Astrocytic ECGF1/TP and VEGF-A drive blood-brain barrier opening in inflammatory CNS lesions.

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Supplementary Materials

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Supplementary Materials and Methods

Antibodies: Anti-CLN-5 and anti-CD11b (both mouse), OCLN (rabbit), and GFAP (rat) were from Invitrogen (Carlsbad, CA). Anti-pan-laminin (rabbit) was from Sigma-Aldrich (St. Louis, MO).Anti-VEGF-A (rabbit), anti-CD31 (goat), anti β-Actin and anti-ECGF1/TP (both mouse) were from Santa Cruz Biotech (Santa Cruz, CA). Rabbit cleaved caspase-3 was from Cell Signaling (Danvers, MA). Anti-fibrinogen and anti-myelin basic protein (both rabbit), anti-FVIIIR and anti-CD31 (both mouse) were from Dako (Carpinteria, CA). Anti-BrdU was from Immunology Consultants (Newberg, OR). Anti-albumin (sheep) and CD8 (rat) were from Abcam (Cambridge, MA). Anti-CD4 (rat or rabbit), CD11b (and CD45 (rat) were from eBioscience (San Diego, CA). Antibodies for NeuN and Olig2 were from Millipore (Billerica, MA). Antibody for neurofilament, SMI 32R (mouse), was from Covance (Princeton, NJ). Biotinylated lectin RCA-1 was from Vector Laboratories (Burlingame, CA.). Human IL-1 (rabbit) was from Cistron (Parsippany, NJ)

Real-time PCR: RNA was harvested using an Absolutely-RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA), and cDNA was generated, and real-time PCR performed and validated, as previously described(Argaw *et al*., 2006). Primer sequences are available upon request.

Western blotting: SDS-PAGE and Western blotting were performed as previously reported(Argaw *et al.*, 2006). For densitometry, nonsaturated developed films were scanned using a Canon LiDE scanner (Canon USA, Lake Success, NY), and mean pixel density of each band measured using ImageJ software (NIH, Bethesda, MD). Data were standardized to actin, and fold change versus control calculated.

Bromodeoxyuridine (BrdU) incorporation: 10μM BrdU (BD, San Diego, CA) was added to cultures 12h prior to fixation. Cells were stained for BrdU as per the manufacturer's instructions.

Immunocytochemistry: Cultures were fixed and stained as previously described(Argaw *et al.*, 2009) unless noted. For claudins, cells were fixed in 95% ethanol at 4°C 30min, then 1:1 methanol/acetone 1min RT. Primary antibodies were used at 1:100 except CLN-5 (1:50). Samples were examined using a Leica Microsystems confocal microscope (Buffalo Grove, IL) and stacks collected with z=0.5μm.

ELISA: Sandwich ELISA for VEGF-A was carried out using a commercial assay (R&D Systems).

Permeability assay: CMVEC were plated onto 0.4µm pore culture inserts (Corning, NY) and left to reach confluence for 10-14d. Cultures were then treated as specified, and at times shown dextran-FITC (100µg/ml 40kD or 4kD, Molecular Probes, Eugene, OR) in growth factor-free CSC medium 0.1% BSA was added to the upper chamber, and accumulation in the lower chamber measured over 1-5hr(Argaw *et al*., 2009).

Migration assay: A wound was made in confluent CMVEC cultures on confocal plates by scraping a soft sterile 200µl pipette tip across the coverslip, and cultures fixed and stained as described above at times shown. All dishes were imaged by immunofluorescence and Kohler illumination at RT with a 20x objective, using the camera system described above. Fifty random 20x fields per culture condition were captured, and mean wound diameter measured in each field using ImageJ software.

Angiogenesis assay: Primary CMVEC cultures were starved in medium containing 0.5% FCS for 4h, trypsinized and replated into the same medium in 24-well plates on growth factor-free Matrigel (BD Discovery Labware), and treated as described. At times specified, live cultures were examined and photographed using a Leica confocal microscope, and image analysis performed via ImageJ software.

CNS microinjection: Mice (C57BL/6, 12wk, at least 3 per condition per timepoint) were anaesthetized and placed into a stereotactic frame (Kopf, Tujunga, CA). Mouse $VEGF_{165}$ (60ng in 3µl PBS/BSA), DDR (400ng in 3µl PBS) or vehicle were microinjected into the cerebral cortex at y=1mm caudal to Bregma, x=2mm, z=1.5mm as described(Argaw *et al.*, 2009). All studies were approved by institutional IACUC.

