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Systemic Inflammation Alters the Neuroinflammatory Response: A Prospective Clinical Trial in Traumatic Brain Injury.

Philipp Lassarén
Karolinska Institutet https://orcid.org/0000-0003-0833-1093

Caroline Lindblad
Karolinska Institutet

Arvid Frostell
Karolinska Institutet

Keri LH Carpenter
University of Cambridge

Mathew R Guilfoyle
University of Cambridge

Peter JA Hutchinson
University of Cambridge

Adel Helmy
University of Cambridge

Eric Peter Thelin (eric.thelin@ki.se)
Karolinska Institutet https://orcid.org/0000-0002-2338-4364

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Abstract

Background: Neuroinflammation following traumatic brain injury (TBI) has been shown to be associated with secondary injury development, however how systemic inflammatory mediators affect this is not fully understood. The aim of this study was to see how systemic inflammation affects markers of neuroinflammation, if this inflammatory response had a temporal correlation between compartments and how different compartments differ in cytokine composition.

Methods: TBI patients recruited to a previous randomized controlled trial studying the effects of the drug anakinra (Kineret®), a human recombinant interleukin-1 receptor antagonist (rhIL1ra), were used (n=10 treatment arm, n=10 control arm). Cytokine concentrations were measured in arterial and venous samples twice a day, as well as in microdialysis-extracted brain extracellular fluid (ECF) following pooling every 6 hours. C-reactive protein level (CRP), white blood cell count (WBC), temperature and confirmed systemic clinical infection were used as systemic markers of inflammation. Principal component analyses, linear mixed-effect models, cross-correlations and multiple factor analyses were used.

Results: The development of a systemic clinical infection results in an altered brain-ECF cytokine response (e.g. increase in G-CSF and decrease in PDGF-ABBB, $p<0.05$ respectively), even if adjusting for injury severity and demographic factors. rhIL1ra administration had a strong effect on the inflammatory response, independently altering different blood (n=6) and brain cytokine (n=3) levels. No substantial delayed temporal association between blood and brain compartments could be detected. Jugular and arterial blood held similar cytokine information content, but brain-ECF was markedly different. No clear arterial to jugular gradient could be seen.

Conclusions: Systemic inflammation, and infection in particular, alters cerebral cytokine levels, and rhIL1ra administration potently affects both systemic and cerebral cytokine levels. Cerebral inflammatory monitoring provides independent information from arterial and jugular samples, which both demonstrate similar information content. These findings could present potential new treatment options in severe TBI patients, and stresses the need of adequate monitoring of inflammatory markers.

Introduction

Traumatic brain injury (TBI) is a devastating condition, with an increasing morbidity and mortality [1, 2]. Following the primary impact, secondary injury mechanisms play a major role in patient deterioration [3], and of these pathologies neuroinflammation is acknowledged as being one of the main drivers [4]. However, the inflammatory response in the damaged brain probably has both detrimental and beneficial effects [5-9]. It serves an important role to clear up debris and to restore homeostasis following tissue injury, but may lead to an over-recruitment of pro-inflammatory mediators, as well as a shift of resident microglia to a more pro-inflammatory state, resulting in neurotoxic conditions and subsequent harm to surviving cells [10-12]. Moreover, the massive inflammatory tissue response seen in TBI also leads to a systemic inflammatory response [13], resulting in e.g. dysfunctional coagulation [14], haemolysis [15] and

a decreased capacity of the adaptive immune system [16]. In fact, patients with TBI have shown to present a peripheral shift in their immune capacity, sometimes referred to as a “TBI induced peripheral immune suppression” [17]. Animal studies support this shift away from an adaptive response by a strong activation of the complement cascade, resulting in secondary poor regulatory inflammation mechanisms, not being able to adapt to invading pathogens, which may lead to a greater susceptibility for infections [17-20]. Up to 50% of severe TBI patients have been suggested to suffer from infections during their hospital stay [21]. A recent study in mice exposed to *Streptococcus pneumoniae* and to either TBI or sham surgery, showed that brain-injured mice were unable to demonstrate an appropriate immune response due to impaired monocytic function in both acute and chronic stages of TBI which resulted in greater pulmonary bacterial loads [22]. In humans, ventilator associated pneumonia (VAP) is present in up to 36% of TBI cases and is associated with a longer hospital stay and increased morbidity [23].

Conversely, a systemic-to-brain inflammatory interaction has also been observed. In preclinical studies, mice systemically injected with endotoxin lipopolysaccharide (LPS), a common sepsis model, respond with subsequent neuroinflammation, primarily via an activated potent microglial response and increased concentrations of pro-inflammatory cytokines, specifically interleukin (IL)-1b, IL-6 and tumour necrosis factor alpha (TNF) [24-28]. An increased microglial activation has similarly been observed on positron emission tomography (PET) scans of LPS-injected non-human primates, and reactive astrocytes have been seen on immunohistochemistry [29]. When using a pre-clinical multiple sclerosis (MS) model, mice administrated with LPS have had an increased degree of axonal injury and a macrophage/microglial shift, suggesting that systemic inflammatory triggers could cause long-term neuroinflammation and injury, especially in susceptible individuals [30]. These systemic inflammatory stimuli usually result in impaired cognitive functions and behavioural changes [27], and are suggested to play an important role in the development in conditions such as long-term neuro-degeneration and psychiatric conditions following inflammatory exposure [31].

In humans, there are several studies describing the cytokine levels in central nervous system (CNS) infections, such as meningitis and encephalitis [32-34]. However, to the best of our knowledge, there are no studies specifically analysing the neuroinflammatory response to peripheral, systemic inflammatory conditions, i.e. to infections originating from outside the CNS, or to systemic inflammatory markers such as white blood cell count (WCC), C-reactive protein (CRP) and high temperature. Studies in relapsing remitting MS show that increased inflammatory markers (soluble intracellular adhesion molecule-1 (sICAM-1)) result in exacerbation of disease, presumably highlighting a distinct interplay between the systemic and cerebral inflammatory response [35].

Due to the inaccessibility of the human CNS, there are obvious logistical and ethical caveats concerning longitudinal sampling and monitoring. Commonly, cerebrospinal fluid (CSF) cytokine levels are used as surrogates for measuring cerebral inflammatory activity, including in TBI [36, 37]. In addition, the jugular blood has been suggested as a surrogate locale to measure neuroinflammation due to its proximity to the brain, as compared to arterial blood [38]. Another method for measuring brain CNS cytokine levels is microdialysis (MD) [39], whereby a catheter with a semi-permeable membrane is implanted in brain tissue

following conditions such as severe TBI where it enables monitoring of cerebral metabolism, assisting in guiding therapy and interventions [40].

Human recombinant IL-1 receptor antagonist (rhIL1ra), a potent immunomodulatory drug currently used for treating auto-immune conditions such as rheumatoid arthritis, has received a lot of interest in treating CNS conditions [41]. Our group has undertaken a phase II trial of rhIL1ra in TBI, where a panel of 42 cytokines were measured in brain microdialysate for five days following injury, noting that the subcutaneously administered drug reached the brain and shifted the cytokine response in the brain extracellular fluid [42]. A similar shift was also seen in the cytokine profile in arterial serum [43], with the CNS cytokine profile shift being time-dependent. Yet, it is still unknown to which extent the inflammatory state of the brain is affected following systemic inflammation by conditions such as severe infections. If it were established that a systemic inflammatory condition affected the brain, it could highlight a potential treatment avenue for improved management of acute CNS conditions, an area where there is a paucity of pharmaceutical options.

We aimed to use safety-data collected in the prospective rhIL1ra trial in order to study if systemic cytokine levels, as well as other markers of inflammation and infection, are associated with cerebral cytokine levels (from MD-retrieved extracellular fluid (ECF)). As secondary aims, we wished to study the temporal interaction to see if systemic inflammation precedes brain inflammation, or vice-versa. Additionally, we studied the jugular cytokine levels to see how they compare to arterial and cerebral levels.

