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

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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 mIL-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.

Keywords
IL-6 stimulation, systemic inflammation, MCP-1, cell phenotype
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Competing interests: No competing interests were disclosed.

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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 Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. It appears that brain endothelial cells may how circulating IL-6 in which is known as classical IL-6 signalling. Cells types such as endothelial cells that do not express mIL-6R are thus dependent on the trans signalling pathway, wherein neutrophils release sIL-6R. 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 chemotactic protein (MCP-1). 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. 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 dementia. Furthermore, a large meta-analysis of 40 cross-sectional studies found Alzheimer’s disease patients who had elevated serum levels of a number of pro-inflammatory cytokines including IL-6 when compared to healthy control subjects. Since sIL-6 trans-signalling is thought to be pro-inflammatory while the classic mIL-6 signalling is mainly anti-inflammatory, 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), 1μg/mL hydrocortisone (Sigma), 3ng/mL hFGF (PeproTech), 10ng/mL hEGF (PeproTech), 10μg/mL heparin (Sigma), 1% GlutaMAX (Gibco), 1% Pen-Strep (Sigma), and 80μM dibutyryl-cAMP (Sigma). All cell culture surfaces were coated with collagen I (Gibco) at a concentration of 1μg/cm².

For treatment, cells were plated in M199 medium supplemented with 2% fetal bovine serum, 110 nM hydrocortisone, 1% GlutaMAX, 1% Pen-Strep, and 80μM 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). 100μL of media was collected at 72 hours post treatment from each well and centrifuged at 420 × G for 10 minutes to remove any cellular debris. 80μL 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 laboratory 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 5 μL of conditioned media was used instead of 50 μL). 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, 156.25pg/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. The bead diluent is supplied by BD Biosciences at a concentration of 5000pg/mL in 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.

Page 3 of 9
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

hCMVEC cells were shown to be responsive to IL-6 treatment alone (Figure 1). A clear concentration dependent effect was observed in

**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 ± 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.
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 1 A–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 virus1,5,10. 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 permeability13,14. 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. These preliminary results open the unexpected possibility that human brain endothelial cells may be able to utilise both the classical (sIL-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-6R/IL-6 complex1 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. 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 sIL-6R/IL-6 complex undergoes very little internalisation prolonging its signalling capabilities, and adding to the differences between sIL-6R-mediated signalling and mIL-6R-mediated signalling.

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 laboratory18 has shown that hCMVEC cells produce substantial amounts of many inflammatory markers at 72 hours post treatment with TNFα 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 TNFα-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 short comings 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 is unlikely to be through the soluble sIL-6R but instead uses the classical mIL-6R signalling pathway in a concentration-dependent manner as measured by the production of monocyte chemotactic 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 an 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.d11498519

**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 we 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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**References**


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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 mIL-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.

Competing Interests: No competing interests were disclosed.

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.

Author Response 18 Apr 2016

Louise Nicholson, University of Auckland, 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 sIL6R. 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

Reviewer Report 11 March 2016

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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-ß 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.

**Competing Interests:** No competing interests were disclosed.

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.

Author Response 15 Mar 2016

Louise Nicholson, University of Auckland, 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 mIL-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
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