EAE: Mice (male, 8wk, at least 10/group, on the C57BL/6 background) were immunized s.c. with 300µg myelin oligodendrocyte glycoprotein-35-55 ($MOG_{35–55}$, Invitrogen) in 200-µl Freund's Adjuvant containing 300µg/ml Mycobacterium tuberculosis H37Ra (Difco) in the dorsum. Mice received i.p. 500 ng of pertussis toxin on day of sensitization and 2d later. Disease was scored as: 0, no symptoms; 1, floppy tail; 2, hind limb weakness; 3, hind limb paralysis; 4, fore- and hind limb paralysis; 5, death.

Immunohistochemistry: Animals were perfused with 4% paraformaldehyde at times specified. Embedding and immunostaining were performed as published(Argaw et al., 2006) except where noted. For claudins, prior to blocking sections were soaked in EDTA pH8 100°C. Primary antibodies were used

at 1:100, except CLN-5 (1:50), fibrinogen (1:1000) and albumin (1:1000). Samples were examined as above and Z-series stacks collected with z=1μm.

Morphometric analysis: Using ImageJ software, pixels positive for CLN-5, OCLN, fibrinogen and albumin immunoreactivity were counted in projections of Z series stacks with the same number of images, from 5 separate animals with EAE, and 5 age- and sex-matched normal controls, at least 5 random 20x fields in 5 sections per animal from lumbar spinal cord by a blinded observer. EAE samples were from mice with paraparesis/paraplegia, 15-18d post-induction.

Immunohistochemistry: paraffin sections: Early postmortem tissues (4–18 h) from 10 MS patients staged as described (Lassmann *et al*., 1998) 5 age- and sex-matched normal controls, and 4 patients with other neurological diseases, were studied.For all antigens except IL-1, the following protocol was used: sections were dewaxed and rehydrated, then incubated in PBS 3% hydrogen peroxide for 30 min, blocked with a commercially available blocking buffer (NEN), and incubated with relevant primary antibodies in blocking buffer for 2 h at RT, then overnight at 4°C. After washing, sections were incubated in relevant goat biotinylated secondary Abs (1/200; Vector Laboratories) for 30 min, then developed using standard ABC protocols (Vector Laboratories) (GFAP) or tyramide signal amplification protocols (NEN) (all other antigens), followed by diaminobenzidine (brown) (Sigma-Aldrich). In the case of RCA-1, sections were incubated in biotinylated lectin, then developed using ABC protocols (Vector Laboratories) (GFAP) or tyramide signal amplification protocols (NEN) (all other antigens) followed by diaminobenzidine. Control sections were processed using species- and isotype-matched irrelevant primary antibodies, IgG fractions, and no primary antibodies controls. All sections were then dehydrated, mounted, and examined as described above.For IL-1, staining was performed using an alternate protocol, as described previously (Griffin *et al.*, 1989).

Supplementary Figure and Table Legends

Figure S1. Astrocytic Ecgf1/TP induction by IL-1β *in vivo* is potentiated by conditional *Vegfa* inactivation. Results presented here extend findings in **Figures 1** and **2**. **(A)** Assessment of purity of primary human astrocyte culture samples used for Illumina HumanHT-12 v4 Beadchip© transcriptional

