Lack of NKG2D in MAGT1-deficient patients is caused by hypoglycosylation

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Human Genetics – Special issue on Inherited Metabolic Diseases

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Supplementary data: yes

1 Supplementary Information file (pdf format)

Abstract

Mutations in the X-linked gene *MAGT1* cause a Congenital Disorder of Glycosylation (CDG), with two distinct clinical phenotypes: a primary immunodeficiency (XMEN disorder) versus intellectual and developmental disability. It was previously established that MAGT1 deficiency abolishes steady state expression of the immune response protein NKG2D (encoded by *KLRK1*) in lymphocytes. Here, we show that the reduced steady state levels of NKG2D are caused by hypoglycosylation of the protein and we pinpoint the exact site that is underglycosylated in MAGT1-deficient patients. Furthermore, we challenge the possibility that supplementation with magnesium restores NKG2D levels and show that addition of this ion does not significantly improve NKG2D steady state expression nor does it rescue the hypoglycosylation defect in knock out human cell lines. Moreover, magnesium supplementation of an XMEN patient did not result in restoration of NKG2D expression on the cell surface of lymphocytes. In summary, we demonstrate that in MAGT1-deficient patients the lack of NKG2D is caused by hypoglycosylation.

Keywords

CDG, OST, STT3B, MAGT1, XMEN, NKG2D

Declarations

Funding

This research was supported by Research Foundation Flanders (FWO): under the frame of E-Rare-3, the ERA-Net for Research on Rare Diseases (ERA-NET Cofund action N°64578) (to GM), a research stay grant (FWO V417818N) (to EB), a senior clinical investigator fellowship (to RS), and GLYCO4DIAG, an International Associated Laboratory grant from National Centre for Scientific Research (CNRS) and FWO (to FF and GM). The work was also supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number GM43768 (to RG), C1 KU Leuven fund (to RS) and by the JAEKEN-THEUNISSEN CDG FUND.

Conflict of interest

The authors declare no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Author contributions

Designed the experiments: EB, NAC, FS, RG and RS. Performed the experiments: EB, NAC, FS and MW. Analysed the data: EB and NAC. EB wrote the manuscript with support from JJ, FF and GM. Clinical evaluation of patient: RS. All authors discussed and revised the manuscript.

Ethics approval

Research on patient cells was approved by the Ethical Committee of the University Hospital of Leuven (approval number S58466).

Consent for publication

The patient and controls in this study provided informed consent.

Introduction

MAGT1 is a controversial protein with two described and intrinsically conflicting roles: it has been shown that MAGT1 is a subunit of the STT3B oligosaccharyltransferase (OST) complex at the endoplasmic reticulum (Kelleher et al. 2003; Cherepanova et al. 2014). However, MAGT1 was also described as a plasma membrane localised magnesium (Mg^{2+}) transporter (Goytain and Quamme 2005; Zhou and Clapham 2009). Patients with mutations in *MAGT1* present with two distinct phenotypes; a primary immunodeficiency characterised by chronic-active EBV infections (XMEN disorder) (Li et al. 2011) and a strikingly different phenotype where patients present with intellectual and developmental disability (Blommaert et al. 2019). Recently, we demonstrated that in both clinical presentations, N-glycosylation is affected. The disorder, MAGT1-CDG, therefore falls under the umbrella of the Congenital Disorders of Glycosylation (CDG) (Blommaert et al. 2019).

CDG are a group of genetic disorders with defects in the synthesis or attachment of glycans onto glycoproteins and glycolipids. They show a great variety of clinical presentations with involvement of multiple organs and tissues (Freeze et al. 2014; Jaeken and Péanne 2017). In the N-glycosylation pathway, the enzymes constituting the OST are responsible for the transfer of glycans from a dolichol carrier onto proteins at specific Asn residues recognised by the Asn-X-Ser/Thr motif (Aebi 2013). The OST is a multimeric complex with two different catalytic subunits, STT3A and STT3B. Each of these assemble into their own complex. Some accessory subunits are present in both and others are specific for each respective catalytic unit (Kelleher et al. 2003). MAGT1 is an isoform-specific subunit of STT3B with an oxidoreductase domain and is crucial for the functioning of the STT3B OST complex (Cherepanova et al. 2014; Cherepanova and Gilmore 2016). Interestingly, TUSC3 is a homologue of MAGT1 that is able to perform the same role, but has a more restricted

88 expression profile (Zhou and Clapham 2009; Matsuda-Lennikov et al. 2019). Thus, mutations
89 in *MAGT1* lead to a glycosylation defect caused by dysfunctional STT3B-dependent
90 glycosylation, but only in absence of TUSC3 expression (Blommaert et al. 2019).
91 Understanding the pathophysiological consequences of a disordered glycosylation
92 machinery is particularly challenging, as it is estimated that 50% of the proteome is
93 glycosylated and many downstream pathways are affected (Petrescu et al. 2006; Jaeken and
94 Péanne 2017). The investigation of two MAGT1-deficient XMEN patients demonstrated that
95 steady state expression of the immune response NKG2D protein is impaired; it was therefore
96 postulated that reduced expression of NKG2D leads to increased susceptibility to EBV
97 infections (Chaigne-Delalande et al. 2013). NKG2D is encoded by *KLRK1,* and expression of
98 this protein is restricted to $CD8^+$ T cells, NK, NKT and subsets of γδ T cells. NKG2D forms a
99 homodimer of two disulphide-linked transmembrane proteins (Garrity et al. 2005). The
100 protein itself does not have signalling properties, instead it associates with a signalling
101 subunit, DAP10; this in turn activates several intracellular cascades such as the
102 phosphatidylinositol-3-kinase pathway (Wu et al. 1999). Cellular stress, *e.g.* due to
103 bacterial/viral infections, or malignantly transformed cells, cause an upregulation of NKG2D
104 ligands (Jamieson et al. 2002; Raulet et al. 2013). As reduced levels of intracellular free Mg^{2+}
105 concentrations were measured in XMEN patients (Li et al. 2011), supplementation with Mg^{2+}
106 was proposed as a potential therapy. Upon addition of an excess concentration of Mg^{2+},
107 NKG2D steady state levels were restored in defective $CD8^+$ and NK cells. Interestingly,
108 patients receiving Mg^{2+} supplementation *in vivo* also presented with increased levels of
109 NKG2D expression (Chaigne-Delalande et al. 2013).
110 In light of the newly gained knowledge that MAGT1 mutations cause a glycosylation defect
111 in XMEN disorder (Blommaert et al. 2019; Matsuda-Lennikov et al. 2019), we studied the

glycosylation status and protein expression of NKG2D, using HEK293 cells knocked out for different subunits of the OST. We hypothesised that the lack of NKG2D expression in patient $CD8^+$ and NK cells is due to hypoglycosylation of the protein, caused by mutations in MAGT1. Furthermore, we assayed the effects of Mg^{2+} supplementation on NKG2D expression in an XMEN patient.

