Supplementary File

mRNA Profiling of Inflammatory Stress Responses after Aquaporin-4 Antibody and Human Complement Treatment Reveals Upregulation of NF-κB and IL6 Pathways

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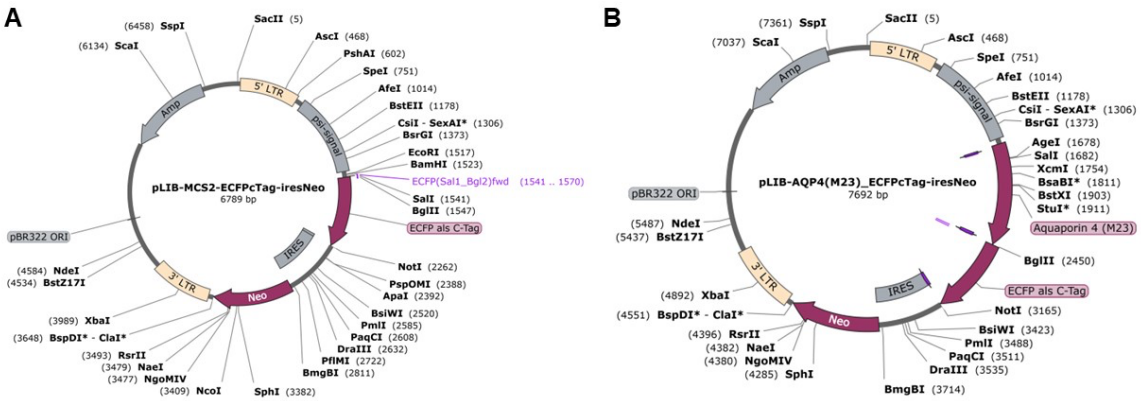
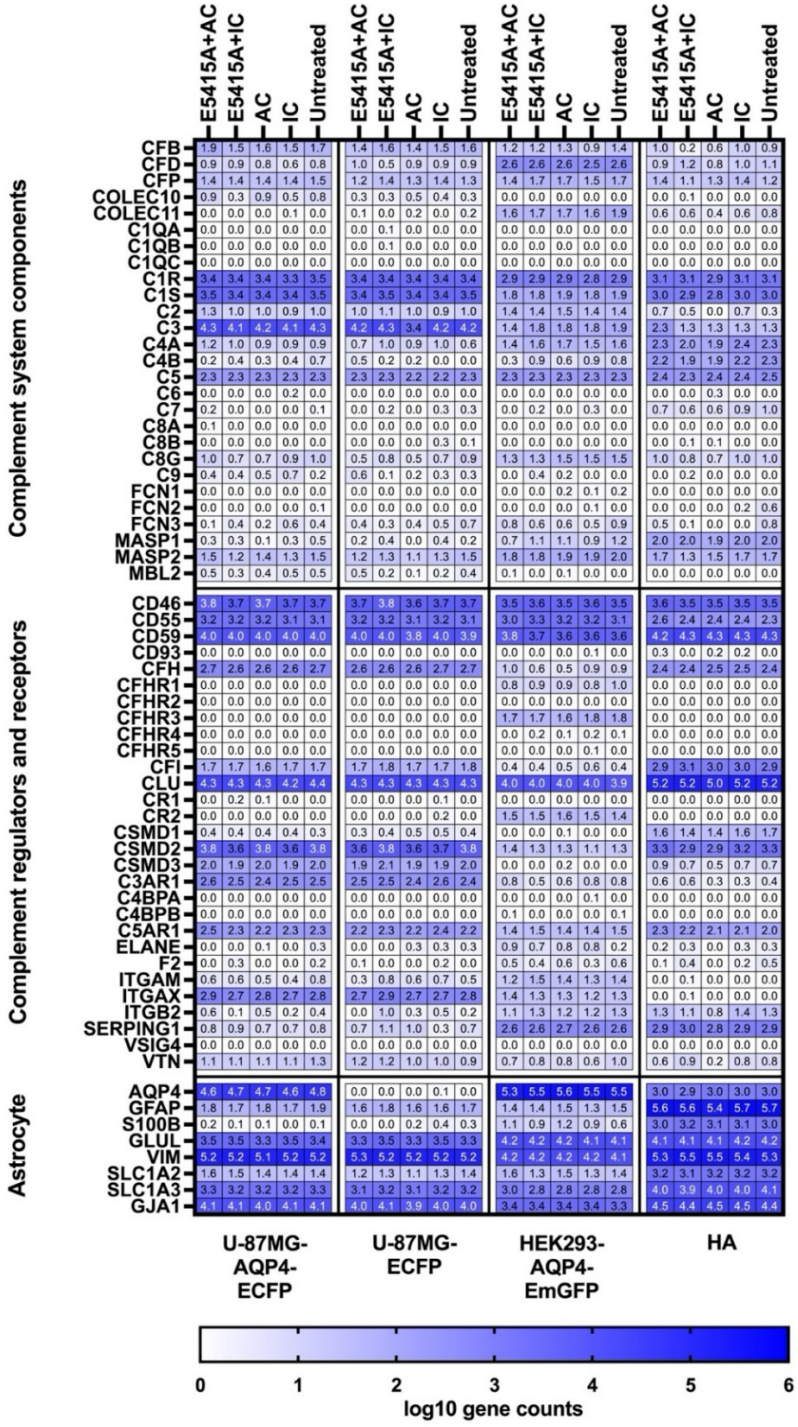
Supplementary Figures

