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Investigation of Kir4.1 potassium channel as putative antigen of multiple sclerosis: a cohort study

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Summary

Background—Antibody-dependent pathogenicity is suggested in multiple sclerosis (MS) by intrathecal immunoglobulin production, IgG and complement deposition in the most common immunopathological lesion subtype (pattern II), and by a recent report that 47% of MS patients' sera contain a glial potassium-channel-specific-IgG(inwardly-rectifying, Kir4.1). Our study's aims were to determine, in MS serum and CSF, the frequency and specificity of Kir4.1-binding-IgG and, in demyelinating MS lesions, whether Kir4.1-immunoreactivity is retained or lost.

Methods—We tested by ELISA(Kir4.1-peptide 83–120) sera from 286 clinically and geographically diverse MS patients (229 population-based and 57 clinic-based),99 healthy controls and 109 disease controls, and cerebrospinal fluid [CSF] from 25 MS and 22 controls. CSFs and clinic-based MS-subset serum (50)were tested on functional Kir4.1-expressing cells, using methodologies validated for detecting clinically-pertinent neural plasma membrane-reactive autoantibodies: immunofluorescence and immunoprecipitation (solubilized recombinant human Kir4.1). We evaluated Kir4.1-immunoreactivity in brain from 15 archival histopathologically-

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Drs. Brickshawana, Buttman, Cabre, Chang, Chen, Guo, and Hinson have nothing to disclose.
Mr. Fryer, Ms. Jenkins, and Mr. Kryzer have nothing to disclose.

Author contributions
Study design and conceptualization: VL, AM, SP, CL, MB, PC, MR; Drafting of manuscript: AB, SH, JF, TK, MR; Acquisition, analysis and interpretation of data: AB, TK, SH, JF, YG, CL, MR, M-HC, A-PC, SJ, PC; Critical revision of the manuscript: VL, AM, CL; Obtained funding: VL, MR, SP, CL

confirmed MS cases(22 plaques: 8 early active, 8 inactive, 6 remyelinated; 13 periplaque regions)and compared 3 non-neurological cases (8 normal-appearing white/gray matter regions).

Findings—Kir4.1-peptide-ELISA reactivity was rare and did not differ significantly for 286 MS or 208 control sera (both 1%); no CSF was positive. IgGin 0/50 clinic-based MS sera immunoprecipitated Kir4.1, but control Kir4.1-specific-IgG did. By immunofluorescence,1/50 MS sera yielded faint plasmalemmal staining on both Kir4.1-expressing and non-expressing cells; 16/50 bound faintly to intracellular components. In all cases, IgG binding was quenched by absorption with liver powder or non-transfected cell lysates. Control Kir4.1-specific-IgG binding was quenched only by Kir4.1 protein-containing lysates. IgG in 0/25 MS CSFs bound to Kir4.1-transfected cells, live or fixed. Glial Kir4.1-immunoreactivity was increased relative to baseline normal brain expression (3 controls) in early active and remyelinated MS lesions, and in periplaque white matter (15 patients).

Interpretation—We did not find Kir4.1-specific-IgG in MS sera or CSF, nor Kir4.1 loss from glial cells in active demyelinating MS lesions. Serological testing for Kir4.1-IgG is unlikely to aid MS diagnosis. The target antigen of MS remains elusive.

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Introduction

Multiple sclerosis etiology and pathogenesis are poorly understood.[1] Interacting genetic and environmental factors[2–4] are implicated as susceptibility determinants and immune mechanisms as the effect or of central nervous system inflammatory demyelination with later neurodegeneration.[5] Assumption of autoimmune pathogenesis rests on inflammatory pathology, intrathecal immunoglobulin production and models of T cell-mediated CNS immunopathology. Reproducible antibody discoveries recently defined two inflammatory demyelinating CNS mimics of multiple sclerosisas autoimmune: neuromyelitis optica spectrum disorders (unified by aquaporin-4-IgG)[6] and relapsing optic neuropathy/ myelopathy accompanied by collapsin response-mediator protein [CRMP]5-IgG(T cell-mediated, usually paraneoplastic).[7, 8]

No neural-autoantigenis validated clinically as target of serum or cerebrospinal fluid (CSF) immunoglobulins in multiple sclerosis.[9] The variable clinical course[1] and inter-patient heterogeneity of active demyelinating lesions suggest multiple sclerosisis not a single entity.[10] Pattern II demyelination, the most common of four defined immunohistopathological patterns, suggests antibody and complement-dependent pathogenicity. Lack of a disease-specific biomarker to aid multiple sclerosis diagnosis confounds therapeutic trial design and outcome interpretation. Srivastava *et al*[11] recently identified a glial inwardly-rectifying potassium channel, Kir4.1, as a putative multiple sclerosis autoantigen by analyzing proteins immunoprecipitated from brain lysates by IgG pooled from 12 patients whose individual sera bound to brain sections and a brain membrane protein extract. Confirmatory evidence involved multiple experimental approaches. The investigators reported detecting Kir4.1-channel-specific-IgGin serum of

47% of adult multiple sclerosis patients(<1% of other neurological patients)[11] and, subsequently, in serum of 57% of children with acquired demyelinating disease.[12]

Here we report investigations of (i) Kir4.1-IgG (by ELISA) in serum of diverse clinic-based and population-based patient cohorts and in serum and CSF of a clinic-based subset of multiple sclerosis patients (by ELISA and cell-based assays and additionally [for serum] immunoprecipitation), and (ii) relative abundance of Kir4.1 protein-immunoreactivity in both astrocytes and oligodendrocytes of early active demyelinating multiple sclerosis brain lesions.

Methods

Subjects

Mayo Clinic's Institutional Review Board approved the study. Mayo Clinic patients signed consent for biospecimen research use at registration. Population-based subjects gave written consent at study entry. We tested by ELISA specimens from all multiple sclerosis patients(appendix) (serum, 286 [57 clinic-based; 229 population-based] and CSF, 25 [clinic-based])and healthy/or disease control subjects(serum, 208 [99 community healthy control donors recruited from registered Mayo employees, 90%, and employees' neighbours/ spouses, 10%; 109 autoimmune neurologic and miscellaneous immunologic disease controls]; CSF, 22 normal pressure hydrocephalus patients). We also tested the 25 MS and 22 control CSFs, and serum of 50 clinic-based MS patients and 31 healthy/disease controls (those with sufficient residual specimens), by Kir4.1-transfected cell-binding assays, and (sera only) by immunoprecipitation. We analyzed, immunohistochemically, archival supratentorial brain tissues (Table) from 15 MS cases (7 biopsies/8 autopsies) with histopathologically diagnosed MS-compatible inflammatory demyelinating lesions (22 plaques and 13 periplaque regions) and 3 control non-neurological cases.[10] Treating physicians obtained patient consent for research studies on biopsies (performed to exclude alternative diagnoses). Diagnoses of acute disseminated encephalomyelitis and neuromyelitis optica were excluded by clinical and pathological published criteria.

