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## Signaling role for Mg<sup>2+</sup> revealed by immunodeficiency due to loss of MagT1

Feng-Yen Li<sup>1,6,†</sup>, Benjamin Chaigne-Delalande<sup>1,†</sup>, Chrysi Kanellopoulou<sup>1</sup>, Jeremiah C. Davis<sup>2</sup>, Helen F. Matthews<sup>1</sup>, Daniel C. Douek<sup>3</sup>, Jeffrey I. Cohen<sup>4</sup>, Gulbu Uzel<sup>5</sup>, Helen C. Su<sup>2</sup>, and Michael J. Lenardo<sup>1</sup>

<sup>1</sup>Molecular Development Section, Lymphocyte Molecular Genetics Unit, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

<sup>2</sup>Human Immunological Diseases Unit, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

<sup>3</sup>Human Immunology Section, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

<sup>4</sup>Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

<sup>5</sup>Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

<sup>6</sup>Medical Scientist Training Program, Biomedical Sciences Graduate Program, University of California-San Francisco, San Francisco, CA

### Summary

The magnesium ion, Mg<sup>2+</sup>, is essential for all life as a cofactor for ATP, polyphosphates such as DNA and RNA, and metabolic enzymes, but whether it plays a role in intracellular signaling similar to Ca<sup>2+</sup> is unknown. In this study, we identify mutations in the magnesium transporter gene, *MAGT1*, in a novel X-linked human immunodeficiency characterized by CD4 lymphopenia, severe chronic viral infections, and defective T lymphocyte activation. We demonstrate that a rapid transient Mg<sup>2+</sup> influx is induced by antigen receptor stimulation in T cells or growth factor

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Correspondence and requests for materials should be addressed to M.J.L. (lenardo@nih.gov).

<sup>†</sup>These authors contributed equally

**Author Contributions** F-Y.L. characterized the *MAGT1* mutations and the TCR activation defect in the patients. B.C.D. characterized the Mg<sup>2+</sup> influx and the signaling defects. B.C.D., F-Y.L., H.S. and M.J.L. conceived and planned the experiments as well as prepared the manuscript. J.D. performed the ionization assay. C.K. performed the RT-PCR experiments. G.U., J.I.C. and H.S. referred patients and provided clinical data. H.M. coordinated clinical protocol and samples collection. D.C.D. provided assistance with sequencing and flow cytometry and guided some patient assessments. All authors discussed and revised the manuscript.

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stimulation in non-lymphoid cells. MagT1 deficiency abrogates the  $Mg^{2+}$  influx leading to impaired responses to antigen receptor engagement including defective activation of phospholipase  $C\gamma$  and a markedly impaired  $Ca^{2+}$  influx in T cells but not B cells. These observations reveal a role for  $Mg^{2+}$  as an intracellular second messenger and identify MagT1 as a possible target for novel therapeutics.

## Introduction

$Mg^{2+}$  is the most abundant divalent cation in mammalian cells and is an essential cofactor for ATP, nucleic acids, and numerous enzymes in animals and plants<sup>1-3</sup>. Whether it serves as a second messenger in intracellular signalling is controversial<sup>4-8</sup>.  $Ca^{2+}$ , another alkali earth metal, is well-established as a second messenger because free intracellular  $[Ca^{2+}]_i$  is 0.1  $\mu M$  and extracellular  $[Ca^{2+}]_e$  is 1 mM, creating a steep gradient that favors voltage- and ligand- gated  $Ca^{2+}$  influx signals<sup>9</sup>. By contrast,  $[Mg^{2+}]_i$  is 10-30 mM and mostly complexed with ATP and other molecules. Only 1-5% (0.2 to 1 mM) is cytosolic free  $Mg^{2+}$ . However, this is 100-fold below its electrochemical equilibrium potential, which theoretically allows regulated  $Mg^{2+}$  influxes<sup>6,10</sup>.  $Mg^{2+}$  has been found to enhance lymphocyte activation in suboptimal  $Ca^{2+}$  concentrations by phytohemagglutinin (PHA) but not ionomycin<sup>11-13</sup>. Since ionomycin bypasses proximal TCR signals, optimal T cell activation could require a magnesium-generated process upstream of  $Ca^{2+}$  signaling.  $Mg^{2+}$  sensitive probes have revealed changes in  $[Mg^{2+}]_i$  in lymphocytes following lectin stimulation<sup>14,15</sup>. Nevertheless, how extracellular  $Mg^{2+}$  promotes cellular activation signals is unknown.

The molecular elucidation of primary immunodeficiencies (PIDs) often yields novel insights into lymphocyte signal transduction<sup>16-18</sup>. T cell receptor (TCR) signaling is critical for T lymphocyte selection during ontogeny and for peripheral responses against foreign pathogens<sup>19,20</sup>. Mutations in the Zeta-chain-associated protein kinase of 70 kDa (ZAP70) tyrosine kinase in severe combined immunodeficiency (SCID) patients illustrated its role in thymic development of CD8+ T cells and peripheral T cell activation<sup>19,21</sup>. Likewise, genetic defects in SCID patients in *Orai1* revealed that it was a critical store-operated  $Ca^{2+}$  channel<sup>22</sup>. Idiopathic CD4 lymphocytopenia (ICL) is a rare immunodeficiency in which circulating CD4+ T cell counts are  $<300/mm^3$  or  $<20\%$  of total T cells in the absence of HIV infection or other causes of lymphopenia<sup>23-25</sup>. The etiology of ICL is heterogeneous with no infectious cause identified and a genetic etiology suspected in some cases<sup>26-29</sup>. Here, we have uncovered a second messenger role for  $Mg^{2+}$  in receptor-induced phospholipase  $C\gamma 1$  (PLC $\gamma 1$ ) activation and  $Ca^{2+}$  signaling by discovering an X-linked mutation in *MAGT1*, a highly selective transporter for  $Mg^{2+}$ , in a subset of ICL patients.

