Megalencephalic leukoencephalopathy with subcortical cysts: defect in chloride currents and cell volume regulation

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Abstract

Megalencephalic leukoencephalopathy with subcortical cysts is a genetic brain disorder with onset in early childhood. Affected infants develop macrocephaly within the first year of life, after several years followed by slowly progressive, incapacitating cerebellar ataxia and spasticity. From early on, MRI shows diffuse signal abnormality and swelling of the cerebral white matter, with evidence of highly increased white matter water content. In most patients, the disease is caused by mutations in the gene MLC1, which encodes a plasma-membrane protein almost exclusively expressed in brain and at lower levels in leukocytes. Within the brain, MLC1 is mainly located in astrocyte-astrocyte junctions adjacent to the blood-brain and cerebrospinal fluid-brain barriers. Thus far, the function of MLC1 has remained unknown. We tested the hypothesis that MLC1 mutations cause a defect in ion currents involved in water and ion homeostasis, causing the cerebral white matter oedema.

Using whole-cell patch-clamp studies we demonstrated an association between MLC1 expression and anion channel activity in different cell types, most importantly astrocytes. The currents were absent in chloride-free medium and in cells with disease-causing MLC1 mutations. MLC1-dependent currents were greatly enhanced by hypotonic pretreatment causing cell swelling, while ion channel blockers, including Tamoxifen, abolished the currents. Down-regulation of endogenous MLC1 expression in astrocytes by small interfering RNA greatly reduced the activity of this channel, which was rescued by overexpression of normal MLC1. The current-voltage relationship and the pharmacological profiles of the currents indicated that the channel activated by MLC1 expression is a volume-regulated anion channel. Such channels are involved in regulatory volume decrease. We showed that regulatory volume decrease was hampered in lymphoblasts from MLC patients. A similar trend was observed in astrocytes with decreased MLC1 expression; this effect was rescued by overexpression of normal MLC1. In the present study we show that absence or mutations of the MLC1 protein negatively impact both volume-regulated anion channel activity and regulatory volume decrease, indicating that MLC is caused by a disturbance of cell volume regulation mediated by chloride transport.
Introduction

Megalencephalic leukoencephalopathy with subcortical cysts (MLC, MIM 604004) is an autosomal recessive brain disorder with infantile onset, first described in 1995. MLC patients develop macrocephaly during the first year of life. After several years, slowly progressive cerebellar ataxia and spasticity occur. Most patients become wheelchair-dependent in their teens. Brain magnetic resonance imaging (MRI) reveals diffuse signal abnormalities and swelling of the cerebral white matter and subcortical cysts (Supplementary Fig.1). Quantitative MRI parameters indicate that the white matter water content is highly increased. In 2001 a gene for MLC, MLC1, was identified. This gene encodes a 377 amino acid plasma-membrane protein with eight transmembrane domains, almost exclusively expressed in the brain and leukocytes. Within the brain the MLC1 protein is mainly expressed in astrocyte-astrocyte junctions adjacent to the blood-brain and cerebrospinal fluid-brain barriers. MLC1 mutations all disrupt the membrane localization of the MLC1 protein. We have recently shown that mutations in GLIALCAM, coding for hepatic and glial cell adhesion molecule GlialCAM, also cause MLC. GLIALCAM mutations also disrupt the localization of MLC1, indicating that MLC1 is central in the pathophysiology of MLC. Thus far, the function of MLC1 has remained unknown. Amino acid sequence analysis reveals a weak similarity with potassium channel Kv1.1, ABC-2 type transporters and sodium:galactoside symporters. Besides, MLC1 contains an internal repeat that is found in several ion channel proteins. These observations and the highly increased white matter water content prompted us to test the hypothesis that MLC1 plays a role in ion transport involved in cell volume regulation.

Supplementary Figure 1. Morphology of the brain. Axial T2-weighted MRI of the brain of a normal child (A) and patient 1 (B), both at the age of 4. The cerebral white matter has a low signal in normal children, whereas it has a high signal in the patient. The normal white matter is swollen, leading to broadening of the gyri. (C) Sagittal T1-weighted MRI of the brain of the normal child and patient 2. (D) The cerebral white matter has a high signal in normal children and is low in the patient. A cyst is seen in the anterior temporal region in the patient. The images in the patient are diagnostic of MLC.
**Materials and Methods**

The study was performed with approval of the Institutional Review Board. Written informed consent was obtained for the use of patient lymphoblasts. The MLC1 mutations of the patients, of whom lymphoblasts or MRIs were used, are given in Table 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutations</th>
<th>Patient material used</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>c.135_136insC, p.Cys46LeufsX34 (homozygous)</td>
<td>Lymphoblasts, MRI</td>
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<tr>
<td>Patient 2</td>
<td>c.736A&gt;C , p.Ser246Arg (homozygous)</td>
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**Cell culture**

Culture media for HEK293 and HeLa cells consisted of DMEM/HAM-F10 (1:1) (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal calf serum (Invitrogen, San Diego, CA, USA). *Spodoptera frugiperda* (Sf9) insect cells were cultured in suspension in serum free HyQ SFX-Insect medium (Perbio, Philadelphia, PA, USA) at 27°C. Isolation and immortalization of human lymphoblasts were performed as previously described. Lymphoblasts were cultured in RPMI-1640 medium (Invitrogen, San Diego, CA, USA), supplemented with 10% fetal calf serum. Primary rat astrocytes were isolated and cultured as described with the exception that we used trypsin and trituration with a glass Pasteur pipette to dissociate the cells instead of passing through needles. For influence of culture conditions on MLC1 expression, see Supplementary Fig. 2.

