

Modulation of NKG2D-Mediated Cytotoxic Functions of Natural Killer Cells by Viral Protein R from HIV-1 Primary Isolates^{▽†}

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HIV-1 viral protein R (Vpr) from laboratory-adapted virus strains activates the DNA damage/stress sensor ATR kinase and induces cell cycle arrest at the G₂/M phase through a process that requires Vpr to engage the DDB1-CUL4A (VprBP/DCAF-1) E3 ligase complex. Activation of this DNA damage/stress checkpoint in G₂ by Vpr was shown to modulate NKG2D-dependent NK cell effector functions via enhancing expression of NKG2D ligands, notably ULBP2. However, it is unknown whether Vpr from HIV-1 primary isolates (groups M, N, O, and P) could modulate NKG2D-mediated cytotoxic functions of NK cells. Here, we report that Vpr from most HIV-1 primary isolates can upregulate ULBP2 expression and induce NKG2D-dependent NK cell killing. Importantly, these activities were always accompanied by an active G₂ cell cycle arrest function. Interestingly, Vpr variants from group P and a clade D isolate of group M were defective at enhancing NKG2D-mediated NK cell lysis owing to their inability to augment ULBP2 expression. However, distinct mechanisms were responsible for their failure to do so. While Vpr from group P was deficient in its ability to engage the DDB1-CUL4A (VprBP/DCAF-1) E3 ligase complex, the Vpr variant from group D was unable to properly localize to the nucleus, underlining the importance of these biological properties in Vpr function. In conclusion, the ability of Vpr from HIV-1 primary isolates to regulate NK cell effector function underscores the importance of this HIV-1 accessory protein in the modulation of the host's innate immune responses.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS and responsible for nearly 33 million infections worldwide according to the latest Joint United Nations Programme on HIV/AIDS (UNAIDS) estimate (27). HIV-1 isolates are divided into four groups: M (main), N (non-M non-O), O (outlier), and P (pending), with the pandemic group M accounting for about 95% of the world's HIV/AIDS cases (reviewed in reference 26). Isolates of the non-pandemic groups N, O, and P account for the remaining HIV infections. Heterogeneity in the structural genes *env* and *gag* classifies group M viruses into at least nine clades (identified by letters A through K) and numerous subclades.

The HIV-1 RNA genome, aside from encoding the structural proteins, also codes for four accessory proteins, one of which is viral protein R (Vpr). The 96-amino-acid Vpr is highly conserved across primate lentiviruses and could be found *in vivo* as an intravirion, an intracellular, and a soluble molecule. The multifunctional Vpr is thought to assist in viral transcription, to promote infection of nondividing cells, and to be involved in cell apoptosis, although it is perhaps best known for its ability to induce a G₂ cell cycle arrest (14). Vpr-mediated G₂ block relies on the execution of a stepwise process thought to require engagement of Vpr to the host DDB1-CUL4A

(VprBP; also designated DCAF-1) E3 ubiquitin ligase complex, degradation of an as yet unknown chromatin-bound cellular target(s), and activation of the DNA damage/stress ataxia telangiectasia-mutated and Rad3-mutated (ATR)-mediated pathway (3, 5, 18). ATR is a DNA damage sensor kinase that is involved in activating the G₂ checkpoint in response to genotoxic stress conditions, preventing cell entry into mitosis (1). Vpr interaction with components of the E3 ligase, especially the substrate specificity receptor VprBP, is crucial to its arrest activity, as Vpr mutants, such as Vpr Q65R, which are unable to bind VprBP fail to induce a G₂ block (3, 18). That being said, it is important to recognize in this context that, although Vpr and VprBP association is essential, binding to VprBP alone is insufficient for Vpr to activate ATR since some Vpr mutants, such as Vpr R80A, display a G₂ arrest-defective phenotype despite their efficient engagement to VprBP (3, 18). Activation of the ATR signaling pathway generally leads to phosphorylation and/or recruitment of several checkpoint mediators, including H2AX (histone 2A, variant X) and 53BP-1 (p53 binding protein 1) as well as to the formation of DNA damage foci containing these molecules (15, 30). On this note, we previously demonstrated that Vpr forms distinct nuclear punctuate structures which partially overlap with γ -H2AX (phosphorylated H2AX) and 53BP-1 and that formation of these foci is critical to the induction of G₂ arrest (5).

