Detection of the HIV-1 accessory proteins Nef and Vpu by flow cytometry represents a new tool to study their functional interplay within a single infected CD4+ T cell

Jérémie Prévost^{1,2,*}, Jonathan Richard^{1,2,*}, Romain Gasser^{1,2}, Halima Medjahed¹, Frank Kirchhoff³, Beatrice H. Hahn⁴, John C. Kappes⁵, Christina Ochsenbauer⁵, Ralf Duerr⁶, Andrés Finzi^{1,2,7,#}

¹Centre de Recherche du CHUM, Montreal, Quebec, Canada

²Département de Microbiologie, Infectiologie et Immunologie, Université de Montréal, Montreal, Quebec, Canada

³Institute of Molecular Virology, Ulm University Medical Center, Ulm, Germany.

⁴Departments of Medicine and Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

⁵Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA

⁶Department of Microbiology, New York University School of Medicine, New York, NY, USA

⁷Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada

*Contributed equally

#Corresponding author:

Andrés Finzi

Centre de recherche du CHUM (CRCHUM)

900 St-Denis street, Tour Viger, R09.420

Montréal, Québec, H2X 0A9, Canada

andres.finzi@umontreal.ca

Phone: 514-890-8000 ext: 35264

Fax: 514-412-7936

Running Title: Nef and Vpu expression dictates ADCC responses

Key Words: HIV-1, Env, Nef, Vpu, CD4, BST-2, ADCC, LucR.T2A, non-neutralizing antibodies, CD4-bound conformation

ABSTRACT

The HIV-1 Nef and Vpu accessory proteins are known to protect infected cells from antibody-dependent cellular cytotoxicity (ADCC) responses by limiting exposure of CD4-induced (CD4i) envelope (Env) epitopes at the cell surface. Although both proteins target the host receptor CD4 for degradation, the extent of their functional redundancy is unknown. Here, we developed an intracellular staining technique that permits the intracellular detection of both Nef and Vpu in primary CD4⁺ T cells by flow cytometry. Using this method, we show that the combined expression of Nef and Vpu predicts the susceptibility of HIV-1-infected primary CD4⁺ T cells to ADCC by HIV⁺ plasma. We also show that Vpu cannot compensate for the absence of Nef, thus providing an explanation for why some infectious molecular clones that carry a LucR reporter gene upstream of Nef render infected cells more susceptible to ADCC responses. Our method thus represents a new tool to dissect the biological activity of Nef and Vpu in the context of other host and viral proteins within single infected CD4⁺ T cells.

IMPORTANCE

HIV-1 Nef and Vpu exert several biological functions that are important for viral immune evasion, release and replication. Here, we developed a new method allowing simultaneous detection of these accessory proteins in their native form together with some of their cellular substrates. This allowed us to show that Vpu cannot compensate the lack of a functional Nef, which has implication for studies that use Nef-defective viruses to study ADCC responses.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) genome encodes four accessory proteins (Vif, Vpr, Vpu and Nef), which are dispensable for viral replication *in vitro* but required for efficient replication, restriction factors counteraction and immune evasion *in vivo* (1-7). Among them, Nef and Vpu are well known for their role in subverting the host cell protein trafficking machinery (8, 9).

HIV-1 Nef is a small cytoplasmic protein of 27 kDa produced from early viral transcripts (10), which requires a myristoyl group on its N-terminus to traffic to intracellular and plasma membranes (11). Nef harbors a highly conserved dileucine motif in its C-terminal flexible loop that is responsible for the interaction with clathrin adaptors protein complexes (AP-1, AP-2 and AP-3) (12). Among these, interaction with AP-2 is required to downregulate the CD4 receptor from the surface of infected cells (13, 14) and target it for degradation in lysosomal compartments (15, 16).

HIV-1 Vpu is a small type-I transmembrane protein of 16 kDa produced late in the viral replication cycle (17, 18) and contains a short luminal N-terminal peptide followed by a single helical transmembrane domain and a C-terminal cytoplasmic domain (19-21). The cytoplasmic domain is comprised of two α -helices linked by a flexible loop known for its interaction with the SCF ^{β TRCP} E3 ubiquitin ligase complex via a conserved phosphoserine motif (DS^PGNES^P) (22, 23). Vpu mainly localizes within intracellular compartments, notably the endoplasmic reticulum (ER) and the *trans*-Golgi network (TGN) (24-26). Like Nef, Vpu also induces degradation of newly synthesized CD4 by directing it through an ER-associated pathway (ERAD) for further

proteasomal degradation (22, 27-29). In addition, Vpu sequesters the restriction factor BST-2 in the TGN using its transmembrane domain, thereby increasing the release of progeny virions (30-33).

CD4 downregulation by Nef and Vpu was previously reported to be critical for efficient viral replication in T cells by enhancing virion release and infectivity, and by preventing superinfection (34-39). CD4 downregulation is critical for immune evasion since the anti-Env antibody (Ab) response is dominated by non-neutralizing antibodies (nnAbs) that target Env in its “open” CD4-bound conformation (40-42). The interaction between CD4 and Env at the surface of HIV-1-infected cells has been shown to promote nnAbs binding to Env, leading to the elimination of infected cells through Fc-mediated effector functions, including antibody-dependant cellular cytotoxicity (ADCC) (41, 43). Nef and Vpu limit the presence of Env-CD4 complexes at the cell surface and thus protect infected cells against ADCC (41, 43, 44).

In previous studies, Nef and Vpu expression was mostly examined in transfected cell lines, frequently using tagged proteins (30, 31, 45, 46) or by performing Western blots and immunofluorescence microscopy in infected primary cells (47-52). However, both proteins are small, intracellularly located and present in low amounts, rendering their detection difficult. To facilitate their analysis in primary CD4⁺ T cells, we developed an intracellular staining technique to detect Nef and Vpu expression by flow cytometry, which allows the simultaneous detection of these proteins together with host and viral proteins within a single infected cell. Using this method, we show that Nef and Vpu expression predicts the susceptibility of HIV-1-infected primary CD4⁺ T cells to ADCC by HIV⁺ plasma. We also explain why decreased Nef

expression in widely-used reporter viruses increase the susceptibility of infected cells to ADCC responses.

RESULTS

Intracellular detection of Nef and Vpu in HIV-1-infected primary CD4⁺ T cells.

To facilitate detection of intracellular Nef, we obtained a polyclonal Nef antiserum through the NIH AIDS Reagent Program, which was generated by immunization of rabbits with a recombinant clade B Nef consensus protein produced in *E. coli* (53). In previous studies, this antibody detected native Nef proteins by Western blot and immunofluorescence microscopy in both transfected and infected cells (47, 54-56). Given the scarcity of anti-Vpu antibodies, we immunized rabbits with a peptide corresponding to the clade B Vpu C-terminal region (residues 69-81). A similar approach was previously used to generate a polyclonal antibody capable of detecting Vpu by Western blot and immunofluorescence microscopy (24, 57).

