Cytokines of the Interleukin-1 superfamily and Interleukin-6 are associated with neuroinflammation and brain injury in Herpes Simplex Virus Encephalitis

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Abstract

Early adjunctive anti-inflammatory therapy could modulate the immune response and improve clinical outcomes in Herpes Simplex Virus encephalitis (HSE). However, data on the array of inflammatory mediators to target and the immunomodulatory drugs to use are limited. This study aimed to determine key cytokines associated with HSV-induced neuroinflammation, brain injury, and clinical outcomes, and the effect of dexamethasone on their blood levels.

We profiled the levels of forty-eight cytokines and four brain injury biomarkers (GFAP, NfL, Tau and UCHL-1) in CSF and serum samples collected from HSE adult patients; recruited in a randomised clinical trial to receive either adjunctive dexamethasone plus intravenous aciclovir or aciclovir alone.

We found that cytokines of the IL-1 superfamily (IL-1, IL-18, IL-1RA) and IL-6 were most consistently associated with neuroinflammation, brain injury and poor clinical outcomes. Spearman correlation analysis revealed positive associations between CSF concentrations of IL-1RA, IL-18, and IL-10 and serum concentrations of IL-1RA and IL-6 with the astrocytic marker, Glial fibrillary acidic protein (GFAP). Levels of CSF IL-1RA and IL-18 were associated with increased volume of cerebral oedema on MRI and were significantly raised ($p < 0.05$) in both patients with abnormal GCS scores (< 15) and worse outcomes (LoS <3). Moreover, the blood levels of these key mediators were unaffected by adjunctive dexamethasone treatment.

Taken together, this work identified key targets for direct, targeted adjunctive anti-inflammatory and neuroprotective therapy to be taken forward in clinical trials, aiming to improve outcomes following HSE.

Introduction

Outcomes following Herpes Simplex Virus encephalitis (HSE) are poor, with less than 30% of patients fully recovering after successful treatment with aciclovir [1,2]. About 30 – 70% of patients end up with long-term sequelae, including cognitive deficits, behavioural difficulties, mood disturbance, hearing and vision loss, motor impairment and seizures, costing a huge economic burden in post-discharge rehabilitation [3]. The exact mechanism of HSV-induced neuroinflammation and brain injury remains elusive. Understanding the mechanism is vital in determining effective strategies to improve outcomes.

Increasing evidence from HSV infection mechanistic studies suggests that inflammatory cytokines and chemokines produced by the CNS play a vital role in the pathology and control of HSE [4–6]. Thus, the interplay between the virus and the host's immune responses profoundly shapes the severity and outcome [7,8]. Determining key mediators associated with neuroinflammation and brain injury provides an avenue to explore new therapeutic targets for adjunctive immunotherapy.

Adjunctive immunotherapy with dexamethasone, a general immunomodulatory drug, is often used to treat patients with HSE in clinical practice. However, there is a paucity of data on its benefits and effects on levels of inflammatory mediators. Also, the array of inflammatory mediators involved in HSV-induced neuroinflammation varies and depends on the patient's immune status. Although increased levels of cytokines of the IL-1 superfamily and IL-6 have been associated with clinical severity and poor outcomes [9–12], there is limited data on their association with HSV-induced oedema and brain injury. Furthermore, data on the role of IL-18, another member of the IL-1 superfamily, in HSVE is limited. Higher levels of IL-18 and IL-1β, released via the NLR family pyrin domain containing 3 (NLRP3) inflammasome pathway, have been associated with poor outcomes in murine HSE studies [9,13–15].

Hence, our study aimed to determine key inflammatory mediators associated with brain swelling, brain injury, clinical severity and outcomes in HSV encephalitis patients, and whether their blood levels were affected by adjunctive dexamethasone treatment.

Results

Cytokines of the interleukin-1 superfamily and interleukin-6 levels in blood are associated with HSV encephalitis clinical severity and poor outcomes

CSF and blood samples were obtained from 55 participants (32 in the treatment group and 23 in the control group). The median ages of the treatment and control groups were 65 (IQR: 56 - 73) years and 70 (IQR: 58 - 80) years, respectively. Both groups had similar clinical characteristics and disease outcomes (Table 1).

Serum and CSF samples were assessed for a broad panel of mediators and four brain injury biomarkers using the Bio-Rad multiplex Luminex and Quanterix single-molecule array panels. The Glasgow Coma Scale score (GCS) at admission was used to assess clinical severity, while modified Rankin scale (mRS), Liverpool outcome score (LoS) and Barthel Index were used to assess the disability and functional outcomes at discharge or 30 days.

We found that the levels of biomarkers in samples collected at admission or the early post-randomisation period (<10 days post-randomisation) were consistently associated with clinical severity, outcomes, and neuroglial injury. Hence, the results and discussions focus on the earliest samples collected after randomisation in the study.

Of the 48 immunological biomarkers assessed in CSF collected at admission, 9 were associated with clinical severity and included predominantly pro-inflammatory cytokines, IL-1RA and IL-18 of the interleukin-1 superfamily; interleukin-12 (IL-12), interferon-γ (IFNγ); leukocyte chemotactic chemokines, C-C motif chemokine ligand 3 (CCL3), C-C motif chemokine ligand 5 (CCL5); and C-C motif chemokine ligand 7 (CCL7); and granulocyte colony-stimulating factor (G-CSF). Most of the levels were significantly raised ($p < 0.05$) in patients with severe disease (GCS score < 15), except for IL-12 and CCL5, where the levels were significantly higher in patients with a GCS score of 15. The

ratios of interleukin-1α, interleukin-1β and interleukin-1RA were also associated with clinical severity (Fig. 1).

Next, we determined which of these 9 mediators were also associated with poor disability and functional outcomes. We found that patients with poor outcomes (LoS <3) had significantly higher CSF levels of IL-1RA (180.7 vs 81.0 pg/mL, p = 0.03) and IL-18 (0.48 vs 0.34 pg/mL; p = 0.01) (Fig. 2 and Suppl Table 1); and both cytokines were significantly negatively correlated with LoS (IL-1RA: tau = -0.43, p =0.009; IL-18: tau = -0.42, p = 0.01) and mRS (IL-1RA: tau = 0.39, p = 0.01; IL-18: tau = 0.39, p = 0.01) Extended Fig. 1. Although baseline CSF HSV viral load showed a positive correlation with proinflammatory cytokines, IFN-γ, IL-6, and IL-10, it was not associated with clinical severity or outcome. Thus, levels of IL-1RA and IL-18 in CSF were associated with clinical severity and poor outcomes following HSV encephalitis.

In serum, the pro-inflammatory cytokines, IL-1RA and IL-6, were associated with both clinical severity (significantly higher in patients with GCS scores <15; p <0.05) and poor outcomes (significantly higher in patients with LoS of 2, and mRS scores of 3 or more), Fig. 1-b,g,i and Fig. 2-b,g,h. Both cytokines showed a negative correlation with GCS and LoS, reiterating their association with clinical severity and poor outcome (Extended Fig. 2).

The serum levels of IL-1β:IL-1RA ratio, Eotaxin, and antiviral cytokine TNF-related apoptosis ligand antagonist (TRAIL) were associated with mild disease and good outcomes (significantly higher in patients with LoS >2), Fig. 1-b,h, and Fig. 2-b.

Altogether, these results showed that the levels of proinflammatory mediators, particularly IL-1 family cytokines in CSF and IL-6 in blood, were associated with both clinical severity and poor outcomes following HSV infection.

Cytokines of the IL-1 superfamily are associated with neuroinflammation and neuroglial injury in HSV encephalitis

Given the significantly increased levels of the predominantly proinflammatory cytokines, including cytokines of the interleukin-1 family, in patients with severe clinical disease

and poor outcomes, our data indicated that these cytokines drive neuroinflammation and neuroglial injury in HSV encephalitis. We thus determined if these cytokines were also associated with brain oedema (MRI scans) and the levels of biomarkers of brain injury. Spearman correlation analysis revealed positive associations between CSF concentrations of cytokines of the IL-1 superfamily, IL-1RA, IL-18, and IL-10 with GFAP, a marker of astrocytic damage, and an inverse association with CSF levels of IL-7, IL-12 and the antiviral cytokine TNF-related apoptosis ligand antagonist (TRAIL), Fig. 3. We also found significant associations between CSF IL-18 with Neurofilament Light chain (NfL) - a biomarker for neuroaxonal damage and neurodegeneration. There were also significant associations between CSF C-X-C motif chemokine ligand 9 (CXCL9), CSF C-X-C motif chemokine ligand 12 (CXCL12), and C-C motif chemokine ligand 7 (CCL7) with GFAP (Fig. 3).

In serum, the levels of IL-1RA, IL-6, and M-CSF were positively associated with GFAP (Fig. 4) and IL-1RA and M-CSF with NfL (Supplementary Table 1).

We did not find any association between these key mediators and brain oedema in 15 participants with an MRI scan at admission (Extended Fig. 3). The median time between lumbar puncture and MRI scans was 3 (range: 1 - 9) days. However, when we limited the analysis to 10 participants who had both their MRI scan and lumbar puncture within 3 days, we found a significant positive association between CSF IL-18 levels and cytotoxic oedema (Fig. 5-e and Supplementary Fig. 1). Similarly, a correlation analysis of all 38 participants with MRI scans and CSF samples, irrespective of when the CSF was collected, showed a positive association between CSF IL-18 and vasogenic oedema (Fig. 5-a,b), and CSF IL-1RA and cytotoxic oedema (Fig. 5-c,d); indicating the potential role of the inflammasome in the HSV-induced neuroinflammation.

Serum levels of IL-1RA, IL-6 and M-CSF predict outcome in HSV encephalitis

We aimed to identify inflammatory mediators in serum and CSF that could predict clinical outcome in HSV encephalitis. For each sample type, a pre-specified subset of biologically plausible predictors was selected to avoid overfitting in this small dataset.

For serum, IL-1RA, IL-6, M-CSF, and CCL2 were chosen based on exploratory analysis and prior literature. For CSF, the subset included IL-1RA, IL-1α, IL-1β, IL-18, and CCL3.

Outcomes were binarized using clinically relevant thresholds: mRS ≤ 1 (favourable), GCS of 15 (favourable), and length of stay ≤ 2 weeks (short). Given the limited sample size and potential for multicollinearity, we evaluated penalised logistic regression models (ridge, lasso, and elastic net) with and without an interaction term between dexamethasone use and time since randomisation. For each model, we used 5-fold cross-validation to estimate the area under the ROC curve (AUC), selecting the model with the highest AUC for each outcome-sample type pair.

The optimal models were then subjected to non-parametric bootstrapping (1,000 resamples) to assess coefficient stability, derive 95% confidence intervals, and calculate empirical p-values. Predictors were scaled prior to modelling, and collinearity was checked using variance inflation factors (VIFs) in unpenalized linear models. To accommodate sparse data, cross-validation was modified to allow folds with fewer than three observations.

Across all outcomes, serum-based models outperformed CSF-based models in terms of cross-validated AUC. Notably, serum IL-1RA, IL-6, and M-CSF emerged as significant predictors of outcome in the ridge regression models selected by cross-validation. These associations remained robust under bootstrapping, with narrow confidence intervals and low p-values. In contrast, none of the CSF cytokines showed statistically significant associations with outcome, and VIFs indicated substantial multicollinearity (e.g., VIF > 6 for CCL3), limiting interpretability.

The serum levels of cytokines were unaffected by adjunctive Dexamethasone treatment

We noted that the CSF and serum levels of all analytes were comparable between the treatment (aciclovir plus dexamethasone) and control (aciclovir only) groups.

Of the 55 participants, 20 had paired serum samples obtained before and after 14 days of trial treatment. We compared the serum levels of key cytokines at pre-trial treatment

(defined as blood collected within 9 days after randomisation) and post-trial treatment (defined as blood collected at day 10 or later after randomisation) time points for both groups. We found that the levels of the mediators were unaffected by dexamethasone treatment. There was no significant difference ($p > 0.05$) between the pre-treatment and post-treatment levels for any of the key mediators in the treatment group (Fig. 6a). However, we noted that levels of IL-1RA were significantly reduced ($p = 0.02$) and the ratios of IL-1α:IL-1RA and IL-1β:IL-1RA were significantly increased ($p = 0.02$ and $p = 0.01$, respectively) in the control group (Fig. 6a).

We also assessed the fold changes in analyte concentrations at different sample collection time points and found that the patterns were similar in both groups (Fig. 6b). For example, the median (IQR) levels of IL-18 for the treatment group were {Day 0-9: 1.01 (0.9 - 1.5) vs Day 10-29: 1.2 (1.0 - 1.4) vs Day 30 plus: 1.4 (1.1 - 1.5) pg/mL}. The corresponding levels for the control group were {Day 0-9: 1.07 (0.9 - 1.3) vs Day 10-29: 1.1 (0.9 - 1.5) vs Day 30 plus: 1.1 (0.98 - 1.3) pg/mL}.