Supplementary Fig. 1



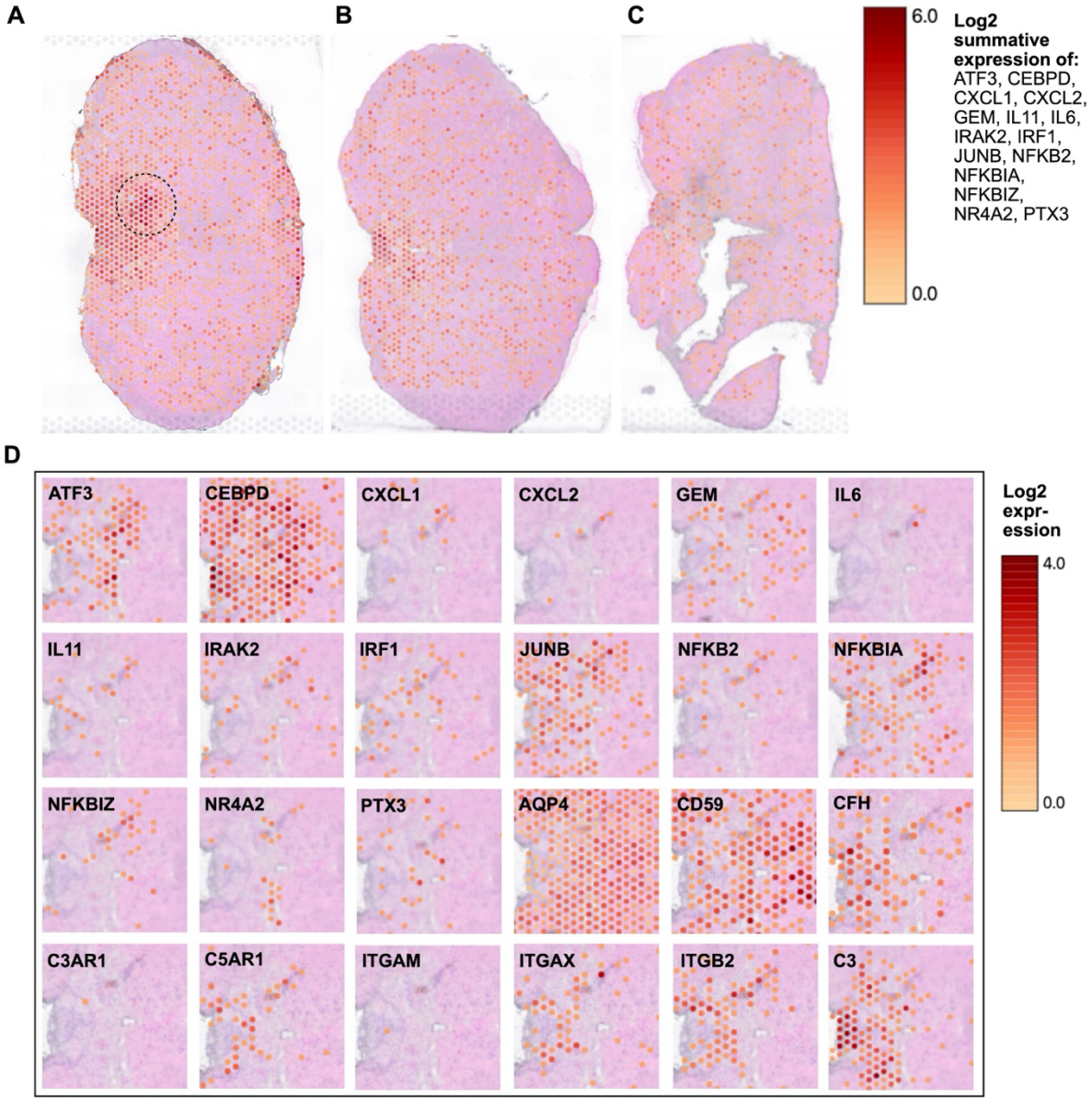
Plasmid maps of pLIB-MCS2-ECFPcTag-iresNeo and pLIB-AQP4M23-ECFOcTag-iresNeo to generate U-87MG-AQP4-ECFP and U-87MG-ECFP cells. Maps were created with the SnapGene software (www.snapgene.com)

Supplementary Fig. 2



Log10-transformed gene counts obtained by mRNA sequence analysis of four different cell lines after treatment with E5415A, and/or human complement. The upper panel shows complement components, the middle panel shows complement regulators and receptors, and the lower panel shows astrocyte-associated genes.