## Immune and gene defects in ICL patients

We examined two young brothers (patient A.1 and A.2) who exhibited recurrent infections, including chronic Epstein-Barr virus (EBV) infection and low CD4+ T cell counts; other lymphocyte populations were normal or elevated, and immunoglobulin levels and vaccine responses were only intermittently defective (Supplementary Table 1, Supplementary Fig. 1a, b). Both patients had an inverted CD4:CD8 ratio and reduced CD31<sup>+</sup> cells in the naïve

(CD27<sup>+</sup>, CD45RO<sup>-</sup>) CD4<sup>+</sup> T cell populations suggesting diminished thymic output<sup>30-32</sup> (Fig. 1a, b). However, we also found pronounced defects in TCR-mediated activation events including CD69, CD25, Fas (CD95), and CTLA-4 upregulation following OKT3 (agonistic  $\alpha$ CD3) stimulation (Fig. 1c, Supplementary Table 1, Supplementary Fig. 2a). Early TCR signaling events, such as NF- $\kappa$ B and NFAT nuclear translocation, were impaired (Fig. 1e, f). By contrast, the patients' T cells were fully activated by the downstream inducers phorbol 12-myristate 13-acetate and ionomycin (PMA/Iono), implying a proximal TCR activation defect (Fig. 1c, e, f). The patients showed no defect in B cell receptor (BCR) or toll-like receptor (TLR) stimulation of B cells (Fig. 1d, Supplementary Fig. 2b).

Given that family A harbored two affected boys (Fig. 2a), we tested for X chromosome linkage by assessing lyonization, the process of X chromosome inactivation by methylation in females<sup>33</sup>. Skewed lyonization reflects the reduced fitness of stem cells due to a deleterious X chromosome mutation. We digested genomic DNA with the methylation-sensitive restriction endonuclease *HpaII* to eliminate all active non-methylated X-chromosome DNA and analyzed the remaining undigested (inactive) X chromosome by PCR at an indicator locus<sup>33</sup>. The mother of the two affected boys exhibited completely skewed lyonization with only the X chromosome inherited by her two sons inactivated in her T cells, strongly suggesting that she carries an X-linked genetic defect (Fig. 2b).

We therefore performed X-chromosome exon-capture and single-end next-generation sequencing on the mother and the two boys, yielding 18-20 million reads per subject with at least 10x coverage in ~90% of target regions. This revealed a 10 bp deletion in the two brothers present in *MAGT1* (Fig. 2c, Supplementary Fig. 3a), a gene encoding a magnesium transporter<sup>34-36</sup>. This deletion was not detected in the mother's cDNA or 100 normal individuals (data not shown). The deletion removes a splice donor site located in the 3' exon-intron junction of exon 7, and was present in the grandmother and great-grandmother of the patients (Fig. 2a, c, Supplementary Fig. 3a). The patients' mutant *MAGT1* splice variant was ~150 bp smaller than the mother's normal splice variant of approximately 1100 bases and missing both exon 7 and 8, leading to a premature stop codon (Supplementary Fig. 3b, c). Apparent nonsense-mediated decay caused markedly decreased mRNA expression (Fig. 2d). The MagT1 protein was undetectable in the patient cells by Western blot or immunofluorescent cell surface staining (Fig. 2e, f).

Additional screening yielded another immunodeficient patient (B.1) with chronic EBV infection and a nonsense mutation in exon 3 of *MAGT1*, leading to a 90% decrease in mRNA expression (Fig. 2a, c, d, Supplementary Table 1, Supplementary Fig. 4a). The patient died five years ago from chronic EBV-associated lymphoma at the age of 45. Like the other two patients, patient B.1 exhibited a similar T cell defect in NF- $\kappa$ B and NFAT nuclear translocation in response to TCR but not PMA/Iono stimulation (Supplementary Fig. 4b, c).

## TCR-induced Mg<sup>2+</sup> and Ca<sup>2+</sup> influx defects

MagT1 is a mammalian Mg<sup>2+</sup>-selective transporter whose physiologic function is not well understood<sup>34,35</sup>. The full-length protein of 367 amino acids encodes a signal peptide, a large

N-terminal segment, four transmembrane domains, and a small C-terminal tail with little similarity to any other known transporter except TUSC3, a nonselective  $Mg^{2+}$  transporter<sup>36</sup>. We measured ion uptake in normal and patients' lymphocytes using fluorescent probes sensitive for  $Ca^{2+}$  (fluo3-AM and Fura red-AM) or  $Mg^{2+}$  (Magfluo4-AM), which exhibited no detectable cross-reactivity (Supplementary Fig. 5a). Initial experiments showed a low basal level of free  $Mg^{2+}$  and defective passive  $Mg^{2+}$  influx, whereas that for  $Ca^{2+}$  was normal (Supplementary Fig. 5a). The total  $Ca^{2+}$  and  $Mg^{2+}$  levels in the patients' T cells determined by inductively coupled plasma mass spectrometry were normal, indicating that MagT1 deficiency chiefly affects free  $Mg^{2+}$  and that general metabolic processes requiring bound  $Mg^{2+}$  should not be affected (Supplementary Fig. 5b).

We next examined whether various TCR stimuli would affect free  $Mg^{2+}$  transport. We observed a robust transient  $Mg^{2+}$  influx together with the well-documented  $Ca^{2+}$  influx in normal T cells stimulated with various TCR agonists (Fig. 3a). The apparent  $Mg^{2+}$  influx was not due to cross-detection of the  $Ca^{2+}$  influx because the specific  $Ca^{2+}$  chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) abolished the  $Ca^{2+}$  fluorescence but not the  $Mg^{2+}$  fluorescence (Supplementary Fig. 6a). The  $Mg^{2+}$  influx was not detectable in the patients' T cells even with the strongest TCR agonist tested (5  $\mu$ g/ml  $\alpha$ CD3) (Fig. 3b, c). Surprisingly, we also found that the  $Ca^{2+}$  influx was severely compromised in the patient T cells across a broad dose range of  $\alpha$ CD3 (Fig. 3b, c). The TCR-induced  $Mg^{2+}$  influx was selective since stimulation of T cells with secondary lymphoid tissue chemokine (SLC/CXCL21), Fas-ligand (FasL), and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) caused no  $Mg^{2+}$  influx and the  $Ca^{2+}$  influxes induced by SLC and FasL were normal in the patient cells (Supplementary Fig. 6b). Also, no  $Mg^{2+}$  influx was discernible in B lymphocytes following anti-IgM and anti-CD40 stimulation, and the ensuing  $Ca^{2+}$  influx was not diminished in patient B cells (Supplementary Fig. 6c). Although the patient B cells exhibited reduced basal free  $Mg^{2+}$ , their B cell activation was normal (Fig. 1d).