profiling studies shown in **Figs.1A,B**. Samples from four cultures have been subjected to SDS-PAGE and immunoblotting for the astrocytic marker GFAP, the CMVEC marker CD31/PECAM-1, and actin. All samples are $GFAP^+$ $CD31^-$. Samples were also all negative for the T lymphocyte marker CD3 (not shown). **(B)** Demonstration that ECGF1/TP induction by IL-1β in primary human astrocytes occurs independently of JAK-STAT signaling. Data complement results showing NFκB-sensitivity of induction, presented in **Fig.1J**. Primary human astrocytes were pretreated with JAK inhibitor 1 at the IC_{50} of 15mM 2h, then activated with IL-1β 24h and harvested for immunoblotting. JAK inhibitor treatment did not impact ECGF1/TP induction. **(C-E)** Data are shown from male 12wk wildtype or V*egfa*$^{fl/fl}$ control or experimental *mGfapCre:Vegfa*$^{fl/fl}$ mice receiving cortical microinjection of 10^7pfu AdIL-1 or AdCtrl, and sacrificed at 7dpi. Sections have been stained for Vegf-a and the astrocytic marker Gfap **(C)**, or Ecgf1/Tp plus Gfap **(D,E)**. These panels complement and in part overlap with findings illustrated in **Figs.2A-F**. In each, data for Vegf-a or Ecgf1/Tp are also shown as a single channel below the main panel. In $Gfap^+$ reactive astrocytes (arrowed) in AdIL-1 lesions at 7dpi, Ecgf1/Tp or Vegf-a are both strongly induced in wildtype or non-*Cre Vegfa*$^{fl/fl}$ control mice **(C,D)**, but not in mice receiving empty vector (AdCtrl) **(C,E)**. In *mGfapCre:Vegfa*$^{fl/fl}$ mice, the *Vegfa* gene is conditionally inactivated in reactive astrocytes(Argaw *et al.,* 2012), thus Vegf-a induction fails in AdIL-1 lesions **(C)**. Notably, this also results in potentiation of astrocytic Ecgf1/TP induction **(D)**, suggesting that expression of the two in inflammatory CNS lesions is inter-related. Results shown are representative of findings from at least 4 mice per condition. **(F)** Immunoblotting of human CMVEC treated with DDR (1-10μM), VEGF-A (100ng/ml), or both, and harvested at 24h. Data have been subjected to densitometry and normalized to actin loading control. DDR and VEGF-A each downregulate OCLN. Similar data showing downregulation of CLN-5 are illustrated in **Fig.2G**. **(G)** Assessment of purity of human CMVEC cultures used for Illumina HumanHT-12 v4 Beadchip© transcriptional profiling shown in **Figs.2I-M**. Samples from five cultures have been subjected to SDS-PAGE and immunoblotting for GFAP, CD31/PECAM-1, CD3 and actin. All samples are $CD31^+$ $GFAP^-$ $CD3^-$. Scalebars, **(C-E)**, 30μm.

Figure S2. DDR and Vegf-a promote BBB disruption and neuronal loss in adult cerebral cortical lesions. (A) Time course morphometric measurements over a 7 day period postinjection from confocal Z series projections of cerebral cortices of 12wk C57BL/6 mice following stereotactic microinjection of murine Vegf-a_{165} (60ng in 3µl PBS/BSA), and/or DDR (400ng in 3µl PBS), or vehicle control. Sections were immunostained for fibrinogen as a marker of BBB breakdown, and the neuronal marker NeuN. Data complement and partially overlap with findings in **Fig.4F.** The results of these studies reveal that BBB disruption, as defined by the increase in area of parenchymal fibrinogen positivity, precedes neuronal loss (increase in area of $NeuN^-$ field) in DDR-induced or Vegf-a-induced lesions. Additionally, co-treatment results in more persistent and extensive BBB breakdown, associated with exacerbation of neuronal loss. **(B,C)** Results of studies in cultures of human CMVEC **(B)** or cortical mouse neurons **(C)**, treated with 1-100µM DDR or vehicle control. In human CMVEC cultures, DDR (1µM) reduces numbers of apoptotic (cleaved caspase-3^+) cells at 16 h **(B)**. **(C)** In isolated mouse neurons, DDR (1-100µM) has no detrimental effect on cell number at 24h post treatment. **(D-G)** Results of analysis of confocal Z-series projections of ventrolateral lumbar spinal cord from 10wk C57BL/6 mice with the MS model EAE (tail paralysis + paraparesis) 15d-18d post-induction. Data shown directly complement findings in **Figure 5**. Panel **(D)** illustrates pathology. Sections are immunostained for the inflammatory leukocyte marker CD45 (upper panel) or fluoromyelin + CD45 (lower panel). Spinal cords of mice with EAE display subpial and perivascular accumulation of $CD45^+$ cells, and multifocal demyelination (lower panel, arrowed). **(E-G)** Morphometric analysis of spinal cord sections from mice with EAE, immunostained for fibrinogen, Cln-5 or Ecgf1/Tp, plus the reactive astrocyte marker Gfap. These data complement findings in **Figs.5F-H**. These analyses demonstrate that Ecgf1/Tp expression is positively correlated in EAE lesions with expression of Gfap. However, unlike Ecgf1/Tp, Gfap does not correlate significantly with extravasation of fibrinogen into the CNS parenchyma, or negatively with endothelial Cln-5 expression. Results in **(A)** and