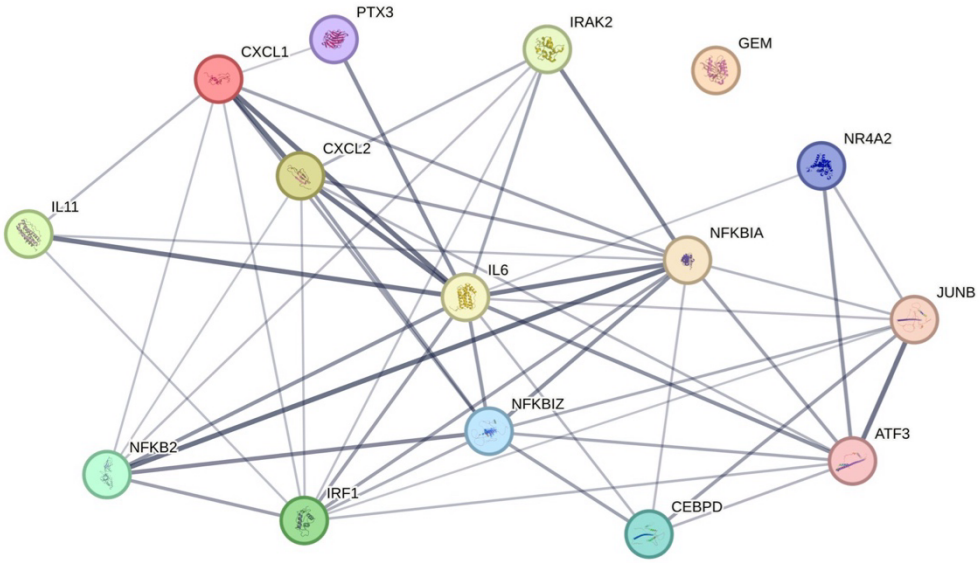
Supplementary Fig. 3



Characterization of AQP4 antibody E5415A induced molecular changes in Lewis rats analyzed by spatial transcriptomics. (**A**) Loupe Browser image of the medulla oblongata of a Lewis rat treated with E5415A at day 0 and day 1 and sacrificed at day 2. The encircled area in this tissue section contains an inflamed blood vessel and was further analyzed by spatial transcriptomics. (**B**) Loupe Browser image of the medulla oblongata of a Lewis rat treated with E5415A at day 0 and sacrificed at day 1. (**C**) Loupe Browser image of the medulla oblongata of a healthy, untreated Lewis rat. The red dots in these tissue sections represent a bar-coded spot in which the differentially regulated transcripts identified in this study were

upregulated. The color scale shows the log2 expression levels of these transcripts. (**D**) Loupe Browser images of the medulla oblongata of the Lewis rat treated with E5415A at day 0 and day 1 and sacrificed at day 2 (shown in A), showing the individual expression of differentially regulated transcripts, AQP4 and complement genes, and their receptors.

