Upregulation of the NKG2D ligand ULBP2 by JC polyomavirus infection promotes immune recognition by natural killer cells.

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Abstract

JC polyomavirus (JCPyV) establishes a chronic infection in 70-90% of the world’s population. In immunocompetent individuals, JCPyV chronic infection is asymptomatic and not associated with diseases. However, JCPyV causes progressive multifocal leukoencephalopathy (PML), a potentially fatal complication of severe immune suppression due to monoclonal antibody treatments for cancer, autoimmune diseases and transplantation, or due to uncontrolled HIV infection. There is currently no effective treatment against PML and novel immunotherapies are urgently needed to decrease the morbidity and mortality caused by JCPyV. The risk of developing PML increases with loss of immune control by JCPyV-specific T cells and antibodies. Natural killer (NK) cells play critical roles in defense against viral infections, yet NK cell contribution to the control of JCPyV infection remains largely unexplored. Here, we first compared NK and T cell responses against JCPyV VP1 peptide pools. In about 40% of healthy donors, we detected robust CD107a upregulation and IFN-γ production by NK cells, extending beyond T cell responses. Next, using a novel flow cytometry-based killing assay, we showed that co-culture of NK cells and JCPyV-infected astrocyte-derived SVG-A cells leads to a 60% reduction in infection, on average. Expression of ligands for the activating NK cell receptor NKG2D was modulated in JCPyV-infected cells, with overall enhanced expression of ULBP2. To evaluate the impact of NKG2D triggering on NK cell-mediated elimination of JCPyV-infected cells, we performed co-cultures in the presence of NKG2D blocking antibodies, which resulted in decreased NK cell degranulation. Altogether, these findings suggest NKG2D-mediated activation may play a key role in controlling JCPyV replication and may be a promising immunotherapeutic target to boost NK cell anti-JCPyV activity.

Author Summary (150-200 words)
The human polyomavirus JC (JCPyV) infects most people for life but only causes disease in persons with a compromised immune system. In particular, JCPyV reactivation in the brain is responsible for the development of progressive multifocal leukoencephalopathy (PML). There is currently no effective treatment for PML, which is often fatal. Natural killer (NK) cells are effector cells of the innate immune system that play critical roles in defense against viral infections, yet their contribution to the control of JCPyV infection remains largely unexplored. The current study shows that NK cells can eliminate cells infected with JCPyV and that immune recognition is partly mediated by NKG2D, an activating ligand expressed on NK cells, and its binding to ULBP2, a stress-induced ligand expressed on infected cells. Our findings provide new insights into immune mechanisms involved in JCPyV immunity, and unveil opportunities to harness NK cell function in future therapeutic strategies to target JCPyV.

Introduction

The majority of the adult human population worldwide carries the human polyomavirus JC (JCPyV) [1]. Primary JCPyV infection occurs early in life and results in a persistent, lifelong, asymptomatic infection within the kidney tubular epithelial cells, where the virus reproduces benignly with occasional shedding in the urine [2]. JCPyV can spread to secondary sites, including the bone marrow, lymphoid tissues and the brain [3, 4]. The precise processes underlying entry of JCPyV in the brain have not been completely elucidated and proposed mechanisms include brain transmigrated leukocytes that are either infected with JCPyV or carry viral particles at their surface [5]. JCPyV infection usually does not have significant clinical consequences in immunocompetent individuals. However, prolonged and severe immunosuppression or immunomodulation promotes viral reactivation and increases the risk of developing progressive multifocal leukoencephalopathy (PML). PML is a rare but often fatal infection of oligodendrocytes - the myelinating cells of the central nervous system - by JCPyV. While PML is most common
among people living with HIV (PLWH) [6, 7], with up to 80% PML patients being HIV-positive [8],
there is a growing number of patients with autoimmune diseases, such as people with multiple
sclerosis, at risk for PML [5, 9-12].