We first evaluated the ability of both Nef and Vpu antisera to recognize their cognate antigen using HEK 293T cells transfected with plasmids expressing the Nef or Vpu proteins from the transmitted/founder (T/F) virus CH058 (58, 59). Forty-eight hours post-transfection, cells were permeabilized and stained with the antisera, followed by detection with a fluorescently-labelled anti-rabbit secondary antibody. As expected, the Nef antiserum recognized only Nef transfected cells, while the Vpu antiserum recognized only Vpu transfected cells (Fig. 1A-C). To evaluate whether our method detected Nef and Vpu when expressed in a biologically relevant culture system, we infected primary CD4⁺ T cells with CH058 infectious molecular clones (IMC) encoding Nef, and/or Vpu proteins. While wildtype (WT)-infected cells were efficiently

recognized by both Nef and Vpu antisera, abrogation of Nef (Fig 1D-E) or Vpu (Fig. 1F-G) expression prevented the recognition of productively-infected cells as identified by Gag protein intracellular staining (p24+). Of note, mock-infected or uninfected bystander cells (p24-) were not detected by either antiserum, further confirming their specificity (Fig 1D-G).

We next examined the antiserum binding to Nef and Vpu proteins from different HIV-1 clades and groups as well as from closely related simian immunodeficiency viruses (SIV). Primary CD4+ T cells were infected with a panel of HIV-1 IMCs representing clades B, C, A1 and CRF01_AE. As expected, both Nef and Vpu antisera recognized their respective antigen in cells infected with clade B viruses since both were raised against clade B immunogens (Fig. 1H-I). The anti-Nef polyclonal antibody was also able to recognize Nef proteins from group M clades C, A1 and CRF01_AE as well as the Nef from a group O isolate. This recognition extended even to the Nef protein of a related SIVcpzPts strain (isolate TAN2) but not to chimeric simian-human immunodeficiency viruses (SHIV) which express a SIVmac Nef (Fig. 1H). The Vpu antiserum was less cross-reactive and failed to detect Vpu from clade C viruses (Fig. 1I). These findings confirmed the specificity and cross-reactivity of the intracellular detection of Nef and Vpu using infected primary CD4+ T cells.

Measuring CD4 and BST-2 downregulation in infected primary CD4+ T cells with or without Nef and Vpu expression.

The efficient detection of Nef and Vpu at the single cell level by flow cytometry allowed us to combine this approach with the quantification of CD4 and BST-2 expression levels on the cell surface. Productively-infected cells (p24+) expressing both Nef and Vpu had little detectable

CD4 and BST-2 compared to uninfected cells (Fig. 2A). In contrast, cells infected with Vpu or Nef mutant viruses differed in the extent of CD4 and BST-2 downregulation (Fig. 2A).

Vpu targets CD4 and BST-2 by different mechanisms. First, Vpu interacts with multiple transmembrane proteins, including BST-2, through its transmembrane domain (TMD), which sequesters these proteins in perinuclear compartments (32, 33, 60-63). Second, Vpu downregulates CD4 by interaction of its cytoplasmic domain with the cytoplasmic tail of CD4 (64-69). Consistent with these different interaction modes, Vpu-mediated CD4 and BST-2 degradation involves independent pathways (proteasomal and lysosomal degradation, respectively), both of which depend on polyubiquitination by the SCF^{βTRCP} E3 ubiquitin ligase complex, recruited by Vpu using its highly conserved phosphoserine motif (22, 26, 70, 71). To examine whether we could measure the expression and activity of Vpu mutants by flow cytometry, we introduced mutations at critical residues of the Vpu TMD (A14L/A18L) or its phosphoserine motif (S52A/S56A). CH058 IMCs coding for wildtype or mutated Vpu proteins were used to infect primary CD4⁺ T cells. While the TMD mutations did not affect Vpu expression, the phosphoserine mutations led to a significant accumulation of intracellular Vpu proteins (Fig. 2B), most likely because Vpu is degraded together with its target protein as a ubiquitinated complex (24, 72, 73). Despite a higher expression, the Vpu phosphoserine mutant was unable to downregulate CD4 and marginally diminished in its capacity to antagonize BST-2 (Fig 2C-F). This is consistent with studies demonstrating that the recruitment of the SCF^{βTRCP} E3 ubiquitin ligase complex and the degradation of BST-2 by Vpu is dissociable from its capacity to antagonize the restriction factor (32, 71, 74-76). In contrast, the Vpu TMD mutations did not affect Vpu's ability to target CD4 but completely abrogated its capacity to downregulate BST-2

(Fig 2C-F). Together, these results emphasize the need of measuring Nef and Vpu expression when studying their biological functions.

Nef and Vpu expression inversely correlates with ADCC responses.

CD4 downregulation by Nef and Vpu, together with Vpu-mediated BST-2 antagonism were found to be critical factors preventing the exposure of vulnerable CD4-induced Env epitopes, thus protecting HIV-1-infected cells from ADCC (41, 43, 44, 77-80). To investigate the link between Nef and Vpu expression and HIV-1-infected cells immune evasion, we infected activated primary CD4⁺ T cells from five HIV-negative individuals with two clade B IMCs, CH058 T/F and JR-FL, encoding functional or defective *nef* and *vpu* genes. Focusing on the productively-infected cells (p24⁺), we performed a comprehensive characterization of the patterns of viral protein expression including cell-surface Env (detected with the conformation-independent Ab 2G12), intracellular Nef and Vpu in combination with cell-surface levels of CD4 and BST-2. We also measured the specific recognition and elimination of infected cells by ADCC using the CD4-induced (CD4i) A32 monoclonal Ab (mAb). This antibody binds the cluster A region of the gp120 which is occluded in the “closed” trimer and therefore can only bind Env in the “open” CD4-bound conformation. We also tested 25 different plasma samples from chronically HIV-1-infected individuals. As expected, Nef was only expressed by WT and Vpu- constructs, while Vpu was only expressed by WT and Nef- constructs (Fig. 3A). Consistent with previous reports (43, 77, 78), deletion of Nef strongly impaired CD4 downregulation by both viruses but did not affect Env or BST-2 cell-surface levels. Vpu deletion mitigated CD4 downregulation to a lesser extent than Nef and abrogated BST-2 downmodulation, resulting in an overall increase in the amount of cell-surface Env (Fig. 3B). We noticed lower levels of the JR-

FL Vpu protein compared to CH058 Vpu, which was linked to a less effective Vpu-mediated CD4 downmodulation (Fig. 3B). The cumulative effect of Nef and Vpu on cell-surface levels of Env, CD4 and BST-2 prevented the recognition of infected cells and protected them from ADCC responses mediated by A32 and HIV+ plasma (Fig. 3C-D). In contrast, abrogation of Nef or Vpu expression resulted in increased recognition and susceptibility of infected cells to ADCC mediated by nnAbs (Fig. 3C-D). We performed correlation analyses to measure the level of association between the different cellular, virological and immunological variables (Fig 3E-F). We found that both Nef and Vpu established a large network of inverse correlations with cellular and immunological factors. Interestingly, Env levels hardly contributed to the network and were poorly associated with the immunological outcome, thus indicating that the overall amount of Env present at the surface does not dictate ADCC responses mediated by CD4i Abs or HIV+ plasma, but rather the conformation Env occupies. Apart from antibody binding, ADCC responses mediated by nnAbs correlated strongly with CD4 and Nef levels (Fig. 3E-F). Overall, Nef and Vpu expression inversely correlates with the susceptibility of HIV-1-infected cells to ADCC mediated by CD4i Abs and HIV+ plasma.

