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MAGT1 deficiency dysregulates platelet cation homeostasis and accelerates arterial thrombosis and ischemic stroke in mice

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Short title: MAGT1 in arterial thrombosis and ischemic stroke

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Abstract:

BACKGROUND: Magnesium transporter 1 (MAGT1) is a subunit of the oligosaccharide (OST) protein complex with thiol-disulfide oxidoreductase activity, supporting the process of N-glycosylation. MAGT1 deficiency was detected in human patients with XMEN syndrome and congenital disorders of glycosylation (CDG), resulting in decreased cation responses in lymphocytes, thereby inhibiting the immune response against viral infections. Curative hematopoietic stem-cell transplantation of XMEN patients causes fatal bleeding and thrombotic complications.

METHODS: We studied the role of MAGT1-deficiency in platelet function in relation to arterial thrombosis and hemostasis using several *in vitro* experimental settings, and *in vivo* models of arterial thrombosis and tMCAO model of ischemic stroke.

RESULTS: MAGT1-deficient mice (*Magt1*^{-/-}) displayed accelerated occlusive arterial thrombus formation *in vivo*, a shortened bleeding time, and profound brain damage upon focal cerebral ischemia. These defects resulted in increased Ca²⁺ influx and enhanced second wave mediator release, which further reinforced platelet reactivity and aggregation responses. Supplementation of MgCl₂ or pharmacological blockade of TRPC6 channels, but not inhibition of store-operated Ca²⁺ entry, normalized the aggregation responses of *Magt1*^{-/-} platelets to the control level. Glycoprotein VI (GPVI) activation of *Magt1*^{-/-} platelets resulted in hyperphosphorylation of Syk kinase, LAT and PLC γ 2, whereas the inhibitory loop regulated by PKC was impaired. A hyperaggregation response to GPVI agonist was confirmed in human platelets isolated from a MAGT1 deficient (XMEN) patient. Haploinsufficiency of TRPC6 in *Magt1*^{-/-} mice could normalize GPVI signaling, platelet aggregation, and thrombus formation *in vivo*.

CONCLUSIONS: These results suggest that MAGT1 and TRPC6 are functionally linked. Therefore, deficiency or impaired functionality of MAGT1 could be a potential risk factor for arterial thrombosis and stroke.

GRAPHIC ABSTRACT: A graphic abstract is available for this article

Key Words: MAGT1, PKC, TRPC6, TUSC3, arterial thrombosis, ischemic stroke

Nonstandard Abbreviations and Acronyms

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
Ca²⁺	Calcium
CDG	Congenital disorder of glycosylation
CRP	Collagen-related peptide
DAG	Diacylglycerol
ER	Endoplasmic reticulum
FVII	Factor VII
FcRγ	Fc receptor γ
GPVI	Glycoprotein-VI
GPCR	G-protein-coupled receptor
HSCT	Hematopoietic stem cell transplantation
IP3	Inositol-triphosphate
IP3R	IP ₃ receptor
I/R	Ischemia/reperfusion
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T cells
Lyn	Src family tyrosine kinase Lyn

MAGT1	Magnesium transporter 1
MCA	Middle cerebral artery
Mg²⁺	Magnesium
MGAT1	Mannosyl (α -1,3-)-glycoprotein β -1,2-N-acetylglucosaminyltransferase
MKs	Megakaryocytes
P2X₁	Purinergic receptor type X, subtype 1
P2Y₁	Purinergic receptor type Y, subtype 1
P2Y₁₂	Purinergic receptor type Y, subtype 12
PAR	Protease-activated receptor
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PRP	Platelet-rich plasma
ROCE	Receptor-operated Ca ²⁺ entry
SERCA	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase
SFK	Src family kinase
SHIP-1	SH2 containing inositol-5-phosphatase
SOCE	Store-operated Ca ²⁺ entry
STIM1	Stromal interaction molecule 1
STT3B	Oligosaccharyltransferase complex catalytic subunit B
Syk	Spleen tyrosine kinase
TCR	T cell receptor
TG	Thapsigargin
TP	Thromboxane A2 receptor
TxA₂	Thromboxane A2
tMCAO	Transient middle cerebral artery occlusion
TRPC6	Transient receptor potential cation channel, subfamily C, member 6
TRPM7	Transient receptor potential melastatin-like 7 channel
TUSC3	Tumor suppressor candidate 3
XMEN	X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection and neoplasia
WES	Whole exome sequencing
WT	Wild-type

INTRODUCTION

Platelets and vascular integrity are critical for normal hemostasis.^{1,2} Excessive platelet activation can lead to vessel occlusion and cause life-threatening conditions such as myocardial infarction and ischemic stroke.³ Changes in free cytoplasmic calcium concentration [Ca²⁺]_i play a critical role in regulating platelet reactivity. Indeed, Ca²⁺ increase in platelets from type 2 diabetes mellitus (T2DM) and sepsis patients contributes to the increased platelet reactivity upon stimulation with collagen.^{4,5}

Magnesium (Mg²⁺) is the second most abundant divalent cation in living cells, and is of major interest for vascular biology due to its widely accepted use as a natural Ca²⁺ antagonist in cardiovascular pathologies.⁶ Altered Mg²⁺ homeostasis has been described in patients with coronary heart disease, arrhythmias, acute myocardial infarction, hypertension and stroke.⁷⁻¹⁰ Moreover, Mg²⁺ is promoted as a natural “platelet antagonist”, since elevated intracellular Mg²⁺ concentration ([Mg²⁺]_i) inhibits platelet aggregation, cyclooxygenase activity and hence thromboxane A2 (TxA₂) release.^{11,12} Platelets from patients with coronary artery disease display decreased [Mg²⁺]_i, which is associated with an increased risk of arterial thrombosis.¹⁰ Although the antithrombotic potential of Mg²⁺ has been known for more than 50 years, the molecular mechanisms regulating Mg²⁺ homeostasis have not been investigated in platelets.