There is currently no effective treatment against JCPyV and attempts to treat PML with
medications previously approved for other diseases, such as mefloquine, have all failed [13-16].
Furthermore, rapidly restoring the immune functions in the central nervous system can also be
fatal due to immune reconstitution inflammatory syndrome [17-20]. Thus, there is an urgent need
for novel immunotherapeutic interventions to specifically enhance immune control of JCPyV.

Impaired immunity is key in the development of PML. In particular, JCPyV-specific CD8+ T cells
are crucial in curtailing JCPyV replication to recover from PML and CD4+ T cell counts are
associated with prognosis in PML [21-27]. Based on these observations, recent efforts in PML
treatment trials have focused on reviving T cell response using T cell checkpoint inhibitor and
allogeneic polyomavirus-specific T cells [28] or infusions of cytokines such as IL-7 and IL-15
superagonist [29, 30]. Results of these studies showed potential efficacy in subgroups of patients,
indicating other immune factors, in addition to T cells, are necessary to treat PML in all patients.

While it is known that risk of PML increases with the loss of immune control by virus-specific T
lymphocytes and antibodies [31, 32], the contribution of natural killer (NK) cells to the containment
of JCPyV replication has been suggested in a few studies but remains overall largely unexplored
[30, 33, 34]. Classically, NK cells are viewed as nonspecific effector cells of the innate immune
system that play critical role(s) in defense against viral infections or nascent neoplasms. Unlike
other lymphocytes, NK cells lack antigen-specific receptors but lyse target cells following the
integration of inhibitory and activating signals. These signals are generated by an arsenal of
germline encoded cell surface molecules, commencing effector functions when activating signals
overcome inhibitory ones [35]. NKG2D represents one of the most potent activating NK cell
receptors that allow NK cells to discriminate between “self” and a variety of pathological cell
states, as engagement with one of its ligands is enough to override inhibitory signals [36]. NKG2D
ligands (NKG2DL) consist of MHC class I related chain (MIC) A and B and six UL-16 binding proteins (ULBP1-6), which are typically not expressed in healthy tissues but rather upregulated by cellular stress such as viral infection [37-41]. NKG2D-mediated NK cell responses have been found critical in the control of several viral infections [42-44]. The NKG2D/NKG2DL axis also plays a pivotal role in tumor immunosurveillance and therefore immunotherapeutic strategies targeting the NKG2D pathway are currently under investigation [45-48].

A plethora of studies has provided compelling evidence supporting the significant contribution of NK cells to the immune control of major human viral infections such as CMV, influenza virus, hepatitis C virus and HIV [44, 49-54]. Nevertheless, investigations to better define the role played by NK cells in JCPyV infection are currently lacking and are needed to improve our understanding of PML pathogenesis. Moreover, identification of NK cell subpopulations with enhanced or reduced anti-viral activity against JCPyV could provide novel targets for immunotherapeutic strategies to prevent and treat PML. To fill an important gap in knowledge, in this study we measured NK cell responses against JCPyV and investigated the role of NKG2D, a major activating receptor on NK cells and a checkpoint target for cancer immunotherapies, in NK cell-mediated elimination of JCPyV-infected targets.

**Results**

**NK cells display robust responses to JCPyV VP1 peptides.**

Viral infection induces changes in the HLA peptide repertoire and such alterations can directly [55, 56] or indirectly [57-59] modulate NK cell function. Therefore, NK cell responses can be measured upon stimulation of PBMCs with viral peptide pools or inactivated pathogens and have been proposed to vastly depend on IL-2-secreting effector memory T cells [57-59]. However, to our knowledge, NK cell responses to JCPyV peptide pools have never been investigated. Using PBMCs from JCPyV-seropositive healthy donors, we compared NK and T cell responses against
2 pools of overlapping peptides derived from the JCPyV capsid protein VP1 by intracellular cytokine staining (ICS) (S1 Fig, Fig 1). Previous studies reported T cell responses against JCPyV using ICS, however optimal detection of T cell responses often relied on ex vivo expansion of JCPyV-specific T cells for 10 to 14 days of culture in the presence of JCPyV peptides and IL-2 [31]. To avoid culture conditions that could significantly alter and misrepresent primary NK cell responses to JCPyV, we performed a 6h assay in the absence of exogenous IL-2. We observed positive responses to VP1 (defined as at least twice above the background) by bulk NK cells from over 40% of donors, with proportions of IFN-γ+ NK cells being overall higher against the peptide pool encompassing the 161-253 and 257-341 regions of VP1. Using this assay, T cell responses could be directly measured without the need for prior amplification of antigen-specific T cells, yet NK cell responses were consistently more potent than T cell responses.

