



RESEARCH NOTE

REVISED IL-6 stimulates a concentration-dependent increase in MCP-1 in immortalised human brain endothelial cells [version 2; referees: 1 approved, 2 approved with reservations]

Jai Min Choi, Odunayo O. Rotimi, Simon J. O'Carroll, Louise F.B. Nicholson

Department of Anatomy and Medical Imaging and the Centre for Brain Research, Faculty of Medical and Health Sciences (FMHS), University of Auckland, Auckland, New Zealand

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Abstract

Systemic inflammation is associated with neurodegeneration, with elevated interleukin-6 (IL-6) in particular being correlated with an increased risk of dementia. The brain endothelial cells of the blood brain barrier (BBB) serve as the interface between the systemic circulation and the brain microenvironment and are therefore likely to be a key player in the development of neuropathology associated with systemic inflammation. Endothelial cells are known to require soluble IL-6 receptor (sIL-6R) in order to respond to IL-6, but studies in rat models have shown that this is not the case for brain endothelial cells and studies conducted in human cells are limited. Here we report for the first time that the human cerebral microvascular cell line, hCMVEC, uses the classical mL-6R signalling pathway in response to IL-6 in a concentration-dependent manner as measured by the production of monocyte chemotactic protein (MCP-1). This novel finding highlights a unique characteristic of human brain endothelial cells and that further investigation into the phenotype of this cell type is needed to elucidate the mechanisms of BBB pathology in inflammatory conditions.

Open Peer Review

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	Invited Referees		
	1	2	3
REVISED			
version 2	report	report	report
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11 May 2016	↑	↑	
version 1			
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03 Mar 2016			

- 1 **Robert B Petersen**, Case Western Reserve University USA
- 2 **Diego Gomez-Nicola**, The University of Southampton UK
- 3 **Mansi R. Khanna**, University of Pennsylvania USA

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Corresponding author: Louise F.B. Nicholson (lfb.nicholson@auckland.ac.nz)

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REVISED Amendments from Version 1

The authors have addressed the reviewers' comments by including, in the Discussion, further experiments suggested by the reviewers that would strengthen the preliminary findings presented in this Research Note. The authors are not in a position to undertake these experiments themselves but believe their preliminary findings reported in this Research Note are of interest and importance to the field. The authors are most grateful for the valuable feedback from the reviewers and have acknowledged this in the revised manuscript.

See referee reports

Abbreviations

AD: Alzheimer's disease; BBB: Blood brain barrier; BD: Becton Dickinson; BSA: Bovine serum albumin; CBA: Cytometric bead array; FACS: fluorescent activated cell sorting; FBS: Fetal bovine serum; gp130: Glycoprotein 130; hCMEC: Human cardiac microvascular endothelial cells; hCMVEC: Human cerebral microvascular endothelial cell; hFGF: human fibroblastic growth factor; HUVEC: Human umbilical vein endothelial cell; IL-6: Interleukin-6; IL-8: Interleukin-8; JAK: Janus kinase; M199: Mediium 199; MCP-1: Monocyte chemoattractant protein-1; mL-6R: Membrane bound interleukin-6 receptor; sIL-6r: Soluble interleukin-6 receptor; STAT: Signal transducers and activators of transcription; TNF α : Tumour necrosis factor alpha; VCAM-1: Vascular cell adhesion protein-1.