Impaired Nef expression from IMC LucR.T2A constructs enhance the susceptibility of infected cells to ADCC.

Infectious molecular clones encoding for the *Renilla* luciferase (LucR) reporter gene upstream of the *nef* sequence, and a T2A ribosome-skipping peptide to drive Nef expression are widely employed to quantify anti-HIV-1 ADCC responses (81-92). Despite evidence that Nef-mediated CD4 downregulation is impaired when using these IMCs (54, 79), a series of recent studies have hypothesized that Vpu can compensate for the absence of Nef expression and completely

downregulate CD4 on its own (93-97). To evaluate this hypothesis, we used our intracellular staining to measure Nef and Vpu expression levels and study their impact on ADCC responses mediated by nnAbs against cells infected with IMC-LucR.T2A constructs. Primary CD4⁺ T cells were infected with NL4.3-based IMCs that do (Env-IMC-LucR.T2A) or do not encode (Env-IMC) a LucR.T2A cassette. These IMCs express the Env ectodomain from two clade B viruses, CH058 T/F and YU-2. Consistent with the lack of Nef detection by Western blot (54, 79), insertion of the LucR.T2A cassette also impaired the detection of Nef by flow cytometry, while Vpu expression remained unchanged (Fig. 4A-B). However, we noted an accumulation of cell-surface CD4 for Env-IMC-LucR.T2A compared to *nef*-intact constructs (~20-fold higher) (Fig. 4C), which resulted in a significantly increased recognition and susceptibility of infected cells to ADCC responses mediated by A32 mAb and HIV⁺ plasma (Fig. 4D-E). Of note, both the binding and the ADCC responses mediated by nnAbs were strongly associated with CD4 levels and inversely correlated with Nef expression (Fig 4F-G). In contrast, these variables poorly correlated with Vpu expression. Based on these data, it seems clear that Vpu expression alone is not sufficient to prevent ADCC-mediated killing of infected cells and that HIV-1 requires both Nef and Vpu for efficient humoral response evasion.

Nef, Vpu and CD4 levels predict ADCC responses mediated by HIV⁺ plasma.

We next used univariate multiple linear regression (MLR) analysis to evaluate the capacity of different variables to predict ADCC responses mediated by HIV⁺ plasma. This model is based on the hypothesis that a linear relationship exists between the dependant variable quantified empirically and the independent variables that serve as predictive variables. In our model, the dependant variable is the ADCC responses mediated by plasma from HIV⁺ donors (ADCC

HIV+ plasma) and the independent variables are the cellular, virological and immunological factors measured on infected cells. To run the MLR model, we combined data obtained with the different viral constructs (Fig. 3 & 4) and plotted the mean ADCC obtained with 25 HIV+ plasma against a single virus on the X axis and the associated predicted ADCC value based on one or more independent variables on the Y axis. When looking at cellular factors, we noticed that only CD4 accurately predicts ADCC responses mediated by HIV+ plasma, independent of the viral strain (Fig. 5A). Even though BST-2 displayed a strong correlation with ADCC responses (Fig 3E), it was not predictive. When focusing on virological variables, we observed that Nef is the only significant ADCC predictive variable, albeit not as good as CD4 (Fig. 5A-B). However, combinations of Nef with Vpu or Env increased its predictive scores, reaching similar levels as CD4 when combined with Vpu (Fig. 5B). Of note, the strength of the prediction was not further improved when combining all three virological variables altogether. As for immunological variables, their capacity to predict ADCC by HIV+ plasma was found to be equivalent or even higher than for cellular and virological factors (Fig. 5C). Indeed, the binding of HIV+ plasma predicted ADCC values with a similar score as CD4 or Nef and Vpu combined, while the binding of A32 predicted ADCC by HIV+ plasma even better (Fig. 5A-C). This could be explained by the fact that A32-like Abs present in plasma from infected individuals are from the main class of Abs (anti-cluster A Abs) mediating ADCC responses against infected cells (41, 80, 81, 91, 98). In line with this interpretation, ADCC mediated by A32 was found to have a near-perfect predictive ability, suggesting that factors, other than antibody binding, are presumably needed to fully explain the ADCC phenotypes observed (Fig. 5C).

DISCUSSION

Unlike simple retroviruses, HIV-1 and related SIVs encode multiple accessory proteins that promote viral replication and immune evasion (99). Among them, Nef and Vpu modulate the expression, trafficking, localization and function of several host cell surface proteins, including the viral receptor CD4, restriction factors and homing receptors (28, 30, 31, 62, 69, 100-104). They also modulate a wide range of immunoreceptors to evade immune responses mediated by CD8⁺ T, NK and NKT cells (105-113). Most of these host cell proteins are naturally expressed on primary CD4⁺ T cells, the preferential target of HIV-1. The detection of Nef and Vpu has previously been done in transfected cells (30, 31, 46, 48, 49, 114), which results in the overexpression of the viral proteins when compared to infected primary CD4⁺ T cells. Moreover, tagged viral proteins are frequently used to facilitate their detection (30, 31, 46, 48, 49, 114). Protein overexpression and/or tag insertion, have the potential to impact the trafficking and functions of these accessory proteins. To study Nef and Vpu's biological activities in a physiologically relevant system, we developed an intracellular staining method to detect native Nef and Vpu proteins in HIV-1-infected primary CD4⁺ T cells by flow cytometry. Using Nef and Vpu antisera, we detected both viral proteins with high specificity in cells productively infected (p24⁺) with multiple IMCs. The Nef antiserum was cross-reactive, detecting Nef from group M (clade B, C, A1 and CRF01_AE), from a group O isolate and from a closely related SIVcpz strain. In contrast, the Vpu antiserum recognized only clade B Vpu proteins, consistent with the fact that we used a peptide from the C-terminal region of clade B Vpu. This region is highly variable among group M viruses (115). More conserved regions of Vpu map to the transmembrane domain of the protein and the β TRCP binding site (116, 117). However, these regions are either buried into the plasma membrane or occluded by cellular partners, and thus are

not readily accessible for antibody recognition. While the generation of a broad Vpu antiserum is challenging, it may be possible to generate clade-specific Vpu antisera by immunization using peptides corresponding to the C-terminal region specific for a given clade.