Recently, we identified transient receptor potential melastatin-like 7 channel (TRPM7) as an important Mg^{2+} permeable channel in megakaryocytes (MKs) and platelets.¹³ Cell type-specific gene deletion of TRPM7 in murine MKs and platelets resulted in altered intracellular Mg^{2+} homeostasis and a higher Ca^{2+} increase. These changes affected the subcellular localization and activity of myosin IIa resulting in aberrant cytoskeletal rearrangements and macrothrombocytopenia.¹³ Moreover, kinase activity of TRPM7 was revealed as a key modulator of phospholipase C (PLC) signaling and store-operated Ca^{2+} entry (SOCE) in platelets.¹⁴ In addition, an increased expression of Mg^{2+} transporter 1 (MAGT1) was observed in TRPM7-deficient MKs. However, this compensatory mechanism could not prevent macrothrombocytopenia and platelet dysfunction in these mutant mice, thus indicating a distinct role of MAGT1 in regulating Mg^{2+} homeostasis in murine MKs and platelets. MAGT1 function has been proposed to be a central regulator of N-glycosylation^{3,15} and cellular Mg^{2+} homeostasis in mammals.¹⁶ The MAGT1 protein contains five transmembrane domains incorporating the protein into membranes, a short N-terminus with a signal peptide and a longer cytoplasmic tail with several putative phosphorylation sites.¹⁶ MAGT1 and its close homolog tumor suppressor candidate 3 (TUSC3) are associated with the multisubunit enzymatic oligosaccharyltransferase (OST) complex which regulates N-glycosylation in the endoplasmic reticulum (ER).^{3,15} MAGT1 and TUSC3 are thioredoxin homologs with a conserved CXXC motif, which regulates the thiol-oxidoreductase activity of these proteins.^{17,18} The oxidized form of the thioredoxin motif (CXXC) interacts with the STT3B-targeted substrates, forming a disulfide bridge supporting N-glycosylation. Consequently, MAGT1 deficiency or mutation of CXXC motif can disturb the process of N-glycosylation.^{15,18} In line with this, MAGT1 deficiency in XMEN patients (X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection and neoplasia) causes hypoglycosylation and is associated with an enhanced expression of TUSC3, indicating a partial compensatory mechanism in the process of N-glycosylation.^{15,18} The glucose transporter GLUT1 is hypoglycosylated in lymphocytes which was confirmed in *Magt1^{-/-}Tusc3^{-/-}* and *Stt3b^{-/-}* HEK293 cell lines,^{3,15,18} indicating that MAGT1 plays a regulatory role in N-glycosylation-dependent energy metabolism. In addition, haptoglobin, hemoglobin and immunoglobulin heavy chain are hypoglycosylated in XMEN patients.¹⁷ Altogether, these results indicated that MAGT1 deficiency is a congenital disorder of glycosylation (CDG).¹⁵ Interestingly, low Mg^{2+} diet in mice enhances MAGT1 expression in different tissues when compared to animals fed with a normal diet.¹⁶ The intracellular activation of MAGT1 was proposed to be regulated by protein kinase C (PKC)-mediated phosphorylation at different serine residues.¹⁹ Downregulation of MAGT1 in mammalian cells results in decreased $[Mg^{2+}]_i$, implying a potential regulatory role of MAGT1-dependent thiol-oxidoreductase activity and N-glycosylation in cellular Mg^{2+} homeostasis.^{19,20} Interestingly, MAGT1 expression was found to be upregulated in *Trpm7* knock-down DT40 chicken bursal lymphoma cells, accompanied by an increased capacity of extracellular Mg^{2+} uptake.²¹ The overexpression of MAGT1 was considered to rescue the severe growth impairment of *Trpm7^{-/-}* cells.²¹ In human T lymphocytes, upon T cell receptor (TCR) stimulation, a rapid but transient elevation of $[Mg^{2+}]_i$ was measured, while MAGT1 deficiency abrogated this Mg^{2+} entry leading to a defective activation of PLC γ 1 and associated Ca^{2+} responses.²² In support of such a role in T cells, patients with hemizygous loss of function in *MAGT1* develop an inborn error of immunity (XMEN syndrome), which is characterized by severe immunodeficiency and chronic viral infections, especially with Epstein-Barr virus.²² Therefore XMEN patients were treated with hematopoietic stem cell transplantation (HSCT), which frequently resulted in fatal bleeding and thrombotic complications during early post-HSCT.²³⁻²⁶ MAGT1-deficient mice have abnormal B cell function and Mg^{2+} and Ca^{2+} homeostasis are severely dysregulated, highlighting an important role for MAGT1 in normal B cell function.²⁷

Here, we address the role of MAGT1 in platelet function during thrombosis and ischemic stroke and provide new insights into the molecular regulation of platelet cation homeostasis.

METHODS

The data supporting the findings of this study are available within article and its Supplemental Material. Additional data that support the findings of this study are available from the corresponding author upon reasonable request. Please see the Major Resources Table in the Supplemental Material.

Animals

C57BL/6J mice were obtained from Janvier (France) and *Magt1*^{-y} mice from TIGM (Texas, USA). *Magt1*^{-y} mice were bred on a C57BL/6J background. *Trpc6*^{-/-} mice were described previously²⁸ and crossed with *Magt1*^{-y} mice. *Unc13d*^{-/-} mice were generated as previously described.²⁹ TUSC3-deficient mice were generated by CRISPR/Cas9 technology (Jackson Laboratories, MGI:1933134). All animal procedures described in this study were performed in accordance with relevant guidelines and regulations and approved by the Regional Administration of Unterfranken (Lower District), Würzburg, Germany in accordance with European equivalent of the NIH's Guide for the Care and Use of Laboratory Animals (directive 2010/63/EU) and are reported according ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>). Animals were maintained under controlled light (12-hr light/dark cycle) and temperature and had access to water and food *ad libitum*. The *Magt1* gene is located on the X chromosome, which means that MAGT1 deficiency and the consequent XMEN syndrome mainly affects males in humans. Therefore, all the *in vivo* and *in vitro* experiments were performed using age-matched wild-type (*WT*) and *Magt1*^{-y} male mice. MAGT1 genotyping protocol provided by TIGM (Texas, USA). MAGT1 specific primers (IST13422E1-F: CCAATCCTGGACTTTGTGCA; IST13422E1-R1: CCTCTTCTGACCTCCAAAAG; IST13422E1-R2: CCAATAAACCTCTTGCAGTTGC) amplify *WT* allele (644 bp) and knockout allele (377 bp). *Trpc6*^{-/-} and *Unc13d*^{-/-} mice were genotyped as previously described.^{28,29}

Patient and healthy subjects

Patient and blood donor volunteers gave free and informed written consent to participate in this study, conforming to the ethical standards adhering to the local Institutional Review Boards and the Declaration of Helsinki. The ethics committee of Hannover Medical School, Hannover, Germany approved the study (approval number 8875-BO-K-2020). The studied XMEN patient was a male born to immunocompetent parents of German descent. He had a history of recurrent respiratory tract infections since his early childhood, including pneumonia. At the age of 14, he was diagnosed with a primary immunodeficiency disorder. Initial immunological investigations were consistent with combined immunodeficiency. Genetic diagnosis was made by means of whole exome sequencing (WES) at the age of 18 years identifying a pathogenic hemizygous variant in *MAGT1* c.128_129delCT; p.(Ser43Cysfs*28), which led to the loss of MAGT1 expression. Blood sample preparation, WES analysis and the assessment of rare single nucleotide variants (SNV) were performed as described previously³⁰⁻³² and detailed in Supplemental Material. Results of immunological investigations of this patient are summarized in Supplemental Material (**Table S1 and Table S2**). Molecular testing for EBV in peripheral blood revealed a chronic low-grade EBV proliferation (3000-6000 copies/mL).

Isolation of platelets, B and T lymphocytes

Mouse and human platelets were isolated from whole blood in the presence of anticoagulants as previously described.^{13,33} For the analysis of B and T lymphocytes, spleens were isolated and smashed and incubated with the biotin-conjugated anti-mouse CD3 or CD45R/B220 antibody followed by streptavidin nanobeads according to MojoSort™ Selection Kit and manufacturer's instructions (Biolegend, Germany).

Measurement of intracellular free magnesium in platelets

Washed platelets (5x10⁵ platelets/μL) in Tyrode's-HEPES buffer containing 5 mM glucose supplemented with 0.02 U/mL apyrase and prostaglandin (PG)I₂ were loaded with Mag-Fluo4

(5 µg/mL of dye in dimethyl sulfoxide (DMSO) mixed 1:1 with 37°C pre-heated pluronic acid) and left shaking at 300 rpm for 30 min at 37°C in the dark. To allow the ester to cleave, loaded platelets were incubated without shaking for an additional 20 min at 37°C in the dark. 25 µL of the loaded platelets were diluted to 1 mL with Tyrode's-HEPES (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0) without CaCl₂, and fluorescence intensity was recorded for an initial 50 s. Subsequently, 2 mM CaCl₂ and the stated agonist were added to 500 µL of the platelet suspension. Fluorescence intensity was recorded for an additional 300 s. To quantify each measurement, geometric mean fluorescence intensity (Geo-MFI) of approximately 10% of the dot plot of each measurement before CaCl₂ and agonist addition was calculated for resting values. Similarly, Geo-MFI of 10% of the dot plot before the stopping each measurement (after agonist addition) was calculated to attain values after activation. Finally, Geo-MFI % loss values were calculated from each measurement and reported. Platelets were pre-incubated with either 1 mM, 2.5 mM or 5 mM MgCl₂, as stated. Flow cytometric measurements were performed on a BD FACS Canto II and results were analyzed by using FlowJo software.

