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MAJOR ARTICLE

Upregulation of the NKG2D Ligand ULBP2 by JC Polyomavirus Infection Promotes Immune Recognition by Natural Killer Cells

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Background. JC polyomavirus (JCPyV) causes progressive multifocal leukoencephalopathy (PML), a potentially fatal complication of severe immune suppression with no effective treatment. Natural killer (NK) cells play critical roles in defense against viral infections; however, NK-cell response to JCPyV infection remains unexplored.

Methods. NK- and T-cell responses against the JCPyV VP1 were compared using intracellular cytokine staining upon stimulation with peptide pools. A novel flow cytometry-based assay was developed to determine NK-cell killing efficiency of JCPyV-infected astrocyte-derived SVG-A cells. Blocking antibodies were used to evaluate the contribution of NK-cell receptors in immune recognition of JCPyV-infected cells.

Results. 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 the NK-cell–mediated killing assay, we showed that coculture of NK cells and JCPyV-infected SVG-A cells leads to a 60% reduction in infection, on average. JCPyV-infected cells had enhanced expression of ULBP2—a ligand for the activating NK-cell receptor NKG2D, and addition of NKG2D blocking antibodies decreased NK-cell degranulation.

Conclusions. NKG2D-mediated activation of NK cells plays a key role in controlling JCPyV replication and may be a promising immunotherapeutic target to boost NK-cell anti-JCPyV activity.

Keywords. JC polyomavirus; NK cell antiviral immunity; NKG2D; ULBP.

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]. 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 [4]. 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 with human immunodeficiency virus (PWH) [5, 6], with up to 80% of PML patients being HIV positive [7], there is a growing number of patients with autoimmune diseases, such as people with multiple sclerosis, at risk for PML [4].

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 [8]. Furthermore, rapidly restoring the immune functions in the central nervous system can also be fatal due to immune reconstitution inflammatory syndrome [9, 10]. 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 [11–13]. Based on these observations, recent efforts in PML treatment trials have focused

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on reviving T-cell response using T-cell checkpoint inhibitor and allogeneic polyomavirus-specific T cells [14] or infusions of cytokines such as interleukin 7 (IL-7) and IL-15 superagonist [15, 16]. 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 [11, 17], 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 [16, 18, 19]. 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 [20]. 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 [21]. NKG2D ligands (NKG2DL) consist of MHC class I related chain (MIC) A and B and 6 UL-16 binding proteins (ULBP1–6), which are typically not expressed in healthy tissues but rather upregulated by cellular stress such as viral infection [22, 23]. NKG2D-mediated NK-cell responses have been found to be critical in the control of several viral infections [24–26]. The NKG2D/NKG2DL axis also plays a pivotal role in tumor immunosurveillance and therefore immunotherapeutic strategies targeting the NKG2D pathway are currently under investigation [27–30].

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 cytomegalovirus, influenza virus, hepatitis C virus, and HIV [31–33]. 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 antiviral 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.

METHODS

Human Subjects

Deidentified blood samples from healthy donors were purchased from Research Blood Components, LLC. Frozen peripheral blood mononuclear cells (PBMC) from healthy donors were purchased from STEMCELL Technologies. Beth Israel Deaconess Medical Center institutional review board approved this study.

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 2 μg/mL of peptide pools consisting of 15-mer sequences with 11 amino acid overlap covering the JCPyV VP1 sequence, split into 2 pools (VP1 1–93 + VP1 97–157 [n = 48]; VP1 161–253 + VP1 257–341 [n = 49]). PBMC were cocultured with peptides at 37°C for 6 hours with CD107a BV786 (H4A3; BD Biosciences) and 1 μg/mL of CD28/CD49d costimulatory reagent (BD Biosciences). One μL/mL GolgiPlug (BD Biosciences) and 0.7 μL/mL GolgiStop (BD Biosciences) were added for the last 2 hours of incubation. Cells were then stained first with the LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen), followed by BD Biosciences CD3 BV510 (UCHT1), CD14 BV421 (M5E2), CD19 BV421 (HIB19), CD16 APC-Cy7 (3G8), CD56 BV605 (NCAM16.2), CD4 BUV395 (L200), and CD8 APC (SK1), and finally fixed, permeabilized (Thermofisher Fix and Perm), and stained with BD Biosciences interferon-γ (IFN-γ) FITC (B27) and interleukin 2 (IL-2) PE-CF594 (5344.11) antibodies to detect intracellular cytokines. Incubation in the presence of 5 μg/mL of phytohemagglutinin was used as positive control and unstimulated cells served as negative controls and for background subtraction. A fluorescence minus one control and phytohemagglutinin-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 version 10.8.1.