Nef and Vpu intracellular detection by flow cytometry represents an excellent tool to study their biological activities in HIV-1-infected primary CD4⁺ T cells. This method allows for the detection of cell-surface substrates or antibody recognition of surface Env and the concomitant detection of Nef and Vpu expression within a single infected cell (Fig. 2A). Infected CD4⁺ T cells represent the most relevant system to study the complex interplay between these two accessory proteins and the wide range of host cell factors naturally expressed by T cells. Recent findings revealed that modulation of BST-2 levels by type I IFN impacts the capacity of Vpu to downregulate NTB-A, PVR, CD62L and Tim-3, thus reducing its polyfunctionality (63, 69). Nef and Vpu also display overlapping functions, as they share the capacity to downregulate several cell-surface proteins, including CD4, PVR, CD62L and CD28 (8, 56, 62, 110, 118). The expression levels of one viral protein could therefore modulate the biological activities of the other, making it essential to study their functions in a context where both viral proteins are expressed simultaneously at physiological levels. Thus, our intracellular staining measuring Nef/Vpu expression and functionality in HIV-1-infected cells represents a new approach to better characterize their functional interplay.

Increasing evidence points towards Env conformation on the surface of infected cells as a critical parameter of ADCC susceptibility to HIV⁺ plasma (41, 119-121). Non-neutralizing antibodies in the plasma from HIV-1-infected individuals target epitopes that are only exposed when Env

interacts with cell-surface CD4, thus adopting the “open” CD4-bound conformation (41, 43). Nef and Vpu contribute to protect HIV-1-infected cells from ADCC by limiting Env-CD4 interaction via CD4 downregulation and BST-2 antagonism (41, 43, 44, 77, 78). Here we confirm and extend previous observations by showing that Nef and Vpu expression predicts the susceptibility of HIV-1-infected primary CD4⁺ T cells to ADCC responses. In agreement with recent studies (44, 79), we found that CD4 accurately predicted the susceptibility of infected cells to ADCC (Fig. 5). Given its enhanced capacity to downregulate CD4 compared to Env or Vpu (34, 41, 43, 118), Nef represents the main viral factor influencing ADCC responses mediated by CD4-induced ligands (Fig. 5B). On the contrary, BST-2 and Env expression, alone or in combination, were unable to accurately predict the susceptibility of infected cells to ADCC. These results are consistent with previous reports suggesting that Env conformation rather than overall cell-surface Env levels, drives ADCC responses mediated by HIV⁺ plasma (41, 43, 120, 121). This is also in agreement with recent work showing that BST-2 upregulation by type I IFN enhances cell-surface Env levels without increasing the susceptibility of infected cells to ADCC mediated by HIV⁺ plasma, unless CD4-mimetics are used to “open-up” Env and stabilize the CD4-bound conformation (122).

A series of recent studies using LucR.T2A IMCs have hypothesized that Vpu can compensate for the absence of Nef expression by fully downregulating cell-surface CD4 (93-97). Our results show that this is not the case. Consistent with its role in targeting CD4 already present at the plasma membrane, the impact of Nef on CD4 downmodulation is more prominent (Fig. 2 & 3) (34, 41, 43, 118). In its absence, Vpu was unable to fully downregulate CD4, thus sensitizing infected cells to ADCC responses. These results highlight the importance of selecting full-length

unmutated IMCs with proper Nef and Vpu expression to generate biologically relevant ADCC measurements. For example, a recent manuscript recently reported no differences in ADCC susceptibility between cells infected with clade B, clade C or CRF01_AE IMCs (123) while previous studies have shown otherwise (120, 124). In this article (123), the authors use functionally Nef defective LucR.T2A IMCs, which results in incomplete CD4 downregulation and therefore exposure of Env in its CD4-bound conformation at the cell surface (Fig. 4) (79). Thus, it is not surprising that the usage of Nef defective viruses skew ADCC responses in favor of nnAbs and mitigate the intrinsic differences that exists between Env from different clades. Fortunately, several alternatives to the use of LucR.T2A IMCs are available to measure ADCC against productively-infected cells (125), including the Infected Cell Elimination (ICE) assay, which measures the loss of productively-infected cells (p24+) by flow cytometry and allows the utilization of unmodified IMCs. Utilization of an NK cells resistant T cell line expressing a Tat-driven luciferase reporter gene (CEM.NKr-CCR5-sLTR-Luc) as target cells also represents an option (126). Finally, luciferase reporter IMCs (referred to as LucR.6ATRi IMCs) expressing similar levels of Nef than those obtained with unmodified IMC are also available. These IMCs utilize a modified encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) element in lieu of T2A (54, 79). Thus, LucR.6ATRi reporter viruses represent a biologically relevant alternative to LucR.T2A IMCs when measuring ADCC mediated by nnAbs and plasma collected from infected or vaccinated individuals.

ACKNOWLEDGMENTS

The authors thank the CRCHUM BSL3 and Flow Cytometry Platforms for technical assistance, Mario Legault from the FRQS AIDS and Infectious Diseases network for cohort coordination

and clinical samples. We thank the following collaborators for kindly providing some infectious molecular clones: Dennis Burton (The Scripps Research Institute) for JR-FL, Malcom A. Martin (NIAID) for SHIV_{AD8-EO}, George M. Shaw (UPenn) for SHIV.AE.40100 and Sodasai Tovanabutra (US MHRP) for the HIV-1_{WR27}, HIV-1₇₀₃₃₅₇, HIV-1₄₀₀₆₁, HIV-1_{CM235} and HIV-1₈₅₁₈₉₁ IMCs. We thank MédiMabs for their scientific and technical support during the development of the Vpu antiserum. This study was supported by grants from the National Institutes of Health to A.F., C.O. and J.C.K. (R01 AI148379), to A.F. (R01 AI129769 and R01 AI150322) and to BHH (R01 AI162646 and UM1 AI164570). This work was also partially supported by 1UM1AI164562-01, co-funded by National Heart, Lung and Blood Institute, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Neurological Disorders and Stroke, National Institute on Drug Abuse and the National Institute of Allergy and Infectious Diseases, a CIHR foundation grant #352417, a CIHR Team grant #422148 and a Canada Foundation for Innovation grant #41027 to A.F. A.F. is the recipient of a Canada Research Chair on Retroviral Entry #RCHS0235 950-232424. F.K. is supported by the Deutsche Forschungsgemeinschaft (CRC 1279 and SPP 1923). J.P. is the recipient of a CIHR doctoral fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

J.P. J.R. and A.F. conceived the study. J.P., J.R., and A.F. designed experimental approaches. J.P., J.R., R.G. R.D. and A.F. performed, analyzed, and interpreted the experiments. H.M., F.K., B.H.H., J.C.K. and C.O. supplied novel/unique reagents. J.P., J.R., BHH, and A.F. wrote the paper. All authors have read, edited, and approved the final manuscript.