Measurement of intracellular free Ca²⁺ in platelets

For intracellular free Ca²⁺ measurement, platelets were loaded with Fura-2/AM (5 µM) in the presence of Pluronic F-127 (0.2 µg/mL) for 30 min at 37°C in Tyrode's-HEPES buffer containing 5 mM glucose. Labeled platelets were washed and suspended in Tyrode's-HEPES buffer without extracellular CaCl₂. Platelets were activated with the indicated agonists and fluorescence was determined with a PerkinElmer LS 55 fluorimeter (excitation at 340 and 380 nm and emission at 509 nm) for Ca²⁺ store release or in the presence of 1 mM CaCl₂ for Ca²⁺ entry. For measurements with thapsigargin (TG), store release was measured until 300 sec in the absence of extracellular CaCl₂ and later 1 mM CaCl₂ was added to the same sample and SOCE was measured until an additional 200 sec. Each measurement was calibrated using Triton X-100 and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA).

Aggregation

Light transmission of washed platelets (1.5x10⁵ platelets per µL) or platelet-rich plasma (PRP) supplemented with 100 µg/mL fibrinogen and 2mM Ca²⁺ was monitored over time using a four-channel aggregometer (APACT, Laborgeräte und Analysensysteme, Hamburg). Aggregation studies with adenosine diphosphate (ADP) were performed in PRP. Platelet aggregation was induced by the addition of the indicated agonists and in the presence of indicated doses of extracellular MgCl₂.

Platelet adhesion under flow

Heparinized whole blood was used for *ex vivo* flow adhesion with or without stated supplementation with MgCl₂. Flow chamber experiments on collagen I were performed at 1000 s⁻¹ and at 1700 s⁻¹ as previously described.³⁴ To carry out von Willebrand factor (vWF) flow chamber, heparinized whole blood diluted 2:1 in Tyrode's buffer without CaCl₂ was perfused on vWF-coated glass coverslips at 1700 s⁻¹ for 2-4 min. Later, the system was washed with Tyrode's buffer without CaCl₂ and 10 bright field images were captured per mouse. Using CellProfiler software, individual platelet adhesion to vWF surface was quantified and finally mean of total number of platelets was reported.

Statistics

The statistical significance of results was analyzed using the GraphPad Prism program, version 8.0 (Prism, GraphPad, LaJolla, CA, USA). The Shapiro-Wilk normality test was used to assess the normality of the data and the statistical difference of the mean was analyzed using the Student unpaired two-tailed t test and 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For data not following a Gaussian distribution, the non-parametric Mann-Whitney test was used. For Bederson score and Grip test, the non-

parametric Mann-Whitney test was used. Data are presented as median with 95% confidence limits or mean \pm SD. *P*-values < 0.05 were considered as statistically significant and *P*-values > 0.05 as not significant.

RESULTS

Normal platelet biogenesis in *Magt1*^{-y} mice

To study the role of MAGT1 function in MKs and platelets, we capitalized a constitutive knockout *Magt1* mouse model (*Magt1*^{-y}). The efficient ablation of *Magt1* expression in platelets isolated from *Magt1*^{-/-} mice was confirmed by immunoblotting (**Figure S1A, S1B**). *Magt1*^{-y} mice were born at a normal Mendelian ratio, viable, fertile and did not display any obvious signs of disease. Histological analysis of hematopoietic organs including spleen, thymus, lymph nodes and bone marrow revealed normal tissue morphology in *Magt1*^{-y} mice (**Figure S1C, S1D**). Platelet count, size, the distribution of red and white blood cells of *Magt1*^{-y} mice were indistinguishable from controls (**Figure S1F, G; Table S3**). Megakaryocyte (MK) number and ploidy in the bone marrow of *Magt1*^{-y} mice were slightly increased compared to controls (**Figure S1E, S2A**), but this alteration did not affect platelet lifespan or morphology (**Figure S2B**). Recently, we reported that in platelet TRPM7-deficient mice (*Trpm7*^{fl/fl-Pt4Cre}), altered cellular Mg²⁺ homeostasis accounts for increased activity of myosin IIa and consequently impaired pro-platelet formation, thus suggesting a critical role of [Mg²⁺]_i in the regulation of actin dynamics.¹³ In contrast to *Trpm7*^{fl/fl-Pt4Cre} platelets, *Magt1*^{-y} platelets spread normally on fibrinogen, thus suggesting normal actin dynamics and excluding a role of MAGT1 in this cytoskeleton-dependent process (**Figure S2C**).

MAGT1 deficiency impairs platelet cation homeostasis

It has been shown that MAGT1 deficiency impairs STT3B-mediated N-glycosylation¹⁵ and strongly influences Mg²⁺ and Ca²⁺ homeostasis in T and B lymphocytes in humans and mice.^{22,27,35} MAGT1 belongs to the thiol-disulfide oxidoreductase family and is essential for the formation of native disulfide bonds in the ER.³ Therefore, we measured the impact of MAGT1 deficiency on cellular thiol homeostasis in platelets and immune cells, quantifying the conversion of thiol-substrate using a fluorometric glutaredoxin activity assay. We found a significant reduction of thiol-oxidoreductase activity in MAGT1-deficient platelets and also in B as well as in T cells (**Figure S2D-S2F**). To dynamically determine changes in cation homeostasis in platelets, first, [Mg²⁺]_i was determined in resting and activated platelets with the Mg²⁺-specific Mag-Fluo4 dye and time-resolved flow cytometry. Interestingly, the basal [Mg²⁺]_i in resting *Magt1*^{-y} platelets as well as total Mg²⁺ and Ca²⁺ concentrations were normal as determined by flow cytometry and inductively-coupled plasma mass spectrometry (ICP-MS), respectively (**Figure S3A, S3B**). Using confocal microscopy, we detected intensive Mag-Fluo4 staining in platelet secretory granules of resting wild-type (*WT*) and *Magt1*^{-y} platelets which was absent in collagen-activated *Magt1*^{-y} platelets, but residual foci were observed in *WT* platelets (**Figure 1A**). Changes of extracellular Mg²⁺ concentrations from 1 to 2.5 or 5 mM can increase [Mg²⁺]_i of both resting *WT* and *Magt1*^{-y} platelets (**Figure S3C**). Interestingly, *Magt1*^{-y} platelets showed normal activation-dependent change in [Mg²⁺]_i in response to thrombin or a high dose of collagen-related peptide (CRP). In contrast, upon stimulation with a lower dose of CRP, an increased Mg²⁺ efflux was detected in *Magt1*^{-y} platelets (**Figure S3D**). Altogether, our data show that MAGT1 does not regulate Mg²⁺ uptake in resting cells and the accelerated [Mg²⁺]_i depletion in activated *Magt1*^{-y} platelets could be the consequence of faster release from a granular-resident Mg²⁺ store. Next, platelets of mice deficient for the exocytosis-priming protein MUNC13-4 (*Unc13d*^{-/-}) in which dense-granule release was inhibited,²⁹ were studied to evaluate the contribution of granular-resident Mg²⁺ stores to the process of Mg²⁺ efflux. In Mag-Fluo4-loaded *Unc13d*^{-/-} platelets, Mg²⁺ efflux was severely impaired (**Figure S4A**), suggesting that the dense-granule-resident Mg²⁺ store is an important part of Mg²⁺ efflux in activated platelets, and it is defective in *Magt1*^{-y} platelets. Interestingly, MgCl₂ supplementation could revert the aberrant Mg²⁺ efflux

in *Magt1*^{-y} platelets upon stimulation with CRP (**Figure S3E** WT: 7.62±2,492; *Magt1*^{-y}: 27.68±11,45 versus **Figure S3F** Mg²⁺ supplemented WT: 19,46±6,756; *Magt1*^{-y}: 15,79±4,633). Altogether, our results suggest a specific role of MAGT1 in Mg²⁺ store release, located in the dense-granules, which is regulated by downstream effectors of GPVI signaling, a function that can be compensated by alternative mechanisms upon thrombin stimulation or in MgCl₂-supplemented platelets. Interestingly, quantitative RT-PCR showed unaltered mRNA expression levels of the major Mg²⁺ channel TRPM7 and the tumor suppressor candidate 3 (TUSC3) protein in *Magt1*^{-y} platelets (**Figure S5A, S5B**), suggesting functional independence of MAGT1 from TRPM7 or TUSC3.