Enzyme-linked immunosorbent assay (ELISA)

Synthetic human Kir4.1-peptide (residues 83–120, N-terminally biotinylated)[11] was purchased from JPT Peptide Technologies, Berlin, Germany; assay plates (Nuncimmobilizer™ streptavidin pre-coated and pre-blocked) from Thermo Scientific Inc., Rockford, IL, USA. Diluent and wash buffer:phosphate buffered saline (PBS) containing 0.05% Tween-20 (plus 3% skimmed milk, Biorad Inc., to dilute serum (1/200, 1/400 and 1/800)and CSF (1/2), 10% normal goat serum to dilute enzyme-conjugated-IgG). Plates were washed before and after adding peptide (1 ug/mL, 1 hr, 20°C), and after each antibody incubation (serum 2 hr, 20°C; alkaline phosphatase-conjugated goat anti-human IgG, IgM and IgA [Rockland, PA], 1/4000 dilution, 1 hr, 20°C). After adding diethanolamine substrate, optical density was measured at 405 nm (Tecan micro plate reader).

Cells and cDNA used to establish cell-based and immunoprecipitation assays

We cloned from whole Human Brain QUICK-Clone cDNA library (Clontech Laboratories, Inc.) cDNA encoding Kir4.1, inserted it into pEGFP-C2 vector (Clontech) and transfected Human Embryonic Kidney cells (HEK 293, American Type Culture Collection)using Effectene Reagent (QIAGEN). Stable transfectants expressing eGFP-tagged human Kir4.1 (eGFP-Kir4.1) were selected in media containing Geneticin (GIBCO), 0.8 mg/mL. Clones expressing eGFP highly were expanded for Kir4.1 protein solubilization. For *Xenopus* oocyte studies, eGFP-Kir4.1 and Kir4.1 cDNAs were cloned into pGEMHE plasmid. cRNA was synthesized from linearized plasmid using T7 RNA polymerase.[13] Primary astrocytes were cultured from newborn mouse cerebral cortices. Astrocytic phenotype was confirmed antigenically (plasma membrane AQP4 and cytoplasmic glial fibrillary acidic protein).[14]

Solubilization of human Kir4.1 protein

At 4°C, stably-transfected HEK293 cells were twice homogenized (PT10–35 Polytron [Kinematica]) in buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1 mM $MgCl_2$, 700 mM NaCl, and protease inhibitors: 0.4 mM phenylmethanesulfonylfluoride, 0.1 μg/mL pepstatin, and 0.1 μg/mL aprotinin, final pH 7.8. After clearing debris (centrifugation 1,000g, 10 min), pooled supernatant membranes were pelleted (100,000g, 30 min); eGFP-tagged recombinant Kir4.1 was extracted in buffer containing 2% Triton X-100 (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl with protease inhibitors; final pH 7.8). Diluted supernatant antigen preparation (100,000g, 30 min) yielded 78,000 relative fluorescence units (RFU)/100μL.

Confirmation of recombinant Kir4.1 Channel Activity

Oocytes were clamped at −60 mV, and pulsed 50 ms(−160 mV to +60 mV, 20 mV steps; Warner OC-725C). Currents were filtered at 2 kHz (8 pole Bessel filter), recorded at 10 kHz and analyzed using Pulse and PulseFit (HEKA Instruments, Germany). *Solutions:* K^+ ringer (98 mMKCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) or K+ ringer + 5 mM $BaCl_2$ to block K^+ currents.[15] For inside-out patch-clamp experiments, HEK293 cells were seeded on cover slips(inverted microscope stage,20–23°C).[16] Patch pipettes pulled from borosilicate glass were back-filled (in mM: 95 K-gluconate, 30 KCl, 4.8 Na_2HPO_4, 1.2 NaH_2PO_4, 5 glucose, 2.38 $MgCl_2$, 0.726 $CaCl_2$, 1 EGTA, and 3 ATP, pH 7.2). Resistances of filled pipettes were 2–6 MΩ. After G Ω-seal was formed, C-Fast compensation circuit was used to cancel fast capacitive currents. Patches were excised from eGFP-Kir4.1-HEK cell membrane, with pipette solution as bath. EPC10-plus amplifier recordings were controlled by PatchMaster (HEKA). Channel activities were recorded in response to 500 ms voltage steps (20 mV, −140 to +60 mV; holding potential, −40 mV). Currents were filtered at 1 kHz with a 4-pole low-pass Bessel filter, sampled at 2–20 kHz and stored directly to hard disk. Records were inspected visually and automatic channel events analyzed by FitMaster software (HEKA).

Immunostaining of Kir4.1-expressing cells

HEK293 cells transiently expressing eGFP-Kir4.1, and primary mouse astrocytes, were seeded on coated coverslips (respectively, poly-L-lysine or poly-D-lysine/laminin, BD Biosciences). Live-cell staining[14] was at 4°C; fixed-cell staining (at 80–90% confluency)

was at 20–23°C. Diluent was PBS containing 10% goat serum and sodium azide, 0.02% w/v; fixation,100% methanol (−20°C, 10 min); permeabilization and blocking, 0.05% Tween-20 in PBS-10% goat serum (30 min); staining, human serum or CSF (unabsorbed, 1/100 or 1/2 dilution; 1 hr [fixed]; 30 min [live]); Kir4.1-specific-IgG (mouse monoclonal [Sigma-Aldrich], 1/500, and/or rabbit polyclonal [Alomone], 1/1200); aquaporin-4 C-terminus-specific-IgG (affinity-purified rabbit polyclonal [Sigma], 1/500).[14]

Bound IgG was detected with fluorochrome-conjugated goat anti-human, mouse or rabbit-IgG, (SouthernBiotech). Coverslips were washed after 30 min, mounted with ProLong Gold Antifade Reagent (Invitrogen) containing 4′, 6-diamidino-2-phenylindole (DAPI), then visualized by LSM510 laser-scanning confocal microscope with C-Apochromat 63×/1.2 na water immersion objective (Carl Zeiss Microscopy, LLC). Two independent blinded observers, experienced in assessing autoantibody staining of cultured cells, scored images for IgG binding patterns (membrane, cytoplasmic compartment, nucleus) and intensity (faint, medium, bright).[14]

For astrocyte studies, all sera and control IgGs were absorbed with bovine liver powder(16 hr, 4°C, or 1 hr, 20–23°C). Sera yielding any staining were absorbed further with lysates of HEK293 cells (non-transfected or eGFP-Kir4.1-transfected; sonicatedin 3 mL PBS/10% goat serum/Complete Protease Inhibitor Cocktail, Roche). Astrocytes were washed after exposure to HEK293-lysate-absorbed antibodies to prevent residual recombinant Kir4.1 blocking subsequently applied Kir4.1-specific-IgG probe.

Immunoprecipitation assay

Serum (15μL), or diluted control antibody, was added to duplicate 100μL aliquots of eGFP-Kir4.1 extract, and shaken overnight.[17] Control monoclonal IgGs were diluted in normal mouse serum. Recombinant Protein G-Sepharose-4B (Invitrogen) was added as precipitant (50% suspension in PBS, 30μL/sample), rotated 2 hr and washed by centrifugation. Fluorescence emission was measured spectrophotometrically.