**Supplementary figure 2. Expression of MLC1 is cell type- and growth condition-dependent.** (A) Total RNA was isolated from HEK293_EBNA (Lanes 1 and 2), HEK293 (lane 3), HEK293T (lane 4) and control lymphoblasts (lane 5) and MLC1 expression analysis by RT-PCR (top panel). Cells were either grown in the presence of 10% High Clone serum (lanes 1 and 5) or 10% Fetal Calf Serum (lanes 2-4). (B) Hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression in the same samples as control for total RNA input. “M” indicates the Invitrogen 1Kb plus ladder. Note the remarkable differences in endogenous MLC1 expression between different types of HEK293 cells (e.g., lanes 3 and 4), which is further affected by growth conditions (lanes 1 and 2). In cells with higher levels of endogenous MLC1 expression (lanes 1 and 4) we were unable to measure differences in chloride currents between MLC1-transfected and control cells.
Transfection and infection

HEK293 and HeLa cells were transfected using calcium phosphate precipitation and lipofectamine (Invitrogen, San Diego, CA, USA), respectively, with a construct expressing green fluorescent protein (GFP) (pEGFPN1, Clontech, Mountain View, CA, USA) to mark transfection either alone or in combination with constructs expressing wild-type or mutant MLC1 (MLC1_Ser93Leu or MLC1_Cys326Arg). HEK293 and HeLa cells were used for patch-clamp experiments 24-48 hours after transfection.

Constructs for expression in Sf9 insect cells were made by cloning the sequences for GFP (to mark transfection) or MLC1 (wild-type or mutant) into pIEX5 (Novagen, Madison, WI, USA.). Sf9 cells were transfected with Insect GeneJuice (VWR, San Diego, CA, USA). Sf9 cells were used 48-72 hours after transfection.

Astrocytes were infected with adenoviruses (multiplicity of infection [MOI= ratio of infectious virus particles to cells] of 4-6) and used after 6-8 days for patch-clamp experiments. Generation of adenoviruses for expression of wild-type or mutant (Ser246Arg) haemagglutinin (HA)-tagged MLC1 has been described 7.

Small interfering RNAs

The generation of adenoviruses for expression of small interfering RNA (siRNA) against nucleotides 405-425 of the coding region of rat MLC1 or a sequence without mRNA target as control has been described 6. Sequences are given in Supplementary Table 1.

Supplementary Table 1. Primers used for qPCR, RT-PCR and siRNA

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<th>Primer</th>
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<td>siRNA against rat MLC1</td>
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MLC1 expression

Total RNA was isolated using RNA-Bee (BioConnect, Huissen, The Netherlands). Complementary DNA was made with 5µg total RNA with SuperScript™ III (Invitrogen, San Diego, CA, USA) and used in qPCR and RT-PCR.
Diego, CA, USA) according to the vendor’s instruction. Expression of MLC1 mRNA was studied by reverse transcription polymerase chain reaction as described, or quantitative PCR using SYBR-green according to the vendor’s protocol (Applied Biosystems, Carlsbad, CA, USA). For primers see Supplementary Table 1. The values obtained were corrected for the amount of glyceraldehyde 3-phosphate dehydrogenase mRNA in each sample (DCt). In addition, the relative expression of MLC1 mRNA in the separate cell types as compared to its expression in the brain sample was calculated.

**Immunocytochemistry**

For immunofluorescence staining, cells were fixed with phosphate buffered saline containing 4% paraformaldehyde for 5 min, permeabilized with 1% goat serum, 0.9% NaCl and 0.1% Saponin in phosphate buffered saline for 10 min at room temperature and blocked with 5% goat serum, 0.9% NaCl and 0.05% Saponin in phosphate buffered saline for 30-60 min. Primary antibodies were diluted in 1% goat serum, 0.9% NaCl and 0.01% Saponin in phosphate buffered saline and cells were incubated over-night at 4°C. Rabbit anti-mouse MLC1 (1:100), rabbit anti-human MLC1 (1:100), chicken anti-glial fibrillary acidic protein (GFAP) (1:1000; Chemicon, Temecula, CA, USA), mouse anti-HA clone HA-7 (1:1000; Sigma, St. Louis, MO, USA), and appropriate secondary antibodies were used. Cells were washed and incubated for 1 hour at room temperature with secondary antibodies: Alexa-488 goat anti-chicken, Alexa-568 goat anti-rabbit, Alexa-594 goat anti-mouse. Coverslips were mounted in Vectashield medium (Vector Laboratories Burlingame, CA, USA) with 1.5 µg/ml DAPI (Sigma, St. Louis, MO, USA) and visualized using a Leica DM6000B microscope.

**Electrophysiology**

Voltage-clamp experiments were performed at 20-22°C using the tight-seal, whole-cell variant of the patch-clamp technique as described previously and currents were amplified using an AXOPATCH-200A amplifier (Axon Instruments, Union City, CA, USA). In all experiments, unless mentioned specifically, we used iso-osmotic chloride-selective bath and pipette solutions. In experiments with HeLa cells, HEK293 cells, lymphoblasts and astrocytes, the iso-osmotic bath solution contained in mM: 140 N-methyl-D-glucamine Cl, 2 CaCl2, 2 MgCl2, 10 HEPES, 0.1 LaCl3 (average osmolarity 295 mOsmol/l, pH 7.4), and the pipette (resistance of 3-7 MΩ) solution contained in mM: 40 N-methyl-D-glucamine-Cl, 100 N-methyl-D-glucamine-gluconate, 2 MgCl2, 10 HEPES, and Ca2+-EGTA buffer (buffered to pH 7.2, average osmolarity 290 mOsmol/l), unless mentioned otherwise.