Material and methods

Patient and cells

CRISPR-Cas9 engineered HEK293 WT cells, depleted for either STT3A, STT3B, MAGT1 and TUSC3 were previously described (Cherepanova and Gilmore 2016). HEK293 cells were cultured in Dulbecco's modified Eagle medium (Life Technologies), supplemented with 10% fetal bovine serum (Clone III, HyClones) at 37°C under 5% CO_2. Peripheral Blood Mononuclear Cells (PBMCs) from patient 1 and healthy controls were isolated using Lymphocyte Separation Medium (MP Biomedicals). Patient cells were isolated during routine outpatient visits, in the absence of intercurrent illnesses, without (at time point 0) and with Mg-gluconate 3 g QD oral substitution (containing 162 mg Mg^{2+}) at different time points. Clinical synopsis is described in greater detail in the supplementary data.

Epstein-Barr Virus quantification

DNA was extracted from whole blood (EDTA) using QIAsymphonySP/AS (Qiagen) followed by quantification using RT-PCR Taqman on QuantStudio Dx (Thermo Fisher Scientific).

Expression vectors

NKG2D-Myc-DDK and DAP10-Myc-DDK expression plasmids were purchased from Origene. Elimination of glycosylation sites was achieved by introducing asparagine to glutamine mutations using site-directed mutagenesis to obtain the NKG2D-Myc-DDK N131AS (N108Q,

N163Q, N202Q), N163QS (N108Q, N131Q, N202Q), N202CS (N108Q, N131Q, N163Q) and ΔN108CS (N108Q) mutants. The mutations were confirmed by Sanger sequencing.

Protein expression

HEK293 cells were seeded in 6-well or 60 mm dishes 24h prior to transfection. Plasmid transfection was performed using 6 µg plasmid (60 mm dish) and Lipofectamine 3000 (Invitrogen) in serum-free Opti-MEM medium (GIBCO) according to the manufacturer's instructions. Cotransfections were performed with 1.5 µg of each vector (6-well). Cells were assayed 24h later for pulse chase labelling, 48h later for immunoblotting.

Antibodies

Anti-NKG2D N20 (sc-9621) polyclonal antibody was purchased from Santa Cruz Biotechnology, goat polyclonal antisera specific for CatC (AF1071) from R&D Systems and mouse monoclonal antisera for β-tubulin (ab101019) from Abcam. Anti-STT3B and anti-STT3A antibodies were described previously (Kelleher et al. 2003). For recognition of epitope tags anti-DDK (F3165 anti-FLAG M2, Sigma) was used.

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH=7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein content was quantified using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). 20 µg of proteins were immunoblotted onto a nitrocellulose membrane (Thermo Fisher Scientific). Signals were detected using Western Lightning-ECL (Perkin Elmer) and autoradiographed with ImageQuant LAS4000, band intensities were measured using ImageQuant TL (both GE Healthcare). To calculate the total NKG2D levels,

156 the whole area –from the fully glycosylated to the completely hypoglycosytlated protein –
157 was taken into account.

158 **Pulse-chase radiolabelling**

159 Expression vectors were pulse-chase labelled with Tran^{35}S label (Perkin Elmer), as previously
160 described (Cherepanova et al. 2014). Dry gels were exposed to a phosphor screen (Fujifilm),
161 scanned in Typhoon FLA 9000, and quantified using ImageQuant TL (GE Healthcare).
162 Endoglycosidase H was used as a mobility marker, according to manufacturers' instructions
163 (New England BioLabs).

164 **Flow cytometry**

165 After thawing of PBMCs, cells were washed in dPBS supplemented with 0.5% BSA. The cells
166 were stained with anti-CD19 (clone 1D3, 557398, BD biosciences) and anti-CD56 (Clone
167 CMSSB, 46-0567-42), anti-CD16 (clone eBioB16, 17-0168-42), anti-CD4 (clone OKT4, 56-
168 0048-41), anti-CD8 (clone SK1, 48-0087-42; clone RPA-T8, 56-0088-41) and anti-CD3 (clone
169 OKT3, 86-0037-42) (all eBioscience); anti-NKG2D (clone 1D11, 320806), anti-CD2 (clone
170 TS1/8, 309214), anti-CD3 PE Cy7 (300420) (all Biolegend); anti-CD56 PerCP e71 (46-0566-41)
171 (Thermo Fisher Scientific). FVD 780 APC-Cy7 (Thermo Fisher Scientific) was used for
172 live/dead staining. Data were collected on a BD LSR Fortessa and analysed using FlowJo
173 V.10.5 (Tree Star Inc.).

174 **Statistics**

175 Statistical analyses were performed in R (version 3.6.2). Individual data points between two
176 groups were analysed by Welch Student's t test, samples before and after treatment by
177 paired Student's t test. $p < 0.05$ was considered significant.

178

Results

1 NKG2D is an STT3B-dependent glycoprotein

It has previously been shown that steady state levels of NKG2D in $CD8^+$ lymphocytes and NK cells from XMEN patients were severely decreased (Chaigne-Delalande et al. 2013). By immunoblotting, it was observed that NKG2D is absent in both $CD8^+$ and NK cell lysates of MAGT1-deficient patients, but that a ~8 kDa lighter isoform was present. From this we hypothesise that this isoform is the hypoglycosylated protein. NKG2D is a 216 residue protein that has four N-glycosylation sites (N_{108}NC, N_{131}AS, N_{163}GS, N_{202}CS), of which one is a suboptimal sequon (N_{108}NC) (**Fig. 1a**).

Different KO HEK293 cell lines ($STT3A^{-/-}$, $STT3B^{-/-}$ and the double KO $MAGT1^{-/-}TUSC3^{-/-}$) (Cherepanova and Gilmore 2016), were used as cellular model to assess the glycosylation status of NKG2D. The $MAGT1^{-/-}TUSC3^{-/-}$ HEK293 cell line was preferred over $MAGT1^{-/-}$ cells, as TUSC3 expression rescues the glycosylation defect caused by MAGT1-deficiency (Cherepanova and Gilmore 2016; Blommaert et al. 2019). Each HEK293 cell line was transfected with a NKG2D-Myc-DDK construct, followed by metabolic pulse-chase labelling. Treatment with endoglycosidase H (EH) was used to remove all glycans and represents a mobility marker for the fully hypoglycosylated form of NKG2D. **Fig. 1b** shows that for NKG2D transfected in WT cells, the most abundant form carries 3 glycans. A faint, slower migrating band can be observed, where the protein carries 4 glycans. This 4^{th} glycosylation site is the suboptimal N_{108}NC sequon, as radiolabelling of NKG2D-ΔN_{108}NC-Myc-DDK showed that the protein carries 3 glycans (**Fig. S2**). In the $MAGT1^{-/-}TUSC3^{-/-}$ and $STT3B^{-/-}$ cells, multiple hypoglycosylated forms appear (average number of glycans is in both cell lines 1.3, compared to 3.1 in WT). In contrast, NKG2D is fully glycosylated in $STT3A^{-/-}$ cells (average number of glycans is 2.9).

