± 2.3%; $P < 0.05$; Figures 2A and 2B), compared with placebo. Although ischemic brain damage was not different between Cp and lean animals (Figures 1 and 2), and despite changes in the inflammatory response in the brain due to IL-1Ra treatment (see below), of the inflammatory cytokines measured in plasma by cytometric bead array, only IL-6 was higher in Cp than lean rats before stroke (lean: 32.4 ± 30.9 pg/ml versus Cp: 61.7 ± 32 pg/ml; $P < 0.05$), and none was affected by IL-1Ra.

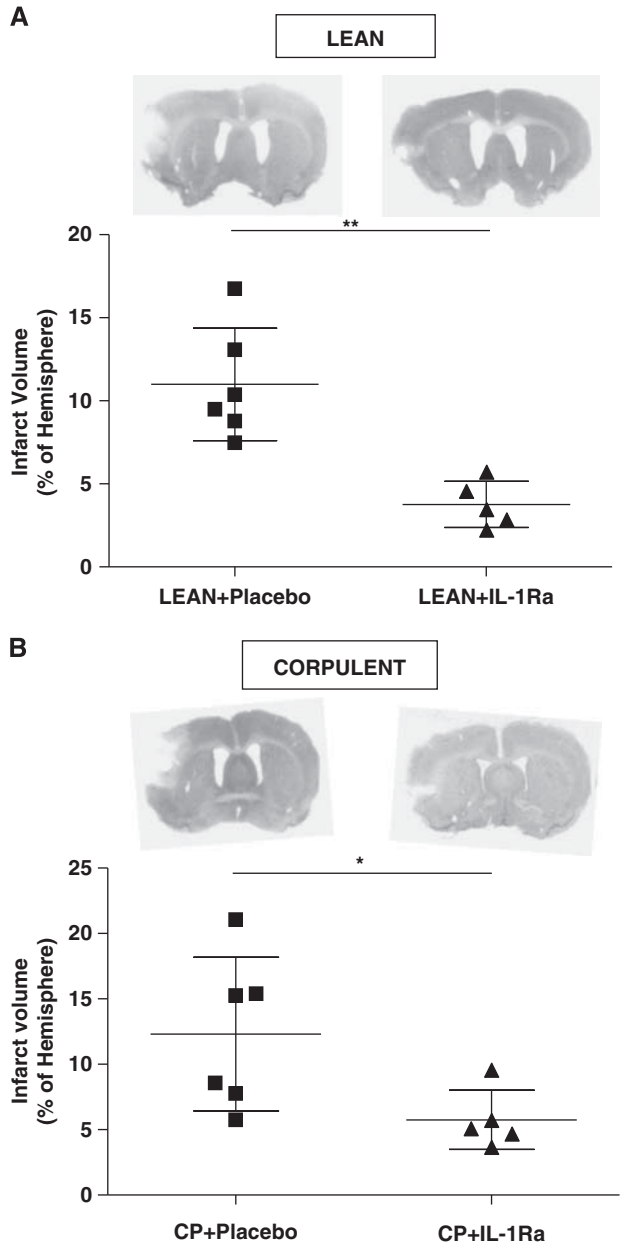

Figure 2 Effect of interleukin-1 receptor antagonist (IL-1Ra)/placebo on brain injury after transient occlusion of the middle cerebral artery (tMCAO), administered at reperfusion. Total infarct volume, expressed as percentage of ipsilateral hemisphere, was measured 24 hours after tMCAO in Nissl-stained brain sections in lean (**A**) and in corpulent rats (Cp, **B**; $n = 6$). $^*P < 0.05$; $^{**}P < 0.01$, Student's *t*-test.

Interleukin-1Ra treatment significantly reduced immunoglobin G immunostaining in lean (55%) and Cp (45%) rats, compared with placebo, indicating a reduction of BBB damage (Figures 3A–C). Cp rats displayed significantly larger BBB damage compared with lean rats, both in placebo groups and with IL-1Ra treatment (Figure 3C).