**NK cells efficiently kill JCPyV-infected SVG-A cells.**

Beyond modulation of NK cell function by changes in the HLA peptide repertoire, recognition of virally infected cells by NK cells mainly rely on downregulation of ligands for inhibitory NK cell receptors (i.e., select HLA class I molecule) and upregulation of ligands for activating receptors (i.e., NKG2DL). To evaluate the overall ability of NK cells to directly recognize and eliminate JCPyV-infected cells, we developed a new flow cytometry-based assay to measure NK cell killing of human fetal astroglial cells immortalized with SV40 (SVG-A) and infected with JCPyV (M1-SVEΔ) (virus originally made in Dr Eugene Major’s lab and provided by Campbell Kaynor, Biogen)[60, 61]. JCPyV M1-SVEΔ is a chimeric polyomavirus derived from the MAD1 strain with replaced regulatory regions from SV40, which display enhanced replication in cell culture [61] (Fig 2A). We show that co-culture of primary NK cells and JCPyV-infected SVG-A cells leads to, on average, a 4-fold decrease (range 1.2-20) in infected cells (Fig 2B) and 60% killing (Fig 2C). While there were inter-individual variations in NK cell-mediated killing of infected SVG-A cells, NK cells
from all donors were able to mediate cytotoxic responses against JCPyV. Fifty percent of the
donors were seronegative for JCPyV, yet as expected, killing of JCPyV-infected SVG-A cells by
NK cells was not influenced by the JCPyV serostatus (data not shown). Of note, CD107a
upregulation in NK cells has been shown to correlate with NK cell-mediated cytotoxicity (41,
42) and accordingly, a significant CD107a upregulation was observed in NK cells exposed to
JCPyV-infected SVG-A cells (Fig 2D), independently of addition of protein transport inhibitors
during the co-culture (S2 Fig). In sum, these data strongly suggest that NK cells significantly
contribute to regulating JCPyV replication.

NKG2D and its ligand ULBP2 play a key role in NK cell responses against JCPyV-infected
cells.

NKG2D is an activating receptor expressed on most NK cells which is a major regulator of NK
cell function [35] and represents a checkpoint target for immunotherapies currently in
development [62]. A role of NKG2D in immune responses against polyomaviruses has been
previously suggested by studies demonstrating that JCPyV and another member of the family,
BKPyV, both express an identical miRNA that target ULBP3 [34]. To evaluate the impact of
NKG2D in NK cell-mediated lysis of JCPyV-infected cells in our system, we compared cell surface
expression of the different NKG2DL on uninfected and JCPyV-infected SVG-A cells and 293T
cells, another cell line permissive for JCPyV infection (Fig 3A-B). SVG-A cells expressed MICA
and MICB independently of infection, which are likely responsible for the background response
by NK cells against uninfected cells (Fig 2D). We consistently found enhanced binding of an
antibody that recognizes ULBP2, ULBP5 and ULBP6 on JCPyV-infected cells, suggesting one or
several of these receptors are upregulated upon JCPyV infection. To precisely determine how
these receptors are independently modulated by JCPyV infection, we quantified mRNA
expression levels in uninfected and infected cells using primers specific for each ULBP by
quantitative real-time PCR and found that only expression of ULBP2 is increased upon infection in both SVG-A and 293T cells (Fig 3C). Enhanced ULBP2 expression levels were also observed in JCPyV-infected human primary renal proximal tubule epithelial (HPRTE) cells, a primary target for JCPyV, upon secondary analysis of data generated in the laboratory of Walter J. Atwood [63] (Fig. 3D). Based on enhanced expression of ULBP2 on JCPyV-infected cells, we then performed co-culture assays in the presence of antibodies blocking the activating NKG2D receptor. Blockade of NKG2D resulted in decreased NK cell degranulation response to JCPyV-infected SVG-A cells (Fig 3E-F). Therefore, one of the mechanisms underlying NK cell-mediated responses to JCPyV-infected SVG-A cells relies on their recognition via NKG2D.

