

# TRPM2 Cation Channels Modulate T Cell Effector Functions and Contribute to Autoimmune CNS Inflammation

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## Abstract

TRPM2, a highly  $\text{Ca}^{2+}$ -permeable member of the transient receptor potential melastatin-related (TRPM) family of cation channels, is expressed in cells of the immune system. We demonstrate firstly that TRPM2 cation channels on T cells critically influence T cell proliferation and proinflammatory cytokine secretion following polyclonal T cell receptor stimulation. Consistently, *trpm2*-deficient mice exhibited an attenuated clinical phenotype of experimental autoimmune encephalomyelitis (EAE) with reduced inflammatory and demyelinating spinal cord lesions. Importantly, *trpm2*-deficient T cells were as susceptible as wildtype T cells to oxidative stress-induced cell death as it occurs in inflammatory CNS lesions. This supports the notion that the attenuated EAE phenotype is mainly due to reduced T cell effector functions but unaffected by potential modulation of T cell survival at the site of inflammation. Our findings suggest TRPM2 cation channels as a potential target for treating autoimmune CNS inflammation.

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## Introduction

Activation, migration and exertion of effector mechanisms of immune cells critically depend on receptor-operated  $\text{Ca}^{2+}$ -entry from the extracellular space. This takes place via cross-linking of different cell-surface receptors, followed by  $\text{Ca}^{2+}$  release from intracellular stores via different  $\text{Ca}^{2+}$  release channels and subsequent  $\text{Ca}^{2+}$  entry through store-operated plasma membrane  $\text{Ca}^{2+}$  channels [1,2].

However, receptor-operated  $\text{Ca}^{2+}$ -entry in immune cells may also occur through non-store operated  $\text{Ca}^{2+}$ -permeable channels [3]. TRPM2 is a highly  $\text{Ca}^{2+}$ -permeable member of the transient receptor potential melastatin-related (TRPM) family of cation channels [4], that is – among others – expressed in various cell types of the innate (dendritic cells [5], monocytes/macrophages [6,7,8], CNS microglia [5]) and adaptive (T cells [6,7,8] and B cells [9]) immune system. Moreover, functional expression of TRPM2 channels was also demonstrated in CNS neurons [10,11,12,13,14].

TRPM2 channels are gated by adenosine diphosphate ribose (ADPR) binding to a c-terminal Nudix-like region with pyrophosphatase activity that cleaves ADPR to ribose 5-phosphate and adenosine monophosphate (AMP) [15]. Under physiologic conditions, ADPR predominantly originates from hydrolysis of nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) by various plasma membrane-associated and cytosolic glycohydrolases as well as a mitochondrial NADase [16,17]. Moreover, two other  $\text{NAD}^+$ -derived molecules, nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic

ADPR (cADPR), together with  $\text{Ca}^{2+}$  function as co-agonists facilitating the activation of TRPM2 channels by ADPR [10,11,16].

In T cells, cross-linking of cell surface receptors induces a rise of ADPR endogenously generated from  $\text{NAD}^+$  followed by a TRPM2-mediated transmembrane inward current and rise of the intracellular  $\text{Ca}^{2+}$  concentration [7]. Moreover, cADPR and NAADP were shown to be capable of eliciting TRPM2-mediated transmembrane currents in T cells either by directly binding to the channel or after metabolic conversion to ADPR [6]. Pharmacological inhibition of NAADP signalling in T cells [18] reduced antigen-induced proliferation and cytokine production and ameliorated clinical symptoms of experimental autoimmune encephalomyelitis (EAE, [18]).

In dendritic cells, TRPM2 functions as intracellular  $\text{Ca}^{2+}$  release channel and is critically involved in differentiation and migration [19].