We next examined the hypothesis that the defective  $Ca^{2+}$  influx in the patients was secondary to the loss of the  $Mg^{2+}$  influx. We first explored the relationship between the TCR stimulated influxes in normal T cells by modulating  $[Mg^{2+}]_e$  and  $[Ca^{2+}]_e$  in the extracellular buffer. Both  $Mg^{2+}$  and  $Ca^{2+}$  influxes were optimal when  $[Mg^{2+}]_e$  and  $[Ca^{2+}]_e = 1$  mM, respectively, but abolished when  $[Mg^{2+}]_e$  and  $[Ca^{2+}]_e = 0$ , respectively (Fig. 4a). Moreover, when  $[Mg^{2+}]_e = 0$ , the  $Ca^{2+}$  influx was decreased, but when  $[Ca^{2+}]_e = 0$ , the  $Mg^{2+}$  influx was unaffected (Fig. 4a). These results show that the TCR-induced  $Ca^{2+}$  influx is partially dependent on  $[Mg^{2+}]_e$ . Thus, we infer that the  $Ca^{2+}$  influx defect in T cells associated with MagT1 deficiency is secondary to the loss of the TCR-stimulated  $Mg^{2+}$  influx. Conversely, when  $[Mg^{2+}]_e = 0$ , the  $Ca^{2+}$  influx in B cells after the BCR stimulation is normal, which is consistent with the absence of BCR-induced  $Mg^{2+}$  influxes (Fig. 4b, Supplementary Fig. 6b). Thus, the loss of the TCR-induced  $Ca^{2+}$  influx in the absence of extracellular  $Mg^{2+}$  is not likely to be due to a deficiency in  $Mg^{2+}$  as a cofactor required for ATP-dependent processes since B cells had no comparable defect. This is underscored by the fact that both B and T cells from the MagT1 deficient patients had the same partial reduction in the free  $[Mg^{2+}]_i$ , but only the latter exhibited functional defects in antigen receptor signaling.

To determine whether  $Mg^{2+}$ -promoted  $Ca^{2+}$  influxes are important in other tissues, we treated two transformed epithelial cell lines, A549 and HepG2, with epidermal growth factor (EGF), which is known to cause a  $PLC\gamma 1$ -dependent  $Ca^{2+}$  influx analogous to that induced by TCR engagement<sup>37</sup>. In each cell type, we observed a clear dose-dependent influx of  $Mg^{2+}$  induced by EGF that was abrogated when  $[Mg^{2+}]_e = 0$  (Fig. 4c, Supplementary Fig. 7). The EGF-induced  $Ca^{2+}$  influx was also decreased, though not abolished, by  $Mg^{2+}$  depletion. By contrast, carbachol, an agonist for acetylcholine receptors that induces a  $Ca^{2+}$  influx through  $PLC\beta$ , failed to induce a  $Mg^{2+}$  influx. In addition, the carbachol-induced  $Ca^{2+}$  influx was not altered when  $[Mg^{2+}]_e = 0$ . Thus, receptor-induced  $Mg^{2+}$  influxes that promote  $Ca^{2+}$  influxes occur in both lymphoid and non-lymphoid tissue types. Moreover, receptors that trigger a  $Ca^{2+}$  influx through  $PLC\gamma 1$ , but not  $PLC\beta$  or  $PLC\gamma 2$  (BCR) can induce a  $Mg^{2+}$  influx to regulate the  $Ca^{2+}$  influx.

### Knockdown and reconstitution of MagT1

To demonstrate that decreased MagT1 expression can account for the immunological and signaling defects observed in the patients, we knocked down MagT1 in normal human T cells by transient siRNA transfections. We found that the TCR-stimulated  $Mg^{2+}$  and  $Ca^{2+}$  influxes were decreased in proportion with the degree of MagT1 mRNA knockdown (Fig. 5a, Supplementary Fig. 8a). Similar to the patients' T cell phenotype, MagT1 knockdown impeded TCR-induced p65 nuclear translocation (Fig. 5b, Supplementary Fig. 8b). These results verify that MagT1 is necessary for normal T cell activation.

To determine whether MagT1 deficiency is sufficient to explain the patients' functional defects, we reconstituted MagT1 expression by lentiviral transduction of patient T cells. Positively transduced cells marked by a coexpressed fluorescent marker (mCherry) were examined by live cell confocal imaging of the influxes. We found that expressing wild type MagT1 in the patients' T cells restored a TCR-stimulated  $Mg^{2+}$  influx (Fig. 5c). MagT1 restoration also improved the TCR-stimulated  $Ca^{2+}$  influx, thereby validating our conjecture that it is contingent upon the  $Mg^{2+}$  influx (Fig. 5c). The expression of MagT1 also augmented other activation events of the patients' T cells, such as TCR-induced CD69 upregulation (Fig. 5d). Thus, MagT1 is necessary and sufficient for the  $Mg^{2+}$  influx required for optimal T cell activation, and MagT1 deficiency is the proximate cause of the T cell activation defect in this PID.

### Loss of MagT1 impairs $PLC\gamma 1$ activation

To understand the molecular mechanism of the  $Ca^{2+}$  influx defect observed in MagT1-deficient patients, we examined proximal TCR signaling components (Fig. 6d). TCR engagement causes clustering and phosphorylation of the  $CD3\zeta$  chain by the Src-family leukocyte-specific protein tyrosine kinase (Lck) and subsequent recruitment of the protein tyrosine kinase ZAP70, which phosphorylates the scaffold proteins Linker of Activated T cells (LAT) and Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP76)<sup>20,38</sup>. These phosphorylated scaffolds then bind Inducible T cell kinase (Itk), which activates  $PLC\gamma 1$  and thereby generates inositol triphosphate ( $IP_3$ ) and diacylglycerol (DAG) as second messengers to trigger  $Ca^{2+}$  mobilization and protein kinase C $\theta$  (PKC $\theta$ ) activation,