In various cell types, including *Trpm7*^{fl/fl-Pf4Cre} platelets and MAGT1-deficient B cells, reduced [Mg²⁺]_i is frequently associated with an increase in [Ca²⁺]_i.^{13,27,36} To investigate whether MAGT1 deficiency leads to abnormal Ca²⁺ responses, resting and activated *Magt1*^{-y} platelets were subjected to a detailed analysis of Ca²⁺ homeostasis. In line with the unaltered [Mg²⁺]_i in resting platelets, the basal [Ca²⁺]_i was normal as well. In addition, agonist-induced Ca²⁺ store release was also normal in *Magt1*^{-y} platelets (**Figure S5C**). Strikingly, Ca²⁺ influx was significantly elevated in response to stimulation with receptor agonists, CRP, ADP or the thromboxane A₂ (TxA₂) analogue U46619 (**Figure 1C**), but not upon thrombin stimulation (**Figure S5D**). MgCl₂ supplementation could normalize the abnormal Ca²⁺ responses in *Magt1*^{-y} platelets (**Figure 1D**). To directly test whether SOCE was dysregulated in *Magt1*^{-y} platelets, thapsigargin (TG), a sarco/endoplasmic reticulum Ca²⁺-ATPase inhibitor, was used to bypass platelet receptor-induced Ca²⁺ store depletion, thereby directly activating STIM1-dependent Orai1 activation in platelets. Interestingly, normal SOCE was detected in *Magt1*^{-y} platelets (**Figure S5E, S5F**), indicating that MAGT1 deficiency could enhance an alternative Ca²⁺ entry mechanism that is independent of STIM1/Orai1-mediated SOCE, which we also observed earlier in MAGT1-deficient B cells.²⁷

MAGT1 deficiency enhances platelet aggregation through the activation of TRPC6

To assess the physiological consequences of *Magt1*-deficiency, *Magt1*^{-y} platelets were activated with different agonists and aggregation responses were monitored. Upon stimulation with collagen, CRP or U46619, *Magt1*^{-y} platelets showed enhanced aggregation responses, which could be normalized by MgCl₂ supplementation (**Figure 2A**). However, at high agonist concentrations or when thrombin or ADP was used, *Magt1*^{-y} platelets showed normal aggregation responses (**Figure S5G**). Interestingly, increased ATP secretion and TxA₂ release were observed in *Magt1*^{-y} platelets upon stimulation with collagen, CRP or U46619, indicating an enhanced purinergic signaling and cyclooxygenase activity, respectively (**Figure 2B-E**). In line with these data, accelerated aggregation response of *Magt1*^{-y} platelets was inhibited using a selective P2Y₁₂ blocker 2MeSAMP, but not with a P2Y₁ blocker (**Figure S5G**). Altogether, these results suggested that enhanced purinergic signaling and Ca²⁺ channel activity and a subsequent increase in [Ca²⁺]_i account for the increased aggregation response of *Magt1*^{-y} platelets. To assess the consequence of the hyperreactivity of *Magt1*^{-y} platelets during thrombus formation, heparinized whole blood from *Magt1*^{-y} mice was perfused over a collagen-coated surface at different shear conditions. At a shear rate of 1000 s⁻¹, surface coverage and thrombus volume were indistinguishable from controls (**Figure S6A-S6C**), whereas at a higher shear rate of 1700 s⁻¹, enhanced thrombus formation was observed in *Magt1*^{-y} samples compared to WT (**Figure 3A-D**). The stimulus-dependent [Ca²⁺]_i increase in platelets plays a pivotal role in several steps of thrombus formation. It is known that most of the activated platelet receptors trigger PLC, mediating the production of diacylglycerol (DAG) as well as inositol triphosphate (IP₃) and subsequent activation of DAG-mediated receptor-operated Ca²⁺ entry (ROCE) through transient receptor potential cation channel C, member 6 (TRPC6) and IP₃-dependent SOCE, respectively.³⁷⁻⁴⁰ Supplementation of blood samples with MgCl₂ diminished thrombus formation in a dose-dependent manner (**Figure 3A-D**). SOCE inhibitor 2-APB or BTP2 was unable to normalize *ex vivo* thrombus formation of *Magt1*^{-y} whole blood, indicating that SOCE plays a dispensable role in this process (**Figure 3B-D**). In sharp contrast, inhibition of TRPC6 activity did normalize the surface coverage and thrombus volume of *Magt1*^{-y} samples to WT level

(**Figure 3B-D**). Consequently, haploinsufficiency of TRPC6 also normalized thrombus formation in *Magt1^{-y}/Trpc6^{+/-}* platelets *in vitro* and *ex vivo* under flow (*WT*: 28,77±2,596; *Magt1^{-y}*: 45,20±5,311; *Magt1^{-y}/Trpc6^{+/-}*: 28,82±5,540), (**Figure 4A-C**). In line with this result, the intracellular loss (%) of Mg²⁺ in *Magt1^{-y}/Trpc6^{+/-}* platelets was normalized to *WT* level after CRP activation (*WT*: 7,621±2,492; *Magt1^{-y}*: 27,68±11,45; *Magt1^{-y}/Trpc6^{+/-}*: 6,2±2,1). Under high shear conditions, glycoprotein Ib (GPIb)-mediated signaling via binding to vWF plays an essential role in thrombus formation.^{41,42} To study the role of vWF-GPIb signaling in the regulation of MAGT1 function, whole blood was perfused over a vWF-coated surface at 1700 s⁻¹. No differences were observed between bloods from *WT* and *Magt1^{-y}* mice, thus excluding a role of GPIb-mediated signaling in this process (**Figure S6D**).