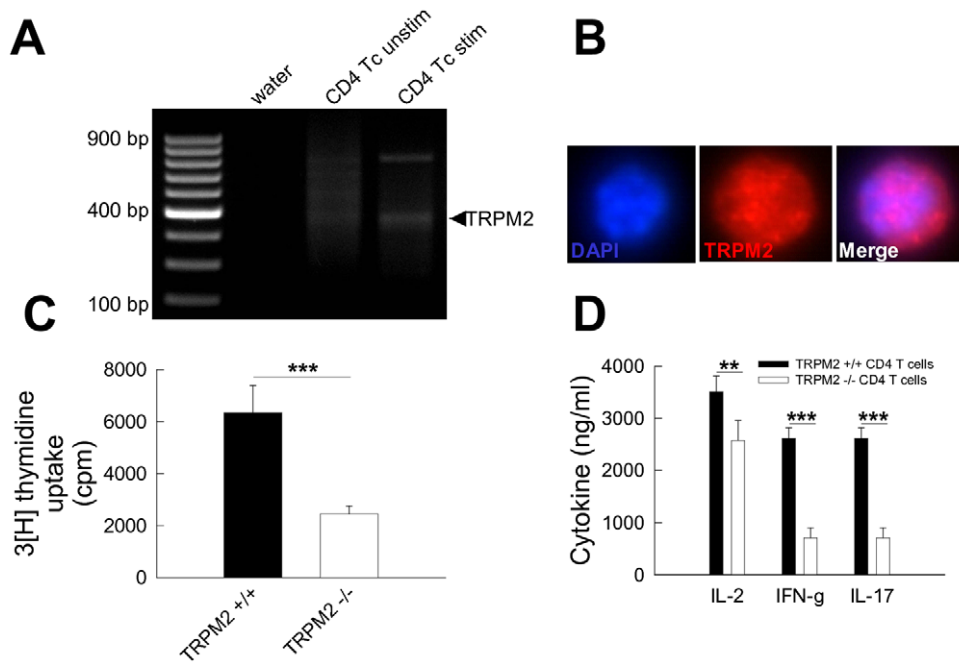
In CNS neurons, intracellular  $\text{Ca}^{2+}$  overload and depolarization of the membrane potential following activation of TRPM2 channels have been implicated in neuronal cell death during oxidative and nitrosative stress [11,14].

Here, we study the role of TRPM2 channels in T cell effector functions and autoimmune CNS inflammation using a combination of in vitro approaches and EAE experiments in wildtype and *trpm2*-deficient mice.

## Materials and Methods

### Mice

Wildtype and *trpm2*-deficient mice both on a mixed 129/SvJ × C57BL/6J genetic background [20] were kindly provided by Dr.



**Figure 1. TRPM2 cation channels are upregulated in CD4<sup>+</sup> T cells upon polyclonal T cell receptor stimulation and critically determine proliferation and proinflammatory cytokine secretion.** (A) RT-PCR performed with naïve (middle) and anti-CD3/CD28 bead-stimulated (right) wildtype CD4<sup>+</sup> T cells using murine TRPM2-specific primers revealed upregulation of TRPM2 expression upon stimulation. As negative control water (left) instead of cDNA was used. An experiment representative of 3 independent trials is shown. (B) Immunocytochemistry using an antibody specific for the c-terminal portion of the TRPM2 protein (red) revealed positive staining in activated bead-stimulated CD4<sup>+</sup> T cells. Cell nuclei were counterstained with DAPI (blue). (C, D) Proliferation as determined by <sup>3</sup>[H]-thymidine uptake (C) and secretion of proinflammatory IL-2, IFN- $\gamma$  and IL-17 determined by ELISA from the supernatants (D) were significantly reduced in CD4<sup>+</sup> T cells from *trpm2*-deficient (TRPM2 -/-) as compared to wildtype (TRPM2 +/+) mice following anti-CD3/CD28 bead stimulation at a bead:cell ratio of 1:4. Moreover, the TRPM2 channel blocker ACA dose-dependently reduced proliferation (filled circles) of bead-stimulated splenocytes (E, bead:cell ratio 1:4) without overt toxic effects on cell viability as assessed using flow cytometry for annexin V and propidium iodide (open circles). ACA at a concentration of 100  $\mu$ M significantly reduced IFN- $\gamma$  secretion of bead-stimulated splenocytes (F, bead:cell ratio 1:4; n = 3 for all experiments) P-values  $\leq 0.05$  were considered significant (\*). P-values  $\leq 0.01$  and  $\leq 0.001$  were considered highly significant (\*\* and \*\*\*, respectively). doi:10.1371/journal.pone.0047617.g001

