Published in final edited form as:

Science. 2013 July 12; 341(6142): 186–191. doi:10.1126/science.1240094.

Mg²⁺ regulates cytotoxic functions of NK and CD8 T cells in chronic EBV infection through NKG2D

Benjamin Chaigne-Delalande^{1,†}, Feng-Yen Li^{1,12,†}, Geraldine M. O'Connor², Marshall J. Lukacs¹, Ping Jiang¹, Lixin Zheng¹, Amber Shatzer³, Matthew Biancalana¹, Stefania Pittaluga⁴, Helen F. Matthews¹, Timothy J. Jancel⁵, Jack J. Bleesing⁶, Rebecca A. Marsh⁶, Taco W. Kuijpers⁷, Kim E. Nichols⁸, Carrie L. Lucas¹, Sunil Nagpal⁹, Huseyin Mehmet⁹, Helen C. Su¹⁰, Jeffrey I. Cohen³, Gulbu Uzel¹¹, and Michael J. Lenardo^{1,*}

¹Molecular Development of the Immune System Section, Lymphocyte Molecular Genetics Unit, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD ²Cancer and Inflammation Program, Laboratory of Experimental Immunology, Frederick National Laboratory for Cancer Research, Frederick, MD ³Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD ⁴Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD ⁵Clinical Center Pharmacy Department, National Institutes of Health, Bethesda, MD ⁶Division of Bone Marrow Transplantation and Immune Deficiency, Cincinnati Children's Hospital Medical Center, OH ⁷Pediatric Hematology, Immunology and Infectious Diseases, Academic Medical Center, Amsterdam, The Netherlands ⁸Division of Oncology The Children's Hospital of Philadelphia, Philadelphia PA ⁹Respiratory & Immunology Division, Merck Research Laboratories, Boston, MA 02115 Human ¹⁰Immunological Diseases Unit, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD ¹¹Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD ¹²Biomedical Sciences Graduate Program, University of California-San Francisco, San Francisco, CA

Abstract

The magnesium transporter 1 (MAGT1) is a critical regulator of basal intracellular free Mg²⁺ levels. Individuals with genetic deficiencies in *MAGT1* have high levels of Epstein-Barr virus (EBV) and a predisposition to lymphoma. We show that decreased intracellular free Mg²⁺ causes defective expression of the natural killer activating receptor NKG2D in natural killer (NK) and CD8⁺ T cells and impairs cytolytic responses against EBV. Remarkably, magnesium supplementation in MAGT1-deficient patients restores intracellular free Mg²⁺ and NKG2D while concurrently reducing EBV-infected cells *in vivo*, demonstrating a link between NKG2D cytolytic activity and EBV antiviral immunity in humans. Moreover, these findings reveal a specific molecular function of free basal intracellular Mg²⁺ in eukaryotic cells.

Divalent metal cations, including Mg²⁺, play important roles in cellular processes. In eukaryotic cells, 95% of intracellular Mg²⁺ ([Mg²⁺]_i) is bound (1). The remaining unbound, free [Mg²⁺]_i (~0.5–1 mM) is tightly regulated, yet its specific molecular functions are unknown (1–3). Magnesium transporter 1 (MAGT1) selectively conducts Mg²⁺ across the plasma membrane (4, 5). We previously characterized a novel primary immunodeficiency,

*Correspondence to: lenardo@nih.gov.

†These authors contributed equally

The data presented in this paper are tabulated in the main paper and in the supplementary materials.

named X-linked immunodeficiency with Mg^{2+} defect, Epstein-Barr virus (EBV) infection, and neoplasia (XMEN) disease, caused by null mutations in the *MAGT1* gene. We found that *MAGT1* was required for a T cell receptor (TCR)-stimulated Mg^{2+} influx that transiently raises free $[Mg^{2+}]_i$ to temporally coordinate T cell activation (2, 3).

