RESEARCH ARTICLE

Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence [version 1; peer review: 1 approved, 1 approved with reservations]

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V1 First published: 10 Jul 2019, 8:1039
<https://doi.org/10.12688/f1000research.19308.1>

Latest published: 16 Oct 2019, 8:1039
<https://doi.org/10.12688/f1000research.19308.2>

Abstract

Background: Blockade of the cytokine interleukin-1 (IL-1) with IL-1 receptor antagonist (IL-1Ra) is a candidate treatment for stroke entering phase II/III trials, which acts by inhibiting harmful inflammatory responses. Infection is a common complication after stroke that significantly worsens outcome and is related to stroke-induced deficits in systemic immune function thought to be mediated by the sympathetic nervous system. Therefore, immunomodulatory treatments for stroke, such as IL-1Ra, carry a risk of aggravating stroke-associated infection. Our primary objective was to determine if factors associated with antibody-mediated antibacterial defences were further compromised in patients treated with IL-1Ra after stroke.

Methods: We assessed plasma concentrations of immunoglobulin isotypes and complement components in stroke patients treated with IL-1Ra or placebo and untreated non-stroke controls using multiplex protein assays. Activation of the sympathetic nervous system (SNS) was determined by measuring noradrenaline, a major SNS mediator.

Results: There were significantly lower plasma concentrations of IgM, IgA, IgG1 and IgG4 in stroke-patients compared to non-stroke controls, however there were no differences between stroke patients treated with placebo or IL-1Ra. Concentrations of complement components associated with the classical pathway were increased and those associated with the alternative pathways decreased in stroke patients, neither being affected by treatment with IL-1Ra.

Open Peer Review

Approval Status

	1	2
version 2 (revision) 16 Oct 2019		 view
version 1 10 Jul 2019	 view	 view

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Noradrenaline concentrations were increased after stroke in both placebo and IL-1Ra-treated stroke patients compared to non-stroke controls.

Conclusion: These data show treatment with IL-1Ra after stroke does not alter circulating immunoglobulin and complement concentrations and is therefore unlikely to further aggravate stroke-associated infection susceptibility through reduced availability of these key anti-microbial mediators.

Keywords

stroke, IL-1Ra, immunoglobulins, complement, infection

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Author roles: **McCulloch L:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; **Allan SM:** Formal Analysis, Writing – Review & Editing; **Emsley HC:** Resources; **Smith CJ:** Conceptualization, Formal Analysis, Writing – Review & Editing; **McColl BW:** Formal Analysis, Funding Acquisition, Project Administration, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was funded by grants from BBSRC [BB/J004332/1] and MRC [MR/L003384/1, MR/R001316/1] assigned to Dr Barry W. McColl and the University of Edinburgh Moray Endowment Fund assigned to Dr Laura McCulloch.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: McCulloch L, Allan SM, Emsley HC *et al.* **Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence [version 1; peer review: 1 approved, 1 approved with reservations]** F1000Research 2019, 8:1039 <https://doi.org/10.12688/f1000research.19308.1>

First published: 10 Jul 2019, 8:1039 <https://doi.org/10.12688/f1000research.19308.1>

Introduction

Blocking the actions of the inflammatory cytokine interleukin-1 (IL-1) using a highly selective IL-1 receptor antagonist (IL-1Ra) reduced injury and improved outcome in multiple experimental animal models of cerebral ischemia and is in ongoing clinical stroke trials¹⁻⁴. The inflammatory-modifying properties of IL-1Ra may confer protective effects to the brain after stroke; however, due to its potential for immunosuppression, it may also compromise systemic immune responses important for defence against infection. Systemic immune dysregulation is particularly important to consider in the context of stroke as patients are highly susceptible to infection, which likely involves roles for stroke-induced impairments in some immune functions⁵.

We have previously shown deficits in early antibody responses, particularly IgM, associated with innate-like B cells in both experimental animals and stroke patients, which may contribute to post-stroke infection susceptibility⁶. IL-1 β is reported to induce IgM production in innate-like B cells⁷; therefore, treatment with IL-1Ra may inhibit these important anti-microbial effects. We assessed whether markers associated with antibody-mediated antibacterial defences were compromised in patients treated with IL-1Ra after stroke. In summary, our data suggests treatment with IL-1Ra is unlikely to aggravate antibody-associated immune function deficits induced by stroke.

Methods

Standard protocol approvals, registrations, and patient consents

This study involved tertiary analysis of plasma samples taken from a randomised, placebo-controlled phase II trial originally designed to determine the safety and biological activity of intravenous (IV) IL-1Ra⁴. The online clinical trials registries ClinicalTrials.gov and ISRCTN went live online during the year 2000, at which time online trial registration was a relatively new recommendation. The original IV IL-1Ra trial was set-up in 2000 and commenced February 2001 and therefore this trial was not officially registered. Ethical approval for reanalysis of the samples was obtained through the Health Research Authority National Research and Ethics Service Committee (16/NW/0853).

Clinical trial protocol

Participants and study procedures. In brief, patients ≥ 18 years of age with a clinical diagnosis of stroke within six hours of stroke onset were eligible. Exclusion criteria included National Institutes of Health Stroke Scale (NIHSS) score of ≤ 4 , pre-stroke modified Rankin Scale (mRS) score of ≥ 4 or rapidly improving neurological deficit. Patients were randomly assigned to treatment with recombinant methionylated human IL-1Ra (n=17) or placebo (n=17) stratified by age (< 70 and ≥ 70 years), baseline stroke severity (NIHSS score 4–9, 10–20, ≥ 21) and time since stroke onset (< 4 or ≥ 4 h), but not by sex. IL-1Ra was initially administered as an IV loading dose of 100 mg over 60 seconds, followed by 72 hours of consecutive infusions at 2 mg/kg/h. Full patient baseline characteristics and stratification of groups are provided as *Extended data*⁸.