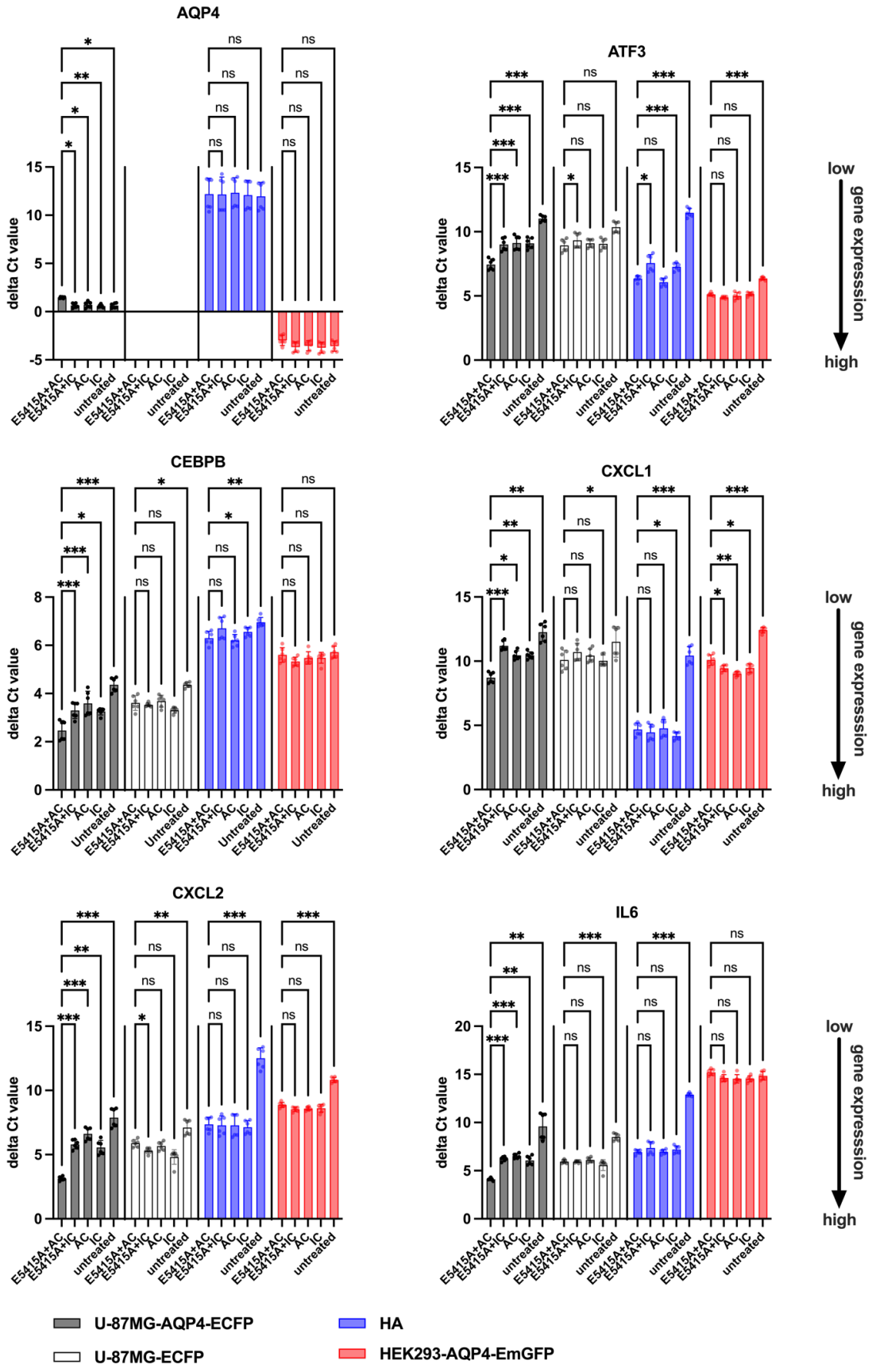
Supplementary Fig. 4

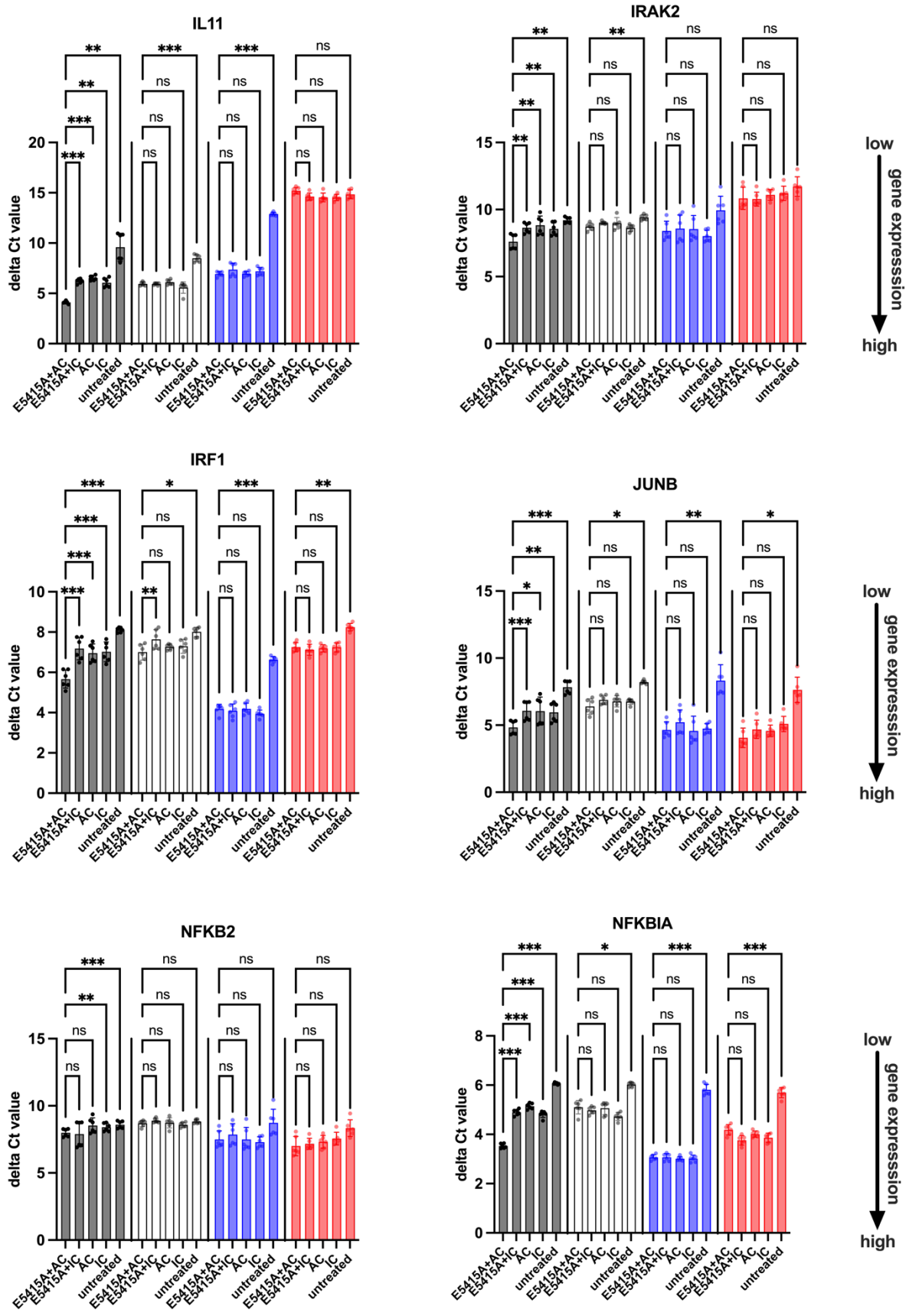


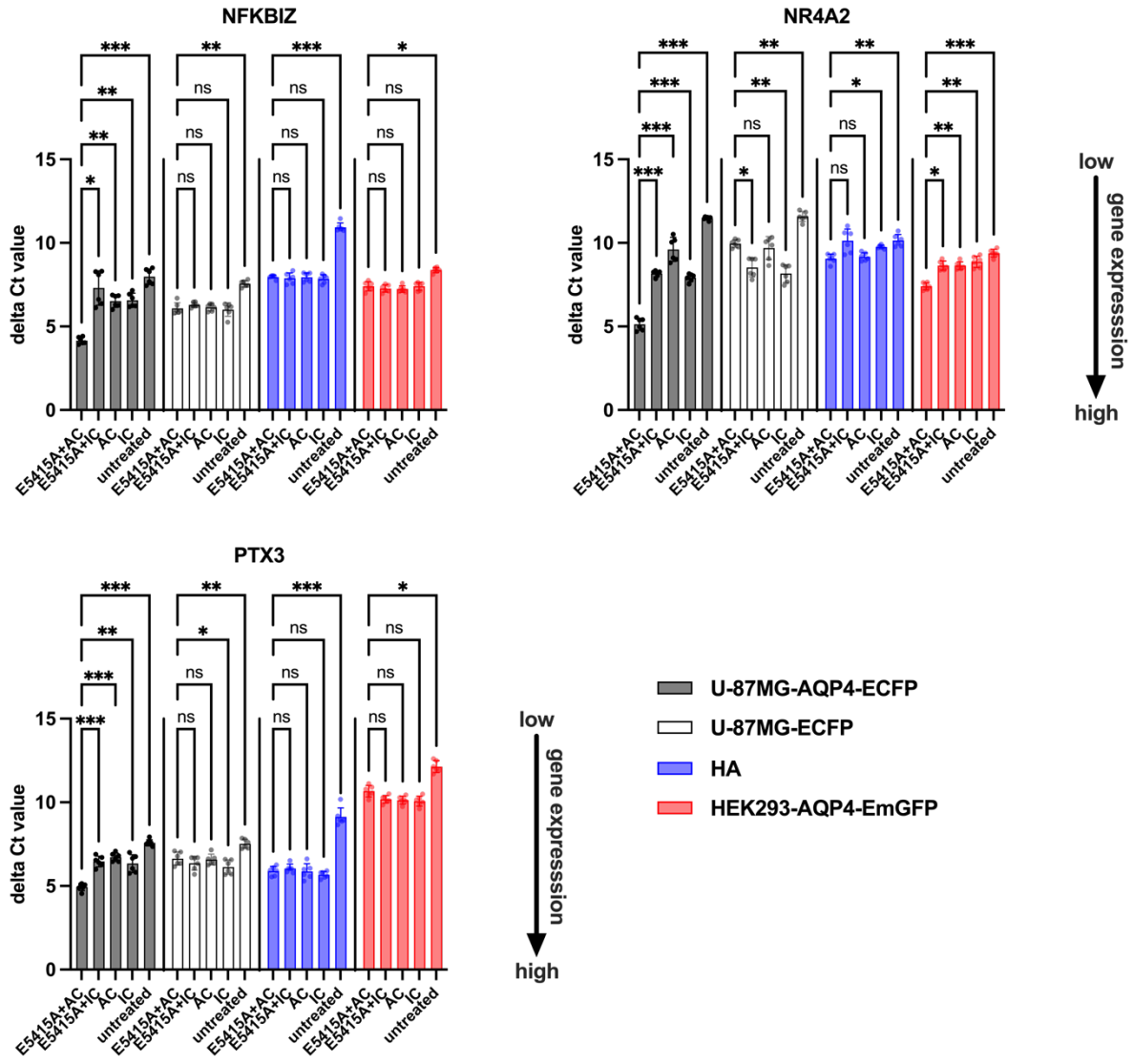
STRING Network analysis of differentially expressed genes, visualized as full STRING network (the edges indicate both functional and physical protein associations). The thickness of connecting lines indicates the strength of data support. Medium confidence (0.400) was selected for minimum required interaction score. Network stats: number of nodes = 15, number of edges = 50, average node degree = 6.67, avg. local clustering coefficient = 0.759, expected number of edges = 5, PPI enrichment p-value = < 1.0^{-16}; STRING database.