Y. Mori, Kyoto University, Japan. Mice were kept under pathogen-free conditions and had access to food and water ad libitum. All experiments were conducted according to the German law of animal protection and were approved by local authorities (TVA-Nr. 87–51.04.2010. A325, University of Münster, Germany).

#### Isolation and culture of splenocytes and T cells

Isolation and culture of splenocytes was performed as described [21]. CD4<sup>+</sup> T cells were purified from isolated splenocytes using the respective MACS T cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) according to manufacturer's instructions and cultured as described [21].

#### T cell stimulation, proliferation and cytokine secretion

Splenocytes and purified CD4<sup>+</sup> T cells from non-immunized mice were stimulated with anti-CD3/CD28 beads (DynaL Biotech, Oslo, Norway) for 72 h at various bead-to-cell ratios. For T cell proliferation analysis, [methyl-<sup>3</sup>H]-thymidine (1.0  $\mu$ Ci/well; Amersham Biosciences, Buckinghamshire, UK) was added 24 h before the end of incubation. Cells were harvested and [methyl-<sup>3</sup>H]-thymidine incorporation was measured using a liquid scintillation counter (Packard BioScience, Meriden, USA). For analysis of cytokine secretion, supernatants were analyzed for IL2, IFN $\gamma$ , and IL17 by ELISA according to the manufacturer's protocol. All experiments were performed as triplicates.

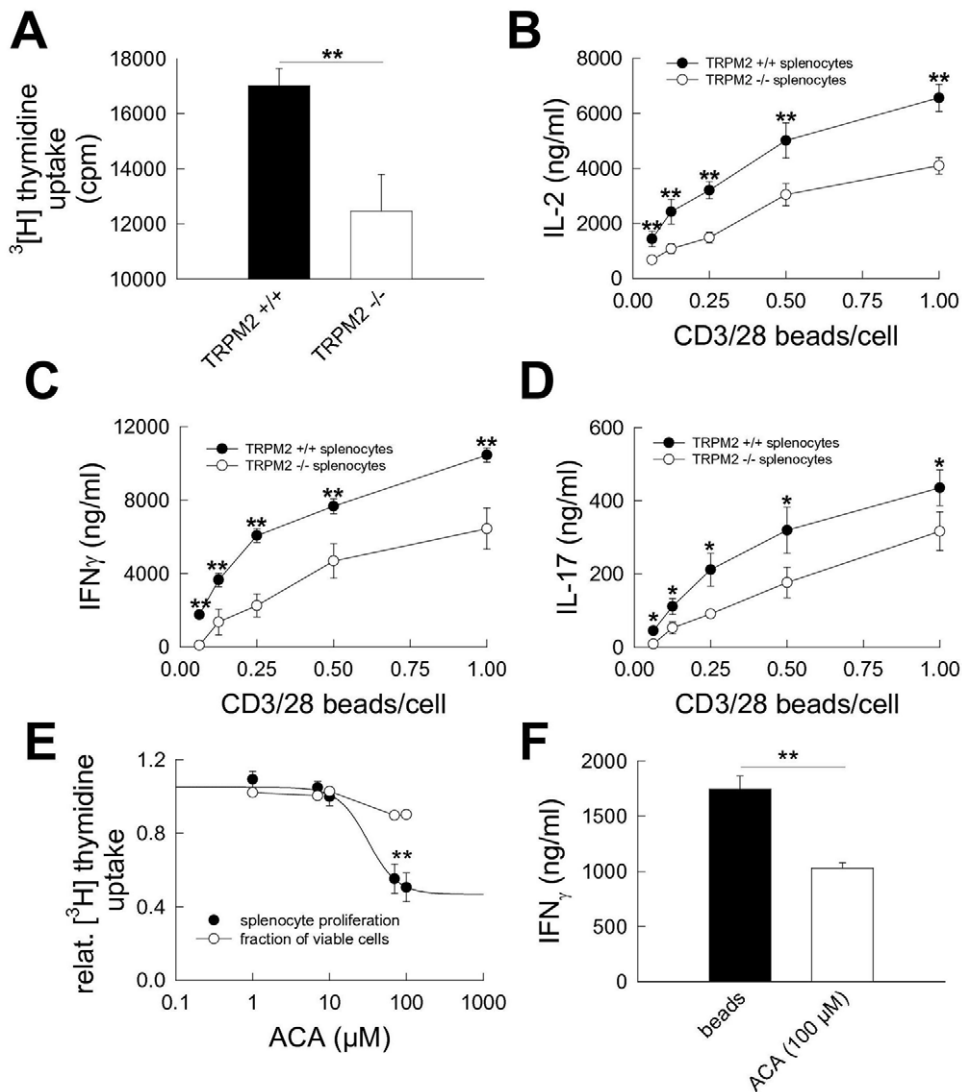
In a subset of experiments, the TRPM2 channel blocker N-(p-amylocinnamoyl)anthranilic acid (ACA; Sigma) [22,23] was dissolved in dimethylsulfoxide (DMSO) giving stock solutions of 50 mM. In final solutions, the concentrations of DMSO did not exceed 0.2%, and final DMSO concentrations without ACA served as controls (data not shown).