Introduction

IL-6 is a pleiotropic cytokine that exerts its effects by binding to an IL-6 receptor, that exists in both a soluble form (sIL-6R) and a membrane-bound form (mIL-6R), that then associates with the membrane-bound glycoprotein 130 (gp130) receptor to initiate intracellular signalling pathways including the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway¹. gp130 is expressed ubiquitously throughout the body whereas mIL-6R is only expressed in a few cell types including hepatocytes, monocytes, B cells and neutrophils. These cells are able to respond to circulating IL-6 in what is known as classical IL-6 signalling. Cell types such as endothelial cells that do not express mIL-6R are thus dependent on the trans signalling pathway, wherein neutrophils release sIL-6R². sIL-6R binds to free IL-6 to form a sIL-6R/IL-6 complex which can then interact with gp130 to initiate cell signalling pathways. This has been demonstrated in studies using human umbilical vein endothelial cells (HUVEC) where IL-6 alone did not elicit a response, while the sIL-6R/IL-6 complex induced the production of IL-6, interleukin-8 (IL-8) and monocyte chemoattractant protein (MCP-1)³. It appears that brain endothelial cells may however exhibit a different phenotype, with a response to IL-6 alone shown in primary cultures of rat brain endothelial cells^{4,5}. Such phenotypic differences are of interest as brain endothelial cells not only form a major component of the blood brain barrier (BBB), but elevated levels of circulating serum IL-6 have been correlated with increased risk of developing dementia⁶. Furthermore, a large meta-analysis of 40 cross-sectional studies found Alzheimer's disease patients had elevated serum levels of a number of pro-inflammatory cytokines including IL-6 when compared to healthy

control subjects⁷. Since sIL-6 trans-signalling is thought to be pro-inflammatory while the classic mIL-6 signalling is mainly anti-inflammatory¹, the nature of the response of brain endothelial cells to IL-6 is very important. We serendipitously found, in a related study, that human brain endothelial cells show a unique response to IL-6. Thus we explored the response of human cerebral microvascular endothelial cells (hCMVEC) following treatment with IL-6, by measuring the concentration of four pro-inflammatory cytokines (IL-6, IL-8, VCAM-1 and MCP-1), in order to better describe the phenotype of these human brain endothelial cells.

Materials and methods**hCMVEC cell culture and IL-6 treatment**

The human cerebral microvascular endothelial cell (hCMVEC) line was purchased from Applied Biological Materials, Inc. The hCMVEC cell line was cultured in M199 media supplemented with 10% fetal bovine serum (HyClone), 1 μ g/mL hydrocortisone (Sigma), 3ng/mL hFGF (PeproTech), 10ng/mL hEGF (PeproTech), 10 μ g/mL heparin (Sigma), 1% GlutaMAX (Gibco), 1% Pen-Strep (Sigma), and 80 μ M dibutyryl-cAMP (Sigma). All cell culture surfaces were coated with collagen I (Gibco) at a concentration of 1 μ g/cm².

For treatment, cells were plated in M199 medium supplemented with 2% fetal bovine serum, 110 nM hydrocortisone, 1% GlutaMAX, 1% Pen-Strep, and 80 μ M dibutyryl-cAMP for 3 days at which time the media was replaced with media supplemented with varying concentrations of IL-6 (PeproTech) (0.1ng/mL, 1ng/mL, 10ng/mL, and 100ng/mL). 100 μ L of media was collected at 72 hours post treatment from each well and centrifuged at 420 \times G for 10 minutes to remove any cellular debris. 80 μ L of the supernatant was stored at -80°C until needed.

Cytometric bead array (CBA)

Soluble IL-6, IL-8, VCAM-1 and MCP-1 were measured by multiplexed cytometric bead array (CBA; BD Biosciences, San Jose, CA, USA; see <http://www.bdbiosciences.com/documents/CBA>). These were selected as previous studies carried out in our laboratory⁸ show that they are highly inducible in the hCMVEC cells, whilst having different functions in the inflammatory response: proinflammatory cytokine (IL-6, IL-8), leukocyte adhesion (VCAM-1) and leukocyte recruitment (MCP-1). The CBA was conducted according to the manufacturer's instructions (except 25 μ L of conditioned media was used instead of 50 μ L)⁹. For each cytokine measured, a 10-point standard curve (1–5000 pg/ml) was included. CBA samples were analysed using a BD Accuri C6 Flow Cytometer (BD Biosciences). Data were analysed using FCAP Array software (version 3.0.1, BD Biosciences) which automatically converts the sample mean fluorescent intensity values to pg/ml concentration based on the standard curve.