respectively. The  $\text{Ca}^{2+}$  influx modulates the protein phosphatase calcineurin, which together with PKC $\theta$ , activates downstream transcription factors such as NF- $\kappa$ B and NFAT<sup>39</sup>. We found that TCR cluster formation and LAT and PLC $\gamma$ 1 recruitment to these clusters were intact in patient T cells after  $\alpha$ CD3 stimulation (Fig. 6a, Supplementary Fig. 9). Moreover, early TCR activation events including the phosphorylation of CD3 $\zeta$ , Lck, ZAP70 and LAT induced by TCR ligation were normal in the patient T cells using flow cytometric staining (Supplementary Fig. 10) and western blots (Fig. 6b). Again, this normal series of activation events excludes a general defect in  $\text{Mg}^{2+}$  as a co-factor for ATP-requiring processes accounting for the TCR signaling defects in MagT1 deficient T cells. By contrast, PLC $\gamma$ 1 activation was markedly delayed by almost one hour in the patient T cells compared to healthy control T cells (Fig. 6b). Moreover, the activating phosphorylation of PKC $\theta$  and IP<sub>3</sub> generation downstream of PLC $\gamma$ 1 were significantly reduced (Fig. 6b, c). On the other hand, we found that TCR signaling events that do not require PLC $\gamma$ 1 such as the phosphorylation of the mitogen activated protein kinases (MAPKs) p38 and Erk1/2 were intact in the patient T cells (Fig. 6b). This deficiency in PLC $\gamma$ 1 and PKC $\theta$  activation following TCR stimulation was recapitulated by RNAi silencing of *MAGT1* in normal T lymphocytes (Supplementary Fig. 11). Thus,  $\text{Mg}^{2+}$  can regulate signal transduction pathways involving PLC $\gamma$ 1 in lymphoid and non-lymphoid cells, and MagT1-deficiency profoundly delays the activation of the PLC $\gamma$ 1 branch of TCR signaling.

## Discussion

$\text{Mg}^{2+}$  is an essential cofactor for polyphosphate compounds, the phosphoryl transfer of ATP-dependent enzymes, and the stability of membrane phospholipids<sup>1-3</sup>. The possibility that free  $\text{Mg}^{2+}$  can act as a second messenger in rapid intracellular signaling has been debated<sup>4-8</sup>. We have found a biologically important transient  $\text{Mg}^{2+}$  influx mediated by MagT1 during T cell activation and EGF stimulation of epithelial cells. Sutherland articulated three fundamental features of a second messenger: 1) its levels must increase rapidly in response to a stimulus which is typically the engagement of a cell surface receptor (first messenger), 2) it needs to alter the rate of one or more cellular processes, and 3) it exerts cell-type specific activity because different cells harbor different complements of enzymes<sup>40</sup>. We find that  $\text{Mg}^{2+}$  fulfills these requirements and is a kinetic regulator of signaling in T cells. We found no difference in total  $\text{Mg}^{2+}$  content between our patients and healthy controls, indicating that MagT1 does not influence general  $\text{Mg}^{2+}$  homeostasis or its cofactor function. Rather, we measured rapid and transient free  $\text{Mg}^{2+}$  influxes after TCR engagement which depend on the MagT1 transporter. These transient  $\text{Mg}^{2+}$  influxes increased the rate of PLC $\gamma$ 1 activation and the corresponding  $\text{Ca}^{2+}$  influx in T cells. B cells, which depend on PLC $\gamma$ 2 for the BCR-induced  $\text{Ca}^{2+}$  influx, have no such requirement for MagT1-mediated  $\text{Mg}^{2+}$  influxes despite their expression of MagT1. Since ions diffuse rapidly as second messengers, a  $\text{Mg}^{2+}$  influx may promote rapid spatial integration of antigen and costimulatory receptor signals critical for T cell activation. Our findings shed new light on older observations that extracellular magnesium promotes lymphocyte activation synergistically with calcium by T cell but not B cell mitogens and that mice fed  $\text{Mg}^{2+}$  deficient diets have lower calcineurin activity in their splenocytes<sup>11-13,41,42</sup>. It will

also be of interest to determine if defective thymopoiesis caused by disruption of the TRPM7  $Mg^{2+}$  channel could be due to the loss of the signaling function of  $Mg^{2+}$  influxes<sup>43</sup>.

The patients examined in this study have a novel X-linked PID impairing thymic production of CD4+ T cells and circulating T cell function which we now denominate XMEN (X-link immunodeficiency with magnesium defect and EBV infection and neoplasia) disease. The defective development and function of T cells in XMEN patients categorizes them as SCID patients, although they have a milder phenotype comprising chiefly uncontrolled viral infections consistent with a T cell specific defect<sup>44</sup>. They also share features with X-linked lymphoproliferative disease (XLP) except for the absence of NKT cell deficits or hemophagocytic lymphohistiocytosis<sup>45</sup>. Like XMEN patients, mice with PLC $\gamma$ 1 deficient T cells have fewer CD4+ T cells and defective TCR activation, but they also have features we did not observe in the patients such as impaired ERK activation, lower CD8+ and FoxP3 regulatory T cells, and inflammatory/autoimmune symptoms<sup>46</sup>. The fact that XMEN patients only have a kinetic block in PLC $\gamma$ 1 activation whereas the mice have a complete deficiency could account for these differences.

Finally, the integrity of early TCR activation events in the patient T cells explains how MagT1 acts as a TCR-gated transporter that controls a new step in the later contingent series of TCR signaling events<sup>47</sup> (Fig. 6d). The selective requirement of this  $Mg^{2+}$  influx for the activation of T cells but not B cells suggests that MagT1 may be a potentially useful therapeutic target for diseases requiring T cell specific immunomodulation.

## Methods Summary

All human subjects in this study provided informed consent in accordance with Helsinki principles to be enrolled in research protocols approved by the institutional review board of the National Institute of Allergy and Infectious Diseases, NIH. Patient and healthy control peripheral blood mononuclear cells (PBMC) were Ficoll-purified and activated with OKT3 for 3 days and then continuously cultured in 10% RPMI media supplemented with 100 U/mL IL-2 for 3-4 weeks. For assessment of primary stimulation, cells were harvested and stained with  $\alpha$ CD2,  $\alpha$ CD4 and  $\alpha$ CD8 antibodies, and various activation markers for 30 min at 4°C and analyzed with FACS Calibur or LSRII flow cytometer (BD Biosciences). Activated T cells were restimulated with various T cell activation agents and processed for immunofluorescence imaging, immunoblotting, and influx assays as described in supplemental methods. Genomic DNA isolated from activated T cells was prepared for ligation assays, SureSelect Human X Chromosome (Agilent) target enrichment, single-read Solexa sequencing on Illumina Genome Analyzer Iix, and Sanger sequencing according to the manufacturers instructions. All P values were calculated with the Students t-test using PRISM software (GraphPad Software), with two-tailed distribution.