XMEN disease is characterized by uncontrolled chronic EBV infection (2, 3). EBV is a B cell-tropic gammaherpesvirus that infects and establishes latency in 90% of humans worldwide (6). Uncontrolled EBV infection can lead to malignancy especially in B cells (7–9). The cytotoxic functions of natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTLs) are essential for control of viral infections and tumor immunosurveillance (10). XMEN patient cells contain normal bound $[Mg^{2+}]_i$ but decreased free $[Mg^{2+}]_i$ (2, 3), and whether the latter contributes to XMEN disease pathogenesis was unknown.

To understand defective EBV control in XMEN disease, we examined seven patients harboring mutations in *MAGT1* (Table S1). Similar to the first three patients characterized (A.1, A.2, and B.1) (2, 3), four additional XMEN patients with repeated minor viral infections and elevated EBV DNA in the blood were identified (Table S1, Fig. 1A). Furthermore, starting in childhood, some patients developed various EBV⁺ Hodgkin's, Burkitt's, and non-Hodgkin's B cell lymphomas or related lymphoproliferative disorders (Table S1, Fig. 1B). Nevertheless, XMEN patients had normal adaptive immune responses generating EBV-specific memory $CD8^+$ T cells (Fig. S1). However, unlike normal controls, EBV-specific CTLs from XMEN patients expanded by serial stimulations with irradiated autologous lymphoblastoid cell lines (LCLs) showed defective cytotoxicity against autologous LCLs (Fig. 1C) despite comparable expansions of EBV-tetramer positive cells (Fig. S2). Moreover, NK cells that were expanded *in vitro* with interleukin-2 (IL-2) exhibited defective cytotoxicity against K562 target cells (Fig. 1D). The NK cytotoxicity defect indicated that the previously described TCR defect could not fully account for the defective EBV immunity in XMEN patients. Decreased cytotoxicity also did not stem from deficient granule effectors or degranulation (Fig. S3).

MAGT1 not only mediates TCR-induced Mg^{2+} flux but also regulates the basal free $[Mg^{2+}]_i$ (2–4, 11, 12). We found that all XMEN patients, but not chronic active EBV (CAEBV) patients lacking *MAGT1* mutations or X-linked lymphoproliferative disease (XLP) patients chronically infected with EBV, had decreased basal free $[Mg^{2+}]_i$ (Fig. 2A and 2B). Given that the bound $[Mg^{2+}]_i$ (95–99% of total) is not reduced in XMEN patients (3, 5), we hypothesized that Mg^{2+} supplementation might restore the free $[Mg^{2+}]_i$ in XMEN patient cells. We therefore cultured CTLs and NK cells from XMEN patients in Mg^{2+} supplemented media and observed a dose-dependent increase in free $[Mg^{2+}]_i$ with nearly full restoration after 5 days in 10 mM magnesium sulfate ($MgSO_4$) (Fig. 2C and D). Magnesium supplementation did not rescue the TCR activation defect (Fig. 2E) or TCR-induced Mg^{2+} and Ca^{2+} fluxes in XMEN T cells (Fig. S4). However, Mg^{2+} supplementation did recover the cytotoxicity defect partially in CTLs and nearly completely in NK cells (Fig. 2F–2G), suggesting that the chronically low free $[Mg^{2+}]_i$ caused impaired cytotoxicity.

NK cells use a combination of inhibitory and activating receptors to specifically recognize target cells altered by transformation or virus infection (13, 14). Certain activating NK receptors are also expressed on CTLs as costimulatory signals for cytotoxic function (15). We surveyed the major NK receptors and found a selective loss of NKG2D expression on XMEN CTLs and NK cells (Fig. 3A and B). This was confirmed by surface staining, intracellular staining, and immunoblotting (Figs. 3A–C; S5A). The NKG2D loss was not secondary to EBV infection, as cells from CAEBV or XLP patients expressed normal NKG2D levels (Fig. 3B). NKG2D is associated with the adapter DAP10 on NK cells, CTLs, and $\gamma\delta$ T cells and mediates anti-viral and anti-tumor cytotoxic responses (16–20). We also

observed decreased DAP10 in XMEN patients which could be a cause or consequence of the NKG2D defect (20) (Fig. 3C). Their defective expression was post-transcriptional, as their steady-state mRNA levels were not reduced in XMEN samples (Fig. S5B). Ubiquitin-conjugated peptides of NKG2D and DAP10 were detected by mass spectrometry implying accelerated protein turnover.