Neuropathological evaluation of multiple sclerosis lesions

We stained formalin-fixed, paraffin-embedded sections (4 μm) with hematoxylin and eosin, luxol fast blue and periodic acid-Schiff, and Bielchowsky silver impregnation. Avidin-biotin immunohistochemistry analysis used primary antibodies specific for: myelin-associated glycoprotein (polyclonal, Prof. Schaeren-Wiemers, Basel, Switzerland), myelin oligodendrocyte glycoprotein (polyclonal, Prof. Merkler, Geneva, Switzerland), 2′3′-cyclic nucleotide 3′ phosphodiesterase (CNPase, clone SMI91, SternbergerMonoclonals, Lutherville, MD), proteolipid protein (clone Plpc1, Serotec, Oxford, UK), activated terminal human complement components (anti-human C9 neo, rabbit polyclonal, Professor Paul Morgan, Cardiff, UK), and Kir4.1-specific polyclonal rabbit IgG (APC035, Alomone Labs, USA). Lesions were first staged for demyelinating activity;[18] early active lesions were further classified into immunopathological patterns I–IV.[10]

Role of the Funding Source

The funding sources had no role in study design, data collection, analysis, and interpretation, or in writing the report. All authors had full access to all data in the study and the corresponding author had final responsibility for submitting the paper for publication.

Results

By the ELISA assay (figure 1A) we found no significant differences between sera from 286 diverse multiple sclerosis patients and sera from 208 controls, with respect to high optical density values (exceeding 1.25, the mean OD of 208 control subjects+ 5 standard deviations; threshold for positivity defined by Srivastava et al[11]). At 1/200 dilution, 3 multiple sclerosis sera and 2 of 99 healthy control sera yielded OD values exceeding the 1.25 threshold. IgG binding did not differ significantly for 25 multiple sclerosis and 22 neurological control CSFs tested at 1/2 dilution (median ODs, 0.1033 vs 0.1019; interquartile ranges, 25^{th} to 75^{th}, 0.0968–0.1074 and 0.0976–0.2104; p= 0.39, Wilcoxon rank-sum test).

Because only 1.1% of 286 diverse multiple sclerosis patients tested had serum IgG that bound to the synthetic Kir4.1-83-120 peptide in an ELISA assay used by Srivastava et al for most testing (reported to yield equivalent results for peptide and recombinant Kir4.1 protein),[11] we tested more extensively specimens with sufficient available volume (50 Mayo Clinic-based multiple sclerosis patients and 31 healthy/disease controls).

To minimize denaturation of potentially critical epitopes, we used an immunoprecipitation assay to investigate serum IgG binding to eGFP-tagged human Kir4.1 protein in solution phase. The average RFU emitted from eGFP-Kir4.1 captured by IgG did not differ significantly between the three groups tested: multiple sclerosis, miscellaneous immunological disorders or healthy controls (p = 0.40; figure 1B). RFU values yielded by all three groups were only slightly higher than yielded by aquaporin-4-specific-IgG (negative control), about 20-fold lower than precipitated by Kir4.1-specific-IgG (positive control). Again, multiple sclerosis patients' sera lacked demonstrable Kir4.1-specific-IgG.

Next we investigated the binding of IgG in serum or CSF of multiple sclerosis patients to human Kir4.1 expressed in transfected cell membranes. To verify eGFP-Kir4.1 location in the plasma membrane, and that the eGFP tag did not alter K^+-channel function, we measured membrane currents, initially in voltage-clamped *Xenopus* oocytes. Oocytes expressing tagged or non-tagged Kir4.1, but not control-injected oocytes, displayed inwardly-rectifying currents (figure 2A [upper panel] and 2B). With Ba^{2+} added as K^+-channel blocker,[15]~90% current was reduced for both tagged and non-tagged Kir4.1-expressing oocytes (figure 2A [lower panel] and 2B). Thus both recombinant proteins were plasma membrane-resident and had Kir4.1 channel function. Inside-out patch-clamp experiments on HEK293 cells (figure 2C) revealed no inwardly-rectifying K^+ current in non transfected cells (black); eGFP-Kir4.1-transfected cells displayed an obvious inwardly-rectifying current (red) which, consistent with Kir4.1 channels, was reduced by Ba^{2+} (green).

To test human serum and CSF for Kir4.1-specific IgG binding to the transfected HEK293 cells, we lacked a control IgG reactive with Kir4.1 extracellular domain. We therefore permeabilized the cells. Those expressing recombinant Kir4.1 in the plasma membrane are green (eGFP fluorescence, middle lanes, figure 3). Bound IgG is red (fluorochrome-conjugated secondary antibody). Non-transfected cells (lacking eGFP fluorescence; identifiable by blue [DAPI] nuclear labeling) served as control for binding of non-Kir4.1-specifichuman IgG. No human IgG co-localized with eGFP(figures 3A,3B lower panel). By contrast, control Kir4.1-specific-IgG bound selectively to transfected cells (plasma membrane and cytoplasm, including perinuclear [consistent with endoplasmic reticulum], figures 3A lower panel, 3B upper panels). Kir4.1-specific control IgG staining was not removed by liver powder, but was removed by HEK293 lysate containing recombinant Kir4.1 protein (figure 3B, upper panel). Of 50 clinic-based multiple sclerosis sera tested, 42 were non-reactive with either transfected or non-transfected cells. IgG in 7 sera bound to all cell nuclei; IgG in one of those, and in the eighth serum, bound to cytoplasmic elements of all cells. All cell-reactive IgG in multiple sclerosis serum was quenched by liver powder absorption (figure 3B, lower panel). No Kir4.1-specific-IgG was evident.

Because pathogenic neural autoantibodies sometimes are detected more readily in CSF than in serum, we tested 25 multiple sclerosis CSF son live and fixed Kir4.1-expressing cells. No IgG bound. We also tested 7 multiple sclerosis patient sera on live Kir4.1-transfected cells, 3 that yielded ELISA results exceeding the “positivity threshold,” and 4 that yielded the highest OD values below threshold (figure 1A). None bound.

To investigate whether lack of multiple sclerosis IgG reactivity with recombinant Kir4.1 in transfected HEK293 cells may reflect subtle antigenic differences between native and recombinant Kir4.1, we tested the sera on cultures enriched for mouse astrocytes expressing native Kir4.1 (green in Figure 4A; co-expressed aquaporin-4 is shown in red). Co-localization of rabbit Kir4.1-specific-IgG (red, left lanes, figure 4B and 4D [upper panel]) and mouse Kir4.1-specific-IgG (green)in the plasma membrane and around the nucleus yielded yellow staining in merged images. Sera were pre-absorbed with liver powder for this study because unabsorbed sera (at 1/100 dilution)yielded high background staining. Pre-absorption did not abrogate control Kir4.1-specific-IgG binding to astrocytes (figure 4B). Of 50 multiple sclerosis sera tested, 38 lacked astrocyte-reactive-IgG; 12 yielded staining patterns (5 nuclear and 7 cytoplasmic). Figure 4C illustrates one non-reactive specimen and two reactive. The single multiple sclerosis serum with IgG apparently binding to both plasma membrane and cytoplasm(figure 4C, lower panel) did not co-localize with mouse Kir4.1-IgG (compare green merge image with yellow merge image yielded by control rabbit Kir4.1-specific-IgG, figure 4B). That serum IgG also bound to small non-astrocytic cells lacking Kir4.1-immunoreactivity (presumptive microglia, arrows, figure 4C lower panel). We absorbed this serum with HEK293 cell lysates (figure 3C, right panel). Non-transfected and Kir4.1-transfected lysates were equivalent in eliminating astrocyte staining. Astrocyte binding by the rabbit Kir4.1-IgG was completely eliminated by absorption with lysate containing recombinant human Kir4.1, but not affected by non-transfected cell lysate(figure 4D, left panel). We concluded that binding of multiple sclerosis IgG to mouse astrocytes was rare and not Kir4.1-specific.

