Antigen-Specific Adaptive Immune Responses in Fingolimod-Treated Multiple Sclerosis Patients

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T cells exit secondary lymphoid organs along a sphingosine1-phosphate (S1P) gradient and, accordingly, are reduced in blood upon fingolimod-mediated S1P-receptor (S1PR)-blockade. Serving as a model of adaptive immunity, we characterized cellular and humoral immune responses to influenza vaccine in fingolimod-treated patients with multiple sclerosis (MS) and in untreated healthy controls. Although the mode of action of fingolimod might predict reduced immunity, vaccine-triggered T cells accumulated normally in blood despite efficient S1PR-blockade. Concentrations of anti–influenza A/B immunoglobulin (Ig)M and IgG also increased similarly in both groups. These results indicate that fingolimod-treated individuals can mount vaccine-specific adaptive immune responses comparable to healthy controls.

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In multiple sclerosis (MS) lesions lymphocytes mediate inflammation, demyelination, and axonal damage.1 Lymphocytes that express the chemokine receptor CCR7 are able to migrate to secondary lymphoid organs (SLO) such as lymph nodes, whereas cells not expressing CCR7 (CCR7-negative) do not recirculate to SLO on a regular basis. In order to egress from SLO to the peripheral blood circulation cells migrate along a sphingosine 1-phosphate (S1P) gradient.2 The oral S1P receptor (S1PR)-agonist fingolimod—which has shown efficacy in the treatment of MS3–5—blocks this egress, thereby reducing peripheral lymphocyte counts, in a dose-dependent manner, to 25% to 40% of baseline values.3,6–8 As a consequence, CCR7-negative cells represent the major T cell population circulating in the blood of fingolimod-treated patients. Despite severe lymphopenia only few infectious complications were observed in fingolimod-treated patients with MS. However, a case of fatal disseminated varicella zoster infection and a case of herpes simplex virus type 1 encephalitis nonetheless raise concern with regard to the immunological competence vis-à-vis viral pathogens in fingolimod-exposed individuals.5,9 So far only animal data are available on the effect of S1PR-blockade on adaptive immune responses following viral antigen exposure. In simian human immunodeficiency virus (SHIV)-infected rhesus macaques treatment with fingolimod did not result in deviations from the natural pattern of viral control.10 Treatment with fingolimod also had no effect on the disease course and T cell exhaustion in mice infected with lymphocytic choriomeningitis virus (LCMV).11 In contrast, treatment with fingolimod lead to a significant reduction of influenza-antigen specific CD8+ T cells in lungs of animals infected with influenza.12 Fundamental in this context, yet never experimentally addressed in humans, is how blocking S1PR impacts on the presence of bulk vs recently antigen-activated T cells in the peripheral circulation. Here we sought to define in a prospective observational study the effect of fingolimod-mediated S1PR-blockade on the development of antigen-specific immune responses in patients with MS.

Patients and Methods

Study Subjects and Procedures
We conducted an open-label, observational, prospective study to assess the adaptive immune response induced by influenza-vaccine in fingolimod-treated patients with MS and in healthy controls (HC). The trial was conducted during the influenza vaccination periods 2008/2009 and 2009/2010 (for inclusion...
and exclusion criteria see the Supporting Information Methods). The institutional review board of both Basel approved the study. After written informed consent, blood samples from study subjects were obtained before and 7, 14, and 28 days after seasonal influenza vaccination with Mutagrip (Sanofi Pasteur SA, Lyon, France). Clinical assessments are described in the Supporting Information Methods.

Flow Cytometry
T cells were analyzed for expression of CD3, CD4, and CD8 using a CyAn cytometer (DakoCytomation, Glostrup, Denmark) according to standard procedures (used antibodies listed in Supporting Information Methods).

Enzyme-Linked Immunospot Assay
Enzyme-linked immunospot (ELISpot) was done as described13 with the modification that we used with Inflexal (Berna Biotech, Kuesnacht, Switzerland) as the source of antigen (year-adjusted) (see also Supporting Information Methods).

Virus-Specific Antigen Presentation Assay
Epstein-Barr virus (EBV)-specific T cell responses were characterized in the presence or absence of fingolimod as described14 (see also Supporting Information Methods).

Reverse-Transcription–Polymerase Chain Reaction Analysis
For reverse-transcription–polymerase chain reaction (RT-PCR) analysis, see Supporting Information Methods.

### TABLE: Characteristics of the Study Population and Tolerability of Influenza Vaccination and Incidence of Influenza-Like Illnesses

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>MS Fingolimod</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Median age, yr (range)</td>
<td>37 (19–46)</td>
<td>44 (31–60)</td>
</tr>
<tr>
<td>Female/male</td>
<td>6/12</td>
<td>8/6</td>
</tr>
<tr>
<td>Median disease duration, yr (range)</td>
<td>NA</td>
<td>12.3 (3–20)</td>
</tr>
<tr>
<td>Median EDSS (range)</td>
<td>NA</td>
<td>2.6 (1.0–4.0)</td>
</tr>
<tr>
<td>Median therapy duration, mo (range)</td>
<td>NA</td>
<td>36 (7–42)</td>
</tr>
<tr>
<td>Fingolimod dosage 0.5mg/1.25mg</td>
<td>NA</td>
<td>6/8</td>
</tr>
<tr>
<td><strong>Tolerability of vaccine/incidence of influenza-like illness</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection-site reactions day 0–3 postvaccination</td>
<td>12/18 (66%)</td>
<td>7/14 (50%)</td>
</tr>
<tr>
<td>General symptoms day 0–3 postvaccination</td>
<td>2/18 (11%)</td>
<td>4/14 (29%)</td>
</tr>
<tr>
<td>MS relapses</td>
<td>NA</td>
<td>0/14 (0%)</td>
</tr>
<tr>
<td>Incidence of influenza-like illness</td>
<td>2/18 (11%)</td>
<td>2/14 (14%)</td>
</tr>
</tbody>
</table>