Non-stroke control patients (n=13) of a similar age range with no previous history of stroke or transient ischemic attack were also recruited. Control patients were living independently at home, free of infection and able to provide written, informed consent. Controls were matched to stroke patients (six to patients receiving IL-1Ra and seven to patients receiving placebo) on a basis of age (± 5 years), sex and degree of atherosclerosis, which was determined using a non-invasive assessment of either ankle-brachial pressure or carotid atherosclerosis using Doppler, as previously described⁹.

Blood sampling. Venous blood samples were collected prior to the initiation of treatment (admission), at the next 9 am time point (if admission was before 7 am or after 11 am), and then at 9 am at 24 hours, 2 days, 3 days, 4 days and at 5–7 days after stroke, into tubes containing a final concentration of 10 μ g/ml pyrogen-free heparin and wrapped in cool packs. Control patients were sampled at 9 am and also at matched patient admission time (two hours) if this was not between 7 am and 11 am. Samples were centrifuged one hour after collection at 2000 $\times g$ for 30 min at 4°C. Plasma was separated and frozen in aliquots at -70°C until further analysis.

Luminex analysis of immunoglobulins and complement components

Immunoglobulins and complement components were measured in plasma samples using MILLIPLEX[®] multiplex assays. Patient details were blinded from samples and coded samples were randomised across plates for analysis. The MILLIPLEX[®]_{MAP} Human Isotyping Magnetic Bead Panel- Isotyping Multiplex Assay (HGAMMAG-301K-06, Merck Millipore Corporation, Billerica, MA, USA) was used to measure IgG1, IgG2, IgG3, IgG4, IgA and IgM. MILLIPLEX[®]_{MAP} Human Complement Panel 1 (HCMP1MAG-19K, Merck Millipore Corporation) was used to measure C2, C4b, C5, C9, Mannose-binding lectin (MBL), Factor D (Adipsin) and Factor I. Many samples had concentrations of Factor D and Factor I below the detection range of the standard curve and so results for these analytes are not reported. MILLIPLEX[®]_{MAP} Human Complement Panel 2 (HCMP2MAG-19K, Merck Millipore Corporation) was used to measure C1q, C3, C3b/ iC3b, C4, Factor B, Properdin and Factor H. Samples were assayed as singlets and all samples, standards and quality controls were prepared in accordance with the manufacturer's instructions. Samples were incubated with beads on a plate for one hour (isotyping assay) or overnight (complement assays) at 4°C and washes carried out using a magnetic plate washer. Plates were analysed using a Magpix[™] Luminex[®] machine and Luminex xPonent[®] software version 4.2, with a sample volume of 50 μ l per well and a minimum of 50 events counted per sample.

Measurement of noradrenaline

Noradrenaline was measured in plasma samples using a Noradrenaline ELISA kit (BA E-5200; LDN[®], Nordhorn, Germany). Patient details were blinded from samples and coded samples were randomised across plates for analysis. Samples were assayed as singlets and all samples, standards and quality

controls were prepared in accordance with the manufacturer's instructions, where noradrenaline is extracted from plasma using a cis-diol-specific affinity gel, acylated, enzymatically converted and then measured by ELISA. Optical density at 450 nm was measured using an MRX microplate Reader (Dynatech Labs, Chantilly, VA).

Statistical analyses

All immunoglobulin and complement components were measured in µg/ml and the D'Agostino and Pearson omnibus test was used to determine Gaussian distribution of sample data. As data were non-normally distributed, sample values were log₁₀-transformed. As the precise kinetics of individual patient responses may vary, the maximal and minimal concentrations of each mediator in the first seven days after stroke were compared to non-stroke controls. Maximal and minimal concentrations from IL-1Ra-treated and placebo-treated stroke patients and non-stroke controls were compared by one-way ANOVA with Bonferroni correction. Noradrenaline concentrations were measured in ng/ml and the D'Agostino and Pearson omnibus test was used to confirm Gaussian distribution of sample data. Maximal and minimal noradrenaline concentration from IL-1Ra-treated and placebo-treated stroke patients and non-stroke controls were compared by one-way ANOVA with Bonferroni correction. Data analysis was performed using GraphPad Prism 6.0 statistical analysis software and for all experiments, values of $P \leq 0.05$ were accepted as statistically significant.

An earlier version of this article can be found on bioRxiv (<https://doi.org/10.1101/587881>).

Results

Plasma IgM concentration is reduced after stroke and is not affected by treatment with IL-1Ra

Immunoglobulin M (IgM) is the predominant immunoglobulin isotype associated with early B cell antibody responses to infection by innate-like B cells, which we have previously shown to be depleted after experimental stroke in mice^{6,10,11}. Lower minimum concentrations of IgM were measured after stroke in comparison to non-stroke controls, and no difference was found between placebo and IL-1Ra treated patients (Figure 1A)¹². Maximum IgM concentrations in the first seven days after stroke were also assessed and did not significantly differ in IL-1Ra or placebo treated patients in comparison to non-stroke controls (Supplementary Figure 1A, *Extended data*)⁸. This indicates that the reduced minimum IgM concentration measured over the first seven days reflects an actual reduction in circulating IgM in stroke patients and is not an artefact of increased variance in IgM concentration after stroke.