Standard preparation

A master cocktail containing the proteins of interest (as supplied by BD Biosciences) at a concentration of 5000pg/mL in CBA assay diluent was prepared. 10 standard solutions of the following concentrations were then prepared using the standard diluent (BD Biosciences): 5000pg/mL, 2500pg/mL, 1250pg/mL, 625pg/mL, 312.5pg/mL, 158.25pg/mL, 78.125pg/mL, 39.0625pg/mL, 19.53125pg/mL and 0pg/mL.

Bead preparation and addition to samples/standards

The capture beads supplied with the kit for the proteins of interest (CBA Flex set numbers; IL-6, 558276; IL-8, 558277; sVCAM-1, 560427; MCP-1, 558287) were vortexed for 15 seconds and diluted 1:100 in bead diluent to make up the bead cocktail. 25µL of the bead cocktail was added to 25µL of sample/standard in a 96-well plate. The plate was mixed on a shaker at 500rpm for 5 minutes before incubating for 1 hour at room temperature. The detector cocktail was prepared in the same way as the bead cocktail, substituting the capture beads for detector antibodies (as per the BD manufacturer's protocol) and the bead diluent for detector diluent. 25µL of the detector cocktail was then added to the wells and the plate mixed as before and left to incubate at room temperature for 2 hours. After incubation was complete 200µL of wash buffer (BD Biosciences) was added to each well and the plate centrifuged at 1300rpm for 10 minutes at room temperature. The supernatant was discarded and the beads re-suspended in 50µL of wash buffer before being transferred into a tube for analysis.

Quantitative analysis

A BD Accuri C6 Flow Cytometer was used to measure the fluorescence of the samples/standards. Using the FCAP Array software version 3.0.1, (BD Biosciences), the mean fluorescent intensities of the standards were used to generate a standard curve for each protein of interest. The mean fluorescent intensities of the samples were measured against the standard curve to give the concentrations of the proteins in pg/mL. This data was exported to Microsoft Excel 2010 (Microsoft Corporation) for processing.

Statistical analysis

Student's t-test with two-tailed distribution and correction for two samples with unequal variances was carried out on data obtained from CBA using Microsoft Excel 2010. Results were deemed to be significant if the p-value was less than 0.05.

Results

Dataset 1. Raw data for effect of IL-6 on hCMVEC expression of inflammatory markers (Figure 1)

<http://dx.doi.org/10.5256/f1000research.8153.d114985>

File shows expression levels of IL-6, IL-8, VCAM-1, and MCP-1 with averages and standard deviations.

hCMVEC cells were shown to be responsive to IL-6 treatment alone (Figure 1). A clear concentration dependent effect was observed in MCP-1 release, with IL-6 concentrations as low as 1ng/mL producing a statistically significant increase in MCP-1 (Figure 1D). Such a response was not found for release of IL-6, IL-8 and VCAM-1 (Figure 1A–C, respectively). Interestingly, no difference was found in IL-6 levels between the media control and treated wells. We also observed that the BSA vehicle caused a decrease in VCAM-1 levels similar to that seen with 100ng/mL IL-6 treatment (Figure 1C). Given that the scale of release is so small (approximately 70pg/mL) it is difficult to reach a meaningful conclusion about the production of VCAM-1.

Discussion

To date, the effects of IL-6 on brain endothelial cells have been studied mostly in cells derived from rats, in which IL-6 was found

