Supplemental Data

Figure S1

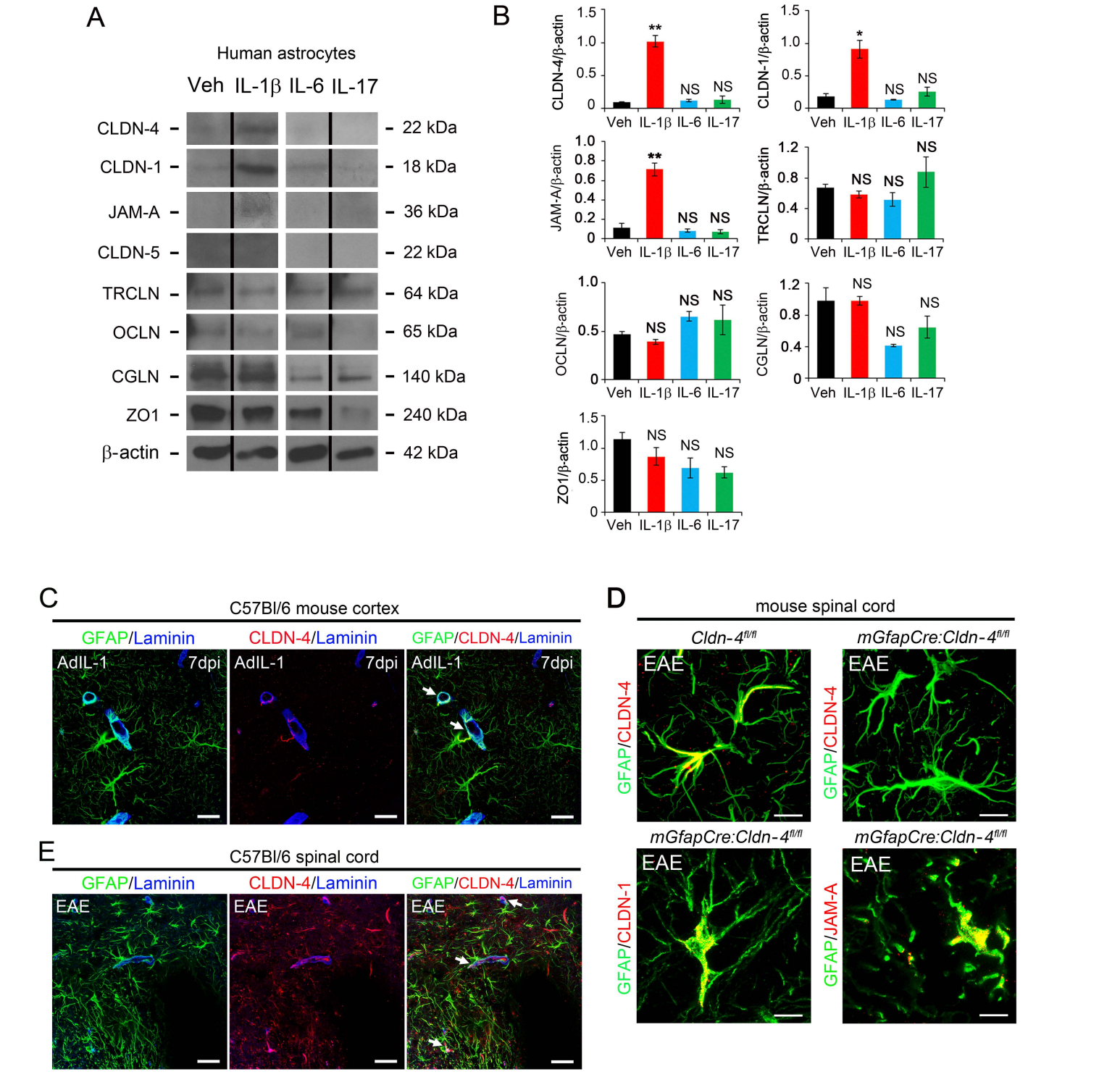

Figure S1. Related to Figures 1 and 2. IL-6 and IL-17 do not induce astrocytic TJ expression (A, B) Western immunoblots from cultured human astrocytes treated with 10ng/ml IL-1β, IL-6 or IL-17 show that neither TJ proteins (CLDN-1, CLDN-4 and JAM-A), nor TJ associated proteins are upregulated by IL-6 or IL-17. Immunoblots in A were run on parallel contemporaneous gels. **CLDN-4 is upregulated in reactive astrocytes of two models of CNS inflammatory disease. (C)** Immunostaining reveals that reactive astrocytes in AdIL-1 injected cortex at 7dpi exhibit CLDN-4 expression (red) enriched at astrocytic endfeet (GFAP, green) surrounding the perivascular spaces (Laminin, blue). Scale bar 15 µm. **(D)** Spinal cord lesions of control (*Cldn-4*$^{fl/fl}$) and astrocyte conditional *Cldn-4* knock-out *mGfapCre:Cldn-4*$^{fl/fl}$ (CKO) mice with EAE, immunostained