EDSS = Expanded Disability Status Scale; MS = multiple sclerosis; MS fingolimod = fingolimod-treated patients with multiple sclerosis; NA, not applicable.

### Anti–Influenza A and Anti–Influenza B Enzyme-Linked Immunosorbent Assay
Concentrations (given as virotech [VE] units/ml) of anti–influenza A and anti–influenza B immunoglobulin (Ig)M and IgG were determined using a quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Genzyme Virotech, Ruesselsheim, Germany). As recommended by the manufacturer, seroprotection was defined as an anti–influenza A/B IgG concentration of ≥10 VE/ml.

### Statistical Analyses
See Supporting Information Methods.

### Results
Characteristics of the study population are summarized in the Table. Rates of local injection site reactions and general tolerability of the vaccine, as monitored by clinical assessments and a patient diary, were comparable in fingolimod-treated patients and HC (see Table). Flow cytometric analyses revealed a reduction of mean lymphocyte counts in fingolimod-treated patients by 64% compared to the lower limit of the reference range (CD4+ by 76–83%, CD8+ by 42–63%) (Fig 1A), an observation that is in line with our previous findings.6 The frequency of T cells producing interferon (IFN)-γ in response to influenza-antigen was assessed by ELISpot. Before vaccination, frequencies of influenza-specific IFN-γ–secreting T cells were comparable in fingolimod-treated patients and HC, as was the number of
individuals with no detectable influenza-specific response. By day 7 postvaccination, frequencies significantly increased in both groups and reached comparable levels (see Fig 1B). Numbers of influenza-specific T cells remained increased and comparable until day 14 postvaccination in both study groups. By day 28 postvaccination, frequencies of IFN-γ secreting cells contracted to prevaccination levels in both groups. No individual mounting a very high frequency of influenza-specific cells was contained in the fingolimod group—a finding which, however, did not reach statistical significance. No statistically significant correlation between lymphocyte counts and vaccine-specific immune responses was found in HC or fingolimod-treated individuals (data not shown). To assess whether fingolimod alters antigen-specific triggering of circulating virus-specific T cells or their in vitro induction, the effect of active (ie, phosphorylated) fingolimod on ex vivo triggering and on in vitro expansion of antigen-specific T cells was assessed. In the presence vs absence of fingolimod (in its active, ie, phosphorylated form) no differences were detected in either experimental system (see Fig 1C–E). Also, the presence of fingolimod did not impact S1P1 mRNA levels in this system (see Fig 1F).

To investigate how S1PR-blockade influences antibody responses, we quantified in these same patients and HC influenza-specific IgM and IgG antibody production by ELISA. Prevaccination levels of anti–influenza A and
anti–influenza B IgM were comparably low in fingolimod-treated patients and HC. Following vaccination, concentrations of anti–influenza A and anti–influenza B IgM increased significantly and comparably in both study groups, and remained increased at comparable levels until day 28 postvaccination (Fig 2A, B). Before vaccination, 71% of the fingolimod-treated patients and 50% of the HC fulfilled the predefined seroprotection criteria (IgG ≥10 VE/ml) for influenza A \( (p = 0.41) \), 71% of the fingolimod-treated patients and 44% of the HC for influenza B \( (p = 0.38) \), indicating previous contact with antigen from these viruses in a substantial proportion of study participants (see Fig 2C, D). At day 7 after vaccination the proportion of individuals fulfilling seroprotection criteria was comparably increased in both fingolimod-treated patients and HC (influenza A: \( p = 0.64 \); influenza B: \( p = 0.53 \)), and remained increased at days 14 and 28 postvaccination in both groups (day 14 and 28: influenza A and B; \( p = 1.0 \)). The proportion of individuals converting from undetectable to protective antibody levels was also similar in fingolimod-treated patients and HC (see Fig 2E, F). Thus, the vaccine-specific production of IgM and, more importantly, IgG in fingolimod-treated individuals was not impaired when compared to levels in HC.

**Discussion**

The key observation of this study was that fingolimod-treated patients with MS—despite severe peripheral lymphopenia—could mount a vaccine-specific adaptive immune response that is comparable to the response observed in HC. Finding a similar postvaccination frequency of influenza-specific peripheral blood T cells in fingolimod-treated patients and HC—in spite of fingolimod-mediated lymphopenia—was unexpected. This observation indicates that in humans lymphocyte egress from SLO is controlled differentially between lymph node–homing T cells interacting with cognate antigen, as opposed to T cells screening for—but not interacting with—cognate antigen. Recent animal data are in line with such a model.15
An impaired antibody response in fingolimod-treated individuals is a concern, as the drug directly impacts germinal center reactions and B cell migration.\(^{16,17}\) Again, the vaccine-specific production of IgM and IgG in fingolimod-treated individuals was, however, not detectably impaired when compared to levels in healthy controls, a finding in line with some,\(^{18}\) but not all,\(^{17}\) data obtained in animal models.