Methods

Patient population and treatment

This patient population has been described in detail in the previous studies [42, 43]. In short, these 20 TBI patients (n=10 drug, n=10 no drug) with predominantly diffused injury were included in the randomized clinical trial to study the efficacy of rhIL1ra on cytokine profiles (REC# 06/Q0108/64). Ethical assent was collected from next of kin. Patients randomized to the treatment arm were administered 100 mg rhIL1ra (Anakinra, brand name Kineret®, Sobi, Stockholm, Sweden) once daily subcutaneously for five days. Apart from this, all patients received standard neuro-critical care, as described [44], for their TBI.

Microdialysis and blood sampling

A detailed description can be found in the original publication [42]. In short, brain ECF was acquired using a microdialysis catheter (CMA 71, 100 kDa molecular weight cutoff) perfused with 3.5% (w/v) Human Albumin Solution (Pharmacy Manufacturing Unit, Ipswich Hospital NHS Trust, Ipswich, UK) composed in central nervous system perfusion fluid. Microdialysate collection vials were changed hourly, and samples were pooled from a 6-hour epoch to allow sufficient volume to assay.

Blood samples were taken concurrently into EDTA tubes, from arterial and jugular venous catheters at 1 hour before and after rhIL1ra administration. The blood was immediately centrifuged at 15 minutes at 4,000 x g. at 4°C and the supernatant extracted and stored in -80°C until analysis. Plasma samples had sufficient volume for analysis without requiring dilution or pooling.

Clinical management and parameter definitions

C-reactive protein (CRP) and white blood cell count (WCC) were analysed simultaneously one hour before and one hour after the daily administration of anakinra or no drug using conventional hospital laboratory equipment at Addenbrooke's Hospital, Cambridge, UK. Core body temperature was measured at the same time using a nasopharyngeal temperature probe. Confirmed clinical infection was defined as an aggregate of clinical, laboratory parameters and cultures, and/or initiation of treatment, and commenced when the condition was defined. No patient suffered from a confirmed infection originating from the CNS.

Acquired clinical and demographic data included age, biological sex, motor score component of the Glasgow Coma Scale (GCSm) [45] on admission, Injury Severity Score (ISS) [46] and computerized tomography (CT) severity scoring assessed as per the Stockholm CT-score [47]. Deterioration on follow-up CT score was noted.

Cytokine assay

Samples were analysed using the Milliplex Multi-Analyte Profiling Human Cytokine/Chemokine 42 analyte premixed kit (Millipore, St Charles, MI, USA) using the manufacturer's instructions as described previously [42]; the 42 cytokines and chemokines assayed are detailed in Additional file 1. All samples were assayed in duplicate wells (25 mL per well) and the mean of the ensuing results was used. The plates were read using a Luminex 200 analyser (Luminex Corporation, Austin, TX, USA) using the STarStation software (Applied Cytometry Systems, Sheffield, UK). Cytokine concentrations were calculated by reference to an eight-point five-parameter logistic standard curve for each cytokine.

Statistical analysis

Statistical analysis was performed using R, version 3.6.2 and RStudio, version 1.3.1073 for Macintosh. The methods included principal component analysis and multiple factor analysis, performed using the package "FactoMineR" [48], cross-correlation analysis, performed using "ccf" from base R, and linear mixed-effects modelling, performed using the package "nlme" [49]. Other packages used include "messageR" [50], "ggplot2" [51] and "gplots" [52]. The full reproducible code is available in Additional file 2.

Linear mixed-effect models

In order to investigate whether systemic inflammation (arterial cytokine levels) is associated with cerebral inflammation (brain-ECF cytokine levels), we used linear mixed-effect models. For each cytokine, we performed a separate regression. The natural logarithms were taken of the cytokine levels in brain-ECF and blood. As fixed effects, we entered the cytokine level in arterial blood, CRP, WCC, temperature, confirmed clinical infection, rhIL1ra treatment, and time from TBI until measurement of these parameters (without any interaction terms). For each model, *p*-values were extracted; no correction for multiple testing was used as we had an unbiased approach and our scope was not to identify a particular cytokine of importance. Finally, the coefficients of the models were normalized using the quotient of their standard deviation and that of the dependent variable, and visualized as heatmaps, using the “massageR" and “gplots” packages. A similar analysis was performed predicting arterial cytokine levels, but here replacing the parameter “arterial cytokine” with the brain-ECF counterpart.

Blood and brain-ECF samples were not acquired simultaneously (Figure 1 in Helmy et al. 2014 [42]). Since the time variable primarily serves as a predictor of the brain-ECF cytokines and as a parameter in the correlation structure of the linear mixed-effect model, we used the correct brain-ECF sampling times and matched the blood sampling times in the analyses to these. As random effects, we had intercepts for patients, as well as by-patient random slope for the effect of time.

Similar models were then used to predict brain-ECF and arterial cytokine levels, with clinical and admission parameters (age, sex, GCSm, ISS and Stockholm CT score) as independent variables. The strongest signals, based on the number of significant cytokines in the linear models of both compartments, were then combined with inflammatory markers into a final analysis.

Further details can be found in the supplementary statistical text (Additional file 3).

Cross-correlations of inter-compartmental dynamics of cytokines

In order to investigate the direction of cytokine level changes between the different compartments, we performed cross-correlation analyses on all cytokines between their respective concentrations in brain-ECF and in arterial blood. Each time lag was approximately six hours; the timings of the blood samples were matched to the appropriate time interval during which the brain-ECF samples were drawn. We used the “ccf” function in R to find the cross-correlations and calculated the signed absolute maximum value for each cytokine and patient time series (plotted using the “ggplot2” package). An exclusion algorithm was employed in order to select robust cross-correlation series (Additional file 4).

Principal Component Analyses and Multiple Factor Analysis of cytokine compartments

In order to investigate whether the cytokines recovered from brain-ECF, and arterial and jugular venous plasma demonstrate different information, we conducted principal component analysis (PCA) and multiple factor analysis (MFA). The analysis was performed using the FactoMineR package in R, applying

default configurations of the PCA function, such as scaling the data to unit variance. Any timepoint with incomplete data was omitted. Furthermore, potential cytokine gradients between jugular and arterial cytokines were studied, using the cytokine levels, grouped by patient.

MFA is an extension to PCA, by which it is possible to analyse groups of variables [53]. In this case, an MFA was deployed on the three fluid compartments as groups. Default settings, including scaling of the data to unit variance, were used. Since every timepoint at which data were collected has at least one cytokine with a missing value, a complete case analysis could not be performed. Instead, missing values were exchanged with zeros.

Results

Patient demographics and missing analytes

The patients included in this study have been previously described in detail [42]. In summary, the twenty recruited patients (10 females, 10 males) had predominantly diffuse injury, all with a post-resuscitation GCS of less than 8 (traditionally considered a “severe” TBI). A relatively large variation can be observed between patients in trends of WCC, CRP and temperature during the first five days following the TBI (Additional file 1). Apart from a more substantial decrease in CRP in the intervention group compared to the control group, there were no significant differences between the groups in terms of inflammatory markers. Both treatment groups experienced four infections each requiring treatment. Five of these were reported as SAEs as per the pre-specified definitions [42]. Median levels of CRP, WCC, Temperature as well as admission CT scores and injury characteristics can be seen in Table 1.

Table 1
Patient characteristics.