for CLDN-1, CLDN-4 or JAM-A (red), plus the reactive astrocyte marker GFAP (green). These images reveal astrocytic expression of CLDN-4 (red) in lesions in controls, but not in *Cldn-4* CKO mice. However, EAE lesions of *Cldn-4* CKO mice show preserved astrocytic expression of CLDN-1 and JAM-A (both red). Scale bar, 7.5µm. **(E)** In EAE lesions of control mice, CLDN-4 (green) expression is localized in astrocytic endfeet (GFAP, green) projecting around the perivascular spaces (Laminin, blue). Scale bar 15 µm.

Figure S2

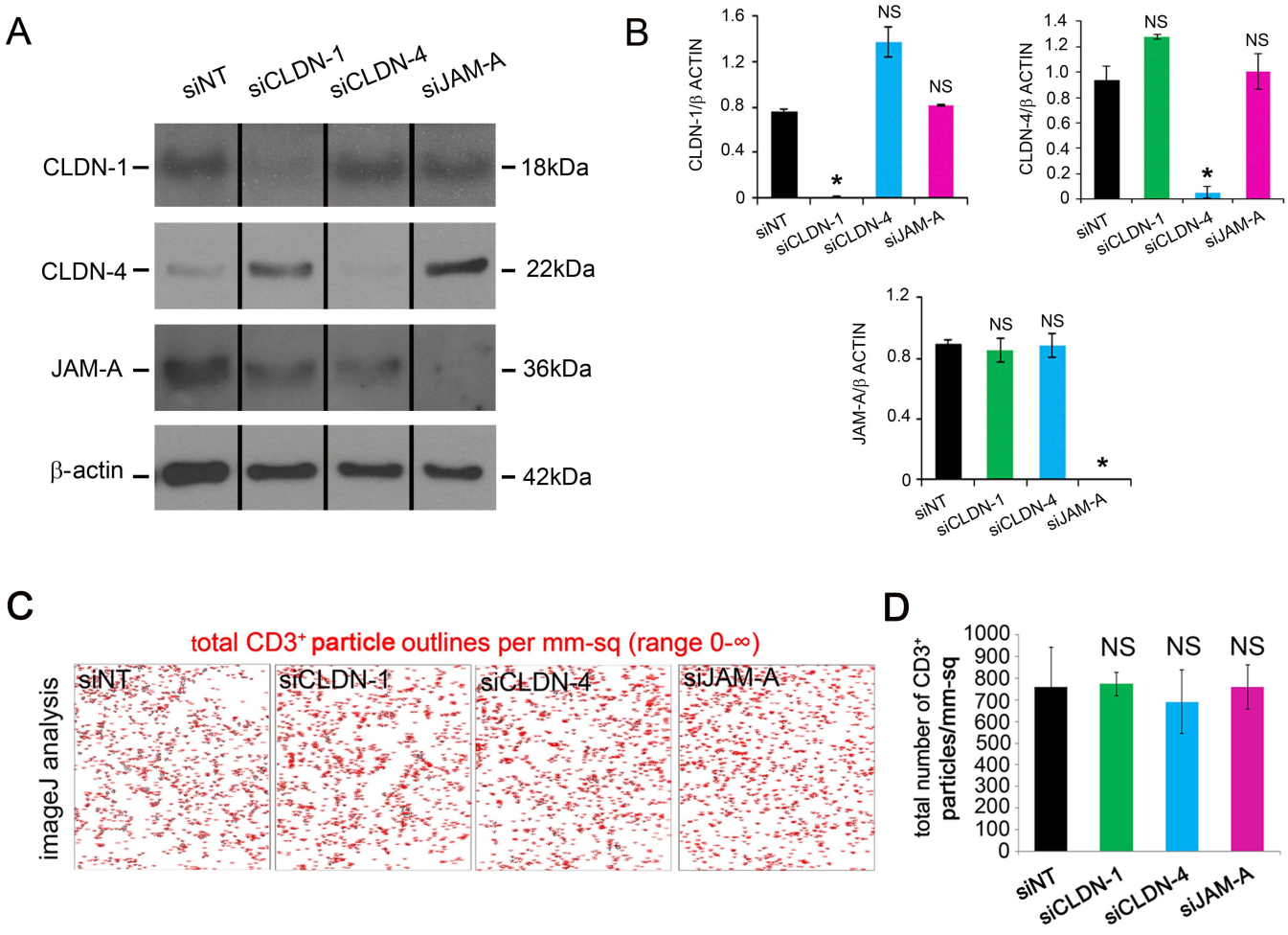
A
siNT
siCLDN-1
siCLDN-4
siJAM-A
CLDN-1
18kDa
CLDN-4
22kDa
JAM-A
36kDa
β-actin
42kDa
B
CLDN-1/β ACTIN
CLDN-4/β ACTIN
JAM-A/β ACTIN
NS
*
C
total CD3⁺ particle outlines per mm-sq (range 0-∞)
imageJ analysis
D
total number of CD3⁺ particles/mm-sq
E
serum starved human astrocytes-activated leukocytes
12h
24h
48h
GFAP/CD3/DAPI
F
NA astro-A leuko
A astro-NA leuko