Our study has limitations, both from an immunological and from a clinical point of view. The vaccination model used does not take into account the complexity brought by an influenza infection or any other virus infection, and our study was underpowered to evaluate clinical endpoints such as protection from influenza infection. Likewise, our experiments detecting unchanged EBV-specific immune responses in vitro cannot directly be extrapolated to indicate intact immune control of other virus infections in fingolimod-treated individuals. However, the data serve as definite proof-of-principle, demonstrating that blocking S1P-dependent lymphocyte migration in humans does not hinder the appearance of antigen-activated T cells in the peripheral circulation, nor does it affect the antibody response quantitatively. Clinical conditions with T cell lymphopenia comparable to that induced by fingolimod (human immunodeficiency virus [HIV] infection, myelotoxic chemotherapy) are associated with a high risk for opportunistic infections.\(^{19,20}\) Our data indicate that fingolimod-treated patients in principle can mount a virus-specific immune response. It remains unclear, however, to what extent these findings in the context of vaccine responses allow extrapolation to immunological competence vis-à-vis infectious pathogens. The molecular basis of the observed bypass of S1PR-dependent SLO egress by vaccine-triggered T cells in humans remains to be determined. For clinicians these data are informative when weighing the grade of immunosuppression inflicted on individuals treated with fingolimod, and they permit a more rational interpretation of infectious complications if they occur.

Acknowledgments
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Authorship
Patricia Hilbert and Stefanie Fritz, as well as Christoph Hess and Ludwig Kappos contributed equally to this work.

Potential Conflicts of Interest
C.H. is supported by the Swiss National Science Foundation (PP00B-114850) and L.K. by the Swiss MS Society. L.K. acted as a consultant, member, or chair of Steering Committees, Data Safety Monitoring Boards, or Advisory Boards in MS clinical trials sponsored by Accordia, Actelion, Allergan, Allozyne, Bayer Schering, Biogen Idec, Biogen-Dompé, Boehringer Ingelheim, Genmab, GlaxoSmithKline, Medicinova, Merck Serono, Novartis, Roche, Sanofi Aventis, Santhera, Teva Pharmaceuticals, UCB Pharma, and Wyeth, and has received lecture fees from Biogen Idec, Helvea, GlaxoSmithKline, Mediservice, and Merck Serono. Payments and consultancy fees have been exclusively used for the support of research activities. L.K. discloses that he has received grant support from Bayer Schering, Biogen Idec, CSL Behring, the European Community Research Fund, Genmab, Genzyme, GlaxoSmithKline, Medicinova, Merck Serono, Novartis, Novartis Foundation, the Rubato Foundation, Roche, Santhera, Sanofi Aventis, and UCB Pharma. Payments and consultancy fees have been exclusively used for the support of research activities. M.M., PH., S.F., J.K., T.K., R.L.P.L., and C.H. have no competing financial interests to declare.

References
Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy syndrome (CADASIL) is a systemic nonamyloid, nonatherosclerotic vasculopathy, with characteristic subcortical lacunar infarcts and white matter lesions. CADASIL is the most common monogenic inherited form of adult-onset stroke and vascular dementia, linked to mutations in Notch 3 expressed predominantly in vascular smooth muscle cells. The incidence of migraine with aura, typically the first disease symptom, is 5 times greater compared to the general population. Classical visual and sensory auras are most common in CADASIL, although 50% of the patients also experience atypical attacks with basilar, hemiplegic, or prolonged aura, and even coma. The mechanisms of this association are unknown. Among more than 150 CADASIL mutations identified to date, the Notch 3 R90C mutation produces a strong phenotype in humans with early onset of disease symptoms.

Animal experiments as well as human imaging studies suggest that migraine aura is caused by cortical spreading depression (CSD), an intense depolarization of neuronal and glial membranes slowly propagating by way of gray matter contiguity. Evoked when extracellular K⁺ concentrations ([K⁺]ₑ) exceed a critical threshold, CSD is associated with massive K⁺ and glutamate efflux, depolarizing adjacent neurons and glia to facilitate its spread. Neuronal mutations in Caᵥ₂.1 channels causing familial hemiplegic migraine type 1 augment CSD susceptibility as a mechanism for the severe migraine with aura phenotype in this syndrome. Using transgenic mice expressing the human archetypal CADASIL mutation Notch 3 mutations in its pathogenesis. Our results link vascular smooth muscle Notch 3 mutations to enhanced spreading depression susceptibility, implicating the neurovascular unit in the development of migraine aura.