In humans, NKG2D ligands include the MHC-class I-like proteins MICA and MICB as well as the UL16-binding protein family of glycoproteins (ULBP1-6) that are induced by infection, cellular transformation, and cell stress (19, 21, 22). Using a ULBP1-expressing P815 target cell, we observed that NKG2D-mediated cytotoxicity by XMEN patient NK cells was abolished (Fig. 3D). Similarly, although defective cytotoxicity against anti-CD3-coated P815 cells could involve the TCR signaling defect previously documented, further enhancement of the anti-CD3-induced cytotoxicity by ULBP1 was defective (Fig. 3E), suggesting that NKG2D loss contributed to the CTL killing defect in XMEN patients (2, 3).

We next examined Mg^{2+} and Ca^{2+} fluxes after stimulating various NK receptors, and found that only CD16 stimulation induced a Mg^{2+} flux (Fig. S6A). This flux was MAGT1-dependent and absent in XMEN NK cells but did not impact the Ca^{2+} flux in XMEN samples (Fig. S6A). Most NK receptors work in synergy with each other (23) and we consistently observed a defect when triggering flux through combinations including NKG2D in XMEN patient cells (Fig. S6B–6C).

Because NKG2D was constitutively reduced in XMEN cells, we hypothesized that this resulted from decreased free $[Mg^{2+}]_i$. Correspondingly, we found that cultivation of normal CTLs in Mg^{2+} -depleted medium caused a reduction of free $[Mg^{2+}]_i$ and NKG2D expression (Fig. S7). We therefore assessed NKG2D in XMEN NK cells and CTLs after 5 days of cultivation in medium with added $MgSO_4$. We observed a rise in free $[Mg^{2+}]_i$ correlating with increased NKG2D expression in both cell types (Fig. 3F–3G). The supplementation did not change the expression or function of other NK receptors or Mg^{2+} fluxes (Fig. S4 and S8A–8B). Consistent with the lack of flux restoration, Mg^{2+} supplementation did not restore the CD16-redirected lysis by XMEN NK cells (Fig. S8C). EBV⁺ LCLs expressed substantial levels of NKG2D ligands (Fig. S9). The enhancement of CTL killing of autologous LCLs by Mg^{2+} supplementation in XMEN CTLs (Fig. 3H) appears to be NKG2D-specific because it was blocked by NKG2D-Fc (Fig. 3H). Thus, NKG2D expression is sensitive to free basal $[Mg^{2+}]_i$ and can be restored in XMEN patient cells by exogenous Mg^{2+} supplementation.

The defective killing by XMEN NK cells and CTLs associated with decreased free $[Mg^{2+}]_i$ might account for the failed immune control of EBV. Therefore, we attempted to test this hypothesis *in vivo* by providing magnesium supplementation to patient A.1. First, we administered oral magnesium gluconate for 2 weeks. We observed a small but significant increase in basal free $[Mg^{2+}]_i$ and a modest restoration of NKG2D expression on both CTLs and NK cells (Fig. S10). The fraction of B cells harboring intra-nuclear EBV-encoded small RNA (EBER) declined during treatment (Fig. S10C). Twenty-one days after treatment cessation (Day 36), we observed that basal free $[Mg^{2+}]_i$ dropped to the pretreatment level and the fraction of EBV-infected peripheral blood mononuclear cells (PBMCs) rebounded. These data indicate that continuous supplementation is required to maintain free $[Mg^{2+}]_i$ and a beneficial effect on EBV control. We also tested the infusion of magnesium sulfate for 3 days and observed stronger and faster responses (Fig. S11). We then administered oral supplementation to patients A.1 and A.2 for 175 days with magnesium threonate (MgT), which has better bioavailability and tolerability. Free $[Mg^{2+}]_i$ and NKG2D expression increased in both patients within 2 days of treatment (Fig. 4A and B). The free $[Mg^{2+}]_i$ reached a plateau after 7 days of treatment and remained stable thereafter, whereas NKG2D expression increased progressively for the first 60 days (Fig. 4A, B and C). The expression