Delayed IL-1Ra administration in aged lean rats, starting 3 hours after the onset of reperfusion, induced a significant reduction of infarct volume (30%) as measured in T_2W images (lean + placebo—3h: 18% ± 4% versus lean + IL-1Ra—3h: 12.4% ± 3%; $P < 0.05$; Figure 4A). When brain damage was measured by histology (Nissl-staining) 24 hours after stroke, also a significant reduction (46%) was observed in the IL-1Ra-treated group with delayed treatment (lean + placebo—3h: 12.5% ± 6.2% versus lean + IL-1Ra—3h: 6.7% ± 2.9%; $P < 0.05$; Figure 4B). Immunoglobin G staining revealed also a significant reduction in BBB damage (24%) in the delayed IL-1Ra-treated group when compared with the placebo group (Figure 4C).

Interleukin-1Ra treatment did not improve functional outcomes in lean and Cp rats when administered at reperfusion, although a trend to improvement was observed in lean rats treated with IL-1Ra in both administration schemes (data not shown). It is important to note that due to the age and size of Cp rats, functional outcome measurements were difficult to perform.

Interleukin-1Ra Reduces Brain Inflammation, Microglial Activation, and Neutrophil Infiltration After Stroke in Comorbid Animals

Immunofluorescence revealed that aged Cp rats had increased neutrophil infiltration in the brain after stroke compared with lean rats, which was reduced by IL-1Ra (Figures 5A and 5B). Although IL-1Ra treatment caused a significant reduction in the number of MMP-9-positive neutrophils when compared with placebo (Figure 5D), it did not change the

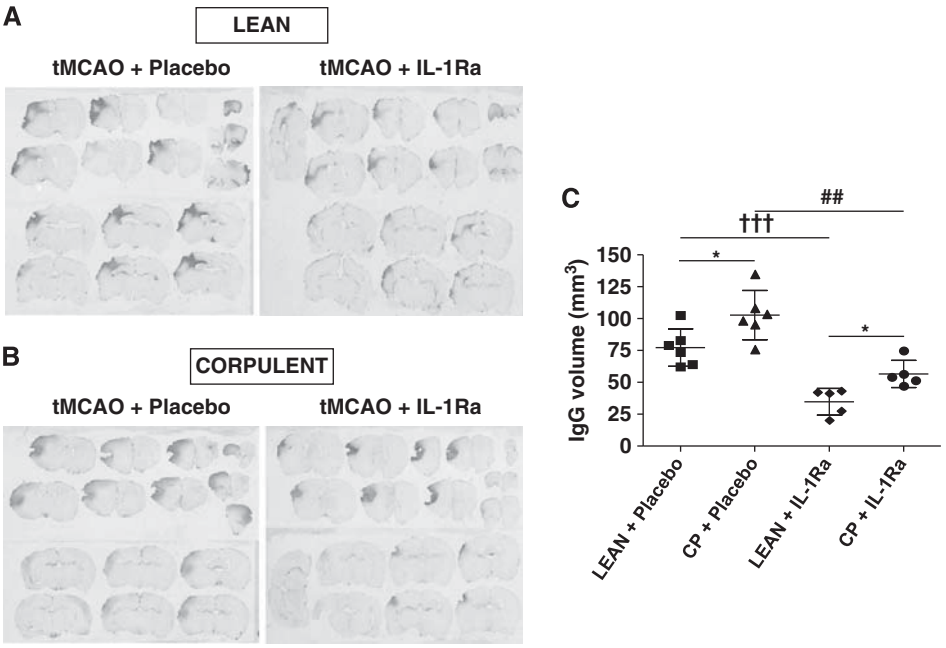

Figure 3 Effect of interleukin-1 receptor antagonist (IL-1Ra) on blood–brain barrier (BBB) damage after transient occlusion of the middle cerebral artery (tMCAO), administered at reperfusion. The volume of BBB damage was calculated on brain sections after immunoglobin G (IgG) staining 24 hours after tMCAO after administration of IL-1Ra/placebo to lean or corpulent (Cp) rats (**A**, **B**; $n = 6$). $^{*}P < 0.05$ lean versus Cp with placebo or IL-1Ra, two-way analysis of variance with Bonferroni correction; $^{\dagger\dagger\dagger}P < 0.001$ lean + placebo versus lean + IL-1Ra; $^{\#\#}P < 0.01$ Cp + placebo versus Cp + IL-1Ra, Student's *t*-test (**C**).