To pinpoint the N-glycosylation sequons that are strictly STT3B-dependent, we proceeded by generating three NKG2D mutant constructs, where all sequons were removed by mutagenesis except one (N_{131}AS, N_{163}GS or N_{202}CS). The different HEK293 cell lines were transfected with these constructs, followed by pulse-chase labelling. **Fig. 1c** shows that the N_{202}CS NKG2D construct is completely hypoglycosylated in the MAGT1$^{-/-}$TUSC3$^{-/-}$ and STT3B$^{-/-}$ HEK293 cells. In the other constructs, N_{131}AS and N_{163}GS, a doublet appears in the STT3B$^{-/-}$ and MAGT1$^{-/-}$TUSC3$^{-/-}$ cells, indicating a partial block in STT3B dependent glycosylation (ranging from 20 to 40% reduction of glycosylation compared to WT cells). In summary, these results indicate that NKG2D is a glycoprotein with one sequon (N_{202}CS) that is strictly STT3B and MAGT1/TUSC3-dependent, and two others that are partially.

2 NKG2D steady state expression is decreased in cells with a defective STT3B complex

To determine whether the observed hypoglycosylation in MAGT1$^{-/-}$TUSC3$^{-/-}$ and STT3B$^{-/-}$ cells has an effect on the protein steady state levels of NKG2D, 48h cotransfection with NKG2D-myc-DDK and DAP10-myc-DDK, an accessory protein of NKG2D, was performed in the different KO cell lines, followed by immunoblotting. This revealed that the expression of fully glycosylated NKG2D is strongly reduced in the MAGT1$^{-/-}$TUSC3$^{-/-}$ and STT3B$^{-/-}$ cell lines (**Fig. 2a, 2c**). In addition, multiple hypoglycosylated bands appear in these KO cell lines (**Fig. 2a**). Taking the total NKG2D (all the glycoforms) into account, a reduction of more than 70% was observed in STT3B$^{-/-}$ and MAGT1$^{-/-}$TUSC3$^{-/-}$ cells, compared to WT. When looking at the biologically most relevant isoform, the fully glycosylated NKG2D (carrying 3 to 4 glycans), only 11% and 14% of this isoform was present respectively in MAGT1$^{-/-}$TUSC3$^{-/-}$ and STT3B$^{-/-}$ mutants compared to the WT control (**Fig. 2c**). This is in contrast with the relative abundance of 53% in STT3A$^{-/-}$ cells. Altogether, we show that the block in STT3B-dependent glycosylation causes a strong reduction in NKG2D steady state levels.

3 Magnesium supplementation does not rescue NKG2D expression

Supplementation of $CD8^+$ and NK cells from XMEN patients with 5 mM $MgSO_4$ restored the defective expression of NKG2D protein levels (Chaigne-Delalande et al. 2013). To test whether Mg^{2+} supplementation could rescue the observed hypoglycosylation for NKG2D in $STT3B^{-/-}$ and $MAGT1^{-/-}TUSC3^{-/-}$ cell lines (**Fig. 2a, 2c**), HEK293 cells were cotransfected with NKG2D-Myc-DDK and DAP10-Myc-DDK, treated with 5 mM $MgSO_4$ for 48h and analysed by immunoblotting. A statistically significant increase in the abundance of the fully glycosylated NKG2D was observed between untreated and Mg^{2+} supplemented cells in $STT3A^{-/-}$ and $STT3B^{-/-}$ cell lines, however for total NKG2D no statistically significant differences were seen for the mutant cell lines (**Fig. 2b, 2c**).

Previously, we identified an XMEN patient with a pathogenic variant (c.938T>G; p.Leu313*) in *MAGT1* (clinical synopsis in **supplementary information** and pedigree shown in **Fig. S1**). We were able to show that STT3B-dependent substrates were underglycosylated in patient-derived cell lines. Furthermore, we demonstrated that NKG2D cell surface expression in NK cells was strongly reduced in P1 compared to healthy controls (Blommaert et al. 2019). The latter experiment was repeated after treatment of P1 with 3 g Mg-gluconate (containing 162 mg Mg^{2+}) per day at 3 and 21 months, and was also performed on $CD8^+$ lymphocytes. The supplementation of P1 with Mg^{2+} did not significantly increase the levels of NKG2D cell surface expression compared to the results before supplementation (**Fig. 3a**). Moreover, EBV viremia levels increased over time and were deemed to be non-responsive to Mg^{2+} supplementation (**Fig. 3b**). Both findings are in contrast with (Chaigne-Delalande et al. 2013), where *in vivo* treatment resulted in increased NKG2D levels on $CD8^+$ lymphocytes and lower EBV^+ B cells. Additional biochemical workup of P1 showed no differences before or after

250 treatment (**Table S1**). However, the patient himself feels better with the magnesium
251 supplementation.

252 Discussion

253 MAGT1-deficiency is a remarkable disorder; it is an immunological disease as well as a
254 glycosylation defect. Therefore, it belongs to the CDG group with major immunological
255 involvement, such as ALG12-CDG, ATP6AP1-CDG, EXTL3-CDG, FUT8-CDG, G6PC3-CDG,
256 JAGN1-CDG, MOGS-CDG, PGM3-CDG, SLC35C1-CDG and SLC37A4-CDG (Pascoal et al. 2020).
257 Since the principal description in 2011 by Li et al., 36 male patients with this X-linked
258 disease, with 21 different pathogenic variants, have been reported (see review in Ravell et
259 al. 2020a). The patients have a combined immunodeficiency, clinically characterized by
260 increased susceptibility to chronic EBV sinopulmonary and ear infections (75%),
261 lymphadenopathy (53%), EBV-associated lymphoproliferative disease/lymphoma (36%),
262 splenomegaly (31%), molluscum contagiosum (25%), severe autoimmune cytopenias (25%),
263 and mouth sores (22%). Other, albeit less frequent, symptoms (19 to 6%) are skin warts,
264 neurological symptoms (including Guillain-Barré syndrome), bleedings, varicella zoster virus
265 and herpes simplex virus infections, intellectual/developmental disability, effusions and EBV
266 negative malignancy. All described patients by Ravell et al. 2020a have decreased NKG2D
267 expression on natural killer and CD8+ T cells, and increased (sustained or fluctuating) serum
268 transaminases.
269 NKG2D is an essential activating receptor in the cytolytic response of immune cells,
270 expressed in NK and $CD8^+$ cells, among other cell types. It has been previously demonstrated
271 that mutations in MAGT1 cause a dramatic decline in NKG2D protein levels (Chaigne-

