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A Proliferation-Inducing Ligand–Mediated Anti-Inflammatory Response of Astrocytes in Multiple Sclerosis

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Objective: The two related tumor necrosis factor members a proliferation-inducing ligand (APRIL) and B-cell activation factor (BAFF) are currently targeted in autoimmune diseases as B-cell regulators. In multiple sclerosis (MS), combined APRIL/BAFF blockade led to unexpected exacerbated inflammation in the central nervous system (CNS) of patients. Here, we investigate the role of the APRIL/BAFF axis in the CNS.

Methods: APRIL expression was analyzed in MS lesions by immunohistochemistry. The *in vivo* role of APRIL was assessed in the murine MS model, experimental autoimmune encephalitis (EAE). Functional *in vitro* studies were performed with human and mouse astrocytes.

Results: APRIL was expressed in lesions from EAE. In its absence, the disease was worst. Lesions from MS patients also showed APRIL expression upon infiltration of macrophages. Notably, all the APRIL secreted by these macrophages specifically targeted astrocytes. The upregulation of chondroitin sulfate proteoglycan, sometimes bearing chondroitin sulfate of type E sugar moieties, binding APRIL, in reactive astrocytes explained the latter selectivity. Astrocytes responded to APRIL by producing a sufficient amount of IL-10 to dampen antigen-specific T-cell proliferation and pathogenic cytokine secretion. Finally, an intraspinal delivery of recombinant APRIL before disease onset, shortly reduced EAE symptoms. Repeated intravenous injections of recombinant APRIL before and even at disease onset also had an effect.

Interpretation: Our data show that APRIL mediates an anti-inflammatory response from astrocytes in MS lesions. This protective activity is not shared with BAFF.

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B Cells are currently targeted in autoimmune diseases, and atacept is one drug targeting B cells.¹ The clinical trial testing atacept in multiple sclerosis (ATAMS) provoked an unexpected exacerbation of inflammation in the central nervous system (CNS) of treated patients, halting the trial.² Atacept is an immunoglobulin-fused soluble form of the transmembrane activator and CAML interactor (TACI, TNFRSF13b), a receptor belonging to the tumor necrosis factor (TNF) receptor superfamily. TACI binds 2 related members of the TNF ligand superfamily, the B-cell activation factor from the TNF family (BAFF; TNFSF13b) and a proliferation-inducing ligand (APRIL; TNFSF13).³ Atacept-mediated progression of a neurodegenerative disease has been reproduced with the ATON trial, testing atacept in optic neuritis (ON).⁴ Concurrently, atacept-mediated worsening was also reported in a variant of experimental autoimmune encephalitis (EAE) resulting in ON in rat.⁵ These results may mean that antagonism of the APRIL/BAFF axis accelerates autoimmune neurodegeneration.

BAFF and APRIL play a nonredundant role in humoral immunity.⁶ APRIL mainly acts on antibody-producing plasma cells by driving isotype switch and survival. In addition to TACI, BAFF and APRIL also share the B-cell maturation antigen (BCMA) as a receptor.³ To efficiently signal in TACI/BCMA-expressing target cells, APRIL needs heparan sulfate proteoglycans (HSPGs) as coreceptors.^{7,8} BAFF does not share this coreceptor binding activity, but has a specific receptor, BAFF-R.⁹ In the present study, we are showing that APRIL, but not BAFF, may have a protective role in the CNS by acting on astrocytes.

Material and Methods

Human Samples and Mouse Preclinical Model

The human autopsies from multiple sclerosis (MS) patients have been previously described.¹⁰ Control brain autopsies were obtained in healthy white matter area from a patient suffering from lateral amyotrophic sclerosis, and 3 patients deceased from non-neurodegenerative disorders. The *APRIL* knockout (KO) mice used were as originally described by Varfolomeev et al.¹¹ Controls were wild-type (WT) mice housed in the same conditions. EAE was induced in female mice with a commercial emulsion with 30 μ g of myelin oligodendrocyte glycoprotein 35–55 peptide in complete Freund adjuvant, followed 24 hours later by an intravenous injection of 100 ng of pertussis toxin (Hooke Laboratories, Lawrence, MA). Mice were scored as follows: 0, no symptoms; 1, decreased tail tone; 2, mild monoparesis or paraparesis; 3, severe paraparesis; 4, paraplegia and/or quadriparesis; and 5, moribund condition or death. Intraspinal injections were performed into the spinal subarachnoidal space at the

L5–L6 level using a 30-gauge needle (MicroFine; BD Biosciences, Franklin Lakes, NJ). The correct delivery was systematically checked by the mouse tail flick reflex. Intravenous injections were performed in the tail vein. Disease scoring was conducted in a randomized manner by 2 independent investigators masked to the mouse genotype and experimental group. All experimentation was prepared in concordance with the ARRIVE guidelines, and was approved by the Grenoble ethical committee.

Immunohistochemistry

Luxol fast blue (Sigma, St Louis, MO) was used to stain myelin. Formalin-fixed paraffin-embedded biopsies were stained with Stalk-1 (rabbit polyclonal) and Aprily-8 (mouse IgG1) detecting APRIL-producing cells and secreted APRIL, respectively, as previously described.¹² Anti-CD68 (clone PGM1, IgG3; Dako, Carpinteria, CA) was used at 10 μ g/ml after heat-induced epitope retrieval (HIER) in Tris 10 mM, ethylenediaminetetraacetic acid 1 mM, pH 9. The polyclonal anti-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been described elsewhere.¹⁰ Anti-chondroitin sulfate proteoglycan (CSPG; CS-56, IgM) was from Sigma and was used at 10 μ g/ml without HIER. Anti-HSPG 3g10 (IgG2b) and 10e4 (IgM) were from Euromedex (Souffelweyersheim, France) and were used at 10 μ g/ml. 10e4 recognizes native HSPG, whereas 3g10 recognizes HSPG after heparinase digestion.¹³ The ScFv anti-CSE, GD3G7, was a kind gift of Dr Th. van Kuppevelt (Nijmegen, the Netherlands). GD3G7 binding was revealed by a polyclonal anti-vesicular stomatitis virus (VSV) tag (Abcam, Cambridge, MA). Detection was based on a horseradish peroxidase (HRP)-labeled polymer conjugated with secondary antibodies (Dako, EnVision+ System-HRP kit). Development was made using 3-amino-9-ethyl-carbazol substrate (Sigma-Aldrich) and counterstain with Mayer hematoxylin (Dako). For multicolor immunofluorescence stainings, the following primary antibodies were used: Stalk-1, Aprily-8, 3g10, 10e4, CS-56, GD3G7, anti-CD68, anti-gial fibrillary acidic protein (GFAP; polyclonal rabbit IgG, 1/500, Dako). Detection was performed with anti-VSV-fluorescein isothiocyanate (FITC), and Alexa Fluor 488, phycoerythrin (PE), Alexa Fluor 350-conjugated anti-Ig secondary reagents. 4,6-Diamidino-2-phenylindole (DAPI; Dako) was used for nucleus staining. Stainings were visualized with an Olympus (Tokyo, Japan) BX-41 microscope.

Recombinant Protein Production

Expression constructs for Fc-tagged muAPRIL_{A88} (88-233), muAPRIL_{H98} (98-233), and muBAFF (134-285) have been described elsewhere.⁸ Fc-tagged human glucocorticoid-induced TNF-related ligand (Fc-hGITRL [47-177]) was

used as negative control. Due to the T-cell activation property of GITRL, Fc-tagged human ectodysplasin A (245-391) was used in the T-cell *in vitro* stimulation assay and in the *in vivo* treatment assay. Recombinant proteins were transiently produced in 293 T HEK cells with serum-free Optimem 1 medium. Purification was performed with Protein-A Sepharose (GE Healthcare, Chicago, IL). The absence of endotoxin contamination was assessed with the limulus amoebocyte lysate kit (Enzo Life Sciences, Farmingdale, NY).

Radioimaging

Proteins were radiolabeled with iodogen and injected intravenously (3.7 MBq/mouse, 6 mice/group). Forty-eight hours later, the animals were euthanized and perfused with saline, and the activity in the spinal cord was expressed as percentage injected dose per gram.