In Sf9 cells, the iso-osmotic bath solution contained in mM: 180 N-methyl-D-glucamine-Cl, 2 CaCl2, 2 MgCl2, 10 MES (average osmolarity 365 mOsmol/l, pH 6.2), and the pipette
(resistance of 3-6 MΩ) solution contained in mM: 50 N-methyl-D-glucamine-Cl, 120 N-methyl-D-glucamine-gluconate, 2 MgCl₂, 10 TES, and Ca²⁺-EGTA buffer (buffered to pH 7.2, average osmolarity 365 mOsmol/l). The extracellular pH and osmolarity mimics that of the HyQ SFX-Insect medium¹²,¹³.

Hypo-osmotic pretreatments were executed as previously described.¹⁴ The hypo-osmotic solution had a 40% reduced osmolarity as compared to the iso-osmotic solution.

Adenovirus-infected astrocytes were treated with 0.025% trypsin for 5 min before recording.

Where indicated, 10 µM Tamoxifen (Tocris Bioscience, Bristol, UK), 1 mM Zinc (Sigma, St. Louis, MO, USA), 100 µM NPPB (Sigma, St. Louis, MO, USA) or 200 µM DIDS (Sigma, St. Louis, MO, USA) was added to the bath solution. For the inhibition of chloride channels in astrocytes, Tamoxifen had to be used in reduced concentration (1 µM instead of 10 µM) and had to be present during both trypsin pretreatment and electrophysiological recording, as described before.¹⁵ The other blockers were added to the bath solution after the trypsin treatment. In all lymphoblast experiments 2 mM ATP (as Mg²⁺ salt) was present in the pipette-filling solution.

The electrophysiology protocols are summarized in figure 1. The holding potential was -60 mV. Currents were measured with voltage ramps of -120 to +120 mV, or -100 to +100 mV for Sf9 cells and astrocytes in steps of 0.14 mV/ms. Sample interval was 250 µs. Data were filtered at 2 kHz. Each current measurement was an average obtained from 5 ramps, with an interval of 1 s between the ramps. Voltage ramps were used for all current-voltage-curves and bar figures (Fig 2-5). In addition to the voltage ramps, we also recorded current traces obtained from a step-voltage protocol (holding potential -60 mV, 250 ms steps ranging from -120 to +60 mV, spaced 10 mV). For Sf9 cells and astrocytes we used a step-voltage protocol ranging from -100 to +100 mV. Supplementary figures 4 and 5B are the only figures incorporating currents from the step-voltage experiments. A tight seal (resistance >1 ΩG) was established prior to the whole-cell patch-clamp recordings. Only cells that showed a stable holding current and a low series resistance (<6 MΩ) throughout the experiment were analysed. Mean capacitance of the different cell types are given in table 2. Voltage-clamp protocol, data acquisition, storage and analysis were carried out using commercial software (pClamp 9.1, Axon Instruments).

Potassium currents were measured, as previously described,¹⁶ using bath solution containing (in mM) 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, and 5 glucose; pH was adjusted to 7.4 with NaOH and osmolarity was adjusted to approximately 315 mOsmol/l with mannitol. The intracellular (pipette) solution contained (in mM) 144 KCl, 2 MgCl₂, 5 EGTA, and 10 HEPES; pH was adjusted to 7.2 with KOH and osmolarity was adjusted to approximately 300 mOsmol/l with mannitol.¹⁶
Figure 1. Electrophysiology protocols. (A) Schematic representation of the voltage ramp protocol used for HEK293 cells, HeLa cells and lymphoblasts. (B) Schematic representation of the voltage ramp protocol used for Sf9 cells and rat primary astrocytes. (C) Schematic representation of the method used for hypotonic pretreatment of cells. The two upper bars represent the standard procedure without hypo-shock, where cells are kept in iso-osmotic bath solution for at least 5 minutes before recording. The lower bars show experimental set-up for incubation in hypo-osmotic bath solutions before recording in standard bath solution. The colours indicate the cells used (transfected with wild-type MLC1: red and purple; transfected with a control GFP construct: black and blue). The osmotic conditions of the bath solutions are indicated by red or black for iso-osmotic, and purple or blue for hypo-osmotic conditions. The arrows indicate the starting point of the measurements. (D) Schematic representation of the step protocols used for measuring chloride currents in HEK293 cells (upper part) and astrocytes (middle part), and potassium currents in astrocytes (lower part).

Imaging of regulatory volume decrease.

Astrocytes and lymphoblasts were loaded with 5 µM Calcein-AM (Molecular Probes) for 20 min at 37°C. Changes in cell volume of single lymphoblasts, plated on poly-ornithine coated glass cover slips, were monitored by measuring cell surface area. Changes in cell volume of single astrocytes, plated on glass cover slips, were analysed by measuring calcein fluorescence using the calcein-quenching method. Chloride selective iso- and hypo-osmotic (-40%) solutions were used, as described in the electrophysiology section. To ensure that differences between cells were dependent on anion permeability rather than cation permeability, lymphoblasts were pretreated with 40 µg/ml and astrocytes with 10 µg/ml gramicidin (Sigma, St. Louis, MO, USA) for 5 min prior to the start of the experiment. Cells were bathed in the iso-osmotic solution (20-22°C) and transferred to a continuously perfused (5 ml/min) recording chamber, equipped with a microscope with a 10x objective. An image
was taken every 30 s. At the beginning of each experiment, images were obtained for 5 min in the iso-osmotic solution to establish the baseline. Cells were perfused with the hypo-osmotic solution for 30 min, after which the iso-osmotic solution was re-introduced and images were taken for another 10 min. The cell surface or average fluorescence of each cell in the acquired images was calculated using ImageJ software (National Institutes of Health, Bethesda, MD). Changes in cell surface and average fluorescence were expressed as $S_t/S_0$ and $F_t/F_0$ ratios, respectively, where $S_0$ and $F_0$ are the average cell surface area and fluorescence under iso-osmotic conditions at the beginning of the experiment. The curve from maximum swelling to the end of the hypo-osmotic treatment was fitted with an exponential curve using Microsoft Excel and the time constant of this exponential decay was determined for each cell.