#### RNA preparation and RT-PCR

RNA was purified from naïve and bead-stimulated wildtype CD4<sup>+</sup> T cells by extraction with peqGold TriFast reagent according to the manufacturer's instructions (peqLab, Erlangen, Germany). cDNA was synthesized from 1  $\mu$ g of the total RNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany). PCR amplification was performed using TRPM2-specific primers under the following conditions: 1 cycle at 94°C for 2 min; 35 or 40 cycles at 94°C for 10 s, 55°C for 10 s, and 72°C for 30 s; and 1 cycle at 72°C for 2 min as described [24]. As negative control water instead of cDNA was used.

#### Immunocytochemistry

Immunocytochemical staining was performed on naïve and bead-stimulated wildtype CD4<sup>+</sup> T cells as described [25]. Cells were placed on coverslips coated with poly-L-lysine (Sigma, Germany) and fixed with 4% paraformaldehyde. Subsequently, cells were blocked with PBS containing 10% horse serum (PAA Laboratories, Cölbe,



**Figure 2. TRPM2 cation channels critically determine proliferation and proinflammatory cytokine secretion following various strengths of polyclonal T cell receptor triggering.** (A–D) Proliferation as determined by  $^3\text{H}$ -thymidine uptake (A; bead:cell ratio of 1:4) and secretion of proinflammatory IL-2 (B), IFN- $\gamma$  (C) and IL-17 (D) determined by ELISA from the supernatants were significantly reduced in splenocytes from *trpm2*-deficient mice as compared to wildtype mice following anti-CD3/CD28 bead-stimulation for 3 days at various bead:cell ratios (1:1 to 1:16;  $n = 3$  for all experiments). P-values  $\leq 0.05$  were considered significant (\*). P-values  $\leq 0.01$  and  $\leq 0.001$  were considered highly significant (\*\* and \*\*\*, respectively).