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Table: Electrophysiological Measures of CSD and Systemic Physiological Parameters

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gender</th>
<th>Age (mo)</th>
<th>CSD Duration (sec)</th>
<th>BP (mmHg)</th>
<th>Systemic Physiology</th>
<th>Electrophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pCO₂ (mmHg)</td>
<td>pH</td>
</tr>
<tr>
<td>WT</td>
<td>Male</td>
<td>12</td>
<td>22</td>
<td>87 ± 3</td>
<td>7.39 ± 0.02</td>
<td>31 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>22</td>
<td>83 ± 7</td>
<td>7.39 ± 0.03</td>
<td>32 ± 2</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>13</td>
<td>14</td>
<td>86 ± 5</td>
<td>7.41 ± 0.02</td>
<td>29 ± 2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>22</td>
<td>76 ± 6</td>
<td>7.36 ± 0.06</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>WT</td>
<td>Male</td>
<td>7</td>
<td>22</td>
<td>76 ± 8</td>
<td>7.39 ± 0.04</td>
<td>33 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>22</td>
<td>82 ± 9</td>
<td>7.40 ± 0.03</td>
<td>30 ± 3</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>17</td>
<td>13</td>
<td>76 ± 8</td>
<td>7.39 ± 0.04</td>
<td>33 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>13</td>
<td>81 ± 9</td>
<td>7.37 ± 0.04</td>
<td>32 ± 2</td>
</tr>
</tbody>
</table>

Values are mean ± SD. The amplitude and duration of the first CSD were measured in each hemisphere. Systemic physiological parameters were averaged over the entire experiment. BP = mean arterial blood pressure; CSD = central spreading depression; pCO₂ = partial pressure of CO₂; pO₂ = partial pressure of O₂; SD = standard deviation; WT = wild type.

Materials and Methods

Experimental procedures strictly followed institutional guidelines for animal care and use for research purposes, and were approved by the institutional review committee. A total of 84 mice were used in this study. Experimental groups and the numbers of mice in each group are shown in the Table. Male and female (6–7 months old) wild type (Notch3WT) and transgenic mice expressing human CADASIL mutation Arg90Cys under the control of the SM22α promoter (TgNotch3R90C), were studied.9 TgNotch3R90C mice overexpress mutant Notch 3, specifically in arterial smooth muscle cells, on a WT Notch 3 background (ie, normal endogenous expression of WT mouse Notch 3); therefore, we compared TgNotch3R90C to transgenic mice overexpressing the human WT Notch 3 (TgNotch3WT; n = 5) in addition to nontransgenic WT controls (Notch3WT). We did not observe a difference between Notch3WT and TgNotch3WT in any parameter, and therefore pooled the data from these 2 groups into a single WT group. We also studied adult (5 months old) male Notch 3 knockout mice (Notch3−/−)9 and compared them to their WT littermates (Notch3WT). Although Notch3−/− mice do not carry a CADASIL mutation per se, they model CADASIL mutations located in the ligand-binding domain that have been shown to abolish Notch 3 receptor function.17,11

To address the importance of brain perfusion, we tested the impact of chronic cerebral hypoperfusion on CSD susceptibility in WT mice after bilateral common carotid stenosis (steel coils 0.18mm internal diameter, n = 6), as described.12 CSD susceptibility was tested 9 ± 1 weeks later, and compared to age-matched sham-operated controls (n = 5).

The femoral artery was catheterized, and the trachea was intubated for mechanical ventilation under isoflurane anesthesia (2.5% induction, 1% maintenance, in 70% N₂O/30% O₂). Arterial blood gases and pH were measured every 20 minutes and maintained within normal limits by adjusting ventilation. Systemic physiological parameters did not differ among groups (see Table). For analysis of CSD susceptibility, 3 burr holes were drilled at the coordinates described.8 Two glass capillary microelectrodes were placed to record extracellular steady (DC) potential and electrocorticogram. On each hemisphere, first the electrical CSD threshold was determined by escalating intensity cathodal square pulses (10–8000μC) via a bipolar electrode placed on the occipital cortex, and then a 1mm cotton ball soaked in 300mM KCl was topically applied onto occipital cortex for 1 hour and the frequency of evoked CSDs was recorded. The amplitude, propagation speed, and duration at one-half amplitude of the first CSD in each hemisphere were also measured. The data obtained from the 2 hemispheres were averaged to serve as a single data point for each animal. In separate experiments using R90C that exhibit cerebrovascular dysfunction and most of the arterial pathological hallmarks of CADASIL, we now show that a CADASIL-associated vascular Notch 3 mutation also augments susceptibility to CSD, implicating CSD susceptibility as one common determinant of migraine with aura phenotype in human syndromes.
**FIGURE**: Increased CSD susceptibility in TgNotch3R90C mutant mice. (A) Left panel: Representative tracings showing that the electrical threshold for CSD induction was significantly lower in TgNotch3R90C compared to WT mice. Stepwise escalating cathodal stimulation was used to determine the CSD threshold. Right panel: Representative 30-minute tracings showing that TgNotch3R90C mutant mice developed significantly higher number of CSDs compared to WT during continuous topical KCl (300mM) application. Calibration bars = 10mV, 5 minutes. (B) Left panel: Electrical threshold for CSD induction in WT and TgNotch3R90C mutant mice. Each data point represents the threshold for 1 animal (square, male; circle, female). Medians and interquartile ranges are also shown. Please note the logarithmic ordinate scale. *p < 0.001 vs WT. Right upper panel: The frequency of CSDs triggered during 60-minute topical KCl application in WT and TgNotch3R90C mutant mice. Topical continuous KCl application (300mM) induced repetitive CSDs with higher frequency in TgR90C than in WT. Data are mean ± SD. *p < 0.001 vs WT. Right lower panel: CSD propagation speed was faster in TgNotch3R90C mutant mice compared to WT in both males and females (*p < 0.05 vs WT), although there was a trend toward even faster propagation speeds in female vs male TgNotch3R90C (*p = 0.07).