(https://string-db.org/cgi/network?taskId=bzHCAHygMp6t&sessionId=blIftJZlPpgh 05.02.25)

Supplementary Fig. 5

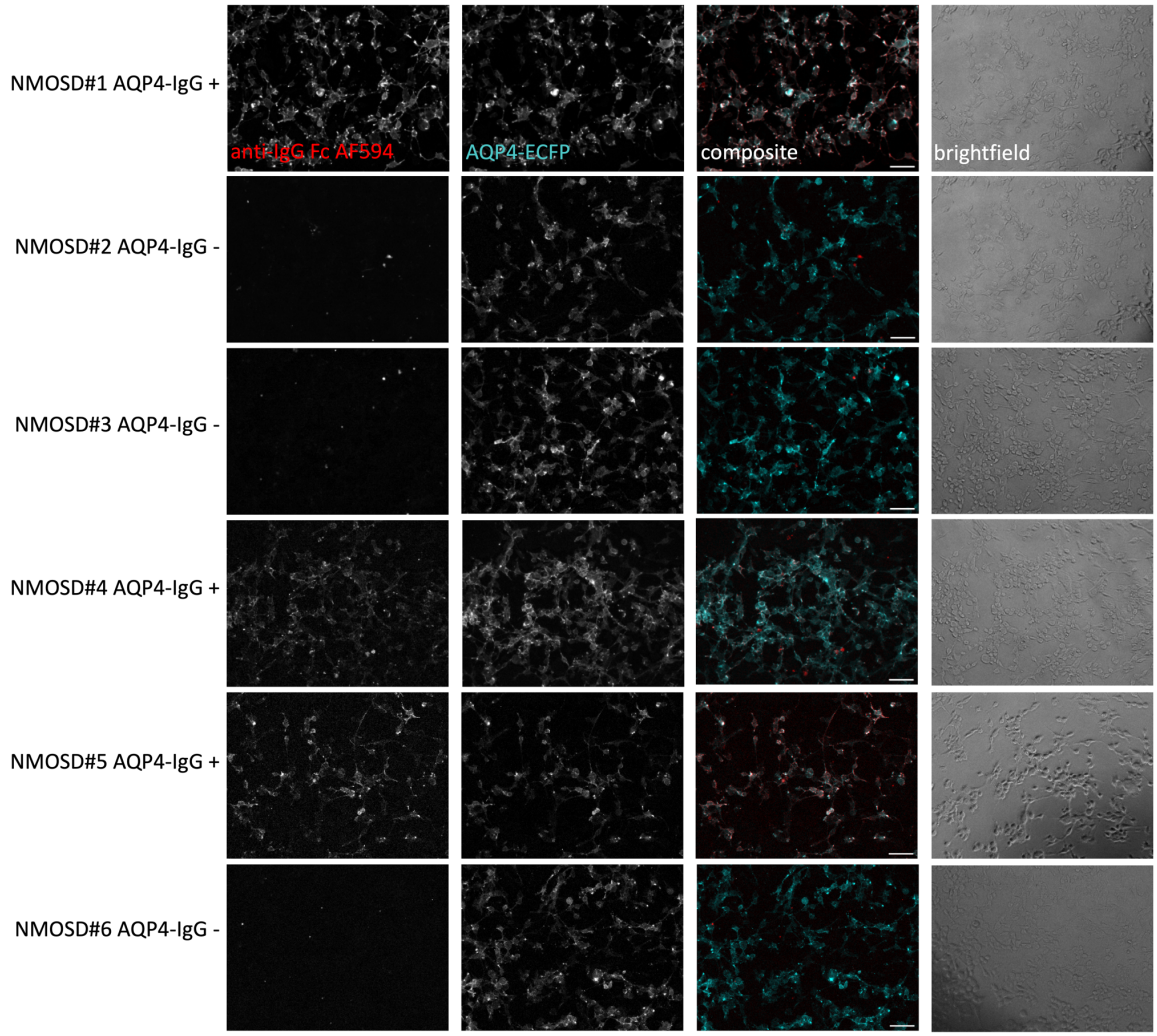



IL11
IRAK2
IRF1
JUNB
NFKB2
NFKBIA
delta Ct value
low
gene expression
high
E5415A+AC
E5415A+IC
AC
IC
untreated
U-87MG-AQP4-ECFP
U-87MG-ECFP
HA
HEK293-AQP4-EmGFP