JCPyV Infection of SVG-A Cells

SVG-A cells were grown in Eagle's minimum essential medium supplemented with 2% fetal bovine serum (FBS) and 2% penicillin/streptomycin. SVG-A cells were plated into 2 T-75 flasks with 0.7 M cells in each flask. After 2–3 days, 1 flask was infected with JCPyV Mad 1/SVEΔ (5.9e9 viral genome/mL) at a 1:100 dilution and 1 flask left uninfected. Percentages of infected SVG-A cells were determined 9–11 days after infection and prior to functional assays on the BD LSRFortessa X14.

Killing Assay

NK cells were enriched from freshly isolated PBMC using the EasySep Human NK Cell Isolation Kit (STEMCELL Technologies), counted, and resuspended at 1 M/mL in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL streptomycin,

and 100 U/mL penicillin. SVG-A cells and NK cells were combined at an effector-to-target (E:T) ratio of 10:1 (NK cells to infected SVG-A cells). To complement measures of direct NK-cell–mediated killing, CD107a BV786 was also added at a 5 µL/mL concentration and served as a surrogate marker of degranulation. In a subset of experiments where only CD107a upregulation but not killing was measured, 1 µL/mL GolgiPlug (BD Biosciences) and 0.7 µL/mL GolgiStop (BD Biosciences) were added for the whole incubation (Supplementary Figure 4). For blockade experiments, purified NK cells were incubated for 10 minutes at room temperature with 2.5 µg of Human BD Fc Block followed by addition of 10 µg/mL of anti-NKG2D purified antibody (clone 1D11; BD Biosciences) or isotype control antibodies prior to addition of SVG-A cells. Cocultures were incubated at 37°C for 6 hours. 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) and NKG2D APC(1D11), 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 version 10.8.1.

Anti-JCPyV IgG ELISA

JCPyV capsid protein VP1 was obtained from Abcam (AB74569). End point titer dilution enzyme-linked immunosorbent assay (ELISA) was performed to determine the JCPyV serostatus of all healthy donors included in our study, as previously published [34]. ELISA end point 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 1 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). Uninfected SVG-A and 293T cells as well as Raji and K562 cells were included as controls.

Quantification of NKG2DL by qPCR

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 polymerase chain reaction (qPCR) was performed using the QuantStudio 3 Flex Real-Time PCR System (Applied Biosystems) and TaqMan Fast Advanced Master Mix. cDNA was amplified with primers for ULBP2 (Hs00607609_mH), ULBP3 (Hs00225909_m1), ULBP5 (Hs01584111_mH), ULBP6 (Hs04194671_s1), and β actin (Hs99999903_m1) with TaqMan MGB probes all conjugated with fluorochrome 6-carboxyfluorescein (FAM; Thermofisher). The cycling conditions were 50°C for 2 minutes, 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 and 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 ± standard error of the mean. Statistical analyses were performed using the GraphPad Prism software version 9.5.0 The nonparametric Wilcoxon signed-rank test was used to assess differences in cytokine production or CD107a 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 <.05 were considered significant.

RNA Transcriptome Analysis

Transcriptome profiling of infected primary human renal proximal tubule epithelial (HRPTE) cells and data analysis were performed as previously described [35]. Deposited data including precomputed $\log_2$ fold changes and *P* values were used to generate volcano plots using ggplot2 version 3.3.2. and are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135833.