Plasma IgA, IgG1 and IgG4 concentrations are reduced after stroke and are not affected by treatment with IL-1Ra

Minimum IgG1 concentration was significantly reduced in both placebo-treated and IL-1Ra-treated stroke patients in comparison to non-stroke controls (Figure 1B)¹². Minimum IgG4 (Figure 1C) and IgA (Figure 1D) concentrations were significantly reduced in placebo-treated stroke patients only. However, there was no significant difference in these immunoglobulins

between placebo-treated and IL-1Ra-treated patients. Minimum concentrations of IgG2 (Figure 1E) and IgG3 (Figure 1F) were not significantly altered in IL-1Ra or placebo treated patients in comparison to non-stroke controls. Maximal circulating concentrations of all immunoglobulin isotypes measured in the first seven days after stroke were also compared to non-stroke controls and no significant differences were measured in any immunoglobulin isotypes (Supplementary Figure 1B-F, *Extended data*)⁸.

Concentrations of complement components are differentially affected by stroke and not affected by treatment with IL-1Ra

As complement components are directly associated with the antibacterial functions of immunoglobulins, we investigated stroke-induced changes in circulating complement components and if any changes observed were further influenced by treatment with IL-1Ra. Stroke induced a significant reduction in the minimum concentrations of C3b/iC3b (Figure 2A), C3 (Figure 2B), C4 (Figure 2C), Factor H (Figure 2D) and Properdin (Figure 2E) measured in the first seven days after stroke in both placebo and IL-1Ra treated patients in comparison to non-stroke controls¹². Maximum circulating concentrations of these complement components measured in the first seven days after stroke were also compared to non-stroke controls and no significant differences were seen (Supplementary Figure 2A-E, *Extended data*)⁸.

In contrast, stroke induced a significant increase in maximal circulating concentrations of C1q (Figure 3A), C5 (Figure 3D) and C9 (Figure 3E) in both IL-1Ra and placebo treated patients measured in the first seven days after stroke in comparison to non-stroke controls. Maximum concentrations of C2 (Figure 3B) and C4b (Figure 3C) were increased in placebo-treated patients only¹². However, no significant difference was apparent between placebo treated and IL-1Ra treated patients for these factors, suggesting IL-1Ra treatment exerts no effects additional to stroke. Minimum concentrations of these complement components measured in the first week after stroke were also compared to non-stroke controls and no significant differences were seen (Supplementary Figure 3A-E, *Extended data*)⁸.

Minimal and maximal levels of Factor B, MBL and C5a measured in the first week after stroke were also compared to non-stroke controls. Concentrations of Factor B (Figure 4A, B) and MBL (Figure 4C, D) were not significantly altered by stroke or by treatment with IL-1-Ra¹².

Plasma noradrenaline concentration is increased after stroke and is not affected by treatment with IL-1Ra

Splenic noradrenaline levels are increased after experimental stroke and may be toxic to IgM producing B cells⁶. Maximum noradrenaline concentration measured in the first seven days after stroke was increased in both placebo and IL-1Ra treated patients in comparison to non-stroke controls (Figure 5A)¹². Treatment with IL-1Ra had no additional effect on noradrenaline concentration when compared to placebo. Minimum noradrenaline concentration in the first seven days after stroke was also

Figure 1. Reduced plasma IgM, IgA, IgG1 and IgG4 after stroke is not affected by IL-1 receptor antagonist (IL-1Ra). (A) Minimum IgM concentration measured in the first seven days after stroke was lower in both placebo and IL-1Ra treated patients in comparison to healthy controls. Data show mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with Bonferroni correction. (B) Minimum concentration of IgG1 and measured in the first seven days after stroke was reduced in both placebo and IL-1Ra treated patients in comparison to healthy controls. Minimum IgG4 (C) and IgA (D) concentrations were reduced in placebo-treated stroke patients in comparison to healthy controls. There was no significant difference between placebo-treated and IL-1Ra-treated stroke patients. No significant difference in IgG2 (E) and IgG3 (F) concentration was detected between placebo-treated and IL-1Ra-treated stroke patients in comparison to healthy controls. Data show mean $\pm$ SD, * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with Bonferroni correction.

measured and was not significantly different to non-stroke controls (Supplementary Figure 4, *Extended data*) or affected by IL-1Ra treatment⁸.

Discussion

In this study, we assessed if markers associated with antibody-mediated antibacterial defences were compromised in patients treated with IL-1Ra after stroke. Plasma IgM, IgG1, IgG4 and IgA immunoglobulin concentrations were reduced after stroke and this was not further altered by treatment with IL-1Ra. Assessment of complement components indicated induction of the classical pathway of complement activation after stroke but inhibition of the alternative pathway without modulation by IL-1Ra. Plasma noradrenaline was increased after stroke and also not influenced by treatment with IL-1Ra. These data suggest

that treatment with IL-1Ra is unlikely to aggravate antibody-associated immune function deficits induced by stroke.

The IL-1 family of cytokines play a critical role in host defence to pathogens by signalling to a variety of host cells to induce downstream effects including, but not limited to, pro-inflammatory cytokine and chemokine production, immune cell recruitment and upregulation of vascular adhesion molecules^{13,14}. However, in conditions of sterile inflammation and tissue injury, such as stroke, these effects can aggravate primary tissue damage and impair injury repair mechanisms. Blocking IL-1 signalling has shown improved outcome in both experimental animal and patient stroke studies^{1,4,15}. However, the immunosuppressive effects of blocking IL-1 signalling after stroke may additionally inhibit systemic responses to infection, further

Figure 2. Treatment with IL-1 receptor antagonist (IL-1Ra) has no effect on complement components downregulated after stroke. Minimum concentrations of (A) C3b/ iC3b, (B) C3, (C) C4, (D) Factor H and (E) Properdin were measured in the first seven days after stroke were reduced in both placebo and IL-1Ra treated patients in comparison to healthy controls. Data show mean $\pm$ SD, * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with Bonferonni correction.