Discussion

Compelling evidence supports a crucial role for NK cells in the control of several major human viral infections as well as in shaping adaptive immune responses. However, there are many gaps in knowledge regarding the contribution of NK cells in the pathogenesis and progression of PML, the disease caused by JCPyV reactivation in the brain of immunocompromised individuals. Herein, we present the first evidence that JCPyV VP1-derived peptide stimulation and JCPyV infection both elicit robust NK cell function, suggesting that NK cells may significantly contribute to the cellular effector response to JCPyV. We also unveil mechanistic insights underlying immune recognition of a JCPyV-infected astrocyte cell line, which implicate NKG2D and its stress-induced ligand ULBP2.

While NKG2D engagement promotes potent and dominant NK cell activation, several viruses have evolved elaborate mechanisms to evade NKG2D-mediated recognition. For instance, studies have shown shedding of NKG2DL by HIV-infected CD4+ T cells, thereby promoting reduced expression of NKG2D on NK cells and impaired NKG2D-mediated NK cell responses [64, 65]. As PLWH represent the majority of PML patients, it is possible that impaired NKG2D
signaling in PLWH is associated with poor NK cell-mediated control of JCPyV replication and development of PML. Future investigations are warranted to examine NK cell surface expression of NKG2D and plasma levels of soluble NKG2DL in PML patients, including both PLWH and HIV-negative patients.

Another reported immune escape mechanism exploited by viruses is the downregulation of NKG2DL expressed on infected cells. Interestingly, an elegant study previously reported that JCPyV encodes microRNAs that downregulate ULBP3, thereby reducing NK cell-mediated recognition of polyomaviruses-infected cells [32]. In our system, we did not observe significant changes in ULBP3 expression upon infection. This discrepancy may be explained by the different cell lines and JCPyV strain used, as well as the later time point for the assessment of NKG2D L expression in our experiments compared to the studies conducted by Bauman et al. Many immortalized cell lines trigger potent NK cell cytotoxicity because they express ligands for activating NK cell receptors or lack HLA-Class I ligands for inhibitory NK cell receptors [66]. SVG-A cells were selected for our experiments because infection of these cells is a long standing and well-established in vitro system to study JCPyV [103, 104] and compared to other cell lines permissive for JCPyV infection such as 293T, 293TT and C33A cells, uninfected SVG-A cells elicited only background levels of NK cell responses (data not shown). Nevertheless, altogether, these findings are consistent with differential expression of the various NKG2DL in different primary cells or cell lines and all support a significant role for the NKG2D pathway in the recognition of JCPyV-infected cells by NK cells. This data opens avenues for therapeutics against PML based on existing strategies currently in development targeting NKG2D [45-48].

Finally, NK cell responses to JCPyV most likely do not solely depend on the NKG2D/NKG2DL axis, and other pathways may be additive or alternative to this mechanism. Several immunotherapeutic strategies to treat PML have been evaluated in individual case reports and small patient series, including stimulation of lymphocytes using IL-7 [29, 67-72] and IL-15 [30]. These approaches have been associated with improved PML outcome in subsets of patients and
since IL-7 and IL-15 are known to activate NK cells, their contribution to the clinical outcome of these therapies cannot be excluded. Notably, successful treatment with IL-15 superagonist in a PML patient with allogeneic stem cell transplant was associated with a rise in peripheral blood natural killer (NK) cells but not in CD3+ T cells [30]. Overall, rationally optimizing therapies under investigation by targeting bulk or subsets of NK cells may offer novel immunotherapeutic approaches against PML.