to stimulate eicosanoid production, increase permeability, and increase trans-cellular transport of the human immunodeficiency virus^{4,5,10}. This would suggest that rat brain endothelial cells are able to utilise the classical pathway of IL-6 signalling by binding directly to the membrane form of the receptor, mIL-6R. Additionally, *in vivo* studies in rats have shown that deletion of mIL-6R on brain endothelial cells attenuated the development of fever, further supporting the involvement of mIL-6R in pathological processes¹¹. However, such findings in animal models do not always translate into humans. Differences in the signalling pathways of IL-6 between humans and rats have been demonstrated in astrocytes. Human astrocytes were found to require the presence of sIL-6R in order to respond to IL-6 whereas rat astrocytes were able to respond to IL-6 alone¹². It is not yet known if similar differences exist between human and rat brain endothelial cells. There are few studies examining the response of human brain endothelial cells to IL-6, mostly focusing on permeability^{13,14}. Nor are there any available studies investigating the immune-modulatory effects of IL-6 in human brain endothelial cells. We have conclusively shown chemokine production in response to IL-6 alone in a human-derived brain endothelial cell line. We found that IL-6 treatment alone resulted in a concentration-dependent release of MCP-1 in hCMVEC cells but did not induce production of IL-6, IL-8 or VCAM-1. The fact that hCMVEC cells respond to IL-6 in the absence of the addition of sIL-6R suggests that hCMVEC cells may be expressing mIL-6R. Future studies confirming the presence of mIL-6R on the surface of hCMVEC cells (or demonstrating the absence of sIL-6R in the system) would add strength to this suggestion. That, notwithstanding, these preliminary results open the unexpected possibility that human brain endothelial cells may be able to utilise both the classical (mIL-6R-mediated) and trans (sIL-6R-mediated) pathways of IL-6 signalling through the presence of mIL-6R and the possible proteolytic cleavage or differential mRNA splicing to form sIL-6R. Furthermore, the lack of IL-6 and IL-8 induction in the hCMVEC response to IL-6 compared to the HUVEC response to the sIL-6/IL-6 complex³ suggests that signalling through mIL-6R and sIL-6R elicits different responses. A possible explanation for this can be found in hepatocytes, where the expression of gp130 is much higher than mIL-6R so that only a small proportion of gp130 receptors will be activated with IL-6 stimulation, whereas the sIL-6R/IL-6 complex can activate all gp130 receptors on the cell resulting in a much stronger effect¹. An investigation of the different inflammatory profiles, including pro versus anti-inflammatory events, following activation of either mIL-6R or sIL-6R in hCMVEC cells would be an excellent complementary study to that presented here. Our finding that hCMVEC cells show a concentration-dependent increase in MCP-1 in response to IL-6 is important, as MCP-1 has been shown to be involved in increasing BBB permeability in an *in vitro* co-culture model of rat brain endothelial cells and astrocytes¹⁵. The production of MCP-1 by brain endothelial cells helps to explain the effects of IL-6 on BBB permeability.

It is worth noting that the IL-6 concentration was similar between IL-6 treatment groups and control groups. One might expect that the higher concentration treatment groups would have a higher concentration of IL-6 since a greater amount of IL-6 was added. It would be interesting to know if the added IL-6 was internalised by the cells via receptor-mediated endocytosis, as is known to occur in hepatocytes¹⁶. While IL-6 is rapidly internalised, the