Patient ID	White cell count	C-reactive protein	Temperature	Infection type	Severe adverse event	Stockholm CT score	Injury severity score
C01	12.4 (8.7–15.3)	94 (63–114)	37.07 (36.82–37.5)	-	-	1.5	45
C02	1.5 (0.6–3.4)	172 (37–250)	37.3 (35.83–37.88)	-	Yes	0.5	38
C03	14.85 (13.5–20.2)	147 (52–250)	36.92 (35.65–37.93)	VAP	-	1	45
C04	10.4 (8.4–15.7)	182 (103–250)	36.17 (33.07–37.75)	VAP	Yes	3	33
C05	21.5 (16.5–26)	216 (26–250)	36.47 (35.45–38.1)	VAP	-	2.5	38
C06	8.1 (5.7–16.7)	136.5 (45–143)	36.03 (34.72–37.15)	-	-	1.9*	38
C07	9.1 (6.8–18.3)	187 (5–250)	35.42 (35.02–37.72)	-	-	2	29
C08	7.1 (6.4–10.4)	131.5 (107–250)	37.19 (36.63–37.4)	-	-	3	36
C09	9.55 (8.2–10.7)	136.5 (47–194)	36.36 (34.48–37.72)	-	-	0.5	38
C10	4.7 (0.9–13.5)	250 (92–250)	33.21 (32.92–35.42)	IAS	Yes	4	30
I01	7.8 (7.8–9.8)	104.5 (48–186)	36.1 (35.88–36.65)	-	-	3	27
I02	12.35 (7.8–22.1)	74 (18–250)	36.92 (36.32–38.3)	CI	Yes	3	43
I03	5.6 (4.4–8.1)	181 (152–211)	35.37 (34.3–35.67)	-	-	2.4*	50

Patient ID	White cell count	C-reactive protein	Temperature	Infection type	Severe adverse event	Stockholm CT score	Injury severity score
I04	10.4 (7.2–16.1)	230 (162–250)	38.13 (37.17–39.68)	-	Yes	3	50
I05	14.3 (4.9–23.4)	238 (128–250)	35.48 (34.95–37.97)	VAP	Yes	1.5*	30
I06	11.45 (7.3–12.7)	102 (26–201)	37.28 (36.83–37.72)	VAP	Yes	3.8	27
I07	7.7 (5.8–14.2)	64 (38–150)	34.55 (33.12–37.02)	-	-	2*	45
I08	6.15 (4–8.3)	96.5 (31–204)	36.92 (36.57–37.17)	-	-	2	66
I09	11 (7–15.3)	113 (2–239)	37.47 (36.87–37.62)	VAP	-	1.5*	43
I10	7.15 (4.7–13.4)	76 (17–158)	36.58 (36.08–37.45)	-	-	3	27

Table 1 — legend: The first ten patients are control (C) patients and the other ten patients are intervention (I) patients receiving rhIL1ra treatment. White cell count, C-reactive protein (CRP) and temperature are displayed as median values, with maximum and minimum values in parentheses; their respective units are number of 10^9 cells per liter, µg/ml and °C. Infection types are ventilator-associated pneumonia (VAP), intraabdominal sepsis (IAS) and chest infection (CI). Stockholm CT score and injury severity score (ISS) are defined as in [47] and [54], respectively. CT scores with an asterisk indicate a deterioration.

On average, cytokines measured in arterial and venous jugular plasma, and brain-ECF compartments, had missing rates of 18.5 %, 30.9 % and 26.3 %, respectively (Additional file 1). There were no apparent differences in distribution of missing data between the two treatment groups, compartments or patients, but there was a difference in missing rate (frequency of absence) between different cytokines. This indicates that missingness might not be random, but presumably related to the Luminex assay's capabilities to measure specific cytokines (such as IL-4, which is known from previous studies [55]). The missingness of IL-4 in microdialysates is unlikely to be due to poor relative recovery, as previous studies in vitro have shown IL-4 to be well-recovered (57%) using the same type of 100 kDa microdialysis catheter used in the present study [39]. Patient C04 had no cytokine measured in jugular blood due to clotting of the catheter.

Markers of systemic inflammation affect brain cytokine levels

Among the systemic inflammatory markers, confirmed systemic clinical infections resulted in the largest relative effects on brain-ECF levels. Infections increased brain-ECF levels of e.g. IL-1b and G-CSF, and decreased levels of e.g. IL-1ra and PDGF-ABBB (Fig. 1A and Additional file 5). Considering that the variance inflation factor (VIF) was low (Additional file 3), the overall collinearity between the variables was also considered low. Therefore, systemic infection is key for altering brain-ECF cytokines. However, “WCC” has ten while “infection” only has seven significant cytokines, but there is a larger variation among cytokines for infection than for WCC. There were several cytokines with distinct temporal patterns in this cohort, as was previously highlighted [43], with a majority decreasing over time in the brain (Fig. 1A). All non-normalized coefficients are available in Additional file 5.

rhIL1ra treatment resulted in significant alterations of both the systemic and neuroinflammatory responses (Fig. 1A). In brain-ECF, EGF, IFN-γ, IL-9 and MDC were statistically significant, all of which were associated with lower cerebral cytokine levels for the treatment group. Overall, this is consistent with previously published results from Helmy et al. 2016 which used a different statistical approach (Partial Least Squares-Discriminant Analysis, PLS-DA), in part validating our model and findings [43].

High WCC and temperature generally showed associations to increased cerebral levels of cytokines, with stronger associations for WCC (Fig. 1A). Levels of CRP was generally showing weaker normalized coefficients, and the clustering was most associated with arterial cytokine levels. Strikingly, increases in arterial cytokines were not necessarily associated with brain-ECF cytokine levels; IL-4, TNFa and EGF had negative normalized coefficients, highlighting the difference between the two compartments.

Using a similar approach to see which parameters affect blood cytokine levels, confirmed systemic infections seemed to have the strongest positive association with several cytokines in blood, notably IL-6, IL-8 and IL-10 (Fig. 1B and Additional file 5). On a group level, CRP was more associated with increased blood cytokine levels than brain cytokine levels. Time from study inclusion also seemed to affect blood cytokine levels, but not as much as brain-ECF levels, and not as many cytokines decreased in blood over time as in the brain (Fig. 1B). Similar to the brain-ECF levels, rhIL1ra treatment resulted in a distinct separation between cytokines that were altered in blood, though these were not the same as those affected in the cerebral compartment. Altogether, these results highlight marked differences in the down-stream cytokine response to inflammatory stimuli in brain compared to its systemic counterparts.

The systemic effect on brain-ECF levels remain following adjustment of baseline and injury severity parameters

Similar mixed-models were used to illustrate cytokine patterns using demographic and injury severity parameters. Interestingly, these quite strongly affected both brain-ECF and blood cytokine levels (Fig. 2A-B and Additional file 5). An increasing age was associated with increasing cytokine levels in brain-ECF, while lower levels in blood (Fig. 2A-B). Similarly, female sex was associated with higher levels (thus, males had lower levels) of cytokines in brain-ECF while the inverse was shown for blood cytokine levels where female sex was associated with a decrease for a majority of cytokines (Fig. 2A-B). Stockholm CT score, ISS and GCSm showed relatively similar cytokine trends, clustering in proximity (Fig. 2A-B).

When combining demographic- and injury severity parameters with “clinical infection” and “rhIL1ra treatment”, there were still five and two cytokines, respectively, that were significantly altered by these inflammation markers (Fig. 3A-B and Additional file 5). Since most patients with infection were female (7/8), an interaction term between sex and infection was added to this model. In brain-ECF, alterations of PDGF-ABBB and G-CSF kept their associations to infections also when adjusting for baseline demographic and injury severity parameters (Fig. 3A). Interactions were not significant in any of the cytokines with significant coefficients for the impact of infection, and were significant only in three cytokines with significant coefficients for the impact of sex (Fig. 3A). In blood, clinical infections now showed a significant increase in fourteen cytokines (Fig. 3B), more than any other variable. rhIL1ra treatment showed similar trends following these types of adjustment for both brain and blood cytokines (Fig. 3A-B).

No temporal trends between compartments could be visualized

In order to investigate the direction of cytokine level changes between the different compartments, we performed cross-correlation analyses on all cytokines between their levels in brain-ECF and in arterial blood. Figure 4 depicts a fairly even distribution of cytokines in the four quadrants of the signed absolute maximum mean cross-correlation plot. The points are distributed such that there is a global symmetry around lag zero for both positively and negatively cross-correlated cytokine and patient series. This indicates that there is no observed global trend in neither directionality in movement between the blood and brain-ECF compartments nor in relative concentration changes between the two compartments.