of NKG2D ligands observed on B cells was reduced in both patients (Fig. 4D). Moreover, the cells expressing NKG2D ligands were mostly EBV⁺ (Fig. 4E and 4F). We noted a reversal of the fraction of EBV⁺ B cells in Patient A.1 and to a lesser extent in Patient A.2 at day 60 of treatment which was later traced to a gap in Mg²⁺ administration. This indicates that the effects of Mg²⁺ supplementation were reversible and that control of EBV levels *in vivo* was linked to the level of NKG2D expression which was continuously regulated by the free [Mg²⁺]_i. The decrease in EBV infected cells may be important since high levels of EBV are correlated with early lymphoma development and mortality in XMEN patients (Table 1).

Our findings demonstrate that basal free [Mg²⁺]_i is regulated at least in part by extracellular Mg²⁺ and has concentration-dependent biological functions in eukaryotic cells besides the cofactor function of bound Mg²⁺ (1, 24, 25). The dynamic control of NKG2D expression by free [Mg²⁺]_i reveals the significance of [Mg²⁺]_i homeostasis for anti-viral and anti-tumor immunity (Fig. 4C). There are over 20 mammalian magnesium transporters, and it will be interesting to determine which control free [Mg²⁺]_i in XMEN patients during supplementation (5). XMEN patients manifest dysgammaglobulinemia, EBV infections, and susceptibility for lymphoma development similar to XLP (9, 26). In XLP1, 2B4 and NTB-A mediated cytotoxicity against EBV-infected B cells is defective (27–29). Given the loss of synergy between NKG2D and 2B4 in XMEN patients (Fig. S6C and S13), 2B4-mediated recognition of EBV-infected cells may be impaired in XMEN patients as well (23). Unlike cytomegalovirus, EBV does not have mechanisms to evade NKG2D recognition (30–32). NKG2D ligands are upregulated by lytic induction of EBV-infected cells and in tumors from EBV⁺ post-transplant lymphoproliferative disorders (22, 33). Moreover, recent studies show that NKG2D plays a critical role in the success of anti-tumor and hematopoietic stem cell transplants (HSCT) (34–36). Given that the only two known HSCT attempts in XMEN disease have been unsuccessful, Mg²⁺ supplementation may provide an adjunctive treatment in XMEN disease. Although the clinical utility of Mg²⁺ supplementation remains to be validated, our data illustrate *in vivo* that NKG2D is regulated by [Mg²⁺]_i and apparently plays a key role in EBV control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Division of Intramural Research, NIAID, NIH, through project ZO1AI001187-01. The authors thank S. Holland, H. Malech, A. Snow, J. Niemela, G. Fahle, and T. Dimaggio for assistance in various aspects of the research and patient evaluation. We also thank R. Germain, P. Schwartzberg, R. Siegel, T. Waldmann, K. Schafer-Weaver, C. Kanellopoulou, S. Muljo, D. McVicar, J. Milner, R. Baker, and S. Holland for critically reading the manuscript. We thank E. Long and S. Rajagopalan for sharing antibodies and cell lines and helpful assistance with NK experiments; we also thank K. Dowdell, R. Orentas and B. Lafont for sharing reagents and D. Douek, D. Price, and M. Quigley for assistance with tetramer staining. The authors thank Merck Research Laboratories for generous support in kind of reagents and materials. F.-Y.L. is in the Medical Scientist Training Program at the University of California–San Francisco and thanks K. Shannon and J. Toutolmin for support and encouragement.