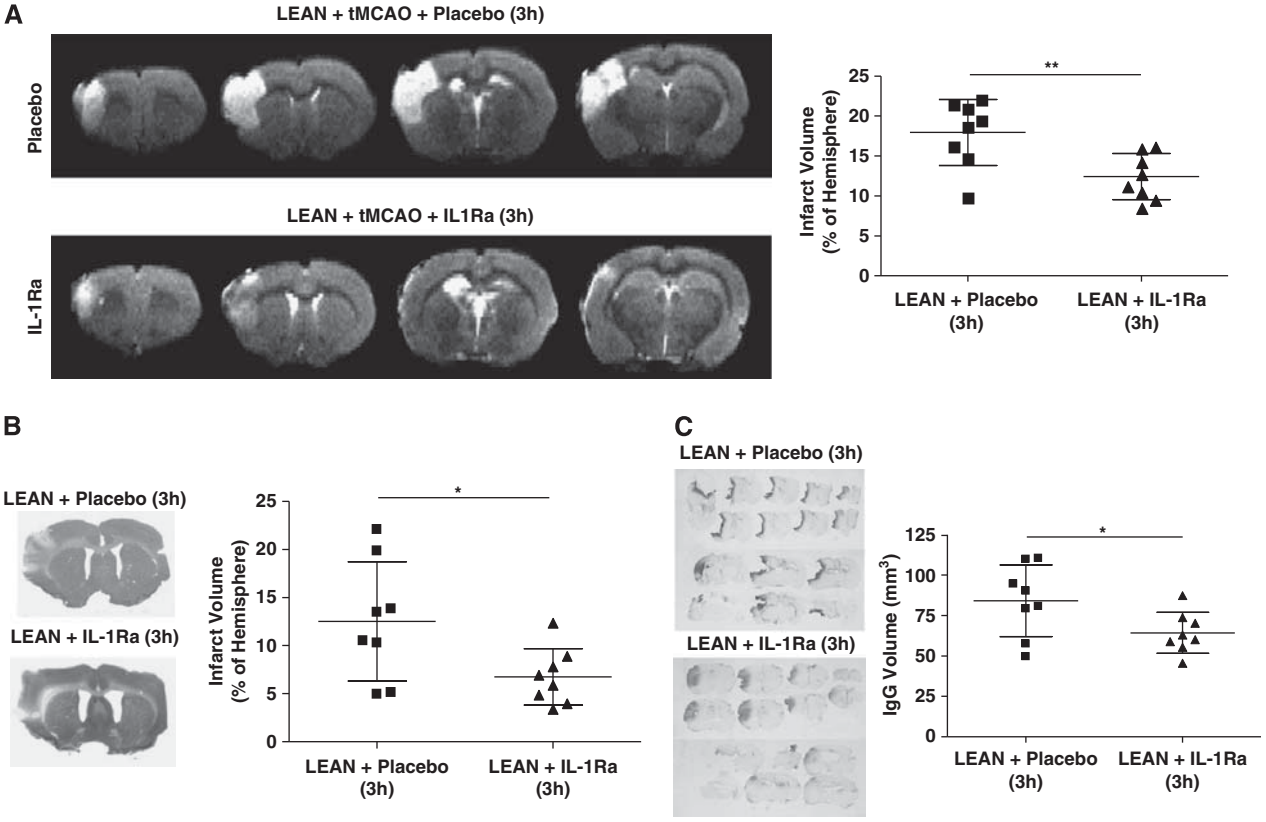

Figure 4 Effect of interleukin-1 receptor antagonist (IL-1Ra)/placebo on brain damage, administered at 3 hours of reperfusion to aged lean rats. Total infarct volume expressed as percentage of ipsilateral hemisphere and measured 23 hours after transient occlusion of the middle cerebral artery (tMCAO) on T_2W images and 24 hours in Nissl-stained sections (**A**, **B**; $n = 8$). Blood–brain barrier damage calculated on brain sections after immunoglobin G (IgG) staining 24 hours after tMCAO in aged leans treated with placebo or IL-1Ra (**C**; $n = 8$). $^{*}P < 0.05$; $^{**}P < 0.01$, Student's *t*-test.