Although there might not be an average global trend for all 42 cytokines in the 20 patients included in this study, individual cytokines or patients could have distinct patterns. In an attempt to address this hypothesis, we visually examined plots for every cytokine, as well as plots for every patient, annotated with the same parameters. Generally, we find a similar picture when visualizing individual cytokines as seen in Fig. 4 when all cytokines were combined (Additional file 2: Fig. 4 code). While no individual cytokine showed any significant delayed temporal association, notable cytokines include eotaxin, IFN-γ and MIP-1a which trended from brain to blood, while others showed a predominant delayed temporal association from blood to brain, such as IL-1α and IL-6 and the anticipated IL1ra which was given systemically. When looking at individual patients, there were some patients leaning toward a brain-to-blood pattern and others oppositely, but there seems to be no clear pattern in regard to the inflammatory parameters studied (Additional file 2: Fig. 4 code).

Arterial and jugular compartments provide similar information

For each cytokine, a variables factor map was plotted (Additional file 2: Fig. 5A code) and visually analysed. Most cytokines exhibit a similar pattern: the axes corresponding to arterial and venous cytokine concentrations are adjacent to each other and near the first principal component axis, while the brain-ECF axis is approximately orthogonal to these and near the second principal component axis. Consequently,

variations seen in arterial blood are generally corresponding to variations found in jugular venous blood, indicating that the two samples carry similar information and that this is different from the information carried by brain-ECF concentrations.

Figure 5 shows the groups' representation of the MFA (Fig. 5A), where the dichotomy between the brain-ECF and the blood plasma samples is evident, as well as the similarity of information in arterial and venous blood samples. The includes the arterial-venous gradient which was localized differently from the blood compartments, but more similar to them as compared to the cerebral compartment (Fig. 5A). As shown by linear correlations, the blood and brain compartments did not correlate (Fig. 5B), while there was a strong correlation between the arterial and jugular compartments for all cytokines (Fig. 5C).

The median arterial-jugular (AJ) gradient for each cytokine is shown in Table 2 (and in Additional file 2: Table 2 code). As can be seen, 28 cytokines had a positive AJ gradient, since they had higher concentrations in arterial blood than in jugular venous blood. Accordingly, 14 cytokines had higher concentrations in the venous compartment. Hence, these results oppose the idea that there is one way flow of cytokines from the brain to the blood at any given timepoint. Furthermore, the arterial cytokine concentrations were plotted against the brain-ECF and venous cytokine concentrations (Additional file 2: Fig. 5B-C code). Apart from the cytokines with low concentrations (e.g. IL-4), there was an almost linear correlation between arterial and jugular cytokines, while this could not be seen between arterial and brain-ECF cytokines.

Table 2
Median difference between arterial (A) and jugular (J) venous cytokine concentrations.

Cytokine	Abbreviation	A – J [pg/mL]
Epidermal growth factor	EGF	1.19
Eotaxin	Eotaxin	-0.207
Basic fibroblast growth factor	FGF.2	0.641
Fms-related tyrosine kinase 3 ligand	FLT.3.ligand	-2.053
Fractalkine/CX3CL	Fractalkine	-0.662
Granulocyte colony stimulating factor	G.CSF	-3.413
Granulocyte-monocyte colony stimulating factor	GM.CSF	-0.388
GRO/CXCL3	GRO	20.593
Interferon alpha-2	IFNa2	0.476
Interferon gamma	IFNg	-0.172
Interleukin-1 alpha	IL.1a	1.654
Interleukin-1 beta	IL.1b	-0.055
Interleukin-1 receptor antagonist	IL.1ra	2.157
Interleukin-2	IL.2	0.313
Interleukin-3	IL.3	-0.004
Interleukin-4	IL.4	0
Interleukin-5	IL.5	0.084
Interleukin-6	IL.6	3.942
Interleukin-7	IL.7	0.139
Interleukin-8	IL.8	1.87
Interleukin-9	IL.9	-1.634
Interleukin-10	IL.10	0.894
Interleukin-12 subunit beta	IL.12.p40	0
Interleukin-12	IL.12.p70	0.004
Interleukin-13	IL.13	0.232
Interleukin-15	IL.15	0.334

Cytokine	Abbreviation	A – J [pg/mL]
Interleukin-17	IL.17	0.061
Chemokine (C-X-C motif) ligand 10	IP.10	-1.628
Monocyte Chemotactic Protein 1	MCP.1	-0.658
Monocyte Chemotactic Protein 3	MCP.3	0
Macrophage Derived Chemoattractant	MDC	19.1
Macrophage Inflammatory Protein-1 alpha	MIP.1a	1.333
Macrophage Inflammatory Protein-1 beta	MIP.1b	2.014
Platelet Derived Growth Factor AA	PDGF.AA	19.988
Platelet Derived Growth Factor AB/BB	PDGF.ABBB	201.807
RANTES	RANTES	88.263
Soluble CD40 Ligand	sCD40L	16.129
Soluble Interleuking-2 Receptor	sIL.2Ra	7.785
Transforming Growth Factor alpha	TGFa	0.47
Tumour Necrosis Factor alpha	TNFa	0.7
Tumour Necrosis Factor beta	TNFb	0.741
Vascular Endothelial Growth Factor	VEGF	2.574

Discussion

We have shown that following a human TBI, there are distinct cytokine profiles that differ between blood and brain in response to systemic markers of inflammation, specifically infections, as well as an effect of anti-inflammatory treatment. To the best of our knowledge, this is the first study to study the interplay between peripheral/systemic markers of inflammation and cerebral cytokine levels in humans. Clinical infections demonstrated a strong cytokine response in both brain-ECF and blood, including when adjusting for demographic and injury severity parameters. Both demographic information, such as age and sex, as well as injury severity markers, altered a wide range of cytokines in both brain-ECF and blood. Systemically administered rhIL1ra treatment induced unique cytokine responses in both blood and brain, with both increased and decreased cytokines. While some cytokines revealed a brain-to-blood delayed temporal association over time or vice-versa, our general interpretation of these cytokine fluxes is that there does not seem to be an apparent delayed temporal association between the compartments. There was no time delay between patterns in the brain and blood, but a peak correlation at lag 0. This is presumably due to rapid dynamics of cytokines in vivo, but could also represent a rapid change between compartments or circumstances that make compartmental comparisons difficult. In addition, we noticed

that monitoring of cytokines in the jugular compartment did not add any explained variance as compared to that of arterial cytokines, and no robust pattern of a jugular-arterial gradient with increased levels from the injured brain could be seen. In summary, systemic infection causes a unique cytokine response in brain as compared to blood in patients suffering from TBI, and anti-inflammatory therapies and infectious treatment could indicate potential therapeutic avenues to modulate the neuroinflammatory response in acute brain injuries.

Peripheral inflammation modulates cerebral inflammation

The cytokine profile in brain was altered following inflammatory marker increase; e.g. IL-1β and G-CSF were specifically increased following a significant infection. Due to the inaccessibility of the human CNS, studies looking at simultaneous cerebral and systemic inflammation in living humans are scarce. Autopsy studies from patients that died with sepsis reveal a distinct increase of microglia activation and astrocytosis as compared to non-sepsis controls [56, 57]. In an autopsy study by Warford et al, they noticed an increase of predominantly chemokines in sepsis patients, while TNF was increased in all patients, IL-1β expression was upregulated in 2 out of 3 patients [57]. Patients with TBI and subarachnoid haemorrhages (SAH) that develop sepsis have been shown to exhibit higher levels of IL-6 in blood during the first week following ictus [58, 59], suggesting that even if the brain may release cytokines, systemic cytokine levels are important to monitor in these patients. Interestingly, CSF level of IL-6 in SAH patients did not increase in patients that developed sepsis [60], potentially highlighting the differences between the CSF and brain-ECF compartments, and that more cytokines are needed to properly detect inflammatory signals. rhIL1ra treatment resulted in a strong alteration of brain and blood cytokine levels. Treatment with rhIL1ra in trials of human SAH have resulted in lower levels of IL-6 in both serum and CSF [61, 62], thus there is evidence that the drug will alter cytokine levels in several compartments following brain injury. Unfortunately, to our knowledge, there are no studies multiplexing cytokines following rhIL1ra treatment, making it difficult to compare our findings, though our new mixed-models based approach in part validates the previous findings using the same material [42, 43]. WCC, temperature and CRP levels were shown to be associated with cytokines. However, clinical infection represents an aggregate of inflammatory markers and a judgement call of the treating physician, why we used it throughout the manuscript to define systemic inflammation. Interestingly, WCC, temperature and CRP, as well as almost all the cytokines explain, a lot of different variance of the data in a principal component analysis (PCA) of the data (Additional file 6). The PCA also shows that, due to collinearity, the marker clinical infection largely encapsulates all the other markers of inflammation. In summary, clinically relevant infections and rhIL1ra treatment were potent factors in our models resulting in altered cytokine levels in both brain-ECF and blood, highlighting that systemic mediators affect neuroinflammation.