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Germany), 2% bovine serum albumin, and 0.3% Triton X-100 overnight. Next, the primary antibody (rabbit anti-mouse TRPM2, 1:200, Novus Biologicals, USA) were added and incubated for 1 h. Cells were washed with PBS containing 0.3% Triton X-100 and incubated with secondary antibodies (Cy3-conjugated rabbit anti-goat, 1:100, Dianova, USA) for another 1 h. Counterstaining of cell nuclei was performed using DAPI (0.5  $\mu\text{g}/\text{ml}$ , Merck). Pictures were collected by immunofluorescence microscopy (Axiophot, Zeiss, Jena, Germany). Negative controls without the primary antibody revealed no positive signals (data not shown).

#### T cell death analysis

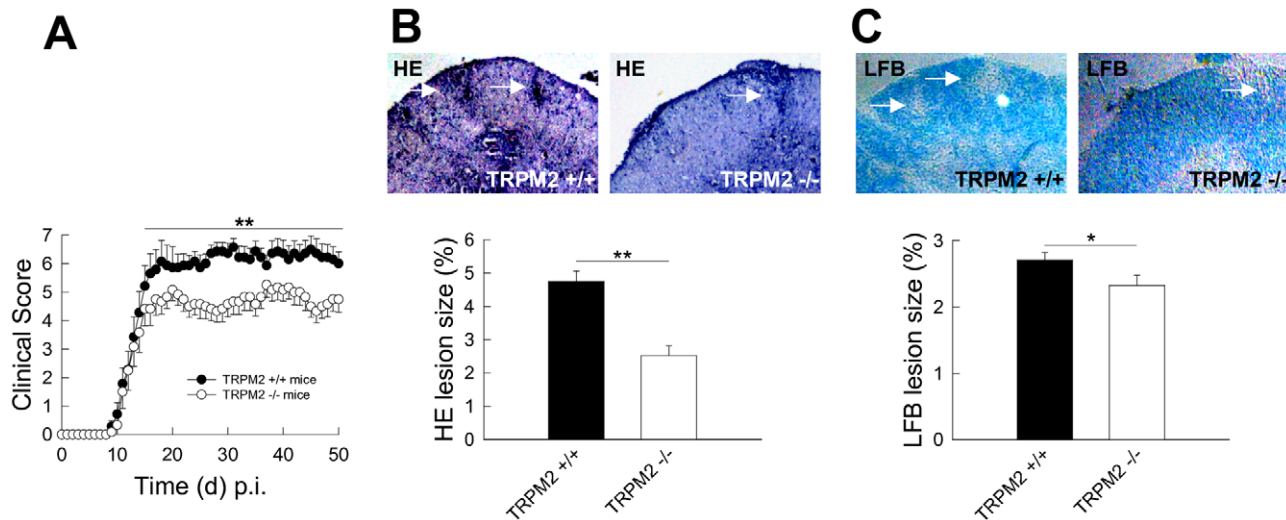
For analysis of  $\text{H}_2\text{O}_2$  induced cell death, 50 000 bead-stimulated  $\text{CD4}^+$  T cells/well were cultured on white 96-well microassay plates (Greiner Bio-One, Frickenhausen, Germany) and incubated for 6 h with different concentrations of  $\text{H}_2\text{O}_2$ .

Afterwards, the amount of ATP in the supernatant following cell lysis was assessed as a parameter of cell viability using the ATPLite<sup>TM</sup> Luminescence Assay System (PerkinElmer, Rodgau-Jügesheim, Germany) according to the manufacturer's instructions. Luminescence was measured on a Topcount NXT (PerkinElmer, Rodgau, Germany). All experiments were performed as triplicates.

In a subset of experiments, cell death analysis was performed using flow cytometry for annexin V and propidium iodide. The viable cell fraction was considered to represent the fraction of annexin V/propidium iodide double-negative cells as described [21].

#### Experimental autoimmune encephalomyelitis (EAE)

Induction and clinical evaluation of myelin oligodendrocyte glycoprotein (MOG<sub>35–55</sub>) peptide-induced EAE was performed as described [21].