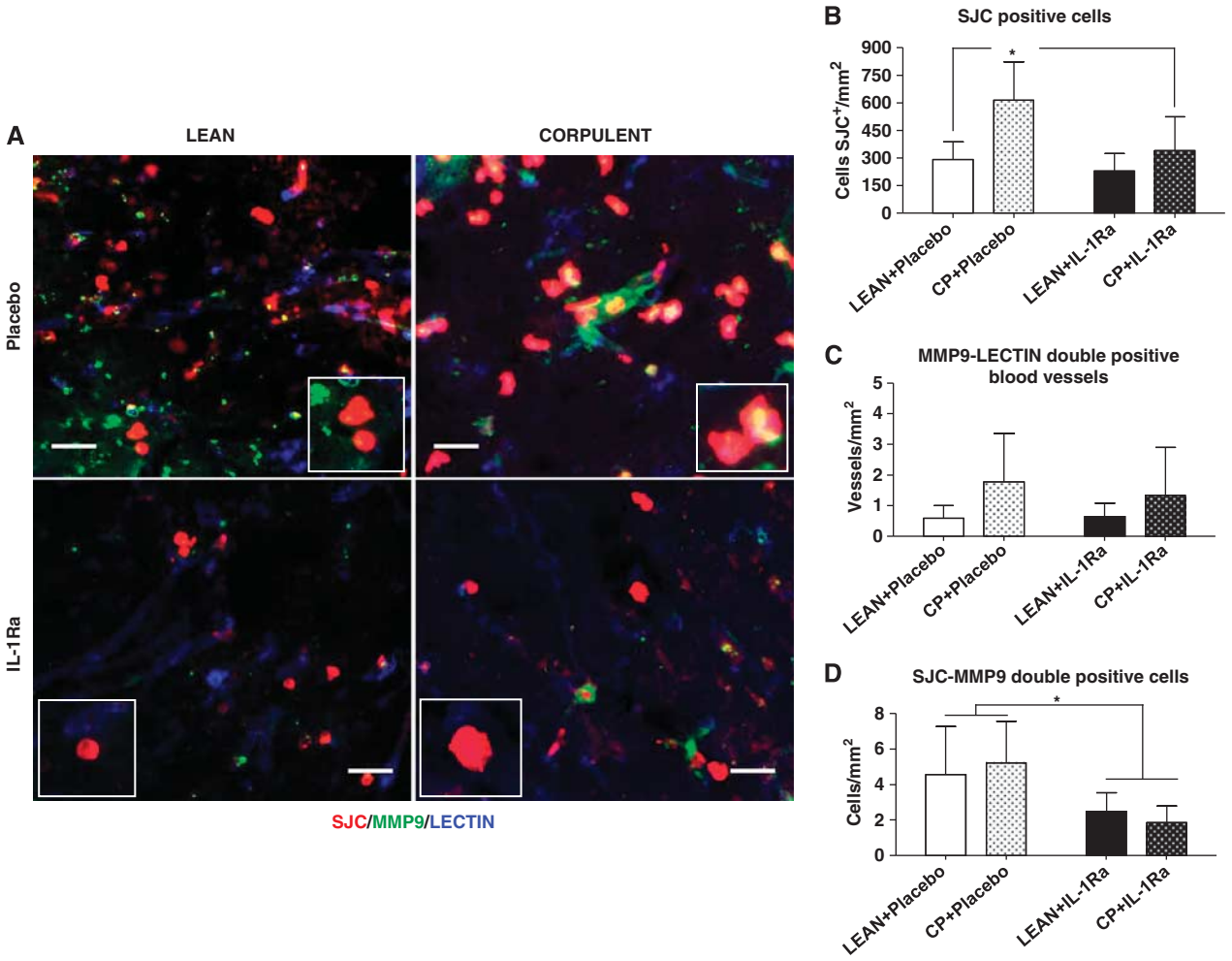

Figure 5 Effect of interleukin-1 receptor antagonist (IL-1Ra) on neutrophil infiltration and metalloproteinase-9 (MMP-9) expression in lean and corpulent (Cp) rats 24 hours after transient occlusion of the middle cerebral artery (tMCAO). (**A**, **B**) Number of neutrophils (SJC^+ (red)); (**A**, **C**) number of blood vessels positive for MMP-9 ($lectin^+$ (blue)/$MMP\text{-}9^+$ (green)-double-positive vessels); and (**A**, **D**) SJC/MMP-9-double-positive cells (neutrophils) in the infarct and peri-infarct of lean and Cp rats receiving placebo or IL-1Ra. $n = 6$/experimental group; $^*P < 0.05$, two-way analysis of variance with Bonferroni correction. Scale bar: 25 μm.

number of MMP-9-positive blood vessels in either leans or Cp (Figure 5C).

In both lean and Cp rats, IL-1Ra treatment resulted in a large (40%) reduction in microglial activation compared with the placebo-treated groups (Figure 6A). Immunofluorescence showed higher CXCL1 and IL-6 levels in the ipsilateral hemisphere in Cp than in lean rats. CXCL1 and IL-6 colocalized with Iba1-positive cells in the infarct and peri-infarct area and this immunoreactivity was reduced by IL-1Ra in Cp and lean animals (Figures 6A and 6B).

In 16-month-old lean rats, IL-1Ra administered at 3 hours of reperfusion significantly reduced the number of MMP-9-positive blood vessels and the number of MMP-9-positive neutrophils when compared with the placebo group, while neutrophil infiltration was unaffected (Figure 7A). Interleukin-1Ra also significantly reduced (22%) the number of activated Iba1-positive cells, although the number of Iba1-CXCL1-double-positive cells was not significantly decreased (Figure 7B). Delayed administration of IL-1Ra also reduced IL-6 expression in microglia (Figure 7C).

Discussion

This study shows that subcutaneous administration of IL-1Ra is protective in aged animals with multiple risk factors for stroke, a key requirement in the most recent STAIR guidelines (Fisher *et al*, 2009) for the development of clinical candidates for the treatment of stroke. More specifically, our results show for the first time, that peripheral administration of IL-1Ra at time of reperfusion or 3 hours delayed reduces the ischemic brain damage in aged lean and Cp rats, as measured by MRI (T_2W images), a clinically relevant primary outcome. The MRI outcome was confirmed by post-mortem histological analysis using Nissl staining, which is used widely in experimental stroke studies.