To determine whether Kir4.1 antigen loss might occur in multiple sclerosis lesions, a potential outcome of IgG binding, we analyzed 22 lesions (8 early active [4 pattern II/4 pattern III], 8 inactive, 6 remyelinated) and 13 periplaque white matter regions in 15 archival cases (7 biopsies/8 autopsies; Table). Astrocytic Kir4.1 and oligodendroglial Kir4.1 immunoreactivity within early active lesions, remyelinated lesions (Figure 5) and in surrounding peri-plaque white matter, were increased relative to control supratentorial brain white matter tissue from three non-neurological patients (Figure 6). Astrocytic Kir4.1 immunoreactivity was variable within inactive plaques, whereas oligodendrocyte expression was reduced, reflecting the scarcity of mature oligodendrocytes (figure 6).

Discussion

This study revealed neither serological nor neuropathological evidence to support an autoantibody attack targeting glial Kir4.1K^+-channels in patients with multiple sclerosis. Srivastava et al,[11] using mainly ELISA as detection method, reported finding Kir4.1-specific-IgG in 47% of 397 patients' sera (and in 63% of 30 multiple sclerosis CSFs), regardless of disease classification. Supplementary data indicated full-length recombinant human Kir4.1 and the synthetic putative extracellular loop peptide-83–120 had equivalent antigenicity. The ELISA in our hands (same methodology and peptide source[11])yielded values exceeding the authors' defined positivity threshold in only 1.0% of sera from 286 multiple sclerosis patients representing diverse ethnicities and disease severity and duration. No CSF was positive. Watanabe et al[19] and Nerrant et al[20] similarly found no significant differences for Kir4.1-peptide-ELISA results in sera of Japanese and European patients with multiple sclerosis or other neurological diseases and healthy donors (see Research in Context). Detailed analyses of sera from 50 Mayo Clinic-based multiple sclerosis patients on cells expressing functional Kir4.1-channels revealed no Kir4.1-specific-IgG. Of the few instances where patient IgG bound to Kir4.1-expressing cells, only one was plasma membrane-reactive and none was Kir4.1-specific.. The discrepancy between Srivastava's observations[11] and our documented lack of multiple sclerosis serum-IgG or CSF-IgG reactivity with human and murine Kir4.1 antigens is not readily explained. ELISA is acknowledged prone to false positive results. In contrast, immunoprecipitation and transfected and native cell binding assays largely preserve autoantigenicity, and are validated internationally as sensitive and specific for detecting clinically-pertinent autoantibodies reactive with neural plasma membrane channels (e.g., aquaporin-4,[6, 14] NMDA receptors[21] and voltage-gated potassium channel-complex components[22–24]). The lack of multiple sclerosis-IgG reactivity with Kir4.1 in plasma membrane, cytoplasm or endoplasmic reticulum of cultured glial cells argues against antigenic differences in recombinant and native glial Kir4.1 (attributable for example to differences in subunit composition or differential glycosylation states). The few immunostaining studies illustrated by Srivastava et al[11] employed biotin-conjugated secondary antibody, a more sensitive assay than the directly fluorochrome-conjugated anti-human IgG reagent that we employed. However, the more sensitive biotin-conjugated secondary antibody would have amplified all signals from bound human IgG, including the non-Kir4.1-specific-IgGs that we observed.

Detection of an apparently pathogenic, aquaporin-4-specific-IgG in neuromyelitis optica enables unambiguous distinction of that inflammatory CNS disorder from multiple

sclerosis.[6] Lesional autoantigen loss is an early histopathological event in neuromyelitis optica.[25,26] If Kir4.1 is a pertinent autoantigen for a form of multiple sclerosis mediated by IgG,[11] one might anticipate Kir4.1 loss from glial cells in a subset of multiple sclerosis patients. Accordingly, the Munich group recently reported loss of Kir4.1-immunoreactivity from oligodendroglia and perivascular (but not reactive) astrocytes in active multiple sclerosis lesional tissues.[27] We evaluated brain tissues from 15 histopathologically-confirmed multiple sclerosis cases (and 3 non-neurological controls).[10] Contrary to the Munich report,[27] but in agreement with Satoh et al. (who analyzed chronic demyelinating lesions of secondary progressive multiple sclerosis),[28] we observed no Kir4.1 loss, but rather an apparent increase in both astrocytic and oligodendrocytic Kir4.1-immunoreactivity in early active, remyelinated and periplaque white matter lesions (Table). Different analytical approaches may in part explain the contradictory results. Kir4.1 immunoreactivity must be analyzed relative to baseline control white matter expression, before drawing quantitative conclusions.

The Munich group's key interpretations were based largely on high power confocal microscopic analysis of Kir4.1 immunoreactivity within small regions. Unlike our study, there was limited assessment of well-defined and well-staged lesions, at both low and high power. The distribution of Kir4.1 immunoreactivity across different staged plaques within a given case was not clearly specified, nor whether the analysis used frozen or paraffin tissues. Our findings overall do not support a disease-pertinent pathogenic basis for the Kir4.1 loss, complement deposition, and glial cell damage reported in brain tissues of mice injected intrathecally with selected multiple sclerosis patients' IgG plus human complement.[11]

Multiple sclerosis is undeniably immune-mediated, with CNS-confined pathology. However it does not fulfill criteria for classification as an organ-specific autoimmune disease, i.e., having a reproducibly defined neural autoantigen. The proposition that at least half of patients diagnosed with multiple sclerosis have a neural-specific autoantibody in common[11, 12] is unprecedented in published observations concerning the frequency of any organ-specific autoimmune manifestations in multiple sclerosis. Patients with neurological autoantigen-specific disorders, both IgG-mediated channelopathies (e.g., myasthenia gravis, Lambert-Eaton syndrome, neuromyelitis optica and NMDA receptor encephalitis) and T cell-mediated inflammatory paraneoplastic CNS disorders,[9,29] commonly have a coexisting non-neurological autoimmune disorder, such as thyroiditis, type 1 diabetes orpernicious anemia). Lupus and Sjogren syndrome are particularly common with neuromyelitis optica. A population-based study involving 5,000 multiple sclerosis patients found no excess of autoimmune disorders compared to spousal controls.[30] In four decades of autoimmune serological studies, the Mayo Clinic Neuroimmunology Laboratory has not observed specific binding of multiple sclerosis-IgG to brain tissue substrates. Our published study reported that neural autoantibodies were as in frequent in 77 patients with immunohistopathologically-confirmed multiple sclerosis(9%) as in 173 healthy control subjects (6%). The frequency in 177 neuromyelitis optica patients (excluding aquaporin-4-IgG) was 34%.[31] Sharing of disease susceptibility loci by multiple sclerosis, Crohn's disease and psoriasis[32] (all lacking a specific autoantibody marker) supports designation of these diseases as immune-mediated inflammatory disorders, but does not infer autoantigen-specific pathogenesis. Insights emerging from genome-wide association studies of large