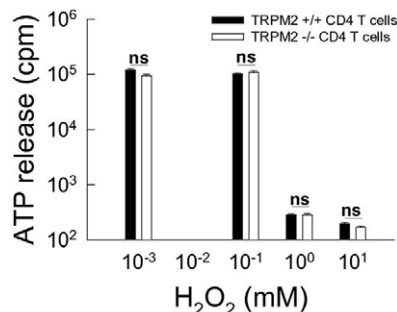


**Figure 3. Trpm2-deficient mice exhibit an ameliorated EAE phenotype with reduced inflammatory CNS infiltrates and demyelination.** (A) Clinical MOG<sub>35–55</sub> peptide-induced EAE disease score in wildtype and trpm2-deficient mice was examined for 50 days post-immunization (p.i.) and revealed a significantly reduced peak- and residual score in trpm2-deficient as compared to wildtype mice (n = 3 independent trials with 10 mice each for both experimental groups). (B, C) Representative H&E (B) and LFB (C) staining (upper panels) of spinal cord sections from wildtype (left) and trpm2-deficient (right) mice obtained at day 20 post-immunization and quantification of respective lesion sizes (lower panels; n = 2 mice from each of the 3 independent trials representing the mean clinical score of the experimental group at day 20 post-immunization, white arrows indicate lesions). P-values  $\leq 0.05$  were considered significant (\*). P-values  $\leq 0.01$  and  $\leq 0.001$  were considered highly significant (\*\* and \*\*\*, respectively).

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### Histopathology

On day 20 of the EAE experiments, mice were sacrificed and transcardially perfused with phosphate-buffered saline (PBS). Spinal cords were carefully excised from the brainstem to lumbar region, fixed in 4% paraformaldehyde (PFA) in PBS for 2 h and then embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, USA). Ensuring that the same regions were analysed for all mice, 10- $\mu$ m cross sections of the beginning of the cervical enlargement were cut [26]. H&E and Luxol fast blue (LFB) stainings were performed on paraformaldehyde-fixed spinal cord cryosections according to standard procedures [2]. Spinal cord inflammatory infiltrates and demyelination were assessed by quantifying the respective lesion area relative to the total section area in 100 H&E and LFB stained sections randomly chosen from the cervical and lumbar part of the spinal cord, respectively.



**Figure 4. TRPM2 channels do not impact survival of activated CD4<sup>+</sup> T cells under altered redox conditions.** Survival of activated bead-stimulated CD4<sup>+</sup> T cells (bead:cell ratio of 1:4) from wildtype and trpm2-deficient mice under various concentrations of H<sub>2</sub>O<sub>2</sub> (0.001–10 mM) using the amount of ATP released following cell lysis as a parameter of cell viability (n = 3; ns = not significant).

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### Statistical analysis

All results are presented as mean  $\pm$  sem. Statistical analysis was performed using the student's t-test for normally distributed datasets or a Wilcoxon rank sum test for non-normally distributed datasets. A one-way ANOVA was used in case of multiple comparisons using SigmaPlot 11.0 software (Sigma Plot). P-values  $\leq 0.05$  were considered significant (\*). P-values  $\leq 0.01$  and  $\leq 0.001$  were considered highly significant (\*\* and \*\*\*, respectively).

### Results

#### TRPM2 channel expression is upregulated in CD4<sup>+</sup> T cells following polyclonal T cell receptor stimulation *in vitro*

To assess the expression and regulation of TRPM2 cation channels, isolated naïve murine CD4<sup>+</sup> T cells were polyclonally stimulated for 3 days *in vitro* using anti-CD3/CD28-coated beads at a bead:cell ratio of 1:4. RT-PCR revealed an upregulation of TRPM2 message following bead-stimulation after 3 days (Fig. 1 A). Moreover, immunocytochemistry using an antibody specific for the c-terminal portion of the TRPM2 protein revealed positive staining in activated bead-stimulated CD4<sup>+</sup> T cells (Fig. 1 B).