Delalande et al. 2013). NKG2D activity is important for the recognition of EBV infection, and subsequent activation (Münz 2017). Therefore, it was concluded that XMEN patients are susceptible to chronic-active EBV viremia, and in some cases the development of EBV-related lymphoma's, due to the lack of NKG2D expression (Chaigne-Delalande et al. 2013). By addition of an excess amount of $MgSO_4$ to NK and $CD8^+$ cells, NKG2D expression was restored to similar levels as control samples. As a consequence, Mg^{2+} supplementation was explored as a possible treatment for MAGT1-deficient patients. This led to an increase in cell surface expression of NKG2D in two tested patients, and a decrease in EBV-infected cells (Chaigne-Delalande et al. 2013).

Recently, we demonstrated that in a XMEN patient, as well as in MAGT1-deficient patients with intellectual and developmental disability, STT3B-dependent glycosylation is affected due to MAGT1 mutations (Blommaert et al. 2019). Later on, our findings were confirmed by Matsuda-Lennikov et al. 2019 and Ravell et al. 2020b, showing N-glycosylation defects in other XMEN patients. Interestingly, the thorough clinical examination of a large XMEN cohort concluded that this is a complex, multisystem glycosylation disorder, going beyond the initially described immunodeficiency. The researchers abandoned the Mg^{2+}-transporter hypothesis, and agreed with the proposed role of MAGT1 in glycosylation (Matsuda-Lennikov et al. 2019). This strengthens the research line that MAGT1 is an OST subunit, and that possibly a downstream Mg^{2+} homeostasis glycoprotein is hypoglycosylated (Cherepanova et al. 2016). Here, we focussed on the link between NKG2D and XMEN, to assess whether its instability is caused by hypoglycosylation.

We demonstrated that NKG2D has one sequon ($N_{202}CS$) that is completely dependent on the STT3B complex (and therefore also on MAGT1) for proper glycosylation, which is in concordance with previous reports showing that glycosylation sites in the last 65 residues of

296 the C-terminus are strictly STT3B-glycosylated (Cherepanova et al. 2019). Moreover, the
297 other sites (N_{131}AS and N_{163}GS) showed a mild dependency on the STT3B complex. We
298 believe that hypoglycosylation causes the described deficient expression of NKG2D in XMEN
299 patients. Upon supplementation of 5 mM Mg^{2+} no significant restoration of NKG2D could be
300 observed in MAGT1$^{-/-}$TUSC3$^{-/-}$ cells transfected with NKG2D and DAP10. This is in sharp
301 contrast with the rescue of NKG2D stability in patient lymphocyte cell cultures in a previous
302 publication (Chaigne-Delalande et al. 2013). We cannot exclude that the high concentration
303 of Mg^{2+} used for this rescue was able to enhance NKG2D expression by indirect effects,
304 which makes it difficult to reproduce such results as environmental factors likely have an
305 influence. Unfortunately, stimulation of P1 PBMBCs with anti-CD3/CD28 did not result in
306 proliferation of lymphocytes, therefore we were not able to perform this experiment on
307 patient cells. Moreover, we showed that *in vivo* treatment of P1 with Mg^{2+} did not cause any
308 significant improvements in NKG2D expression, supporting the data obtained in cell lines.
309 This is in contrast with the study showing that treatment with Mg-threonate leads to an
310 increase in NKG2D expression on the cell surface of $CD8^+$ and NK cells (Chaigne-Delalande et
311 al. 2013), although in our case Mg-gluconate was used.
312 As NKG2D is important in EBV recognition, the lack of expression of this protein is probably
313 an important modulator for the typical EBV infections in XMEN patients. In addition, a
314 glycoproteomic assay in XMEN lymphocytes showed that multiple important immune
315 regulators, such as CD28 and CD70, were hypoglycosylated, further contributing to the
316 phenotype. In line with our results shown here, NKG2D was also hypoglycosylated in this
317 dataset (Ravell et al. 2020b).
318 Remarkably, the previously described MAGT1-CDG patients do not have an immune
319 phenotype to this date (Blommaert et al. 2019). It would be interesting to study immune

glycoproteins from these patients, to determine whether the difference in clinical phenotype is caused by differences in steady state levels of these proteins. Unfortunately, no lymphocyte material was available from these patients, leaving room for speculation.

In conclusion, we demonstrated that NKG2D glycosylation is dependent on STT3B, and by extension, of MAGT1. Moreover, we pinpoint the exact sequon that is underglycosylated. The strongly reduced NKG2D expression in patients is, at least partially, responsible for the susceptibility of MAGT1-deficient patients to EBV infections. Thus, we were able to link the pathological mechanism (hypoglycosylation) in XMEN patients to the clinical phenotype.