populations of multiple sclerosis patients may generate fresh leads and testable hypotheses to explain the intractable mystery of the immunologic target in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Dr. Pittock is a named inventor on patents that relate to functional AQP4/NMO-IgG assays and NMO-IgG as a cancer marker; receives research support from Alexion Pharmaceuticals, Inc., the Guthy-Jackson Charitable Foundation, and the National Institutes of Health (NS065829). Dr. Pittock has provided consultation to Alexion Pharmaceuticals, MedImmune LLC, and Chugai Pharma but has received no personal fees or personal compensation for these consulting activities. All compensation for consulting activities is paid directly to Mayo Clinic.

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Dr. Lennon is a named inventor on a patent (#7101679 issued 2006) relating to aquaporin-4 antibodies for diagnosis of neuromyelitis optica and receives royalties for this technology; is a named inventor on patents (#12/678,350 filed 2010 and #12/573,942 filed 2008) that relate to functional AQP4/NMO-IgG assays and NMO-IgG as a cancer marker; receives research support from the National Institutes of Health (NS065829).

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Research in Context Panel

Systematic review

We searched PubMed from January, 2011 until June 3, 2014, for studies published in English with the search terms “multiple sclerosis” and “antibody”, “Kir4.1” and “potassium channel”. Subsequent to the original report from Munich of finding Kir4.1-specific IgG in 47% of multiple sclerosis patients’ sera (Srivastava et al, ***New Engl J Med 2012***, 367:115–23), there has not yet been a confirmatory report apart from 2 studies published by the same group: one reporting detection of Kir4.1-IgG exclusively in, and in 57% of, children with acquired demyelinating CNS disease (Kraus V, Srivastava R et al., ***Neurology 2014;*** 82:470–3); the second reporting loss of Kir4.1-immunoreactivity from glial cells in active lesions of autopsied multiple sclerosis patients’ brain tissues (Schirmer L et al., ***Ann Neurol 2014, April 29;).*** Two brief serological publications from Japan and France reported (from Kir4.1 peptide-based ELISA and tissue-based immunofluorescence studies) that the frequency of Kir4.1-IgG in serum does not differ significantly for multiple sclerosis patients and control subjects (Watanabe M et al, ***Clin Exp Neuroimmunol 2013***; 4:241–2, and Nerrant E et al, ***Neurology*** 2014; 82 [April] Supplement P4.130), and a single case immunohistopathological study reported from Japan by Satoh J-I et al. showed that chronic demyelinating brain lesions of a patient with secondary progressive multiple sclerosis lacked evident Kir4.1 loss (***Clin Exp Neuroimmunol*** 2013; 5:1–10).

Interpretation

Our comprehensive study revealed neither Kir4.1-specific-IgG in serum or CSF of multiple sclerosis patients, nor Kir4.1 loss from glial cells in supratentorial brain white matter lesions of 15 archival multiple sclerosis cases (22 plaques: 8 early active, 8 inactive, 6 remyelinated; 13 periplaque regions) compared with 3 non-neurological cases (8 normal-appearing white/gray matter regions). Our serological data were obtained using methodologies validated for detecting clinically-pertinent neural autoantibodies:immunoprecipitation and cell binding antibody studies (human Kir4.1-transfected HEK293 cells and cultured glia). Differential glycosylation of Kir4.1 does not seem a plausible explanation for the different serological results obtained by the Munich laboratory because: i) the frequency of positive ELISA results reported by Srivastava et al. did not differ significantly using as antigen recombinant human Kir4.1 produced in HEK293 cells or the synthetic Kir4.1 peptide, which is not glycosylated; ii) our photomicrographs demonstrate that, by confocal immunofluorescence, Kir4.1 protein in both transfected HEK293 cells and cultured glial cells is abundant on the plasma membrane (anticipated to be most glycosylated), in the perinuclear region (endoplasmic reticulum, anticipated to be least glycosylated) and in the cytoplasm (trafficking vesicles, variably glycosylated).

We conclude that serological testing for Kir4.1-IgG is unlikely to aid multiple sclerosis diagnosis. The target of the immune response in multiple sclerosis remains elusive.

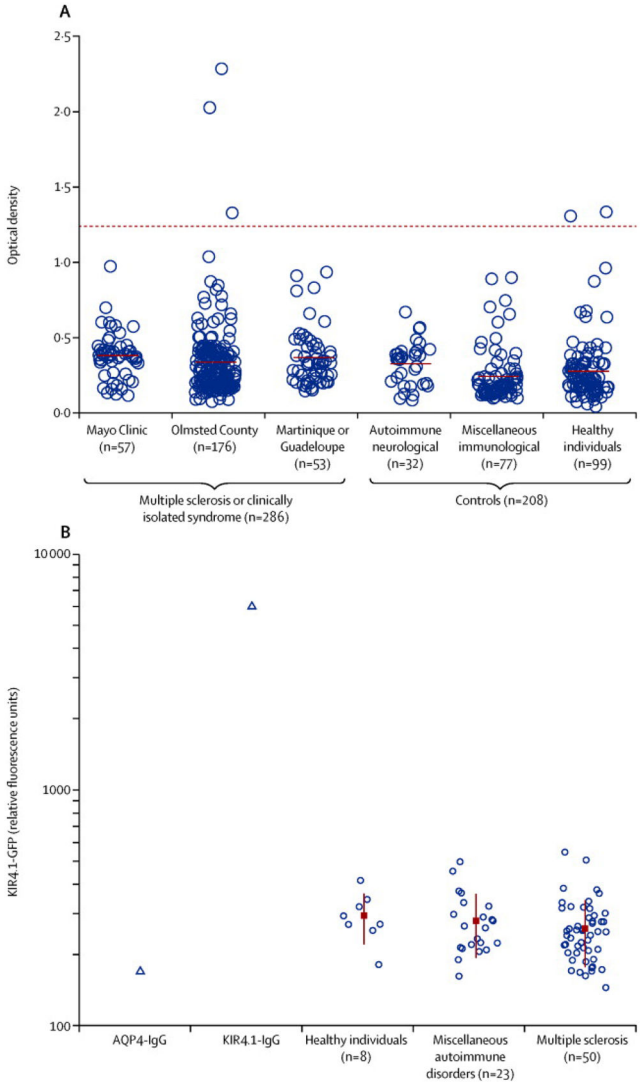

Figure 1.