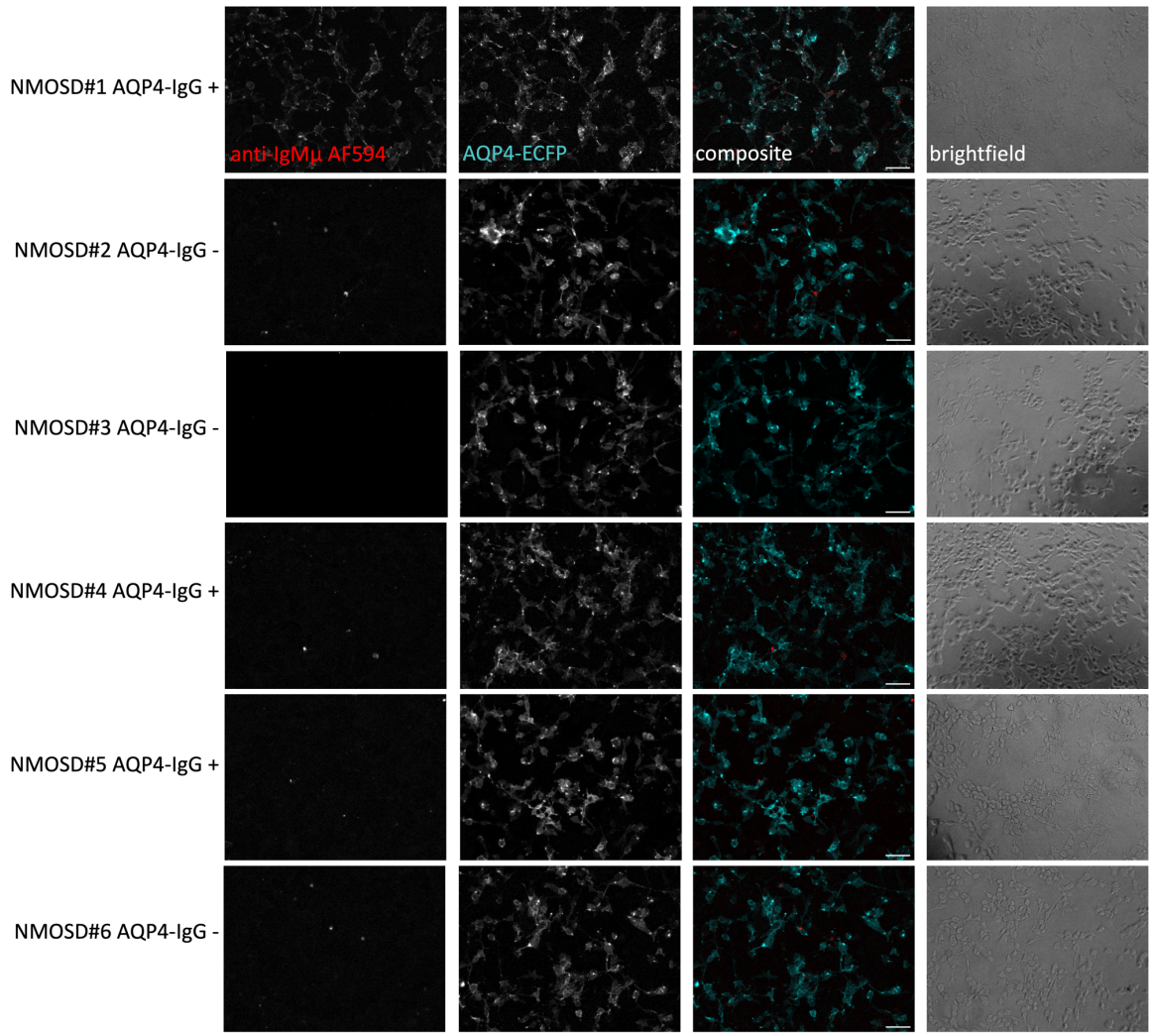
Validation of results from RNA-seq by quantitative real-time reverse transcriptase-PCR analysis. Gene expression is visualized with ΔCt values. Ct values of GAPDH housekeeping gene were subtracted from the Ct values of the respective genes. Higher values indicate a lower gene expression. Samples with no detectable gene expression are not shown. The data represent two biologically independent experiments (triplicates each) and are shown as means ± SD and individual data values. Data were analyzed using two-way ANOVA Šídák's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to E5145A+AC of each cell type. AC = active complement, AQP4 = aquaporin-4, ECFP = enhanced cyan fluorescent protein, EmGFP = emerald green fluorescent protein, HA = human astrocytes, IC = inactive complement, ns = not significant.