**Statistical analysis**

Group measures were expressed as mean ± standard error of the mean; error bars also indicate standard error of the mean. We used the Student $t$-test to assess the statistical significance of differences between control and experimental conditions.

**Results**

*MLC1-expression related chloride currents in transfected cells.*

To address the question whether MLC1 could have a role in ion transport, we transfected different cell types with constructs expressing wild-type MLC1 and used the whole-cell patch-clamp technique to test whether ionic currents were induced. Figures 2-5 illustrate current profiles in the cell types investigated, each figure representing a specific cell type. Quantitative results of all electrophysiological experiments are given in Table 2.

Initial patch-clamp data in transfected HEK293 cells expressing either GFP as a control, or wild-type MLC1 and using different recording media indicated that MLC1 contributed to small ionic currents that become visible in particular at highly positive membrane potentials and are carried either by an influx of anions or an efflux of cations (data not shown). To test which ion carries the currents observed in MLC1-transfected HEK293 cells, we first eliminated the contribution of the monovalent cations sodium and potassium in the pipette and bath solutions by the impermeant cation N-methyl-D-glucamine. Under these conditions, a small inward current was observed at negative potentials in HEK293 cells transfected with the control GFP construct that reversed at 0 mV (Fig. 2A, Table 2). In MLC1-transfected HEK293 cells, a stronger inward current was observed at negative potentials, which also reversed around 0 mV and showed outward rectification (Fig. 2A, Table 2). Anion channels have a very low permeability for gluconate $^{19}$. Replacing chloride by gluconate in bath and pipette filling solutions almost completely abolished the inward and outward currents (Fig. 2B), demonstrating
that the MLC1 expression-induced current is carried by chloride. Given the nature of the voltage clamp protocol, the current measured in MLC1-overexpressing cells appeared to be a steady state current.

Figure 2. MLC1-expression related chloride currents in HEK293 and HeLa cells. (A) Current-voltage ($I$-$V$) relationships of control (black trace, n=9) and MLC1-transfected HEK293 cells (red trace, n=7). (B) $I$-$V$ relationship of control HEK293 cells (black trace, n=3) and MLC1-transfected HEK293 cells (red trace, n=4) with replacement of chloride in both bath and pipette filling solutions by equimolar amounts of gluconate. (C) $I$-$V$ relationships of control (black trace, n=4) and MLC1-transfected HeLa cells (red trace, n=8). (D) $I$-$V$ relationship of MLC1-transfected HEK293 cells (red trace, n=7) after adding 10µM Tamoxifen (light blue trace, n=4) to the bath solution. (E) The average $I$-$V$ relationship of whole-cell chloride current in control (black trace, n=4), MLC1_Ser93Leu (dark green trace, n=6) and MLC1_Cys326Arg (light green trace, n=5) expressing HEK293 cells. (F) The amplitude of the chloride current at 120 mV in control HEK293 cells (black bar, n=6), HEK293 cells overexpressing wild-type (wt) MLC1 (red bar, n=6), MLC1_Ser93Leu (dark green bar, n=6) and MLC1_Cys326Arg (light green bar, n=5). Currents measured in HEK293 cells overexpressing wild-type MLC1 were significantly different from those measured in
control cells and cells overexpressing mutant MLC1. The I-V profiles of the MLC1-transfected cells were outwardly rectifying. Statistically significant differences are indicated by asterisks (*p<0.05; **p<0.01).

HEK293 cells have a low endogenous MLC1 expression (Supplementary Table 2), which was undetectable in antibody staining (data not shown). To exclude influence of the low endogenous MLC1 expression in HEK293 cells, we also tested HeLa cells, which lack endogenous MLC1 expression (Supplementary Table 2). In HeLa cells the current profiles were highly similar to those observed in HEK293 cells (Fig. 2A and C). For practical reasons we used HEK293 cells for further experiments.

Supplementary Table 2. Expression of MLC1 mRNA

<table>
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<tr>
<th>Cell/tissue type</th>
<th>∆Ct (Ct_{human MLC1}-Ct_{human GAPDH})</th>
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<td>HEK293</td>
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<table>
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<tr>
<th>Cell</th>
<th>∆Ct (Ct_{rat MLC1}-Ct_{rat GAPDH})</th>
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<tr>
<td>Astrocyte MLC1 siRNA</td>
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</table>

a compared to expression of MLC1 mRNA in brain
b compared to expression of MLC1 mRNA in cells with non-targeted siRNA