References

1. Grubbs RD, Maguire ME. Magnesium as a regulatory cation: criteria and evaluation. *Magnesium*. 1987; 6:113. [PubMed: 3306178]
2. Li FY, Lenardo MJ, Chaigne-Delalande B. Loss of MAGT1 abrogates the Mg²⁺ flux required for T cell signaling and leads to a novel human primary immunodeficiency. *Magnes Res*. 2011 Sep. 24:S109. [PubMed: 21983175]

3. Li FY, et al. Second messenger role for Mg²⁺ revealed by human T-cell immunodeficiency. *Nature*. 2011 Jul 28;475:471. [PubMed: 21796205]
4. Zhou H, Clapham DE. Mammalian MagT1 and TUSC3 are required for cellular magnesium uptake and vertebrate embryonic development. *Proc Natl Acad Sci U S A*. 2009 Sep 15;106:15750. 2009. [PubMed: 19717468]
5. Quamme GA. Molecular identification of ancient and modern mammalian magnesium transporters. *Am J Physiol Cell Physiol*. 2010 Mar;298:C407. [PubMed: 19940067]
6. Cohen JI. Epstein-Barr virus infection. *N Engl J Med*. 2000 Aug 17;343:481. [PubMed: 10944566]
7. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med*. 2004 Mar 25;350:1328. [PubMed: 15044644]
8. Rezaei N, Hedayat M, Aghamohammadi A, Nichols KE. Primary immunodeficiency diseases associated with increased susceptibility to viral infections and malignancies. *J Allergy Clin Immunol*. 2011 Jun;127:1329. [PubMed: 21514636]
9. Filipovich AH, Zhang K, Snow AL, Marsh RA. X-linked lymphoproliferative syndromes: brothers or distant cousins? *Blood*. 2010 Nov 4;116:3398. [PubMed: 20660790]
10. Orange JS. Human natural killer cell deficiencies and susceptibility to infection. *Microbes and infection / Institut Pasteur*. 2002 Dec;4:1545. [PubMed: 12505527]
11. Deason-Towne F, Perraud AL, Schmitz C. The Mg(2+) transporter MagT1 partially rescues cell growth and Mg(2+) uptake in cells lacking the channel-kinase TRPM7. *FEBS Lett*. 2011 Jul 21;585:2275. [PubMed: 21627970]
12. Schweigel M, Kolisek M, Nikolic Z, Kuzinski J. Expression and functional activity of the Na/Mg exchanger, TRPM7 and MagT1 are changed to regulate Mg homeostasis and transport in rumen epithelial cells. *Magnes Res*. 2008 Jun;21:118. [PubMed: 18705540]
13. Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev*. 2006 Dec;214:73. [PubMed: 17100877]
14. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nature reviews. Immunology*. 2001 Oct;1:41.
15. Groh V, et al. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol*. 2001 Mar;2:255. [PubMed: 11224526]
16. Guerra N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity*. 2008 Apr;28:571. [PubMed: 18394936]
17. Hayakawa Y, Smyth MJ. NKG2D and cytotoxic effector function in tumor immune surveillance. *Semin Immunol*. 2006 Jun;18:176. [PubMed: 16675266]
18. Jamieson AM, et al. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity*. 2002 Jul;17:19. [PubMed: 12150888]
19. Ogasawara K, Lanier LL. NKG2D in NK and T cell-mediated immunity. *Journal of clinical immunology*. 2005 Nov;25:534. [PubMed: 16380817]
20. Park YP, et al. Complex regulation of human NKG2D-DAP10 cell surface expression: opposing roles of the gammac cytokines and TGF-beta1. *Blood*. 2011 Sep 15;118:3019. [PubMed: 21816829]
21. Obeidy P, Sharland AF. NKG2D and its ligands. *The international journal of biochemistry & cell biology*. 2009 Dec;41:2364. [PubMed: 19631280]
22. Zhang B, et al. Immune surveillance and therapy of lymphomas driven by Epstein-Barr virus protein LMP1 in a mouse model. *Cell*. 2012 Feb 17;148:739. [PubMed: 22341446]
23. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*. 2006 Jan 1;107:159. [PubMed: 16150947]
24. Rubin H. Central role for magnesium in coordinate control of metabolism and growth in animal cells. *Proc Natl Acad Sci U S A*. 1975 Sep;72:3551. [PubMed: 171666]
25. Wolf FI, Trapani V. TRPM7 and magnesium, metabolism, mitosis: An old path with new pebbles. *Cell Cycle*. 2010 Sep 1;9:3399. [PubMed: 20861666]