Figure S2. Related to Figure 4. Silencing of astrocytic tight junction proteins *in vitro*. (A) Western immunoblots of CLDN-1, CLDN-4 and JAM-A on lysates of primary human astrocyte cultures nucleofected with siRNA for *CLDN-1*, *CLDN-4*, *JAM-A*, or non-targeting (NT) control, then exposed to 10ng/ml IL-1β for 24h followed by washout. **(B)** Densitometric analysis of Western immunoblots demonstrates that inactivation of each transcript with siRNA is effective and does not impact induction of the others (3 biological replicates each group, one way ANOVA with Bonferroni correction, $p<0.005$). **(C, D)** ImageJ analysis of co-cultures of $CD3^+$ T lymphocytes and reactive human astrocytes pre-treated with siCLDN-1, siCLDN-4, or siJAM-A, or non-targeting (siNT) control. **(C, D)** Immunostaining for CD3 at 48h validates evenly distributed loading of $CD3^+$ cells onto astrocytic monolayers. **(E)** Confocal images of immunostained co-cultures of activated (A) human $CD3^+$ T lymphocytes with non-activated (NA) (ie. untreated and serum starved) human astrocytes demonstrates an absence of CD3+ cell clustering in co-culture of at 12h, 24h and 48h. Scale bars 20μm **(F)** Similarly, co-culture of activated (A, ie. IL-1β treated) astrocytes with non-activated (NA) leukocytes also demonstrates an absence of CD3+ cell clustering (figures from co-cultures at 24h; a cropped version of the 24h panel from E is redemonstrated in the NA astro-A leuko panel of F). In sum, clustering requires activation of both cell populations. Scalebars, 10μm.