Full Methods and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

## Supplementary Material

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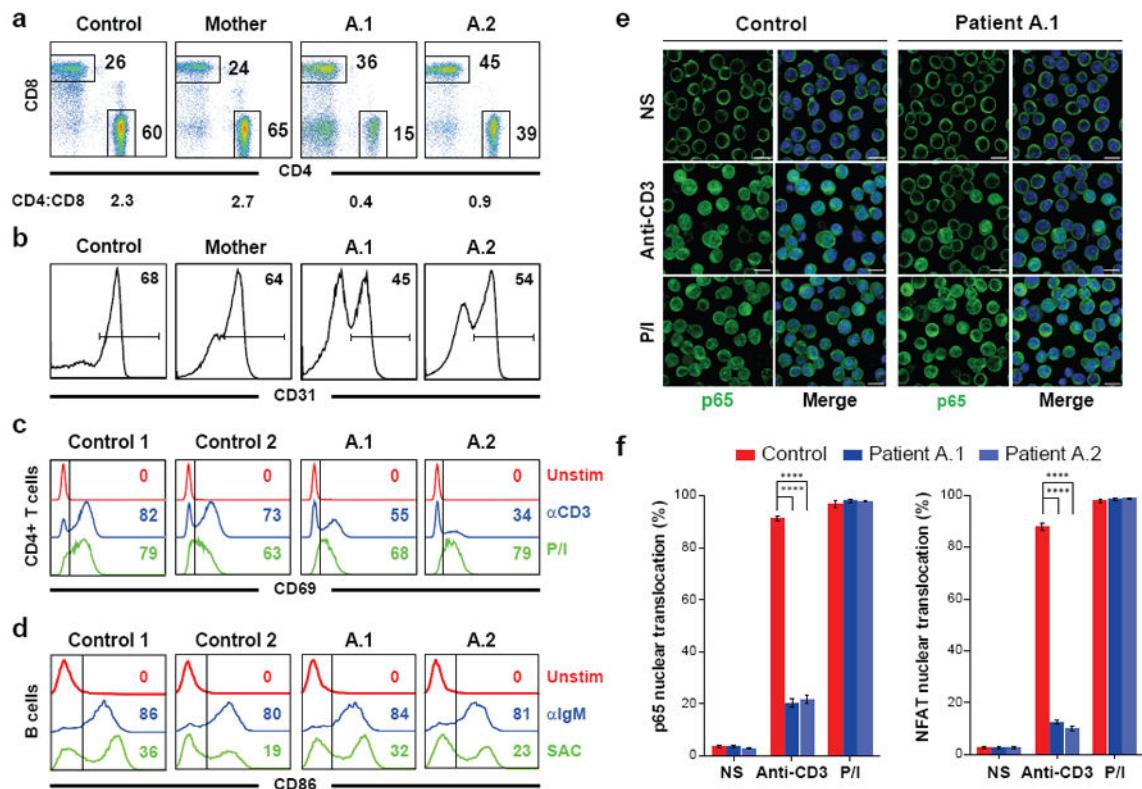
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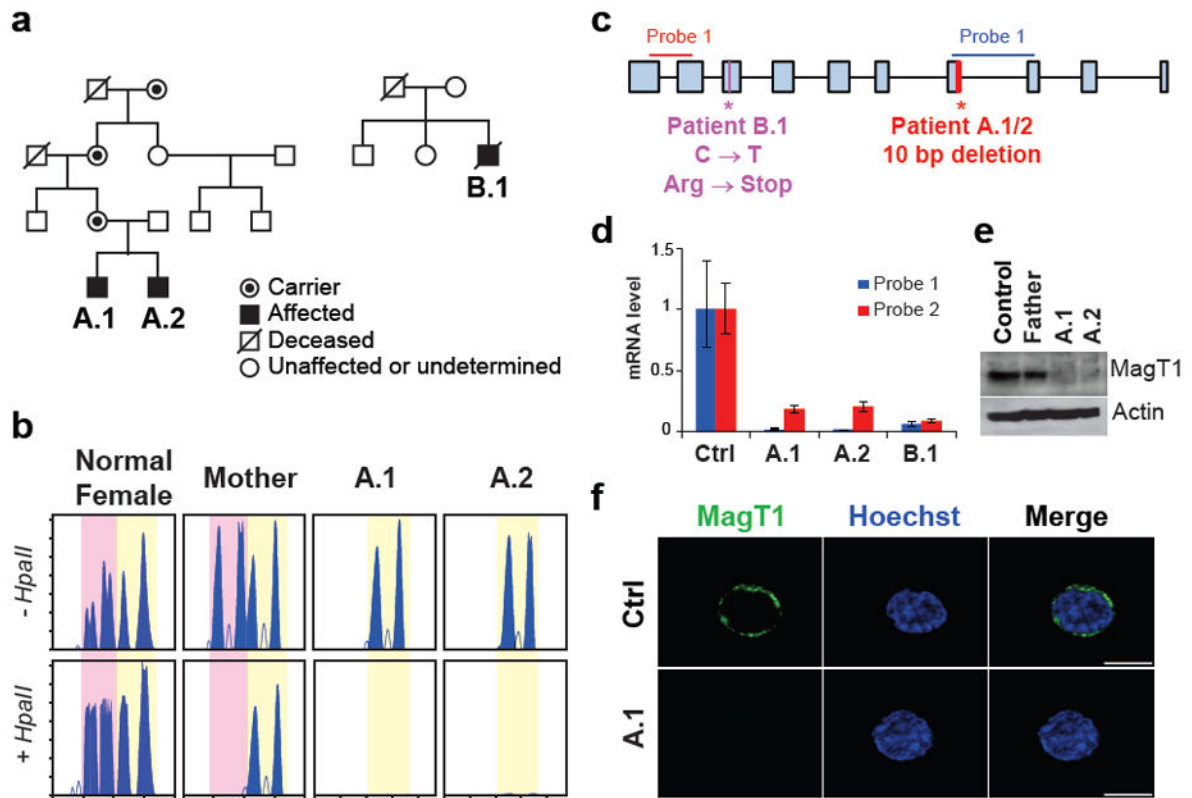
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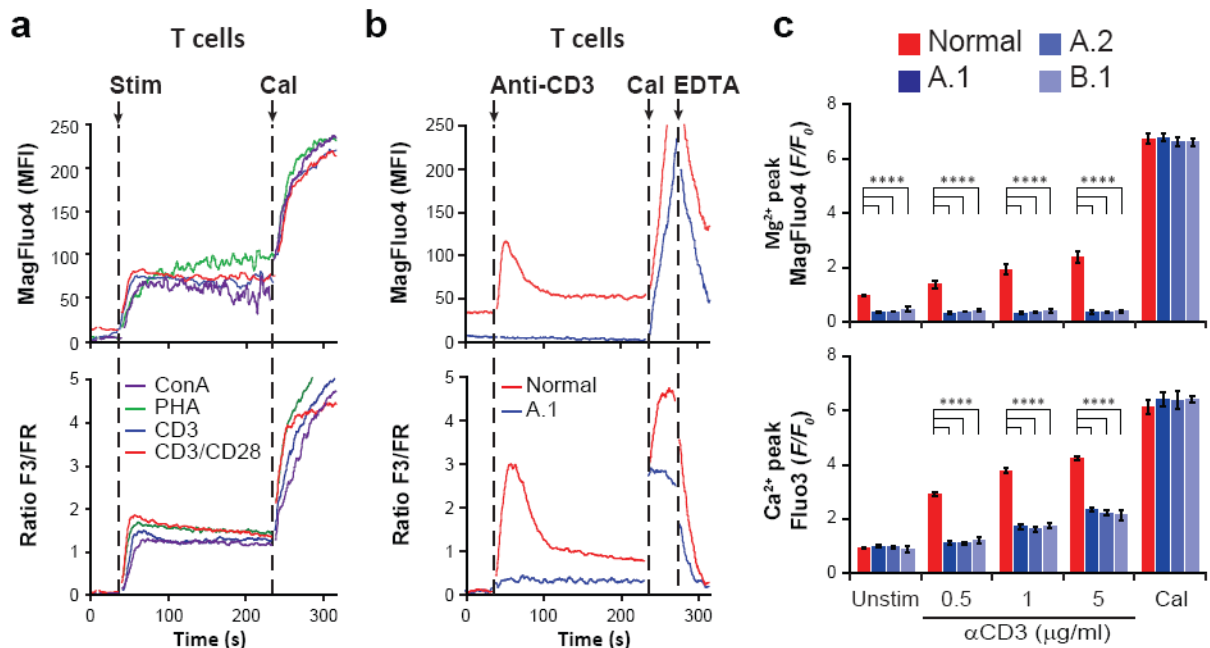
**Figure 1. Patients have a proximal TCR activation defect**