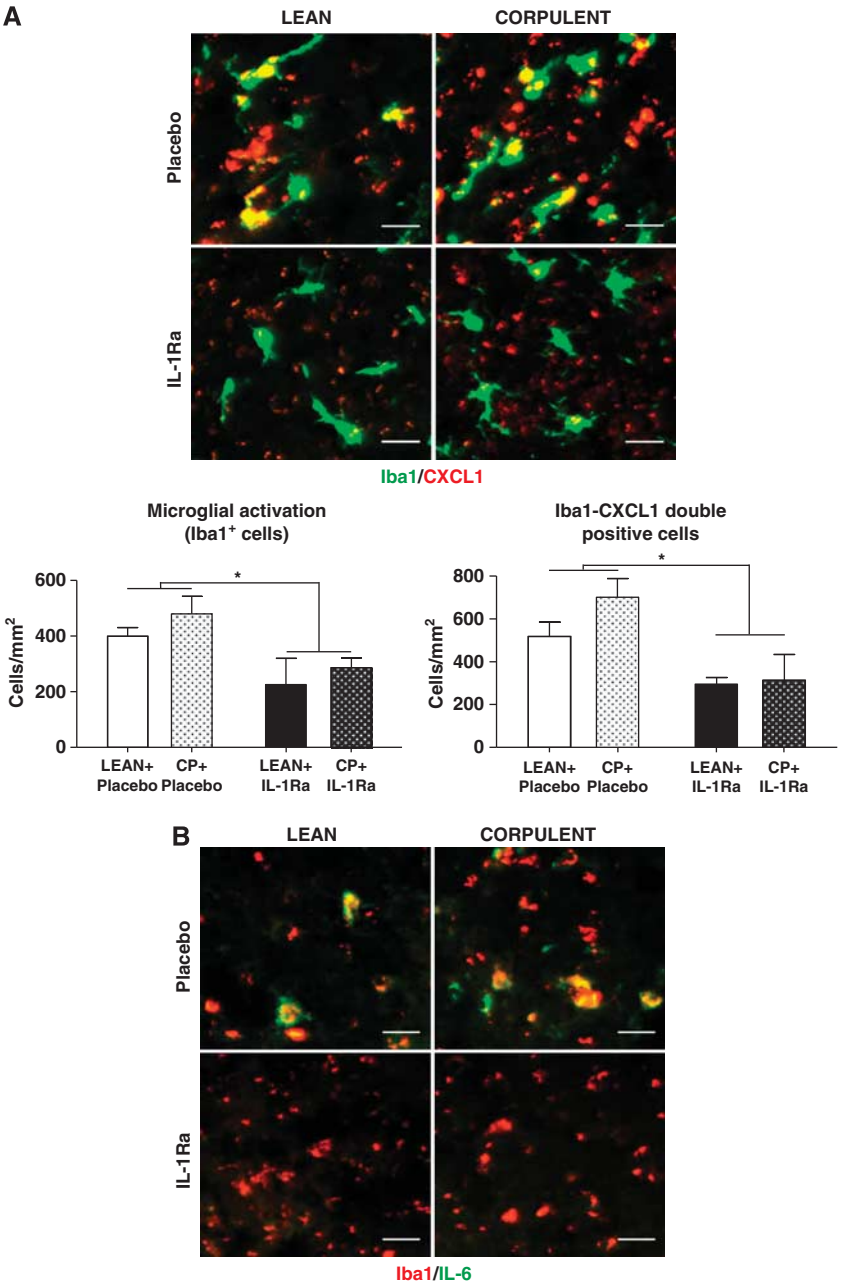

Figure 6 Effect of interleukin-1 receptor antagonist (IL-1Ra) in CXCL1 and IL-6 expression and colocalization with microglia in lean and corpulent (Cp) rats 24 hours after transient occlusion of the middle cerebral artery (tMCAO). (**A**) Activated microglia as identified by increased Iba1 immunopositivity (green), thickened processes and irregular cell bodies and number of Iba1 (green)/CXCL1 (red)-double-positive cells (CXCL1$^+$ microglial cells), and (**B**) microglial cells (Iba1$^+$, red) expressing IL-6 (IL-6$^+$, green) in the infarct and peri-infarct of lean and Cp rats. $n = 3$/experimental group; $^*P < 0.05$, two-way analysis of variance with Bonferroni correction. Scale bar: 25 μm.

Our data also show that although 15-month-old Cp rats have multiple risk factors for stroke and evidence of increased systemic inflammation, infarct volume was similar to lean animals of around the same age. This is in contrast to previously published work that reports increased ischemic injury with chronic systemic infection or obesity (Denes *et al*, 2010*a*, 2010*b*; McColl *et al*, 2009; McColl *et al*, 2010). However, in our study it is likely that the extent of ischemic damage is close to maximal due to the duration of MCAO, thus no further increase was possible (Ginsberg and Busto, 1989; Mohamed *et al*, 1985). This is supported by our own unpublished observation that an increase in infarct volume is seen in lean rats between the ages of 13 and 15 months, with no further increase between 15 and 16 months of age.