Vpr-mediated cell cycle arrest is widely believed to be preserved in all primary lentiviruses during viral evolution (22), implying that this Vpr function is potentially significant for the overall pathogenesis of HIV *in vivo*. Since the relation between Vpr-induced G₂ block and increased viral production has been

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implied in many studies (9, 12), the abnormal accumulation of cells arrested at G₂ in HIV-infected patients (31) may seem paradoxically beneficial to the virus. On the other hand, recent data from our group and others revealed that HIV-1 infection of primary CD4⁺ T lymphocytes with a laboratory-adapted virus enhanced expression of specific ligands of the NKG2D (natural killer [NK] group 2, member D) receptor on T cells in a Vpr-dependent manner (24, 28). These studies further showed that expression of Vpr alone was sufficient to induce an upregulation of NKG2D ligands. Indeed, ULBP-2, a member of the human cytomegalovirus UL16 binding protein (ULBP) family, was most predominantly upregulated on Vpr-expressing T cells, and such an increase promoted T cell killing by NKG2D receptor-expressing NK cells (24, 28). Intriguingly, the enhancement of ULBP2 expression requires Vpr to engage the DDB1-CUL4A (VprBP/DCAF-1) E3 ligase complex and activate the ATR pathway, the exact prerequisite for Vpr-induced G₂ arrest. Indeed, the R80A and Q65R Vpr mutants, which are defective for G₂ arrest, are also attenuated for ULBP2 upregulation (24). Taken together, the data highlight the essentiality of ATR activation for both activities mediated by Vpr.

Given that NKG2D ligands have been shown to be expressed on infected cells of HIV-infected patients (10), the current study aimed to dissect whether Vpr from HIV-1 primary isolates could modulate NK cell cytotoxic function through the NKG2D pathway and whether there was an association between ULBP2 upregulation and G₂ arrest in the context of primary Vpr. Herein, we document that Vpr from most HIV-1 groups can alter NKG2D-mediated NK cell cytotoxic functions through regulating ULBP2 expression on target cells. Coincidentally, we also find that the G₂ arrest-inducing activity is preserved only in primary Vpr variants (interchangeably referred to in the text as primary Vprs) that can modulate the NKG2D pathway in NK cells, strengthening the link with the DNA damage sensor ATR-induced pathway. Overall, our data indicate that the ability of Vpr to modulate NK cell activation extends beyond laboratory-made HIV-1, lending further support to its role as an immunomodulator of the host's innate immune responses.

MATERIALS AND METHODS

Cell lines and isolation of primary cells. CEM.NKR T, HEK293T, and HeLa cells were cultured as described previously (24). Natural killer cells and CD4⁺ T cells were purified from peripheral blood mononuclear cells (PBMC) by negative selection using appropriate immunomagnetic beads (Stem Cell Technologies) and cultured as detailed elsewhere (24). PBMC were obtained from individuals with no documented HIV or hepatitis C virus infections. The study was approved by the IRCM Research Ethic Review Board. Samples were collected after written informed consent had been obtained.

Plasmids and antibodies. All primary *vpr* alleles were cloned into a green fluorescent protein (GFP)-marked pWPI lentiviral vector (generously provided by D. Trono, School of Life Sciences, Swiss Institute of Technology, Lausanne, Switzerland) as described previously (3). psvCMV-HA-tagged Vpr and pCMV-myc-tagged VprBP were generated as detailed previously (3, 29).

Mouse anti-human ULBP2 monoclonal antibody (MAb) was obtained from R&D Systems (Minneapolis, MN). Anti-Vpr MAb (clone 8D1) was a generous gift from Y. Ishizaka (National Center for Global Health and Medicine, Tokyo, Japan). Rabbit anti-Vpr polyclonal antibody (pAb) was prepared as described previously (17). The mouse anti-hemagglutinin (anti-HA) tag and anti-Myc tag MAbs (clones 12CA5 and 9E10, respectively) were produced from hybridomas (ATCC). Rabbit pAbs against DDB1 and actin were purchased from Santa Cruz Biotechnology. Mouse anti-phosphoS139-H2AX MAb (clone JBW301) was ob-

tained from Upstate Biotechnology. All fluorochrome-conjugated secondary antibodies were from Molecular Probes (Invitrogen).

Viral protein R from HIV-1 primary isolates and laboratory-adapted virus. Nine primary *vpr* alleles spanning four HIV-1 groups (M, N, O, and P) were selected. For pandemic group M, two alleles were from clade B (termed M/B-13C and M/B-18E alleles) (21), one from clade C (M/C allele), two from clade D (M/D-lo and M/D-sh alleles), and one from clade H (M/H allele). For non-pandemic groups N, O, and P, one *vpr* allele was selected for each group. Except for the M/D-sh, N, O, and P alleles, all alleles were amplified by PCR from the respective molecular clones. Three of these clones (94UG114 for the Vpr M/D-lo allele, 98IN022 for the Vpr M/C allele, and 90CF056 for the Vpr M/H allele) were obtained through the NIH AIDS Research and Reference Reagent Program from Beatrice Hahn and Feng Gao (11). The GenBank accession numbers for these three alleles are U88824, AF286232, and AF005496, respectively. For this study, a truncated version of Vpr M/D-lo, termed Vpr D-lo-12, was also generated by PCR from Vpr M/D-lo. The Vpr M/D-sh and N, O, and P alleles were synthetically made (GenScript) using published prototype sequences for the respective primary isolates (GenBank accession numbers AB485650, AJ006022, AB485667, and GQ328744, respectively). In all experiments, the activity of Vpr variants from primary isolates was compared to that of laboratory-adapted HxBRU (HxB) Vpr (7).