HSV infection induced neuronal and microglial expression of IL-1β IL-6, and IL-18, and drove downstream inflammatory pathways in a murine model

Given the consistent associations between CSF levels of cytokines of the IL-1 superfamily and IL-6 with clinical severity, poor outcomes, and neuroglial injury, we hypothesised that neuronal and glial (astrocytes and microglial) expressions of these cytokines are upregulated following HSV infection. To test our hypothesis, a parallel HSV encephalitis murine model (Michael et al., 2020) was used. C57BL/6 female mice aged 8-12 weeks were intracranially infected with 20μl of 10^4 PFU HSV-1-KOS (Colgrove et al., 2006) or sham-infected with sterile PBS. Three days post-infection, mice were euthanised with CO2 and death was confirmed by transection of the great arteries. A sample of about 0.5 cm of the cerebral cortex was collected into RNAlater and immediately processed for single-nuclei RNAseq by Chromium 10x. Differentially expressed genes in various brain cell types were identified using the FindMarkers

function from the Seurat package. We used 0.25 log fold threshold and the Wilcoxon rank sum test to compare expressed genes between HSV-1-infected and control mice.

Single-cell RNA-seq data was mapped to Reference UMAP embeddings using the TransferData and MapQuery functions from Seurat package to identify cell types; Fig. 7a.

Then, we investigated which brain cell types expressed the mouse HSV gene transcript to denote active infection. We noted the presence of HSV gene transcripts in various mouse brain cells, including neurons, microglia, and astrocytes (Fig. 7b).

Next, we determined the expression of 6 selected cytokine transcripts, Il1a, Il1b, Il1rn, Il18, Il6, and ccl3 in these brain cells. We noted that neurons, microglia, and astrocytes exhibited the highest number of differentially expressed genes compared to the other brain cells in both the HSV-infected and control mice (Fig. 7c). We then compared the expression levels of these selected cytokines in various brain cells between the HSV-1-infected and control mice. HSV-1 infection led to significantly higher expression of Il18rap (in microglia; log2fold change = 3.80, p value = 0.0056), Il1rap and Il6ra (in astrocytes; Il1rap: log2fold change =0.94, p value <0.001; Il6ra: log2fold change =2.11, p value = 0.00012), and Il6ra (in neurons; log2fold change = 2.11, p value = 0.00012), Fig. 7d. This data indicates that HSV infection induces neuronal, astrocytic, and microglial expression of IL-1, IL-6, and IL-18.

KEGG analysis

To gain biological insights into the potential pathways key mediators associated with clinical severity, a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was done. All significantly different mediators in participants with clinical disease severity (GCS < 15) compared to a GCS score of 15 ($p < 0.05$) were compared with pathways in the KEGG database.

The analysis revealed pathways with many commonalities with other inflammatory syndromes, such as Influenza A. The most significantly clustered pathways included cytokine-cytokine receptor interaction, viral protein interaction with cytokine and

cytokine receptor, JAK-STAT signalling pathway, Herpes simplex virus 1 infection, and NOD-like receptor. (Table 2b and Fig. 8).

NOD-like inflammasome pathways highlight the potential role of the inflammasome in HSV-induced encephalitis.

Discussion

We have identified key mediators associated with HSV-induced inflammation and neuroglial injury that could be explored as direct targets for adjunctive immunotherapy to improve outcomes in HSV encephalitis. Raised levels of the IL-1 superfamily cytokines (IL-18 and IL-1RA), and IL-6, were consistently associated with increased brain injury, volume of cerebral oedema on MRI, clinical disease severity and poor outcomes in HSV encephalitis patients. Moreover, the levels of these key mediators were unaffected by adjunctive dexamethasone treatment. Although other proinflammatory mediators, such as IFNγ, CCL3, CCL7, and G-CSF were also associated with clinical severity, they were not consistently associated with outcomes or neuroinflammation (volume of cerebral oedema on MRI), indicating that the IL-1 superfamily cytokines (IL-1β, IL-18 and IL-1RA), and IL-6 play an important role in determining outcomes following HSV infection. Previous work highlighted the association between inflammatory cytokines with disease severity and outcome in HSVE [7,12]. Our work confirms this central role and adds new evidence that they are also involved in HSV-induced neuroinflammation and brain injury, suggesting they are potential targets for immunomodulatory therapy.

Worse outcomes were associated with increased CSF levels of IL-1RA and IL-18, but not with HSV viral load. We also observed that HSV induces neuronal, astrocytic, and microglial expression of the IL-1 superfamily cytokines and IL-6 in an HSV murine model, suggesting that these cytokines, and not the virus, are likely key drivers of the damaging inflammatory cascade within the central nervous system (CNS). This aligns with previous reports that the overly immune response, rather than the virus itself, often mediates the worst outcomes in viral encephalitis [9,10]. We also found that raised levels of both IL-1RA and IL-18 in acute CSF samples positively correlated with raised levels

of the astrocytic protein, GFAP and the biomarkers of neuronal injury, Tau protein, and UCHL-1, together with increased volume of cerebral oedema on MRI, suggesting that they may be driving the associated neuroinflammation and brain injury in HSVE. CSF and Serum levels of GFAP, NfL and UCHL-1 are elevated in several neurological disorders, including HSE, SARS-CoV-2, and traumatic brain injury [16–19]. It is thought that HSV infects the CNS, leading to the activation of resident brain cells and the subsequent release of proinflammatory cytokines, which may cause persistent inflammation, blood-brain-barrier damage, or direct neuronal injury [7,8]. Therefore, inflammatory pathways and the cytokines produced are vital in determining the outcome in HSVE.

KEGG analysis using cytokines and chemokines associated with abnormal GCS scores identified inflammatory pathways, including influenza A, NOD-like inflammasome, as the most likely pathways mediating HSV-induced inflammation in our study. Our KEGG analysis results and the association between increased levels of IL-18 and IL-1 in CSF with increased cerebral volume oedema suggest that the severe neuroinflammation observed in HSV encephalitis may be driven by this highly regulated, lytic inflammatory pathway. NLRP3 inflammasome is a multi-protein signalling platform that mediates IL-1β and IL-18 production and has been experimentally shown to activate the NLRP3-mediated IL-1β-dependent pathway [15], and to play a key role in many inflammatory diseases [20,21]. Hence, modulating the immune response during acute infection, before severe irreversible brain injury occurs, may reduce inflammation and improve clinical outcomes in HSVE patients [22].

Dexamethasone, a generalised immune modulator, neither affected changes in levels of key mediators nor improved outcomes in our study. This is consistent with a recent review that found no substantial benefits of adjunctive corticosteroid therapy in HSVE patients [23] and a case report of the failed use of corticosteroids to treat an autoinflammatory condition [24]. A plausible explanation could be that dexamethasone has no direct substantial suppression of these specific, key inflammatory mediators in the primary CNS cells: neurons, astrocytes, and microglia. Perhaps more direct targeted immunomodulatory drugs could be more effective alternatives. Anakinra, the pharmacological IL-1R antagonist, for example, has been shown in a murine model of

virus-induced neuroinflammation to reduce neurological injury [10]. Also, a combination therapy of anti-IL1 and anti-IL-18 was successfully used to treat an infectious autoinflammatory disease case that high doses of corticosteroids failed to treat [24]. Thus, drugs that cross the blood-brain barrier and directly reduce IL-1 and IL-18 levels, such as Anakinra and Tadekining alfa (a recombinant IL-18BP), could be more effective in reducing neuroinflammation and hence, improve outcomes in HSVE.

Our finding that the predominantly anti-inflammatory cytokine IL-1RA was associated with worse outcomes, neuroglial injury, and neuroinflammation contradicts previous reports. The high levels observed in our study could be a delayed compensation of initial high levels of IL-1α and IL-1β, which have been shown in murine models to rapidly increase immediately following HSV CNS infection [8,10].

Conclusion

Inflammatory mediators of the IL-1 superfamily and IL-6 were associated with brain swelling, brain injury, clinical disease severity and worse outcomes, and their blood levels were unaffected by dexamethasone treatment. Taken together, this work demonstrates the key targets for focused adjunctive anti-inflammatory and neuroprotective therapy to be taken forward in clinical trials to improve patient outcomes. Also, more experimental studies to determine the role of IL-18 in HSV-induced neuroinflammation are encouraged.

Limitations:

Inconsistencies in the timing of sample collection across participants could have introduced variability in the observed differences in pre- and post-treatment mediator levels, potentially affecting the comparability of results. In addition, the small sample size of 12 and 8 participants for the treatment and control groups, respectively, may limit the statistical power of the effect of dexamethasone on mediator levels.

Methods

Study Participants and Sample Collection

Samples were collected from adults with PCR-confirmed Herpes Simplex Virus (HSV) encephalitis from 53 National Health Service hospitals in the UK. The participants were recruited in a clinical trial and randomised to receive either adjunctive dexamethasone plus intravenous aciclovir (treatment group) or aciclovir alone (control group). The clinical trial is described in detail elsewhere ([25]).

From each participant, up to 2.5 mL of CSF was collected in a universal container, and 5 mL of venous blood in a BD Vacutainer Serum Separator Tube (SST). Serum was separated by centrifugation, and both samples were frozen at the sites per local hospital policy. All frozen specimens were couriered in batches to the Infection Neuroscience Laboratory (INL), Clinical Science Centre, University of Liverpool, for analysis. The specimens were stored at −80°C at INL until analysed, and freeze-thaw cycles were minimised. CSF and blood samples were obtained at recruitment, 14 and 30 days after randomisation in the study or when the patient was discharged from the hospital.

Measurement of Mediators and Brain Injury Biomarkers

A Bio-Plex Pro Human Cytokine 48-plex Assay (cat. no. 12007283, Bio-Rad Laboratories) was used for mediators profiling. Serum samples were diluted fourfold in sample dilution, then 50 µl was pipetted in triplicate into each well. Standards and CSF samples were analysed undiluted, and plates were processed according to the manufacturer's instructions. Four biomarkers of neuroglial injury: GFAP, NfL, Total Tau protein, and Ubiquitin Carboxyl-terminal Hydrolase L1 (UCHL-1) were assessed using a Quanterix Simoa Human Neurology 4-Plex B Advantage kit (Product no. 103345, Quanterix Corporation). Serum and CSF samples were diluted fourfold and fortyfold, respectively, in sample diluent. Then, 100 µL of samples, standards, and controls were pipetted into each well, and the plates were processed according to the manufacturer's instructions. Plates were read on the Quanterix Simoa SR-X (ultrasensitive digital ELISA) benchtop reader. We used the manufacturer's calibrators, provided with the kit, to calculate concentrations.

Clinical and functional outcome measures

Clinical severity of HSV encephalitis was assessed using the Glasgow coma scale (GCS) score obtained at admission; a score of 15 (out of 15) was defined as normal, and scores of <15 were defined as abnormal or severe clinical disease. Disability and functional outcome assessments were: Modified Rankin Score (mRS); scores less than 3 were defined as good, and scores of 3 and above were poor, the Liverpool Outcome Score (LoS); scores of 3 and above were defined as good, while a score of 2 was defined as poor, and the Barthel Index score. mRS is widely used as an outcome measure of disability and dependence of patients following stroke or neurological infections, or non-infectious encephalitis [26]. LoS assess outcome following Japanese Encephalitis, but has been successfully used in adolescents and adults in a previous HSV encephalitis study [27].

Magnetic resonance imaging

Magnetic Resonance Imaging (MRI) scans were obtained from patients' standard medical care records between admission and 7 days post-randomisation. A second scan was done two weeks after randomisation. Volumes of total, cytotoxic and vasogenic oedema and temporal lobe volumes were assessed as previously described and established for patients with HSV encephalitis [25]. Temporal lobe volume was calculated as a percentage of intracranial volume.

Herpes Simplex Virus Encephalitis Mouse Model

C57BL/6 female mice aged 8 -12 weeks were intracranially infected with 20 µl of 10^4 PFU HSV-1-KOS [28] or sham-infected with sterile PBS. Mice were euthanised with CO2 three days post-infection, and death was confirmed by transection of the great arteries. About 0.5 cm of the cerebral cortex was sampled into RNAlater and immediately processed for single-nuclei isolation.

Single Nuclei Isolation: Samples were delicately dounced homogenised in 2ml of ice-cold Nuclei EZ lysis buffer (Sigma, #EZ PREP NUC-101), transferred into a different

tube containing an additional 2ml of ice-cold Nuclei EZ lysis buffer and incubated on ice for 5min. Samples were then filtered through a 30 µm cell strainer (MACS Smart Strainer 130-098-458) and centrifuged at 500 g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in fresh 4 mL of Nuclei EZ lysis buffer. This process was repeated three times. After the last centrifugation, the pellets were resuspended in 300 µL of NSB (1xPBS, 5% BSA, 0.25% Glycerol and 40U/µL RNAse inhibitor from #Clontech Takara 2313A/B) and filtered through a 40 µm Flowmi Cell Strainer (H13680-0040), followed by filtration through a 10 µm cell strainer. The single nuclei suspension was stained with 1 µg/ml DAPI (Fisher, Cat # D1306) and DAPI+ events were sorted. The concentration of single nuclei was adjusted to 1000 single nuclei per µL and immediately loaded on the 10X Chromium controller instrument (10X Genomics) for sequencing.