379

380 **CONFLICT OF INTEREST**

381 The authors declare no competing interests.

382

383 **DATA AVAILABILITY**

384 Data and reagents are available upon request.

385

386

387 **METHODS**

388

389 **Ethics Statement**

390 Written informed consent was obtained from all study participants [the Montreal Primary HIV
391 Infection Cohort (127, 128) and the Canadian Cohort of HIV Infected Slow Progressors (129-
392 131), and research adhered to the ethical guidelines of CRCHUM and was reviewed and
393 approved by the CRCHUM institutional review board (ethics committee, approval number CE
394 16.164 - CA). Research adhered to the standards indicated by the Declaration of Helsinki. All
395 participants were adult and provided informed written consent prior to enrolment in accordance
396 with Institutional Review Board approval.

397

398 **Cell lines and isolation of primary cells**

399 HEK293T human embryonic kidney cells (obtained from ATCC) were grown as previously
400 described (132). Primary human PBMCs and CD4⁺ T cells were isolated, activated and cultured
401 as previously described (43). Briefly, PBMCs were obtained by leukapheresis from HIV-

negative individuals (4 males and 1 female) and CD4⁺ T lymphocytes were purified from resting PBMCs by negative selection using immunomagnetic beads per the manufacturer's instructions (StemCell Technologies, Vancouver, BC) and were activated with phytohemagglutinin-L (10 µg/mL) for 48 hours and then maintained in RPMI 1640 complete medium supplemented with rIL-2 (100 U/mL).

Plasmids and proviral constructs

The vesicular stomatitis virus G (VSV-G)-encoding plasmid was previously described (133). Transmitted/Founder (T/F) and chronic infectious molecular clones (IMCs) of patients CH040, CH058, CH077, CH131, CH141, CH167, CH185, CH198, CH236, CH269, CH293, CH440, CH470, CH505, CH534, CH850, CM235, MCST, REJO, RHGA, RHPA, STCO, SUMA, TRJO, WARO, WITO, WR27, 40061, 703357 and 851891 were inferred, constructed, and biologically characterized as previously described (120, 134-143). The IMCs encoding for HIV-1 reference strains AD8, JR-FL, JR-CSF, NL4-3, YU-2 were described elsewhere (144-149). HIV-1 group O (RBF206), SIVcpz (TAN2) and chimeric SIVmac/HIV-1 IMC constructs (SHIV_{AD8-EO} and SHIV.AE.40100) were generated as previously published (150-153). CH058 IMCs defective for Vpu and/or Nef expression were previously described (58). To generate a *nef*-defective JR-FL IMC, a frameshift mutation was introduced at the unique XhoI restriction site within the *nef* gene, resulting in a premature stop codon at position 47. To generate *vpu*-defective JR-FL IMCs, two stop-codons were introduced directly after the start-codon of *vpu* using the QuikChange II XL site-directed mutagenesis protocol (Agilent Technologies, Santa Clara, CA). The presence of the desired mutations was determined by automated DNA sequencing. Proviral constructs, collectively referred as Env-IMCs, comprising an HIV-1 NL4.3-based isogenic backbone

engineered for the insertion of heterologous *env* strain sequences and expression in *cis* of full-length Env (pNL.CH058.ecto and pNL.YU-2.ecto), were previously described (47). In the same study, isogenic proviral constructs encoding *Renilla* luciferase (LucR) followed in frame by a ribosome-skipping T2A peptide intended to drive Nef expression were also reported (collectively referred to as Env-IMC-LucR.T2A) (47). Construction of plasmids encoding for CH058 Vpu and CH058 Nef in the pCGCG-IRES-eGFP expression vector was previously described (58, 59).

Viral production and infections

To achieve a similar level of infection in primary CD4⁺ T cells among the different IMCs tested, VSV-G-pseudotyped HIV-1 viruses were produced and titrated as previously described (120). Viruses were then used to infect activated primary CD4⁺ T cells from healthy HIV-1 negative donors by spin infection at 800 × *g* for 1 h in 96-well plates at 25 °C.

Antibodies and plasma

The following Abs were used to assess cell-surface Env staining: A32, 2G12 (NIH AIDS Reagent Program) and PGT135 (IAVI). Mouse anti-human CD4 (clone OKT4; Thermo Fisher Scientific, Waltham, MA, USA) and mouse anti-human BST-2 (clone RS38E, PE-Cy7-conjugated; Biolegend, San Diego, CA, USA) were also used as primary antibodies for cell-surface staining. Goat anti-mouse and anti-human antibodies pre-coupled to Alexa Fluor 647 (Invitrogen, Rockford, IL, USA) were used as secondary antibodies in flow cytometry experiments. Plasma from HIV-infected individuals were collected, heat-inactivated and conserved at −80 °C until use. Rabbit antisera raised against a Nef consensus protein (NIH AIDS Reagent Program) or against a Vpu C-terminal peptide (69) were used as primary antibodies in

intracellular staining. BrilliantViolet 421 (BV421)-conjugated donkey anti-rabbit antibodies (Biolegend) were used as secondary antibodies to detect Nef and Vpu antisera binding by flow cytometry. To avoid any potential cross-reactivity with the anti-rabbit secondary antibodies used for intracellular staining, mouse monoclonal antibodies were used to detect CD4 and BST-2 proteins.

Flow cytometry analysis of cell-surface and intracellular staining

Cell-surface staining of infected cells was performed as previously described (41). Binding of cell-surface HIV-1 Env by anti-Env mAbs (5 µg/mL) or HIV+ plasma (1:1000 dilution) was performed at 48h post-infection. Infected cells were then permeabilized using the Cytofix/Cytoperm Fixation/ Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) and stained intracellularly using PE-conjugated mouse anti-p24 mAb (clone KC57; Beckman Coulter, Brea, CA, USA; 1:100 dilution) in combination with Nef or Vpu rabbit antisera (1:1000 dilution). The percentage of infected cells (p24⁺) was determined by gating on the living cell population according to a viability dye staining (Aqua Vivid; Thermo Fisher Scientific). Alternatively, intracellular staining was assessed on 293T expressing Nef or Vpu proteins. Briefly, 2x10⁶ 293T cells were transfected with 7µg of Nef or Vpu expressor with the calcium-phosphate method. At 48 h post transfection, 293T cells were stained intracellularly with rabbit antisera raised against Nef or Vpu (1:1000). Samples were acquired on an LSRII cytometer (BD Biosciences), and data analysis was performed using FlowJo v10.5.3 (Tree Star, Ashland, OR, USA).