Our data also show NK cell activation, and particularly IFN-γ production, upon stimulation with VP1-derived peptides. Interestingly, there was no positive correlation between the proportions of IL-2+ T cells and those of IFN-γ+ NK cells (data not shown), indicating that in this assay, NK cell responses may not mainly rely on IL-2 produced by JCPyV-specific memory T cells. NK cell activation could be directly triggered by VP1 peptides presented by HLA class I that either disrupt the interaction between HLA class I molecules and their inhibitory ligand on NK cells, or engage NK cell activating receptors. Further investigations to explore the role played by inhibitory and activating killer cell immunoglobulin like receptors (KIRs) or by CD94-NKG2A/C heterodimers in these responses are warranted.

Collectively, these findings suggest that the loss of cellular immunity associated with enhanced JCPyV replication and progression towards PML may encompass impaired NK cell function, and that boosting NK cell activity may potentiate the overall immune control of JCPyV. To our knowledge, these results provide the first evidence for a direct effect mediated by NK cells against JCPyV and have important implications for the design of future immunotherapeutic interventions aimed at enhancing JCPyV immunity in immunocompromised individuals.

**Materials and Methods**

**Human Subjects**
Deidentified and coded blood samples from healthy donors used in this study were collected under IRB-approved protocols and delivered to us by Research Blood Components, LLC (Watertown, Massachusetts). Frozen PBMC vials from healthy donors were purchased from STEMCELL Technologies. Beth Israel Deaconess Medical Center institutional review board approved this study, and all subjects gave written informed consent.

Analysis of primary NK cell responses to JCPyV VP1-derived peptides by intracellular cytokine staining

To measure NK and T cell responses to the JCPyV capsid VP1, cryopreserved PBMCs from healthy donors that tested seropositive for JCPyV were thawed and directly stimulated with 2µg/mL of sequential peptide pools consisting of 15-mer sequences with 11 aa overlap covering the whole JCPyV VP1 sequence and combined into 2 pools (VP1 1-93 + VP1 97-157 (n=48); VP1 161-253 + VP1 257-341 (n=49). PBMC were co-cultured with peptides at 37°C for 6h with CD107a BV786 (BD Biosciences, H4A3) and 1µg/mL of CD28/CD49d costimulatory reagent (BD Biosciences). 1µL/mL GolgiPlug (BD Biosciences) and 0.7µL/mL GolgiStop (BD Biosciences) were added for the last 2h of incubation. At the end of the incubation, cells were stained first with the LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Invitrogen), then with BD Biosciences CD3 BV510 (UCHT1), CD14 BV421 (M5E2), CD19 BV421 (HIB19), CD16 APC-Cy7 (3G8), CD56 BV605 (NCAM16.2), CD4 BU395 (L200) and CD8 APC (SK1), and finally fixed, permeabilized (Thermofisher Fix and Perm) and stained with BD Biosciences IFN-γ FITC (B27) and IL-2 PE-CF594 (5344.11) antibodies to detect intracellular cytokines. In all assays described above, incubation in the presence of 5 µg/mL of phytohemagglutinin (PHA) was used as positive control and unstimulated cells served as negative controls and for background subtraction. A Fluorescence Minus One (FMO) control and PHA-stimulated PBMCs were used to set the gates
for positive cytokine responses. Acquisition of data was performed on a BD LSRII instrument (BD Biosciences). Data was analyzed using Flow Jo v.10.8.1.

**JCPyV infection of SVG-A cells**

SVG-A cells were grown in EMEM supplemented with 2% FBS and 2% Penicillin/streptomycin. 2-3 days prior infection, SVG-A cells were plated into two T-75 flasks with 0.7M cells in each flask. After 2-3 days, one flask was infected with JCPyV M1-SVEΔ (5.9e9 viral genome/mL) at a 1:100 dilution and one flask left uninfected. Cells in both flasks were cultured for a total of 9-11 days at 37°C, with addition of fresh media one-week post-infection. Percentages of infected SVG-A cells were determined prior to functional assays by intracellular staining using Thermofisher antibodies against JCPyV VP1 DyLight488 (PAB597) and Large T antigen DyLight594 (PAB2003) on the BDLSRFortessa X14.