A. Binding of multiple sclerosis and control subjects' serum IgG to Kir4.1 peptide 83–120.

Using the ELISA methodology and peptide antigen described by Srivastava et al,[11] we defined the threshold for Kir.4.1-IgG positivity as 5 SD above the mean optical density (OD) yielded by the binding of control subjects' IgG (n=208, horizontal line; horizontal red lines are group means). No serum IgG exceeding this threshold (nor in 4 multiple sclerosis sera yielding the highest OD values below threshold) bound to either live or fixed Kir4.1-transfected cells. The probability for exceeding the threshold did not differ significantly in comparing each multiple sclerosis patient cohort with the controls (Fisher's exact test; sera tested at 1/200 dilution): 2/208 controls exceeded this threshold as compared to Mayo Clinic patients (0/57, p=1.0), Olmsted County patients (3/176, p=0.66), Afro-Caribbean patients (0/53, p=1.0). The median OD (interquartile ranges, 25th, 75th) for each group was: Mayo Clinic MS, 0.3804 (0.3022, 0.4247); Olmsted County MS, 0.2718 (0.1896, 0.4095); Afro-Caribbean MS, 0.3273 (0.2208, 0.4549); autoimmune neurologic controls, 0.3616 (0.1950, 0.41110); miscellaneous immunologic controls, 0.1866 (0.1344, 0.2567); healthy subjects,

0.2221 (0.1713, 0.3279). Clinical and demographic details for the 286 multiple sclerosis patients (and diagnoses of the 109 disease controls) are in Table 1.

B. Immunoprecipitation of solubilized recombinant eGFP-tagged human Kir4.1 protein by sera. Results for healthy subjects and patients with miscellaneous autoimmune disorders or multiple sclerosis did not differ significantly. Human sera or control mouse monoclonal IgGs were held with eGFP-Kir4.1 extracted from HEK293 cells transfected with vector encoding eGFP-Kir4.1 fusion protein. Differences between average values for relative fluorescence units (RFU) precipitated by protein G-agarose from the 3 serum groups (open circles; duplicate or quadruplicate assays) were compared for statistical significance by analysis of variance (ANOVA, p=0.40, degrees of freedom =2). Filled squares and error bars are means and standard deviations (control healthy subjects, n=8: SD=68.5; multiple sclerosis, n=50: SD=80.2; miscellaneous autoimmune disorders, n=23: SD=82.6). Aquaporin-4 (AQP4)-specific and Kir4.1-specific IgGs (triangles; average of 8–10 assays) served as negative and positive controls, respectively. The plot is shown on log base-10 scale, but analyses, means, and standard deviations were computed on the original scale.

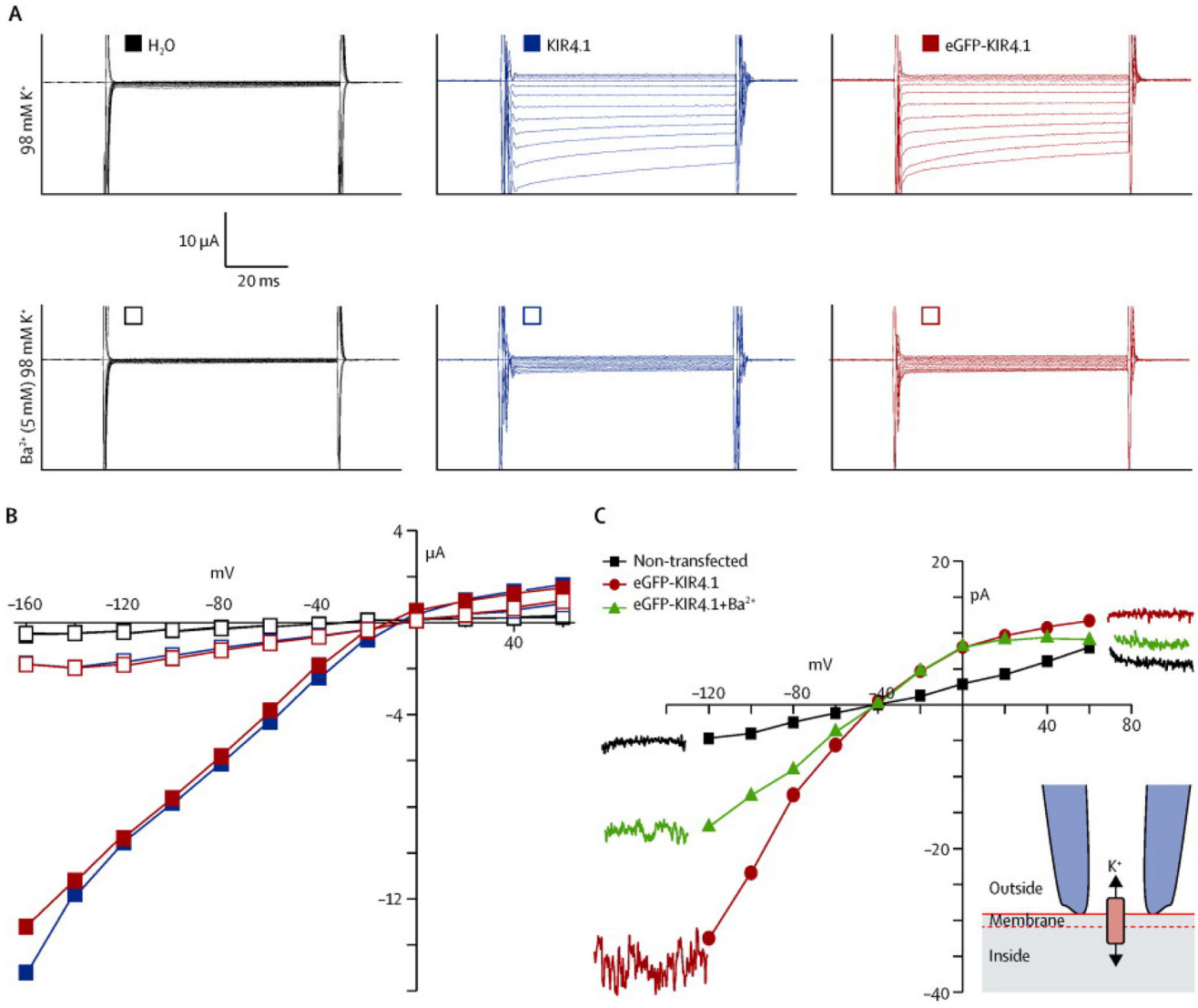

Figure 2. Functional assessment of Kir4.1

K^+-channel activity was assessed using two-electrode voltage clamping, 24–36 hrs after injecting cRNA (1 ng) into *Xenopus* oocytes (A, B) and using inside-out patch-clamp for stably transfected HEK293 cells (C). Panel A compares the resultant, inwardly rectifying K^+ currents in oocytes injected with water (black), or cRNA (1 ng/oocyte) encoding Kir4.1 (blue) or eGFP-Kir4.1 (red). Sweeps in the upper row (solid symbols) are from 3 individual oocytes in a 98 mM K^+ buffer. The lower row (open symbols) shows the same oocytes in presence of 5 mM Ba^{2+} (K^+-channel blocker). Panel B presents the average current for the last 5 ms of each sweep in A, as current-voltage (IV) relationship. Panel C shows the IV curves from inside-out patch-clamped HEK293 cells: non-transfected (black squares); stably transfected with eGFP-Kir4.1 (high K^+ mammalian buffer, red circles; and high K^+ mammalian buffer + 1 mM Ba^{2+}, green triangles; rather than completely blocking, Ba^{2+} reduces the open probability of Kir4.1 channels[15]). Colored lines at −120 mV and +60 mV are the raw current traces for the condition at those voltages. The cartoon (lower right)

illustrates the patch pipette tip and a Kir4.1 channel in the lipid bilayer membrane (intracellular leaflet facing out).