Figure S3

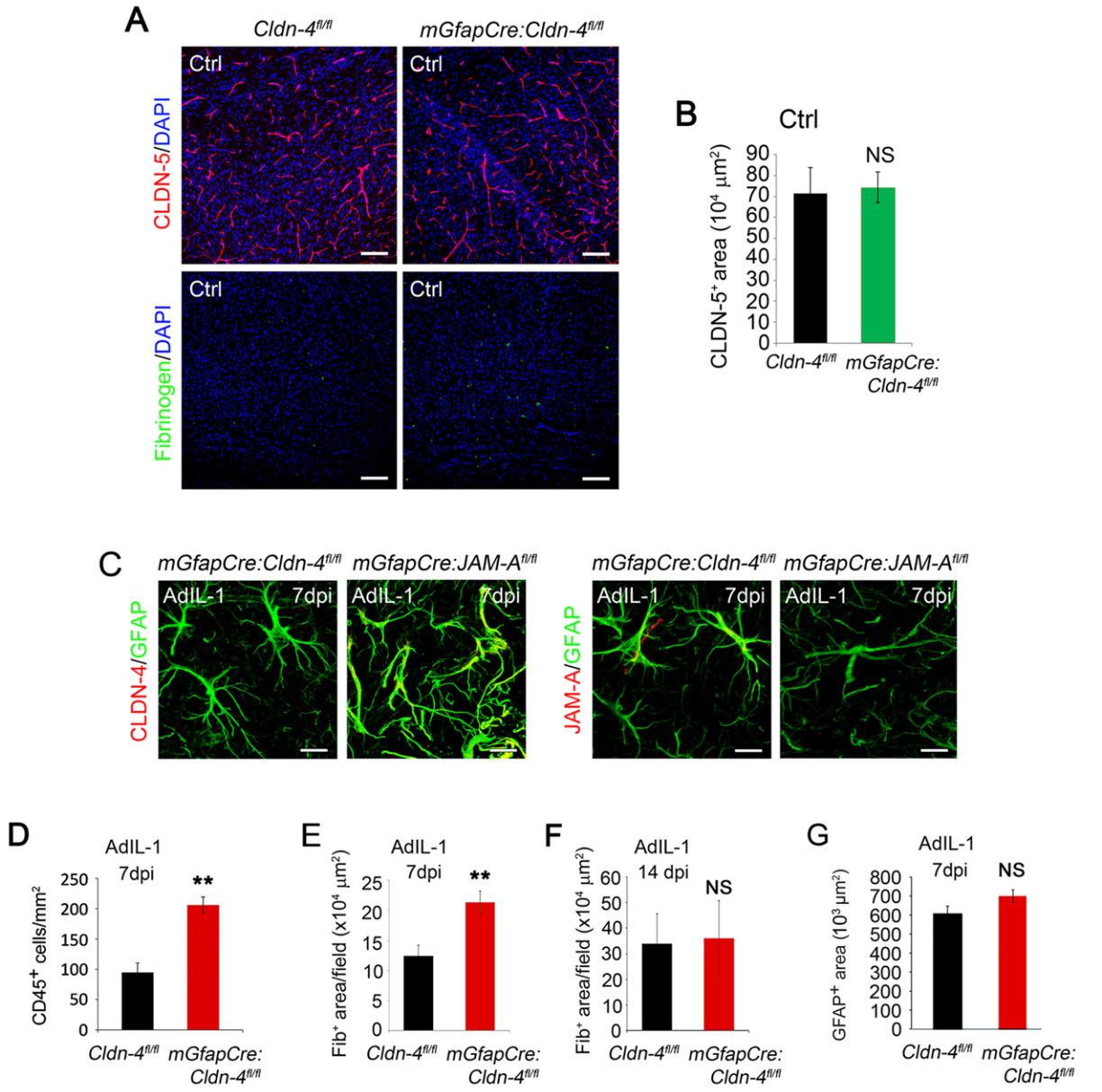

Figure S3. Related to Figure 5. Conditional in vivo deletion of *Cldn-4* in reactive astrocytes results in exacerbated leukocyte infiltration and humoral factor entry into CNS inflammatory lesions. (A,B) In the unchallenged CNS (cerebral cortices shown) of adult (12 week) *Cldn-4* CKO (*mGfapCre:Cldn-4*$^{fl/fl}$) mice and controls (*Cldn-4*$^{fl/fl}$), areas of endothelial CLDN-5 expression are not significantly different between the two groups, and no fibrinogen leakage into the CNS is detected, indicating that there are no differences between *Cldn-4* CKO and control mice in BBB integrity (CLDN-5: KO=5, WT=5, p=0.84, two-tailed t-test). **(C)** In the *mGfapCre:Cldn-4*$^{fl/fl}$ CKO mouse line, reactive astrocytes within AdIL-1 lesions upregulate JAM-A but not CLDN-4, whereas in controls, both CLDN-4 and JAM-A upregulation occurs (see **Fig. 1E**). Conversely in *mGfapCre:JAM-A*$^{fl/fl}$ CKO mouse line, CLDN-4 is upregulated but not JAM-A. Scale bar, 20µm. **(D)** At 7dpi, *Cldn-4* CKO mice have increased numbers of CD45^{+} lymphocytes in AdIL-1 lesions (n=4 CKO, n=6 WT, p<0.0012, two-tailed t-test). See also **Figs.5E-H**. **(E,F)** Area of fibrinogen entry is also increased in *Cldn-4* CKO compared to controls in AdIL-1 lesions at 7dpi (n=13 CKO, n=14 WT, p<0.0005, two-tailed t-test) but this difference does not persist at 14 dpi (n=5 CKO, n=6 WT, p=0.91, two-tailed t-test). See also **Figs.5I,J**. **(G)** Reactive astrocytic expression of GFAP is not significantly different between *Cldn-4* CKO mice and controls at 7dpi (KO=8, WT=11, p=0.09, two-tailed t-test), indicating that levels of astrocyte activation induced by AdIL-1 within lesions are similar.