TgNotch3R90C mice and WT controls (n = 6 each), we measured and compared blood flow changes during KCl-induced CSD (300mM) by laser speckle flowmetry, as described.15

All experiments were done in a blinded fashion. Data were analyzed with SPSS (version 11.0). Using a general linear model of covariance analysis (ANCOVA), we tested for an
effect of the independent variables genotype and age on the dependent variables cortical SD threshold, frequency, and propagation speed. Other electrophysiological measures of CSD and systemic physiological data were compared among groups using 1-way ANOVA. Blood flow changes during CSD were compared between groups by 2-way ANOVA for repeated measures. A \( p \) value of <0.05 was considered statistically significant.

**Results**

Direct cortical cathodal stimulation with stepwise escalating intensities triggered a CSD in all mice. The electrical threshold for CSD was approximately 10-fold lower in TgNotch3R90C compared to WT mice (Fig A,B). Female TgNotch3R90C tended to exhibit a lower CSD threshold compared to males (mean = 25 [range = 21–55] vs 55 [range = 53–55] \( \mu \text{C}; \ p = 0.066 \)). Continuous epidural KCl application evoked repetitive CSDs in all mice. Consistent with increased CSD susceptibility, TgNotch3R90C mice developed approximately 40% more CSDs during KCl application (see Fig A,B). Propagation speed of CSD was also higher in TgNotch3R90C mice compared to WT (see Fig B); however, CSD duration and amplitude did not differ among groups (see Table). Moreover, Notch3\(^{-/-}\) mice developed even higher numbers of CSDs in response to KCl (15 ± 2 vs 9 ± 1 CSDs/hour, in Notch3\(^{-/-}\) and WT littermates; \( p < 0.001 \)).

CADASIL patients, as well as mouse mutants, develop chronic vascular dysfunction and cerebral hypoperfusion. To test whether chronic hypoperfusion might explain enhanced CSD susceptibility, we induced bilateral common carotid stenosis in WT mice. However, we found that both the frequency of KCl-induced CSDs and their propagation speed were lower compared to sham controls (6.0 ± 1.4 CSDs/hour vs 9.2 ± 1.8 CSDs/hour, 1.8 ± 0.5 mm/minute vs 2.5 ± 0.2 mm/minute, \( n = 6 \) and 5, respectively; \( p < 0.05 \); Supporting Information Fig 1). Moreover, setting another contrast with the TgNotch3R90C phenotype, CSD durations tended to be prolonged in the stenosis group, probably reflecting reduced cerebral perfusion pressure (see Table). To test an alternative hypothesis that enhanced CSD susceptibility might be caused by altered vascular response to CSD, we measured CSD-induced blood flow changes using laser-speckle flowmetry and did not find a significant difference between TgNotch3R90C and WT mice (\( n = 6 \) each; Supporting Information Fig 2).

**Discussion**

Genetic and epidemiological evidence suggests a strong association between migraine with aura and adult-onset genetic vasculopathies, such as CADASIL. Using 2 independent but complementary techniques (electrical and KCl stimulation), we found that 2 different Notch 3 mutations (TgNotch3R90C and Notch3\(^{-/-}\)) enhance cortical susceptibility to spreading depression as a potential mechanism for this clinical association. Enhanced CSD susceptibility in CADASIL may also explain more frequent and severe aura features (eg, coma), similar to familial hemiplegic migraine type 1 associated with mutations in the neuronal Ca\(_{2.1}\) channels. Enhanced CSD susceptibility in both the Notch3\(^{-/-}\) and the TgNotch3R90C appears paradoxical, because Notch3\(^{-/-}\) mutation is a loss-of-function, whereas Notch3R90C is probably neomorphic, as it retains Notch 3 function and is expressed on a WT Notch 3 background. However, consistent with our data in mutant mice, both neomorphic and loss-of-function CADASIL mutations are known to be associated with a migraine phenotype in affected individuals.

Cerebrovascular dysfunction is a common phenotype in both mouse mutants as well as in CADASIL patients regardless of Notch 3 gain-of-function or loss-of-function, implicating vascular mechanisms for the enhanced CSD susceptibility phenotype in Notch 3 mutants. TgNotch3R90C mice do develop vascular changes characteristic of CADASIL, including disruption of smooth muscle anchorage to adjacent extracellular matrix and cells, cytoskeletal changes, and later smooth muscle degeneration. Even before the morphological changes become conspicuous, TgNotch3R90C mice display abnormal myogenic and flow-mediated vascular responses, impaired autoregulation and diminished hypocapnic hyperemia. Further supporting a vascular link, mutations in TREX1, another vascular locus linked to the retinal vasculopathy with cerebral leukodystrophy syndrome, are also frequently associated with a migraine phenotype.