**Killing assay**

NK Cells were enriched from freshly isolated PBMC using the EasySep™ Human NK Cell Isolation Kit (STEMCELL Technologies). NK cells were then counted and resuspended at 1M/mL in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin. Based on the number of infected SVG-A determined beforehand on the same day by flow cytometry, SVG-A cells and NK cells were combined at an E:T ratio of 10:1 (NK cells: infected SVG-A cells). To complement measures of direct NK cell-mediated killing, CD107a BV786 was also added at a 5µL/mL concentration and served as a surrogate marker of degranulation. In a subset of experiments where only CD107a upregulation but not killing was measured, 1µL/mL GolgiPlug (BD Biosciences) and 0.7µL/mL GolgiStop (BD Biosciences) were added for the whole incubation (S2 Fig). For blockade experiments, purified NK cells were incubated for 10 minutes at room temperature with 2.5µg of Human BD Fc Block
followed by addition of 10 µg/mL of anti-NKG2D purified antibody (BD Biosciences, clone 1D11) or isotype control antibodies prior to addition of SVG-A cells. Co-cultures were incubated at 37°C for 6 hr. At the end of the incubation, cells were stained first with the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen), then with BD Biosciences CD3 A700 (UCHT1), CD16 APC-Cy7 (3G8), CD56 BV605 (NCAM16.2), NKG2D APC (1D11) and Beckman Coulter NKG2A PE-Cy7 (Z199), fixed, permeabilized (Thermofisher Fix and Perm), and stained with Thermofisher VP1 DyLight488 (PAB597) and Large T antigen DyLight594 (PAB2003) to quantify SVG-A infected cells. Acquisition of data was performed on a BD FACSymphony A5. Data was analyzed using Flow Jo v.10.8.1.

**Anti-JCPyV IgG ELISA**

JCPyV capsid protein VP1 was obtained from Abcam (AB74569). Endpoint titer dilution ELISA was performed to determine the JCPyV serostatus of all healthy donors included in our study as previously published [73]. Briefly, polystyrene ELISA plates were coated overnight with 1 µg/well of JCPyV VP1 protein. After blocking with 1% casein (Thermofisher Blocker™ Casein) and washing with PBS containing 0.05% Tween 20, plasmas were added in serial dilutions and incubated for 1 hr at room temperature. The plates were then washed three times with PBS containing 0.05% Tween 20 and incubated for 1 hr with a dilution of a 1/1,000 horseradish peroxidase (HRP)-conjugated goat anti-human secondary antibody (Jackson ImmunoResearch Laboratories), developed with TMB Peroxidase Substrate (SeraCare), and stopped by addition of stopping solution (SeraCare), and analyzed at 450/550 nm with Spectramax Plus ELISA plate reader using Softmax Pro 4.7.1 software. ELISA endpoint titers were defined as the highest reciprocal plasma dilution that yielded an absorbance > 2-fold over background values.

**Quantification of NKG2DL by flow cytometry**
SVG-A and 293T cells were infected with JCPyV M1-SVEΔ for 9-11 days and stained first with the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen), then cells were split and stained in parallel with one of the following antibodies: R&D Systems ULBP1 PE (170818), ULBP3 PE (166510), ULBP-2/5/6 PE (165903) or BD Biosciences MICA/B PE (6D4) prior to be fixed, permeabilized and stained with Thermofisher VP1 DyLight488 (PAB597) and Large T antigen DyLight594 (PAB2003) with. Uninfected SVG-A and 293T cells as well as Raji and K562 cells known to express low and high levels of NKG2DL, respectively [66], were included as controls.