Figure S4

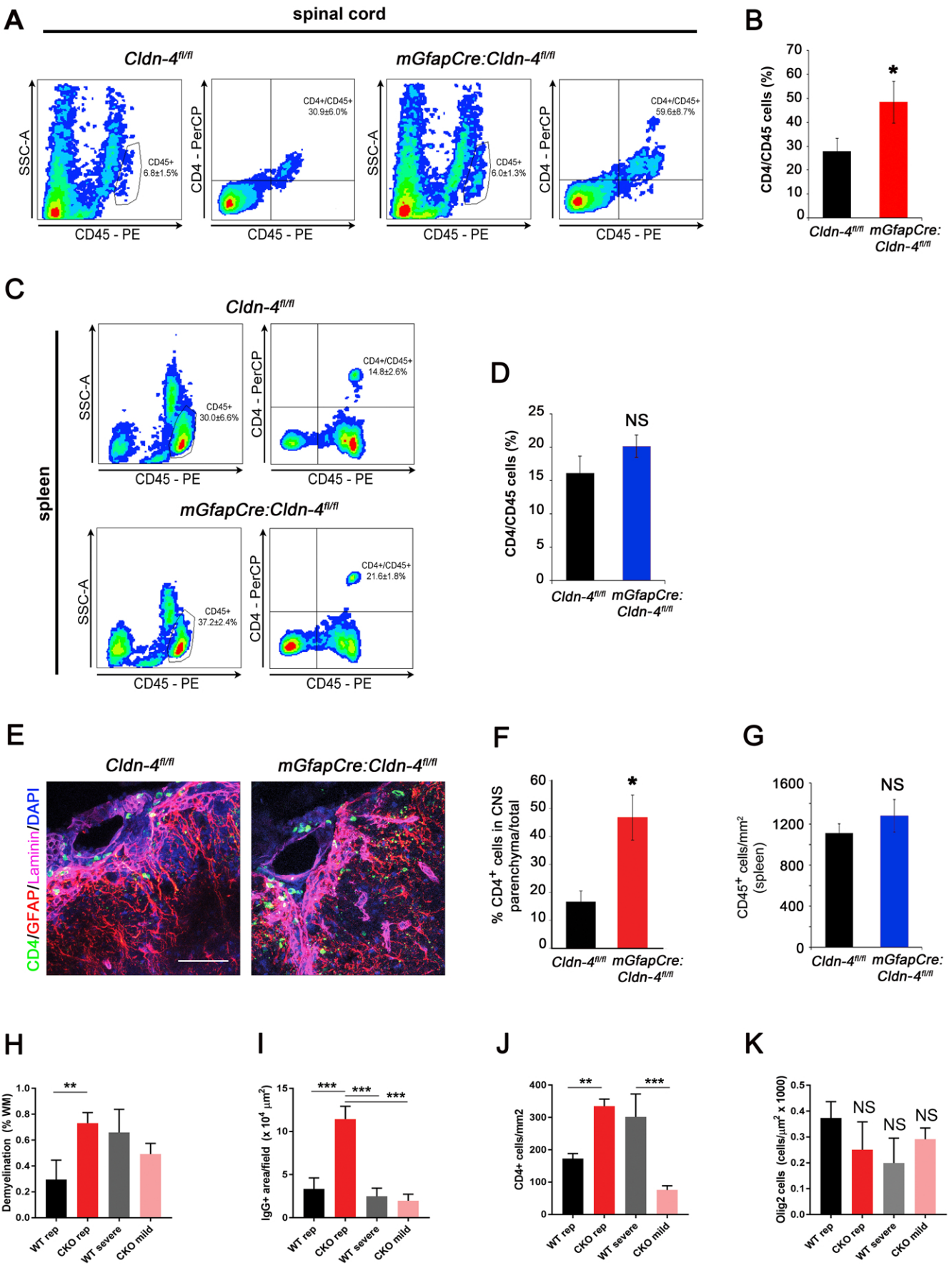
A
spinal cord
Cldn-4fl/fl
mGfapCre:Cldn-4fl/fl
SSC-A
CD45 - PE
CD45+ 6.8±1.5%
CD4 - PerCP
CD4+/CD45+ 30.9±6.0%
CD45+ 6.0±1.3%
CD4+/CD45+ 59.6±8.7%
B
CD4/CD45 cells (%)
C
spleen
CD45+ 30.0±6.6%
CD4+/CD45+ 14.8±2.6%
CD45+ 37.2±2.4%
CD4+/CD45+ 21.6±1.8%
D
NS
E
CD4/GFAP/Laminin/DAPI
F
% CD4+ cells in CNS parenchyma/total
G
CD45+ cells/mm2 (spleen)
H
Demyelination (% WM)
I
IgG+ area/field (x 10^4 µm2)
J
CD4+ cells/mm2
K
Olig2 cells (cells/µm2 x 1000)
WT rep
CKO rep
WT severe
CKO mild