Patient demographics affect cytokine levels

The demographic and injury severity markers all showed some significant associations with alterations on cytokine levels in both brain-ECF and blood. Female sex was associated with higher cytokine levels in brain-ECF and lower levels in blood, as compared to males, after adjusting for interaction with infection,

in which female patients were overrepresented. Except for one, all patients with an infection were female, which stresses the need to adjust for sex in the linear models. Sex differences concerning cytokine responses in humans have previously been shown [63, 64], but we could not find any studies that specifically studied our cytokine panel or compartments following TBI. Mellergård and colleagues demonstrated in a cohort of SAH and TBI patients that IL-1β and IL-6, extracted from brain-ECF using MD, were higher and increased for a longer period in females compared to males [65]. Similarly, these cytokines were higher in females in our study as well, though several other cytokines exhibited even higher concentrations. Majetschak and co-workers could not see a sex difference in post-traumatic cytokine release in blood in a trauma cohort of n = 89 patients [66]. However interestingly, male patients developing sepsis presented with higher cytokine levels in the early phase after injury compared with males without infections, something that could not be seen in females, which partly confirms our findings with females exhibiting lower cytokine levels. Older age was shown to increase levels of cytokine in brain-ECF, but decrease cytokine levels in blood. In the pre-clinical literature, TBI in the aged brain is often considered more detrimental due to a maladaptive neuroinflammatory response [67, 68], which could explain the increased cytokine response seen in brain-ECF in our study. In mild TBI, patients > 55 years old (compared to 21 – 54 years) levels of e.g. IL-6, TNF and fractalkine were elevated in blood up to 6 months following injury [69]. TBI and polytrauma patients exhibit clinically relevant cytokine patterns in blood [70 – 72], CSF and brain-ECF [36], which was similar to what we noticed in our cohort, and speaks for a pronounced cytokine release from brain and other injured tissues. Altogether, we saw a relatively strong association between cytokines and age, sex as well as injury severity markers. These factors should be acknowledged, and properly adjusted for in similar experiments.

No clear delayed temporal association was seen between brain and blood cytokines

In general, we could not see any clear trends for cytokines moving from brain to blood, or vice versa. This could indicate that the movement between compartments occurs in a time frame much shorter than the sampling interval, and half-lives *in vivo* have been suggested to be short and thus cytokine concentrations very dynamic and could be difficult to assess [73]. However, the levels of some selected cytokines, like IL-6, IL-8 and TNF have been shown to be relatively stable for days following TBI in both serum and CSF, with selected cytokine concentrations higher in CSF than in serum [74, 75]. There are several studies that have serially sampled cytokines following human TBI in both serum and blood [55, 74, 76], but to the best of our knowledge we are the first to attempt a temporal cross correlation between brain-blood compartments. As is seen in these TBI studies, it is entirely possible that the gradient between compartments is such that the cytokine movement could follow a cerebral to systemic direction, as well as a systemic to brain as suggested by the preclinical studies using systemic LPS stimulation [25]. While it is possible that the 6h pooled epochs of brain microdialysates are too long to detect rapid cytokine fluxes, our results could also indicate that there are inherent differences and circumstances concerning cytokine generation in brain-ECF and blood, respectively. This is supported by the cytokine concentration similarities found in both arterial and jugular blood vs brain-ECF. Some cytokines showed a predominant delayed temporal association from brain to blood and vice-versa, but altogether, while we cannot rule out

that temporal correlations and fluxes are absent due to intrinsic compartmental differences, future techniques might make more frequent sampling possible, though this is not currently possible.

Both arterial and jugular compartments hold similar information content

There was no difference in cytokine levels between jugular- and arterial blood, as compared to the distinctly different brain-ECF compartment. A jugular-arterial gradient has been suggested with higher levels of cytokines and brain protein biomarkers in blood, indicative of a cerebral source of these markers [38, 77]. In fact, we noticed that more cytokines were higher in arterial blood as compared to jugular blood, which could indicate that a systemic inflammation masked any increase of cytokines in the jugular compartment. Jugular samples had a higher degree of missingness, but it is uncertain how this would affect the analyses. McKeating and colleagues looked at the jugular-arterial gradient in a mixed cohort of TBI and SAH patients, and noted a trans-cranial gradient of IL-6 with higher levels in jugular as compared to arterial blood the first 48 hours after injury [38] (this could not be seen in our material (data not shown)). They could not see any difference for IL-1β, TNF or IL-8, which could be due to a significant amount of missing data in their study (86%, 88% and 52%, respectively) [38]. Possibly, the effective half-life of any brain-released cytokines is longer than the time it takes to reach the arterial compartment. Additionally, any marker that can only be accessed in a jugular compartment is in all likelihood too erratic to act as a good marker of inflammation clinically. Altogether, arterial and jugular compartments presented almost identical data in terms of explained variance for the measured cytokines suggesting that arterial blood is sufficient for cytokine monitoring in TBI patients.

Clinical implications

The clinical implications of our main findings could have great importance in the care of patients suffering from acute brain injuries. It is currently unclear how a systemic inflammation impairs cerebral functions and causes neuroinflammation, though it has been shown that concomitant infections in TBI increase the risk of an unfavourable outcome [21]. Several theories suggest that cytokines in blood targets different cerebral receptors, resulting in, for example, drowsiness, effects on the circulation and respiration, hyperthermia and cognitive implications [78, 79]. However, we can demonstrate in our study that systemic infections not only result in extensive increase of cytokines in blood, but also alters the cytokine levels in brain-ECF. This suggests that infections in TBI patients could benefit from more aggressive treatment, as their brain is presumably more susceptible to this potentially detrimental neuroinflammatory response [21] with potential impacts on both the immunological response to infection and a detrimental inflammatory response with respect to the TBI. Interestingly, levels of IL-6 and IL-8 seen in patients suffering from Covid-19 have been shown to be similar to those of trauma patients [80]. As the notorious “cytokine-storm” in this disease, as well as many other septicaemias, have been attributed to an unfavourable outcome we believe that cytokine monitoring of both brain and blood compartments are crucial in order to better understand the pathophysiology and to device new anti-inflammatory treatment strategies for brain injured patients.

Limitations

One limitation, due to ethical restraints, is the lack of proper controls without a concomitant brain injury. This limits us to use TBI patients where microdialysis catheters are inserted as part of clinical management (while usually harmless, insertion of these catheters may result in haemorrhages and infections [81]). Thus, it becomes difficult to assess exactly what cytokine signal that comes from the injured brain and what may be the result of a peripheral infection. However, we did attempt to adjust for both ISS as a marker of extracranial injury severity and Stockholm CT-score as a marker of intracranial injury severity (as well as other demographic factors) in order to filter out the exact contribution of the systemic inflammation. Furthermore, we tried to address the small sample size of only 20 patients with sophisticated statistical methods, where we parametrized the inter-patient variability (Additional files 3 and 4). Furthermore, for each block in the heatmaps (Fig. 1–3), 382 data points were used in generating the results. In short, our methods allow us to include all the acquired brain-ECF samples, adjusted for individual patients, in order to conduct our analyses.