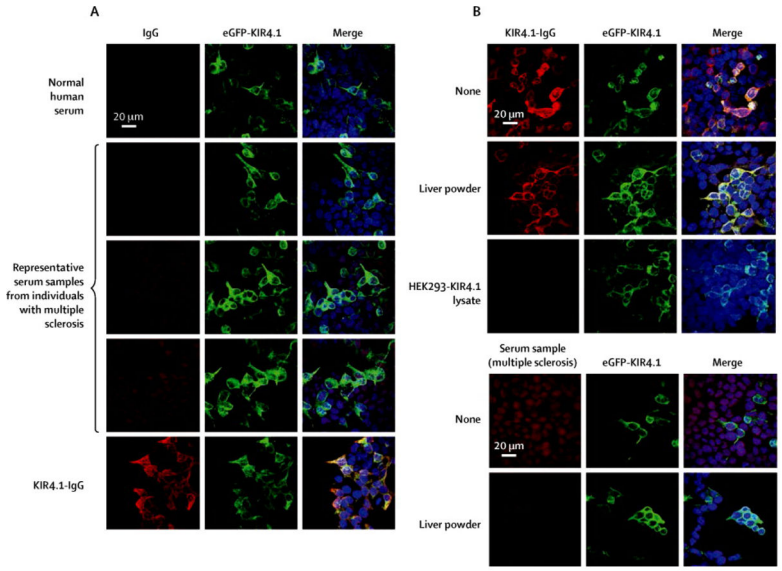

Figure 3. Binding of IgG in serum of multiple sclerosis (MS) patients to HEK293 cells expressing eGFP-tagged recombinant human Kir4.1

Confocal microscopic images of IgG binding to fixed, permeabilized HEK293 cells transiently transfected with plasmid containing human Kir4.1 cDNA. Transfected cells expresse GFP-tagged Kir4.1 in plasma membranes and cytoplasm, including perinuclear endoplasmic reticulum region (green, middle lanes) i. Bound secondary antibodies (anti-human or anti-mouse IgGs) are seen as red (left lanes). Nuclei of all cells, transfected and non-transfected, are stained blue by DAPI (right lanes, merged images). Panel A, from top: NHS, normal human serum; 3 representative MS sera (non-reactive, nuclear-reactive and nuclear-cytoplasmic-reactive); Kir4.1-IgG, a commercial mouse (ms) monoclonal antibody. Panel B shows staining patterns yielded by control Kir4.1-specific mouse IgG and by a reactive MS patient's serum IgG, unabsorbed or absorbed with liver powder or with HEK293 lysate containing recombinant Kir4.1 protein. Images visualized with a 63x water immersion objective. White scale bars represent 20 µm.

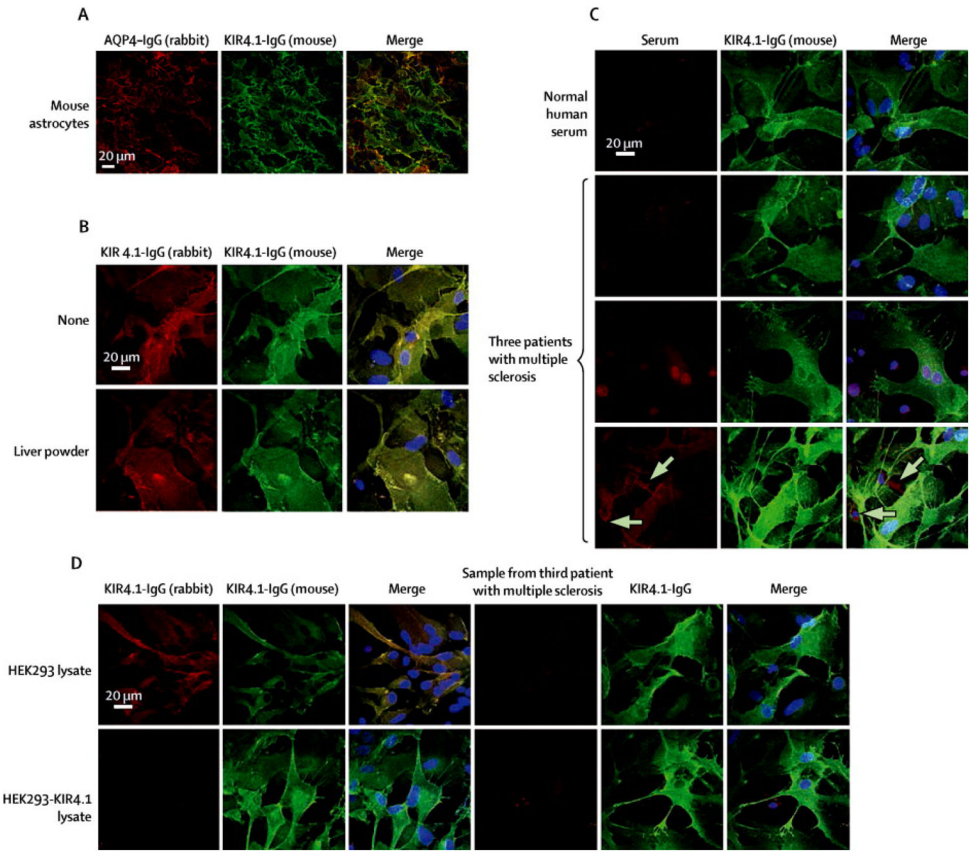

Figure 4. Binding of IgG in serum of multiple sclerosis (MS) patients to in cultured glial cells

Confocal microscopic images of paired IgGs binding to fixed, permeabilized mouse astrocytes on cover slips. Kir4.1-specific monoclonal mouse (ms) IgG probe localizes Kir4.1 in astrocytic plasma membranes and perinuclear cytoplasm (middle lanes, green). Kir4.1 extracellular domain is 98% identical to human in amino acid sequence; overall 99% identity. Nuclei are stained blue by DAPI (right lanes, merged images). Panel A. Astrocyte phenotype confirmed by dual staining with aquaporin-4-IgG. Panel B illustrates co-localization of a positive control Kir4.1-specific rabbit (rb) antibody (red) with the Kir4.1-specific mouse IgG probe (merged images appear yellow) and demonstrates that binding of the positive control antibody is not abolished by pre-absorption with liver powder. Panel C illustrates, from top, non-reactivity of normal human serum (NHS) and the different patterns of staining yielded by 3 representative MS sera (non-reactive, nuclear, and plasma membranous/cytoplasmic); arrows indicate IgG in third MS specimen binding additionally to 2 small red cells not expressing Kir4.1 (presumptive microglia; bottom lane). Panel D illustrates specificity of the control rabbit Kir4.1-IgG binding to astrocytes (abrogated only by absorption with HEK293 lysate containing recombinant Kir4.1). By contrast, binding of MS serum IgG reactive with membranous/cytoplasmic elements(same specimen as panel C, bottom lane) was reduced equally by HEK293 lysates containing and not containing recombinant Kir4.1 protein (panel D, right side). Images visualized with a 63x water immersion objective. White scale bars represent 20 μm.