The mechanisms linking vascular dysfunction to enhanced CSD susceptibility, however, are not clear. Disturbances in the microcirculation caused by platelet dysfunction, inflammation, microemboli, and intermittent hypoperfusion have been implicated. It has been speculated that ischemic neuronal injury could facilitate the occurrence of CSD and migraine attacks in CADASIL. However, migraine with aura is often the first symptom in CADASIL and typically precedes ischemic events by more than a decade. Moreover, migraine attacks gradually recede at later stages of disease when ischemic injury manifests. One possible mechanism is that blood flow dysregulation in CADASIL brains may limit the ability of cerebral vasculature to maintain energy and ionic homeostasis during intense brain activation, facilitating \([\text{K}^+]_o\) to rise above the CSD threshold. However, in separate
experiments we found that chronic forebrain hypoperfusion alone, induced by bilateral common carotid artery stenosis, did not enhance KCl-induced CSD susceptibility, and that CSD-induced blood flow changes did not differ between WT and CADASIL mutants, both arguing against a primary vascular mechanism for enhanced CSD susceptibility. Additional mechanisms are therefore needed to explain enhanced CSD susceptibility in CADASIL syndrome.

Importantly, whether Notch 3 CADASIL mutations impact other cell types in the neurovascular unit has not been investigated in detail. For example, astrocyte end feet are in close proximity to cerebral blood vessels, and abnormal Notch 3 signaling may disrupt normal astrocyte-smooth muscle communication. Indeed, impaired functional neurovascular coupling was recently reported in a CADASIL mouse model expressing the Notch 3 R169C mutation. Alternatively, Notch 3 mutations expressed in neural progenitor cells and transiently in newly-born neurons may lead to enhanced CSD susceptibility phenotype later in life.

In summary, we found that Notch 3 mutations modulate cortical susceptibility to spreading depression, the electrophysiological substrate of migraine aura. These data are the first to link a monogenic vasculopathy to a spreading depression phenotype, and provide an explanation for the frequent and severe migraine with aura phenotype in CADASIL patients.

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Potential Conflicts of Interest
A.J. has one or more patents with Athenadiagnostics for diagnostic assay of CADASIL disease.

References
Autoantibodies to Low-Density Lipoprotein Receptor–Related Protein 4 in Myasthenia Gravis

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Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction, where acetylcholine receptor (AChR), muscle-specific kinase (MuSK), and low-density lipoprotein (LDL) receptor-related protein 4 (Lrp4) are essential. About 80% and 0% to 10% of patients with generalized MG have autoantibodies to AChR and MuSK, respectively, but pathogenic factors are elusive in others. Here we show that a proportion of AChR antibody-negative patients have autoantibodies to Lrp4. These antibodies inhibit binding of Lrp4 to its ligand and predominantly belong to the immunoglobulin G1 (IgG1) subclass, a complement activator. These findings together indicate the involvement of Lrp4 antibodies in the pathogenesis of AChR antibody-negative MG.

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The neuromuscular junction (NMJ) is a synapse between the motor nerve terminal and the skeletal muscle end-plate.1 Postsynaptic clustering of the neurotransmitter receptor acetylcholine receptor (AChR) is controlled by muscle-specific kinase (MuSK) and low-density lipoprotein (LDL) receptor-related protein 4 (Lrp4), which form an essential postsynaptic receptor complex for its ligand, neural agrin.2–4 Myasthenia gravis (MG) is an autoimmune disease of the NMJ.5 About 80% of patients with generalized MG have AChR antibodies, which is a causative factor for the disease, and a variable proportion of the remaining patients (0–50% throughout the world) have MuSK antibodies.6–12 However, diagnosis and clinical management remain complicated for patients who are negative for MuSK and AChR antibodies, giving rise to a need for unveiling the hidden causative factors in MG. Given the essential role and postsynaptic localization of Lrp4 in the NMJ, we hypothesized that Lrp4 autoantibodies might be a pathogenic factor in MG. In this study, we developed a simple technique termed luciferase-reporter immunoprecipitation (LUCIP), which takes advantage of the strong luminescence of *Gaussia* luciferase (GL) to detect protein-protein interactions with high sensitivity. Using this method, we evaluated the titers and pathogenic properties of serum antibodies to the extracellular portion of Lrp4 in patients with AChR antibody-negative MG.

Materials and Methods

We studied serum samples from 300 patients with AChR antibody-negative MG diagnosed by typical clinical features, the edrophonium test, and/or the repetitive nerve stimulation test. Sera from 100 healthy volunteers, 100 patients with AChR antibody-positive MG, and 101 patients with Lambert-Eaton myasthenic syndrome (LEMS) were also studied. All MG sera were tested for AChR and MuSK antibodies using the standard radioimmunoprecipitation assay (RIA).7–13

For the Lrp4-LUCIP assay, HEK293 cells were transfected with expression plasmid for Lrp4-GL, in which the entire extracellular portion of Lrp4 was fused to GL,14 and the fusion protein was purified from the culture supernatant (Supporting Information Methods). The specific luciferase activities of Lrp4-GL were 1.25 × 105 relative light units [RLU]/pmol using the BioLux *Gaussia* luciferase assay kit (BioLabs) and a Lumat LB 9507 luminometer (Berthold Technologies).

To titrate Lrp4 antibodies, 5μl serum was added to 24fmol Lrp4-GL in 700μl phosphate-buffered saline with 0.05% Tween 20 and 3% bovine serum albumin for overnight incubation at 4°C. Immunoglobulin G (IgG)-bound Lrp4-GL was precipitated with 15μl protein G-Sepharose (GE Healthcare). The precipitates were washed and their luciferase activities were determined to calculate the amount of Lrp4-GL protein, whose value was used to represent the titer of Lrp4 antibodies.