increasing the risk of infection in patients who are already immune compromised^{16,17}. Indeed, meta-analysis studies have shown an increased risk of serious infection in rheumatoid arthritis patients treated for prolonged periods with the IL-1 blocking drug anakinra¹⁶. However, as of yet this has not been observed in stroke patients, potentially reflecting differences in the duration of treatment. No statistically significant differences in infection incidence were seen between IL-1Ra and placebo treated patients in this study, with 5/17 IL-1Ra treated patients experiencing infection between admission and day seven and 4/17 infections in placebo treated patients. Consistent with this pattern, we have shown here that relatively short duration of treatment with IL-1Ra after acute stroke did not further affect stroke-induced changes to circulating immunoglobulin, complement or noradrenaline concentrations and is therefore unlikely to further compromise immune defence against infection through reducing the availability of these antibacterial mediators.

IL-1 cytokine family members are reported to have variable effects on B cell antibody production. IL-1 β was reported to be important for the rapid production of anti-bacterial IgM

by innate-like B cells important for early containment of infection prior to the induction of adaptive immune responses^{7,18}. This would suggest treatment with IL-1Ra after stroke could further compromise the early production of IgM in innate-like B cells which are already known to be reduced in number after stroke⁶. However, this effect of IL-1Ra on IgM concentrations was not seen. We know that experimental stroke results in a significant loss of many populations of B cells and associated IgM⁶; therefore, it is possible that the effects of the stroke itself on B cells overwhelm any additional effects of cytokines that could moderately enhance or inhibit immunoglobulin production. Furthermore, we do not know if remaining B cells are functionally impaired and therefore able to respond to IL-1 β signalling as they would under normal homeostatic conditions. We have previously reported that stroke is associated with reduced circulating IgM concentrations in comparison to non-stroke controls⁶, an effect reproduced here. Further studies will be required to determine if IgM, or any of the mediators assessed in this study, would be useful as biomarkers to determine which patients are likely to develop infection after stroke.

Figure 3. Treatment with IL-1 receptor antagonist (IL-1Ra) has no effect on complement components upregulated after stroke. Maximum concentrations of (A) C1q, (B) C2, (C) C4b, (D) C5 and (E) C9 were measured in the first seven days after stroke were increased in both placebo and IL-1Ra treated patients in comparison to healthy controls. Data show mean $\pm$ SD, * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with Bonferonni correction.

We have shown for the first time that circulating IgG1, IgG4 and IgA concentrations were reduced in the first seven days after stroke in comparison to non-stroke controls. This is in agreement with previous data showing that pan-IgG concentrations were reduced in patients after stroke, although subclasses of IgG were not assessed in that study and no reduction in IgA was found at the seven day time point assessed¹⁹. IgA is the most predominant immunoglobulin isotype at mucosal surfaces including the respiratory tract and is crucial for antibacterial protection at these sites²⁰. Given the early reduction of IgA in placebo-treated stroke patients, determining the effect of stroke on IgA-producing B cells at infection susceptible sites, such as the lung mucosa, could further elucidate if this has an important role in post-stroke infection susceptibility.

In contrast to the short half-life of IgA and IgM^{20–22}, the half-lives of IgG1 and IgG4 are reported to be 21 days and therefore, an early reduction in IgG concentration is not compatible with a lack of *de novo* production after stroke due to loss

of B cells²³. Previous studies have suggested that reduced total-IgG after stroke may be associated with increased loss or catabolism of IgG, which could account for reductions in concentration occurring more rapidly than its natural half-life²⁴. An alternative explanation could be that reduced IgG concentration is indicative of vascular risk factors and inflammatory changes preceding stroke that are associated with stroke risk. However, control patients in this study were matched for risk factors including their degree of atherosclerosis and would be expected to show similar changes to stroke patients if these were associated with risk factors. Understanding that the kinetics of an individual immunoglobulin subset changes both preceding and as a result of stroke, and their associations with post-stroke infections, could be invaluable in providing new therapeutic targets to reduce incidence of infection and improve outcome in patients.

The complement system has a crucial role in enhancing humoral immune defence and protecting from bacterial infection via interactions with both the innate and adaptive immune

Figure 4. Treatment with IL-1 receptor antagonist (IL-1Ra) has no additional effect on complement components unaffected by stroke. Minimal and maximum concentrations of (A, B) Factor B and (C, D) mannose-binding lectin (MBL) were measured in the first seven days after stroke were unchanged in both placebo and IL-1Ra treated patients in comparison to healthy controls. Data show mean $\pm$ SD, one-way ANOVA with Bonferonni correction.

Figure 5. Plasma noradrenaline concentration is increased after stroke and is not affected by treatment with IL-1 receptor antagonist (IL-1Ra). Maximal noradrenaline concentration measured in the first seven days after stroke was significantly higher in both placebo and IL-1Ra treated patients in comparison to controls. Data show mean $\pm$ SD * P <0.05; ** P <0.01; one-way ANOVA.

systems²⁵. As activation of complement is closely associated with efficient immunoglobulin-mediated clearance of pathogens, we determined whether these pathways were compromised by stroke. We have assessed, for the first time, individual concentrations of multiple complement components covering all pathways of complement activation after stroke. These exploratory data suggest there are no overall deficits in complement activation after stroke. Complement activation pathways converge at multiple points; however, their initial activation mechanisms are distinct. The classical complement pathway is activated when IgM or IgG immune complexes bind to C1 (composed of C1q, C1r and C1s)^{25,26}. Maximum circulating concentration of complement components associated with the classical and lectin pathways of activation, C1q, C2 and C4b, and end stage mediators common to all pathways, C5 and C9, were increased in the first seven days after stroke in comparison to non-stroke controls. As concentrations of MBL itself was not significantly altered by stroke, this suggests the classical complement pathway is specifically activated after stroke.