Brain Cell Culture

Human astrocytes were purchased from ScienCell Research Laboratories (Carlsbad, CA). The astrocytoma cell line CRT was purchased from American Tissue Culture Collection (Manassas, VA). Primary mixed glial cultures were established from the forebrains of C57Bl/6J newborn mice. Amoeboid microglia floating cells were detached from the astroglial monolayer by manual shaking. The remaining adherent monolayer contained astrocytes and adherent microglia. The cells were detached with trypsin. The microglia were removed by adhesion on plastic over a period of 30 minutes. Lipopolysaccharide (LPS) and Poly-IC were from Sigma. For the endocytosis experiment, the CRT cell line was incubated with 1 µg/ml of human FLAG-tagged APRIL for 30 minutes at 4°C, washed, and further incubated at either 4°C or 37°C for 45 minutes. Cells were then fixed in paraformaldehyde 4% followed by quenching in phosphate-buffered saline (PBS) glycine 0.1 M. Astrocyte-bound APRIL was detected with a biotinylated anti-FLAG antibody (1 µg/ml, Sigma) and PE-conjugated streptavidin (BD Biosciences) in PBS 2% bovine serum albumin 0.2% saponin. Cholera toxin B subunit FITC (10 µg/ml, Sigma) and DAPI (5 µg/ml) were used to label plasma membrane and nucleus, respectively. Cells were analyzed on an LSM510 confocal microscope (Carl Zeiss) with a plan-Apochromat ×63/1.4 oil objective.

Flow Cytometry

Cells were incubated for 30 minutes on ice with Fc-APRIL_{A88}, Fc-APRIL_{H98}, or Fc-GITRL, each at 10 µg/ml. PE-conjugated anti-IgG secondary antibody was used to detect the binding. The 10e4 and CS-56 monoclonal antibodies (mAbs) were also used at 10 µg/ml, followed by FITC-conjugated goat antimouse IgM. Heparinase, chondroitinase, and NaClO₃ treatments were performed as previously described.¹⁴ 7AAD (BD Biosciences) was used at 50 µg/ml to exclude dead cells.

Cytometry bead arrays (CBA) for mouse cytokines were from Becton Dickinson (Franklin Lakes, NJ). Fluorescence was analyzed by flow cytometry on an Accuri C6 apparatus (BD Biosciences).

For the analysis of spinal cord cells, mice were first perfused with saline and infiltrating cells, and microglia were obtained at the interphase between the 75% and 35% Percoll fractions and washed with Hank balanced salt solution 3% fetal calf serum. Cells were stained with fluorochrome-conjugated CD45, CD11b, MHC-II (I-Ab), CX3CR-1, and Ly6C from BD Biosciences, and sorted on a FACSaria III cell sorter (BD Biosciences).

Surface Plasmon Resonance

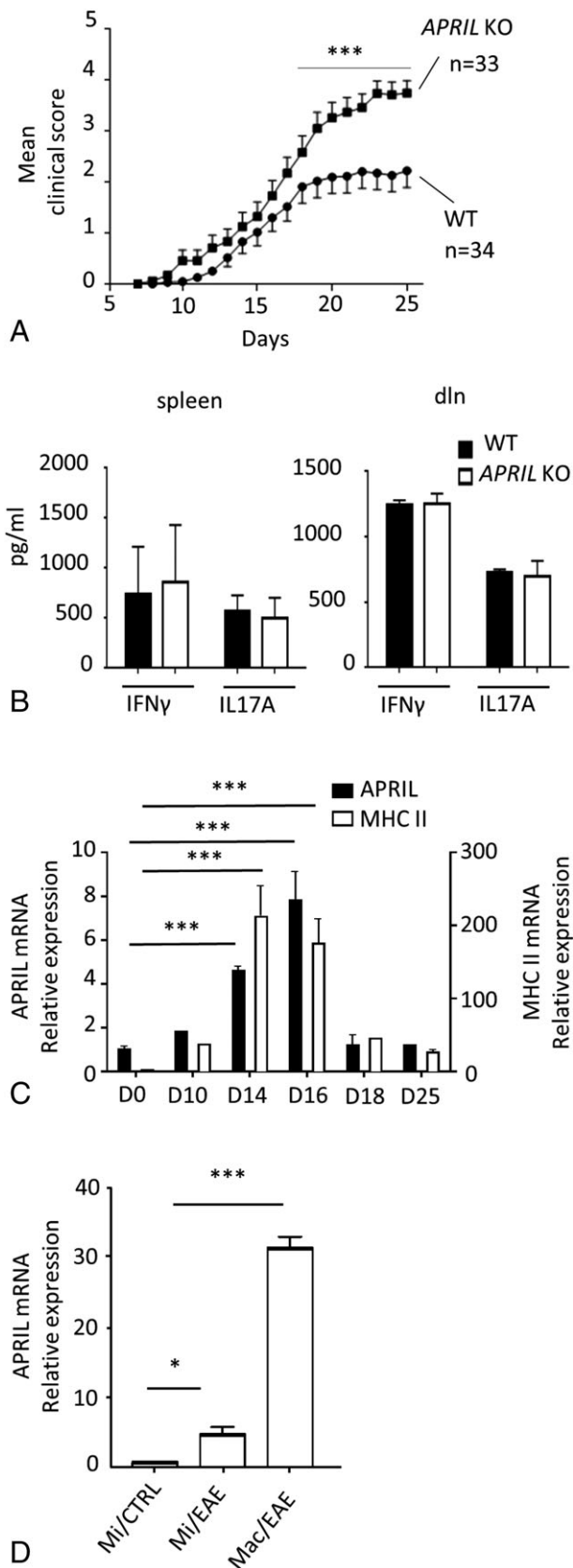
SPR analyses were performed on a BIAcore 3000 (GE Medical Systems, Milwaukee, WI). Binding assays on glycosaminoglycan surfaces were performed as previously described.¹⁵ Trimeric human FLAG-tagged APRIL_{A88} was used.⁸ Data were analyzed using BIAeval 3.1 software.

In Vitro T-Cell Activation Assays

2D2 TCR transgenic mice were obtained from Charles River (Wilmington, MA). After red blood cell lysis, spleen cells from 2D2 mice were stimulated with the MOG 35-55 peptide (3 µg/ml, polypeptide). Semiconfluent mouse primary astrocytes were prestimulated for 48 hours with Fc-APRIL_{A88} at 10 µg/ml. T-Cell proliferation was assessed after standard carboxyfluorescein succinimidyl ester labeling and flow cytometry analysis (LSR-II, Becton Dickinson). Cytokine secretion was assessed by enzyme-linked immunosorbent assay (ELISA) in the supernatant 3 days after stimulation. Rat antimouse IL-10 (clone JES5-2a5; BioLegend, San Diego, CA) was used at 10 µg/ml.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA extractions were performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The reverse transcription was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. Primers were 5' to 3' forward GTGAATATTTTGACAGTCTGCT and reverse ATCTAGTGTGAGTTGGCTTC for muBCMA, forward CCAAAGATCAGTACTGGGAC and reverse AAGCTACACGTTTCCACAG for muTACI, and forward GGGCAGTGCTCCCAAAT and reverse TCGTTTTTCG TGGTGACAAGA for huBCMA. Polymerase chain reaction (PCR) was performed using a Taq Platinum polymerase kit (Thermo Fisher Scientific, Waltham, MA). The amplification procedure included a 3-minute step at 94°C followed by 35 cycles with 30 seconds at 94°C as a denaturation step, 30 seconds at 55°C as a hybridization step, and 1 minute at 72°C as an elongation step. A final 4-minute, 72°C step was



used for final elongation. PCR products were visualized on 1% agarose gel using Gelgreen (Ozyme, Montigny-le-Bretonneux, France) dye under ultraviolet illumination. Quantitative PCR (qPCR) was performed on a 7500 Real Time PCR System with Power Syber Green (Applied Biosystems, Foster City, CA). The primers forward CTGGAGGC-CAGGGAGACAT and reverse GCACGGTCAGGATCA-GAAGG for muAPRIL, forward GTGCGGCGGAGAGTT GAG and reverse ACCGTCTCCAGCATCACCAG for the muMHC class II I-Ab, forward AGCATGGCCCAGAAA TCAAGGA and reverse GCCTTGTAGACACCTTGGTC TTG for muIL-10, and forward TTGTCAAGCTCATTTTCTGGTATG and reverse GGATAGGGCCTCTCTTG CTCA for glyceraldehyde-3-phosphate dehydrogenase (muGAPDH) were used.