Production of Vpr-expressing lentiviral vectors, transduction of T cells, and analysis of ULBP2 and Vpr expression. Production and titration of pWPI lentiviral vectors were performed as described previously (3).

CEM.NKR T cells and CD4⁺ T cells were transduced with GFP-marked pWPI.Vpr (HxB or primary Vpr) or pWPI (no Vpr) and analyzed 48 h later as described previously (24). ULBP2 cell surface expression and Vpr intracellular levels were analyzed on GFP⁺ cells by flow cytometry as previously reported (24).

Cell cycle analysis. Vpr-expressing GFP⁺ T cells were sorted directly into staining buffer (0.1% sodium citrate, 0.3% NP-40, 0.05 mg/ml propidium iodide, and 0.02 mg/ml RNase) using an Influx cell sorter (BD Biosciences) and analyzed for DNA content by flow cytometry. Alternatively, cell cycle analysis was performed on HEK293T cells transfected with psvCMV-HA-tagged Vpr (HxB, M/D-lo, M/D-sh, and M/D-lo-12) as described previously (3, 29). The ModFit mathematical model (Verity Software House) was used to enumerate proportions of cells in G₁ and G₂ phases.

Natural killer cell-mediated cell lysis. Sorted GFP⁺ T cells were used as targets in a standard chromium release assay (6), while purified unactivated NK cells from healthy individuals were used as effector cells. Percent specific lysis was determined according to the formula [(experimental lysis – spontaneous lysis)/(total lysis – spontaneous lysis)] × 100. Data analysis was done using GraphPad (San Diego, CA) Prism 4.0 software.

Immunoprecipitation and immunoblotting. HEK293T cells were cotransfected for 48 h using a standard calcium phosphate method with (i) pCMV plasmid expressing myc-tagged VprBP (or, as a control, pCMV-myc) and (ii) pWPI.Vpr lentiviral vector (or, as a control, pWPI). Immunoprecipitation was performed on total cell lysates using anti-myc antibody followed by a purification step on protein A-conjugated Sepharose beads. Western blotting was carried out as described previously (4).

Fluorescence microscopy. HeLa cells (50,000) were seeded on coverslips in 24-well plates and transfected with psvCMV-HA-tagged Vpr (or empty vector psvCMV-HA) as detailed previously (5). Transfected cells were processed 48 h later for laser scanning confocal microscopy (8). Images were acquired using the Zeiss LSM10 system with ZEN 2009 software and processed using AxioVision version 4.7.

RESULTS

Heterogeneity of Vpr in different HIV-1 groups. To determine whether Vpr from HIV-1 primary isolates can modulate NK cell functions as does that from laboratory-adapted virus (24, 28), we examined nine variants spanning all HIV-1 groups: M, N, O, and P (Fig. 1). Six came from 4 clades (B, C, D, and H) of group M, and these were specifically chosen to reflect different levels of amino acid sequence variations compared to laboratory-adapted HxB Vpr (belonging to clade B). The two variants from clade B, termed M/B-18E and M/B-13C, and isolated from patients with or without HIV-related dementia, respectively (21), showed 93 to 95% sequence similarity to