We studied chloride currents in transfected Sf9 insect cells, which lack the MLC1 gene and therefore any possible endogenous MLC1 expression. Expression of MLC1 in Sf9 cells did not affect chloride currents under standard conditions (Fig. 3A). Because MLC1 is localized in astrocytes 4,5,20 and many chloride currents in astrocytes are volume-sensitive 21, we applied pretreatment with hypo-osmotic solutions (Fig. 1C) in transfected Sf9 cells to induce cell swelling. Hypotonic pretreatment robustly increased the currents at negative and positive voltages in MLC1-expressing Sf9 cells (Fig. 3B, Table 2). As observed in MLC1-transfected HEK293 and HeLa cells (Fig. 2A and C) the current profiles of the MLC1-transfected Sf9 cells after hypo-osmotic pretreatment also showed outward rectification with a small negative reversal current.
Figure 3. MLC1-expression related chloride currents in Sf9 cells. (A) Current-voltage (I-V) relationships of control (black trace, n=8) and MLC1-transfected Sf9 cells (red trace, n=9). (B) I-V relationship obtained after hypo-osmotic pretreatment of control (dark blue trace, n=8) and MLC1-transfected Sf9 cells (purple trace, n=12). (C) I-V relationship obtained after hypo-osmotic pretreatment of MLC1-transfected Sf9 cells (purple trace, n=12) and after adding 2µM Tamoxifen (light blue trace, n=8) to the bath solution during the hypotonic pretreatment and to the isotonic medium during recording. (D) The amplitude of the chloride current obtained after hypo-osmotic pretreatment at 100 mV in Sf9 cells overexpressing wild-type MLC1 (purple bar, n=12), in control Sf9 cells expressing GFP (blue bar, n=8), and in Sf9 cells overexpressing mutant MLC1_Ser93Leu (dark green bar, n=10) and mutant MLC1_Cys326Arg (light green bar, n=10). Currents measured in Sf9 cells overexpressing wild-type MLC1 were significantly different from those measured in control cells and cells overexpressing mutant MLC1. The I-V profiles of the MLC1-transfected cells are outwardly rectifying. Statistically significant differences are indicated by asterisks (*p<0.05; **p<0.01).

**MLC1-expression related chloride currents in primary astrocytes.**

In the brain MLC1 is predominantly expressed in astrocytes. We therefore studied chloride currents in cultured rat primary astrocytes. We chose rat astrocytes, because these are readily available and have been used before for similar patch-clamp experiments. In these cells, MLC1 was demonstrated in the cell plasma membrane and the endoplasmic reticulum (Supplementary Fig. 3). In uninfected astrocytes small-amplitude chloride currents were observed that were increased by hypo-osmotic pretreatment (data not shown). To alter expression of MLC1 we infected the astrocytes with adenoviruses expressing human HA-tagged MLC1 to increase expression or with adenoviruses expressing siRNA directed against
MLC1 to reduce expression (Supplementary Fig. 3A and G). Astrocytes infected with adenoviruses expressing respectively LacZ or siRNA without mRNA target served as controls (Supplementary Fig. 3E).

**Supplementary Figure 3. Immunostaining of MLC1 expression in rat primary astrocytes.** MLC1 expression in cultured rat astrocytes either infected with adenovirus expressing HA-tagged wild-type MLC1 (A and B), HA-tagged mutant MLC1 (Ser246Arg) (C and D), non-targeted siRNA (E and F), or siRNA against MLC1 (G and H). In panels A-D, HA-tagged human MLC1 (HA-hMLC1) expression was visualized using monoclonal anti-HA antibodies and Alexa594-coupled secondary antibodies (green). In panels E-H, endogenous rat MLC1 (rMLC1) expression was detected with anti-MLC1 antibodies and Alexa568-coupled secondary antibodies (red). The adenoviruses expressing siRNA also express GFP (green, panels F and H). In all panels nuclei were stained with DAPI (blue). Scale bars in upper panels correspond to 50 µM.

Adenovirus-infected astrocytes were less viable after hypo-osmotic treatment than uninfected cells, showed greater difficulty to seal to the patch pipette and were generally very difficult to record from. Instead of hypo-osmotic pretreatment we, therefore, used a mild treatment with trypsin, which causes morphological changes in the astrocytes and induces the same chloride currents as hypo-osmotic pretreatment. In trypsin-treated astrocytes expressing LacZ or non-targeted siRNA, the chloride current profiles were similar to those seen in MLC1-transfected Sf9 cells after hypo-osmotic pretreatment (Fig. 4A and B), although the peak current amplitudes of the astrocytes were higher.
Figure 4. MLC1-expression related chloride currents in primary rat astrocytes. (A) Chloride currents in astrocytes overexpressing human MLC1 (red trace, n=11) or LacZ as a control (black trace, n=10) after mild trypsin treatment to activate chloride currents. (B) Current-voltage (I-V) relationship of astrocytes expressing siRNAs (non-targeted siRNA as control [nt siRNA, dark blue trace, n=11] or directed against MLC1 [purple trace, n=8]) after mild trypsin treatment. (C) Amplitudes at 100 mV for astrocytes under diverse conditions: expressing non-targeted siRNA (dark blue, n=12), expressing siRNA against rat MLC1 (purple, n=14), expressing lacZ (black, n=11), overexpressing human wild-type MLC1 (red, n=11), overexpressing mutant human MLC1 [Ser246Arg] (green, n=12) or expressing siRNA against rat MLC1 and overexpressing human wild-type MLC1 together (orange, n=8). (D) Amplitudes of chloride currents at 100 mV after mild trypsin pre-treatment of astrocytes expressing HA-MLC1 either in the absence (control, red, n=16) or in the presence of chloride channel blockers (DIDS [light brown, n=5], NPPB [dark brown, n=6], Zinc [yellow, n=6] or Tamoxifen [light blue, n=6]). The average amplitude of the control cells was set as 100%. For all panels: statistically significant differences are indicated by asterisks (*p<0.05; **p<0.01).