In contrast, the alternative pathway of complement activation is initiated by microbial cell surfaces and polysaccharide antigen and results in a cascade that generates C3^{25,26}. Complement components that were significantly downregulated after stroke, C3b/iC3b, C3, Factor H (fH) and Properdin, are more associated with the alternative pathway of complement activation, suggesting that the alternative pathway is suppressed. These data are in agreement with previous studies investigating systemic C-reactive protein, C3c and C4 complement concentrations in the serum of patients 24 h after ischemic stroke, which concluded that the classical pathway of complement activation was activated in the first 24 h after ischemic stroke, whereas C3c, associated with the alternative pathway, was reduced^{27,28}. The roles of individual pathways of complement activation in infection susceptibility after stroke remains to be determined, but these data suggest overall deficits in complement concentration are unlikely to contribute to reduced antibody-mediated clearance of pathogens that may occur after stroke, further supporting reduced circulating immunoglobulins as an important influence on infection susceptibility.

In this study, circulating noradrenaline concentrations measured in the first week after stroke were increased in comparison to non-stroke controls but were not influenced by treatment with IL-1Ra. This is in agreement with previous studies showing activation of the sympathetic nervous system in both stroke and subarachnoid haemorrhage patients that resulted in increased plasma noradrenaline concentrations that persisted up to 10 days^{29–31}. Our previous studies have shown that after experimental stroke, activation of the sympathetic nervous system and release of noradrenaline within the spleen is toxic to resident B cells and preventing noradrenaline signalling using the β -blocker propranolol prevented B cell and IgM loss and resulted in reduced infectious burden⁶. The cytokine IL-1 β is also increased in the spleen after stroke and is reported to activate

peripheral nerves, including the splenic nerve, and increase production of splenic noradrenaline^{32,33}. However, blockade of IL-1 β signalling did not alter circulating concentrations of noradrenaline after stroke.

In summary, we have shown that treatment with IL-1Ra after stroke does not affect circulating concentrations of immunoglobulins, complement components or noradrenaline and is therefore unlikely to further increase patient susceptibility to infection via pathways in which these mediators are key participants. This is in agreement with data from IL-1Ra Phase II trials, in which treatment of stroke patients with IL-1Ra did not aggravate incidence of infection^{4,12}. These data suggest that blocking IL-1 in a stroke context may not be concerning from the perspective of increasing infection risk in patients. Additionally, the reductions in circulating immunoglobulin concentrations detected after stroke in this study further support that antibody mediated immune defence may be an important therapeutic target to reduce the burden of infection after stroke.

Data availability

Underlying data

Edinburgh Data Share: Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence. <https://doi.org/10.7488/ds/2570>¹²

This project contains the following underlying data:

- Full noradrenaline data by patient and timepoint.xlsx (noradrenaline concentrations in ng/ml)
- Full complement data by patient and timepoint.xlsx (complement concentrations in μ g/ml)
- Full immunoglobulin data by patient and timepoint.xlsx (immunoglobulin concentrations in μ g/ml)
- **Figure 1A** dataset.pzf – **Figure 5A** dataset.pzf (raw data underlying **Figure 1–Figure 5** in GraphPad Prism format)
- **Figure 1** full data excel format.xlsx (minimum concentrations of immunoglobulins in μ g/ml)
- **Figure 2** full data excel format.xlsx (minimum concentrations of complement components C3b/iC3b, C3, C4, Factor H and Properdin in μ g/ml)
- **Figure 3** full data excel format.xlsx (maximum concentrations of complement components C1q, C2, C4b, C5 and C9 in μ g/ml)
- **Figure 4** full data excel format.xlsx (concentrations of complement components Factor B and MBL in μ g/ml)
- **Figure 5** full data excel format.xlsx (maximum concentrations of noradrenaline in ng/ml)

Extended data

Edinburgh Data Share: Supplementary Figures: Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence. <https://doi.org/10.7488/ds/2545>⁸

This project contains the following extended data:

- Supplementary Figure 1.tif (figure showing maximum concentrations of immunoglobulin)
- Supplementary Figure 2.tif (figure showing maximum concentrations of complement components C3b/iC3b, C3, C4, Factor H and Properdin)
- Supplementary Figure 3.tif (figure showing minimum concentrations of complement components C1q, C5, C9, C2 and C4b)
- Supplementary Figure 4.tif (figure showing minimum concentrations of noradrenaline)
- Supplementary Table 1.tif (table of baseline characteristics of stroke patients and stratification of groups)
- Data Supplementary Fig 1.xlsx - Data Supplementary Fig 4.xlsx (data underlying Supplementary Figures 1-4)

- Data Supplementary Fig 5.xlsx (Supplementary Table 1 in spreadsheet format)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Grant information

This work was funded by grants from BBSRC [BB/J004332/1] and MRC [MR/L003384/1, MR/R001316/1] assigned to Dr Barry W. McColl and the University of Edinburgh Moray Endowment Fund assigned to Dr Laura McCulloch.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank all the participating patients and controls for their participation and consent. We also thank Sharon Hulme for assistance with ethical applications and sample transfer. We thank Merck Millipore Corporation, Billerica, MA, USA for kind provision of the Milliplex[®] MAP immunoglobulin isotyping and complement panel kits used in this study.