Figure S4. Related to Figure 6. Flow cytometry of EAE tissue demonstrates increased $CD4^+$ infiltration in *Cldn-4* CKOs. (A-B) Flow cytometry on spinal cord tissues harvested after peak EAE disease showed an increased $CD4^+/CD45^+$ ratio in *Cldn-4* CKO mice compared to controls (CKO=7, WT=7, $p<0.05$, two tailed t-test). **(C-D)** Flow cytometry on spleen showed no difference (CKO=7, WT=6, $p=0.13$, two tailed t-test). ***Cldn-4* CKO show increased CD4+ cell infiltration past the glia limitans superficialis and perivascular spaces. (E, F)** Immunostaining for CD4 (green), GFAP (red) and Pan-laminin (pink) performed on spinal cord meninges revealed a higher proportion of $CD4^+$ cells located past the glia limitans superficialis and perivascular spaces in the CNS parenchyma of CKOs compared to controls (CKO=5, WT=6, $p<0.05$, two tailed t-test). **(G)** In *Cldn-4* CKO mice and controls with EAE at 21d post-induction, $CD45^+$ cell numbers in spleen are not significantly different (KO=3, WT=3, two-tailed t-test). **Histochemical analysis of *Cldn-4* CKO and WT with comparable EAE disability scores reveals increased IgG infiltration in CKO but disability-dependent $CD4^+$ cell infiltration**. **(H-K)** A group of mild CKO (n=3, mean score 2.6) and severe WT (n=3, mean score 3.9), sacrificed at 21 dpi, were compared to representative CKO (n=3, mean score 3.6) and WT (n=3, mean score 2.0). **(H)** Severe WT had similar degrees of demyelination to representative CKO (n=3 each group, one way ANOVA with Bonferroni correction). **(I)** IgG was significantly higher in representative CKO compared to both representative and severe WTs ($p<0.0005$). **(I)** Similar numbers of $CD4^+$ cells were found between severe WT and representative CKO; there were fewer $CD4^+$ cells in the mild CKO compared to the severe WT ($p<0.005$). **(J)** Finally, there were no differences in Olig2 numbers across the 4 groups. Of note, these data confirmed our initial analysis comparing representative CKO and WT groups.