26. Rezaei N, Mahmoudi E, Aghamohammadi A, Das R, Nichols KE. X-linked lymphoproliferative syndrome: a genetic condition typified by the triad of infection, immunodeficiency and lymphoma. *Br J Haematol*. 2011 Jan;152:13. [PubMed: 21083659]
27. Palendira U, et al. Molecular pathogenesis of EBV susceptibility in XLP as revealed by analysis of female carriers with heterozygous expression of SAP. *PLoS Biol*. 2011 Nov;9:e1001187. [PubMed: 22069374]
28. Nakajima H, et al. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur J Immunol*. 2000 Nov;30:3309. [PubMed: 11093147]
29. Sharifi R, et al. SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease. *Blood*. 2004 May 15;103:3821. [PubMed: 14726378]
30. Slavuljica I, Krmpotic A, Jonjic S. Manipulation of NKG2D ligands by cytomegaloviruses: impact on innate and adaptive immune response. *Frontiers in immunology*. 2011; 2:85. [PubMed: 22566874]
31. Wang R, et al. Structural basis of mouse cytomegalovirus m152/gp40 interaction with RAE1gamma reveals a paradigm for MHC/MHC interaction in immune evasion. *Proc Natl Acad Sci U S A*. 2012 Dec 18;109:E3578. [PubMed: 23169621]
32. Pievani A, et al. Dual-functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood*. 2011 Sep 22;118:3301. [PubMed: 21821703]
33. Pappworth IY, Wang EC, Rowe M. The switch from latent to productive infection in epstein-barr virus-infected B cells is associated with sensitization to NK cell killing. *J Virol*. 2007 Jan;81:474. [PubMed: 17079298]
34. Reiners KS, et al. Rescue of impaired NK cell activity in hodgkin lymphoma with bispecific antibodies in vitro and in patients. *Mol Ther*. 2013 Apr;21:895. [PubMed: 23459515]
35. Zingoni A, Ardolino M, Santoni A, Cerboni C. NKG2D and DNAM-1 activating receptors and their ligands in NK-T cell interactions: role in the NK cell-mediated negative regulation of T cell responses. *Frontiers in immunology*. 2012; 3:408. [PubMed: 23316196]
36. Rettinger E, et al. The cytotoxic potential of interleukin-15-stimulated cytokine-induced killer cells against leukemia cells. *Cytotherapy*. 2012 Jan;14:9.
37. Siegel AM, et al. A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. *Immunity*. 2011 Nov 23;35:806. [PubMed: 22118528]
38. Comoli P, et al. Successful in vitro priming of EBV-specific CD8+ T cells endowed with strong cytotoxic function from T cells of EBV-seronegative children. *Am J Transplant*. 2006 Sep;6:2169. [PubMed: 16796723]
39. Savoldo B, et al. Autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for the treatment of persistent active EBV infection. *Blood*. 2002 Dec 1;100:4059. [PubMed: 12393655]
40. Ciarallo L, Brousseau D, Reinert S. Higher-dose intravenous magnesium therapy for children with moderate to severe acute asthma. *Arch Pediatr Adolesc Med*. 2000 Oct;154:979. [PubMed: 11030848]
41. Ciarallo L, Sauer AH, Shannon MW. Intravenous magnesium therapy for moderate to severe pediatric asthma: results of a randomized, placebo-controlled trial. *J Pediatr*. 1996 Dec;129:809. [PubMed: 8969721]
42. Glover ML, Machado C, Totapally BR. Magnesium sulfate administered via continuous intravenous infusion in pediatric patients with refractory wheezing. *J Crit Care*. 2002 Dec;17:255. [PubMed: 12501154]
43. Scarfone RJ, et al. A randomized trial of magnesium in the emergency department treatment of children with asthma. *Annals of emergency medicine*. 2000 Dec;36:572. [PubMed: 11097697]
44. Calattini S, et al. Detection of EBV genomes in plasmablasts/plasma cells and non-B cells in the blood of most patients with EBV lymphoproliferative disorders by using Immuno-FISH. *Blood*. 2010; 116:4546. [PubMed: 20699441]