This difference can mainly be explained by the difference in size and, thus, the membrane capacitance of the different cell types (Table 2). Note that within experiments with a certain cell type, the sizes of the cells were similar to each other (Table 2); differences in cell size therefore cannot underlie the observed changes in currents. In trypsin-treated astrocytes expressing LacZ or non-targeted siRNA, the chloride current profiles were similar to those seen in MLC1-transfected Sf9 cells after hypo-osmotic pretreatment (Fig. 4A and B), although the peak current amplitudes of the astrocytes were higher. This difference can mainly be explained by the difference in size and, thus, the membrane capacitance of the different cell types (Table 2). Note that within experiments with a certain cell type, the sizes of the cells were similar to each
Overexpression of wild-type MLC1 by infection of astrocytes resulted in a large increase in the amplitude of the currents after trypsin pretreatment compared to cells overexpressing LacZ as control (Fig. 4A-C, Table 2). The expression of the endogenous MLC1 protein was reduced by approximately 90% by siRNA directed against MLC1 (Supplementary Fig. 3E-H, Supplementary Table 2). In these silenced astrocytes chloride currents recorded after trypsin pretreatment were significantly reduced (Fig. 4B and C, Table 2). In cells with reduced MLC1 levels due to siRNA, overexpression of human wild-type MLC1 restored the reduced chloride currents to high levels (Fig. 4C, Table 2), indicating that the effects were specific to the loss of MLC1.

In these experiments with primary astrocytes the chloride current profiles showed heterogeneity (Supplementary Fig. 4), similar to previously reported findings in astrocytes. Heterogeneity was seen under all conditions (MLC1 overexpression or knock-down and in the respective controls). The different activation and inactivation characteristics are most likely explained by the presence of other chloride channels.

Supplementary Figure 4. Heterogeneity of MLC1-induced current profiles in primary astrocytes. This figure illustrates whole-cell chloride currents (A) with a delayed activation, most apparent at depolarized potentials, (B) with instantaneous activation, and (C) with instantaneous activation with depolarization-dependent inactivation, most evident at 100 mV. Variability in the kinetics of current activation and inactivation were independent of changes in MLC1 expression.

Potassium and chloride are the most important ions in the regulation of water homeostasis by astrocytes. We therefore tested whether potassium currents would be influenced by MLC1 in astrocytes. Potassium currents were not affected by increased or decreased expression of MLC1 (Supplementary Fig. 5), showing that the currents observed are not caused by side effects of the altered MLC1 expression on, for example, voltage-gated potassium channels.
Supplementary Figure 5. Rectifier potassium currents in primary astrocytes.

(A) Typical whole-cell potassium currents were recorded in physiological intra- and extracellular saline with a ramp protocol in cultured astrocytes expressing non-targeted siRNA (nt siRNA), MLC1 siRNA, lacZ or wild-type MLC1. (B) Rectifier potassium currents elicited by a voltage-step protocol. (C) Quantitative analysis of current densities at −120 and +60 mV of cultured astrocytes expressing non-targeted siRNA (blue, n=11), MLC1 siRNA (purple, n=8), lacZ (black, n=10) or wild-type MLC1 (red, n=13). The expression of non-targeted siRNA, siRNA directed against MLC1 or increased expression of wild-type MLC1 did not cause significant changes in potassium currents at negative or positive voltages as compared to infected cells expressing LacZ.

MLC1-expression related chloride currents in lymphoblasts.
MLC1 is expressed in the brain and in white blood cells. White blood cells are much easier to obtain from human patients and control subjects than astrocytes. We used human lymphoblasts endogenously expressing MLC1 for further studies. In control lymphoblasts, in which chloride currents were small under normal conditions, hypotonic pretreatment increased the currents at negative and positive voltages (Fig. 5A, Table 2) with current profiles similar to the profiles observed in MLC1-transfected Sf9 cells after hypotonic pretreatment (Fig. 3B).
Figure 5. Chloride currents in lymphoblasts. (A) Current-voltage (I-V) relationship of control lymphoblasts obtained after iso- (red trace, n=13) and hypo-osmotic (purple trace, n=9) pretreatment. (B) I-V relationship of control lymphoblasts obtained after hypo-osmotic pretreatment (purple trace, n=9) and after adding 10µM Tamoxifen (light blue trace, n=5) to the bath solution during the hypotonic pretreatment and to the isotonic medium during recording. (C) I-V relationship of lymphoblasts derived from patient 1 obtained after iso- (black trace, n=9) and hypo-osmotic (dark green trace, n=7) pretreatment. (D) I-V relationship of lymphoblasts derived from patient 2 obtained after iso- (black trace, n=10) and hypo-osmotic (light green trace, n=9) pretreatment. (E) The amplitude of the chloride currents obtained at 120 mV for control and patient lymphoblasts after iso- and hypotonic pretreatment and in the absence or presence of Tamoxifen, as indicated. Statistically significant differences with control cells and conditions (red) are indicated by asterisks (*p<0.05; **p<0.01).
**Effect of ion channel blockers.**
Tamoxifen is known to block volume-regulated anion channels (VRACs)\(^{15}\). Added to the bath solution it strongly reduced the chloride currents in MLC1-transfected HEK293 cells (Fig. 2D, Table 2). Tamoxifen also strongly inhibited the currents induced by hypotonic conditions in MLC1-transfected Sf9 cells (Fig. 3C), astrocytes overexpressing MLC1 (Fig. 4D) and control lymphoblasts (Fig. 5B). In all cases currents were reduced approximately 80% at 100 mV (Table 2).

The rat astrocytes expressing human MLC1 were also used to test other ion channel blockers, i.e. Zinc, NPPB, and DIDS\(^{15,16}\). All blockers significantly reduced the chloride currents (Fig. 4D, Table 2).