**a**, T cell CD4 and CD8 expression and ratio. **b**, CD31 expression in naïve CD4<sup>+</sup> CD3<sup>+</sup> T cells. **c**, CD69 expression in CD4<sup>+</sup> T cells after 5  $\mu$ g/ml anti-CD3 ( $\alpha$ CD3) stimulation, PMA/Ionomycin (P/I) or unstimulated (Unstim). **d**, CD86 surface expression in purified B cells after stimulation with anti-IgM, SAC or unstimulated (Unstim). **e**, Confocal imaging of p65 nuclear translocation after  $\alpha$ CD3 or P/I stimulation (scale bar: 10  $\mu$ m). **f**, Percent cells with p65 (left) and NFAT (right) nuclear translocation. Numbers represent percent cells in indicated gates. Error bars represent s.e.m. (n=3), \*\*\*\* (P<0.0001).



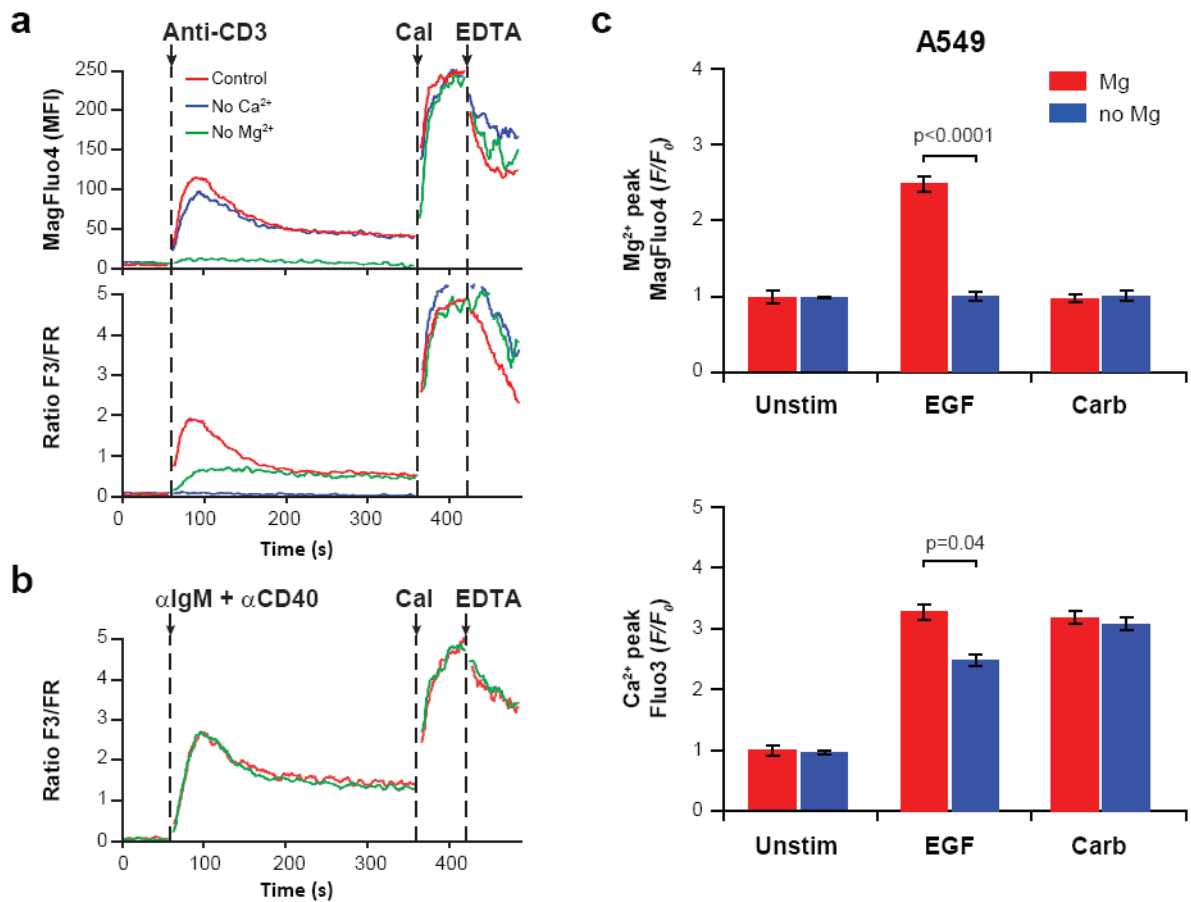
**Figure 2. Patients have MAGT1 null mutations and defective uptake of  $Mg^{2+}$**