FACS-based ADCC assay

Measurement of ADCC using the FACS-based assay was performed at 48h post-infection as previously described (43, 119). Briefly, HIV-1-infected primary CD4⁺ T cells were stained with AquaVivid viability dye and cell proliferation dye eFluor670 (Thermo Fisher Scientific) and used as target cells. Autologous PBMC effectors cells, stained with cell proliferation dye eFluor450 (Thermo Fisher Scientific), were added at an effector: target ratio of 10:1 in 96-well V-bottom plates (Corning, Corning, NY). A 1:1000 final dilution of plasma or 5µg/mL of A32 mAb was added to appropriate wells and cells were incubated for 5 min at room temperature. The plates were subsequently centrifuged for 1 min at 300 *x* g, and incubated at 37°C, 5% CO₂ for 5h before being fixed in a 2% PBS-formaldehyde solution. Samples were acquired on an LSRII cytometer (BD Biosciences) and data analysis was performed using FlowJo v10.5.3 (Tree Star). The percentage of ADCC was calculated with the following formula: (% of p24⁺ cells in Targets plus Effectors) – (% of p24⁺ cells in Targets plus Effectors plus plasma) / (% of p24⁺ cells in Targets) by gating on infected lived target cells.

Software Scripts and Visualization

Correlograms were generated using the corrplot package in program R v.4.1.012 and RStudio v.1.4.1106 (154, 155) . Correlation networks were created using the ggraph and igraph packages in R in undirected mode, clustered based on the igraph layout “star”. Edges are weighted according to P-values (inversely). Edges are only shown if $P < 0.05$, and nodes without edges were removed. Nodes are sized according to the r-values of connecting edges. Multiple linear regression analyses were performed using the GraphPad Prism software (version 9.1.0). The coefficient of determination (R^2) was used as a metric to measure the proportion of the variation observed with the dependant variable that can be explained by the variation in the independent

variables. Since R^2 values usually increases when more predictive variables are added to the model, we also measured the adjusted R^2 (adj. R^2) to account for this caveat.

Statistical analysis

Statistics were analyzed using GraphPad Prism version 9.1.0 (GraphPad, San Diego, CA, USA). Every data set was tested for statistical normality and this information was used to apply the appropriate (parametric or nonparametric) statistical test. P values <0.05 were considered significant; significance values are indicated as * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

FIGURE LEGENDS

Figure 1. Intracellular detection of Nef and Vpu in infected primary CD4+ T cells.

(A-C) 293T cells transfected with an empty vector or a plasmid expressing either CH058 Nef or CH058 Vpu. 48 hours post-transfection, cells were permeabilized and stained with rabbit polyclonal antisera raised against Nef and Vpu to detect their respective intracellular expression. Antiserum binding was detected using donkey anti-rabbit BV421 secondary Abs. (A) Histograms depicting representative staining and (B-C) Median fluorescence intensities (MFI) obtained for multiple independent stainings using (B) anti-Nef or (C) anti-Vpu. (D-G) Primary CD4+T cells mock-infected or infected with CH058 T/F WT, Nef- or Vpu-, were stained to detect the intracellular expression of Nef or Vpu. (D,F) Dot plots (left) and histograms (right) depicting representative (D) Nef and (F) Vpu staining. (E,G) The graphs show the MFI obtained from different cell populations using cells from five different donors using (E) anti-Nef or (G) anti-Vpu. Error bars indicate means $\pm$ standard errors of the means (SEM). Statistical significance was tested using an unpaired t test or a Mann-Whitney U test based on statistical normality (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant). (H-I) Primary CD4+ T cells were infected with a panel of viruses from different clades (A1, B, C, CRF01_AE), group (M, O) and host (HIV-1, SIVcpz, SHIV). The radar plots indicate the level of specific recognition of infected cells (MFI normalized to uninfected cells) using the (H) anti-Nef or (I) anti-Vpu antisera. The limit of detection was determined using (H) cells infected with CH058 Nef- for Nef staining and using (I) cells infected with CH058 Vpu- for Vpu staining.

Figure 2. Concomitant detection of intracellular Nef and Vpu and cell-surface CD4 and BST-2.

Primary CD4⁺T cells infected with CH058 T/F WT, Nef-, Vpu-, Vpu A14L/A18L or Vpu S52A/S56A viruses were stained for cell-surface CD4 and BST-2 prior to detection of intracellular Nef or Vpu expression. (A,C,E) Contour plots depicting representative cell-surface CD4 or BST-2 detection in combination with Nef or Vpu intracellular detection. Mock-infected cells were used as a control and are shown in grey. (B,D,F) The graphs show MFIs obtained from five independent experiments. Error bars indicate means $\pm$ standard errors of the means (SEM). Statistical significance was tested using an unpaired t test or a Mann-Whitney U test based on statistical normality (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant).

Figure 3. Nef and Vpu intracellular detection inversely correlates with the recognition of infected cells and their susceptibility to ADCC responses mediated by HIV+ plasma.

Primary CD4⁺T cells were mock-infected (grey), infected with CH058 T/F (red) or JR-FL (Blue) viruses (WT, Nef-, Vpu-, Nef- Vpu-) and stained for (A) intracellular Nef or Vpu expression in combination with (B) cell-surface staining of Env (using the anti-Env 2G12 mAb), CD4 and BST-2. The ability of the anti-Env A32 mAb and 25 different HIV+ plasma to (C) recognize infected cells and (D) eliminate infected cells by ADCC was also measured. (A-D) The graphs show the MFI obtained on the infected (p24⁺) cell population using cells from five different donors. Error bars indicate means $\pm$ standard errors of the means (SEM). Statistical significance was tested using an unpaired t test or a Mann-Whitney U test based on statistical normality (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant). (E) Correlograms summarize pairwise correlations among all immunological, virological and cellular variables obtained from infected

primary CD4⁺ T cells (shown in A-D). Squares are color-coded according to the magnitude of the correlation coefficient (r) and the square dimensions are inversely proportional with the P-values. Red squares represent a positive correlation between two variables and blue squares represent negative correlations. Asterisks indicate all statistically significant correlations (*P < 0.05, **P < 0.01, ***P < 0.005). Correlation analysis was done using nonparametric Spearman rank tests. (F) Correlation networks were generated using data shown in (E). Each node (circle) represents a cellular (red), an immunological (green) or a virological (blue) feature measured on infected cells. Nodes are connected with edges (lines) if they are significantly correlated (P < 0.05); nodes without edges were removed. Edges are weighted according to P-values (inversely). Red edges represent a positive correlation between two variables and blue edges represent negative correlations. Nodes are sized according to the r-values of connecting edges.