Single-cell RNA sequencing

Cell suspensions were loaded along with reverse transcriptase reagents, 3′ gel beads, and emulsification oil onto separate channels of a 10X Single Cell B Chip, which was loaded into the 10X Chromium instrument to generate emulsions. Emulsions were transferred to polymerase chain reaction (PCR) strip tubes for immediate processing and reverse transcription. Library preparation was performed according to the manufacturer's recommendations. Expression libraries were generated using Chromium Single Cell Next GEM 3′V3.1 chemistry (10X Genomics PN-1000268). DNA and library quality were evaluated using an Agilent 2100 Bioanalyzer, and concentration was quantified using the Qubit dsDNA high-sensitivity reagents (Thermo Fisher Scientific). Gene expression libraries were sequenced on the Illumina NextSeq 500/550 High Output Kit v2.5 (75 Cycles) (Illumina #20024906).

Ethical approval

All participants provided informed consent before being recruited into the study. All ethical approvals were in place before the start of the clinical trial. Ethics approval was obtained from the UK National Research Ethics Committee (reference number: 15/NW/0545).

Statistical Analysis

R statistical software version 4.5.0 (R Core Team 2025) was used for the statistical analysis. Categorical and numerical variables were compared between groups using the Chi-Squared and independent samples Wilcoxon rank-sum (Mann-Whitney) tests, respectively. Correlations were assessed with either Spearman rho or Kendall tau rank correlation tests. A *P* value of 0.05 was considered statistically significant. Graphs were generated using the `ggplot2` package (Wickham, 2016), and statistical annotations were added using the `ggpubr` package (Kassambara, 2024).

To avoid undetectable levels or missing data bias, only mediators detected in CSF or serum specimens from ≥80% of the samples were analysed. Also, mediators with low variability, for which over 90% of the measured concentrations were at or below the limit of quantification, were excluded from all analyses. To minimise any influence of sample storage, concentrations of each mediator were median-centred for each participant. Therefore, the concentration of each mediator was expressed and analysed as a value relative to the median concentration of all of the mediators in that participant's sample, as described previously [29,30].

Differential gene expression analysis, for murine data, was done using the Seurat R package (version 5.2.0). Gene expression between groups was compared with the Wilcoxon rank sum test using the FindMarkers() function. The p values were corrected by the Bonferroni method to control for multiple comparisons.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Protein Enrichment Analysis

The UniProt IDs of mediators that were significantly different between participants with GCS scores <15 and a GCS score of 15 were obtained from uniprot.org. Before the enrichment analysis, Uniprot names were first converted to 'Entrez IDs' using the 'org.Hs.eg.db' annotation package (Carlson, 2024). KEGG pathway enrichment analysis was done using the enrichKEGG() function from the clusterProfiler R package (Yu et al., 2012). Parameters of the analysis were a *P* value cutoff of 0.05 and the Benjamini-Hochberg method for multiple testing correction. Pathway classifications from the KEGG

map search results were ranked by the highest number of mapped candidates and exported in the KGML format. Key pathway diagrams were generated and visualised using the `pathview` package (Luo & Brouwer, 2020). It overlaid the log2 fold-change values of each mediator, providing a visual representation of their regulation within biological contexts.

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Table 1: Characteristics of Participants

Characteristics	All participants (n = 55)	Aciclovir + Dex Group (n = 32)	Aciclovir only Group (n = 23)	p value
Sex-Male	25 (45.5)	19 (59.3)	11 (47.8)	0.57
Age*	67 (57 - 76)	65 (56 - 73)	70 (58 - 80)	0.19
Clinical				
CSF-WCC (per mm3)*	76 (14.9 - 275.3)	141 (33.5 - 288.0)	36 (11.1 - 114.3)	0.1
CSF-RCC (per mm3)*	29 (4.3 - 205.3)	18 (5.0 - 116.0)	32 (4.1 - 240.0)	0.64
CSF-protein (mg/dL)*	18 (15.0 - 23.0)	17.0 (15.0 - 18.0)	22.5 (18.8 - 23.5)	0.19
Had seizures at admission	25 (49.02)	15 (50)	10 (47.6)	0.61
HSV viral load (Log copies/mL)*	4.66 (4.2 – 5.1)	4.3 (4.0 – 5.1)	4.7 (4.4 – 5.0)	0.67
Pre-treatment GCS				
Normal (GCS = 15)	27 (50)	17(53.1)	10(45.5)	0.78
Abnormal (GCS < 15)	27 (50)	15(46.9)	12(54.5)	
Outcome Scores				
mRS (30 days)				
Good Outcome (mRS<3)	27 (51.9)	17(56.7)	8(36.4)	0.24
Poor Outcome (mRS>2)	25 (48.1)	13(43.3)	14(63.7)	
LoS (30 days)				
Good recovery (LoS > 2)	29 (58)	18(64.29)	11(50)	0.47
Poor recovery (LoS =2)	21 (42)	10(35.7)	11(50)	
GoS (26 weeks)				
Good recovery (GoS =7|8)	15 (33.3)	10(40)	5(25)	0.46
Poor recovery (GoS <7)	30 (66.7)	15(60)	15(75)	
Barthel (30 days)				
Independent (score =20)	21(40.38)	15(50)	6(27.3)	0.17
High dependency (score <20)	31(59.62)	15(50)	16(72.7)	

Summary statistics are **counts(column %)** or ***median(IQR)**

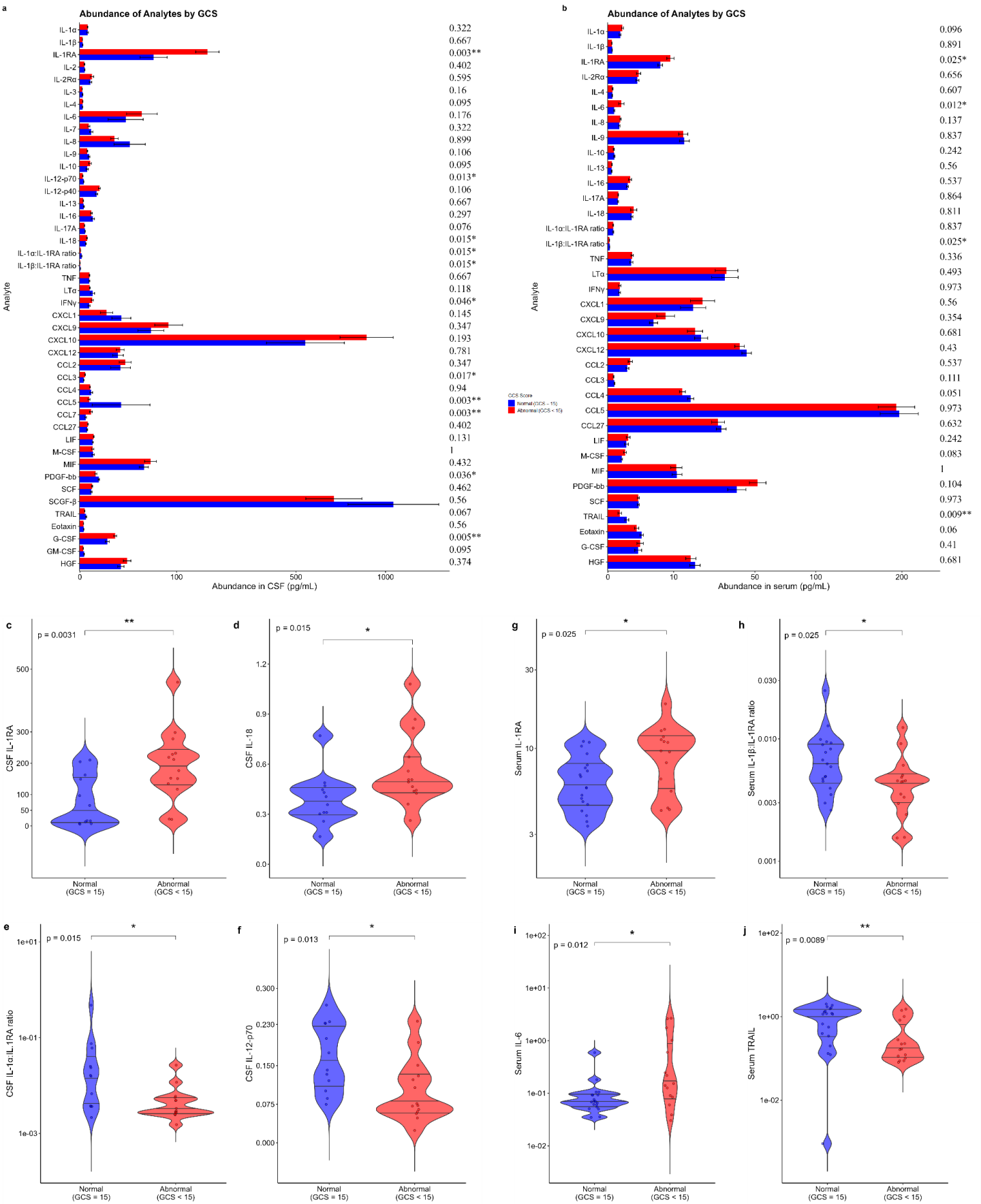


Fig. 1. Inflammatory mediators of the IL-1 superfamily and serum IL-6 levels are associated with disease severity. a,b, Analyte abundance plots showing concentrations of mediators in CSF and serum, respectively from encephalitis patients according to disease severity. Each analyte represents median-centred and normalised values. **c–j**, levels of mediators stratified by GCS scores; **CSF: (c)** IL-1RA, (**d**)IL-18, (**e**) IL1α:IL-1RA ratio, **(f)** IL-12 **Serum: (g)** IL.1RA ratio **(h)** IL1α:IL-1RA ratio, **(i)** IL-6, **(j)** TRAIL. Means were compared using the Mann-Whitney test. The violin plots show the median and interquartile values; each data point represents one patient's clinical sample. . Disease severity is defined as a normal versus an abnormal Glasgow Coma Scale score on admission.

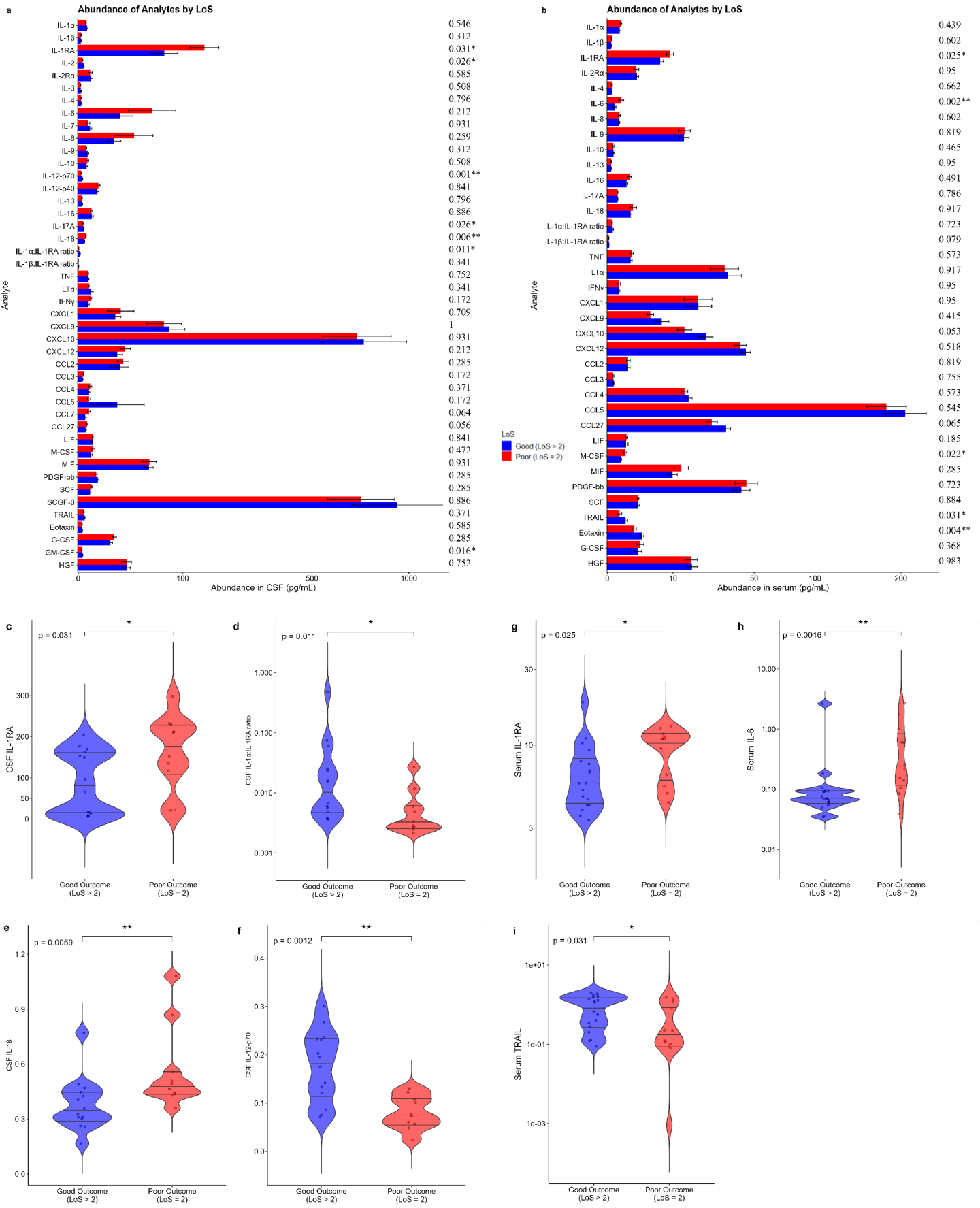


Fig. 2 . Inflammatory mediators of the IL-1 superfamily and serum IL-6 levels are associated with worse outcomes following HSV encephalitis. a,b, Analyte abundance plots showing concentrations of mediators in CSF and serum, from encephalitis patients according to disease severity. Each analyte represents median-centred and normalised values. **c–i**, levels of mediators stratified by LoS; **CSF**: **(c)** IL-1RA, **(d)** IL1α:IL-1RA ratio **(e)** IL-18, **(f)** IL-12. **Serum**: **(g)** IL.1RA **(h)** IL-6, **(i)** TRAIL. Means were compared using the Mann-Whitney test. The violin plots show the median and interquartile values; each data point represents one patient's clinical sample. Clinical outcome is defined based on the LoS; a better outcome is an LoS >2, and a worse outcome is an LoS of 2.