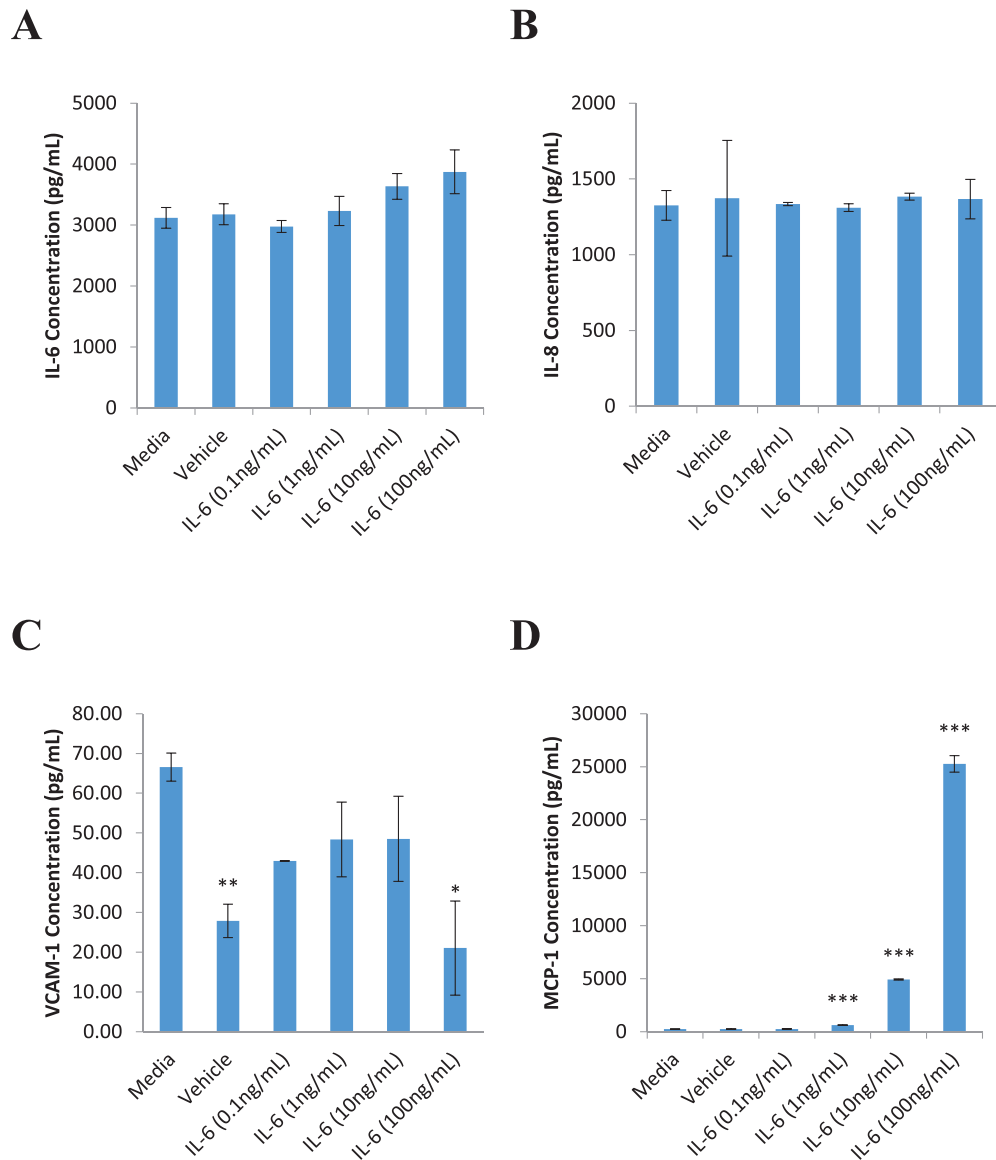


Figure 1. Effect of IL-6 on hCMVEC expression of inflammatory markers. hCMVEC cells were treated with varying concentrations of IL-6 for 72 hours before the media was collected for CBA analysis. The media was assayed for markers of endothelial inflammation IL-6 (A), IL-8 (B), VCAM-1 (C) and MCP-1 (D). Each graph represents the mean \pm SD (n=3). Statistical significance was evaluated using Student's t-test by comparison to the media control; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

sIL-6R/IL-6 complex undergoes very little internalisation prolonging its signalling capabilities, and adding to the differences between sIL-6R-mediated signalling and mL-6R-mediated signalling¹.

In BB19 cells, another immortalised brain endothelial cell line, VCAM-1 production is reported to be increased 2-fold compared to baseline after 8 hours of IL-6 stimulation. This then declines 24 hours post treatment¹⁷. While it is possible that we simply did not observe an increase in VCAM-1 production after IL-6 stimulation it had declined back to baseline by our 72 hour time point, our