For subclass-specific titration of IgG antibodies to Lrp4, 1μl serum was incubated with Lrp4-GL as described above in the presence of 10, 5, 2, and 2μg biotinylated anti-human IgG1, IgG2, IgG3, and IgG4 antibodies (Binding Site), respectively.10 Immune complexes comprised of these subclass-specific antibodies, serum IgGs to Lrp4, and Lrp4-GL were precipitated with 15μl NeutrAvidin-Agarose (Thermo Scientific). The precipitates were washed and their luciferase activities were determined to calculate subclass-specific antibody titers.

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To evaluate agrin-Lrp4 interaction, HEK293 cells were transfected with expression plasmid for 3×FLAG-agrin in Opti-modified Eagle medium (Opti-MEM) and the culture supernatant containing secreted 3×FLAG-agrin was harvested. The culture supernatant containing 11fmol 3×FLAG-agrin was incubated with 6fmol Lrp4-GL, and then 3×FLAG-agrin was immunoprecipitated with anti-FLAG antibody-conjugated agarose (Sigma). The precipitates were washed and luciferase activities were determined to calculate the amount of Lrp4-GL bound to 3×FLAG-agrin (see Supporting Information Methods for details). To further investigate the effects of serum or serum IgGs on agrin-Lrp4 interaction, 11fmol 3×FLAG-agrin and different volumes of serum or corresponding amounts of IgGs were subjected to this assay. Serum IgGs were purified using the IgG Purification Kit-G (Dojindo), or IgGs were depleted from serum using an excess amount of protein G-Sepharose.

To validate the significance of the observed differences, we analyzed simple pairwise comparisons with the Student t test (2-tailed distribution with 2-sample equal variance).

Results

We tested for Lrp4 autoantibodies in sera from patients with AChR antibody-negative MG by the Lrp4-LUCIP assay, which uses a fusion protein of the entire extracellular portion of Lrp4 and GL (Lrp4-GL) as a reporter (Supporting Information Data; Supporting Information Figs 1 and 2). From a cohort of 300 patients, 9 patients were positive for antibodies to the extracellular portion of Lrp4, where the cutoff value (0.015nM) was determined based on the mean + 4 standard deviations (SDs) obtained with 100 healthy control sera (Fig 1A). The control GL-LUCIP assay confirmed that these patients were negative for serum antibodies to GL (Supporting Information Table 1). Titers of Lrp4 antibodies in the 9 patient sera ranged from 0.019nM to 2.07nM (median, 0.65nM; Table). These titers are statistically significant because the minimum titer value (0.019nM) in the Lrp4 antibody-positive sera occurs with a probability of less than 6.312 × 10^-10 under the null hypothesis that the AChR antibody-negative MG data follow the same distribution as healthy controls.

Next, we tested sera from 100 MG patients positive for AChR antibodies and all were negative for the Lrp4 antibodies, suggesting that the autoantibodies are mutually exclusive in MG (see Fig 1A). Furthermore, Lrp4-LUCIP testing of sera from 101 patients with LEMS, a different form of NMJ autoimmune disease, revealed that the patients were negative for Lrp4 antibodies aside from one who showed weak positivity (see Fig 1A). In addition, among 28 patients with MuSK antibody-positive MG in the cohort of 300 AChR antibody-negative patients, 3 patients were also positive for Lrp4 antibodies (index case nos. 6, 8, and 9; see Table).

FIGURE 1: Serum autoantibodies to the extracellular portion of Lrp4 were found in patients with AChR-Ab− MG and recognized native Lrp4. (A) Scatter plot for the calculated titers (nM) of Lrp4 autoantibodies in sera from patients and the HC as indicated. The red line indicates the cutoff value, calculated as the mean + 4 SDs of the healthy control values. (B) IP of full-length Lrp4 protein with sera from patients (index cases nos. 1–3). Immunoprecipitates or WCL of HEK293 cells that had been transfected with expression plasmid for Lrp4 (+) or the empty vector (−) were subjected to IB with rat antiserum to the cytoplasmic portion of Lrp4 (aL4C) (Supporting Information Methods). The blank lane contained size markers (sm). (C) Immunostaining of cell surface Lrp4 with sera from patients (index case nos. 1 and 3). Intact (nonpermeabilized) HEK293 cells that had been transfected with expression plasmid for Lrp4 or the empty vector (mock) were stained with sera from patients or the HC (see Supporting Information Methods). Anti-human IgG-Alexa488 was used as a secondary antibody to visualize cell surface Lrp4 (arrowheads). Scale bars 50µm. AChR = acetylcholine receptor; AChR-Ab+ = AChR antibody–positive; AChR-Ab− = AChR antibody-negative; HC = healthy control; IB = immunoblotting; IgG = immunoglobulin G; IP = immunoprecipitation; Lrp4 = low-density lipoprotein receptor-related protein 4; LEMS = Lambert-Eaton myasthenic syndrome; MG = myasthenia gravis; SD = standard deviation; WCL = whole cell lysates. [Color figure can be viewed in the online issue, which is available at annalsofneurology.org.]
We next examined whether the serum antibodies to Lrp4 present in these MG patients recognize the native form of Lrp4. HEK293 cells transfected with full-length Lrp4 expression plasmid and C2C12 myotubes expressing endogenous Lrp4 were subjected to immunoprecipitation with Lrp4 antibody-positive sera (index case nos. 1–3) or healthy control sera (see Fig 1B and Supporting Information Fig 3A). In Supporting Information Figure 3A, index case no. 2 was excluded due to paucity. Sera from these patients but not serum from the healthy control precipitated Lrp4 proteins. Likewise, cell surface Lrp4 ectopically expressed in HEK293 cells could be visualized by immunostaining of intact cells with sera from these patients, but not the healthy control or antiserum to the cytoplasmic portion of Lrp4 (see Fig 1C and Supporting Information Figs 4 and 5), demonstrating that the serum antibodies to Lrp4 can recognize its native form. However, sera from the remaining patients (index case nos. 4–9) failed to visualize cell surface Lrp4 (data not shown), and these sera, aside from index case no. 4, also failed to immunoprecipitate Lrp4 (see Supporting Information Fig 3B). Unlike the healthy control, however, sera from these patients reacted with the LA domain of the extracellular portion of Lrp4 in immunoblots (see Supporting Information Fig 3C).