**a**, Pedigree of the families A (left) and B (right). **b**, X-chromosome inactivation assay. Peaks of PCR products from the different alleles are highlighted in yellow and pink. **c**, Schematic representation of the *MAGT1* gene exons (boxes) and introns (lines), the mutations (\*) and the probes used for RT-PCR. **d**, RT-PCR showing decreased expression of *MAGT1* mRNA in T cells. **e**, Expression of MagT1 and actin control in T cells by immunoblot. **f**, Confocal images of T cells stained with anti-MagT1 antibody (scale bar: 5  $\mu$ m).



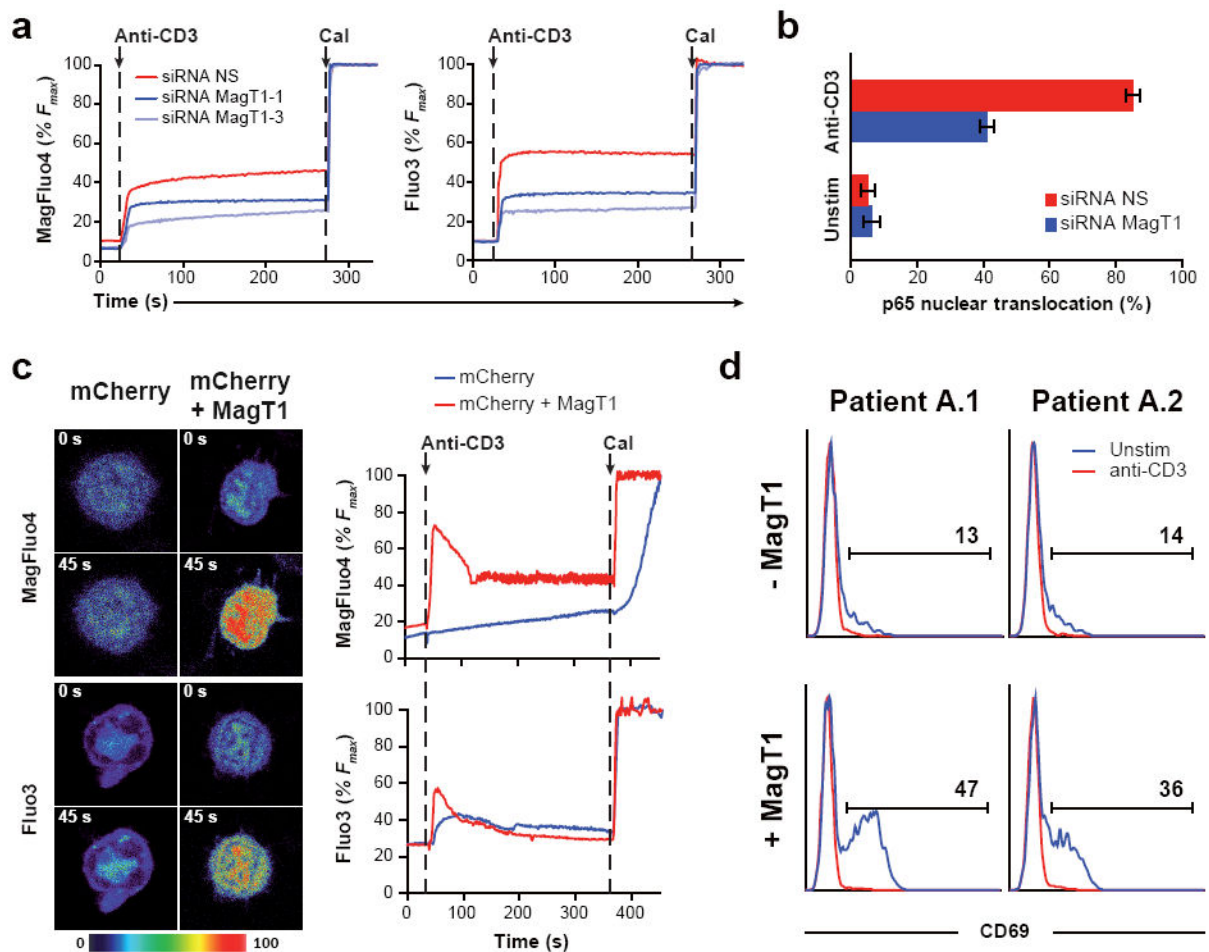
**Figure 3. TCR stimulation induces a MagT1-dependent Mg<sup>2+</sup> influx**

Mg<sup>2+</sup> (upper panels) and Ca<sup>2+</sup> (lower panels) flux: **a**, Fluxes in normal PBMC stimulated with ConA, PHA, αCD3, or αCD3/αCD28. **b**, Fluxes in healthy control T cells or Patient A. 1 after 5 μg/ml αCD3 stimulation. **c**, Peak value of the fluxes in healthy control T cells and the 3 patients upon stimulation with indicated αCD3 concentrations. Error bars represent s.e.m. (n=3), \*\*\*\* (P<0.0001).