As has been highlighted in previous microdialysis studies, the relative recovery (levels of cytokines extracted through the pores in the microdialysis catheter / actual level of cytokines in a fluid) will strongly influence the levels measured in brain-ECF [39, 82]. As can be seen in Additional file 1, there are a few cytokines with a lot of missing data from the brain-ECF compartment (e.g. IL-4), highlighting the limitations of the current method for these selected cytokines. However, there are no other methods available to access the brain-ECF *in vivo* than microdialysis, and the Luminex technology is still amongst the more sensitive methods for multiplexing small volumes while also retaining a standard curve for absolute concentrations. The technique to measure cytokines in brain-ECF is also largely unavailable outside of the research setting, and is currently associated with substantial costs, obstructing translation into main-stream clinical use.

We have deliberately chosen to focus on more broader cytokine signals in this manuscript, and very seldom highlight specific cytokines. Previous studies studying cytokines in TBI often select one or a few, and assign them attributes being “pro-“ or “anti-inflammatory” [36]. The inflammatory pathways are often more complex than this, so we believe that this simplification could potentially skew our understanding by attributing biological function to cytokines that are easy to measure using a particular technique. By multiplexing > 40 cytokines from several compartments, we instead chose to focus on them all in a more unbiased fashion. However, in the Additional files provided, it is possible for researchers and clinicians to highlight dynamics and trends for specific cytokines.

Conclusions

Systemic inflammation alters the cerebral inflammatory response, which remains after adjusting for demographic and injury severity markers that were shown to be affecting the brain cytokine levels as well. rhIL1ra treatment affected systemic and cerebral cytokine levels, demonstrating the effect of anti-inflammatory treatment following TBI. Venous jugular and arterial compartments contain similar cytokine information following TBI, while the cerebral compartment has a unique profile. Improved monitoring of the neuroinflammatory response could improve treatment options in acute brain injuries.

Abbreviations

CNS — central nervous system

CRP — C-reactive protein

CSF — cerebrospinal fluid

CT — computerized tomography

ECF — extracellular fluid

EGF — epidermal growth factor

G-CSF — granulocyte colony-stimulating factor

GCSm — Glasgow coma scale, motor score

IFN — interferon

IL — interleukin

ISS — injury severity score

LPS — lipopolysaccharide

MD — microdialysis

MDC — macrophage-derived chemokine

MFA — multiple factor analysis

MS — multiple sclerosis

PCA — principal component analysis

PDGF — platelet-derived growth factor

PET — positron emission tomography

PLS-DA — partial least squares-discriminant analysis

rhIL1ra — recombinant human interleukin-1 receptor antagonist

SAH — subarachnoid haemorrhage

sICAM-1 — soluble intracellular adhesion molecule-1

TBI — traumatic brain injury

TNF — tumour necrosis factor

VAP — ventialtor-associated pneumonia

VIF — variance inflation factor

WBC — white blood cell count

WCC — white cell count

Declarations

Ethics approval and consent to participate

Study conduct and reporting are consistent with Good Clinical Practice and CONSORT Guidelines. The protocol was approved by the 'Cambridgeshire (2) Local Research Ethics Committee' (06/Q0108/64) and by the appropriate regulatory authorities. Please see the original article for further details [42].

Consent for publication

As patients were incapacitated due to injuries, the next of kin was approached for assent in line with the prospective ethical approvals. For further details, please see original article [42].

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to local regulations, but are available from the corresponding author on reasonable request.

Competing interests

The authors declare no relevant conflict of interest.

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Authors' contributions

EPT, AH, PJAH and CL devised the study. PL and AF carried out the analysis. CL and MRG supervised and verified the analysis. KLHC verified laboratory aspects. EPT and PL wrote the manuscript. All authors reviewed the manuscript.

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Figures

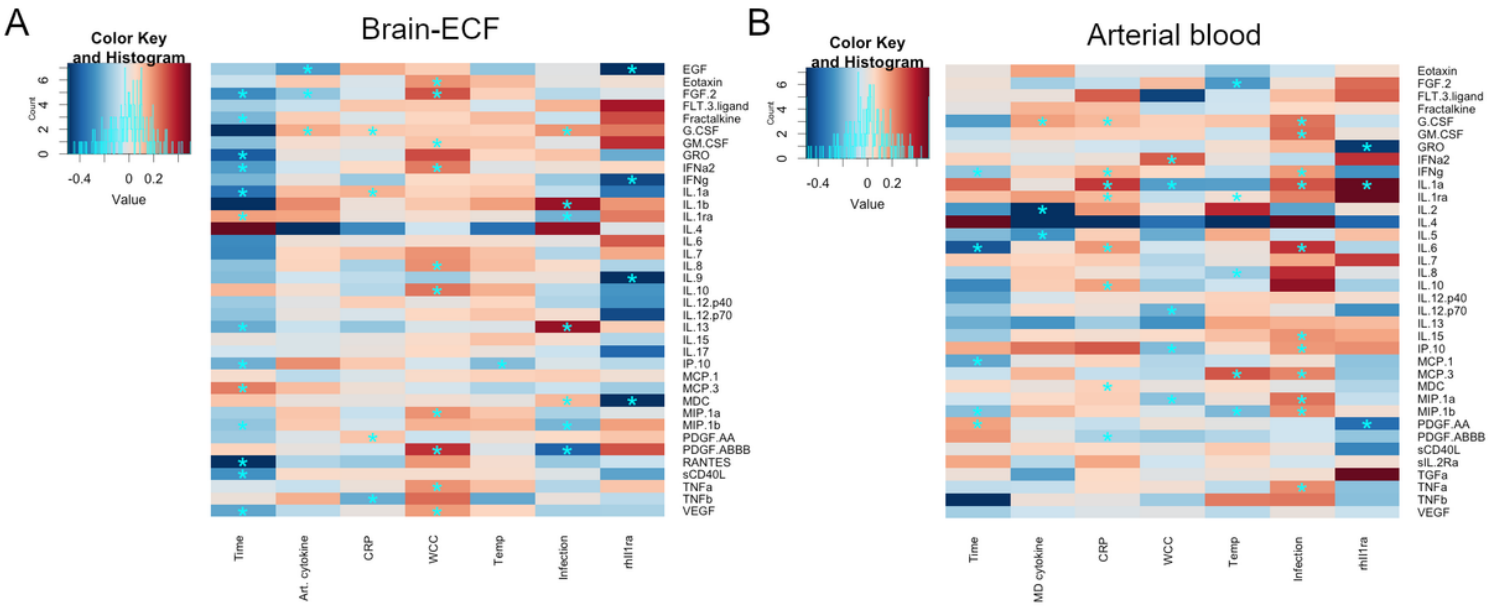

Figure 1

Coefficients of linear mixed effect models, displayed as heatmaps. The colours of the heatmaps are graded such that red represents positive coefficients and blue represents negative coefficients. All coefficients are normalized using the quotient of their standard deviation and that of the dependent variable. Significant coefficients are highlighted with an asterisk. Independent variables are along the x-axis and the dependent variable for each model is the cytokine of the respective row on the y-axis in either (A) the brain extracellular fluid or (B) arterial blood. Differences in cytokines displayed between the two subfigures is due to insufficient data to generate all coefficients from the model. EGF, epidermal growth factor; FGF.2, basic fibroblast growth factor; FLT.3.ligand, Fms-related tyrosine kinase 3 ligand; G.CSF, granulocyte colony stimulating factor; GM.CSF, granulocyte-monocyte colony stimulating factor; IFNa2, interferon alpha-2; IFNg, interferon gamma; IL, interleukin; IL-1R, interleukin 1 receptor; IL1ra, interleukin-1 receptor antagonist; IL12p40, interleukin 12 subunit beta; IL12p70, interleukin-12; IP10, chemokine (C-X-C motif) ligand 10; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemoattractant; MIP1a, macrophage inflammatory protein-1alpha; MIP1b, macrophage inflammatory protein-1beta; PDGF, platelet- derived growth factor; RANTES, chemokine (C-C motif) ligand 5; sCD40L, soluble CD40 ligand; sIL.2R, soluble interleuking-2 receptor; TGFa, transforming growth factor alpha; TNFa, tumor necrosis factor alpha; TNFb, tumor necrosis factor beta; VEGF, vascular endothelial growth factor.