Quantification of NKG2DL by qPCR

Two and a half μg of total RNA isolated using Qiagen RNeasy kit, was used for cDNA first-strand synthesis in a 20-μL reaction volume using Thermofisher SuperScript™ VILO™ cDNA Synthesis Kit. Real-time quantitative PCR was performed using the QuantStudio™ 3 Flex Real-Time PCR System (Applied Biosystems) and TaqMan™ Fast Advanced Master Mix. cDNA was amplified with specific commercially available primers for ULBP2 (Hs00607609_mH), ULBP3 (Hs00225909_m1), ULBP5 (Hs01584111_mH), ULBP6 (Hs04194671_s1) and beta actin (Hs99999903_m1) with TaqMan MGB probes all conjugated with fluorochrome FAM (Thermofisher). The cycling conditions were 50°C for 2 minutes, polymerase activation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second, and 60°C for 20 seconds. Data were analyzed using Design & Analysis Software (version 2.6.0, Applied Biosystems) and R version 4.2.3 (https://www.Rproject.org).

Statistical Analyses

All bars in scatter dot plots represent median values. Column bar graphs represent mean ± SEM. Statistical analyses were performed using the GraphPad Prism software version 9.5.0 The non-parametric Wilcoxon signed-rank test was used to assess differences in cytokine production or CD107α upregulation in response to VP1-derived peptides, to JCPyV infection and in the
presence or absence of blocking antibodies, or differences in proportions of JCPyV-infected cells in the presence or absence of NK cells. Unpaired t tests were used to compare expression of NKG2DL between uninfected and JCPyV-infected cells. P-values of <0.05 were considered significant.

RNA transcriptome analysis
Transcriptome profiling of infected HRPTE cells and data analysis were performed as previously described [63]. Deposited data including pre-computed log2 fold changes and p-values were used to generate volcano plots using ggplot2 v. 3.3.2. and are available here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135833.

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Figure legends

Fig 1. NK and T cell responses to JCPyV VP1 in healthy donors by ICS. PBMC from 24 healthy donors were incubated for 6h with 2µg/mL of pools of peptides spanning JCPyV VP1 in the presence of conjugated anti-CD107a antibodies. BD GolgiStop and GolgiPlug were added for the last 2h of culture. The peptides were divided into two sequential pools as follows: VP1 1-93 + VP1 97-157 (n=48); VP1 161-253 + VP1 257-341 (n=49). Dead cells were excluded using a viability dye. Dot plots represent proportions of CD107a+, IFN-γ+ or IL-2+ T or NK cells in response to JCPyV after subtracting proportions of unstimulated cells positive for each marker. Bars represent the median. * p<0.05 compared to unstimulated.

Fig 2. Development of a flow-cytometry-based assay to measure NK cell cytotoxicity against JCPyV-infected cells. SVG-A cells were infected with M1-SVEΔ, which on average leads to 25% cells infected after 9-10 days of culture as assessed by the proportions of SVG-A cells positive for the early protein Large T (TAg) and/or the late capsid protein VP1 by intracellular flow cytometry staining. SVG-A cells were co-cultured with purified NK cells from 16 healthy donors at 10:1 E:T ratio for 6h. (A) Representative flow cytometry plots of uninfected and infected SVG-A cells stained with antibodies targeting TAg and VP1 after gating on live cells in the presence or absence of NK cells. (B) Proportions of TAg+, VP1+ or Tag+VP1+ SVG-A cells after co-culture with NK cells compared to those cultured without NK cells. (C) Relative proportions of JCPyV-infected SVG-A cells in the presence of NK cells. (D) Proportions of CD107a+ NK cells
from 13 healthy donors co-cultured with uninfected (NI) SVG-A cells or SVG-A cells infected with M1-SVEΔ (JCPyV) for 6h in the presence of conjugated anti-CD107a antibodies. **p<0.01; ****p<0.0001.