Statistics

EAE was analyzed by using a Mann–Whitney *U* test either day by day or for the entire course of the experiment. qPCR results were analyzed by an analysis of variance-based test with a Tukey multiple comparison. Probability values <0.05 were considered statistically significant. ELISA and proliferation were analyzed by parametric unpaired *t* test.

Results

Exacerbated EAE in the Absence of APRIL

To unveil the putative role of APRIL in MS, we first compared EAE in WT and *APRIL* KO mice. Figure 1 shows an exacerbated EAE starting around disease peak. In the periphery of EAE mice, we did not detect any difference in the level of disease-inducing IFN- γ and IL-17A cytokines measured after *in vitro* restimulation of spleen and draining of lymph node cells with the MOG priming peptide. Regarding APRIL

FIGURE 1: A proliferation-inducing ligand (APRIL) modulates the severity of experimental autoimmune encephalitis (EAE). (A) EAE induced in wild-type (WT) and *APRIL* knockout (KO) mice was monitored over time. The mean \pm standard deviation for the clinical score from a pool of 3 independent experiments is shown. Total number of mice is also shown. (B) Seven days postimmunization, total cells from spleen and lymph nodes draining (dln) the priming site were restimulated *ex vivo* with the MOG peptide. IFN- γ and IL-17A secretions are shown. (C) The relative expression to GAPDH of APRIL (left axis, solid bars) and MHC II (right axis, open bars) mRNA is shown for the spinal cord of EAE mice at different time points of disease evolution. A score of 1 was given to the spinal cord of healthy mice. (D) APRIL mRNA expression was determined by quantitative polymerase chain reaction on the indicated cells sorted by flow-activated cell sorting (Mac = macrophages; Mi = microglia). Results are presented as the mean of 4 mice. Microglia from naive mice were given a value of 1. **p* < 0.05, ****p* < 0.001.

TABLE. Pattern of APRIL Production and Secreted APRIL Observed in All MS Patients Analyzed

| Age, yr | Gender | Lesion | MS | APRILp | | | | APRILs | |
|---------|--------|---------|------|--------|-----------|-----------|-----------|--------|--------|
| | | | | NAWM | PPWM | Edge | Center | NAWM | Center |
| 35 | M | Ac | AMS | No | MM | Mod G, MM | Mod MM, G | No | Astro |
| 51 | F | Ac | AMS | No | Few MM | MM, G | MM | No | Astro |
| 40 | F | Ac | RRMS | No | No | Few G, MM | Few G, MM | No | Astro |
| 52 | M | Chr Act | AMS | No | Few MM, G | MM | Few MM | No | Astro |
| 49 | F | Chr Act | RRMS | No | Few G, MM | MM | Few MM | No | Astro |
| 41 | M | Chr Act | SPMS | No | Few MM, G | Weak MM | Few MM | No | Astro |
| 46 | F | Chr Act | SPMS | No | Mod G, MM | Mod G, MM | Few MM | No | Astro |
| 61 | F | SE | SPMS | No | Few G, MM | Few G, MM | Few MM | No | Astro |
| 67 | M | SE | PPMS | No | Few G, MM | Mod MM | Few MM | No | Astro |
| 56 | M | SE | SPMS | No | Few G, MM | Few G, MM | MM | No | Astro |
| 53 | M | Inact | PPMS | No | MM | MM | MM | No | Astro |
| 71 | F | Inact | PPMS | No | MM | MM | MM | No | Astro |
| 34 | M | Inact | PPMS | No | Few G, MM | Few MM | MM | No | Astro |
| 62 | F | Inact | SPMS | No | MM | MM | MM | No | Astro |
| 77 | F | Inact | PPMS | No | No | Few G, MM | Few MM | No | Astro |

Ac = acute; AMS = active MS; APRIL = a proliferation-inducing ligand; APRIL-producing cells (APRILp); secreted APRIL (APRILs); Astro = astrocytes; Chr Act = chronic active; F = female; G = granulocyte; Inact = inactive; M = male; MM = monocyte/macrophage; Mod = moderate; MS = multiple sclerosis; NAWM = normal-appearing white matter; PPMS = primary progressive MS; PPWM = periplaque white matter; RRMS = relapsing-remitting MS; SE = slowly expanding; SPMS = secondary progressive MS.

expression in mouse spinal cord, we observed a strong upregulation of APRIL transcripts, also around disease peak, that went back down to control levels afterward. The expression profile of APRIL mRNA was highly similar to that of major histocompatibility complex (MHC) class II, a marker of CNS inflammation commonly used in EAE. The myeloid lineage is the major source of APRIL.¹⁶ In EAE, infiltrating macrophages join the pool of brain-resident myeloid microglial cells. We examined APRIL expression in these 2 myeloid subsets. APRIL mRNA was barely detectable in Ly6C⁻CX3CR1⁺CD11b⁺CD45^{int} microglial cells from naive mice. During EAE, activated microglia cells defined by MHC class II expression slightly upregulated APRIL expression, whereas Ly6C⁺CX3CR1^{int}CD11b⁺CD45^{high} CNS-infiltrating macrophages were an abundant source of APRIL. These results demonstrated that upregulation of APRIL occurs during EAE in mouse CNS, and identified infiltrating macrophages as the major source of APRIL in EAE. In the absence of APRIL, the disease is exacerbated. We observed this exacerbation without a concomitant enhanced T-cell priming in the periphery, strongly suggesting a local role for APRIL in the CNS.

APRIL Expression in MS Lesions

We next studied APRIL expression in MS patients, and also observed a strong expression of APRIL *in situ*. In chronic active lesions, which are characterized by a dense rim of activated macrophages at the active lesion edge and a diffuse infiltration of macrophages with late myelin degradation products in the lesion center, immunohistochemical studies revealed a subset of macrophages producing APRIL at the active edge (Fig 2). There, we did not detect full-length APRIL, theoretically detectable with our pair of antibodies by a colocalization of APRIL production and secreted APRIL in producing cells, as in all the other human tissues analyzed so far. Rather, most if not all of the secreted APRIL localized throughout the entire lesion and in part also in the surrounding periplaque white matter. APRIL production was different according to disease stages and lesion types as defined previously.¹⁷ Fulminant acute lesions are present in patients with acute MS and relapse/remitting MS.¹⁸ In these lesions, active demyelination is predominantly associated with microglia activation, expressing high levels of NADPH oxidase, and macrophages in the lesions, which contain myelin degradation products.¹⁹ There,