72 hour time point was chosen specifically as an earlier study from our laboratory⁸ has shown that hCMVEC cells produce substantial amounts of many inflammatory markers at 72 hours post treatment with TNF α in comparison to 24 and 48 hours post-treatment. We also found that our BSA vehicle caused a significant decrease in VCAM-1. The amount of BSA in the vehicle treatment group is equivalent to that of the 100ng/mL IL-6 treatment group raising the possibility that the decreased VCAM-1 production with 100ng/mL IL-6 treatment might be due to the BSA. Although there is a study documenting the inhibitory effect of BSA on

TNF α -induced endothelial VCAM-1 production¹⁸, these authors used much higher concentrations of BSA and only measured the decrease in relation to the induced levels of VCAM-1, not basal levels. Despite the shortcomings of our study, the findings are significant and pave the way for future studies to further develop this research. For example, HUVEC could be included as a control, specific receptor blocking antibodies used to test the specificity of IL-6 supplementation and repeating this work using microvascular cells of a different origin (for example, human cardiac microvascular endothelial cells; hCMEC) would expand this study and thus provide further evidence supporting the proposal of a unique human brain endothelial cell phenotype.

Conclusion

There is a paucity of studies investigating the effects of IL-6 in human brain endothelial cells. Our study is the first to report findings suggesting that the human cerebral microvascular cell line, hCMVEC, responds to IL-6 in a way that it is unlikely to be through the soluble sIL-6R but instead uses the classical mL-6R signalling pathway in a concentration-dependent manner as measured by the production of monocyte chemoattractant protein (MCP-1). This is in contrast to previous studies in HUVEC cells that do not respond to IL-6 in the absence of sIL-6R. Thus there is no doubt that hCMVEC cells show a different immunoreactive phenotype to other endothelial cells. This highlights the heterogeneity in immunoreactive phenotypes among endothelial cell subtypes. The response of human brain endothelial cells to pro-inflammatory molecules should be

further explored to elucidate the mechanisms of BBB pathology in inflammatory diseases such as Alzheimer's disease.

Data availability

F1000Research: Dataset 1. Raw data for effect of IL-6 on hCMVEC expression of inflammatory markers (Figure 1), [10.5256/f1000research.8153.d114985](https://doi.org/10.5256/f1000research.8153.d114985)¹⁹

Author contributions

LN conceived the study. SO designed the experiments. OR and JC carried out the research. JC wrote the first draft of the manuscript. SO and LN contributed to the experimental design and all authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

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Acknowledgements

The authors wish to thank Dr Fabiana Kubke for her advice and extend their special thanks to the reviewers, acknowledging the valuable contributions made to the Discussion.

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19. Choi JM, Rotimi OO, O'Carroll SJ, *et al.*: **Dataset 1 in: IL-6 stimulates a concentration-dependent increase in MCP-1 in immortalised human brain endothelial cells.** *F1000Research.* 2016.
[Data Source](#)

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Current Referee Status:



Version 2

Referee Report 06 June 2016

doi:10.5256/f1000research.9413.r13974



Mansi R. Khanna

Center For Neurodegenerative Disease Research, University of Pennsylvania, Philadelphia, PA, USA

The authors have attempted to determine the phenotype of human brain endothelial cells in response to a pro-inflammatory cytokine IL-6. This study is of significance given the paucity of data in this regard. The data on the induction of MCP-1 in response to IL-6 are compelling. However, I have the following suggestions/questions to lend credibility to some of the claims the authors make:

1. Even though the authors have discussed future experiments to study the response of other pro- and anti-inflammatory events in the presence of IL-6, this is one of the short-comings of the study. They might want to consider adding some anti-inflammatory molecules to the repertoire. This will also build a case for the type of signaling mechanism in play, ie classical or trans IL-6 signaling.
2. In order to determine the effect of IL-6 alone on the secretion of IL-6, IL-8, VCAM-1 and MCP-1, statistics should be performed to compare all treatment groups to the vehicle. Currently, the effects reported are those of IL-6+vehicle (since comparison has been done to media control). This will be particularly helpful in reaching a meaningful conclusion on the VCAM-1 result.
3. The authors should cite the serendipitous study mentioned in the introduction. It sounds like it might provide some meaningful background for this piece of work.
4. The vehicle and its concentration must be mentioned in the materials and methods section.