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Figures

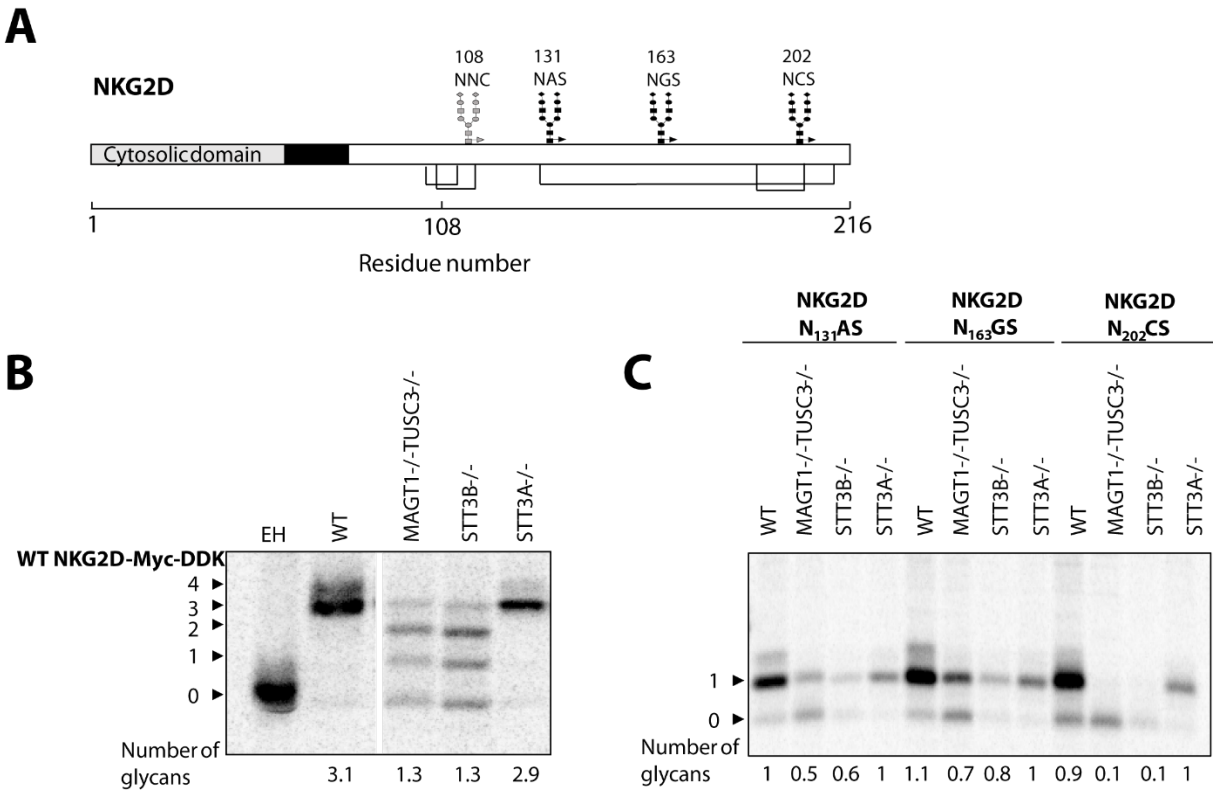


Fig. 1 NKG2D is hypoglycosylated in cells lacking STT3B activity

(a) Diagram showing the glycosylation sites of NKG2D. Grey glycan indicates a suboptimal glycosylation sequon (N_{108}NC). The transmembrane domain is depicted in black, cysteine residues by black lines. **(b)** WT and KO HEK293 cells were transfected with NKG2D-Myc-DDK followed by metabolic pulse-chase labelling. The figure was spliced between the WT and MAGT1$^{-/-}$TUSC3$^{-/-}$ samples. **(c)** Metabolic pulse chase labelling of different NKG2D constructs, containing just one glycosylation site. Quantified values below gel lanes represent the average number of glycans for the respective reporter (n = 3). EH indicates endoglycosidase H treatment.

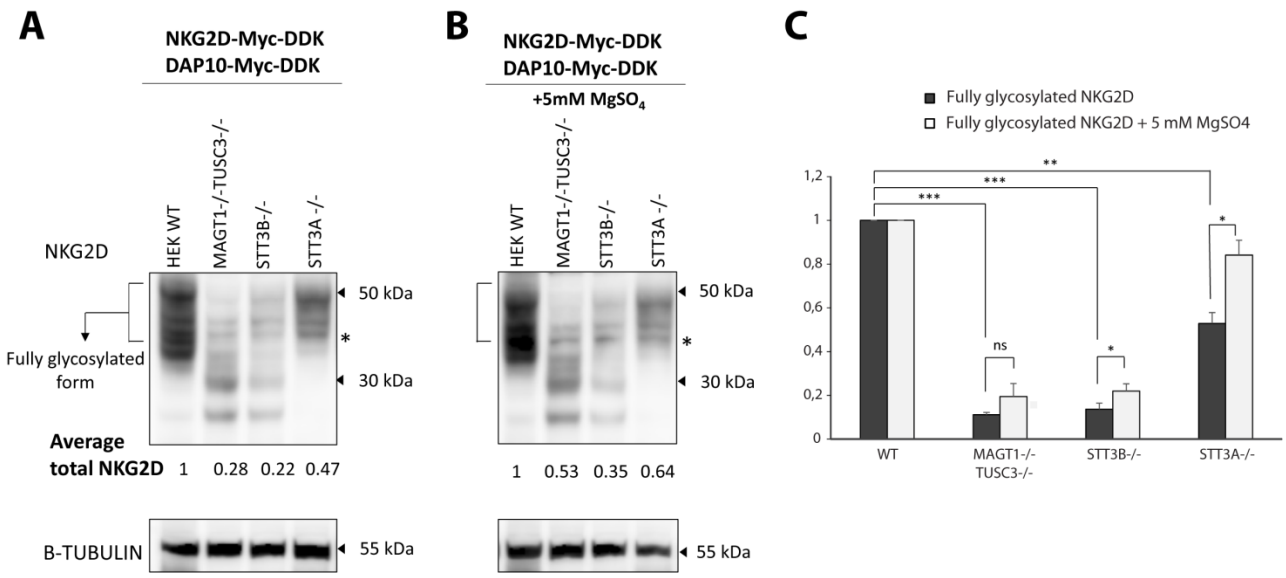
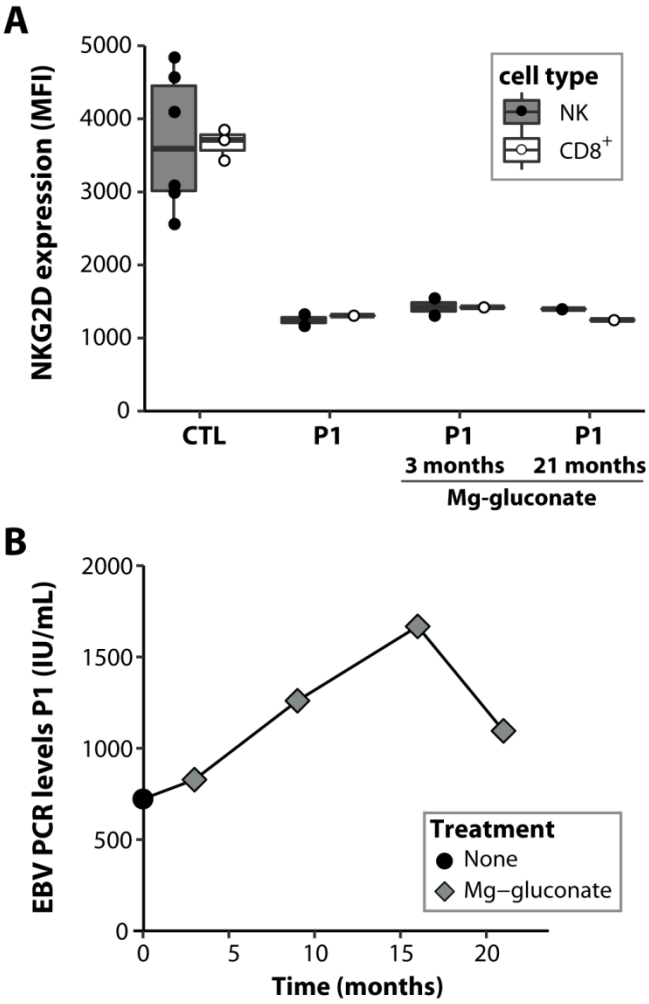


Fig. 2 Steady state levels of NKG2D

(a) The different HEK293 cell lines were cotransfected in regular DMEM with NKG2D-Myc-DDK and DAP10-Myc-DDK constructs and analysed for protein steady state levels of NKG2D 48h later by immunoblotting **(b)** in DMEM supplemented with 5 mM $MgSO_4$. The asterisk depicts a nonspecific band co-migrating with NKG2D. β-Tubulin was used as a loading control. Values represent averaged values normalised to the WT control of each condition (n = 3). **(c)** Relative abundance of the fully glycosylated NKG2D isoform in untreated and Mg^{2+} treated cells, normalised to the respective WT. Error bars depict the standard deviation. (Paired Student's t test: *= $p<0.05$; **= $p<0.005$, ***= $p<0.0005$; ns = not significant).