Figure 1. XMEN patients exhibit high EBV levels, lymphoma development, and defective cytotoxicity

Levels of EBV DNA in XMEN patient blood (n=7) expressed as copies/mL blood (†), copies/ 10^6 cells (*), or copies/ug DNA (§) depending on the specific clinical laboratory. Each point represents an independent blood draw measurements at different times. Chronic active EBV (CAEBV) patient values are shown for comparison. Shaded area indicates normal range, EBV DNA is typically undetectable in EBV⁺ subjects with less than 10% of normal individuals usually showing no more than 20 – 80 copies/mL in the blood (37). (B) Histopathology of the various EBV⁺ lymphoproliferative disorders (LPD) in XMEN patients. The upper panel shows the hematoxylin and eosin (H&E) staining and the lower panel shows the EBV-encoded small RNA (EBER) staining by *in situ* hybridization. Yellow arrowheads show Reed-Sternberg cells. (Magnification: 400X for patients B.1 and E.1 (bottom panel) and 1000X for patient F.1 and E.1 (top panel)). (C) Cytotoxicity of EBV-specific CTLs from father (■), mother (◆), and XMEN patients A.1 (●) and A.2 (○) on autologous LCLs. (D) Cytotoxicity of IL-2-expanded NK cells from normal control (■) or XMEN patients A.1 (●) and A.2 (○) against K562 cells. Results shown in C and D are representative of results of all XMEN patients tested (n=4) in at least three independent experiments (Two-Way ANOVA, ***: p<0.001; ****: p<0.0001).

Figure 2. Mg^{2+} supplementation restores intracellular Mg^{2+} and enhances cytotoxicity but does not restore TCR activation

(A) Flow cytometry profile of the ratio of the mean fluorescent intensity (MFI) of Mg^{2+} -specific fluorescent probe MagFluo4 to the MFI of the Ca^{2+} -sensitive probe Fura Red (MF4/FR) using PBMC from normal control (red line) and XMEN patient A.1 (blue line). The ratio was arbitrarily set at 1 for normal control (dotted line). (B) Dot plot of the MF4/FR ratios from PBMCs of normal controls (red squares, $n=5$) and XMEN patients (blue circles, $n=6$), non-XMEN CAEBV patients (green triangles, $n=5$) or XLP patients (orange diamonds, $n=4$). (C) Flow cytometry profiles of MF4/FR ratio on normal control and XMEN patient CTLs cultured in media supplemented with indicated amounts of $MgSO_4$ for 5 days. (D) MF4/FR ratio of CTLs from normal control (red bars, $n=4$) or XMEN patients (blue bars, $n=4$). (E) Percentage of $CD69^+$ in $CD8^+$ cells after stimulation with anti-CD3 ($1 \mu g/ml$) for 24h in presence of indicated amount of $MgSO_4$ or unstimulated. (F) Cytotoxicity of normal or XMEN patient EBV-specific CTLs, supplemented with or without 2 mM of $MgSO_4$ for 5 days, on autologous LCLs. (G) Cytotoxicity of normal or XMEN patient NK cells, supplemented with or without 2 mM of $MgSO_4$ for 5 days, on K562 targets. Results shown are representative of all XMEN patient tested ($n=4$) in at least three independent experiments (Two-Way ANOVA, *: $p=0.01$, **: $p=0.0038$, ****: $p<0.0001$).