Supplementary Fig. 6



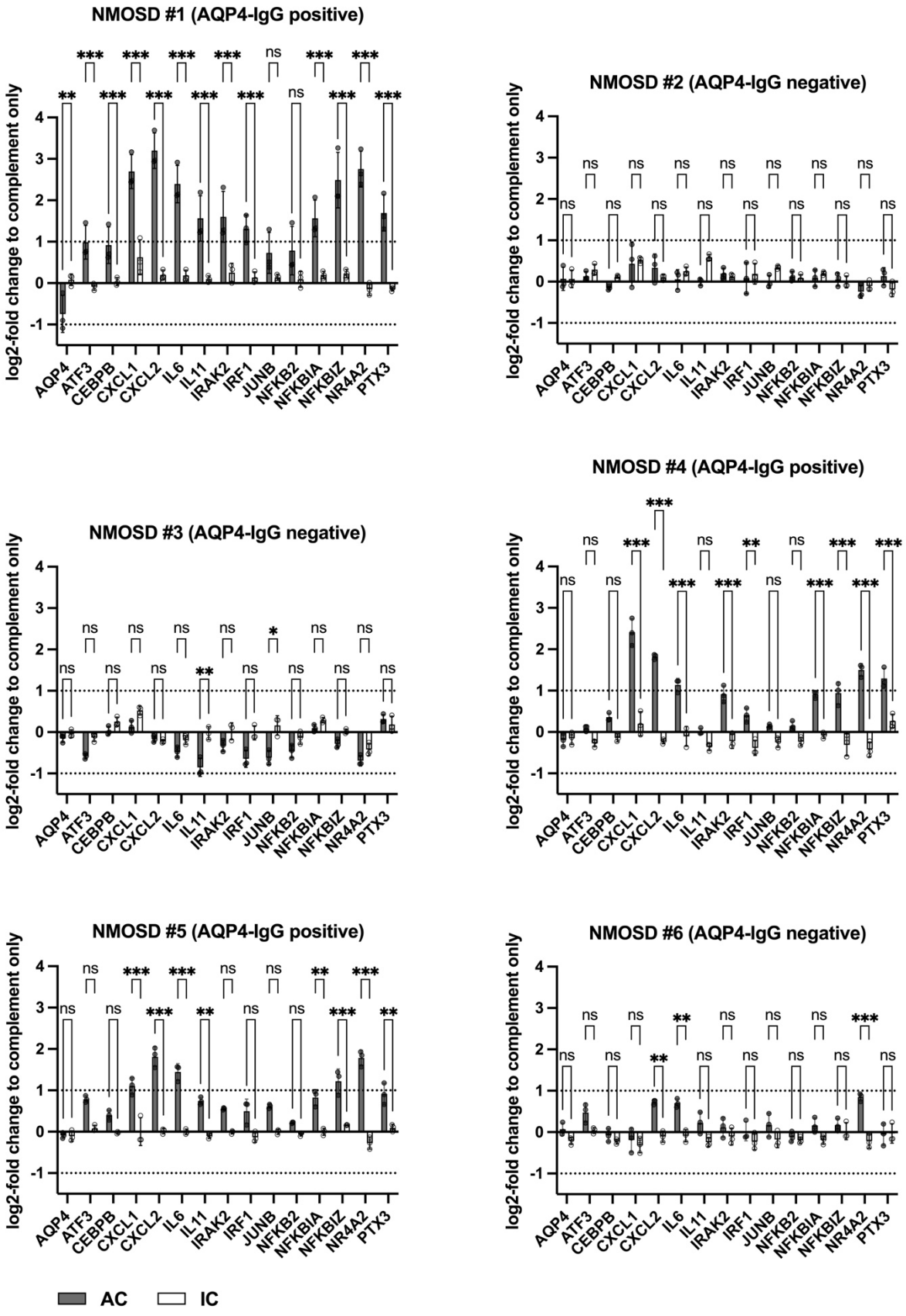
IgG-Fcγ staining of U-87MG-AQP4-ECFP cells after incubation with NMOSD patient sera #1-#6. U-87MG-AQP4-ECFP mixed with U-87MG cells in a ratio of 1:1 were grown to confluence. Then, NMOSD patient sera with or without AQP4-IgG were applied in a dilution of 1:20 in PBS with 10% heat-inactivated FCS. After an incubation of 1 hour at 4 °C, cells were washed and Alexa Fluor® 594 AffiniPure™ Goat Anti-Human IgG, Fcγ fragment specific (Jackson ImmunoResearch, West Grove, PA, USA) was applied at 1:750 for 30 min. at room temperature. Cells were washed and fixed. Microscopy was performed with a Zeiss Axiovert200M microscope (Zeiss, Oberkochen, Germany) (10x objective). Scale bar = 100 µm. AF = Alexa Fluor, AQP4 = aquaporin-4, ECFP = enhanced cyan fluorescent protein.