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<https://doi.org/10.5256/f1000research.21167.r51311>

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Stefano Fumagalli

Department of Neuroscience, Mario Negri Institute for Pharmacological Research, IRCCS, Milan, Italy

McCulloch *et al.* addressed whether stroke patients treated with IL-1 receptor antagonist (IL-1Ra) had factors associated with antibody-mediated antibacterial defences compromised. Based on their results, the authors conclude that IL-1Ra does not alter circulating immunoglobulin and complement factors in patients' blood, thus the treatment is not expected to further aggravate stroke-associated infection.

The addressed issue is of pivotal importance for stroke patient treatment, especially for treatment protocols using anti-inflammatory agents. Post-treatment complications have contributed to the unsuccessful clinical translation of promising anti-inflammatory compounds that proved effective in experimental models.

In its present form, the work presents some weakness that the authors should tackle.

1. Data are presented as the minimum or maximum levels of immunoglobulins and complement factors observed in a 7 day-period after stroke. Blood was sampled at admission (prior to treatment) and at 1, 2, 3, 4 and 5-7 days after stroke. I think that, being so many sampling times available, and post-stroke inflammation quickly changing over time, a picture of the temporal changes in treated vs. non-treated patients would be very informative. As such, I suggest:
 - to add the information on when min (or max) concentrations were reached in each condition, comparing them;
 - to add a statistical analysis for repeated measures comparing IL-1Ra patients' sampling at each time after treatment vs. admission (prior to treatment).
2. Had patients received recanalization therapies? Since the temporal window of IL-1Ra for some patients is still within that of thrombolytic therapies (<4.5h), the authors should

explain why they did not receive them if so. From what I can understand, the specimens used come from a trial registered in 2000, thus likely explaining the use of the placebo in patients eligible for recanalization therapies according to present guidelines. I think that this needs clarification in the text.

3. There is a big difference in placebo vs. IL-1Ra treated patients in the frequency of haemorrhagic stroke (0% vs. 29.4%). Did the authors consider to correct for this potentially impacting difference?
4. The measure of complement components may be biased. Indeed blood specimens were collected in the presence of heparin, which does not represent the standard for running unbiased complement protein analysis. Complement proteins and activation products should be measured in blood sampled in the presence of EDTA (Bergseth *et al.*, 2013¹).
5. In complement system biology, the decrease of circulating levels of recognition molecules in acute phases is generally regarded as an index of complement activation (through consumption of the circulating proteins binding their targets). On later times, circulating levels also mirror increased synthesis and they may increase. This should be considered when discussing the observations relative to complement measures. Moreover, complement system activation in stroke patients is reported in the first 6h (Zangari *et al.*, 2016²), therefore a comparison of admission vs. later sampling in IL-1Ra treated patients would be informative (do they recover to control physiological complement protein levels as expected?).
6. There is now a general consensus that the lectin pathway of complement activation is that mainly involved after ischemic stroke. This has been reviewed in Orsini *et al.* (2014³) and Fumagalli and De Simoni (2016⁴). Besides MBL, the lectin pathway comprises other recognition molecules such as ficolins and collectin-11, that play a major role in ischemic patients (especially ficolin-1 and ficolin-3; refer to the above mentioned reviews). In this regard the conclusions drawn by the authors that: 'As concentration of MBL itself was not significantly altered by stroke, this suggests the classical complement activation is specifically activated after stroke' sounds incorrect. Of note, MBL concentrations are genetically determined and about 20% of the general population bears variants causing MBL deficiency (Garred *et al.*, 2006⁵). Indeed in figure 4C the authors show MBL levels below the detection limit. It is acknowledged that a circulating level of MBL < 500 ng/mL is associated with MBL genetic variants (Eisen *et al.*, 2008⁶). Thus it is not surprising that no MBL changes were observed. A more clear analysis could be done excluding the MBL-deficient patients. Interestingly, these latter patients develop less ischemic lesion volume and deficits than those with normal MBL circulating levels (Osthoff *et al.*, 2011⁷), thus further supporting lectin pathway critical contribution to stroke pathophysiology.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Stroke neuroimmunology, complement system

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 Oct 2019

Laura McCulloch, University of Edinburgh, Edinburgh, UK

We are pleased the reviewer recognises the relevance of this study to the future treatment of stroke patients and thank them for their insightful and constructive comments which we have addressed as detailed below. We hope that these changes meet with the reviewer's approval.

1. Data are presented as the minimum or maximum levels of immunoglobulins and complement factors observed in a 7 day-period after stroke. Blood was sampled at admission (prior to treatment) and at 1, 2, 3, 4 and 5-7 days after stroke. I think that, being so many sampling times available, and post-stroke inflammation quickly changing over time, a picture of the temporal changes in treated vs. non-treated patients would be very informative. As such, I suggest:

- to add the information on when min (or max) concentrations were reached in each condition, comparing them;**
- to add a statistical analysis for repeated measures comparing IL-1Ra patients' sampling at each time after treatment vs. admission (prior to treatment).**

We appreciate that there are limitations in analysing the data as the minimum or maximum concentration reached over a sampling time course. However variation in the kinetics of individual patient immune responses to stroke can be affected by many factors including stroke severity, presence of infection and timing of infection onset. In a small patient cohort as reported here (n=17), this variation can have a large impact at individual sampling time points. Assessing minimal or maximal concentrations over a relatively short period of time (e.g. 1 week) within which there are multiple sampling time points can enable discovery of temporal responses to stroke that are biologically meaningful but where the peaks/troughs may be occurring at slightly different time points in individual patients.