RESULTS

NK Cells Display Robust Responses to JCPyV VP1 Peptides

Viral infection induces changes in the HLA peptide repertoire and such alterations can directly [36, 37] or indirectly [38] 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 be highly dependent on IL-2–secreting effector memory T cells [38–40]. 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) (Supplementary Figure 1 and Figure 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

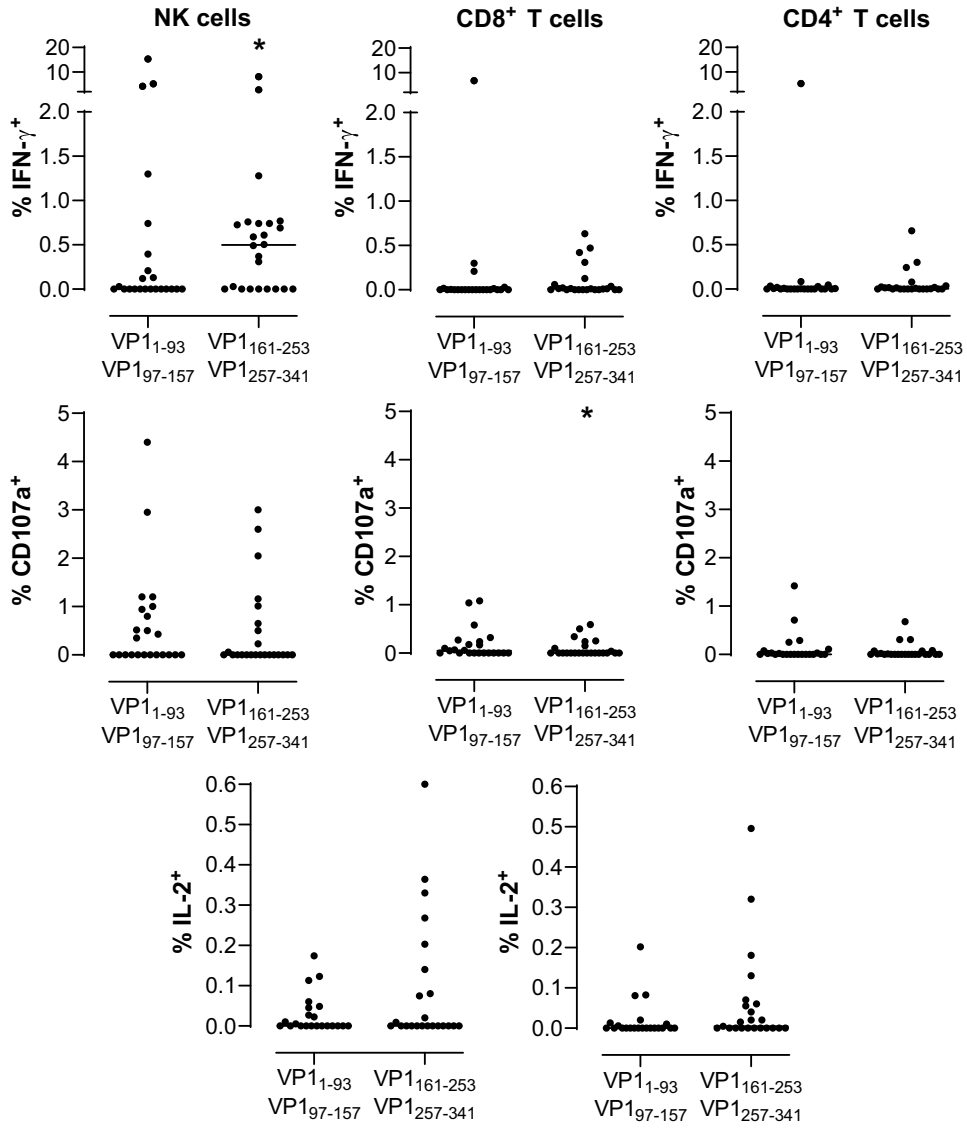

Figure 1. NK- and T-cell responses to JCPyV VP1 in healthy donors by intracellular cytokine staining. Peripheral blood mononuclear cell from 24 healthy donors were incubated for 6 hours with 2 μg/mL of pools of peptides spanning JCPyV VP1 in the presence of conjugated anti-CD107a antibodies. BD GolgiStop and GolgiPlug were added for the last 2 hours of culture. The peptides were divided into 2 sequential pools as follows: VP1 1–93 + VP1 97–157 (n = 48) and 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. * $P < .05$ compared to unstimulated. Abbreviations: IFN-γ, interferon-γ; IL-2, interleukin 2; JCPyV, JC polyomavirus; NK cell, natural killer cell.