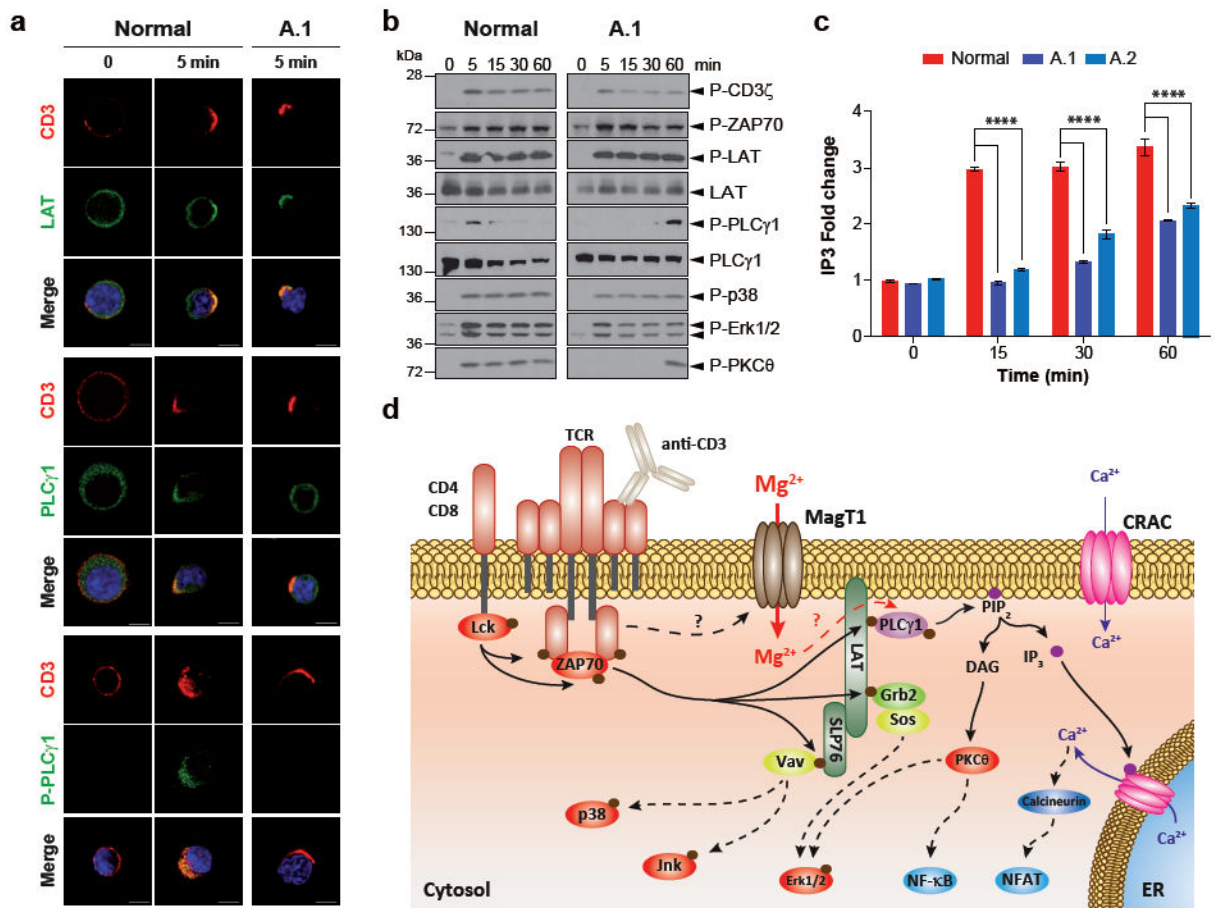
**Figure 4. Requirement of receptor-stimulated Mg<sup>2+</sup> flux for signaling**

**a**, Mg<sup>2+</sup> (MagFluo4, upper panel) and Ca<sup>2+</sup> (Ratio F3/FR, lower panel) flux in healthy control T cells stimulated with  $\alpha$ CD3 in buffers containing 1mM Mg<sup>2+</sup> and Ca<sup>2+</sup> (control) or lacking either ion. **b**, Ca<sup>2+</sup> flux in B cells stimulated with  $\alpha$ IgM and  $\alpha$ CD40. Calcimycin (Cal) and EDTA addition display control influx and ion chelation, respectively. **c**, Graphs represent the fold change of the peak of Mg<sup>2+</sup> (upper panels) and Ca<sup>2+</sup> (lower panels) flux in A549 cells either unstimulated (Unstim) or stimulated with epidermal growth factor (EGF) or carbachol (Carb) as indicated. Error bars represent s.e.m. (n=3).



### Figure 5. Knockdown and rescue of MagT1

Healthy T cells transfected with non-specific (NS) or MagT1 siRNAs. **a**,  $Mg^{2+}$  (MagFluo4, left) and  $Ca^{2+}$  (Fluo3, right) flux upon  $\alpha$ CD3 stimulation. **b**, Percent nuclear p65 after MagT1 knockdown. Error bars represent s.e.m. ( $n=3$ ). **c**, Time-lapse imaging (left, s = sec) or cytometry (right) of  $Mg^{2+}$  (upper) and  $Ca^{2+}$  (lower) flux in T cells transduced with lentiviruses expressing mCherry or mCherry + MagT1. **d**, Flow cytometry of CD69 expression on CD4+ T cells transduced with lentiviruses expressing MagT1 or not, either unstimulated (Unstim) or after anti-CD3 stimulation. Percent cells are shown for the indicated gates. Calcimycin (Cal) displays control influx.



**Figure 6. MagT1 deficiency impairs PLC $\gamma$ 1 activation upon TCR stimulation**

**a**, Confocal images of TCR clustering induced by  $\alpha$ CD3. Cells were stained for LAT, PLC $\gamma$ 1 or phospho-PLC $\gamma$ 1. (Scale bar: 5  $\mu$ m). Immunoblot of the indicated signaling proteins and phosphoproteins (**b**) and quantification of cellular IP3 level (**c**) in healthy control and patient T cells stimulated with  $\alpha$ CD3 for indicated times. Error bars represent s.e.m. (n=3). \*\*\*\*(P<0.0001). **d**, Hypothetical schematic depicting how the MagT1 mediated Mg $^{2+}$  influx participates in TCR signaling. Solid arrows indicate direct effects; dotted arrows indicate indirect effects.