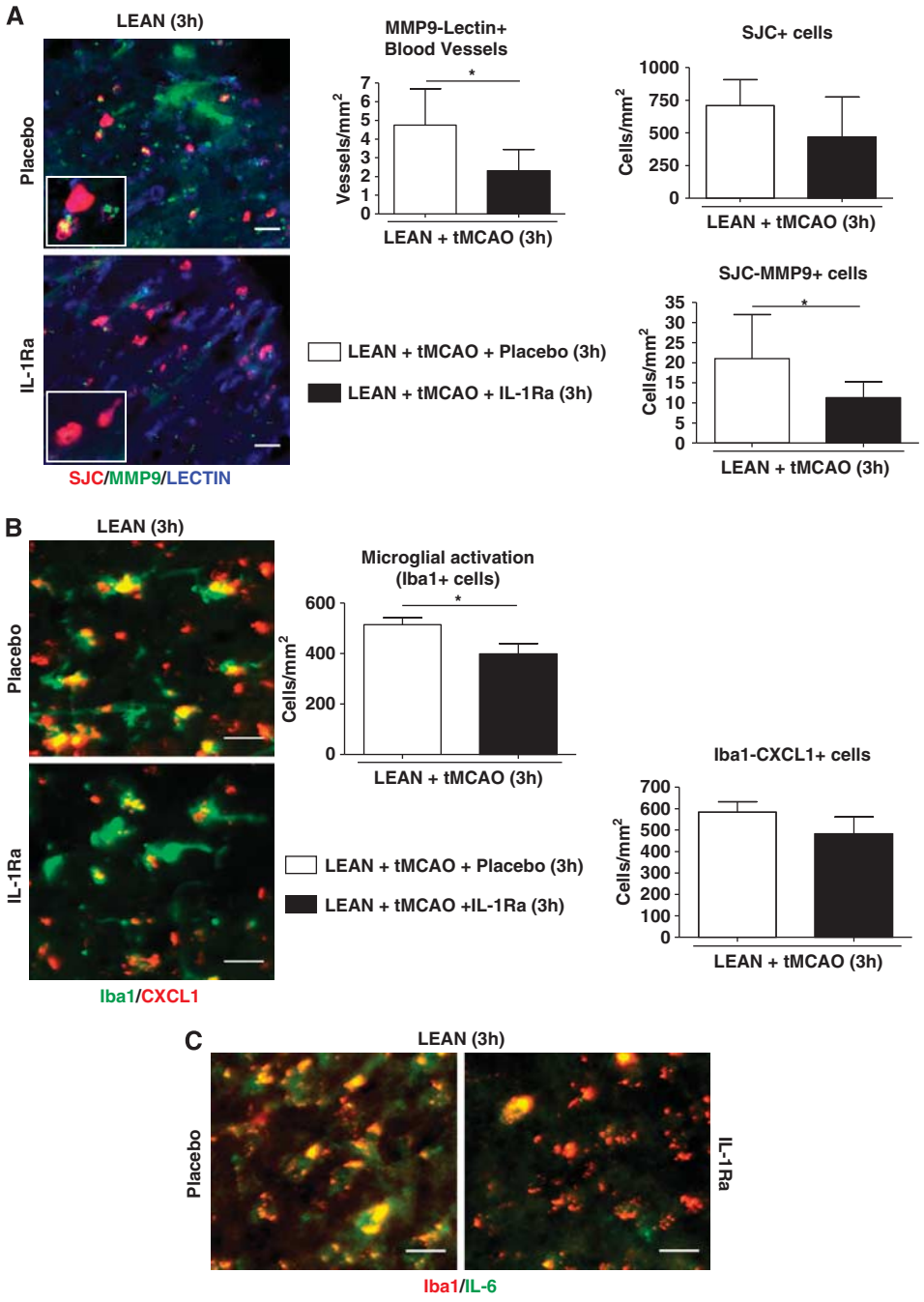

Figure 7 Effect of delayed interleukin-1 receptor antagonist (IL-1Ra) treatment (3 hours of reperfusion) on neuroinflammatory changes in aged lean rats 24 hours after transient occlusion of the middle cerebral artery (tMCAO). (**A**) Number of blood vessels positive for metalloproteinase-9 (MMP-9; lectin$^+$ (blue)/MMP-9$^+$ (green)-double-positive vessels), total number of neutrophils SJC$^+$ (red) and double SJC/MMP-9-positive cells (neutrophils) in aged lean rats treated with IL-1Ra or placebo. (**B**) Activated microglia identified by increased Iba1 immunopositivity (green), thickened processes and irregular cell bodies and double Iba1 (green)/CXCL1 (red)-positive cells (CXCL1$^+$ microglial cells), and (**C**) microglial cells expressing IL-6 (Iba1$^+$ (red)/IL-6$^+$ (green)) in the infarct and peri-infarct of aged lean rats receiving IL-1Ra or placebo. $n = 3$–8/experimental group; $^*P < 0.05$, Student's *t*-test. Scale bar: 25 μm.

It is well known that disruption of the BBB damage markedly influences the pathogenesis of stroke. Although no differences were observed in infarct volume between lean and Cp rats, the latter showed increased BBB damage after stroke. This difference in BBB damage may be explained by increased systemic and cerebrovascular inflammation in the presence of comorbidity (Drake *et al*, 2011). Indeed, peripheral IL-6 is implicated in atherosclerosis (Schuett *et al*, 2009), which, together with increased

neuroinflammation, may lead to alterations in vascular permeability before stroke and/or influence BBB breakdown after stroke. This is supported by our recent data indicating that an acute systemic inflammatory challenge alters the kinetics of BBB disruption after experimental stroke, an effect that is both neutrophil and MMP-9 dependent (McColl *et al*, 2007; McColl *et al*, 2008), and by previous studies confirming the role of MMP-9 in BBB disruption and hemorrhagic transformation experimentally and clinically (Castellanos *et al*, 2003; Gasche *et al*, 1999; McColl *et al*, 2010). An important role for neutrophils and MMP-9 in ischemic injury in the presence of systemic inflammation is confirmed in this study, more neutrophils being seen in Cp rats. Importantly, IL-1Ra treatment reduces the expression of IL-6 in microglia, reduces the increase in neutrophils number, and the expression of neutrophil associated