Figure 3. Effect of Mg²⁺ on NKG2D expression and function *in vitro*

(A) Flow cytometry profiles of the surface expression of NK receptors on gated NK cells (CD3⁺CD56⁺) from normal controls (— and —) and XMEN patient A.1 (—). Isotype antibody staining (■) is shown as a reference for background fluorescence. Results are representative of all XMEN patients tested (n=5). (B) Normalized MFI of NKG2D surface staining on gated CTLs from whole blood of normal controls (■, n=5) and XMEN patients (●, n=6), non-XMEN CAEBV patients (▲, n=5) or XLP patients (◆, n=4). (C) Immunoblots of CTLs and NK cells from normal control and XMEN patient with indicated antibodies. (D) NKG2D-specific redirected lysis of normal or XMEN patient IL-2-expanded NK cells on P815 cells expressing the NKG2D ligand, ULBP1. (E) NKG2D-specific redirected lysis of normal or XMEN patient CTLs on P815 expressing the NKG2D ligand ULBP1 or not in presence of anti-CD3 antibodies. (F) Flow cytometry profiles of MF4/FR ratio in PBMCs (left) and NKG2D expression on CTLs (middle) and NK cells (right) from normal control or XMEN patient supplemented with the indicated concentrations of MgSO₄ for five days. (G) Immunoblot of cells lysates from CTLs from a normal control or an XMEN patient supplemented with or without 5 mM of MgSO₄ for 5 days. (H) Cytotoxicity of normal control or XMEN patient EBV-specific CTLs, supplemented with or without 2 mM of MgSO₄ for 5 days, on autologous EBV-transformed LCLs pre-treated with or without soluble NKG2D-Fc. In C and G, black arrows heads show the various forms of the indicated protein. “nNKG2D” indicates the normal form of NKG2D, “pNKG2D” the patient’s forms and * indicates a nonspecific band detected by the anti-NKG2D antibody. Results shown are representative of all XMEN patient tested (n=4) in at least three independent experiments (Student t-test (B), Two-Way ANOVA (D-E-H), ****: p<0.0001).

Figure 4. *In vivo* Mg²⁺ supplementation of XMEN patient restored NKG2D expression and decreased EBV⁺ PBMCs

Flow cytometry profiles of MF4/FR ratio in PBMC (**A**) and NKG2D expression on gated CTLs (CD3⁺CD8⁺) (**B**) from a normal control (Mother, untreated), patient A.1 (treated) and patient A.2 (treated) at the indicated times of magnesium supplementation in days (D). Mother was not available for sample on D14. (**C**) Quantification of the MF4/FR ratio (top), NKG2D MFI (middle) and percentage of EBV⁺ B cells measured by EBV-encoded small RNA (EBER) fluorescence in situ hybridization (FISH) (bottom) during the magnesium supplementation. The arrowhead and dotted line indicates the starting point of the magnesium supplementation. NKG2D-Fc staining on gated B cells (CD19⁺) from freshly isolated PBMC of a normal control, XMEN patients (before D-63 and after D175 Mg²⁺ supplementation) alone by flow cytometry (**D**) or co-stained with EBER FISH in confocal microscopy (**E**). (**F**) Quantification of the EBER⁺/NKG2D-Fc⁺ cells shown as percentage of EBV⁺ cells that are NKG2D-Fc⁺ or NKG2D-Fc⁻ in the patient's PBMCs during *in vivo* Mg²⁺ supplementation as measured by EBV-specific FISH. (scale bar: 5 μ m).