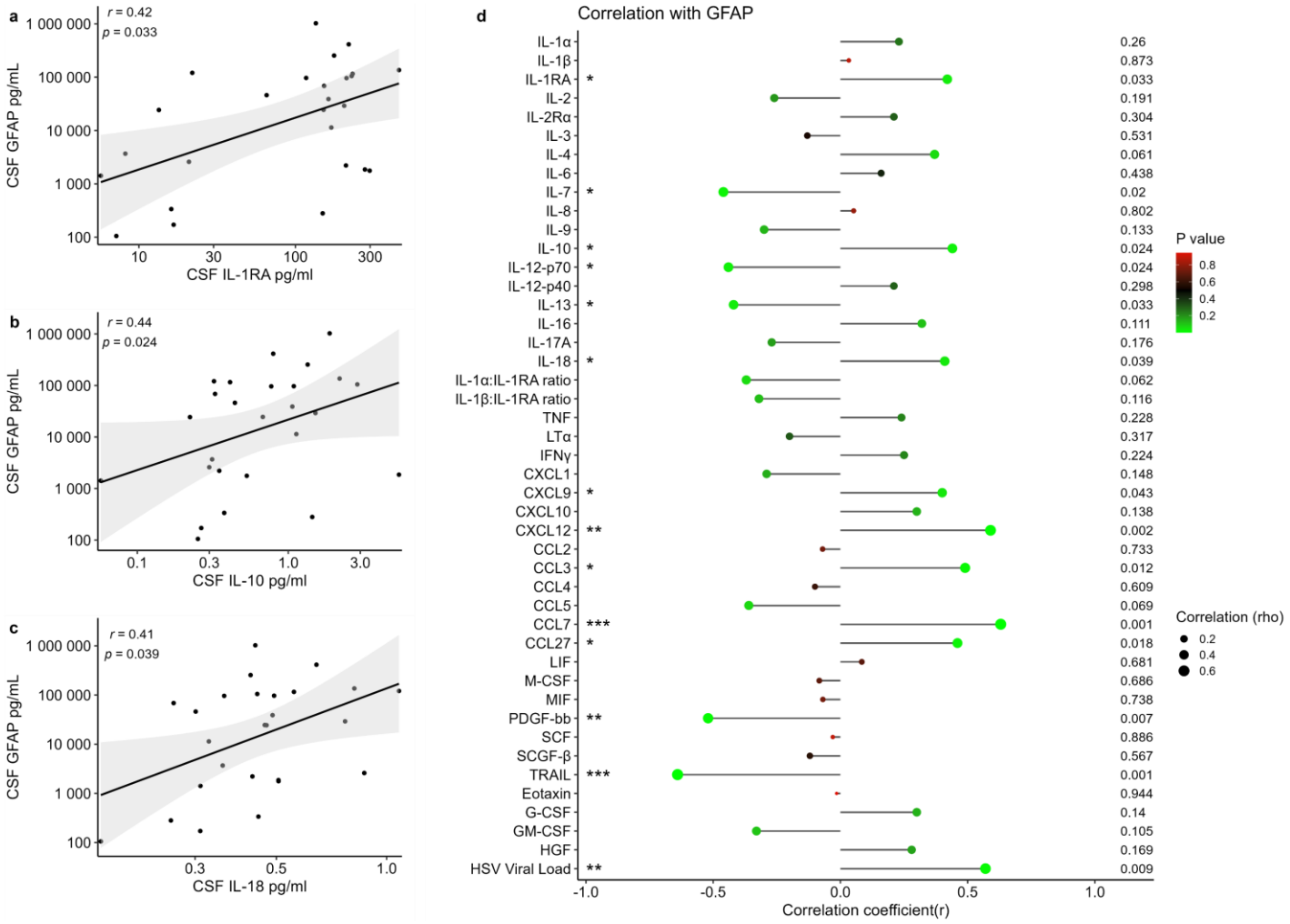


Fig. 3. CSF levels of Cytokines of the Interleukin-1 superfamily are associated with neuroglial injury in HSV encephalitis. a-c, Spearman correlation between the concentration of mediators and GFAP, a marker of astrocytic damage in CSF. **(a),** IL-1Ra **(b)**, IL-10 **(c)**, IL-18. The shaded areas represent the 95% confidence intervals (CIs). **d,** Forest plot of Spearman correlations between mediators and GFAP in **CSF**. Each data point represents one patient. Dot size corresponds to the Spearman correlation coefficient, while colour represents the P value.

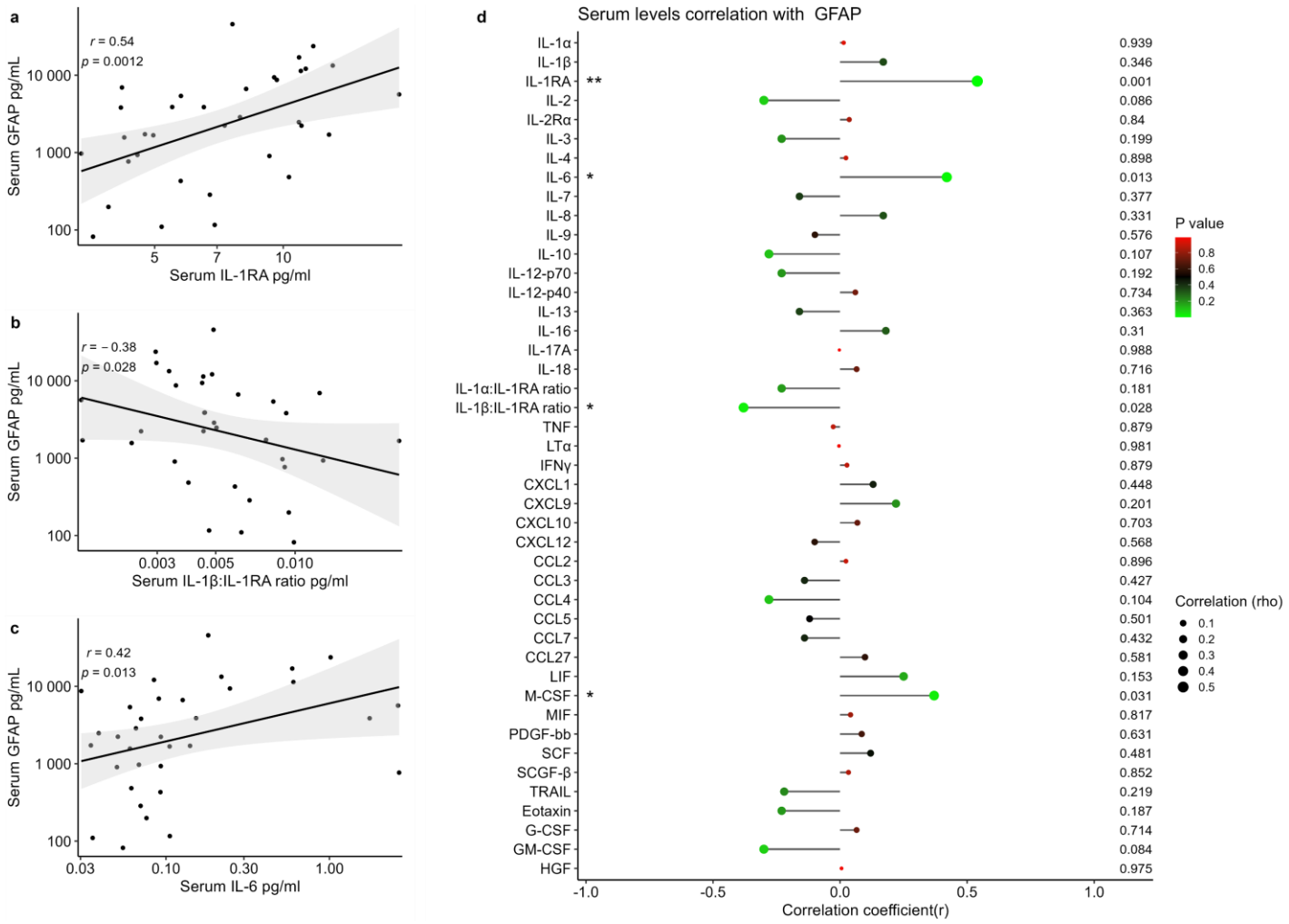


Fig. 4. Blood levels of cytokines of the Interleukin-1 superfamily and IL-6 are associated with neuroglial injury in HSV encephalitis. a-c, Spearman correlation between the concentration of mediators and GFAP, a marker of astrocytic damage in Serum. **(a)**, IL-1Ra **(b)**, IL1α:IL-1RA ratio **(c)**, IL-6. The shaded areas represent the 95% confidence intervals (CIs). **d**, Forest plot of Spearman correlations between mediators and GFAP in **Serum**. Each data point represents one patient. Dot size corresponds to the Spearman correlation coefficient, while colour represents the P value.

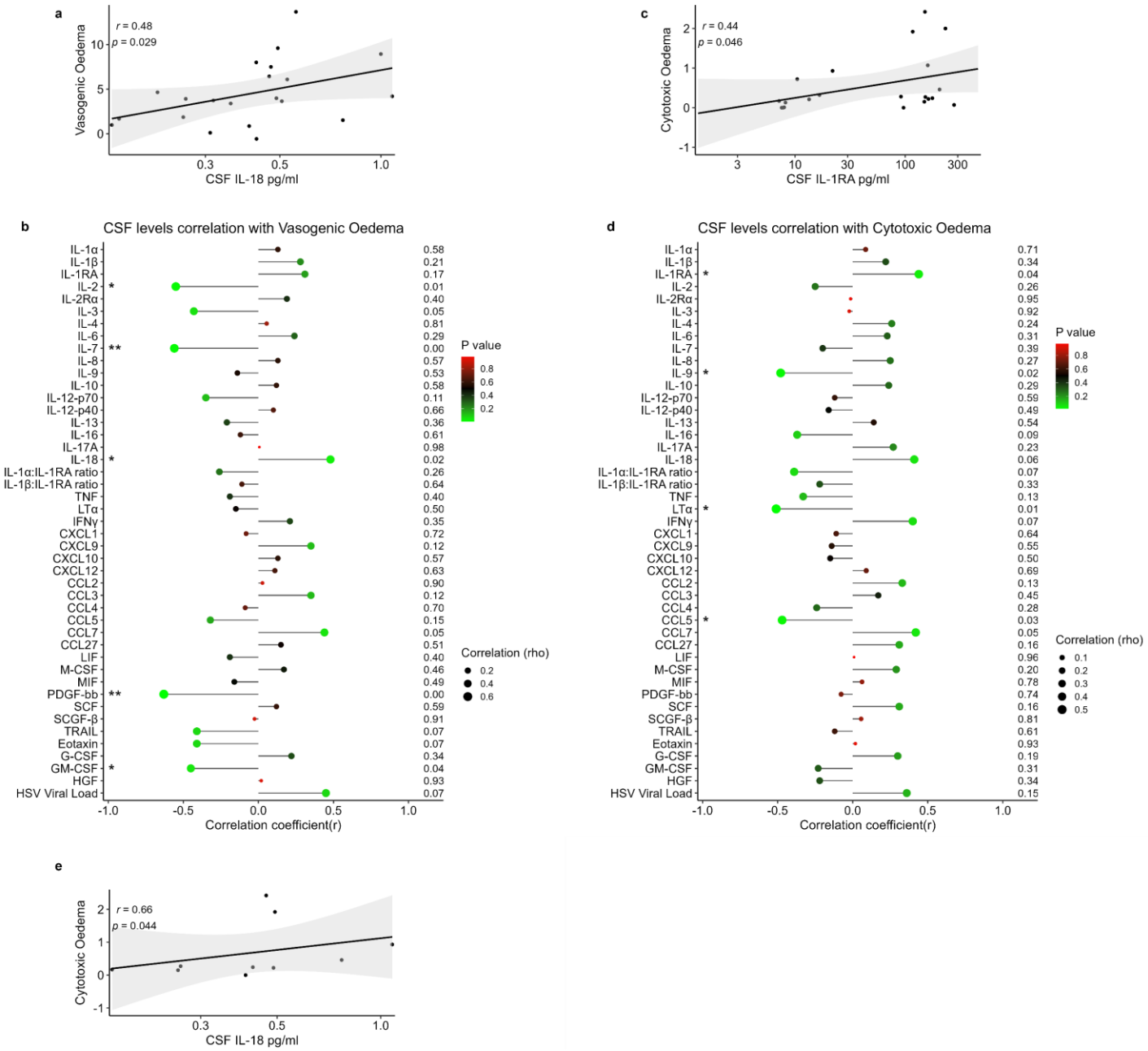


Figure 5. Cytokines of the Interleukin-1 superfamily are associated with neuroinflammation in HSV encephalitis. a, c, Spearman correlation between CSF concentration of mediators and MRI scans for all 38 participants with an *MRI scan and CSF sample collected at any time point*. **(a)**, IL-18 **(c)**, IL-1RA. The shaded areas represent the 95% confidence intervals (CIs). **b,d**, Forest plot of Spearman correlations between mediators and MRI scans. Each data point represents one patient. Dot size corresponds to the Spearman correlation coefficient, while colour represents the P value. **e**, Spearman correlation between CSF concentration of mediators and Cytotoxic oedema for **10 participants** with an MRI scan and CSF sample collected within 3 days.