MAGT1 deficiency dysregulates GPVI and protein kinase C dependent signaling

To further investigate the effects of abnormal TRPC6 function at a molecular level, *WT*, *Magt1^{-y}* and *Magt1^{-y}/Trpc6^{+/-}* platelets were stimulated with CRP in the presence of extracellular CaCl₂, and tyrosine phosphorylation of the GPVI/LAT signalosome was analyzed. We observed a hyperphosphorylation of spleen tyrosine kinase (Syk), linker for activation of T cells (LAT) and PLCγ2 in *Magt1^{-y}* platelets, which was normalized to *WT* levels in *Magt1^{-y}/Trpc6^{+/-}* platelets (**Figure 5A, Figure S7A-S7C**). The PLCγ2-mediated DAG production activates not only TRPC6, but also enhances the enzymatic activity of a subset of PKC isoforms, which transduce the signal from the GPVI/LAT signalosome to integrins and Ca²⁺ channels. However, the various PKC isoforms can either reinforce GPVI signaling or inhibit its signaling at a late step of platelet activation.⁴³ In addition, the channel activity of TRPC6 is negatively regulated by PKC-mediated phosphorylation.⁴⁴ To unravel this complex signaling pathway, we analyzed the phosphorylation status of different PKC isoforms in CRP-stimulated platelets and found normal PKC epsilon (ε) and PKC delta (δ) activation at an early stage (after 15 seconds), while pronounced hypophosphorylation was observed at later time points of platelet activation in *Magt1^{-y}* samples (**Figure 5B, Figure S7D, S7E**). On the other hand, in line with the central role of TRPC6 in this signaling pathway, the phosphorylation defect was normalized to *WT* levels in *Magt1^{-y}/Trpc6^{+/-}* platelets (**Figure 5B**). Interestingly, in the absence of extracellular CaCl₂ (excluding TRPC6 function or other Ca²⁺ channels located in the plasma membrane), CRP-induced phosphorylation of Syk, LAT, PLCγ2 was normal in *Magt1^{-y}* platelets (**Figure 5A, right panel, Figure S7F-S7H**), and also normal phosphorylation of PKCδ was detected (**Figure 5B, right panel, Figure S7I**). However, PKCε phosphorylation was similarly defective (**Figure 5B, right panel, Figure S7J**), as observed in the presence of extracellular CaCl₂ (**Figure 5B, left panel**). This result suggests that hypophosphorylation of PKCδ at later step of GPVI activation is caused by abnormal Ca²⁺ influx, probably through TRPC6, but hypophosphorylation of PKCε is a Ca²⁺-independent process. Of note, mRNA expression of TRPC6 in *Magt1^{-y}* platelets was similar to *WT* control (**Figure S8A**). Taken together, these results suggest that MAGT1 deficiency dysregulates PKC function, thereby influencing the signaling pathway of GPVI signalosome and probably channel activity of TPRC6 during platelet activation.

MAGT1 deficiency enhances arterial thrombosis

To assess the pathological consequences of altered MAGT1 function in platelets, we challenged mice in various models of arterial thrombosis and hemostasis. Chemical injury of the endothelial layer in mesenteric arterioles by ectopic application of a suboptimal concentration FeCl₃ (13%) revealed a significantly shortened time to vessel occlusion in *Magt1^{-y}* mice (*WT*: 18.44±5.833 min versus *Magt1^{-y}*: 10.76±3.419 min; **Figure 6A**). To exclude a possible contribution of MAGT1 in non-hematopoietic cells, bone marrow (BM) cells from either *Magt1^{-y}* or *WT* mice were transferred into lethally irradiated *WT* recipient mice, which were then subjected to a FeCl₃ (6%)-mediated thrombosis model on the carotid artery. Strikingly, *Magt1^{-y}* mice or irradiated *WT* mice which were transplanted with *Magt1^{-y}* BM cells displayed accelerated occlusion times as compared with the respective control mice (*WT*: 680,8±138,8 sec and *WT BM^{WT}*: 860±351,6 sec versus *Magt1^{-y}*: 346,9±116,9 sec and *WT BM^{Magt1^{-y}}* mice: 366,3±113,3 sec; **Figure 6B**). To establish the pathological role of

enhanced channel activity of TRPC6 in this process, *Magt1^{-/-}* and *Magt1^{-/-}/Trpc6^{+/-}* mice were challenged in the thrombosis model of carotid arteries, and prolonged occlusion time was observed in *Magt1^{-/-}/Trpc6^{+/-}* mice compared to *Magt1^{-/-}* mice (**Figure 6C**). Tail bleeding times were measured to answer the question of whether MAGT1 deficiency can influence hemostasis. In line with our results from the thrombosis models, *Magt1^{-/-}* and *WT BM^{Magt1^{-/-}}* chimeric mice showed shortened bleeding times as compared to the respective controls (*WT*: 265±98 sec and *WT BM^{WT}*: 319±89 sec versus *Magt1^{-/-}*: 120±81 sec and *WT BM^{Magt1^{-/-}}*: 176 ± 85 sec, **Figure 6E**). In addition, clot retraction was also enhanced in PRP isolated from *Magt1^{-/-}* mice (**Figure 6D**), but coagulation factors and antithrombin levels in the blood plasma were nearly similar between *WT* and *Magt1^{-/-}* mice (**Table S4**). Altogether, these results indicate that hematopoietic cells and hyperreactive platelets account for the pro-thrombotic phenotype in *Magt1^{-/-}* mice.

MAGT1 deficiency increases ischemic brain infarction

As platelets play an important role in ischemia/reperfusion (I/R) injury of the brain,⁴⁵ *WT* and *Magt1^{-/-}* mice were subjected to a transient middle cerebral artery occlusion (tMCAO) model of stroke.⁴⁶ Of note, blood flow of the middle cerebral artery (MCA) was obstructed only for 30 min, which leads to small infarctions in *WT* mice. Strikingly, *Magt1^{-/-}* mice developed larger infarcts with a strongly increased number of thrombi in ipsilesional vessels 24h after onset (**Figure 7A-E**). To dissect the role of platelet MAGT1 in this process, BM chimeric mice were subjected to tMCAO. Whereas *WT BM^{WT}* and *Magt1^{-/-} BM^{WT}* mice showed only small ischemic lesions in the brain, *WT BM^{Magt1^{-/-}}* mice showed fully-grown brain infarcts with a significantly worsened neurological outcome (**Figure 7F-I**). These data indicate that platelet hyperresponsiveness by MagT1 deficiency could enhance I/R injury in the brain.

MAGT1 deficiency impairs platelet cation homeostasis and enhances platelet aggregation in human platelets

To determine whether the observations in *MAGT1^{-/-}* mice are relevant to humans, we analyzed an XMEN syndrome patient, lacking the functional *Magt1* gene (*MAGT1* c.128_129delCT; p.(Ser43Cysfs*28)), (**Table S1, S2**). This patient developed persistent Epstein-Barr viremia, and did not receive Mg²⁺ supplementation prior to the study, and his platelet count and size were normal as compared to a healthy donor (**Table S1**). Using confocal microscopy and Mag-Fluo4 staining, we analyzed the levels of Mg²⁺ in both resting and activated MAGT1-deficient human platelets. Both control and MAGT1-deficient platelets showed Mg²⁺-positive staining at resting state, which was strongly decreased in MAGT1-deficient platelets adhered to a collagen-coated surface (**Figure 9SA, 9SB**). We also found that at a lower dose of CRP, MAGT1-deficient human platelets aggregated, while control platelets did not aggregate during this experimental condition, indicating that cytoplasmic Mg²⁺ deficiency could influence platelet aggregation responses, similar to *Magt1^{-/-}* mouse platelets (**Figure 9SC, 9SD**). The hyperaggregation responses were similar in both washed platelets (**Figure 9SC**), and PRP (**Figure 9SD**), from the MAGT1-deficient patient, excluding the contribution of plasmatic proteins. Furthermore, flow analysis of MAGT1-deficient human platelets also showed enhanced inside-out activation of αIIbβ3 integrin and degranulation in response to stimulation with CRP (**Table S5**), but not upon thrombin stimulation. Altogether our results show that Mg²⁺ homeostasis is dysregulated in MAGT1-deficient human platelets, indicating similar defects to those observed in *Magt1^{-/-}* mice. Further studies are necessary to confirm these results in a large cohort of MAGT1-deficient patients.