Figure S3. In human MS white matter lesions, ECGF1/TP and VEGF-A localize specifically to reactive astrocytes. Details of patient samples used for immunohistochemical analyses are in **Table S4.** Panels **(A-E)** show images taken from serial sections of a chronic active MS lesion centered on a vessel, from a 32 year old female patient. Panel **(A)** is a low-magnification photograph illustrating the

extent of the lesion, stained with Luxol Fast Blue, while **(B-E)** show higher magnification views of an area of the lesion and its border stained with Luxol Fast Blue and for MBP to illustrate demyelination **(B,C)**, and with Hematoxylin and Eosin to illustrate pathology **(D)**, and for ECGF1/TP **(E)**. In panel **(B),** note demyelination within the lesion (pink), contrasting with myelin at the lesion border and in normal-appearing white matter (blue). Myelin appears as parallel blue lines. Panel **(C)** directly illustrates demyelination, as evidenced by loss of MBP within the lesion. Panel **(D)** illustrates activity as evidenced by hypercellularity at the lesion border. Additionally, some cells in white matter and at the border have small round nuclei characteristic of oligodendrocytes (blue arrows), but these are largely absent within the lesion center. At the active border, a major site of BBB disruption in MRI studies(Absinta *et al.*, 2013), and to a lesser extent within the lesion, are large, hypertrophic cells with morphology matching that of reactive astrocytes (black arrows). Importantly, ECGF1/TP immunoreactivity also localizes to these hypertrophic cells, within the lesion and particularly at the border **(E)**. Panels **(F-J)** show high magnification images of individual cells immunostained for ECGF1/TP, VEGF-A, IL-1 and cell lineage markers. Similar to findings from MS models in **Figure 4**, higher magnification analysis confirms ECGF1/TP localization to hypertrophic cells corresponding to reactive astrocytes **(F)**, which are also positive for GFAP **(G)**. Compatible with previous reports(Proescholdt *et al.*, 2002, Argaw *et al.*, 2006), VEGF-A also localizes specifically to reactive astrocytes in active areas of MS lesions **(H)**, but not in adjacent normal-appearing white matter. Also matching previous data(Brosnan *et al.*, 1995), IL-1 localizes to cells with the typical morphology characteristic of RCA-1^+ macrophages and microglia **(I,J)**. Immunoreactivity for ECGF1/TP, VEGF-A and IL-1 was not observed in normal control tissue. Magnifications, panels **(A)** x30, **(B-E)** x100, **(F-J)** x500.

Tables S1-3. In human CNS microvascular endothelial cells, VEGF-A and DDR elicit transcriptional patterns linked to plasticity and permeability. Tables S1-3 illustrate results of Illumina HumanHT-12 v4 Beadchip© transcriptional profiling of human CMVEC treated with 1μM/130ng/ml DDR and/or 100ng/ml VEGF-A for 24h. Data shown complement and extend findings in **Figure 2 panels G-M**. Data from three separate cultures per condition have been filtered as regulated by $>\log_2 0.5$ fold and statistically significant using Student's t test with FDR correction $P<0.05$. Results

are color-coded using the scheme illustrated in **Fig.2G**. All significantly induced and repressed transcripts and their associated functions (with accompanying PMID numbers for relevant references) are shown for cultures exposed to VEGF-A **(Table S1)**, DDR **(Table S2)**, and both factors in combination **(Table S3a and S3b)**. These data demonstrate that DDR and VEGF-A each induce distinct transcriptional patterns which are both indicative of endothelial plasticity/remodeling, suggesting functional convergence. VEGF-A regulates 80 genes, while DDR regulates a more limited cohort of 41 genes. Co-treatment produces a larger transcriptional profile of 119 genes. Indicative of combinatorial outcomes, co-treatment produces an additional novel cohort of 50 genes which are not regulated by either factor alone.

Table S4. Summary of patient samples used for immunohistochemical studies. Table S4 presents details of 9 MS patients, 5 age- and sex-matched normal controls, and 4 patients with other neurological diseases, Early postmortem (4 to 18hr) cortical tissue sections from each were subjected to immunohistochemistry for ECGF1/TP, VEGF-A, IL-1, lineage-specific markers, and standard histochemical stains (Luxol fast blue, hematoxylin and eosin) as described in **Supplementary Materials and Methods.**. Results of these studies are shown in **Figure S3**.