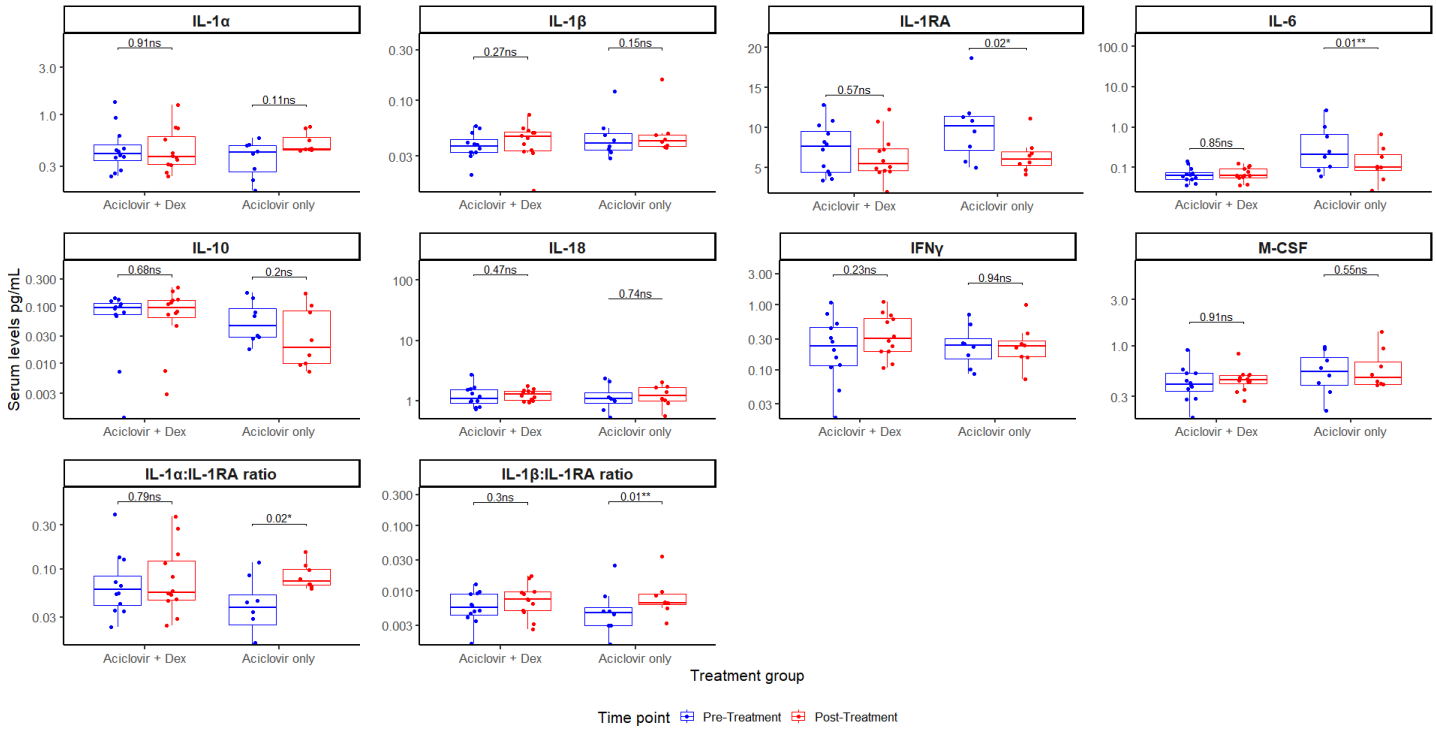


Fig. 6a. Blood mediator levels were unaffected by adjunct dexamethasone treatment. **Boxplot** comparing serum mediator levels before and after dexamethasone treatment. Means were compared using the Mann-Whitney paired test. The boxplots show the median and interquartile values; each data point represents one patient's clinical sample. n=12 and 8 for the aciclovir + Dex and aciclovir only groups, respectively.

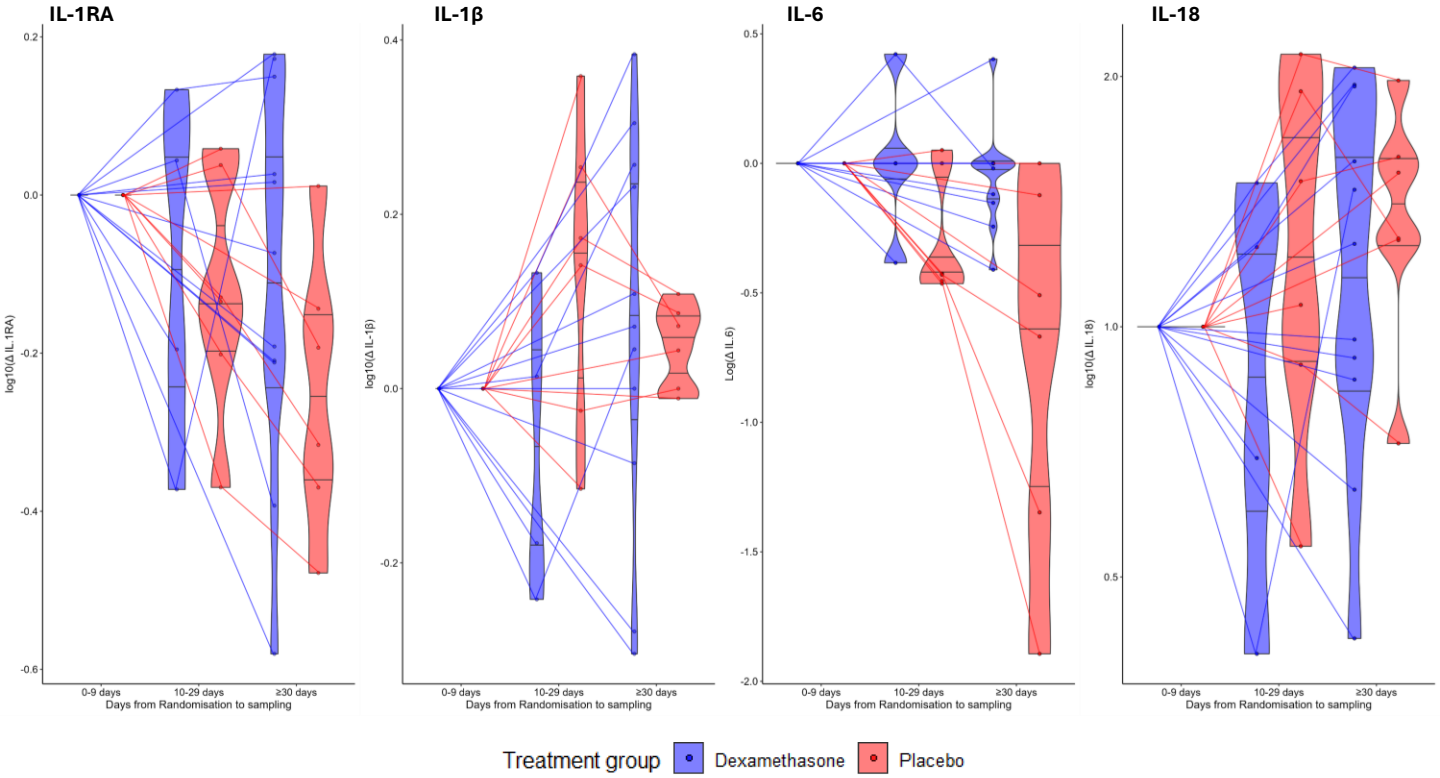


Figure 6b. Serum levels of cytokines were unaffected by adjunct dexamethasone treatment. A-d, Log fold change of serum mediator levels before (0-9 days after randomisation) and after dexamethasone treatment (>10 days post-randomisation), stratified by the treatment group. Each line represents paired samples from one participant. **(a)** IL-1RA, (**b**) IL-1β, (**c**) IL-6, **(d)** IL-18. Levels at 0-9 days were normalised to 0, and subsequent levels were expressed as a ratio of it. The violin plots show the median and interquartile log fold change levels; each data point represents one patient's clinical sample.

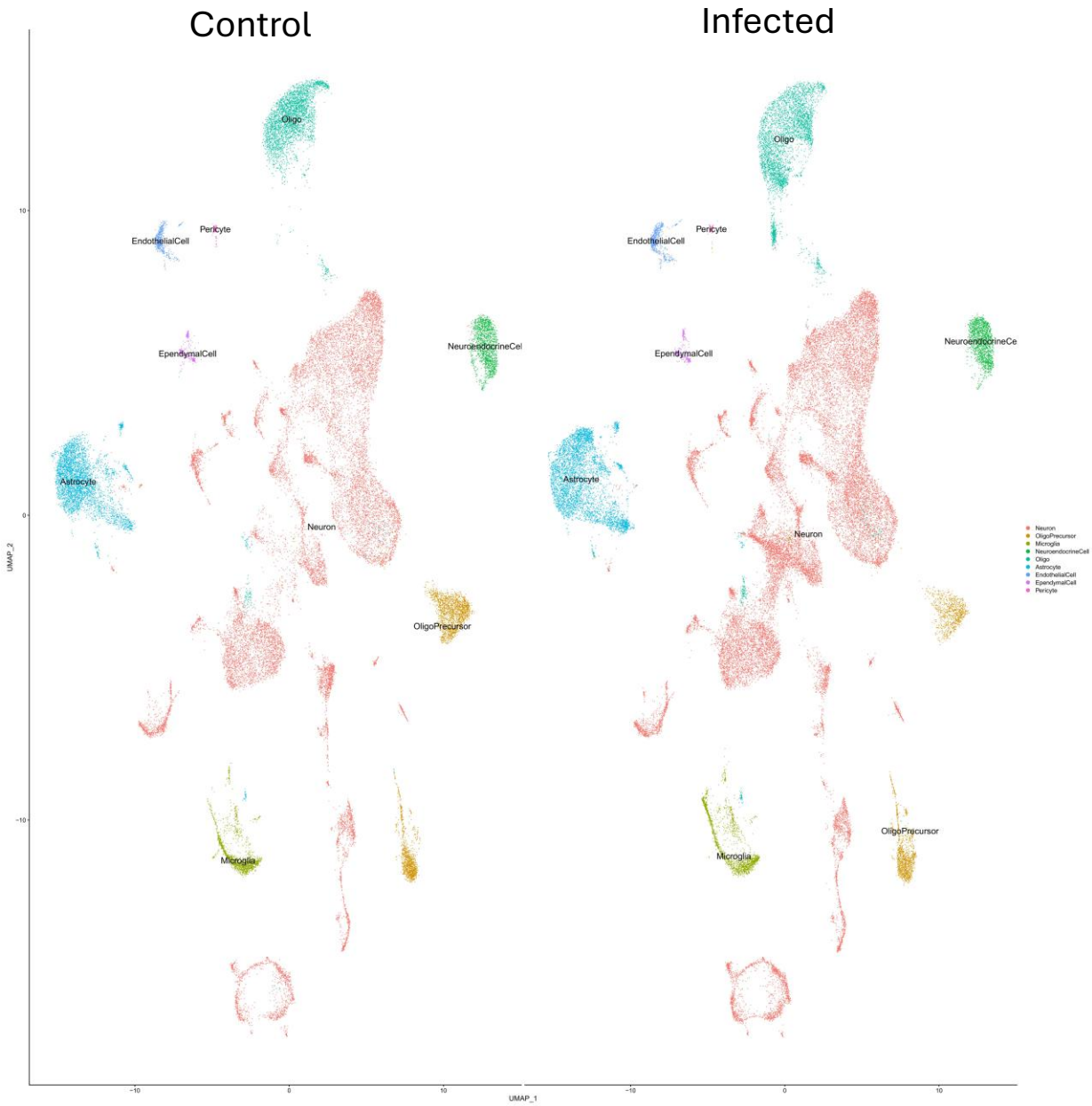


Fig 7. HSV infection induced neuronal and glial expression of IL-1β and IL-18 in a murine model

Fig 7a. UMAP plot of scRNAseq data. Colours are mapped to the clusters (annotated on the right); broader cell-type definitions are overlayed on the plot