We do agree, however, that it is important to ensure more granular temporal patterns are not missed. To ensure this, we have analysed the time points at which minimal or maximal concentrations of mediators significantly changed by stroke were reached, as suggested by the reviewer. The percentage of patients reaching their minimum circulating concentration of immunoglobulins (**Supplementary Figure 5, Extended Data**) and minimum (**Supplementary Figure 6, Extended Data**) or maximum circulating concentration of complement components (**Supplementary Figure 7, Extended Data**) at each time point was compared in placebo and IL-1ra treated groups. These analyses have been added to the relevant results sections (Results, lines 187-190, 203-206, and 217-220). No differences in kinetics were apparent in IL-1Ra versus placebo treated groups further confirming the key findings that IL-1Ra has no significant additional effect on these mediators over and above the effects of stroke. This analysis has been described in the manuscript in Methods section under Statistical analyses section, lines 145-148.

We thank the reviewer for the suggestion to include some statistical analyses to account for the repeated sampling that occurred in individual patients. As there were missing samples in a few patients, a mixed effects model with Dunnett's post-test was used to analyse each sampling time point compared to admission in both IL-1Ra treated patients, as suggested by the reviewer, and placebo treated patients. As expected, due to the variance when

minimum and maximum concentrations were reached in individual patients, there were few significant changes at individual time points in comparison to admission (**Supplementary Figure 9-12, Extended data**). However, many immunoglobulin subsets were significantly reduced at d4 post stroke, and at some other sampling time points, in both IL-1Ra and placebo treated patients showing that the loss of immunoglobulins after stroke can still be detected in a small cohort with high variance in the kinetics of individual responses (**Supplementary Figure 8, Extended data**). This reanalysis has been acknowledged in the manuscript in Methods section, under statistical analyses. Again, there were no major differences in responses between IL-1ra and placebo treated patients, with the exception of IgM which showed significantly reduced concentrations at d4 In the IL-1Ra treated group but not in the placebo treated group. Overall, these analyses further confirm the overall conclusion that IL-1Ra does not exacerbate changes to circulating immune mediators induced by stroke.

2. Had patients received recanalization therapies? Since the temporal window of IL-1Ra for some patients is still within that of thrombolytic therapies (<4.5h), the authors should explain why they did not receive them if so. From what I can understand, the specimens used come from a trial registered in 2000, thus likely explaining the use of the placebo in patients eligible for recanalization therapies according to present guidelines. I think that this needs clarification in the text.

The samples used in this study were tertiary analysis of plasma samples taken from a randomised, placebo-controlled phase II trial originally designed to determine the safety and biological activity of intravenous (IV) IL-1Ra and recruitment for this study began in 2001, completing in 2003. tPA was not approved in the UK for treatment for patients <3 h from stroke onset until 2003 and for patients 3- 4.5 h from onset until 2012, therefore recanalization therapies were not an option for patients in this original study. We thank the reviewer for raising this important point and have provided details in the manuscript (Methods section Standard protocol approvals, registrations and patient consents lines 76-80).

3. There is a big difference in placebo vs. IL-1Ra treated patients in the frequency of haemorrhagic stroke (0% vs. 29.4%). Did the authors consider to correct for this potentially impacting difference?

Due to the blinding and randomisation of patient recruitment in the original study all haemorrhagic stroke patients were by chance allocated to the IL-1Ra treatment group. We agree with the reviewer that as little is known about the differential effects of haemorrhagic versus ischemic stroke on the immune system, the frequency of haemorrhagic strokes in the IL-1Ra treatment group may have the potential to bias the data. To address this, we identified and highlighted the data points that represent haemorrhagic stroke patients in each mediator analysed in the main figures. The data points representing the minimum circulating concentrations of immunoglobulins measured in the first week after stroke in haemorrhagic stroke patients were distributed throughout the range of concentrations measured in the IL-1Ra treated group suggesting that in this data set, the high proportion of haemorrhagic stroke patients in one treatment group did not skew the interpretation of these results (**Figure 1**). A similar distribution of haemorrhagic stroke patients was

apparent in the majority of complement components significantly reduced (**Figure 2**), increased (**Figure 3**) or unchanged (**Figure 4**) and in increased noradrenaline levels (**Figure 5**) measured in the first week after stroke. The exception to this was in the minimum concentrations of C3b/ iC3b and C3 (**Figure 2A and 2B**) where haemorrhagic stroke patient data points localised to the higher end in the range of measured minimum circulating concentrations within the IL-1Ra treated group. However, when data points from haemorrhagic stroke patients were excluded from the IL-1Ra treated data set and statistical analysis was performed, there were no significant differences in minimal circulating concentrations of C3b/ iC3b or C3 between the IL-1Ra and placebo treated groups. Indeed, reanalysis of all mediators excluding the haemorrhagic stroke patient data points did not alter the statistical results or conclusions drawn in the results section.

We have amended our discussion to acknowledge the greater frequency of haemorrhagic stroke patients within the IL-1ra treated patient group, highlight the reanalysis we have performed and confirm that future experiments designed to determine if differences exist between immune responses to ischemic versus haemorrhagic stroke are required to further our understanding of post stroke immune suppression (Discussion).

4. The measure of complement components may be biased. Indeed blood specimens were collected in the presence of heparin, which does not represent the standard for running unbiased complement protein analysis. Complement proteins and activation products should be measured in blood sampled in the presence of EDTA (Bergseth *et al.*, 2013¹).