Figure S5

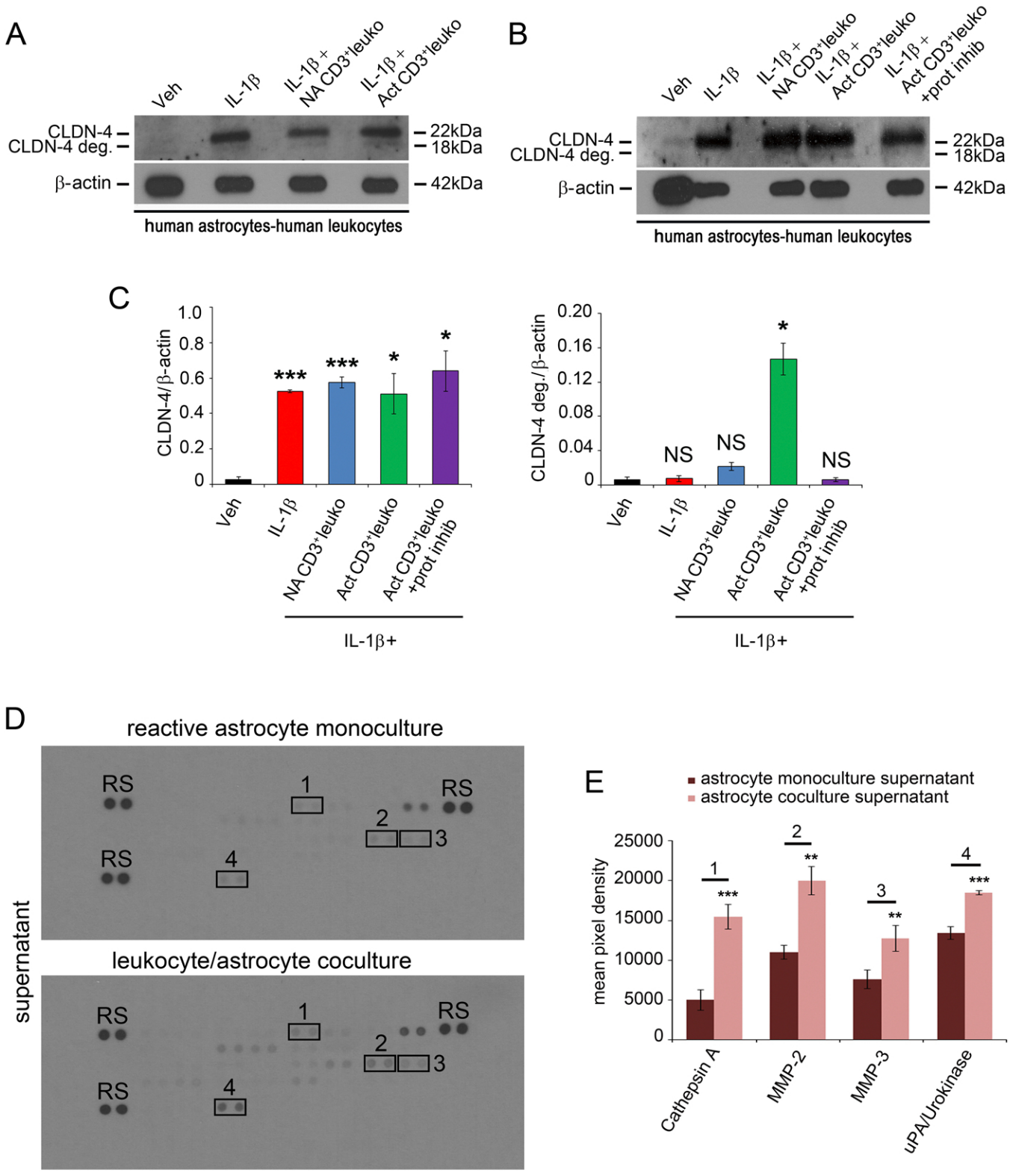

Figure S5. Related to Figure 7. CLDN-4 degradation occurs when activated astrocytes are co-cultured with activated CD3$^+$ lymphocytes and is blocked by a protease inhibitor cocktail. (A-C) Immunoblotting and densitometry of CLDN-4 and CLDN-4 degradation product from co-cultures of vehicle and IL-1β treated human astrocytes in co-culture with non-activated and activated CD3$^+$ lymphocytes. Co-culture of IL-1β treated astrocytes with activated CD3$^+$ lymphocytes leads to degradation of astrocytic CLDN-4 at 24h. See also **Fig.7A-D**. CLDN-4 degradation requires activation of both astrocytes and CD3$^+$ lymphocytes and is blocked by a cocktail of protease inhibitors. Data from 3 biological replicates of each condition. **Human Protease Arrays reveal unique protease expression patterns of astrocytes and CD3$^+$ cells in co-culture (D, E)** Supernatant from astrocyte monocultures were compared to those from co-culture with CD3$^+$ cells. Co-culture supernatant demonstrated higher levels of cathepsin A, MMPs-2 and 3 and uPA compared to monoculture supernatant (n=3 each group, t-test, $p<0.005$).