The redundant function of TUSC3 in platelet activation

It has been shown that MAGT1 deficiency is associated with enhanced expression of TUSC3, which could compensate for the lack of MAGT1 function in different cell types.³⁹ Therefore, we investigated a constitutive knockout TUSC3 mouse line (*Tusc3^{-/-}*) and studied its function compared to *Magt1^{-/-}* platelets. TUSC3 deficiency was detrimental, as knockout mice died in the prenatal or early postnatal periods. To obtain TUSC3-deficient platelets, we generated chimeric mice by using fetal liver-resident hematopoietic cells (FLC) isolated from

Tusc3^{-/-} or *WT* embryos at embryonic day 14 (E14) and transferred them into lethally irradiated adult *WT* recipient mice (*WT-FLC*^{*TUSC3*^{-/-}}, *WT-FLC*^{*WT*}). Platelet count, size, expression of major platelet glycoproteins and also blood cell parameters were found to be normal in *WT-FLC*^{*TUSC3*^{-/-}} mice (**Figure S10A, Table S6**), suggesting a dispensable role of TUSC3 in platelet biogenesis. Flow cytometry analysis of *WT-FLC*^{*TUSC3*^{-/-}} mice also showed normal inside-out activation of $\alpha\text{IIb}\beta\text{3}$ integrin and degranulation in response to different platelet agonists (**Figure S10B, S10C**). Consistently, normal thrombus growth was observed in *ex vivo* whole blood from *WT-FLC*^{*TUSC3*^{-/-}} mice at shear rate of 1700s⁻¹ (**Figure S11A-S11C**). Altogether, these results suggest that TUSC3 function is important in early postnatal development, but it has a redundant function in mouse platelets, probably compensated by MAGT1.

DISCUSSION

In mammalian cells, $[\text{Mg}^{2+}]_i$ is regulated by Mg^{2+} influx through plasma membrane-resident cation channels and transporters, intracellular Mg^{2+} stores and Mg^{2+} efflux. Although intracellular Mg^{2+} store have not yet been identified in MKs and platelets, the ER, mitochondria, secretory granules and the Mg^{2+} -ATP complex could be a potential Mg^{2+} stores, similar to other cell types.⁴⁷ Using Mag-Fluo4-labeled platelets, our study confirmed that platelet dense granule-resident Mg^{2+} stores significantly contribute to Mg^{2+} efflux upon platelet activation. The present results show that basal $[\text{Mg}^{2+}]_i$ and Mg^{2+} efflux are normal in resting *Magt1*^{-/-} platelets as well as upon stimulation of protease-activated receptors (PAR) by thrombin. However, in response to CRP or collagen, a more pronounced granular resident Mg^{2+} store depletion was detected in activated *Magt1*^{-/-} platelets. Using platelets of a MAGT1-deficient XMEN patient, we found a similar hyperaggregation response upon stimulation with collagen or CRP, as we observed in *Magt1*^{-/-} mouse platelets. The Mg^{2+} depletion from dense granule-resident Mg^{2+} stores was strongly decreased in MAGT1-deficient human platelets upon collagen-induced activation. These results need to be confirmed in the future in a large cohort of patients. In *Drosophila* cells PKC has been shown to bind and phosphorylate MAGT1 on serine residues which enhances Mg^{2+} uptake in this cell type.¹⁹ The identified PKC phosphorylation sites on MAGT1 appeared to be highly conserved between different species, suggesting an evolutionally conserved function of PKC in the regulation of MAGT1 function. We recently showed that MAGT1 deficiency strongly influences cation homeostasis in resting and activated B cells.²⁷ We showed that genetic ablation of MAGT1 in mice leads to an enhanced B cell receptor (BCR)-induced Ca^{2+} response and abnormal PKC activation, which disturbs the development of marginal B and plasma cells.²⁷

We further studied the physiological consequence of MAGT1 deficiency on PKC signaling in platelets, because downstream effectors in platelet GPVI/immunoreceptor tyrosine-based activation motif (ITAM) signalosome are similar to BCR/ITAM signalosome of B cells.²² Platelet activation is regulated by different isoforms of PKC.⁴⁸⁻⁵¹ PKC δ negatively regulates collagen-induced platelet activation by the complex interplay between the Src family tyrosine kinase Lyn (Lyn), SH2 containing inositol-5-phosphatase (SHIP-1) and PKC δ , which control downstream effectors of the GPVI receptor.⁵² Our results show that MAGT1 deficiency reduces PKC δ phosphorylation on an Y311 residue downstream of GPVI, thereby losing an inhibitory signaling component, which consequently accelerates phosphorylation of Syk, LAT and PLC γ 2. Others have shown that PKC δ exerts a negative feedback loop on TRPC6 channel through phosphorylation of serine residue 448.⁴⁴ Based on this result, we speculate that dysregulated PKC function in MAGT1-deficient platelets enhances TRPC6 channel activity. We were able to demonstrate that PKC δ phosphorylation was normalized in *Magt1*^{-/-}/*Trpc6*^{+/-} platelets in response to GPVI activation, indicating a complex interplay between PKC and TRPC6. Platelet aggregation responses were also normalized in the *Magt1*^{-/-}/*Trpc6*^{+/-} samples *in vitro* and *ex vivo* under flow, as well as thrombus formation in the carotid arteries *in vivo*, suggesting a contribution of abnormal TRPC6 channel activation as a

result of the loss of MAGT1 activity. It has been shown that PKC δ deficiency enhances collagen-dependent aggregation response, which is further accelerated by increased ATP and TxA₂ secretion.⁵³ In line with these results, we also detected increased TxA₂ and ATP secretion upon collagen or CRP stimulation in *Magt1*^{-y} platelets. Furthermore, application of 6% FeCl₃ was efficient to detect a significantly prolonged occlusion time in the carotid artery of *WT* mice, while an accelerated occlusion was observed in *Magt1*^{-y} mice. Altogether, these results suggest a potential contribution of increased cyclooxygenase as well as an enhanced ATP-gated P2X₁ channel activity as a response to MAGT1 deficiency. Therefore, future investigations are necessary to better understand the role of purinergic signaling in arterial thrombus formation of *Magt1*^{-y} mice.

The phosphorylation of other PKC isoform (PKC ϵ) was reduced in *Magt1*^{-y} platelets in response to CRP. This alteration was normalized in *Magt1*^{-y}*Trpc6*^{+/-} platelets, thus further supporting the notion of functional crosstalk between MAGT1 and PKC isoforms. Interestingly PKC ϵ negatively regulates collagen-induced platelet activation through inhibition of cyclooxygenase activity.⁴⁹ Therefore, reduced PKC ϵ activity probably accounts for the increased cyclooxygenase activity and TxA₂ production in *Magt1*^{-y} platelets, which further amplifies the collagen-induced aggregation response in mutant mice. Consequently, this translated into a shortened time to vessel occlusion in a model of thrombosis and hemostasis in both *PKC ϵ* ^{-/-} mice⁴⁹ or *Magt1*^{-y} mice. Altered N-glycosylation impairs the coagulation cascade, hemostasis and blood clotting.³ Several N-glycosylation sites (N145, N322 and N360) were identified in factor VII (FVII), and mutation of these glycosylation sites inhibits FVII secretion and induces partial protein degradation.^{54,55} Interestingly, the residue of N360 on FVII is glycosylated by the STT3B-MAGT1 complex.¹⁸ However, in MAGT1-deficient blood plasma, normal levels of inactive and active forms of FVII were detected, indicating a compensatory mechanism, probably regulated by TUSC3. Nevertheless, the recapitulation of the prothrombotic phenotype in *WT BM*^{*Magt1*^{-y}} mice clearly revealed that a MAGT1 deficiency in the hematopoietic system is the primary causative factor of the accelerated thrombus growth.

Inhibition of TRPC6 function in *Magt1*^{-y} mice with both genetic and pharmacological tools reduces platelet aggregation, and also thrombus formation *in vivo*, suggesting a pathophysiological contribution of dysregulated TRPC6 function in diseases with MAGT1 dysfunction. In the present study, we show for the first time that MAGT1 deficiency strongly increased platelet aggregation in ipsilesional vessels of the ischemic brain and thereby accelerates brain infarct progression. Using bone marrow chimeric mice, we could demonstrate that MAGT1-deficient blood cells, including platelets, are the major contributing factors of enhanced stroke development. The findings of our *in vitro* and *in vivo* platelet analyses suggest that Mg²⁺ supplementation or partial inhibition of TRPC6 channel activity could be a promising therapeutic intervention to limit platelet reactivity and possibly infarct progression in functionally deficient MAGT1 patients.