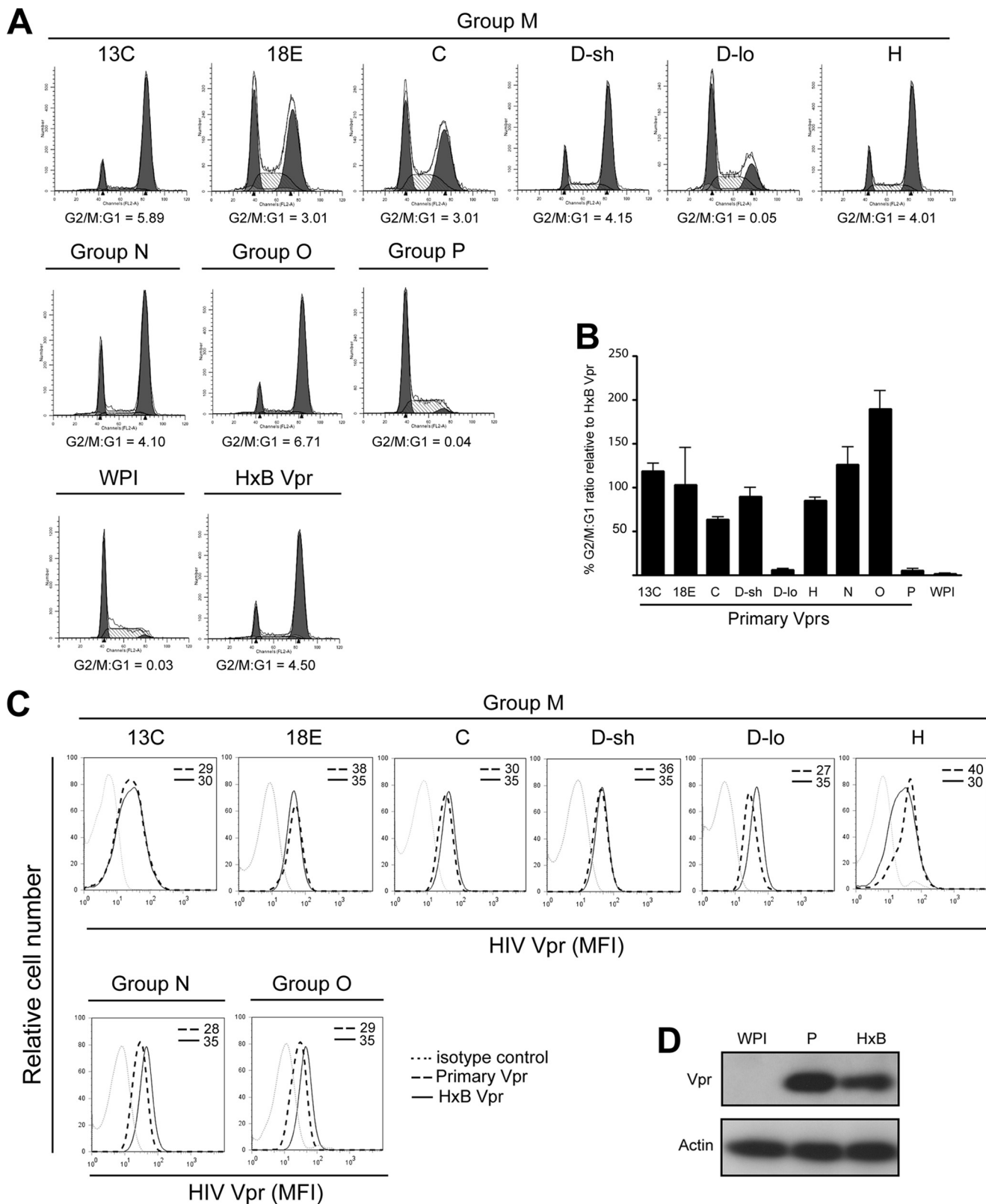


FIG. 2. Most HIV-1 primary Vpr variants can efficiently induce a G₂ cell cycle arrest in T cells. CEM.NKR T cells were transduced with GFP-marked lentiviral vectors expressing Vpr (primary laboratory-adapted [HxB]) or not expressing Vpr (WPI). (A and B) GFP⁺ cells were sorted and analyzed for cell cycle profiles by flow cytometry. Proportions of cells in G₁ and G₂ phases were enumerated using the ModFit mathematical model. (A) Representative results from one experiment. (B) Compilation of 3 experiments in which mean G₂/G₁ ratios \pm standard deviations (SD) for primary Vprs were expressed as percentages of that obtained for HxB Vpr. (C) CEM cells were also stained with anti-Vpr MAb (8D1) and analyzed by flow cytometry for Vpr expression in GFP⁺ cells. MFI values were obtained after subtraction of signals given by cells stained with a relevant isotype control antibody. Shown are data representative of 3 experiments. (D) HEK293T cells were transfected with lentiviral vectors expressing no Vpr (WPI), Vpr P, or HxB Vpr and assessed for Vpr expression by Western blotting using anti-Vpr pAb.