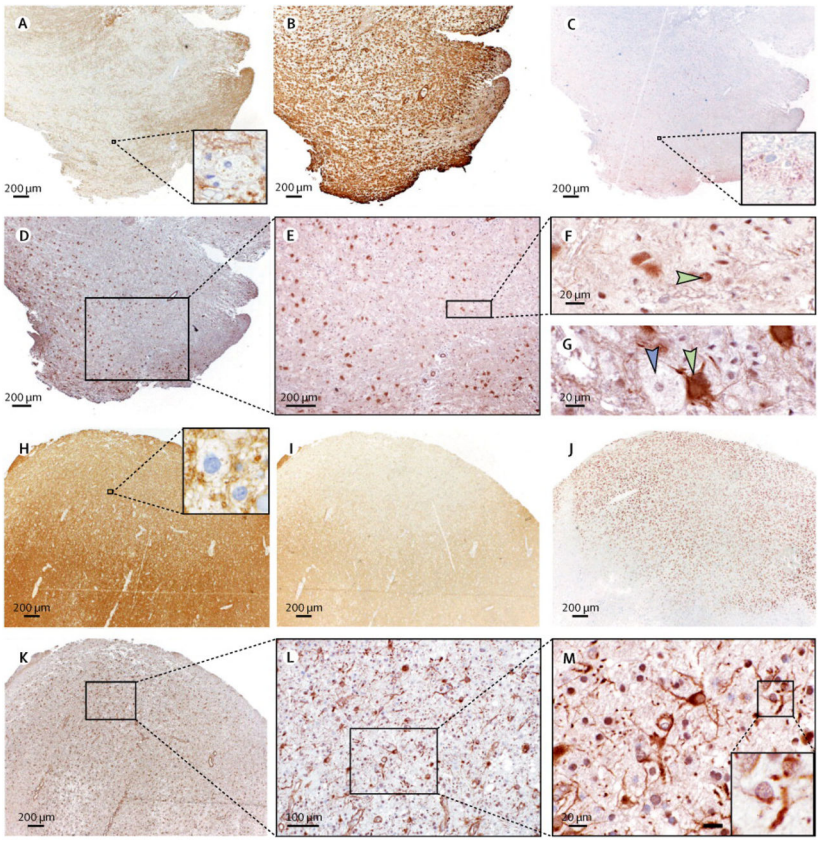

Figure 5. Kir4.1 immunoreactivity in early active multiple sclerosis lesions

MS pattern II lesion (A–G). A: Loss of myelin oligodendrocyte glycoprotein (MOG) is extensive. MOG-laden macrophages (inset) indicate early active demyelinating activity. B: Kim1p staining reveals an abundance of macrophages in the active demyelinated regions. C: Complement C9 neoantigen in cytoplasm of a macrophage (inset) is characteristic of pattern II MS lesions. D: Kir4.1 immunoreactivity is seen in glial cells throughout the lesion. E: Higher magnification of the panel D framed region. F and G: Arrow heads in enlarged views of panel E show, respectively, Kir4.1 glial cells (black) with morphologies of an oligodendrocyte (from framed region) and an intact astrocyte (from another region of the same plaque) with an adjacent foamy macrophage (blue).

MS pattern III lesion (H–M)

H/I: An active pattern III lesion in supratentorial white matter demonstrates characteristic loss of myelin-associated glycoprotein (MAG) immunoreactivity (I) relative to MOG (H). MOG-laden macrophages (H: inset) are present throughout the lesion. J: Macrophage infiltration is extensive (CD68). K: Pronounced Kir4.1 immunohistochemistry is evident. L: Enlarged view of the framed regions in panel D indicates extensive Kir4.1 immunoreactivity. M: Higher magnification of framed panel E shows intense Kir4.1 immunoreactivity in both astrocytes and oligodendrocytes (inset). Bars in A–E and H–K= 200 µm; Bars in F, G and M = 20 µm. Bar in L = 100 µm.

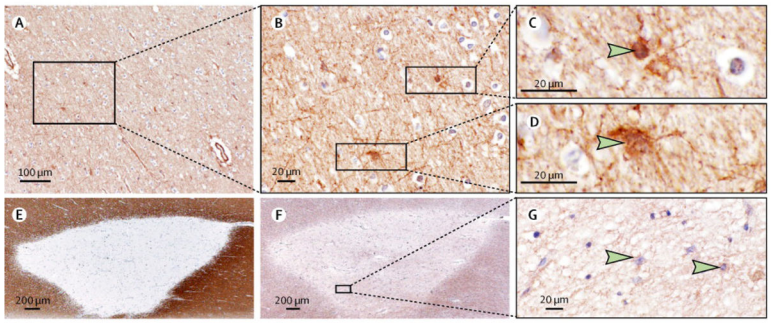

Figure 6. Kir4.1 immunoreactivity in normal brain and inactive MS

A:Kir4.1 immunoreactivity is abundant in normal brain. B: Higher power of framed panel A shows Kir4.1 positive glial cells. C and D: Both Kir4.1-positive oligodendrocytes and astrocytes are present (arrow heads). E: Inactive demyelinated plaque shows complete loss of myelin (proteolipid protein). F: Kir4.1 immunoreactivity is minimal. G: Higher power of framed panel F demonstrates faint Kir4.1 immunoreactivity in some astrocytes (arrowheads). Bars in A = 100 µm; B, C, D, G = 20 µm; E, F = 200 µm.

Table

Kir4.1 immunoreactivity in multiple sclerosis brain tissue

Plaque Type	Number	Astrocyte Kir4.1 Immunoreactivty	Oligodendrocyte Kir4.1 Immunoreactivity
Early Active (Pattern II)	4	+++	++
Early Active (Pattern III)	4	+++	++
Inactive	8	+/−	−
Remyelinated	6	++	+/++
Periplaque White Matter	13	+++	+/++
Normal Appearing White Matter	8	+	+

Kir4.1 immunoreactivity in archival tissues from 15 cases (7 biopsies/8 autopsies) with histopathologically compatible MS was analyzed relative to baseline Kir4.1 expression (scored +) in supratentorial white matter of 3 non-neurological control cases. The median age of the patients (12 women/3 men) was 42 years (range 9–75 years). Clinical diagnosis at last follow-up was MS (11) and an isolated demyelinating syndrome in 4 whose clinical follow-up was < 2 years. Loss of oligodendrocyte Kir4.1 immunoreactivity corresponded to inactive plaques largely devoid of mature oligodendrocytes.