417

418 **Fig. 3 Effects of *in vivo* Mg^{2+} supplementation for patient 1**

419 **(a)** Median fluorescence index (MFI) of NKG2D cell surface expression on NK (n=6) and $CD8^+$ (n=3) control (CTL)
420 and patient (P1) cells. For P1, levels were measured before treatment and after 3 or 21 months of 3g Mg-
421 gluconate per day supplementation. Gating strategy is described in **Fig. S3**.
422 **(b)** EBV (Epstein-Barr virus) PCR levels (IU/mL) of P1 measured over time (diamond squares indicate
423 magnesium supplementation).
424

Abbreviations

CDG, Congenital Disorders of Glycosylation; EH, endoglycosidase H; EBV, Epstein-Barr virus; KO, knock out; MFI, median fluorescence index; Mg^{2+}, magnesium; OST, oligosaccharyltransferase; WT, wild type; XMEN, X-linked immunodeficiency with Mg^{2+} defect, Epstein-Barr virus infection and neoplasia

Supplementary data

Title:

Lack of NKG2D in MAGT1-deficient patients is caused by hypoglycosylation

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Figure S1-3
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Clinical synopsis of patient 1

Patient 1 (also referred to as patient 3 (P3) in Blommaert et al. 2019) is a 20-year-old Caucasian male, born from non-consanguineous parents (pedigree depicted in **Fig. S2**). The patient presented with an Epstein-Barr virus (EBV) primary infection at the age of 2, necessitating admission to the paediatric intensive care unit due to deterioration of his general condition, a petechial rash and pancytopenia with hypertriglyceridemia and mild coagulation abnormalities. Next, also a maculopapular rash and generalized oedema was noted. Bone marrow aspirate demonstrated absence of malignancy and mostly abundant granulocyte formation. Oxygen, antibiotics and IV fluid treatment was installed with a favourable evolution except for persistent hepatomegaly. A liver biopsy, performed after his stay at the paediatric intensive care unit, did not show hepatitis or hematophagocytosis. Serum ferritin, soluble CD25 was not determined. This episode was interpreted as a primary EBV infection, complicated with a macrophage activation syndrome. Afterwards, a persistent EBV viremia was observed (**Table S1** and **Fig. 3B**). Since the age of 8, recurrent herpes simplex virus (HSV) stomatitis episodes, treated with acyclovir, and HSV labialis (every 2-3 months) were noted. During childhood (age 10 years and onwards) he had recurrent warts (predominant on the hands and arms) that subsided later in adolescence. Recurrent furuncles were noted in childhood and adolescence, and post-appendectomy infection required IV antibiotics (age 10 years). Finally, he mentioned upper respiratory tract infections, sporadically leading to antibiotic treatment via his general practitioner (GP).

Clinical examination was normal at time point T0 (17 years, 10 months). Of note, no palpable glands were noted although recurrent enlarged cervical lymph nodes were mentioned and ultrasound revealed a spleen diameter at the upper limit of normal (cranial-caudal diameter of 12.4 cm, age 19, body height 1.77 m). Laboratory results demonstrated mild neutropenia, thrombocytopenia, lowered IgG (7.46 g/L, normal range (NR) 7.51-15.60), elevated transaminases, and persistent EBV-viremia (**Table S1** and **Fig. 3B**). Further immunological evaluation demonstrated normal total lymphocyte, T-, and NK-cell count, elevated $CD19^+$ B-cell count (1542/µL, NR 82 - 0.476), consisting predominantly of naive (94% $CD27^-IgM^+$) B-cells, next to lowered IgM-memory ($CD27^+IgM^-$, 2.2%) and switched memory ($CD27^+IgM^-$, 2.8%) B-cells. CD4, CD8 T-cell, regulatory T-cell (8.4% $CD4^+CD25^+CD127_{low}$) counts were normal. CD4:CD8 ratio was normal (0.89, NR 0.80-3.50). Double negative T-cells (DNT, alpha-beta T-cell receptor $CD3^+CD4^-CD8^-$) were elevated (2.8% of CD3), naive T-cell ($CD27^+CD45RA^+$) count was 62% of CD3 positive cells. Lymphocyte proliferation rate was normal using HSV, varicella zoster virus, or PHA (index for HSV was 14.03, for varicella zoster virus 159.41, for PHA 61.12; normal > 5.0). Stimulation with anti-CD3/CD28 on frozen or fresh PBMCs did not result in proliferation of patient lymphocytes.

The patient received standard vaccinations including vaccination with life attenuated measles, mumps, and rubella uneventfully and resulting in normal or near-normal serologic responses (anti-measles IgG 650 mIU/mL; anti-rubella IgG 8.4 IU/mL, normal >10.0 mIU/mL; anti-hepatitis B surface antigen IgG 11.5 mIU/mL, normal ≥ 10.0 mIU/mL; measured at the age of 19 years). Pneumococcal polysaccharide vaccination was refused. Varicella zoster virus and CMV serology demonstrated past infection, PCR for persistent CMV viremia was negative. PCR for polyoma virus (BK/JC virus) in urine was positive (> 8 log copies/mL).

Next generation sequencing revealed a pathogenic variant in the X-linked *MAGT1* gene (c.938T>G, p.Leu313*) at 17 years of age (Blommaert et al. 2019). Given the clinical features of persistent EBV viremia, macrophage activation syndrome during primary infection, decreased NKG2D expression (Blommaert et al. 2019) and the rare, previously unreported nor identified (ExAC database, gnomAD, 1000 genomes) variant in *MAGT1,* a diagnosis of X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia (XMEN) disease was made.