#### CD4<sup>+</sup> T cells from TRPM2-deficient mice show reduced proliferation and proinflammatory cytokine secretion upon polyclonal T cell receptor stimulation *in vitro*

To assess the impact of TRPM2 channels on T cell effector functions, we first determined proliferation and secretion of various proinflammatory cytokines in CD4<sup>+</sup> T cells isolated from wildtype and trpm2-deficient mice following bead-stimulation for 3 days at a fixed bead:cell ratio of 1:4. CD4<sup>+</sup> T cells from trpm2-deficient mice showed significantly reduced proliferation (Fig. 1 C) and secretion of different effector cytokines such as IL-2, IFN- $\gamma$ , and IL-17 (Fig. 1 D) as compared to those from wildtype mice.

To assess this effect in the whole population of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, we measured proliferation and cytokine secretion in

splenocytes from wildtype and *trpm2*-deficient mice following bead-stimulation for 3 days. To mimick different strengths of T cell receptor triggering, the bead:cell ratio was varied between of 1:1 and 1:16 during these experiments. Again, splenocytes from *trpm2*-deficient mice displayed significantly reduced proliferation (**Fig. 2 A**, bead:cell ratio 1:4) and secretion of IL-2 (**Fig. 2 B**), IFN- $\gamma$  (**Fig. 2 C**), and IL-17 (**Fig. 2 D**) as compared to those from wildtype mice under all experimental conditions.

The TRPM2 channel blocker N-(p-aminocinnamoyl)anthranilic acid (ACA) is known to inhibit TRPM2 channel activity at concentrations between 10 and 100  $\mu$ M [22,23]. Hence, we aimed to reproduce the effect of TRPM2-deficiency on T cell effector functions using different concentrations of ACA. Indeed, ACA dose-dependently reduced proliferation of bead-stimulated splenocytes (**Fig. 1. E**, bead:cell ratio 1:4) without overt toxic effects on cell viability as assed using flow cytometry for annexin V and propidium iodide. Consistently, ACA at a concentration of 100  $\mu$ M significantly reduced IFN- $\gamma$  secretion of bead-stimulated splenocytes (**Fig. 1. F**, bead:cell ratio 1:4). Thus, genetic deficiency and pharmacological inhibition of TRPM2 channels dampened proliferation and proinflammatory cytokine secretion in splenocytes following polyclonal T cell receptor stimulation *in vitro*.

### Trpm2-deficient mice exhibit an ameliorated EAE phenotype with reduced inflammatory CNS infiltrates and demyelination *in vivo*

Given these effects of TRPM2 channels on T cell effector function, we performed MOG<sub>35–55</sub>-peptide induced EAE experiments comparing wildtype and *trpm2*-deficient mice (**Fig. 3 A–C**). As compared to wildtype mice, *trpm2*-deficient mice displayed an attenuated EAE phenotype: although the mean time until disease onset was identical between wildtype and *trpm2*-deficient mice (9 days vs. 9 days), *trpm2*-deficient mice displayed a delayed time to peak disease (20 days vs. 18 days). Moreover, *trpm2*-deficient mice displayed significantly reduced peak- and residual disease scores (**Fig. 3 A**).

Consistent with the ameliorated clinical phenotype, H&E (**Fig. 3 B**) and LFB (**Fig. 3 C**) staining of spinal cord sections from EAE mice representative of the mean clinical score of the respective experimental group at day 20 post-immunization displayed reduced inflammation (**Fig. 3 B**) and demyelination (**Fig. 3 C**) in the spinal cord of *trpm2*-deficient as compared to wildtype mice.