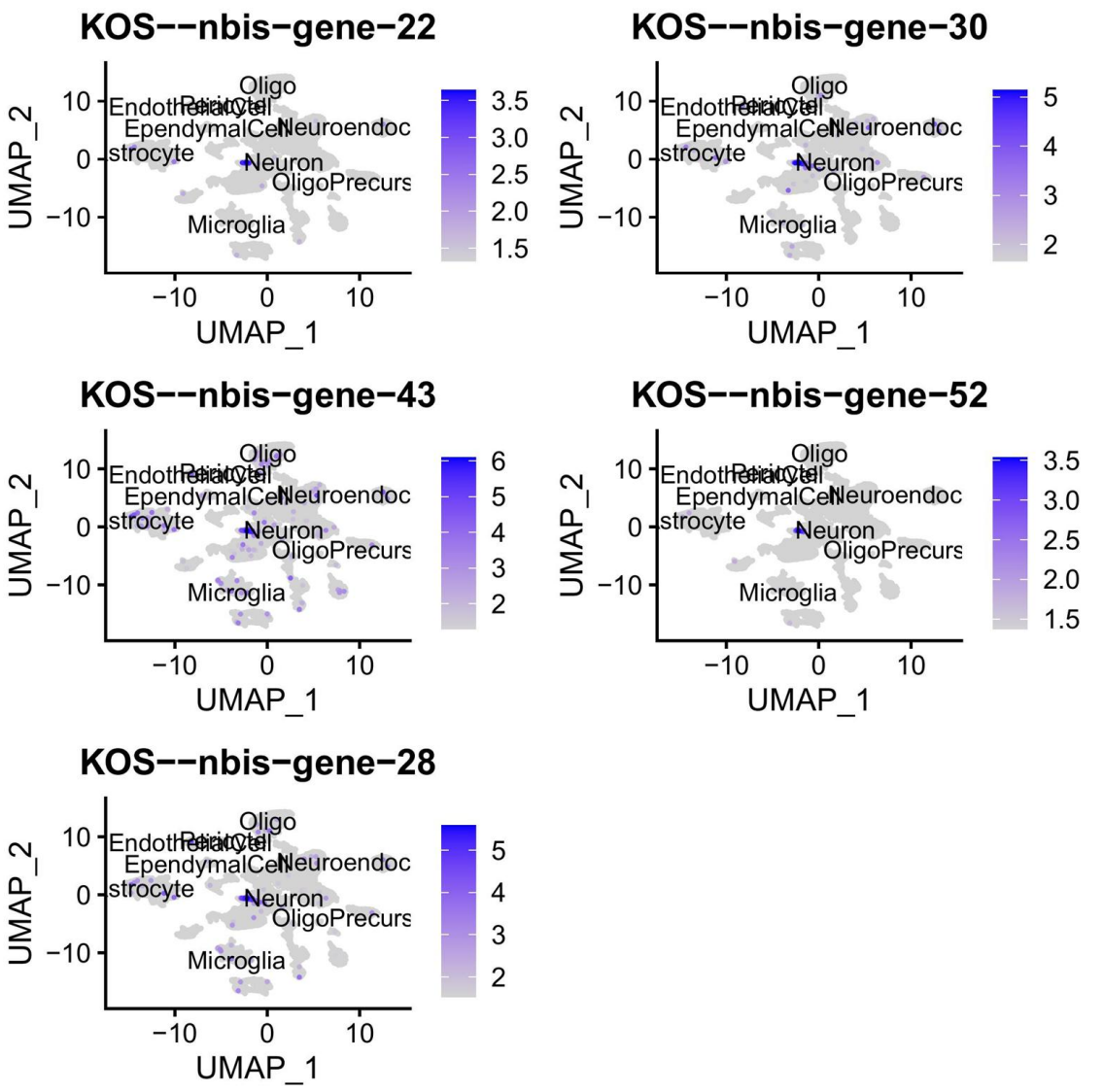


Fig 7b. Active viral infection were observed in neuronal and glial cells.
UMAP plot of scRNAseq data showing the expression of HSV infected mouse genes in different cell-types

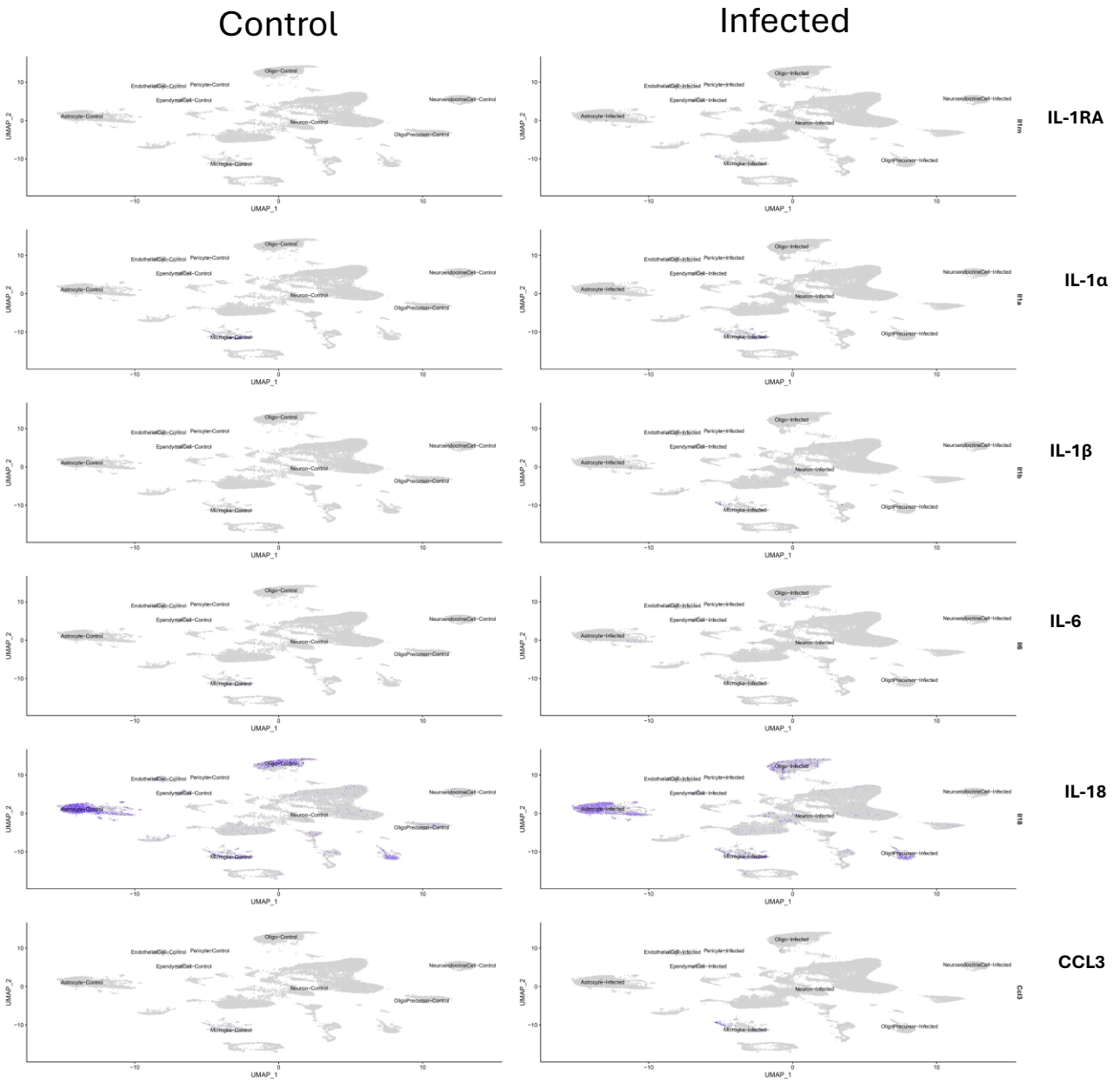


Fig 7c. Mediators were abundantly expressed in neuronal and glial cells. Abundance of 6 selected gene expression levels in both control (left) and infected (right) mouse cell types. Colour intensity represents the level of gene expression of each mediator in the specific cell type. Genes selected were: Il1a, Il1b, Il1ra, Il16, Il118, and ccl3.

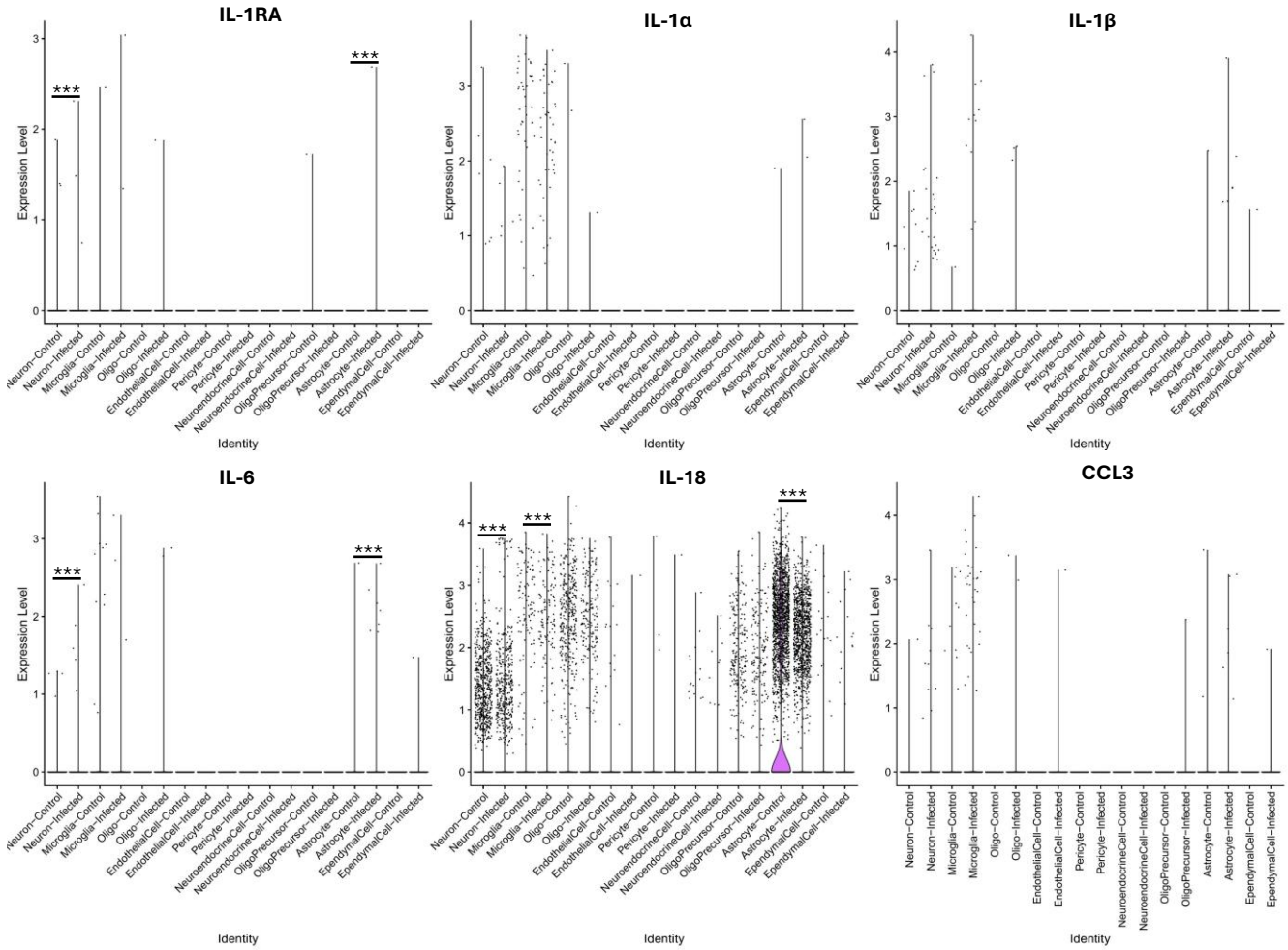


Fig 7d. HSV infection induced neuronal and microglial expression of the interleukin-1 cytokines and IL-6.