He was treated with oral magnesium supplementation from the age of 17 years and 10 months onwards using 3 g of Mg^{2+} gluconate (containing 162 mg Mg^{2+}, Ultra-Mg®, Melisana) per day. Increasing the frequency to twice or three times per day led to gastro-intestinal intolerance. Magnesium threonate, as was previously reported as a therapy for XMEN patients (Chaigne-Delalande et al. 2013), was not initiated because of unavailability via the local hospital pharmacy. Since magnesium supplementation, he noted a drop in upper respiratory tract infections and HSV stomatitis episodes (although from hindsight this was already absent the year before). EBV viremia was deemed to be non-responsive to magnesium supplementation (**Fig. 3B**). Of note, elevated transaminases were observed throughout the follow-up with a temporary flare-up coinciding with the start of magnesium supplementation. No changes in total serum magnesium were noted throughout the follow-up (**Table S1**). Subjectively, he felt his general condition was comparable to that of his peers. Although he was reluctant for a frequent clinical follow-up, adherence to magnesium supplementation was high, possibly suggesting (at least subjective) efficacy.

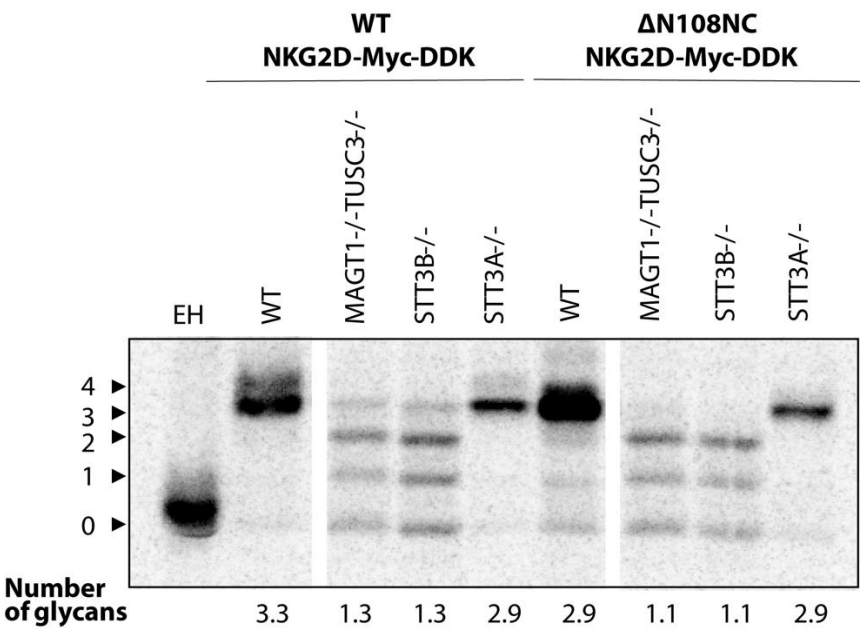

Figure S1: The N_{108}NC glycosylation site is suboptimal.

Metabolic pulse chase labelling of the WT NKG2D-Myc-DDK and the ΔN_{108}NC NKG2D-Myc-DDK construct, in different HEK293 engineered cell lines. Quantified values are shown below gel lanes and represent the number of glycans for the respective reporter. EH indicates Endoglycosidase H treatment, and serves as a mobility marker. White lanes between samples indicates where the figure was spliced.

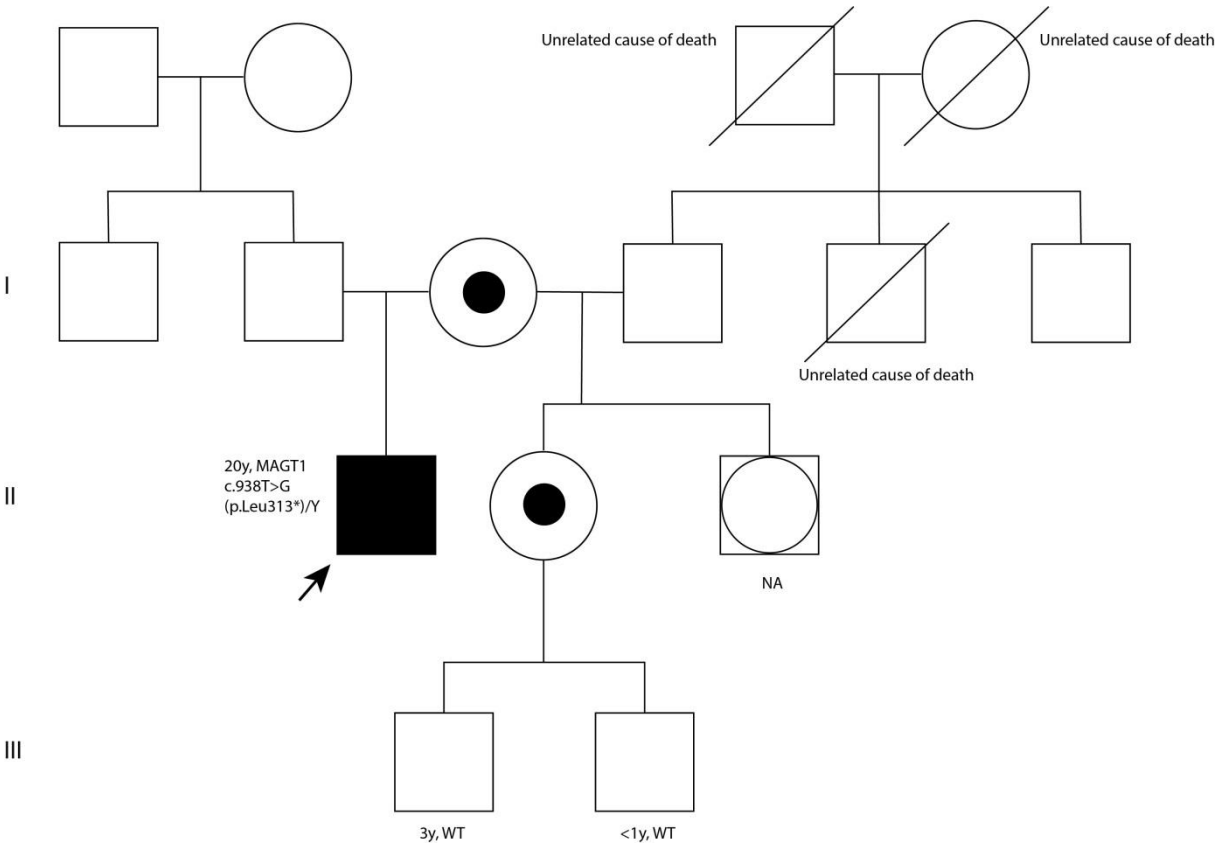

Figure S2: Pedigree of patient 1.

Index case (II.1) is from Caucasian, European descent. No consanguinity was noted and no other affected male family members were identified. Medical history of the other family members was negative except for recurrent nasal and labial herpes simplex virus infection in his half-sibling (II.2, carrier of *MAGT1* c.938T>G), which was regarded as non-exceptional.