Recent studies showed that MAGT1 is located in the ER membrane and regulates N-glycosylation within the STT3B complex.^{15,17,18} Therefore, MAGT1 deficiency in human patients was proposed as a new type of CDG. It has been shown that TUSC3 may compensate for the lack of MAGT1 function within the STT3B complex since strongly defective N-glycosylation was only found in double deficient *Magt1*^{-/-}*Tusc3*^{-/-} cells, but not in single knockouts.^{15,18} Interestingly, we found normal N-glycosylation of the transferrin receptor in *Magt1*^{-y} platelets (**Figure S12**). Future studies are required to determine the N-glycosylation status of hemostatic factors in MAGT1-deficient mice. Several CDGs with abnormal platelet numbers and dysfunction and abnormal hemostatic response have been described,³ although abnormal N-glycosylation has not been investigated in platelets isolated from these patients. Recently, decreased N-glycosylation was found in plasma cell membrane receptors and blood plasma proteins from MAGT1-deficient patients.^{15,17} HSCT can be a curative treatment in XMEN syndrome.²⁵ However, from 7 patients reported to have undergone HSCT, 4 died of transplant-related complications, including severe infections, hemophagocytic syndrome but also severe mucosal bleeding.²³⁻²⁶ Furthermore, XMEN syndrome has been proposed to cause a qualitative platelet disorder.²⁴ Our results rather

point to prothrombotic effects of MAGT1 deficiency and suggest that bleeding complications in XMEN patients may be the consequence of HSCT-associated aplasia. Additional patient studies are required to understand these hemostatic effects. Interestingly, it has been also shown that elimination of a distal glycosylation site on TRPC6 leads to the constitutive activation of this channel.^{56,57} Thus, we speculate that TRPC6 hypoglycosylation or TRPC6-MAGT1 interaction may account for a prothrombotic response in *Magt1*^{-y} platelets. Using docking studies we modeled the interaction between human MAGT1 and TRPC6 *in silico*,^{58,59} and found a positive correlation (**Figure S13, Table S7**), indicating that direct biochemical interaction may exist, which probably regulates N-glycosylation and channel activity of TRPC6 in megakaryocytes and platelets. Recently, gain of function mutations of TRPC6 showed abnormal platelet reactivity with increased phosphatidylserine exposure and activation of prothrombinase complex.⁶⁰ In HEK 293 cells, the deletion of the mannosyl (α -1,3-)-glycoprotein β -1,2-N-acetylglucosaminyltransferase (MGAT1), which is involved in the process of N-glycosylation, could abrogate increased channel activity of gain of function mutant TRPC6.⁶¹ Hydrogen peroxide increases the channel activity of TRPC6 by modification of thiol groups.⁶² Altogether, these results suggest a complex regulatory role of ROS production, thiol-oxidoreductase activity and N-glycosylation in TRPC6 channel function.

In summary, we conclude that the abolished MAGT1 function strongly enhances signaling via the GPVI signalosome resulting in abnormal function of TRPC6 and PKC. In addition, MAGT1 deficiency could enhance TxA₂ production and ATP release thereby increasing TxA₂ (TP) receptor and purinergic signaling, respectively. Our results suggest that defective MAGT1 function could be a risk factor for the development of arterial thrombosis and stroke triggered by hyperactive platelets. Although TUSC3 plays a unique role in early postnatal development, MAGT1 could compensate for the lack of TUSC3 function in platelets.

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Disclosures

None

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Highlights

- MAGT1 deficiency causes XMEN syndrome in humans, resulting in abnormal platelet function.
- MAGT1 deficiency enhances the release of dense-granule resident Mg^{2+} stores and ATP, and also cyclooxygenase activity in mouse platelets. These abnormalities lead to hyperactivation of the GPVI signaling, dysregulation of PKC function, and consequent acceleration of arterial thrombus formation *in vivo*.
- MAGT1 deficiency increases TRPC6 channel activity in mouse platelets. The observed platelet abnormalities and thrombus formation are normalized by partially inhibiting TRPC6 function, indicating a functional crosstalk between MAGT1, PKC, and TRPC6.
- MAGT1 deficiency leads to platelet hyperresponsiveness and thereby aggravates I/R injury in the brain.

Figure legends

Figure 1. Altered cation homeostasis in *Magt1*^{-/-} platelets downstream of GPVI. (A, B) Detection of granular-resident Mg^{2+} stores in MagFluo4-loaded resting and activated *WT* and *Magt1*^{-/-} platelets by confocal microscopy. Platelets were allowed to adhere to a poly-L-lysine (PLL), (resting) or collagen-coated surface (activated). Phal-647: Alexa Fluor 647 conjugated phalloidin. **(A, B)** Scale bar: 2 μ m. **(C, D)** Fura-2-loaded *WT* or *Magt1*^{-/-} platelets were activated with the indicated agonists in the presence of 2 mM extracellular $CaCl_2$ and changes in $[Ca^{2+}]_i$ were monitored by fluorimetry **(C)** in the presence of 1 mM or **(D)** 5 mM extracellular $MgCl_2$. **(C, D)** Data are presented as median with 95% confidence limits. **(C, D)** Each dot represents one individual mouse per each group. *WT* n=6 mice and *Magt1*^{-/-} n=6 mice. **(C, D)** A nonparametric Mann-Whitney U test was used to identify significant different values. MFI: mean fluorescent intensity, Geo-MFI: geometric mean fluorescence intensity, CRP: collagen-related peptide, ADP: adenosine diphosphate, U46619: stable thromboxane A2 analogue.

Figure 2. Magnesium supplementation normalizes enhanced GPVI-induced aggregation response of *Magt1*^{-y} platelets. (A) Washed platelets from either *WT* or *Magt1*^{-y} mice were stimulated with the indicated agonists and extracellular MgCl₂. Platelet aggregation was measured with light transmission aggregometry and recorded for 10 min. (A) Representative aggregation curves. (B) Washed *WT* or *Magt1*^{-y} platelets were incubated with luciferase-luciferin reagent followed by the addition of the indicated agonists. ATP release was measured by lumi-aggregometry. (C-E) Thromboxane A₂ production of platelets was measured by ELISA quantifying the stable thromboxane B₂ (TxB₂) metabolite in (C) resting conditions and upon activation with (D) CRP and (E) thrombin. (B-E) Data are presented as median with 95% confidence limits. (B-E) Each dot represents one individual mouse per each group. (B) *WT* n=6 mice and *Magt1*^{-y} n=6 mice were analyzed for the measurement of total ATP released in response to collagen, CRP and U46619 and *WT* n=4 mice and *Magt1*^{-y} n=4 mice for the stimulation with thrombin. (C-E) *WT* n=6 mice and *Magt1*^{-y} n=6 mice. (B-E) A nonparametric Mann-Whitney U test was used to identify significant different values.