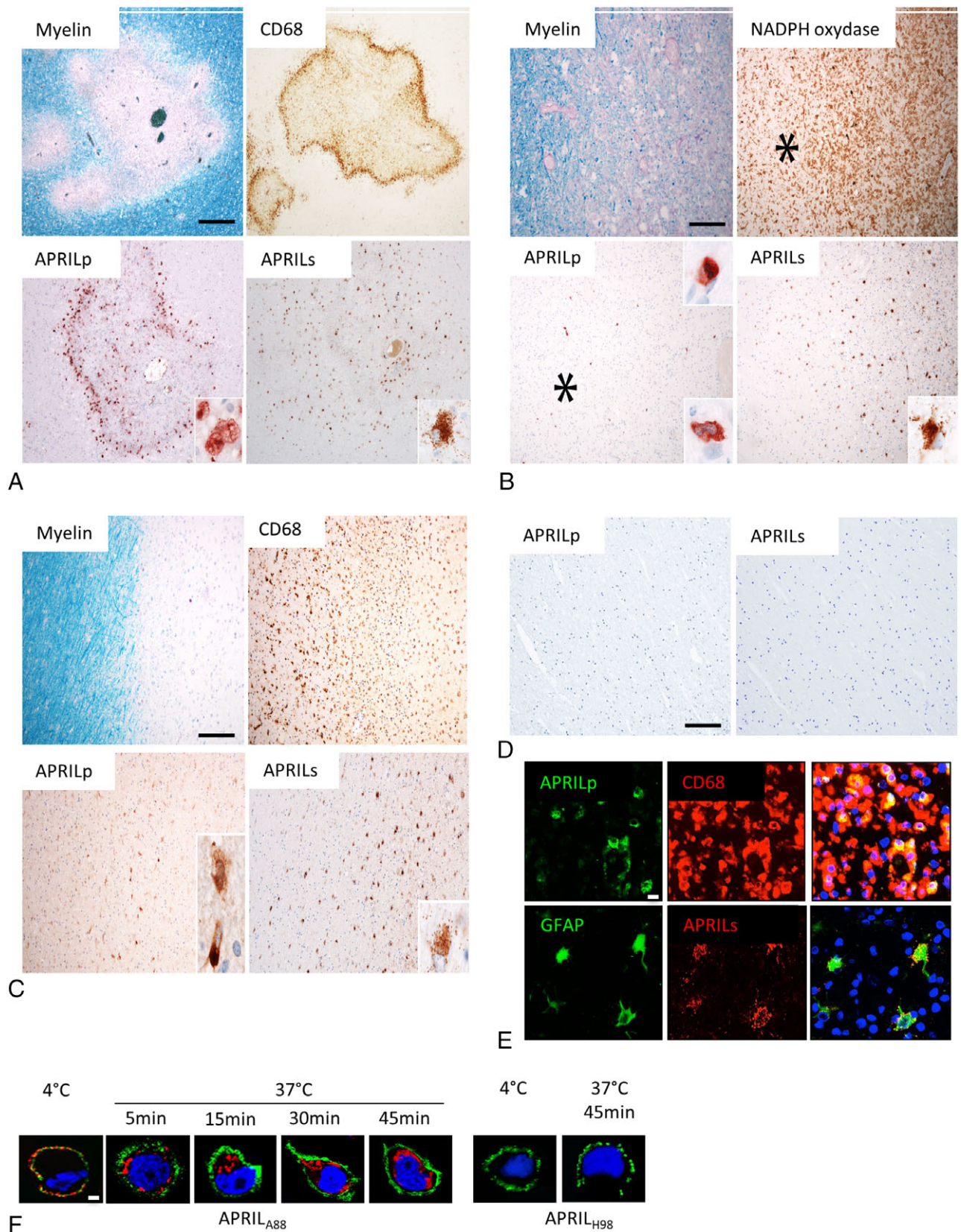


FIGURE 2. Legend on next page.

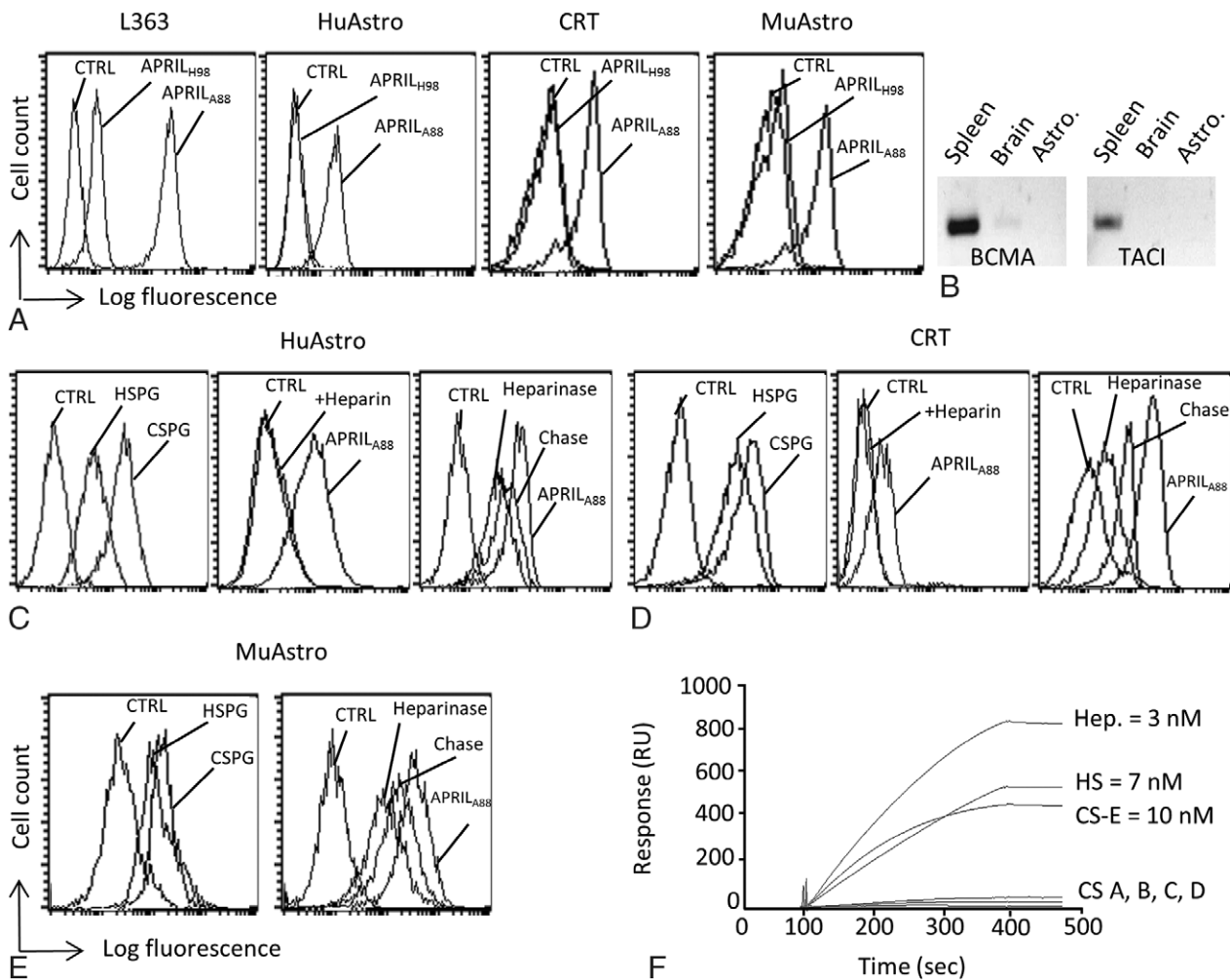


FIGURE 3: Chondroitin sulfate (CS) type E expressed on astrocytes is a new binding partner for a proliferation-inducing ligand (APRIL). (A) Binding of Fc-APRIL_{A88}, Fc-APRIL_{H98}, and control Fc-tagged glucocorticoid-induced TNF-related ligand (CTRL) was assessed by flow cytometry on control L363 cells, and human primary (huAstro), CRT astrocytoma, and mouse primary (muAstro) astrocytes. (B) Expression of B-cell maturation antigen (BCMA) and transmembrane activator and CAML interactor (TACI) mRNA was determined by reverse transcriptase polymerase chain reaction in adult mouse spleen, brain, and primary astrocytes. (C, D) Human primary astrocytes (C) and the CRT astrocytoma (D) were tested by flow cytometry for their reactivity with control IgM (CTRL), anti-heparan sulfate proteoglycan (HSPG; 10e4), and anti-chondroitin sulfate proteoglycan (CSPG; CS-56; left panel), for the binding of Fc-APRIL_{A88} in the presence of heparin (middle panel), and after heparinase/chondroitinase (Chase) treatment (right panel). (E) Mouse primary astrocytes were tested as in C for HSPG/CSPG reactivity and heparinase/chondroitinase treatment. Data are representative of at least 4 experiments. (F) Surface plasmon resonance analysis for the binding of trimeric APRIL_{A88} to the indicated coated heparin (Hep.) heparan sulfate (HS) and chondroitin sulfate (CS). Measured affinities are indicated. Results are representative of at least 3 independent experiments. RU = resonance units.