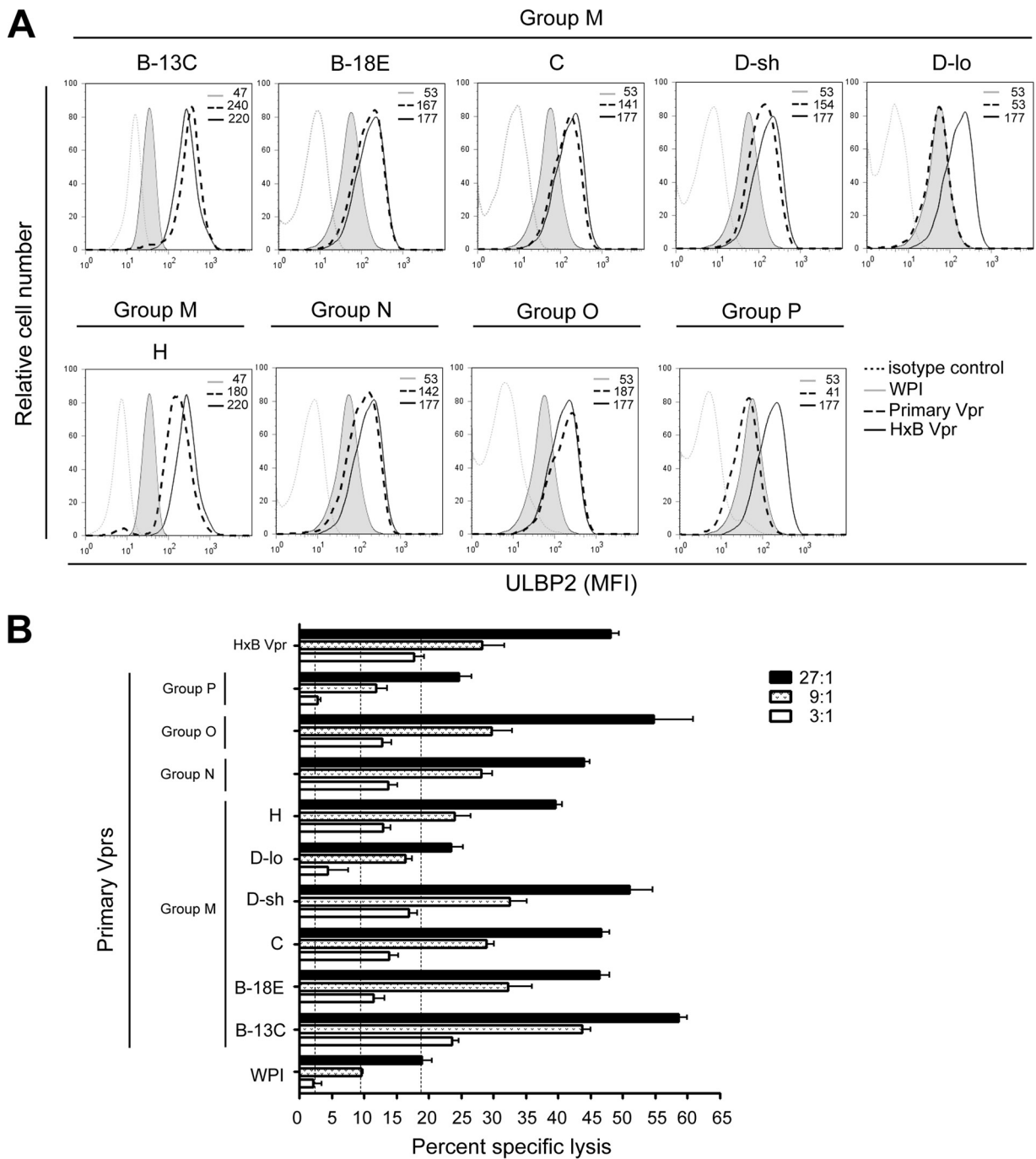


FIG. 3. G_2 arrest-competent primary Vpr variants can modulate NK cell functions through the NKG2D pathway. (A) CEM.NKR T cells were transduced with GFP-marked Vpr-expressing (or non-Vpr-expressing) lentiviral vectors as indicated in the Fig. 2 legend and stained for ULBP2. Expression levels were gated on GFP⁺ cells. MFI values were obtained after subtraction of signals given by cells stained with a relevant isotype control antibody. (B) GFP⁺ cells were sorted and used as targets in a standard ⁵¹Cr release assay. Purified resting NK cells from healthy donors were used as effector cells at the indicated effector/target ratios. Shown is mean percent specific cell lysis \pm SD (triplicate samples) representative of three experiments with different donors. Vertical dotted lines indicated background killing.

was comparable to that of cells expressing the WPI control (Fig. 3B). Nonetheless, the extent of ULBP2 enhancement *per se* did not necessarily predict the degree of NK cell killing. It is important to mention here that, although CEM.NKR T cells were the model cells for this study owing to their easy access and the lack of donor variability, the ability of primary Vprs to

modulate ULBP2 expression has also been observed in primary CD4⁺ T cells (see Fig. S1 in the supplemental material).

Distinct mechanisms responsible for the lack of ULBP2 modulation by Vpr M/D-lo and Vpr P. At this point, our data revealed that two of the nine primary Vprs, namely, Vpr P and Vpr M/D-lo, were unable to upregulate ULBP2 expression (or