Abbreviations: WT, wild type; NA, not available.

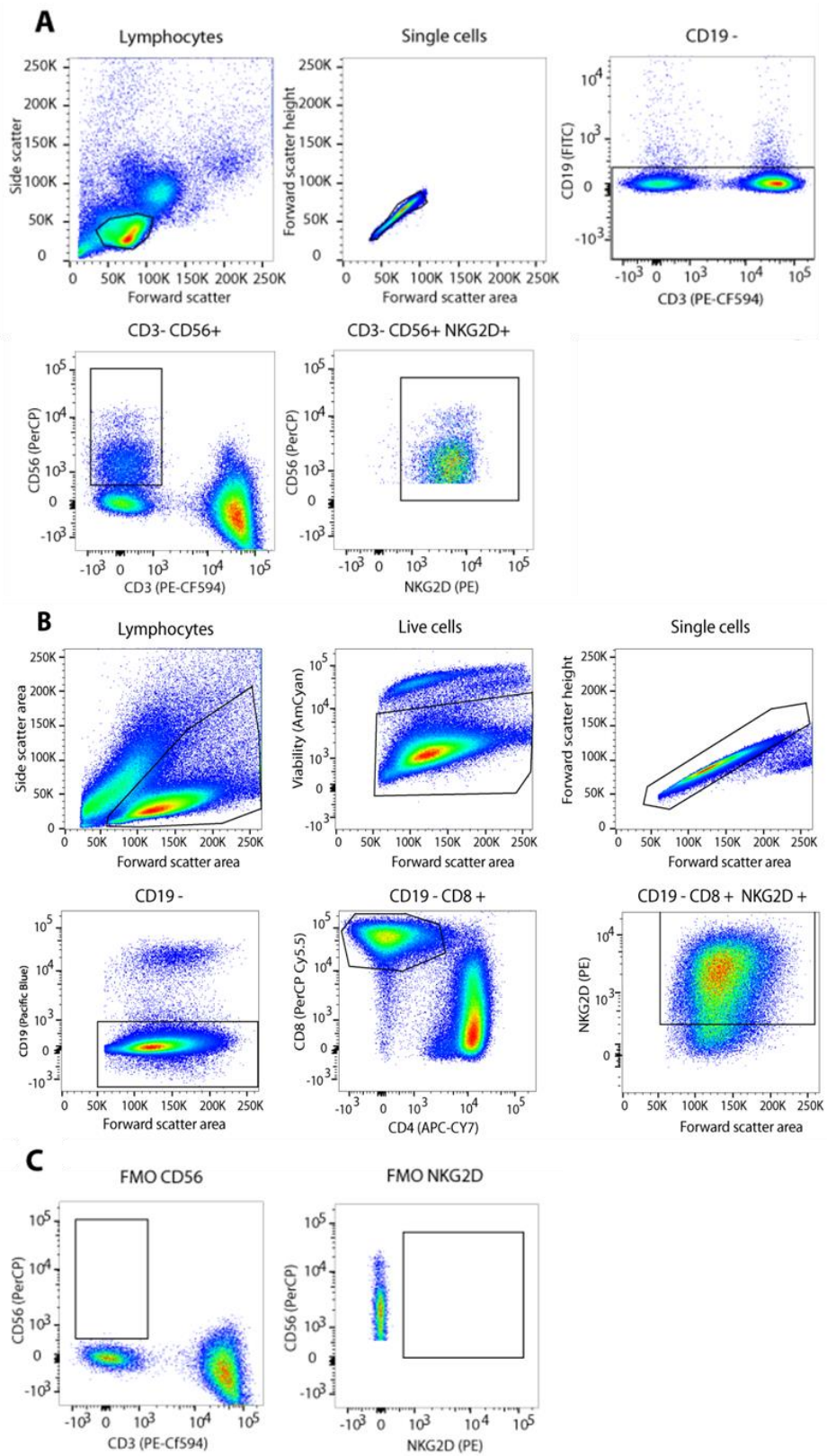

Figure S3: Gating strategy for NKG2D expression levels on NK cell surface.

(A) First, PBMC were selected on both size and singularity. Next, CD19 and CD3 negative cells were gated on expression of CD56 to identify NK cells. Finally, NK cells were gated for expression of NKG2D.

(B) First, PBMC were selected on size, living cells and singularity. Next, CD19 negative cells were gated on expression of CD8 to identify CD8+ cells. These cells were gated for expression of NKG2D;

(C) Fluorescence Minus One (FMO) for CD56 and NKGD2 to determine gates for both proteins.

Table S1: Summary of clinical values for P1 at different time points.

Values outside the normal range are marked in red. EBV = Epstein-Barr Virus, Treg = T regulatory cells

	Reference value	+644 days	+483 days	+287 days	+93 days	+63 days	T0 (17y 10months)
White blood cells	4-10 x10^9/L	4.46	6.43	5.17	4.58	4.59	4.92
Platelets	150-450x10^9/L	132	196	142	177	179	179
Hemoglobin	13.0 -16.0 g/dl	14.0	14.4	14.0	13.8	15.0	14.6
C-reactive protein	≤5 mg/L	0.9	1.0	0.5	1.9	0.8	0.3
Neutrophils	2.5-7.8 x10^9/L	1.5	2.6	1.8	1.4	1.2	1.6
Lymphocytes	20-50 %	58.7	51.6	55.7	59.2	68	59.6
CD19$^+$	0.082-0.476 x10^9/L	1.261	1.619	1.443			1.542
CD4$^+$	0.455-1.885 x10^9/L	0.572	0.646	0.554			0.531
CD8$^+$	0.219-1.124 x10^9/L	0.578	0.741	0.632			0.599
CD4/CD8 ratio	0.8-3.5	0.99	0.87	0.88			0.89
NK (CD3$^-$,CD56$^+$)	4-30 %	4.3	5.7	4.7			4.6
Treg (CD4$^+$, CD125$^+$CD127$_{low}$)	5-12 % of CD4+						8.4
IgG	7.51-15.6 g/l	8.72	8.39	7.46			8.44
IgA	0.82-4.53 g/L	0.46	0.60	0.48			0.52
IgM	0.46-3.04 g/L	0.52	0.48	0.41			0.41
Serum magnesium	0.63-1.05 mmol/L	0.78	0.82	0.78		0.82	
EBV viremia	EBV PCR IU/ml	1095	1667	1260		828	772
Aspartate transaminase	≤38 U/L	38		33	57	81	24
Alanine transaminase	≤41 U/L	76		57	95	100	42
Gamma glutamyl transferase	≤60 U/L	20		19	20	21	19
Alkaline phosphatase	40-130 U/L	112		121	133	149	145
Body mass index	18.5-25 kg/m^2	22.4	22.9	23.2	22.9		22.1