The blood samples were collected in heparin containing tubes for the original study, whereas collection in EDTA would have provided optimal conditions for subsequent measurements of complement components^{1, 2}. Heparin is known to have an effect on baseline levels of complement components in serum as complement can spontaneously activate during the clotting process. However, plasma from blood samples collected in EDTA or heparin were reported to have similar baseline levels of complement components¹. There may be a minimal increase in absolute measures of complement concentrations in the samples used in this study due to original sample collection methods. However as all plasma samples were collected by this method, any artefact of the heparin collection should be comparable across all samples and therefore not alter our conclusions on the effects of stroke on complement components in the manuscript. We have acknowledged this caveat of the study in our discussion and thank the reviewer for this comment (Discussion).

5. In complement system biology, the decrease of circulating levels of recognition molecules in acute phases is generally regarded as an index of complement activation (through consumption of the circulating proteins binding their targets). On later times, circulating levels also mirror increased synthesis and they may increase. This should be considered when discussing the observations relative to complement measures. Moreover, complement system activation in stroke patients is reported in the first 6h (Zangari *et al.*, 2016²), therefore a comparison of admission vs. later sampling in IL-1Ra treated patients would be informative (do they recover to control physiological complement protein levels as expected?).

As reported in response to point 1 from the reviewer, individual patient trajectories were highly varied with patients reaching their maximal or minimal concentrations at various time points from admission to 5-7 d (*Extended data*, Supplementary Figures 7 and 8). Due to this variance, no individual time points after stroke showed significantly different concentrations of any complement component in comparison to control samples, including the later 5-7 d sampling time point. Our analysis has detected common changes to complement components after stroke across this time window. We agree with the reviewer our method limits the capacity to discuss these findings in terms of activation of complement pathways as we cannot associate these changes to a specific time post stroke or post infection. We have altered our discussion of the data to remove any speculation about activation or suppression of complement pathways and instead have reported the overall changes in concentration and their relationship to the different arms of the complement cascade only and have added information that reduced concentration of some complement mediators may reflect pathway activation through sequestration (Discussion). We have acknowledged that to determine if the changes to complement components were associated with increased early activation or increased synthesis of components, further studies would be required in a larger patient cohort with more stringent inclusion and exclusion criteria to account for the variance in the kinetics of individual patient responses (Discussion).

6. There is now a general consensus that the lectin pathway of complement activation is that mainly involved after ischemic stroke. This has been reviewed in Orsini et al. (20143) and Fumagalli and De Simoni (20164). Besides MBL, the lectin pathway comprises other recognition molecules such as ficolins and collectin-11, that play a major role in ischemic patients (especially ficolin-1 and ficolin-3; refer to the above mentioned reviews). In this regard the conclusions drawn by the authors that: 'As concentration of MBL itself was not significantly altered by stroke, this suggests the classical complement activation is specifically activated after stroke' sounds incorrect. Of note, MBL concentrations are genetically determined and about 20% of the general population bears variants causing MBL deficiency (Garred et al., 20065). Indeed in figure 4C the authors show MBL levels below the detection limit. It is acknowledged that a circulating level of MBL < 500 ng/mL is associated with MBL genetic variants (Eisen et al., 20086). Thus it is not surprising that no MBL changes were observed. A more clear analysis could be done excluding the MBL-deficient patients. Interestingly, these latter patients develop less ischemic lesion volume and deficits than those with normal MBL circulating levels (Osthoff et al., 20117), thus further supporting lectin pathway critical contribution to stroke pathophysiology.

We thank the reviewer for this insightful comment. In response to this we have reanalysed our data to determine which patients in the study did not reach a circulating level of >500 ng/ml at any sampling time point and excluded these patients from our analysis of MBL concentration. We identified two patients in each of the control, IL-1Ra and placebo groups that met this criteria and reanalysed our minimum and maximum circulating concentration of MBL with these patients excluded (**Figure 4**). Remaining data points on this graph that are <500 ng/ml were from patients whose minimum circulating MBL level fell to this value, but had read at >500 ng/ml at other sampling time points, preventing the possibility that they had a genetic MBL deficiency. With the exclusion of these patients there were still no

significant differences in maximum or minimum MBL concentration measured in the first week after stroke in placebo or IL-1Ra treated patients. The potential for these genetic variants to confound our analysis and the results of our reanalysis excluding these patients was added to the discussion. However as we have not measured other components specific to the lectin pathway of complement activation we have amended the manuscript to show that we cannot draw any conclusions on the effect of stroke on the lectin pathway from the data within this manuscript (Discussion).

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Competing Interests: No competing interests were disclosed.

Reviewer Report 22 July 2019

<https://doi.org/10.5256/f1000research.21167.r51097>

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María Ángeles Moro

Neurovascular Research Unit, Department of Pharmacology, Faculty of Medicine, Instituto de Investigación Hospital 12 de Octubre (i+12), Complutense University of Madrid, Madrid, Spain

The beneficial properties of IL-1ra have been widely demonstrated in numerous experimental models of cerebral ischemic injury. For this reason, the molecule is currently under evaluation in ongoing clinical trials. However, given the mechanism of action of IL-1ra, possible adverse effects of this drug and derived from its immunosuppressive action, such as the potentiation of stroke-related infections, might hamper its clinical use. In this context, the aim of the work by McCulloch *et al.* is highly relevant by trying to ascertain whether factors associated with enhanced susceptibility to bacterial infections are increased in IL-1ra-treated patients after stroke. The authors explored a wide array of very well selected parameters, ranging from Ig isotypes, components of the complement system, up to noradrenaline, as a parameter of the stress response. The results show that whereas these factors, as expected, are affected by stroke, none of them was affected by the treatment, clearly supporting that, at least with the protocol of administration used for this study, IL-1ra is safe as regards to immunosuppression-derived complications.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: stroke and vascular cognitive impairment

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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