Figure 3. Magnesium supplementation or inhibition of TRPC6 normalizes enhanced thrombus growth of *Magt1*^{-y} samples *ex vivo*. (A-D) Heparinized whole blood (anti-GPIX-DyLight-488 labeled) of either *WT* or *Magt1*^{-y} mice was perfused at 1700s⁻¹ on a collagen (0.2 mg/mL) coated surface in the presence of (A) MgCl₂ or (B) inhibitors. (A, B) Representative bright field (upper panel) and fluorescence microscopy (lower panel). (A, B) Scale bar: 20 μm. (C) Mean surface coverage and (D) relative thrombus volume (right) were quantified using MetaVue software. (C, D) Data are presented as median with 95% confidence limits. (C, D) Each dot represents one individual mouse per each group. *WT* n=6 mice and *Magt1*^{-y} n=6 mice. (C, D) A nonparametric Mann-Whitney U test was used to identify significant different values. T6i: Aminoindane derivative as TRPC6 inhibitor, 2-APB: 2-Aminoethoxydiphenyl borate, BTP2: [N-(4-methyl-1,2,3-thiadiazole-5-carboxamide), Rel. thr. vol.: relative thrombus volume.

Figure 4. TRPC6 heterozygosity normalizes hyperreactivity of *Magt1*^{-y} platelets. (A) Washed platelets from either *WT*, *Magt1*^{-y} or *Magt1*^{-y}/*Trpc6*^{+/-} mice were stimulated with the indicated agonists in the presence of 1 mM MgCl₂. Platelet aggregation was measured with light transmission aggregometry and recorded for 10 min. Representative aggregation curves are shown for *WT*, *Magt1*^{-y} and *Magt1*^{-y}/*Trpc6*^{+/-} mice. (B-D) Whole blood of *WT*, *Magt1*^{-y} or *Magt1*^{-y}/*Trpc6*^{+/-} mice was perfused on a collagen-coated surface (0.2 mg/mL) at 1700 s⁻¹ and representative bright field (upper panel) and fluorescence microscopy (lower panel) images are shown. (B) Scale bar: 20 μm. (C) Mean surface coverage and (D) relative thrombus volume were quantified using MetaVue software. (C, D) Data are presented as mean ± SD. (C, D) Each dot represents one individual mouse per each group. *WT* n=6 mice, *Magt1*^{-y} n=6 mice and *Magt1*^{-y}/*Trpc6*^{+/-} n=6 mice. (C, D) 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to identify significant different values. Rel. thr. vol., relative thrombus volume.

Figure 5. GPVI-induced and PKC-specific protein phosphorylation in platelets from *WT*, *Magt1*^{-y} and *Magt1*^{-y}/*Trpc6*^{+/-} mice. (A, B) Protein phosphorylation patterns analyzed in Western blots of (A) the GPVI signaling cascade and (B) PKC isoforms in *WT*, *Magt1*^{-y} and *Magt1*^{-y}/*Trpc6*^{+/-} platelets upon CRP stimulation in the presence (left) and absence (right) of 2 mM extracellular CaCl₂. Blots are representative of four independent experiments. See Supplementary Figure 7 for a statistical analysis. CRP, collagen-related peptide.

Figure 6. Increased arterial thrombus formation, clot retraction and shortened bleeding time in *Magt1*^{-y} mice. (A) A filter paper soaked in 13% FeCl₃ was topically applied to mesenteric arterioles to induce a chemical injury. Time to full vessel occlusion was monitored by intravital microscopy of fluorescently labeled platelets in *WT* and *Magt1*^{-y} mice. (A) Data are presented as median with 95% confidence limits. (A) Each dot represents one

individual mouse per each group. *WT* n=19 mice and *Magt1^{-y}* n=21 mice. (A) A nonparametric Mann-Whitney U test was used to compare the mean of vessel occlusion times (40 min maximum, *WT* n=9 mice and *Magt1^{-y}* n=21 mice). (A) Each triangle represents an individual mouse with vessel occlusion after 40 min. (B, C) A filter paper soaked in 6% FeCl₃ was topically applied to the carotid artery to induce a chemical injury and blood flow was monitored with a Doppler flow probe. (B, C) Time to full vessel occlusion was determined and compared in (B) *WT*, *Magt1^{-y}*, *WT BM^{WT}* and *WT BM^{Magt1^{-y}}* and (C) *Magt1^{-y}* and *Magt1^{-y}/Trpc6^{+/-}* mice. (B) Data are presented as mean ± SD. (B) Each dot represents one individual mouse. *WT* n=13 mice, *Magt1^{-y}* n=13 mice, *WT BM^{WT}* n=9 mice and *WT BM^{Magt1^{-y}}* n=9 mice. (B) 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare the mean of vessel occlusion times (1800 sec maximum, *WT* n=13 mice, *Magt1^{-y}* n=13 mice, *WT BM^{WT}* n=8 mice and *WT BM^{Magt1^{-y}}* n=8 mice). (B) Each triangle represents an individual mouse with vessel occlusion after 1800 sec. (C) Data are presented as median with 95% confidence limits. (C) Each dot represents one individual mouse per each group. *WT* n=6 mice and *Magt1^{-y}* n=8 mice. (C) A nonparametric Mann-Whitney U test was used to compare the mean of vessel occlusion times (1800 sec maximum, *WT* n=6 mice and *Magt1^{-y}* n=6 mice). (C) Each triangle represents an individual mouse with vessel occlusion after 1800 sec. (D) Clot retraction in PRP of *WT* and *Magt1^{-y}* mice. (E) Hemostatic function was assessed by resecting 1 mm of the tail tip and collecting blood every 20 sec on filter paper. (E) Data are presented as mean ± SD. (E) Each dot represents one individual mouse per each group. *WT* n=23, *Magt1^{-y}* n=23, *WT BM^{WT}* n=10 and *WT BM^{Magt1^{-y}}* n=10 mice. (E) 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare the mean of tail bleeding times.

Figure 7. *Magt1^{-y}* platelets enhance damage of the brain after focal cerebral ischemia. (A) Infarct volumes after 30 min tMCAO were quantified by planimetry in 2,3,5-triphenyltetrazolium chloride (TTC)-stained coronal brain sections of *WT* or *Magt1^{-y}* mice. Data are presented as median with 95% confidence limits. Each dot represents one individual mouse per each group. *WT* n=6 mice, *Magt1^{-y}* n=6 mice. (A) A nonparametric Mann-Whitney U test was used to identify significant different values. (B) Representative confocal pictures of (tMCAO-induced) brain cryosections stained for nuclei (DAPI - blue), platelets (GPIX - green) and the endothelium (CD105 - red) and (C) quantification of the number of thrombi in the ipsilateral hemisphere are presented. (B) Scale bar: 25 μm (left) and 50 μm (right). Frames indicate magnified area. GPIX-positive staining was quantified in every 5th cryosection (field of view). (C) Data are presented as median with 95% confidence limits. (C) Each dot represents one individual mouse per each group. *WT* n=6 mice and *Magt1^{-y}* n=6 mice. (C) A nonparametric Mann-Whitney U test was used to identify significant different values. (D) Representative hematoxylin and eosin (H&E) stained pictures of (tMCAO-induced) brain cryosections and (E) quantification of thrombi are presented. (D) Scale bar: 100 μm. Asterisk symbols (*) indicate magnified insets. (E) Data are presented as median with 95% confidence limits. (E) Each dot represents one individual mouse per each group. *WT* n=6 mice and *Magt1^{-y}* n=6 mice. (E) A nonparametric Mann-Whitney U test was used to identify significant different values. (F) TTC staining of coronal brain sections of BM chimeric mice of the indicated genotypes 24 h after 30 min tMCAO and (G) planimetric quantification of the infarct volumes in *WT BM^{WT}*, *Magt1^{-y} BM^{WT}* and *WT BM^{Magt1^{-y}}* mice are presented. (G) Data are presented as mean ± SD. (H) Bederson score and (I) Grip test were performed to assess neurological and motoric functions 24 h post tMCAO. (G-I) Each symbol represents one individual mouse per each group. *WT* n=7 mice and *Magt1^{-y}* n=7 mice. (G-I) A nonparametric Mann-Whitney U test was used to identify significant different values.