FIGURE 2: A proliferation-inducing ligand (APRIL) secreted by a subset of macrophages is internalized by reactive astrocytes in multiple sclerosis (MS) lesions. Immunohistochemical analysis of different types of MS lesions from patients' autopsies is shown. (A) Chronic active lesion. Scale bar = 1 mm. (B) Acute lesion. Asterisks label an area of profound microglia activation at the edge of the active lesion. Scale bar = 400 μ m. (C) Slowly expanding lesion. Scale bar = 400 μ m. Inserts in A–C show high magnification of APRIL-producing cells APRIL_p and cells positive for secreted APRILs. Note that we did not always use strictly serial sections for the sake of saving material. (D) Serial sections from a brain control autopsy were immunostained for APRIL_p and APRILs. Scale bar = 300 μ m. Pictures are representative of 4 cases. (E) Sections from MS biopsies were costained for APRIL_p/CD68 (upper panel), and APRILs/gial fibrillary acidic protein (GFAP; bottom panel). The costainings shown are representative of 3 chronic active and 2 slowly expanding lesions. Scale bar = 5 μ m. (F) APRIL_{A88}-binding astrocytoma CRT cells were incubated at 4°C or 37°C for the indicated times (left panel). The figures show costaining for APRIL (red) and the plasma membrane (CTxB staining, green). Nuclear 4,6-diamidino-2-phenylindole staining (blue) is shown on the merged pictures. An experiment with APRIL_{H98} is also shown (right panel). Scale bar = 2 μ m. The results are representative of 4 experiments. NADPH = nicotinamide adenine dinucleotide phosphate.

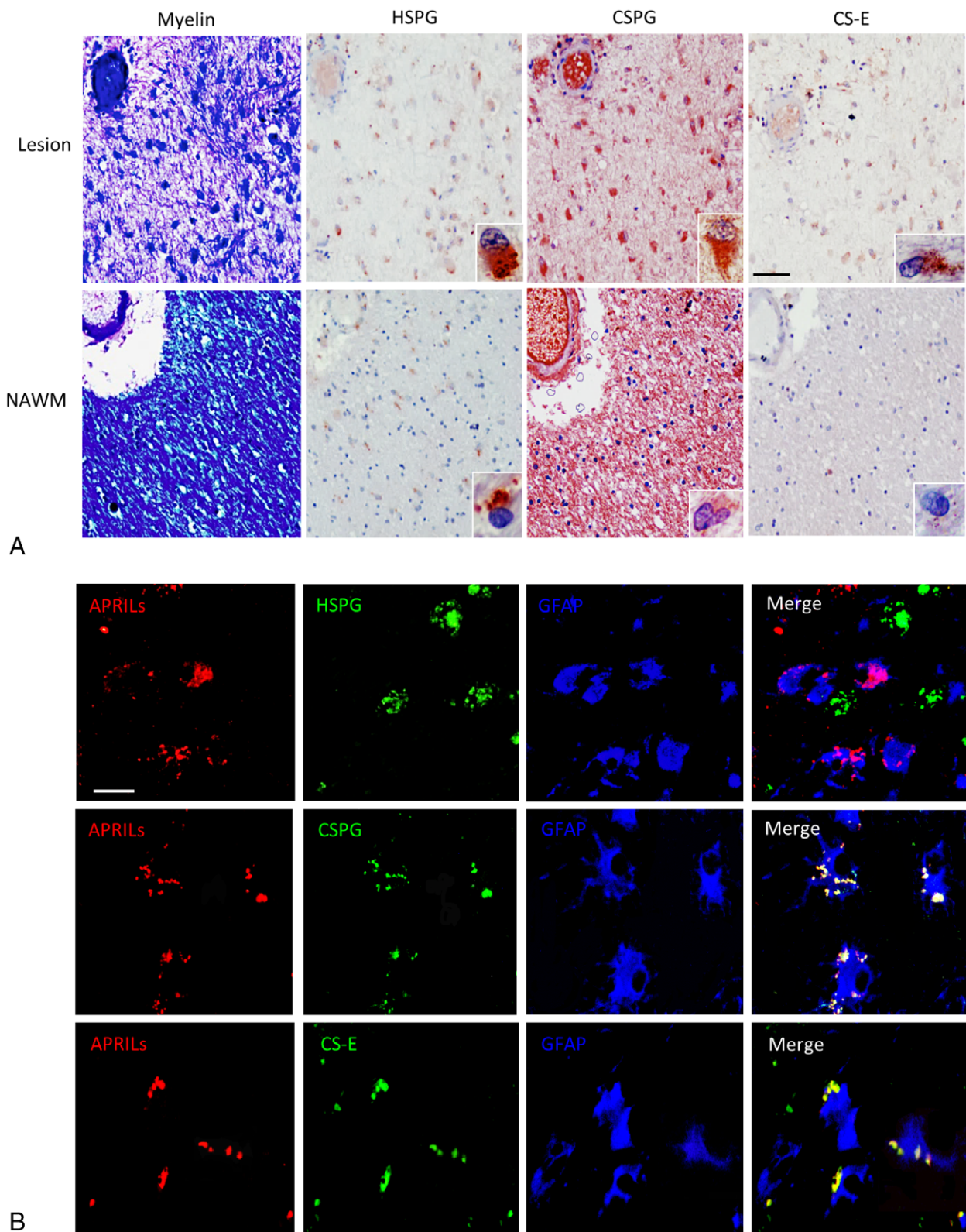


FIGURE 4. Legend on next page.

APRIL production was low and restricted to some scattered macrophages in the lesions and in the perivascular space. There was no evidence for APRIL production by activated microglial cells dominating such lesions. The typical feature

of slowly expanding lesions from patients with primary or secondary progressive MS is the presence of a rim of CD68⁺ activated microglia with some scattered macrophages, which contain early myelin degradation products. In these lesions,

the APRIL-producing cells concerned only a subset of the total CD68⁺ population. In inactive lesions, APRIL production was sparse or absent (data not shown). In most cases, we also observed APRIL production in granulocytes within blood vessel lumina, and it was occasionally present within the pathological tissue. Regarding secreted APRIL, it was found in every active plaque (acute/chronic active plaques and slowly expanding lesions), accumulating in cells with a morphology reminiscent of astrocytes, but only rarely in astrocytes from inactive lesions. No reactivity for APRIL production and secreted APRIL was detected in the normal-appearing white matter (NAWM) of these patients, defined as a zone at least 1 cm distal from the lesion edge. We did not detect APRIL production and secreted APRIL in the white matter from control brain autopsies. The Table summarizes the pattern of APRIL production and secreted APRIL observed in all MS patients analyzed. Costaining experiments confirmed that a subset of CD68⁺ myeloid cells produced APRIL, and that secreted APRIL bound to GFAP⁺ astrocytes. The apparent intracellular localization of APRIL in astrocytes indicated a potential *in situ* internalization of APRIL that we demonstrated *in vitro* upon incubation of astrocytes with recombinant soluble APRIL at 37°C. We used 2 recombinant soluble forms of APRIL, APRIL_{A88} and APRIL_{H98}. APRIL_{A88} has the potential to bind the signaling receptors TACI and BCMA and the coreceptor HSPG. In contrast, APRIL_{H98} binds only TACI and BCMA because of the deletion of the HSPG-binding site. In this experiment, APRIL_{A88} internalization was a rapid process, detectable as early as 5 minutes after incubation at 37°C, and internalized APRIL was quite stable, as we did not detect degradation within a time frame of 45 minutes at 37°C. By contrast, APRIL_{H98} did not become internalized. We did not detect APRIL mRNA by qPCR in resting mouse primary astrocytes, even upon activation by the Toll-like receptor ligands LPS and Poly-IC (data not shown). Taken together, our *in situ* analysis shows that a subset of macrophages infiltrating lesions from MS patients produces APRIL, and the secreted product accumulates into astrocytes.

CS Type E from Astrocytes Is a New Binding Partner for APRIL in MS Lesions

We next analyzed expression of APRIL receptors on astrocytes. APRIL_{A88} and to a lesser extent APRIL_{H98} bound to the control multiple myeloma cell line L363, expressing

the HSPG CD138 (also known as syndecan-1) as well as TACI and BCMA (Fig 3). Human/mouse primary astrocytes and the astrocytoma cell line CRT bound only APRIL_{A88}, excluding surface expression of TACI and BCMA on primary astrocytes. As another control, BAFF did not bind to these astrocytes (data not shown). Reverse transcriptase PCR analysis for TACI and BCMA mRNA further excluded a putative intracellular location of these 2 receptors. Human primary astrocytes and the CRT astrocytoma cell line expressed the known coreceptor for APRIL, HSPG, as revealed by 10e4 mAb reactivity. Additionally, they expressed another sulfated receptor, CSPG, as revealed by CS-56 mAb reactivity. The presence of heparin, a low molecular weight HSPG, fully inhibited APRIL_{A88} binding to these astrocytes, confirming that APRIL binding was strictly dependent on a sulfated receptor. Finally, heparinase and chondroitinase treatment inhibited APRIL_{A88} binding. Similar results were obtained with mouse primary astrocytes. Taken together, these show that APRIL is also interacting with CSPG, in addition to HSPG. The CS chain of CSPG is heterogeneous regarding the sulfation level and position on the disaccharide core unit. This is the case in the CNS, and that alters CSPG ligand binding properties.²⁰ In surface plasmon resonance analysis, APRIL showed a strict specificity to disulfated CS type E but neither to disulfated type D nor to the monosulfated types A, B, and C. The affinity of interaction of trimeric APRIL to CS type E was similar to the one measured with heparin or HSPG, in the nanomolar range.