Expression levels of selected mediators in uninfected and infected mouse brain cell types. P values were determined by Bonferroni corrected non-parametric Wilcoxon rank sum test. Bar plots show mean expression levels. Each dot represents an expression in one cell

Table 2a

Clinical severity: GCS<15/GCS=15

Protein	p_value	Log2FC
IL-1RA	0.00305	1.27553
IL-12p70	0.01267	-0.72198
IL-18	0.01485	0.517
IFNγ	0.04635	0.8114
CCL3	0.01733	0.86715
CCL5	0.00305	-6.81086
CCL7	0.00305	1.80031
PDGFbb	0.0356	-0.4507
G-CSF	0.00539	0.68826

Outcome: LoS=2/LoS>2

Protein	p_value	Log2FC
IL-12p70	0.00196	-1.06134
GM-CSF	0.02552	-0.66528
IL-2	0.03584	-0.58144
IL-17A	0.03033	-0.404
IL-18	0.00989	0.56206
IL-1RA	0.02136	0.97609
CCL7	0.03584	1.43173

Table 2b

ID	Description	Ratio	Adjusted p value	qvalue	Count
hsa04060	Cytokine-cytokine receptor interaction	8/9	1.10368E-09	6.64E-10	8
hsa05144	Malaria	4/9	4.25913E-06	2.56E-06	4
hsa05323	Rheumatoid arthritis	4/9	3.191E-05	1.92E-05	4
hsa04061	Viral protein interaction with cytokine and cytokine receptor	4/9	3.191E-05	1.92E-05	4
hsa05142	Chagas disease	4/9	3.191E-05	1.92E-05	4
hsa05143	African trypanosomiasis	3/9	6.93039E-05	4.17E-05	3
hsa04630	JAK-STAT signaling pathway	4/9	0.000157347	9.46E-05	4
hsa05164	Influenza A	4/9	0.000157347	9.46E-05	4
hsa05321	Inflammatory bowel disease	3/9	0.000268015	0.000161	3
hsa05417	Lipid and atherosclerosis	4/9	0.00030189	0.000182	4
hsa04657	IL-17 signaling pathway	3/9	0.000653316	0.000393	3
hsa04620	Toll-like receptor signaling pathway	3/9	0.000901681	0.000542	3
hsa05330	Allograft rejection	2/9	0.003268126	0.001966	2
hsa05152	Tuberculosis	3/9	0.003268126	0.001966	3
hsa05168	Herpes simplex virus 1 infection	3/9	0.003268126	0.001966	3
hsa04062	Chemokine signaling pathway	3/9	0.003636029	0.002187	3
hsa04940	Type I diabetes mellitus	2/9	0.003662212	0.002203	2
hsa05134	Legionellosis	2/9	0.005594161	0.003365	2
hsa05140	Leishmaniasis	2/9	0.010471975	0.006299	2
hsa04623	Cytosolic DNA-sensing pathway	2/9	0.010964371	0.006595	2
hsa04658	Th1 and Th2 cell differentiation	2/9	0.013056483	0.007854	2
hsa05146	Amoebiasis	2/9	0.015220957	0.009155	2
hsa05145	Toxoplasmosis	2/9	0.0171446	0.010313	2
hsa05418	Fluid shear stress and atherosclerosis	2/9	0.025682608	0.015448	2
hsa04621	NOD-like receptor signaling pathway	2/9	0.043241673	0.02601	2

Table 2. KEGG pathway enrichment analysis on the significantly different CSF cytokines revealed commonalities to other inflammatory conditions, including the JAK-STAT signalling pathway, NOD-like receptor signalling pathway, Inflammatory bowel disease, and HSV-1 infection

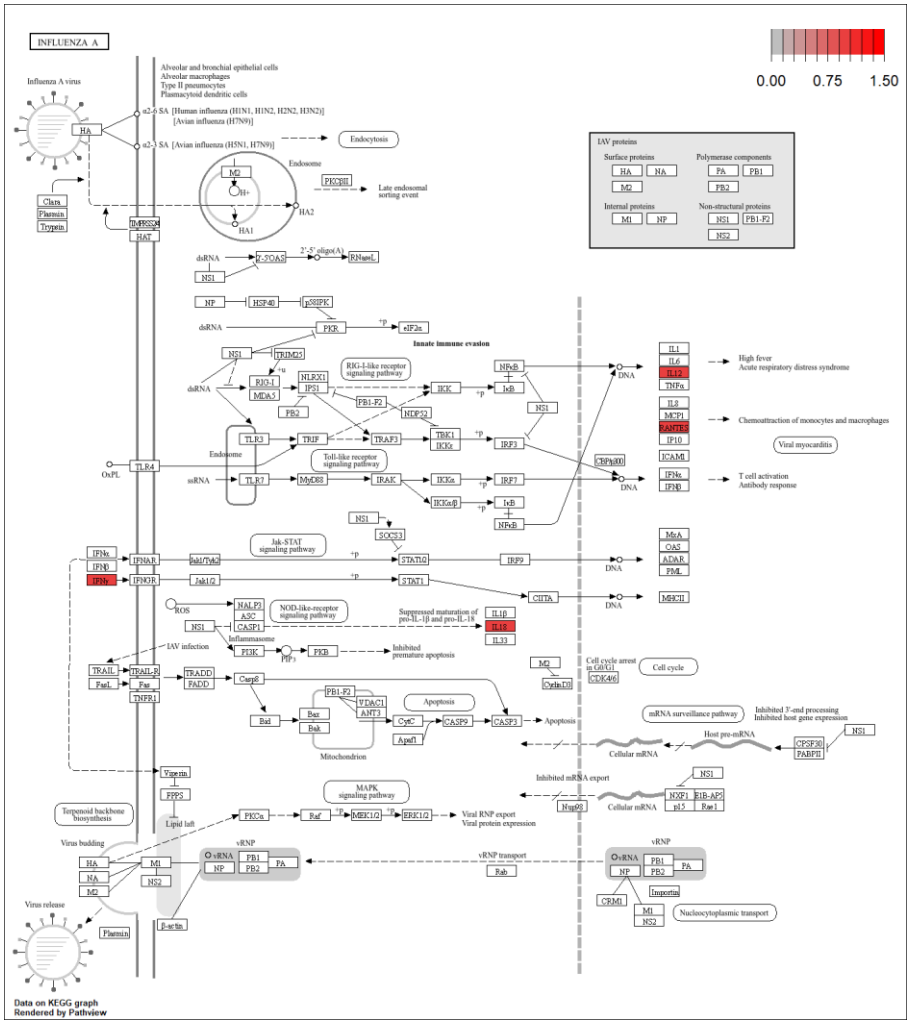
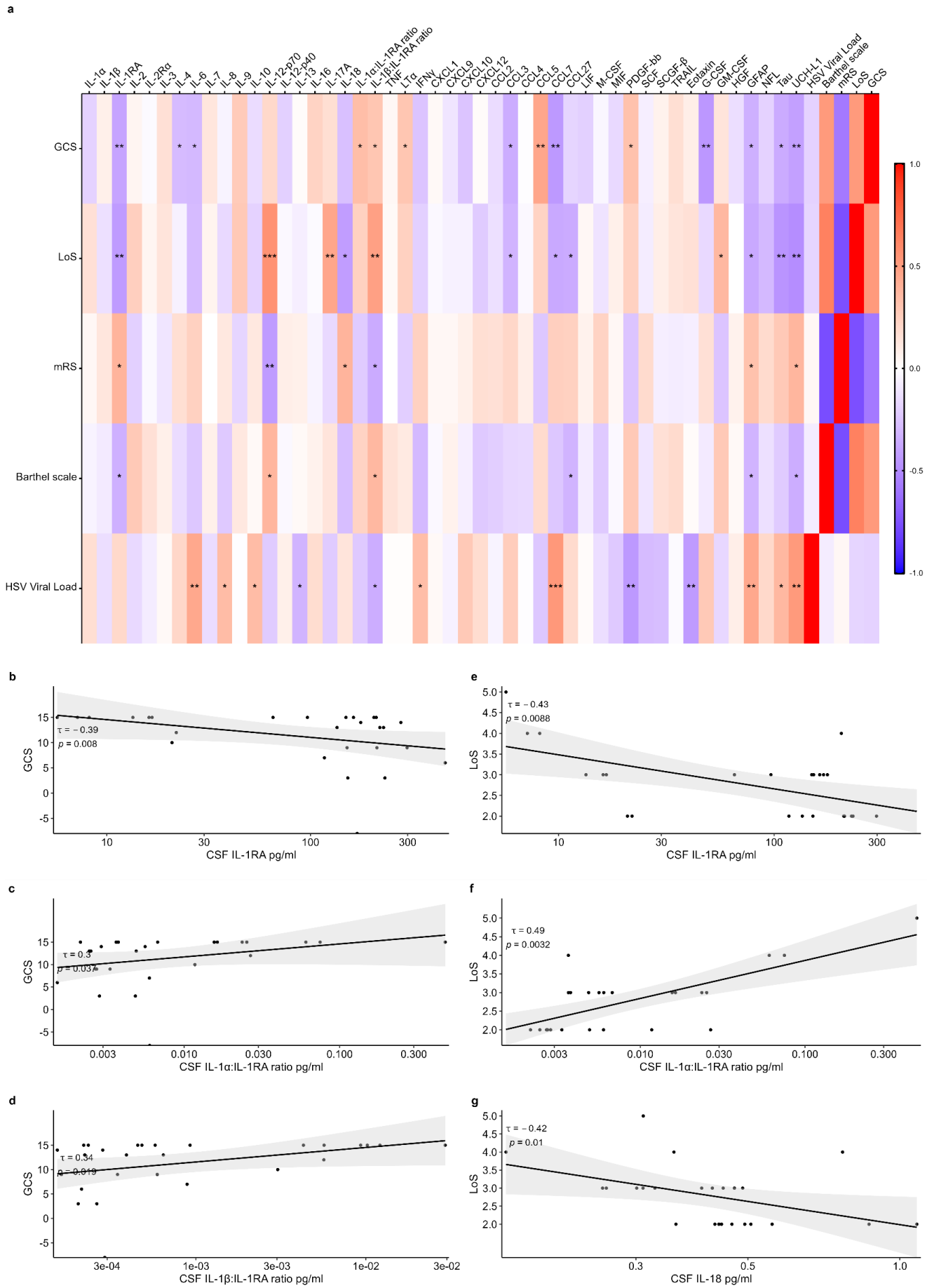
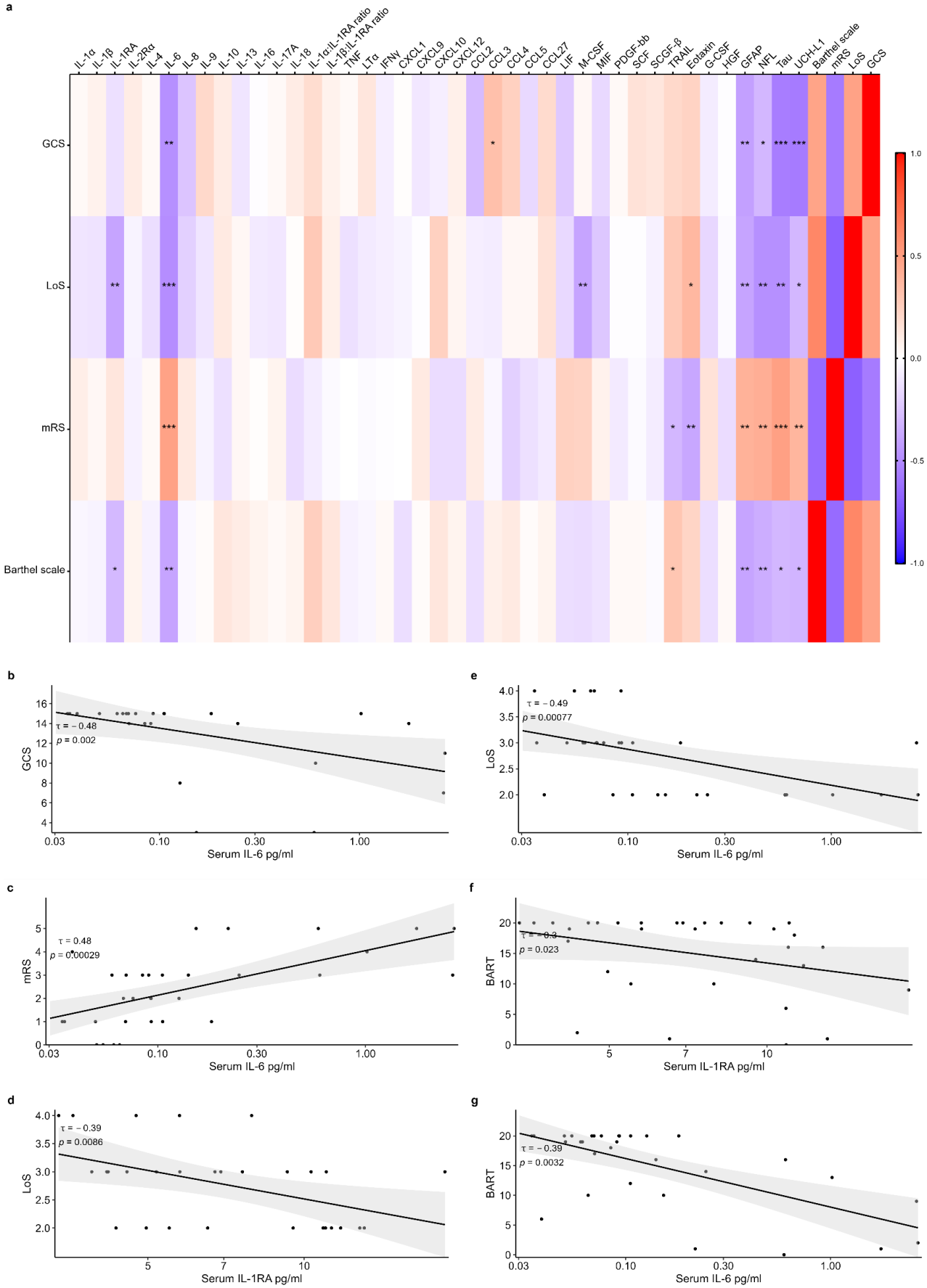