IL-2 [11]. To avoid culture conditions that could significantly alter and misrepresent primary NK-cell responses to JCPyV, we performed a 6 hours 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. Donors displaying the highest T-cell responses against VP1 peptide pools also had robust NK-cell responses (Supplementary Figure 2). Interestingly, in a few donors (ie, Supplementary Figure 2 dark blue and grey circles), elevated NK-cell responses were observed while no or marginal T-cell responses could be detected. Altogether these results show that using this assay, T-cell responses could be directly measured without the need for prior amplification of antigen-specific T cells, however 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 relies on downregulation of ligands for inhibitory NK-cell receptors (ie, select HLA class I molecule) and

upregulation of ligands for activating receptors (ie, 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 (Mad-1/SVEΔ) (virus originally made in Dr Eugene Major's laboratory and provided by Campbell Kaynor, Biogen) [41, 42]. JCPyV Mad-1/SVEΔ is a chimeric polyomavirus derived from the MAD1 strain with replaced regulatory regions from SV40, which displays enhanced replication in cell culture [42] (Figure 2*A*). We show that coculture 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 (Figure 2*B*) and 60% killing (Figure 2*C*). While there were interindividual 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; however as expected, killing of JCPyV-infected SVG-A cells by NK cells was not influenced by the JCPyV serostatus (Supplementary Figure 3). Of note, CD107a upregulation in NK cells has been shown to correlate with NK-cell–mediated cytotoxicity [43, 44] and accordingly, a significant CD107a upregulation was observed in NK cells exposed to JCPyV-infected SVG-A cells (Figure 2*D*), independently of addition of protein transport inhibitors during the coculture (Supplementary Figure 4). In summary, 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 [20] and represents a checkpoint target for immunotherapies currently in development [45]. 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 targets ULBP3 [19]. 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 (Figure 3*A* and 3*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 (Figures 2*D* and 3*B*). 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 (Figure 3*C*). Enhanced ULBP2 expression levels were also observed in JCPyV-infected HRPTE cells, a primary target for JCPyV, upon secondary analysis of data generated in the laboratory of Walter J. Atwood [35] (Figure 3*D*). Based on enhanced expression of ULBP2 on JCPyV-infected cells, we then performed coculture 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 (Figure 4). 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 [46, 47]. As PWH represent the majority of PML patients, it is possible that impaired NKG2D signaling in PWH 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 PWH 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 [19]. 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 NKG2DL expression in our experiments compared to the