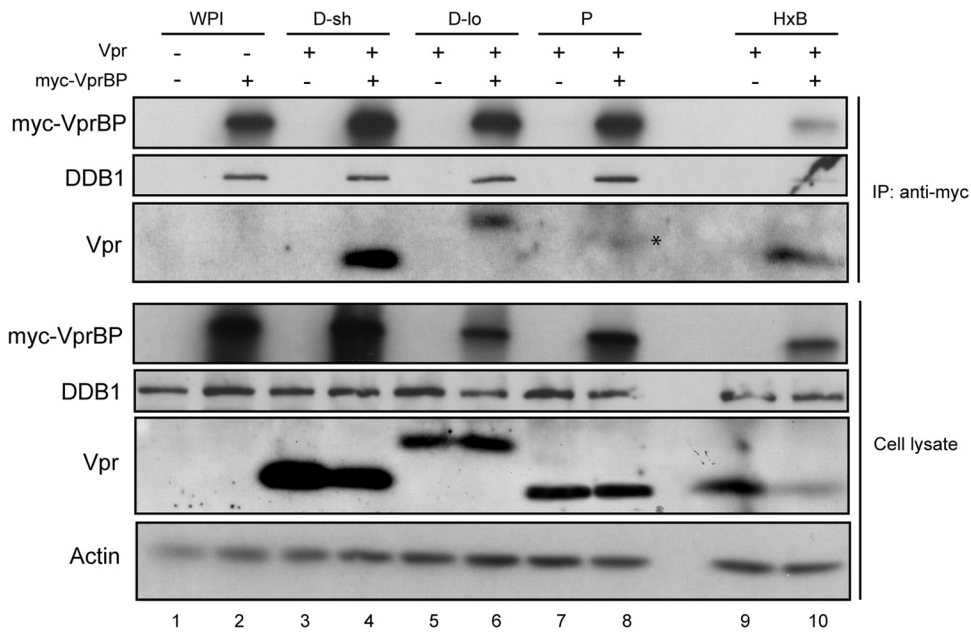


FIG. 4. Analysis of engagement of Vpr M/D-lo and Vpr P to the DDB1-CUL4A (VprBP/DCAF-1) E3 ubiquitin ligase. HEK293 T cells were transfected with plasmids expressing Vpr (D-sh, D-lo, P, or HxB) or no Vpr (WPI). Cells were transfected with plasmids expressing myc-VprBP (lanes 2, 4, 6, 8, and 10) or a myc tag alone (lanes 1, 3, 5, 7, and 9). Immunoprecipitation (IP) of total cell lysates was performed using anti-myc antibody. Resulting immunocomplexes (and total lysates, as a control) were analyzed for myc-VprBP, DDB1, and Vpr by Western blotting using the respective antibodies. The asterisk indicates an unidentified species having a higher molecular size than the expected size (~14 kDa) for Vpr P. Shown are data representative of 3 experiments.

induce a G₂ block). Since Vpr binding to the VprBP adaptor of the DDB1-CUL4A (VprBP/DCAF-1) E3 ubiquitin ligase is instrumental to both activities (24), we asked whether the dual defect could be attributed to their failed association with VprBP. For this purpose, cells expressing Vpr M/D-lo or Vpr P were evaluated for their ability to interact with VprBP by coimmunoprecipitation. As with the positive controls Vpr M/D-sh and HxB Vpr (Fig. 4, lanes 4 and 10, respectively), Vpr M/D-lo was present in the immunocomplexes containing VprBP and DDB1 (lane 6, IP). Nevertheless, putative Vpr P was not detected under the same experimental conditions (lane 8). This indicated that the inability of Vpr P to modulate ULBP2 expression was likely due to failed engagement to the said E3 ligase. As for Vpr M/D-lo, binding to the ligase was clearly insufficient to promote ATR activation and G₂ arrest.

To gain a further insight as to why Vpr M/D-lo was unable to activate ATR, we took advantage of our earlier finding demonstrating the importance of formation of Vpr’s nuclear foci for G₂ arrest activity (5) and assessed the capability of Vpr M/D-lo to make similar structures. By confocal microscopy, we found that, unlike HxB Vpr or Vpr M/D-sh, M/D-lo could not make these nuclear foci (Fig. 5A). Instead, this Vpr variant appeared highly diffused (Vpr shown in red) and mislocalized to either the perinuclear membrane [Fig. 5A, D-lo (a)] or even external to the nucleus [Fig. 5A, D-lo (b)]. A multiple-field analysis revealed that Vpr M/D-lo was outside the nucleus approximately 70% of the time. Further, while the structures made by HxB Vpr and Vpr M/D-sh appeared to partially overlap with γ -H2AX (shown in green), a surrogate marker of ATR activation, this feature was clearly not observed for Vpr M/D-lo.

From the sequence standpoint, Vpr M/D-lo is unique in that it has a 12-amino-acid extension at the C terminus (Fig. 1). To explore whether the tail would somehow hinder Vpr M/D-lo’s ability to activate the ATR—a prerequisite for both ULBP2 upregulation and G₂ cell cycle arrest—a truncated version of the allele (without the extra 12 amino acids), termed D-lo-12, was examined for its ability to induce a G₂ block. Intriguingly, unlike Vpr M/D-lo, D-lo-12 could promote cell cycle arrest essentially as efficiently as does Vpr M/D-sh (Fig. 5B). In addition, Vpr D-lo-12 (Fig. 5A) could form nuclear foci, similar to those observed for G₂-competent HxB Vpr or Vpr M/D-sh. These structures were found, unsurprisingly, in the proximity of phosphorylated γ -H2AX, a marker of ATR activation. Taking these results together, we submit that the C-terminal extension of Vpr M/D-lo is likely responsible for its failure to trigger ATR and G₂ arrest. These findings thus explain why this particular Vpr variant is unable to upregulate ULBP2.