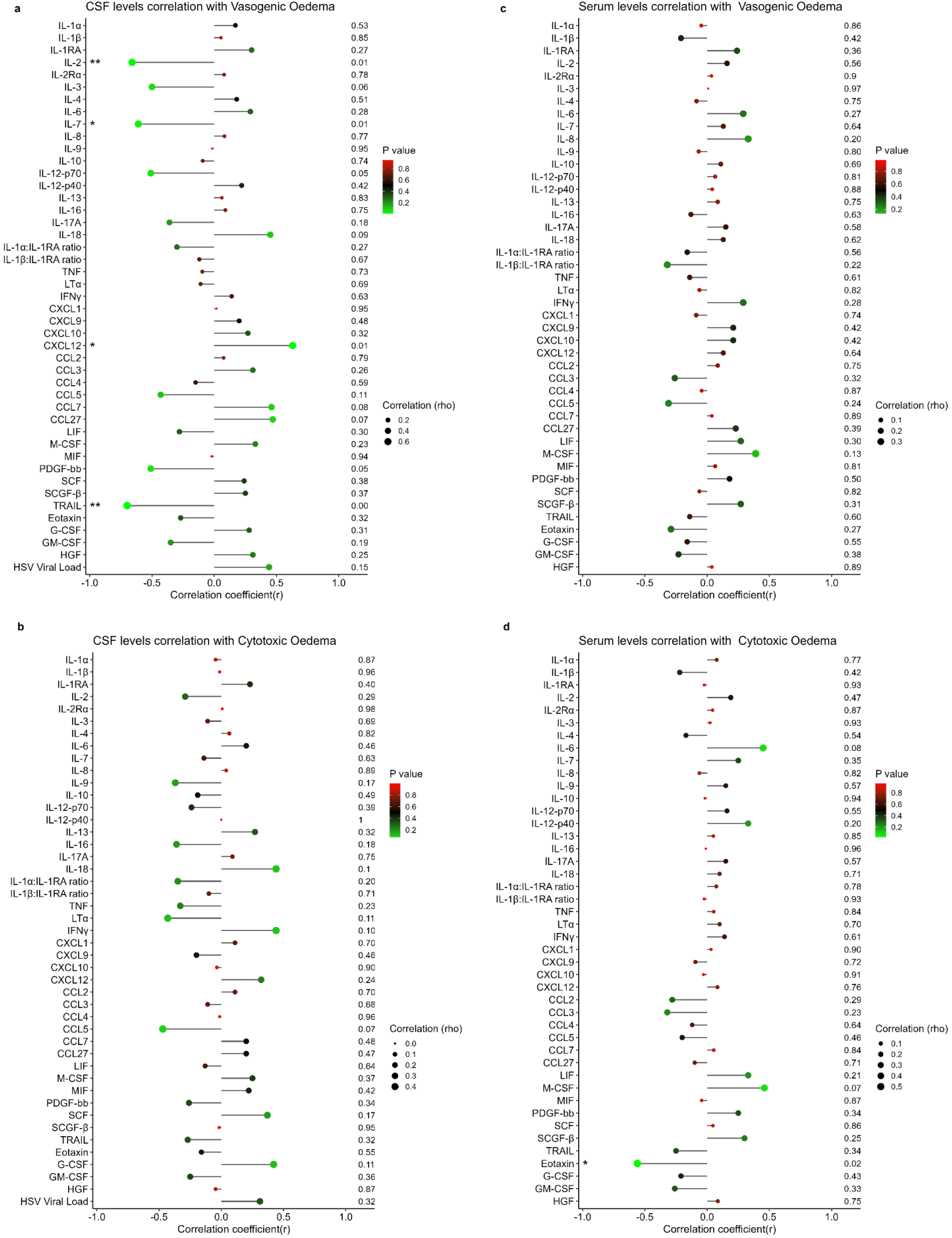
Figure 8. Influenza A pathways highlighting the cytokines associated with clinical severity from our study. Diagram was generated and visualised using the `pathview` package (Luo & Brouwer, 2020). It overlaid the log2 fold-change values of each mediator, providing a visual representation of their regulation within biological contexts



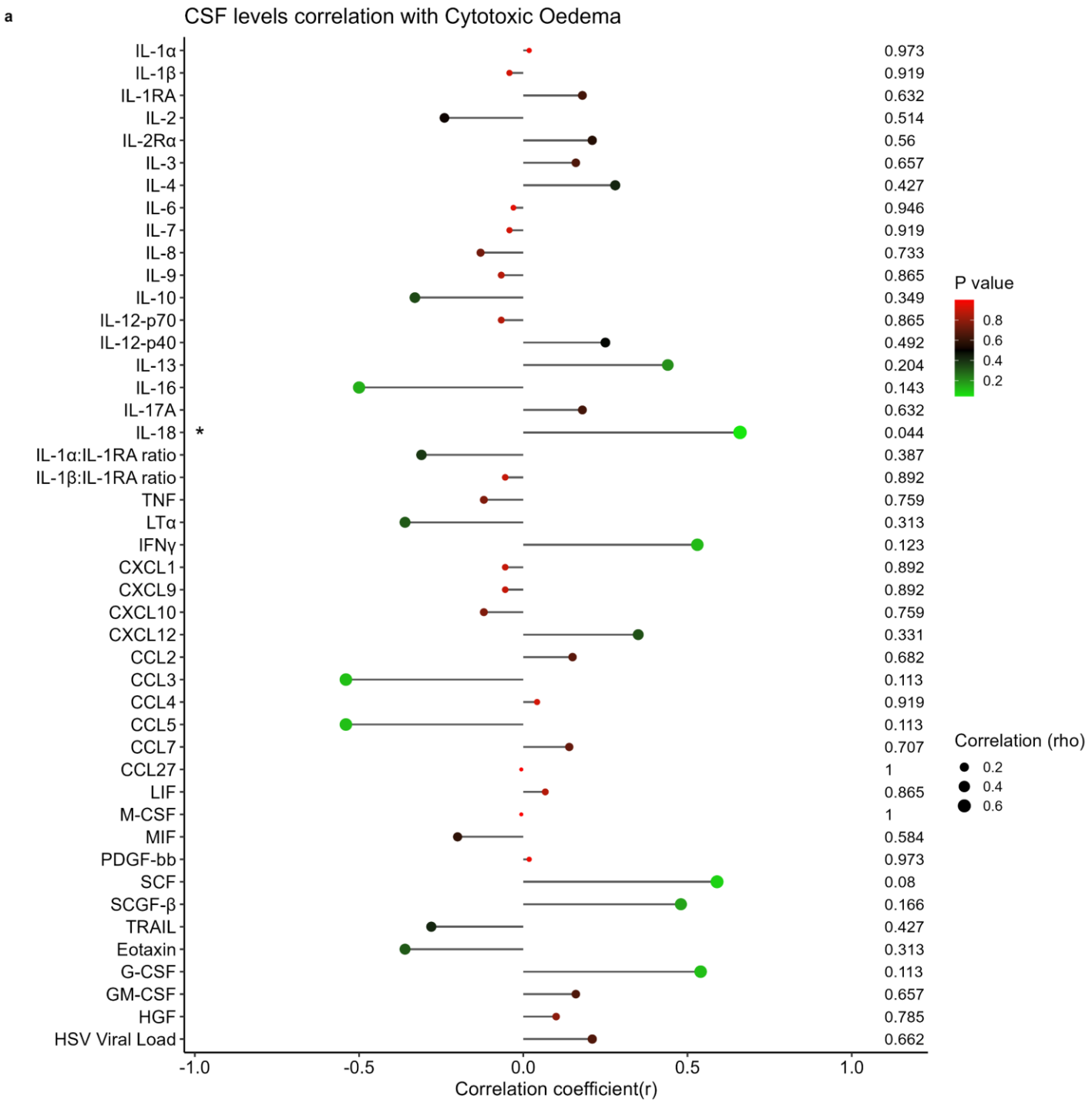
Extended Figure 1. Association between clinical severity, outcome scores and biomarkers in CSF. a) Kendall's correlation matrix of mediators, HSV viral load, GFAP, NFL, T-Tau, UCHL1, GCS, LoS, mRS, and the Barthel Scale. **b – d)** correlation between GCS scores and (b) IL-1RA, (c) IL-1α:IL-1RA ratio, and (d) IL-1β:IL-1RA ratio ; **e-g)** association between LoS and (e) IL-1RA, (f) IL-1α:IL-1RA ratio, and (g) IL-18



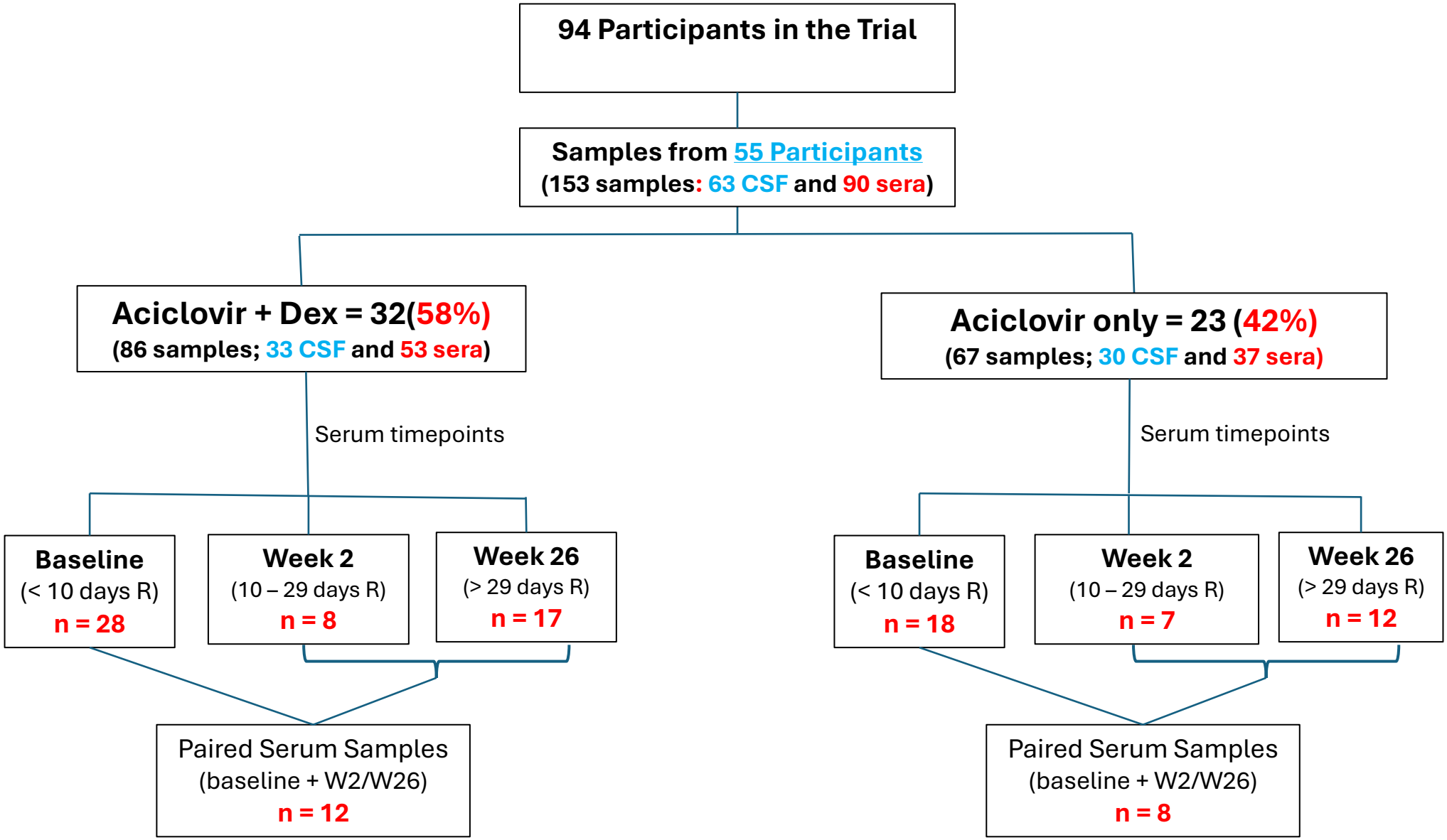
Extended Figure 2. Association between clinical severity, outcome scores and biomarkers in blood. a) Kendall's correlation matrix of mediators, HSV viral load, GFAP, NFL, T-Tau, UCHL1, GCS, LoS, mRS, and the Barthel Scale. **b-g)** correlation plots between (b) GCS and IL-6, (c) mRS and IL-6; (d) Los and IL-1β:IL-1RA ratio; (e) LoS and IL-6; (f) Barth scale score and IL-1RA, and (g) Barth scale score and IL-6



Extended Figure 3. Correlation between mediators and MRI scans. a-d, Forest plot of Spearman correlations between mediators and MRI scan. a, correlation between CSF mediator levels and Vasogenic oedema; b, correlation between CSF mediator levels and Cytotoxic oedema; c, correlation between Serum mediator levels and Vasogenic oedema; d, correlation between Serum mediator levels and Cytotoxic oedema. Each data point represents one patient. Dot size corresponds to the Spearman correlation coefficient, while colour represents the P value.



Supplementary Figure 1. Interleukin-18 drives neuroinflammation in HSV encephalitis. a**,** Forest plot of Spearman correlation between CSF concentration of mediators and Cytotoxic oedema for **10 participants** with an *MRI scan and CSF sample collected within 3 days*. Each data point represents one patient. Dot size corresponds to the Spearman correlation coefficient, while colour represents the P value.



Supplementary Figure 2. Flow chart of participants and samples collected.
R = Randomisation; W= Week