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.

Referee Report 13 May 2016

doi:10.5256/f1000research.9413.r13788



Diego Gomez-Nicola

CNS Inflammation Group, Centre for Biological Sciences, Southampton General Hospital, The University of Southampton, Southampton, UK

The revised version does not include any of the suggested additional experiments. While I understand that my suggestion of performing a deeper analysis of the inflammatory response could go beyond the scope and format of this research note, I still consider that my first comment, focused to clarifying the mL6R is a reasonable ask.

The format of this communication would allow the publication of preliminary findings, but I think it is for the author's interest to provide a clear message with their findings, rather than a source of further questions.

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.

Referee Report 11 May 2016

doi:[10.5256/f1000research.9413.r13789](https://doi.org/10.5256/f1000research.9413.r13789)



Robert B Petersen

Department of Pathology, Case Western Reserve University, Cleveland, OH, USA

The modification of the discussion adequately addresses previous concerns.

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

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 14 April 2016

doi:[10.5256/f1000research.8769.r13376](https://doi.org/10.5256/f1000research.8769.r13376)



Diego Gomez-Nicola

CNS Inflammation Group, Centre for Biological Sciences, Southampton General Hospital, The University of Southampton, Southampton, UK

The reported observations are of interest, to understand immune to brain communication. Given the background of the experiments and the potential interest, I have two suggestions of further experimental work that would provide strong support to the authors claims:

1. As acknowledged by the authors, if IL6 alone can elicit a response, this would indicate the potential expression of mL-6R by hCMVECs. This needs to be confirmed, as its needed to understand the already reported data.

2. The authors comment on the different inflammatory profile caused by activation from mIL-6R or sIL6R. Seems like the current findings just scratch on the surface of that response, and further analysis of pro- vs anti-inflammatory mediators seems a sensible complement to this study.

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.

Author Response 18 Apr 2016

Louise Nicholson, University of Auckland, New Zealand

We thank the reviewer for the constructive feedback and are pleased that our observations add to an understanding of the immune to brain communication. We accept that these preliminary findings need to be explored further and agree that the two suggested experiments are very valuable indeed in confirming the expression of mIL-6R and the inflammatory profile resulting from the activation of mIL-6R or sIL-6R. We shall definitely include these recommendations in the discussion of our revised manuscript as a way of advancing these preliminary findings.

We selected the category of a 'Research note' for publication of our observations because they are preliminary and do indeed 'just scratch on the surface'. We believe however, that the observations are important and of value to the field paving the way for future studies as suggested by the reviewer. At this stage we are not in a position to undertake any further experiments ourselves but would be delighted if someone else picked up the challenge.

Competing Interests: No competing interests

Referee Report 11 March 2016

doi:10.5256/f1000research.8769.r12723



Robert B Petersen

Department of Pathology, Case Western Reserve University, Cleveland, OH, USA

This is an interesting preliminary study on the effect of IL6 on an immortalized human cerebral microvascular cell line. The authors show convincingly that addition of IL6, in the absence of exogenous sIL6R, induces production of MCP-1, a chemotactic protein. This induction is a subset of possible responses shown previously in response to IL1- β and TNF-alpha by the same group. The result also contrasts with an earlier report on the response of HUVEC cells in which the authors showed that the response required sIL6R (the cell culture conditions were quite different than the present study).

The report would be stronger if the authors had showed either the presence of mIL6R on the cell surface or demonstrated the absence of sIL6R in the system.

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

Competing Interests: No competing interests were disclosed.

Author Response 15 Mar 2016

Louise Nicholson, University of Auckland, New Zealand

Thank you for your very positive and constructive feedback. We shall definitely include a sentence or two in our manuscript acknowledging the benefits of future work to confirm the presence of mL-6R on the surface of our hCMVEC or the absence of sIL-6R in the system we have used.

Competing Interests: There are no competing interests