DISCUSSION

In this study, we show that Vprs from primary isolates from three of the four HIV-1 groups are capable of inducing a G₂ arrest and upregulating ULBP2 expression on T cells. The enhanced presence of this NKG2D ligand promotes T cell killing by NK cells through the NKG2D receptor. Further, in all variants investigated, the observation of Vpr-induced augmentation of ULBP2 is invariably accompanied by evidence of activation of DNA damage sensor ATR.

Nine primary Vprs from HIV-1 groups M, N, O, and P were initially evaluated for their G₂ arrest activity. Vpr P and Vpr

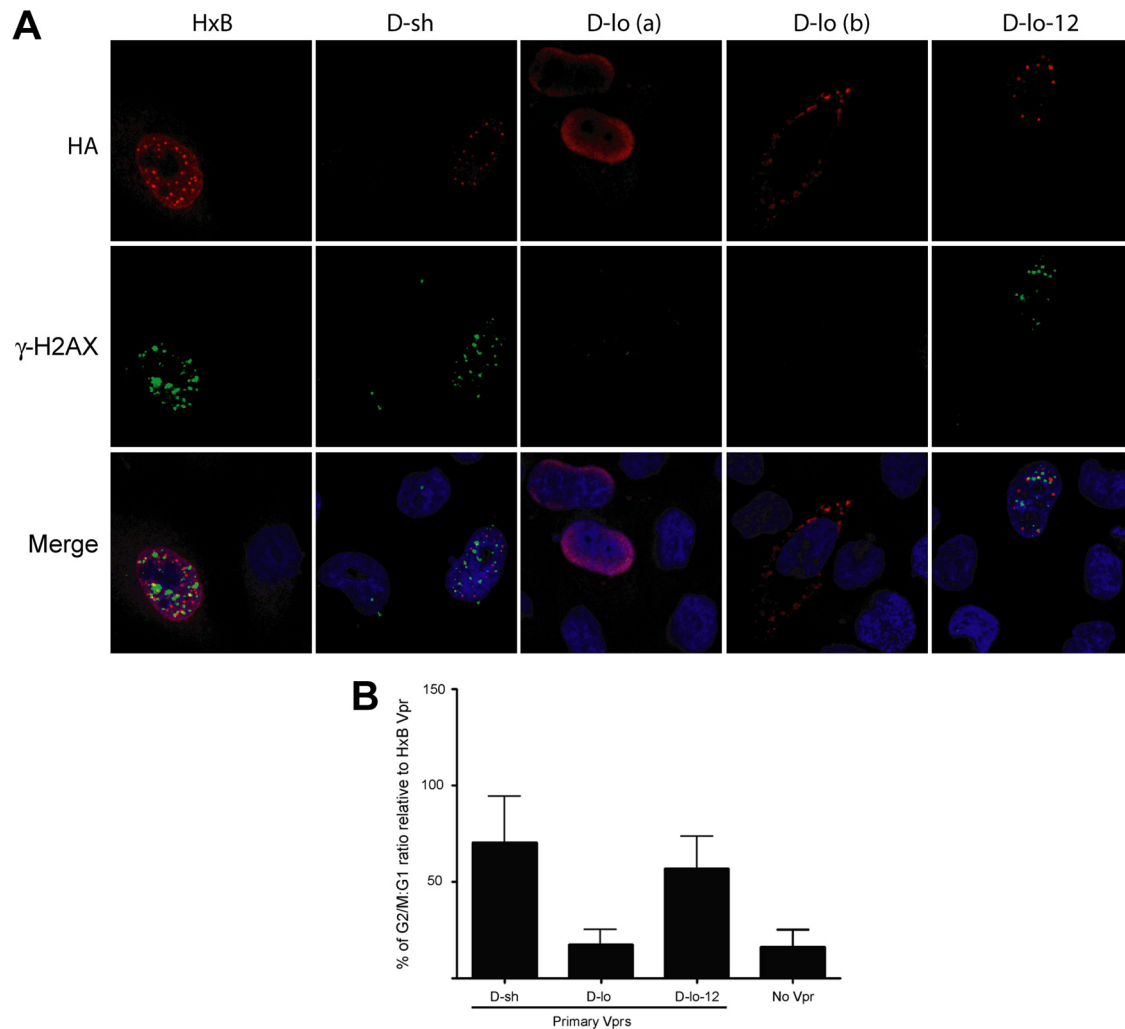


FIG. 5. Formation of DNA damage nuclear foci by primary Vprs competent for G_2 arrest. (A) HeLa cells were transfected with plasmids expressing HA-tagged Vpr, immunostained for Vpr (red) and γ -H2AX (green), and analyzed by confocal microscopy. Nuclei are shown in blue (DAPI [4',6-diamidino-2-phenylindole] staining). Merge images reveal partial colocalization between nuclear Vpr foci and γ -H2AX. Images were acquired by confocal microscopy with a $63\times$ objective. Shown are representative images of multiple fields. (B) HEK293T cells were cotransfected with plasmids expressing GFP- and HA-tagged Vpr. GFP⁺ cells were analyzed for cell cycle profiles as described in the Fig. 2 legend. Shown are mean G_2/G_1 ratios \pm SD ($n = 2$ experiments) expressed as percentages of that obtained for HxB Vpr.

M/D-lo are clearly defective in this regard but differ greatly in how this effect is brought about. For Vpr P, the inactivity appears to be due to its inability to engage the substrate specificity receptor VprBP (Fig. 4) of the DDB1-CUL4A (VprBP/DCAF-1) ubiquitin E3 ligase complex; it is thus unable to activate ATR and induce a G_2 arrest. The same, however, cannot be said for Vpr M/D-lo, as it is clearly able to directly associate with VprBP (Fig. 4). Since triggering ATR requires engagement of Vpr to the E3 ligase and the recruitment of an as-yet-unknown cellular target(s) for polyubiquitination and proteasomal degradation, the unique C-terminal extension of Vpr M/D-lo (Fig. 1) may hinder such a process. In that regard, the fact that this extension causes a mislocalization of the protein from the nucleus most probably underlies the defect since recent indirect evidence from our laboratory suggests that the cellular target(s) of Vpr is likely to be a chromatin-associated protein(s) (5). Indeed, we demonstrate herein that

removal of the extra end renders Vpr M/D-lo able to activate ATR, as evidenced by its formation of DNA damage nuclear foci and mediation of a G_2 arrest (Fig. 5).