### TRPM2 channels do not impact the survival of activated CD4<sup>+</sup> T cells under altered redox conditions

Given the potential role of TRPM2 channels in cell death of T cells in an inflammatory CNS lesion due to its activation by reactive oxygen species [27] we tested survival of activated bead-stimulated CD4<sup>+</sup> T cells from wildtype and *trpm2*-deficient mice under various concentrations of H<sub>2</sub>O<sub>2</sub> (0.001–10 mM; **Fig. 4**) using the amount of ATP released following cell lysis as a parameter of cell viability. Activated CD4<sup>+</sup> T cell viability did not differ between wildtype and *trpm2*-deficient mice under all experimental conditions refuting a role of TRPM2 channels in T cell death under altered redox conditions.

## Discussion

Taken together, we firstly demonstrate here a role for TRPM2 cation channels in T cell proliferation and proinflammatory cytokine secretion following polyclonal T cell receptor stimulation.

The effects of TRPM2-deficiency on T cell effector function could be mimicked by application of the TRPM2 channel blocker N-(p-aminocinnamoyl)anthranilic acid (ACA; [22,23]) providing an opportunity for pharmacological modulation of TRPM2 channel function in T cells. Moreover, TRPM2 channels have been shown to dramatically impact the maturation and chemokine-activated directional migration of dendritic cells, which function as antigen-presenting cells [19]. Both findings, strongly imply an attenuated clinical EAE phenotype with reduced inflammatory and demyelinating spinal cord lesions in *trpm2*-deficient mice as observed.

Moreover, TRPM2 expression within the CNS is in large parts attributed to microglia, the CNS resident monocyte-lineage derived cell type [5]. In microglia cells TRPM2 currents and Ca<sup>2+</sup>-entry could be evoked by triggering of toll-like receptors (LPS) and cytokine receptors (TNF $\alpha$ ) as well as by intracellular ADPR and play a pivotal role in the activation of these cells [5]. Hence, it is very likely that reduced activation of CNS microglia contributes to the attenuated clinical EAE phenotype in *trpm2*-deficient mice.

There are also several lines of evidence that TRPM2 channels are expressed in CNS neurons: TRPM2-mediated currents evoked either by extracellular application of H<sub>2</sub>O<sub>2</sub> or OONO<sup>-</sup> or by intracellular perfusion with NAD<sup>+</sup>-derived second messengers like ADPR have been identified in cultured striatal neurons [10,14], acutely isolated cortical neurons [11] and hippocampal neurons in culture and slice preparations [12,13]. These currents and the associated Ca<sup>2+</sup> overload have been implicated in neuronal cell death during oxidative and nitrosative stress [11,14], which is a dominant mechanism of neuronal cell death in EAE. Hence, lack of TRPM2 channels is very likely to protect neurons and axons from degeneration during EAE and thus may also contribute to the ameliorated clinical EAE phenotype. These findings and considerations strongly suggest that pharmacological inhibition of TRPM2 cation channels might be regarded as a strategy for treating autoimmune CNS inflammation.

However, TRPM2 channels have been suggested to confer susceptibility to cell death following changes in redox status as they occur in inflammatory CNS lesions [27]. However, under conditions of oxidative stress, we did not observe sustained survival of *trpm2*-deficient T cells as compared to wildtype T cells. This is consistent with the unaltered number of T cells in wildtype and *trpm2*-deficient mice in a model of colon inflammation [20] and suggests that TRPM2 channels do not overtly affect lymphocyte survival at the site of inflammation.

Hence, inhibition of TRPM2 channels in autoimmune inflammatory CNS disorders likely dampens the adaptive T cell-mediated immune response without favouring prolonged T cell survival and inflammatory tissue damage. Moreover, TRPM2 channel inhibition likely attenuates microglia activation and neurodegeneration during autoimmune CNS inflammation.

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## Author Contributions

Conceived and designed the experiments: NM HW. Performed the experiments: NM GH KG. Analyzed the data: NM GH KG HW. Contributed reagents/materials/analysis tools: NM KG HW. Wrote the paper: NM KG HW.

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