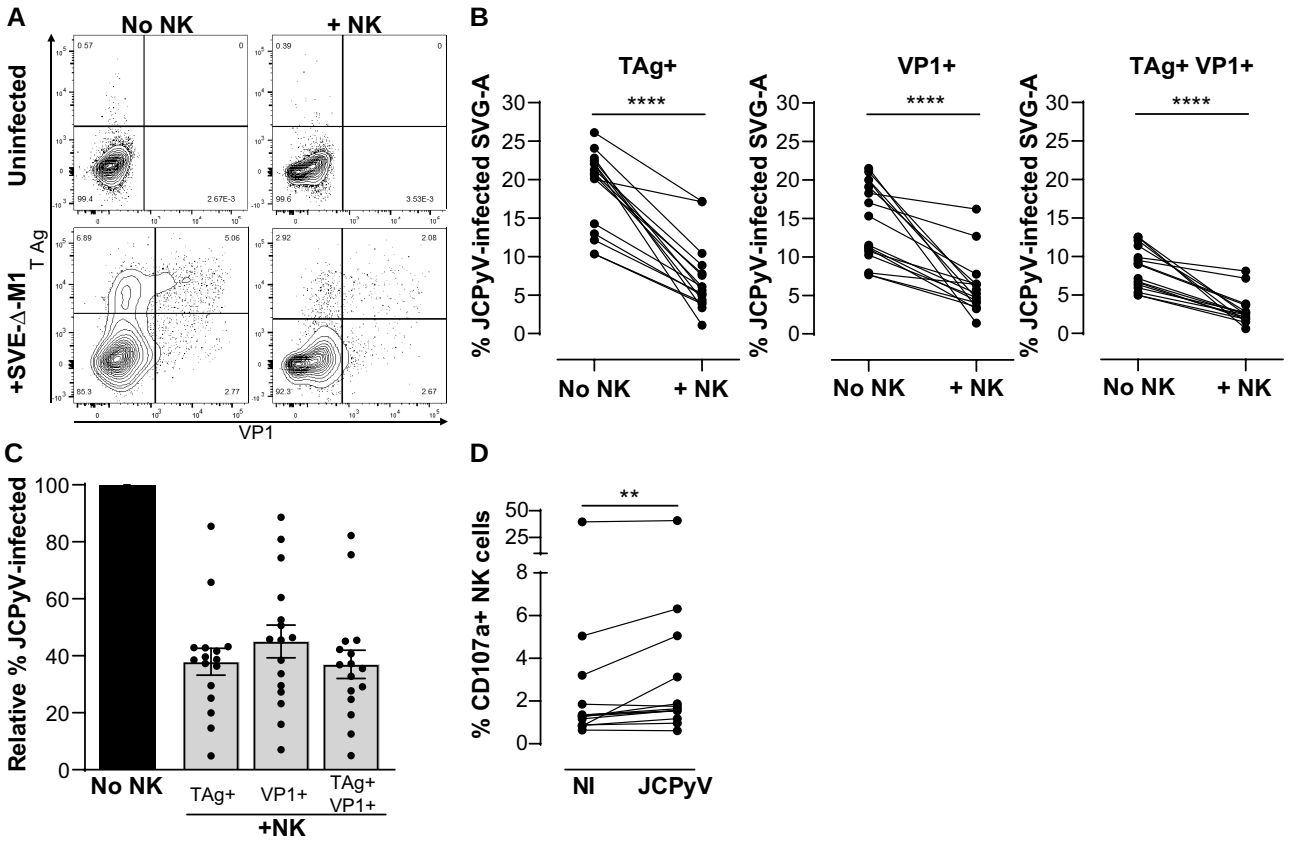

Figure 2. Development of a flow-cytometry-based assay to measure natural killer (NK)-cell cytotoxicity against JC polyomavirus (JCPyV)-infected cells. SVG-A cells were infected with M1-SVEΔ, which on average leads to 25% cells infected after 9–10 days of culture as assessed by the proportions of SVG-A 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 cocultured with purified NK cells from 16 healthy donors at 10:1 E:T ratio for 6 hours. *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 coculture 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 cocultured with uninfected (NI) SVG-A cells or SVG-A cells infected with JCPyV Mad-1/SVEΔ (JCPyV) for 6 hours in the presence of conjugated anti-CD107a antibodies. $^{**}P < .01$, $^{****}P < .0001$.

studies conducted by Bauman et al [19]. 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 [48]. 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 [41, 42] 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 (Supplementary Table 1). 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. These data open avenues for therapeutics against PML based on existing strategies currently in development targeting NKG2D [27, 29, 30].

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 [49, 50] and IL-15 [16]. These approaches have been associated with improved PML outcome in subsets of patients and because 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 NK cells but not in $CD3^+$ T cells [16]. 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 (Spearman with IL-2^+CD8^+ T cells, $r = 0.2466$, $P = .26$ and with IL-2^+CD4^+ T cells, $r = 0.2569$, $P = .24$), indicating that in this assay, NK-cell responses may not mainly rely on IL-2 produced by