Figure 4. Lack of Nef expression in primary CD4⁺ T cells infected with LucR.T2A IMCs results in enhanced ADCC.

Primary CD4⁺T cells mock-infected (grey) or infected with chimeric IMCs expressing CH058 Env (red) or YU-2 Env (green) and expressing or not the LucR reporter gene. (A) Dot plots depicting representative stainings of intracellular Nef or Vpu expression. (B-C) Detection by flow cytometry of (B) intracellular Nef or Vpu expression in combination with (C) cell-surface staining of Env (using anti-Env mAbs 2G12 (CH058) or PGT135 (YU-2)), CD4 and BST-2. The ability of the A32 mAb and 25 HIV⁺ plasma to (D) recognize infected cells and (E) eliminate infected cells by ADCC was also measured. (B-E) The graphs show the MFI obtained on the infected (p24⁺) cell population using cells from five different donors. Error bars indicate means ± standard errors of the means (SEM). Statistical significance was tested using an unpaired t test

or a Mann-Whitney U test based on statistical normality (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant). (F) Correlograms summarize pairwise correlations among all immunological, virological and cellular variables obtained from infected primary CD4⁺ T cells (shown in B-E). Squares are color-coded according to the magnitude of the correlation coefficient (r) and the square dimensions are inversely proportional with the P-values. Red squares represent a positive correlation between two variables and blue squares represent negative correlations. Asterisks indicate all statistically significant correlations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). Correlation analysis was done using nonparametric Spearman rank tests. (G) Correlation networks were generated using data shown in (F). Each node (circle) represents a cellular (red), an immunological (green) or a virological (blue) feature measured on infected cells. Nodes are connected with edges (lines) if they are significantly correlated ($P < 0.05$); nodes without edges were removed. Edges are weighted according to P-values (inversely). Red edges represent a positive correlation between two variables and blue edges represent negative correlations. Nodes are sized according to the r -values of connecting edges.

Figure 5. Prediction of ADCC responses mediated by HIV+ plasma using multiple linear regression models.

(A-C) Multiple linear regression analysis to identify variables that can predict the ADCC responses mediated by HIV+ plasma against primary CD4⁺ T cells infected by different viral constructs (WT, Nef-, Vpu-, Nef-Vpu-, Env-IMC, Env-IMC-LucR.T2A) from different HIV-1 strains (CH058, JR-FL, YU-2). Each dot represents a single virus where the average of ADCC obtained with 25 different HIV+ plasma (dependent variable) is plotted on the X axis and the predicted ADCC value based on one or more independent parameters is plotted on the Y axis.

Predictors include (A) cellular variables, (B) virological variables and (C) immunological variables. Multiple linear regression analyses were performed using the GraphPad Prism software (version 9.1.0). P values below 0.05 are considered significant and are highlighted in bold. The coefficient of multiple correlation (R^2) indicates the goodness of fit of the multiple regression linear model. The adjusted R^2 (Adj. R^2) is used to compare the fits of models across experiments with different numbers of data points and independent variables.

REFERENCES

1. Desrosiers RC, Lifson JD, Gibbs JS, Czajak SC, Howe AY, Arthur LO, Johnson RP. 1998. Identification of highly attenuated mutants of simian immunodeficiency virus. *J Virol* 72:1431-7.
2. Dave VP, Hajjar F, Dieng MM, Haddad E, Cohen EA. 2013. Efficient BST2 antagonism by Vpu is critical for early HIV-1 dissemination in humanized mice. *Retrovirology* 10:128.
3. Sato K, Misawa N, Fukuhara M, Iwami S, An DS, Ito M, Koyanagi Y. 2012. Vpu augments the initial burst phase of HIV-1 propagation and downregulates BST2 and CD4 in humanized mice. *J Virol* 86:5000-13.
4. Sato K, Misawa N, Iwami S, Satou Y, Matsuoka M, Ishizaka Y, Ito M, Aihara K, An DS, Koyanagi Y. 2013. HIV-1 Vpr accelerates viral replication during acute infection by exploitation of proliferating CD4+ T cells in vivo. *PLoS Pathog* 9:e1003812.
5. Crotti A, Neri F, Corti D, Ghezzi S, Heltai S, Baur A, Poli G, Santagostino E, Vicenzi E. 2006. Nef alleles from human immunodeficiency virus type 1-infected long-term-nonprogressor hemophiliacs with or without late disease progression are defective in enhancing virus replication and CD4 down-regulation. *J Virol* 80:10663-74.
6. Rucker E, Grivel JC, Munch J, Kirchhoff F, Margolis L. 2004. Vpr and Vpu are important for efficient human immunodeficiency virus type 1 replication and CD4+ T-cell depletion in human lymphoid tissue ex vivo. *J Virol* 78:12689-93.
7. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. 1995. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Engl J Med* 332:228-32.
8. Haller C, Muller B, Fritz JV, Lamas-Murua M, Stolp B, Pujol FM, Keppler OT, Fackler OT. 2014. HIV-1 Nef and Vpu are functionally redundant broad-spectrum modulators of cell surface receptors, including tetraspanins. *J Virol* 88:14241-57.
9. Tokarev A, Guatelli J. 2011. Misdirection of membrane trafficking by HIV-1 Vpu and Nef: Keys to viral virulence and persistence. *Cell Logist* 1:90-102.
10. Purcell DF, Martin MA. 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. *J Virol* 67:6365-78.
11. Bentham M, Mazaleyrat S, Harris M. 2006. Role of myristoylation and N-terminal basic residues in membrane association of the human immunodeficiency virus type 1 Nef protein. *J Gen Virol* 87:563-571.
12. Craig HM, Reddy TR, Riggs NL, Dao PP, Guatelli JC. 2000. Interactions of HIV-1 nef with the mu subunits of adaptor protein complexes 1, 2, and 3: role of the dileucine-based sorting motif. *Virology* 271:9-17.
13. Chaudhuri R, Lindwasser OW, Smith WJ, Hurley JH, Bonifacino JS. 2007. Downregulation of CD4 by human immunodeficiency virus type 1 Nef is dependent on clathrin and involves direct interaction of Nef with the AP2 clathrin adaptor. *J Virol* 81:3877-90.
14. Ren X, Park SY, Bonifacino JS, Hurley JH. 2014. How HIV-1 Nef hijacks the AP-2 clathrin adaptor to downregulate CD4. *Elife* 3:e01754.
15. Aiken C, Konner J, Landau NR, Lenburg ME, Trono D. 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* 76:853-64.
16. Rhee SS, Marsh JW. 1994. Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *J Virol* 68:5156-63.