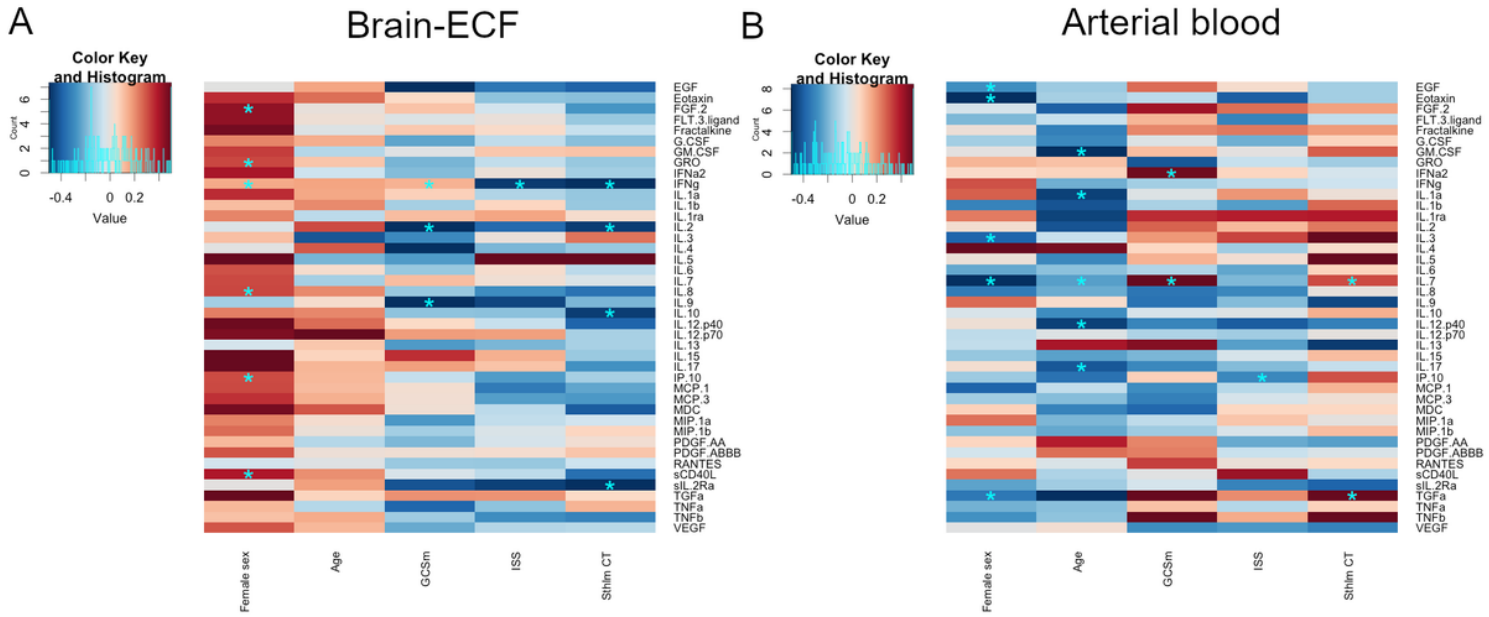

Figure 2

Coefficients of linear mixed effect models, displayed as heatmaps. The colours of the heatmaps are graded such that red represents positive coefficients and blue represents negative coefficients. All coefficients are normalized using the quotient of their standard deviation and that of the dependent variable. Significant coefficients are highlighted with an asterisk. Independent variables are along the x-axis and the dependent variable for each model is the cytokine of the respective row on the y-axis in either (A) the brain extracellular fluid or (B) arterial blood. Differences in cytokines displayed between the two subfigures is due to insufficient data to generate all coefficients from the model.

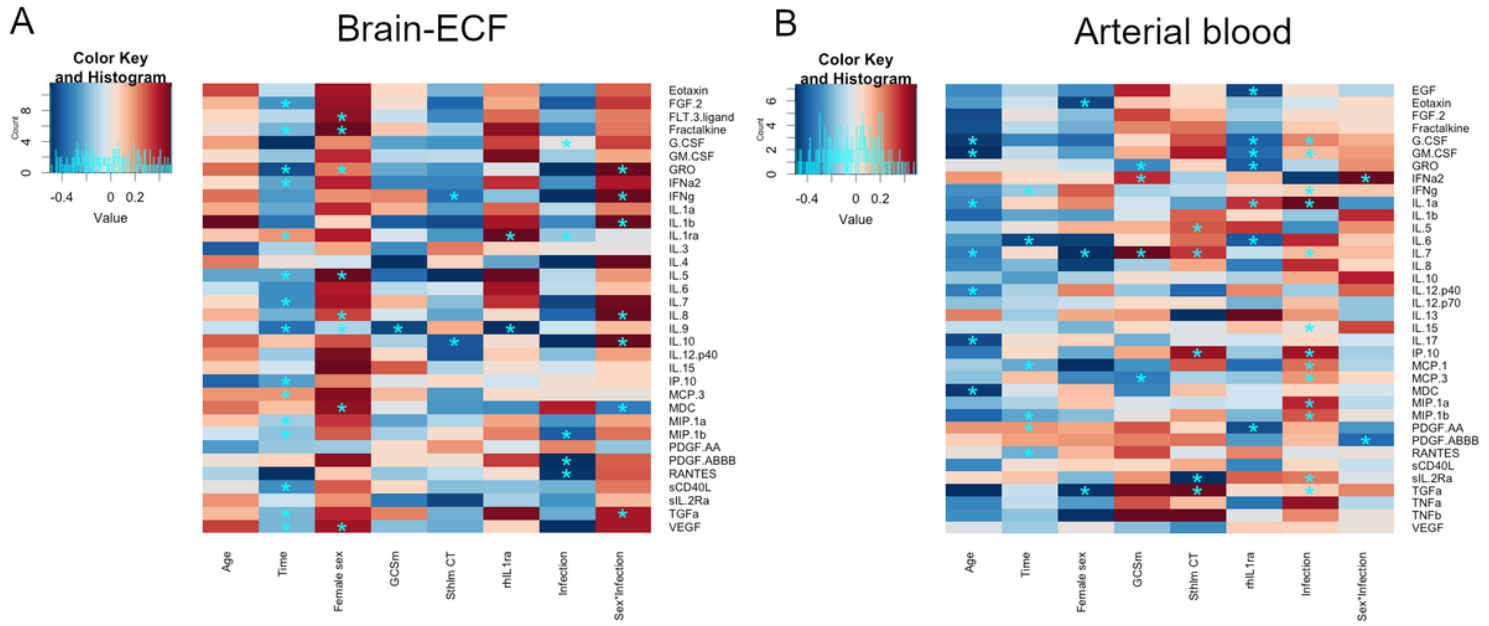

Figure 3

Coefficients of linear mixed effect models, displayed as heatmaps. The colours of the heatmaps are graded such that red represents positive coefficients and blue represents negative coefficients. All coefficients are normalized using the quotient of their standard deviation and that of the dependent variable. Significant coefficients are highlighted with an asterisk. An interaction term between female sex and infection. Independent variables are along the x-axis and the dependent variable for each model is the cytokine of the respective row on the y-axis in either (A) the brain extracellular fluid or (B) arterial blood. Differences in cytokines displayed between the two subfigures is due to insufficient data to generate all coefficients from the model.