From the sequence standpoint (Fig. 1), seven of the nine primary Vpr variants that we studied coincidentally had a substitution at position 77, with six carrying the R77Q mutation. This substitution, found in a higher proportion of HIV-infected long-term nonprogressive patients (19, 20), has been shown to correlate with impaired apoptosis in some studies (19, 23) but not in others (2, 13). In the context of our current study, it is evident that R77Q is unlikely to have a negative effect on G_2 arrest activity as 6 of the 7 Vprs with this mutation were still able to induce a cell division block. On this note, our latest data are in agreement with those previously reported (2, 16, 19).

Consistent with earlier findings reported for laboratory-adapted HIV-1 Vpr (24, 28), we show here that ULBP2 up-

regulation on T cells by primary Vprs is universally correlated with an active G₂ arrest function. Given that it was only with Vpr M/D-lo and Vpr P that we observed no such modulation in ULBP2 expression (Fig. 3), the data point toward ATR activation as the common thread between the two events. Since T cells with elevated ULBP2 levels were more susceptible to NK cell-mediated lysis (Fig. 4), augmentation of this NKG2D ligand, in the context of HIV infection, may initially serve as a host's attempt to contain the viral presence. Nonetheless, prolonged engagement of the NKG2D receptor to its ligand(s) could conceivably lead to gradual deregulation of NK cell activity over time. In this context, it would be interesting to determine whether the lack of ULBP2 upregulation on T cells expressing Vpr P or the unique Vpr M/D-lo would inadvertently be beneficial to the integrity of NK cells in the long haul. Interestingly, the notion of derailed NKG2D-mediated NK cell effector functions in the face of NK cell activation was recently described to be a possible approach exploited by another HIV-1 accessory protein, namely, Vpu, to evade the host's immune responses. In this setting, Vpu appears to avoid NK cell activation but selectively downregulates NTBA—an NKG2D costimulatory receptor for degranulation—to ultimately impair this particular function in NK cells (25).

With our latest findings, it is now well established that, even in the absence of a bona fide infection, Vpr can modulate NK cell effector functions through the NKG2D, implying a potential effect of Vpr on bystander healthy cells (24). Since depletion of CD4⁺ T cells during HIV-1 infection is largely ascribed to the death of uninfected cells, it is conceivable that, through enhancing the expression of ULBP2 on target cells and subsequently promoting their lysis by NK cells, Vpr could contribute to the overall loss of healthy bystander CD4⁺ T cells during HIV infection. Further, the fact that Vpr-mediated cell surface ULBP2 upregulation can now be extended to most HIV-1 groups sheds light on the possibility of ULBP2 as a potential marker in the identification of low-level and chronically infected cells, a population that currently hampers the swift eradication of HIV.

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