The clinical features of 9 patients with Lrp4 antibody-positive MG (index case nos. 1–9) are summarized in Table. Generalized MG was diagnosed in these patients, who showed severe limb muscle weakness or progressive bulbar palsy or both. Thymoma was not observed in any of these patients, unlike the situation in patients with AChR antibody-positive MG. Because Lrp4 is the agrin-binding subunit of the Lrp4:MuSK receptor complex,3,4 and serum antibodies to Lrp4 in MG patients bound to the molecule’s extracellular portion, we speculated that those antibodies might compete with agrin for binding with Lrp4. Indeed, sera from Lrp4 antibody-positive patients, but not the healthy control, inhibited interaction of Lrp4-GL with neural agrin (Fig 2A). We confirmed that serum and IgGs prepared from the same patient (index case no. 3) showed comparable inhibition (see Fig 2B). Conversely, when IgGs were depleted from the patient’s serum, it lost its inhibitory activity (Supporting Information Fig 6). Thus, autoantibodies to Lrp4 could exert pathogenicity through their potential to inhibit agrin and Lrp4:MuSK signaling required for NMJs. Furthermore, to assess potential involvement of the complement system in Lrp4 antibody-positive MG, we investigated the IgG subclass composition of Lrp4 antibodies in patients (see Fig 2C and Supporting Information Fig 7). The Lrp4-LUCIP assay in combination with subclass-specific immunoprecipitation of IgGs revealed that Lrp4 autoantibodies were predominantly comprised of IgG1, a complement activator, in each patient, suggesting the potential for these antibodies to cause complement-mediated impairment of NMJs.

Discussion
We identified the novel antigen Lrp4 as a target for autoantibodies in AChR antibody-negative MG. These antibodies are mainly IgG1 and have the potential to inhibit interaction between neural agrin and the extracellular
portion of Lrp4. Therefore, these findings suggest patho-
genic involvement of the complement system and reduced agrin:Lrp4 signaling in Lrp4 antibody-
positive MG. However, it is important to carefully evalu-
ate contributions of these antibodies to myasthenia, espe-
cially those of the antibodies with lower titers (index case nos. 4–9; see Table), which failed to visualize cell surface Lrp4 likely due to their lower titers. Interestingly, Lrp4 antibodies were found in 3 of 28 patients with MuSK antibody-positive MG and 1 of 101 patients with LEMS. Because MuSK antibodies predominantly belong to the IgG4 subclass, which does not activate complement, and Lrp4 is a postsynaptic protein, antibodies to Lrp4 might contribute differently to pathogenesis than antibodies to MuSK or the P/Q-type presynaptic Ca2+ channel, a target for autoantibodies in LEMS.16 However, since MuSK antibodies, though predominantly IgG4, are partially IgG1 subclass capable of activating complement,18 MuSK and Lrp4 antibodies might also contribute similarly to pathogenesis in a complement-de-
pendent manner. Given that titters of Lrp4 antibodies were relatively low in sera from patients with MuSK antibody-
positive MG or LEMS, again contributions of Lrp4 anti-
bodies to each myasthenia must be carefully evaluated. The LUCIP assay developed in this study is a simple in vitro system using no radioisotope. Moreover, the MuSK-LUCIP assay, in which MuSK-GL was used as a reporter, showed roughly a 50-fold lower cutoff value than that determined with the conventional RIA for MuSK autoantibodies, indicating greater sensitivity for the LUCIP assay (Supporting Information Fig 8). Therefore, this assay system could be used for routine diagnosis and clinical management of various autoimmune disorders, including MG.

It should be noted that the proportion of MuSK antibody-positive patients within an AChR antibody-nega-
tive MG cohort varies from 0% to 50% throughout the world.12 Given the number and narrow ethnic origins of patients in the current study, further clinical and exper-
imental data on greater numbers of patients worldwide are required to fully understand the etiology and pathol-
ogy of Lrp4 antibody-positive MG.

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The study was approved by the ethics committees of the Graduate School of Biomedical Sciences, Nagasaki University (no. 09031864), and the Institute of Medical Science, the University of Tokyo (no. 20-60-210403).

Potential Conflicts of Interest

Nothing to report.

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