**MLC1 mutations abolish chloride currents**
We introduced missense mutations, which have been observed in a homozygous state in patients, in MLC1 expression constructs\(^{10}\). The chloride currents observed in cells expressing wild-type MLC1 were absent in HEK293 cells expressing MLC1_Ser93Leu or MLC1_Cys326Arg (Fig. 2E and F), and after hypotonic pretreatment in Sf9 cells expressing MLC1_Ser93Leu or MLC1_Cys326Arg (Fig. 3D) and rat astrocytes expressing MLC1_Ser246Arg (Fig. 4C).

Lymphoblasts were obtained from MLC1 patients, who were either homozygous for an insertion resulting in a frameshift and a premature stop or homozygous for a missense mutation (Table 1). In both lymphoblast lines hypo-osmotic pretreatment failed to induce chloride currents (Fig. 5C-E).
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pF, picoFarad; pA, picoAmpere; mV, milliVolt; GFP, green fluorescent protein; wt, wild-type; nt siRNA, non-targeted silencing RNA; iso, iso-osmotic treatment before electrophysiological measurements; hypo, hypo-osmotic treatment before electrophysiological measurements; trypsin, mild trypsinization before electrophysiological measurements; tmx, Tamoxifen
Chapter 2

Volume regulation is impaired in patient-derived lymphoblasts and MLC1-silenced astrocytes.

We studied whether the failure to induce chloride currents in lymphoblasts of patients and the reduced chloride currents in MLC1-silenced astrocytes upon hypotonic shock was associated with an impaired regulatory volume decrease that follows hypotonia-induced cell swelling. Lymphoblasts and astrocytes were exposed to the chloride-selective hypotonic solution in the presence of gramicidin. Gramicidin forms pores for monovalent cations. With that, it allows free exchange of mainly potassium, excluding this ion from playing a role in the regulatory volume decrease. Cell volume recovery was significantly slower (p=<0.001) in lymphoblasts from patients 1 and 2 (Table 1) than in the three control lymphoblast lines (Fig. 6F). The same trend of decreased rate of cell volume recovery was observed (p=0.067) in MLC1-silenced astrocytes (Fig. 6B-D and F). Although we observed a trend towards reduced maximum swelling after exposure to the hypotonic solution in MLC1-silenced astrocytes compared to control astrocytes (p=0.056) (Fig. 6B), we did not observe a difference in maximum swelling between patient-derived and control lymphoblasts (p<0.001; Fig. 6A). The normal cell volume recovery was restored in MLC1-silenced astrocytes by overexpression of human wild-type MLC1 (Fig. 6B, C, E and F). After reintroduction of chloride selective isotonic solution, regulatory volume increase was nearly absent in all lymphoblasts and astrocytes (Fig. 6A and B). Regulatory volume increase is highly dependent on influx of monovalent cations, which were absent in our solutions.
Figure 6. MLC1-dependent regulatory volume decrease. Cells were bathed in a chloride selective iso-osmotic solution that was switched to a hypo-osmotic chloride selective solution for 30 minutes as indicated in A–E (black bar). Gramicidin was used in all experiments. (A) Average relative cell surface, obtained under iso- and hypo-osmotic conditions, from lymphoblasts of three controls (control 1, red trace, n=34; control 2, dark grey, n=14; and control 3, black trace n=15) and two MLC patients (patient 1, dark green trace, n=39; patient 2, light green trace n=15). (B) Average relative fluorescence, obtained under iso- and hypo-osmotic conditions, from astrocytes expressing non-targeted siRNA (dark blue, n=11), siRNA against rat MLC1 (purple, n=9) or siRNA against rat MLC1 in combination with expression of human wild-type MLC1 (orange, n=7). Individual typical regulatory volume decrease fluorescence quenching traces fitted with an exponential curve are depicted for an astrocyte expressing non-targeted siRNA (C), an astrocyte expressing siRNA against rat MLC1 (D) and an astrocyte expressing siRNA against rat MLC1 in combination with expression of human wild-type MLC1 (E). (F) Average regulatory volume decrease decay time constants of all lymphoblasts used for panel A (control 1, red; control 2, grey; control 3, black; patient 1, dark green; patient 2, light green) are indicated on the left. Average regulatory volume decrease decay time constants of all astrocytes used for panel B (non-targeted siRNA, dark blue; siRNA against rat MLC1, purple; siRNA against rat MLC1 in combination with expression of human wild-type MLC1, orange) are indicated on the right. Statistically significant differences are indicated by asterisks (**p<0.01).
Discussion

MLC patients are normal at birth and develop macrocephaly during the first year of life. MRI at that time reveals abnormal and swollen cerebral white matter with highly increased water content. Electron microscopy of brain tissue from a MLC patient demonstrates that fluid-filled vacuoles within myelin sheaths and, to a lesser extent, astrocytic endfeet form the anatomic substrate of the white matter edema. Cerebral white matter contains little myelin at birth and acquires most of its myelin during the first year of life. Thus, the macrocephaly and neuroradiological characteristics of MLC develop during a period of rapid myelin deposition, suggesting that the vacuoles are formed during this process. We have shown previously that MLC1 mutations cause MLC. The MLC1 protein is located mainly in the brain in astrocyte-astrocyte junctions adjacent to the blood-brain and cerebrospinal fluid-brain barriers. Its function has remained unknown.