In MS lesions, there was no reactivity with 10e4, recognizing native HSPG (data not shown). 3g10, recognizing HSPG after heparinase digestion, is believed to be of wider reactivity than 10e4. 3g10 staining revealed a similar expression of HSPG in the NAWM and inside lesions (Fig 4). CSPG as defined by CS-56 reactivity was highly present in the NAWM in cells and the extracellular matrix. In lesions, CSPG expression was less abundant, mostly confined to cells. On the contrary, CS type E was barely present in the NAWM, but highly upregulated in cells from lesions. Costaining experiments revealed that GFAP⁺ astrocytes from active (n = 3), slowly expanding (n = 2), and inactive (n = 2) lesions did not express HSPG (3g10 reactivity). The nonastrocytic HSPG found in the extracellular matrix did not colocalize with secreted

FIGURE 4: Chondroitin sulfate (CS) type E expressed by reactive astrocytes is the unique binding partner for a proliferation-inducing ligand (APRIL) in multiple sclerosis (MS) lesions. (A) A biopsy from an MS patient was stained for myelin (Luxol fast blue), heparan sulfate proteoglycan (HSPG; 3g10), chondroitin sulfate proteoglycan (CSPG; CS-56), and CS type E. Pictures representative of a demyelinated lesion and the normal-appearing white matter (NAWM) are shown. Scale bar = 100 μm. (B) The MS biopsy was also costained for secreted APRIL (APRILs) (red)/HSPG (3g10, green)/glial fibrillary acidic protein (GFAP; blue; upper panel), APRILs (red)/CSPG (CS-56, green)/GFAP (blue; middle panel), and APRILs (red)/CS-E (green)/GFAP (blue; lower panel). Right panels show the merged staining. Scale bar = 10 μm. The results are representative of 7 MS patients.

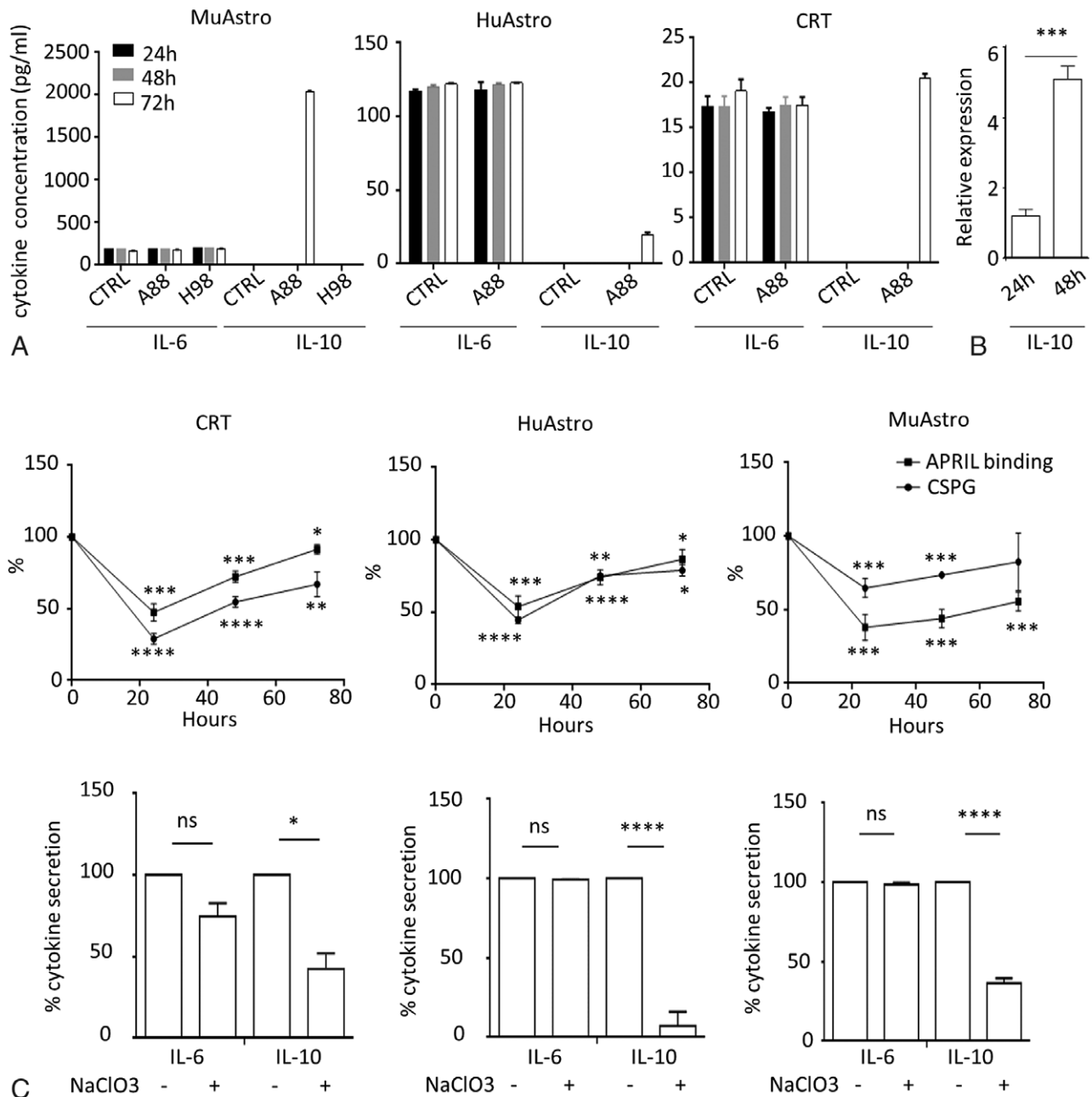


FIGURE 5: A proliferation-inducing ligand (APRIL) induces IL-10 secretion by astrocytes. (A) Enzyme-linked immunosorbent assay analysis of cytokines in supernatants of mouse and human primary astrocytes and CRT astrocytoma treated with 10 $\mu\text{g/ml}$ of control Fc-tagged glucocorticoid-induced TNF-related ligand (Fc-GITRL; CTRL), Fc-APRIL_{A88}, and Fc-APRIL_{H98}. IL-6 and IL-10 productions are shown. The mean of a duplicate test with minimum–maximum values is shown. **(B)** Transcript expression of IL-10 in mouse primary astrocytes was measured by quantitative reverse transcriptase polymerase chain reaction. The results are expressed as the fold change relative to the expression in presence of Fc-GITRL. **(C)** Chondroitin sulfate proteoglycan (CSPG; CS-56 reactivity) and APRIL_{A88} binding on NaClO₃-treated CRT astrocytoma and human and mouse primary astrocytes is shown (upper panels). IL-6 and IL-10 production by NaClO₃-treated cells in the presence of Fc-APRIL_{A88} stimulation is shown (bottom panels). One hundred percent represents untreated cells. Means \pm standard deviation from 3 independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns = not significant.

APRIL. In all cases, including active (n = 3), slowly expanding (n = 2), and inactive (n = 2) lesions, CSPG (CS-56 reactivity) was expressed within GFAP⁺ astrocytes and colocalized with secreted APRIL for active and slowly

expanding lesions. We further observed expression of CS type E within astrocytes in 2 of 3 active, 1 of 2 slowly expanding, and 0 of 2 inactive lesions. When expressed, CS type E colocalized with secreted APRIL in astrocytes.

Taken together, our data show a selective binding of APRIL to CSPG. This binding occurs *in situ* in MS lesions at the level of reactive astrocytes. In some lesions,

but not all, we identified CS type E as the APRIL binder. In MS lesions, APRIL does not bind to HSPG.