MMP-9, which may explain the reduction in BBB breakdown with IL-1Ra in lean and Cp animals. Our results also show a reduction in the expression of vascular- and neutrophil-associated MMP-9 and a reduction in BBB damage with a delayed administration of IL-1Ra (3 hours of reperfusion) in older lean animals. Increases in neutrophil infiltration after stroke could be driven by microglial-derived IL-6 and CXCL1 (Chapman *et al*, 2009; McColl *et al*, 2007; Rodriguez-Yanez and Castillo, 2008), both of which are increased by systemic inflammation. This is also demonstrated by our results, where aged and comorbid animals show higher expression of microglial-associated IL-6 and CXCL1 after stroke, an effect again reversed centrally but not peripherally by IL-1Ra in this study.

In this study, functional (sensori-motor) outcome was assessed before stroke, and at a single acute time-point poststroke, using conventional scales. Despite IL-1Ra treatment having previously been shown to improve stroke outcome using other models of stroke (Garcia *et al*, 1995), no significant effect of IL-1Ra was observed on behavior in either lean or Cp rats using this approach. It is important to note that the age and size of the Cp rats determined the experimental model (distal MCA occlusion) used to induce transient ischemia in this study, an approach that only produced damage in the somatosensory cortex with no injury in the basal ganglia (Chen *et al*, 1986). Hence, functional deficits may be minimal and/or acute assessment may not be the optimal approach. Therefore, future studies in comorbid animals and in female and/or male animals when possible will determine longer-term effects of IL-1Ra, with delayed treatment and with an exhaustive assessment of the outcome.

In summary, our results show that IL-1Ra protects against brain injury and reduces neuroinflammation when administered peripherally to aged and comorbid animals at reperfusion or 3 hours later. These findings address concerns raised in a recent systematic review on IL-1Ra in stroke (Banwell *et al*, 2009), and provide further supporting evidence for IL-1Ra as a lead candidate for the treatment of ischemic stroke.

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Disclosure/conflict of interest

NJR is a non-executive director of AstraZeneca, but there was no involvement of the company in these studies.

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