We demonstrate an association between MLC1 expression and chloride currents in different cell types, most importantly in patient-derived lymphoblasts and astrocytes, the cells that normally mainly express the MLC1 protein. Previous negative results to find ion channel activity related to MLC1 may be due to the inability to identify the opening conditions of the channel or to the specific experimental conditions. The ion substitution experiments, current-voltage profile, the activation by hypo-osmotic pretreatment causing cell swelling, and the sensitivity to inhibition by Tamoxifen and other ion channel blockers indicate that the increase in current amplitude induced by MLC1 expression is due to increased volume-regulated anion channel (VRAC) activity. Water homeostasis and osmotic balance are vital in the brain and astrocytes are central in this process. They are highly sensitive to changes in extracellular osmolarity and can display prominent cell volume changes as part of the osmoregulatory process. Any physiological or pathological osmotic perturbation induces a transmembrane flow of ions and water that rapidly restores the osmotic equilibrium and induces temporary swelling or shrinkage of cells, followed by regulatory volume decrease or regulatory volume increase, respectively, to normalize cell volume. The regulatory volume decrease is caused by activation of ion channels and transporters that allow effluxes of potassium, chloride, organic osmolytes, and water. VRACs, which are activated by water fluxes, changes in cell shape and signal transduction events, play an important role in the regulatory volume decrease. VRAC function is most likely dependent on multiple channels, which could be different for different cell types. The molecular identity of most of these channels is unknown. Our study with patient-derived lymphoblasts shows that a defect in MLC1 not only decreases VRAC-related chloride currents but also the rate of the regulatory volume decrease. Similar findings were obtained from MLC1-silenced astrocytes, with the addition of a rescue experiment with human wild-type MLC1.
The variation in the rate of the regulatory volume decrease was larger in MLC1-silenced astrocytes than in patient lymphoblasts. Consequently, the p-value for the difference between MLC1-silenced and wild-type astrocytes was higher than for the difference between patient and control lymphoblasts. The larger variation in astrocytes is most likely due to variation in the degree of MLC1 knockdown in MLC1-silenced astrocytes, a problem not shared by patient lymphoblasts. As such, patient-derived lymphoblasts are a more consistent system to study effects of mutant or absent MLC1. The difference in degree of swelling of MLC1-silenced and wild-type astrocytes after hypotonic exposure was not observed in patient versus control lymphoblasts. Currently we have no explanation for this observation in astrocytes.

We performed our experiments in the presence of gramicidin and used chloride selective medium, both to enhance the experiments to be dependent on chloride. Yet, with this experimental set-up the regulatory volume decrease is not exclusively dependent on the MLC1-related chloride channel function; it depends in part on the efflux of organic osmolytes and on other chloride channels, not related to MLC1. This explains why the regulatory volume decrease is slower and not abolished in the absence of normal MLC1. MLC is a not immediately life-threatening disease. Complete abolition of the regulatory volume decrease is probably not compatible with life. At present it is still an open question whether MLC1 is a VRAC, a component of a VRAC or a protein that activates a VRAC, either directly or indirectly.

Recessive MLC1 mutations are responsible for approximately 75% of the MLC patients. We have recently shown that mutations in GLIALCAM, coding for hepatic and glial cell adhesion molecule GlialCAM, also cause MLC. Patients with recessive GLIALCAM mutations have classical MLC, while patients with dominant GLIALCAM mutations show transient clinical and MRI features of MLC. GlialCAM is an immunoglobulin-like cell adhesion molecule that is required for the proper localization of MLC1 in astrocytes-astrocyte junctions. All types of MLC1 mutations cause decreased membrane expression of the MLC1 protein and GLIALCAM mutations disrupt the localization of MLC1, which explains that MLC1 and GLIALCAM mutations cause the same disease. Why patients with dominant GLIALCAM mutations have transient features of MLC remains to be explained. In any case, this observation suggests that MLC1 has its most important role during the process of myelin deposition. This conclusion is in line with another observation that all myelin producing organisms have the MLC1 gene, whereas organisms that do not produce myelin do not.

Brain ion channel defects have been associated with epilepsy syndromes, migraine, dyskinesia and episodic ataxia. There is no known human leukoencephalopathy related to ion channel dysfunction. There is also no known human disease caused by a defect in a chloride channel involved in cell volume regulation. The CLCN2-knockout mouse, however, has a leukoencephalopathy. CLCN2 encodes the chloride channel CIC-2, which, like MLC1, is
localized in the brain in astrocytic endfeet lining blood vessels. Remarkably, CLCN2-knockout mice display widespread cerebral white matter oedema and intramyelinic vacuole formation, similar to what is seen in the brain of human MLC patients. Because of the striking similarities between MLC patients and the CLCN2-knockout mice, CLCN2 was investigated as a possible candidate gene for MLC, but CLCN2 mutations were not found among MLC patients without MLC1 mutations. CIC-2 has a more widespread expression than MLC1 and CLCN2-knockout mice have involvement of other organs than the brain. MLC1 is only present in leukocytes and the brain, but patients only have a brain disease. This exclusive brain involvement is most likely explained by the much higher MLC1 expression in the brain than in leukocytes (Supplementary Table 2).

MLC is characterized by chronic cerebral white matter oedema and slow neurological deterioration. The fact that the CLCN2-knockout mice also display widespread cerebral white matter oedema and intramyelinic vacuole formation supports our conclusion that a disturbance of volume-regulated chloride channel activity underlies the white matter disease in MLC. At this point we do not know how a defect in cell volume regulation leads to water accumulation within astrocytic endfeet and myelin sheaths during myelination and to delayed onset neurological deterioration. A better understanding of disease mechanisms is essential for the development of treatment. The transient MLC phenotype of the patients with a dominant GLIALCAM mutation suggests that there is a window of time, in which rescue of MLC1 function may prevent the disease or modify its course.

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