Supplementary Fig. 7



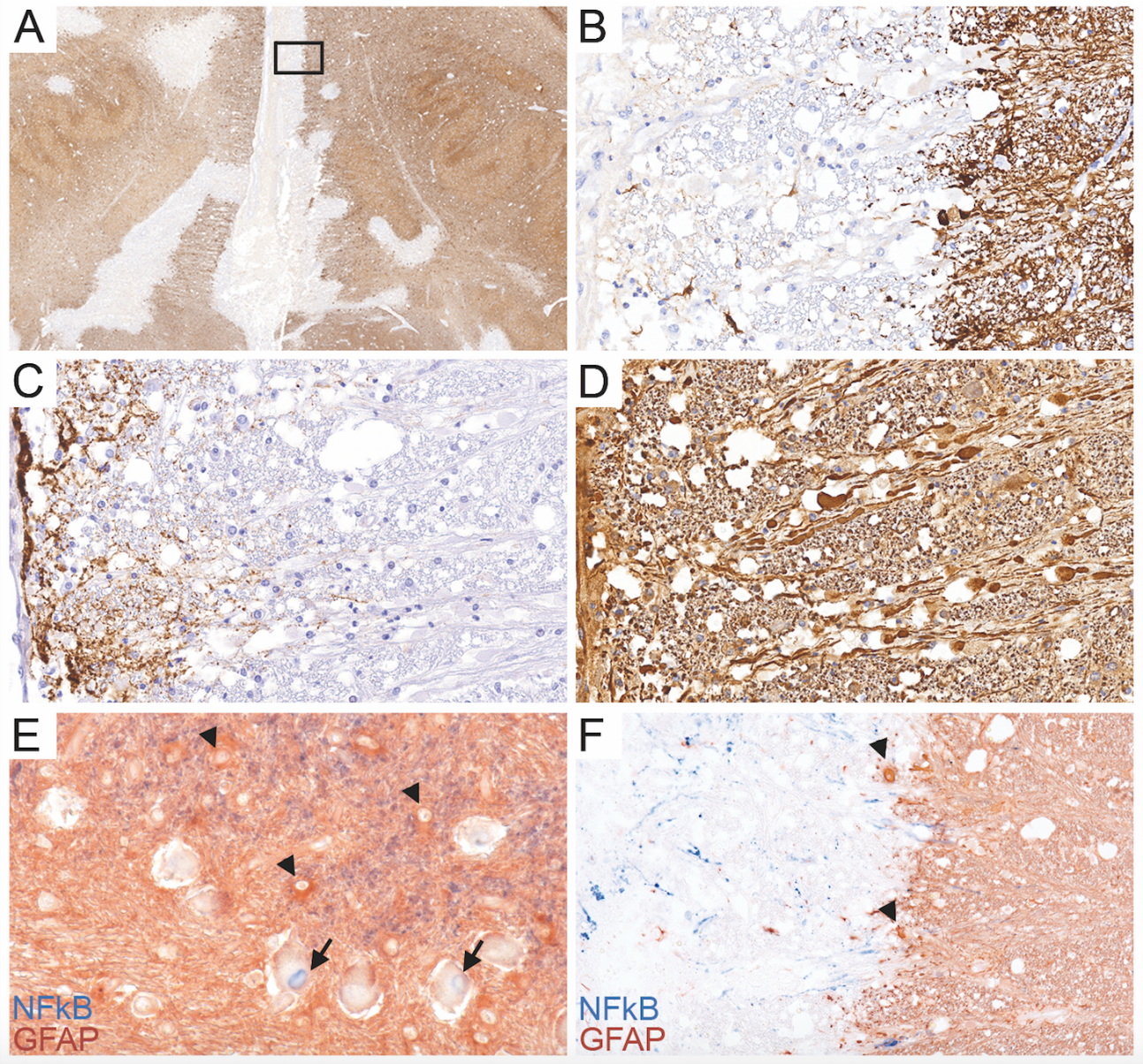
IgM-Fc5μ staining of U-87MG-AQP4-ECFP cells after incubation with NMOSD patient sera #1-#6. U-87MG-AQP4-ECFP mixed with U-87MG cells in a ratio of 1:1 were grown to confluence. Then, NMOSD patient sera with or without AQP4-IgG were applied in a dilution of 1:20 in PBS with 10% heat-inactivated FCS. After an incubation of 1 hour at 4 °C, cells were washed and Alexa Fluor® 594 AffiniPure™ Goat Anti-Human IgM, Fc5μ fragment specific (Jackson ImmunoResearch, West Grove, PA, USA) was applied at 1:750 for 30 min. at room temperature. Cells were washed and fixed. Microscopy was performed with a Zeiss Axiovert200M microscope (Zeiss, Oberkochen, Germany) (10x objective). Scale bar = 100 µm. AF = Alexa Fluor, AQP4 = aquaporin-4, ECFP = enhanced cyan fluorescent protein.

Supplementary Fig. 8



Effects of treatment with AQP4-IgG positive and negative NMOSD serum samples on U-87MG-AQP4-ECFP. U-87MG-AQP4-ECFP cells were treated with human NMOSD patient sera #1-6 in combination with active (AC) or heat-inactivated (IC) human complement. After the treatment, cells were harvested for RNA isolation. Log2-fold changes of $2^{-\Delta\Delta Ct}$ values of human sera plus AC or IC. ΔCt values were calculated with the Ct values of the respective genes minus the Ct values of housekeeping gene GAPDH. To assess serum-specific effects (ΔΔCt), ΔCt values of complement only samples were subtracted from those with serum plus complement. Next, 2-way ANOVA with Šídák's multiple comparisons test was performed comparing each serum treated with AC vs. IC. Bar graphs for each serum respectively were created with GraphPad Prism 10.4. $p<0.05$, ** $p<0.01$, *** $p<0.001$. AC = active complement, IC = inactive complement, ns = not significant.

Supplementary Fig. 9



Nuclear translocation of NF-κB in neurons with acute axonal injury. The medulla oblongata of an NMOSD patient shows multiple active lesions affecting the raphe with loss of astrocytes (**A**, rectangle enlarged in **B**; GFAP), deposition of activated complement complex (**C**, C9neo), and acute axonal injury of fibers derived from the inferior olivary nucleus (**D**; SMI31). (**E**, **F**) Double staining for NF-κB (blue) and GFAP (red): nuclear translocation of NF-κB is found in neuronal nuclei of the inferior olivary nucleus (**E**; arrows) but not in surrounding (**E**; arrowheads) or perilesional astrocytes (**F**; arrowheads). Magnification: A: 13x; B-D, F: 200x; E: 600x.

Supplementary Table. TaqMan™ Gene Expression Assay (FAM) (Applied Biosystems, Thermo Fisher Scientific Waltham, MA, USA, Cat no. 4331182)

Gene Symbol	Assay ID
AQP4	Hs00242342_m1
ATF3	Hs00231069_m1
CEBPB	Hs00942496_s1
CXCL1	Hs00236937_m1
CXCL2	Hs00601975_m1
GAPDH	Hs99999905_m1
IL6	Hs00174131_m1
IL11	Hs00174148_m1
IRAK2	Hs00176394_m1
IRF1	Hs00971965_m1
JUNB	Hs00357891_s1
NFKB2	Hs01028890_g1
NFKBIA	Hs00355671_g1
NFKBIZ	Hs00230071_m1
NR4A2	Hs01117527_g1
PTX3	Hs00173615_m1