APRIL Induces an IL-10-Dependent Anti-Inflammatory Response in Astrocytes

Astrocytes secrete a wide array of cytokines acting on T-cell-mediated autoimmune responses in the CNS.²¹ We screened several of these cytokines by ELISA and CBA after stimulation of murine primary astrocytes with APRIL. *in vitro* cultured primary astrocytes are considered to be non-fully resting astrocytes as judged by their constitutive expression of proinflammatory cytokines.²² APRIL stimulation did not change the expression of the proinflammatory IL-6 by these *in vitro* expanded astrocytes, whereas it induced them to produce the anti-inflammatory IL-10 72 hours after stimulation (Fig 5). APRIL_{H98} had no effect in this experiment. We also observed a late induction of IL-10 with no change in IL-6 by APRIL_{A88} in human primary astrocytes and the human astrocytoma cell line CRT. Other cytokines reported to be produced by astrocytes, including IL-2, IL-4, IL-12, IFN- γ , and TNF, were not detectable at any time point over the course of the experiment. Quantitative reverse transcriptase PCR analysis confirmed the late upregulation of IL-10 mRNA in mouse primary astrocytes 48 hours after stimulation with APRIL. CRT astrocytoma treatment with 25 mM NaClO₃, a transient inhibitor of sulfation, reduced CSPG surface expression and APRIL binding. HSPG decrease on CRT cells required a higher NaClO₃ concentration (75 mM, data not shown). With 25 mM NaClO₃, CRT cell mortality never reached more than 15%. In this condition, IL-10 induction decreased by more than 50% after APRIL stimulation, while IL-6 was not significantly modulated. We observed a similar

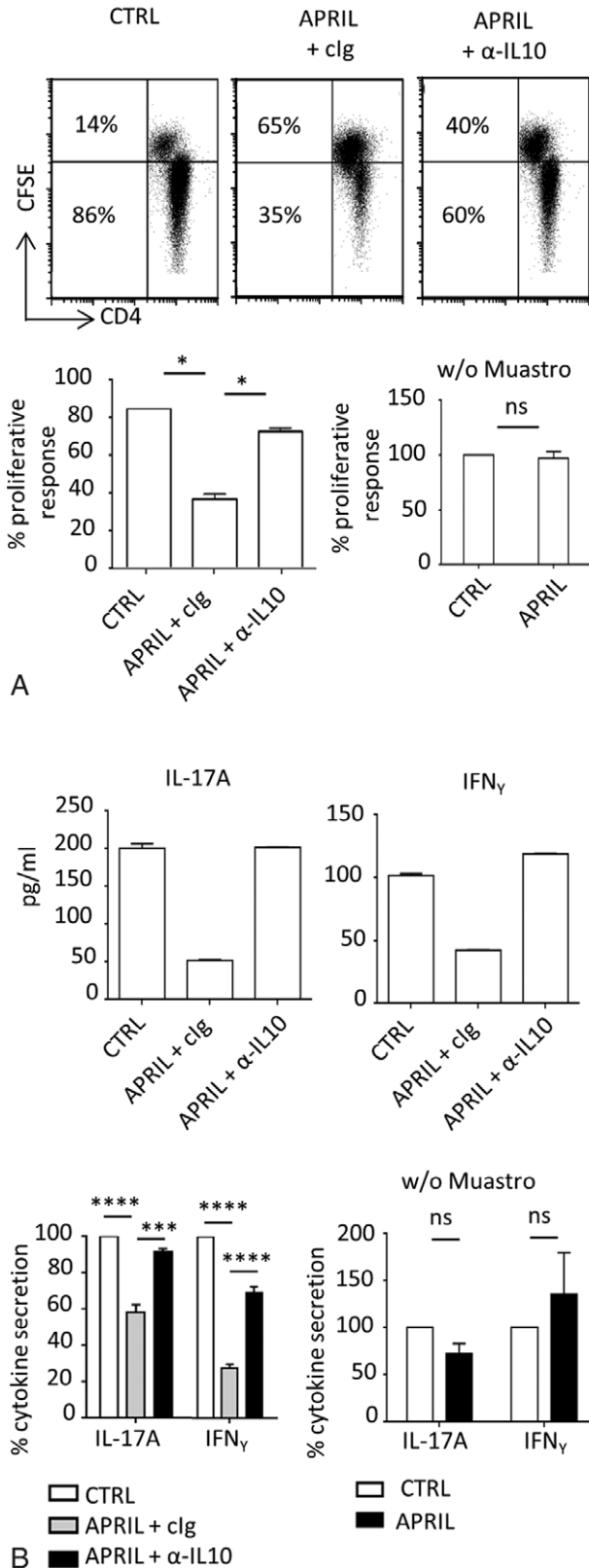
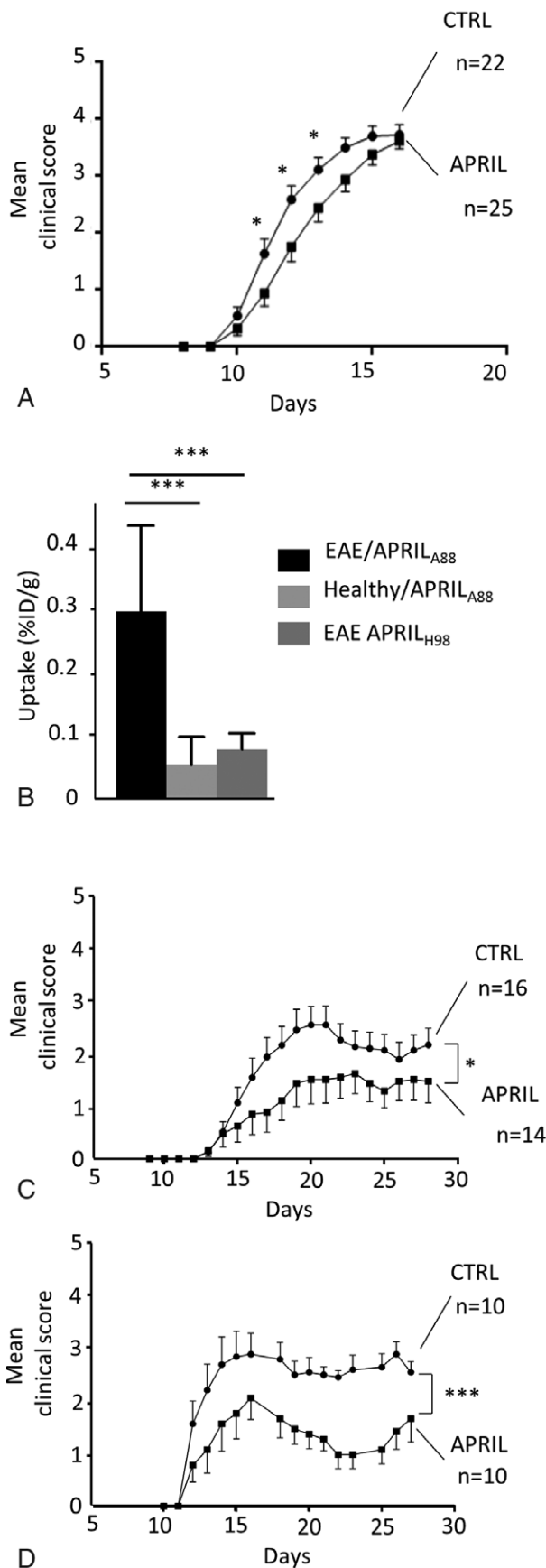


FIGURE 6: IL-10 produced by a proliferation-inducing ligand (APRIL)-stimulated astrocytes inhibits MOG-specific responses. Total splenocytes from 2D2 TCR transgenic mice were stimulated with the MOG peptide (3 μ g/ml) in the presence of control Fc (Fc-ectodysplasin A) and Fc-APRIL_{A88}-treated mouse primary astrocytes. The Fc-APRIL-treated conditions were performed in the presence of a control monoclonal antibody (clg) and antimouse IL-10. In some conditions, the stimulation was performed in the absence of astrocytes. (A) 2D2 T-cell proliferation based on Carboxyfluorescein succinimidyl ester (CFSE) staining is shown (upper panel). The percentages of nondividing/dividing CD4⁺ T cells are indicated. Mean percentage \pm standard deviation (SD) of the proliferative response obtained in 3 independent experiments is also shown (bottom panel). (B) IL-17A and IFN- γ production is shown (upper panel). The mean of a duplicate test with minimum-maximum values is shown. Mean percentage \pm SD obtained in 3 independent experiments is also shown (bottom panel). One hundred percent represents the response obtained in the presence of control-treated astrocytes. * p < 0.05; *** p < 0.001; **** p < 0.0001. ns = not significant.



inhibition of CSPG expression associated with reduced APRIL binding with 25 mM NaClO₃ treatment in human and mouse primary astrocytes. NaClO₃ treatment also reduced by >50% the production of IL-10 in APRIL-treated human and mouse primary astrocytes, while leaving IL-6 secretion unchanged.