Fig 3. NKG2D and its ligand ULBP-2 play a key role in NK cell responses against JCPyV-infected cells. SVG-A (A) and 293T (B) cells were infected with M1-SVEΔ or cultured without virus and after 9 days, cell surface expression of ligands for NKG2D was assessed by flow cytometry. (C) RNA was extracted from uninfected and infected SVG-A and 293T cells to quantify mRNA expression levels using primers specific for each ULBP by TaqMan qPCR. (D) Volcano plot displaying fold-changes in transcripts levels for the indicated NKG2D ligands in HRPTE cells 9 days following infection with M1-SVEΔ compared to uninfected epithelial cells, using pre-computed log2 fold changes and p-values available here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135833. N.S., not significant, defined as absolute value of log 2-fold change less than one and adjusted p-value less than 0.05; Sig., significant, defined as absolute value of log2 fold change greater than 1 and adjusted p-value less than 0.05. (E) Representative flow cytometry plots of CD107a+ NK cells following co-culture with SVG-A cells infected with SVE-Delta-M1 for 6h in the presence of isotype control (IgG1k) or blocking antibodies against NKG2D. (F) Compiled results for NK cells isolated from 7 healthy donors. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Supporting information captions

S1 Fig. Gating strategy to measure NK and T cell responses to JCPyV VP1 by ICS. (Related to Fig 1). (A) Flow cytometry gating strategy to analyze intracellular expression of CD107a and IFN-y on NK, CD4+ T and CD8+ T cells as well as IL-2 on CD4+ T and CD8+ T cells. Gates are set to exclude doublets, dead cells, CD14+ and CD19+ cells. (B) Representative primary flow cytometry plots showing CD107a upregulation as well as IFN-y and IL-2 production
by NK cells, CD8+ T and CD4+ T cells as indicated following stimulation with the JCPyV peptide pool covering VP1 161-253 + VP1 257-341. Expression of functional markers on various lymphocyte subsets was defined by using appropriate unstimulated controls.

S2 Fig. NKG2D blockade results in decreased NK cell response against JCPyV-infected cells.

(Related to Fig 2D and 3E). (A) Proportions of CD107a+ NK cells from 8 healthy donors co-cultured with uninfected (NI) SVG-A cells or SVG-A cells infected with JCPyV M1-SVEΔ. Proportions of CD107a+ NK cells from 6 healthy donors following co-culture with SVG-A cells infected with M1-SVEΔ in the presence of isotype control (IgG1k) or blocking antibodies against NKG2D. NK cells and SVG-A cells were co-cultured for 6h in the presence of conjugated anti-CD107a antibodies and 1µL/mL GolgiPlug (BD Biosciences) and 0.7µL/mL GolgiStop (BD Biosciences). **p<0.01; **** p<0.0001.
**Fig 3**

**A**  
SVG-A  
Uninfected | JCPyV-infected  
--- | ---  
MICA/B | **Uninfected** | **JCPyV-infected**  
ULBP-1 | **Uninfected** | **JCPyV-infected**  
ULBP-3 | **Uninfected** | **JCPyV-infected**  
ULBP-2/5/6 | **Uninfected** | **JCPyV-infected**  
MFI of NKG2D Ligands  

**B**  
293T  
Uninfected | JCPyV-infected  
--- | ---  
MICA/B | **Uninfected** | **JCPyV-infected**  
ULBP-1 | **Uninfected** | **JCPyV-infected**  
ULBP-3 | **Uninfected** | **JCPyV-infected**  
ULBP-2/5/6 | **Uninfected** | **JCPyV-infected**  
MFI of NKG2D Ligands  

**C**  
mRNA expression (Relative to uninfected)  
SVG-A | 293T  
--- | ---  
ULBP-2 |  
ULBP-5 |  
ULBP-6 |  

**D**  
log2 Fold Change  
ULBP1 | MICA | ULBP2  

**E**  
IgG1k | αNKG2D Ab  
CD56 | CD107a  

**F**  
%CD107a+ NK cells  
IgG1k | αNKG2D  
*
S2 Fig

A

\[ \text{%CD107^a+ NK cells} \]

\begin{tabular}{c c}
NI & JCPyV \\
\end{tabular}

B

\[ \text{%CD107^a+ NK cells} \]

\begin{tabular}{c c}
IgG1k & \(\alpha\)NKG2D \\
\end{tabular}

\(p=0.05\)

\[ * \]