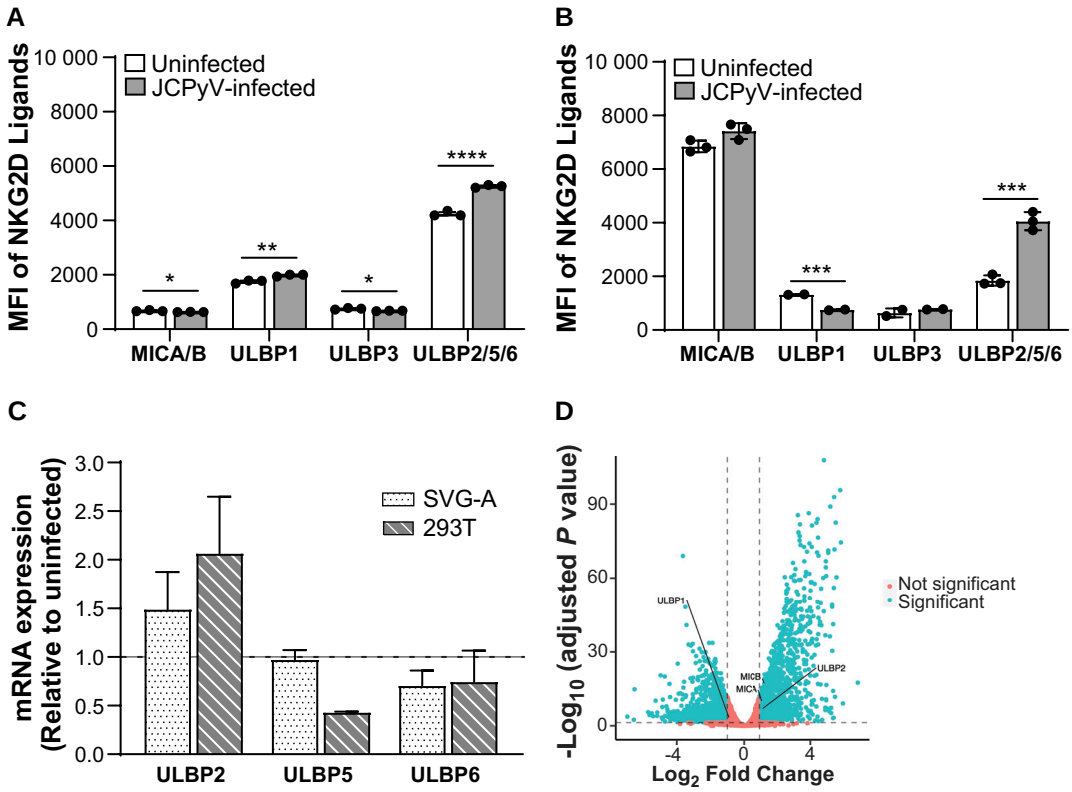

Figure 3. The NKG2D ligand ULBP2 is upregulated in JCPyV-infected cells. 293T (*A*) and SVG-A (*B*) cells were infected with M1-SVEΔ or cultured without virus and after 9 days, cell surface expression of ligands for NKG2D was assessed by flow cytometry. **P* < .05, ***P* < .01, *** *P* < .001, **** *P* < .0001. *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 JCPyV Mad-1/SVEΔ compared to uninfected epithelial cells, using precomputed $\log_2$ fold changes and *P* values available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE135833. Not significant defined as absolute value of $\log_2$ fold change less than 1 and adjusted *P* value less than .05; significant defined as absolute value of $\log_2$ fold change greater than 1 and adjusted *P* value less than .05. Abbreviations: JCPyV, JC polyomavirus; MFI, mean fluorescence intensity.

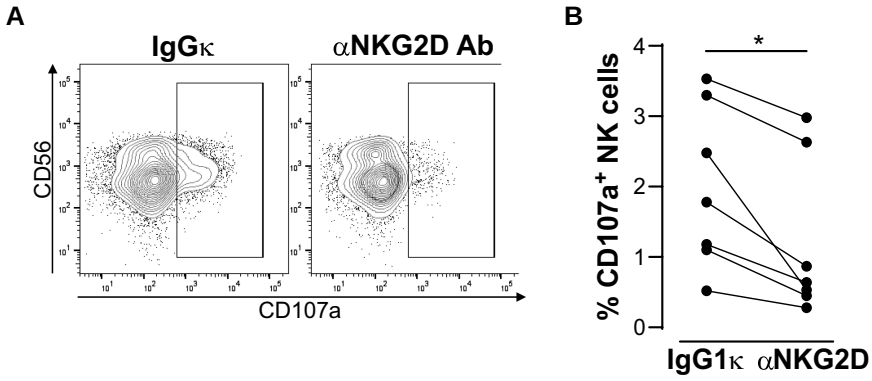

Figure 4. NKG2D blockade impairs NK-cell response against JCPyV-infected SVG-A cells. *A*, Representative flow cytometry plots of $CD107a^+$ NK cells following coculture with SVG-A cells infected with JCPyV Mad-1/SVE-Δ for 6 hours in the presence of isotype control (IgG1κ) or blocking antibodies against NKG2D. *B*, Compiled results for NK cells isolated from 7 healthy donors. **P* < .05. Abbreviations: Ab, antibody; IgG, immunoglobulin G; JCPyV, JC polyomavirus; NK cell, natural killer cell.

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.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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