We next performed *in vitro* peptide stimulation assays with MOG-specific TCR transgenic 2D2 CD4⁺ T cells. In the presence of primary astrocytes treated with APRIL, 2D2 T-cell proliferation was significantly inhibited (Fig 6). Notably, IL-10 antagonism in this assay partially reversed the APRIL-mediated inhibition. Regarding IL-17A and IFN- γ , the presence of APRIL-treated primary astrocytes also inhibited their production by 2D2 T cells. As for proliferation, addition of a blocking anti-IL-10 partially reversed the APRIL-mediated inhibition. In these experiments, the effect of APRIL_{A88} stimulation vanished in the absence of astrocytes. Hence, APRIL induces an anti-inflammatory response in astrocytes with the production of IL-10 that may protect from an autoimmune neurodegenerative insult.

EAE Treatment with Recombinant APRIL

We next sought to assess the effect of recombinant APRIL injection in EAE mice. We first performed a local intraspinal delivery of APRIL twice in the presymptomatic phase. APRIL injections significantly lowered disease severity for 3 consecutive days (Fig 7). This route did not allow us to perform injections in the symptomatic phase due to tail paralysis in EAE mice. We then switched to the intravenous route. Nuclear imaging with iodine-labeled recombinant APRIL showed that APRIL_{A88} accumulated compared to APRIL_{H98} in the spinal cord of EAE mice. No such level of detection was achieved in the spinal cord of healthy mice. This result may reflect leakage of the blood-brain barrier and specific retention of APRIL_{A88} by CSPG upregulation in EAE lesions. Figure 7C shows that repeated intravenous injections of APRIL_{A88} before and during disease significantly reduced disease severity. We

FIGURE 7: Recombinant a proliferation-inducing ligand (APRIL) injection lowers experimental autoimmune encephalitis (EAE). (A) Recombinant APRIL_{A88} and control ectodysplasin A (EDA) were injected intraspinally at day 7 and 9 postimmunization. The clinical score was monitored as in Figure 1A. (B) The biodistribution of the indicated radiolabeled molecules is shown for the spinal cord. (C, D) Fifty micrograms of recombinant Fc-APRIL_{A88} and control Fc-EDA were injected intravenously every 3 days for 10 days starting on day 2 before disease onset (C) and at disease onset (D). Results are shown as in A. Results shown are a pool of 3 independent experiments. Total number of mice is shown. **p* < 0.05, ****p* < 0.001. % ID/g = percentage injected dose per gram.

observed similar reduction when APRIL_{A88} treatment was started at disease onset (see Fig 7D). Taken together, these data show that injection of recombinant APRIL may constitute a valuable treatment for MS.

Discussion

Antagonism of BAFF and/or APRIL with a soluble form of one of their common receptors, TACI, exacerbates neuroinflammatory diseases such as MS and ON. It could be postulated that BAFF and/or APRIL positively regulate the subset of B cells producing IL-10 and IL-35 that was shown to dampen EAE.^{23,24} However, a protective role for BAFF and/or APRIL on nonimmune cells present in the CNS cannot be excluded. BAFF production by astrocytes is upregulated in MS lesions, and EAE is exacerbated in *BAFF-R* KO mice.^{25,26} However, a role for CNS BAFF on CNS-resident cells appears unlikely, as BAFF-R expression was only reported in infiltrating immune cells.²⁵ We confirmed that result with the MS patients analyzed here, and extended it by showing the absence of BAFF binding and BAFF-R expression on primary astrocytes (data not shown). Hence, CNS BAFF produced by astrocytes may exert a local role, but on immune infiltrating cells. Less is known regarding APRIL in MS. In the cerebrospinal fluid (CSF) of MS patients, APRIL is either slightly upregulated or unmodulated compared to control CSF from patients suffering from noninflammatory neurodegenerative disorders.^{27,28} Thangarajh et al reported that reactive astrocytes produced APRIL.²⁹ The authors used the same tissue-reactive antihuman APRIL antibody, Aprily-8. However, Aprily-8 is directed against the secreted part of APRIL, whereas Stalk-1 (used here) reacts with the part of the molecule that remains anchored in producing cells after processing. Hence, Stalk-1 reactivity clearly detects APRIL-producing cells, whereas Aprily-8 reactivity may detect either APRIL-producing cells before APRIL processing or the secreted product. Our combination of Stalk-1 and Aprily-8 reactivity indicates that in MS lesions it is a subset of macrophages that secretes APRIL, and reactive astrocytes selectively bind paracrine APRIL. The absence of APRIL mRNA detection in resting and activated primary astrocytes is also evidence arguing against production of APRIL by astrocytes. Our study also revealed that APRIL expression is variable according to the type of lesions. This is true for APRIL-producing cells. Only very few APRIL-producing macrophages were seen in initial demyelinating lesions of acute MS. APRIL-producing macrophages were also in low numbers in slowly expanding lesions. In contrast, APRIL-producing macrophages were numerous in chronic active lesions. Despite variation in the number of producing cells, reactive astrocytes

homogeneously contained a high concentration of secreted APRIL. APRIL present in reactive astrocytes may originate from infiltrating macrophages but also from the blood. The blood–brain barrier is permeable to serum components in MS,³⁰ and myeloid cells constitutively secrete APRIL in the blood.^{12,16,31} These analyses demonstrate that APRIL acts locally on CNS-resident astrocytes.

We did not find evidence for expression of TACI and BCMA in *in vitro* expanded astrocytes. We only found expression of the APRIL coreceptor, HSPG, and identify CSPG, a related sulfated proteoglycan, as a new APRIL binding partner in the CNS. The dual expression of HSPG and CSPG in *in vitro* cultured astrocytes is consistent with the previous study from Johnson-Green et al.³² *In situ* expression of proteoglycans by astrocytes is different. HSPG is not detected, whereas CSPG is present in reactive astrocytes. We identified CS type E, upregulated following different kinds of brain injury,^{33–35} as one of the binding partners of APRIL in reactive astrocytes. Astrocytes internalized paracrine APRIL. The reason for this internalization process is presently not known, warranting further investigation, but this process is likely due to the ligand internalization property described for proteoglycans.³⁶

Astrocyte function in MS has long been a matter of debate.^{37,38} This might be due to the important plasticity of these cells.³⁹ Nevertheless, astrocyte depletion has been shown by 3 independent research groups to exacerbate EAE, strongly arguing for a dominant protective function, at least in EAE.^{40–42} We are showing here that APRIL induces the production of IL-10 by astrocytes. Our data complement the signaling property of APRIL in astrocytes reported by others.⁴³ This IL-10 induction fits well with a protective function for APRIL and astrocytes in autoimmune MS, owing to the anti-inflammatory activity of this cytokine.⁴⁴ This is a likely explanation for the failure of clinical trials assessing the BAFF/APRIL antagonist, atacicept, in the autoimmune neurodegenerative disorders, MS and ON. To our knowledge, there is currently no trial in MS with an APRIL-specific antagonist, and such a trial may be unwanted. By contrast, exogenous APRIL lowered severity in a mouse model of MS. Taken together, the present bed- to benchside translation leads to the identification of APRIL as a potential new therapeutic compound for autoimmune neurodegenerative disorders.

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Author Contributions

All authors contributed to acquisition and analysis of the data and performed research. P.L. and B.H. contributed to the conception and design of the study. L.B., P.L., and B.H. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

P.S. and B.H. report receiving a commercial research grant and speaker bureau honoraria, respectively, from Merck Serono, which is developing atacept. The other authors have nothing to report.

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