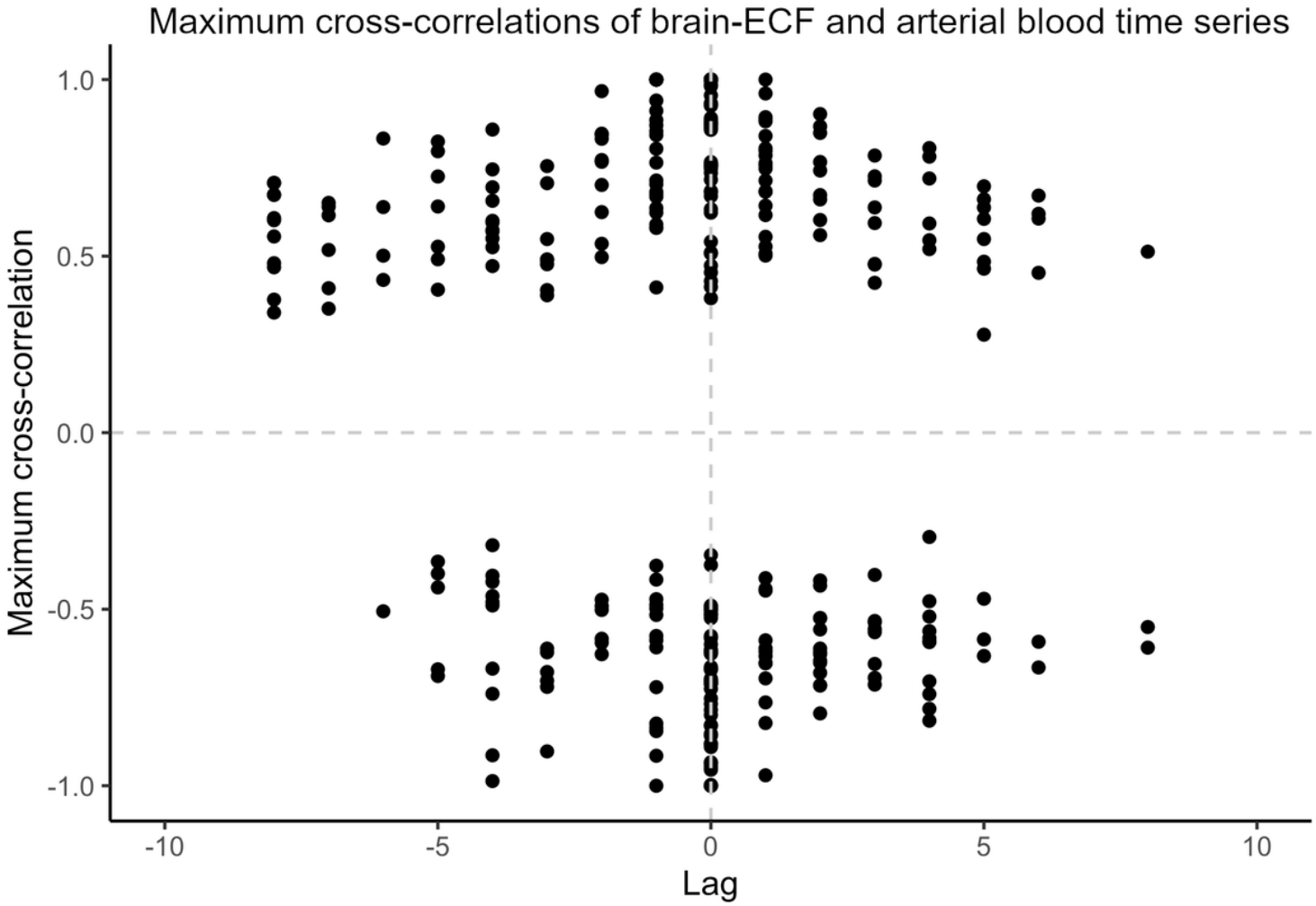

Figure 4

Signed absolute maximum cross-correlations of brain and blood cytokine time series. For every cytokine and patient, the signed absolute maximum of the cross-correlation series of cytokines in extracellular brain fluid and arterial blood was recorded as the y-value, and the lag at which this occurred was recorded as the x-value. Each lag represents six hours.

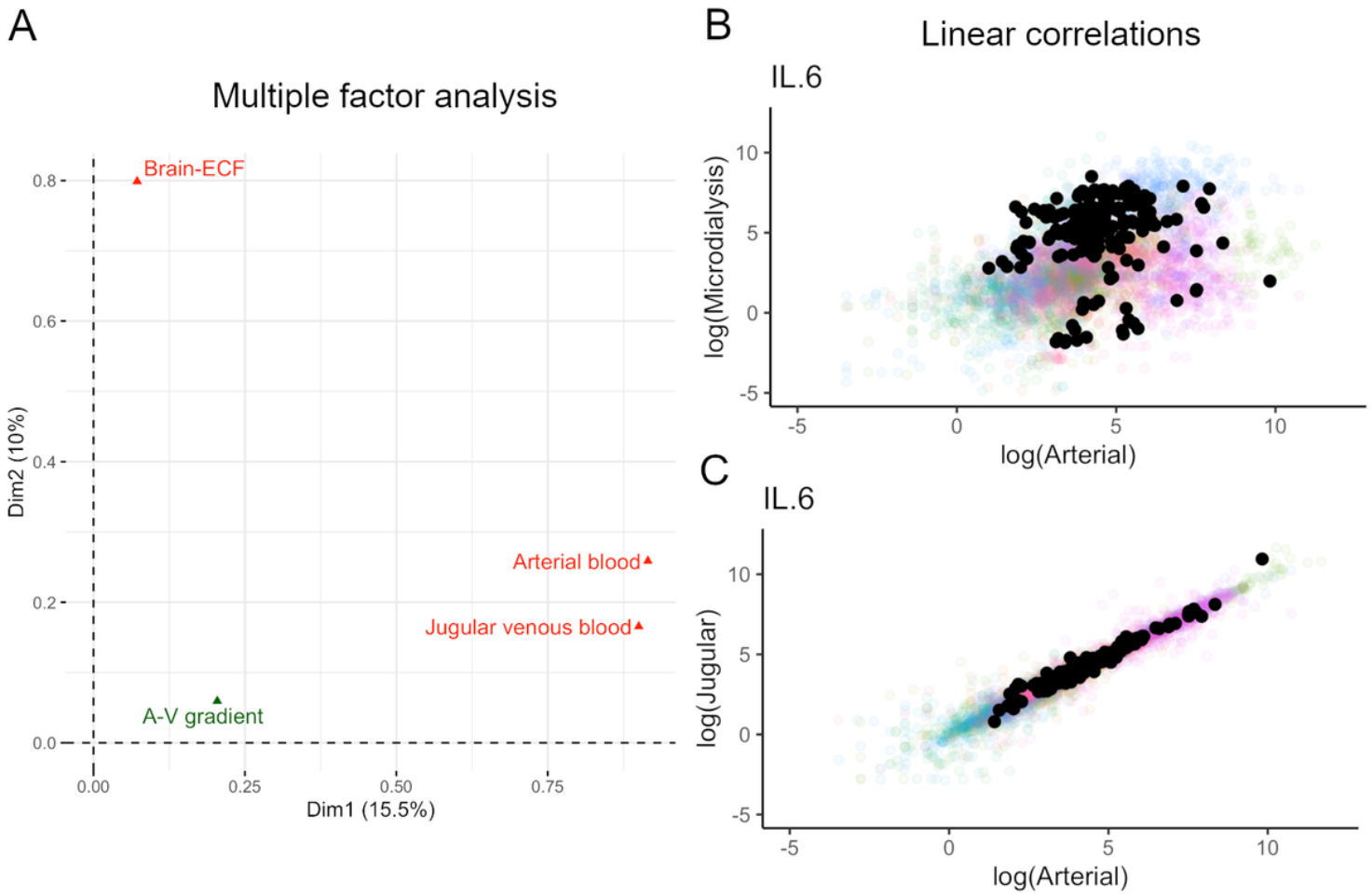

Figure 5

Relationship between brain extracellular fluid (ECF) and blood compartments. (A) Multiple factor analysis of compartments as groups. The arterio-jugular venous (A-V) gradient is plotted as a supplementary variable, while the other variables are active. Displayed on the right, there is an example of the log-log plot of (B) brain ECF vs arterial blood and of (C) jugular venous vs arterial blood concentration of IL-6 — the cytokine with least missing data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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