17. Schwartz S, Felber BK, Pavlakis GN. 1992. Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. *Mol Cell Biol* 12:207-19.
18. Krummheuer J, Johnson AT, Hauber I, Kammler S, Anderson JL, Hauber J, Purcell DF, Schaal H. 2007. A minimal uORF within the HIV-1 vpu leader allows efficient translation initiation at the downstream env AUG. *Virology* 363:261-71.
19. Wray V, Federau T, Henklein P, Klabunde S, Kunert O, Schomburg D, Schubert U. 1995. Solution structure of the hydrophilic region of HIV-1 encoded virus protein U (Vpu) by CD and ¹H NMR spectroscopy. *Int J Pept Protein Res* 45:35-43.
20. Maldarelli F, Chen MY, Willey RL, Strebel K. 1993. Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein. *J Virol* 67:5056-61.
21. Marassi FM, Ma C, Gratkowski H, Straus SK, Strebel K, Oblatt-Montal M, Montal M, Opella SJ. 1999. Correlation of the structural and functional domains in the membrane protein Vpu from HIV-1. *Proc Natl Acad Sci U S A* 96:14336-41.
22. Margottin F, Bour SP, Durand H, Selig L, Benichou S, Richard V, Thomas D, Strebel K, Benarous R. 1998. A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. *Mol Cell* 1:565-74.
23. Coadou G, Evrard-Todeschi N, Gharbi-Benarous J, Benarous R, Girault JP. 2002. HIV-1 encoded virus protein U (Vpu) solution structure of the 41-62 hydrophilic region containing the phosphorylated sites Ser52 and Ser56. *Int J Biol Macromol* 30:23-40.
24. Dube M, Roy BB, Guiot-Guillain P, Mercier J, Binette J, Leung G, Cohen EA. 2009. Suppression of Tetherin-restricting activity upon human immunodeficiency virus type 1 particle release correlates with localization of Vpu in the trans-Golgi network. *J Virol* 83:4574-90.
25. Klimkait T, Strebel K, Hoggan MD, Martin MA, Orenstein JM. 1990. The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *J Virol* 64:621-9.
26. Schubert U, Strebel K. 1994. Differential activities of the human immunodeficiency virus type 1-encoded Vpu protein are regulated by phosphorylation and occur in different cellular compartments. *J Virol* 68:2260-71.
27. Schubert U, Anton LC, Bacik I, Cox JH, Bour S, Bennink JR, Orlowski M, Strebel K, Yewdell JW. 1998. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J Virol* 72:2280-8.
28. Willey RL, Maldarelli F, Martin MA, Strebel K. 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J Virol* 66:7193-200.
29. Magadan JG, Perez-Victoria FJ, Sougrat R, Ye Y, Strebel K, Bonifacio JS. 2010. Multilayered mechanism of CD4 downregulation by HIV-1 Vpu involving distinct ER retention and ERAD targeting steps. *PLoS Pathog* 6:e1000869.
30. Neil SJ, Zang T, Bieniasz PD. 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451:425-30.
31. Van Damme N, Goff D, Katsura C, Jorgenson RL, Mitchell R, Johnson MC, Stephens EB, Guatelli J. 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* 3:245-52.
32. Dube M, Roy BB, Guiot-Guillain P, Binette J, Mercier J, Chiasson A, Cohen EA. 2010. Antagonism of tetherin restriction of HIV-1 release by Vpu involves binding and sequestration of the restriction factor in a perinuclear compartment. *PLoS Pathog* 6:e1000856.

33. Hauser H, Lopez LA, Yang SJ, Oldenburg JE, Exline CM, Guatelli JC, Cannon PM. 2010. HIV-1 Vpu and HIV-2 Env counteract BST-2/tetherin by sequestration in a perinuclear compartment. *Retrovirology* 7:51.
34. Wildum S, Schindler M, Munch J, Kirchhoff F. 2006. Contribution of Vpu, Env, and Nef to CD4 down-modulation and resistance of human immunodeficiency virus type 1-infected T cells to superinfection. *J Virol* 80:8047-59.
35. Ding S, Gasser R, Gendron-Lepage G, Medjahed H, Tolbert WD, Sodroski J, Pazgier M, Finzi A. 2019. CD4 Incorporation into HIV-1 Viral Particles Exposes Envelope Epitopes Recognized by CD4-Induced Antibodies. *J Virol* 93.
36. Bour S, Perrin C, Strebel K. 1999. Cell surface CD4 inhibits HIV-1 particle release by interfering with Vpu activity. *J Biol Chem* 274:33800-6.
37. Cortes MJ, Wong-Staal F, Lama J. 2002. Cell surface CD4 interferes with the infectivity of HIV-1 particles released from T cells. *J Biol Chem* 277:1770-9.
38. Lama J, Mangasarian A, Trono D. 1999. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. *Curr Biol* 9:622-31.
39. Levesque K, Zhao YS, Cohen EA. 2003. Vpu exerts a positive effect on HIV-1 infectivity by down-modulating CD4 receptor molecules at the surface of HIV-1-producing cells. *J Biol Chem* 278:28346-53.
40. Decker JM, Bibollet-Ruche F, Wei X, Wang S, Levy DN, Wang W, Delaporte E, Peeters M, Derdeyn CA, Allen S, Hunter E, Saag MS, Hoxie JA, Hahn BH, Kwong PD, Robinson JE, Shaw GM. 2005. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. *J Exp Med* 201:1407-19.
41. Veillette M, Coutu M, Richard J, Batrville LA, Dagher O, Bernard N, Tremblay C, Kaufmann DE, Roger M, Finzi A. 2015. The HIV-1 gp120 CD4-Bound Conformation Is Preferentially Targeted by Antibody-Dependent Cellular Cytotoxicity-Mediating Antibodies in Sera from HIV-1-Infected Individuals. *J Virol* 89:545-51.
42. Guan Y, Pazgier M, Sajadi MM, Kamin-Lewis R, Al-Darmarki S, Flinko R, Lovo E, Wu X, Robinson JE, Seaman MS, Fouts TR, Gallo RC, DeVico AL, Lewis GK. 2013. Diverse specificity and effector function among human antibodies to HIV-1 envelope glycoprotein epitopes exposed by CD4 binding. *Proc Natl Acad Sci U S A* 110:E69-78.
43. Veillette M, Desormeaux A, Medjahed H, Gharsallah NE, Coutu M, Baalwa J, Guan Y, Lewis G, Ferrari G, Hahn BH, Haynes BF, Robinson JE, Kaufmann DE, Bonsignori M, Sodroski J, Finzi A. 2014. Interaction with cellular CD4 exposes HIV-1 envelope epitopes targeted by antibody-dependent cell-mediated cytotoxicity. *J Virol* 88:2633-44.
44. Alsahafi N, Ding S, Richard J, Markle T, Brassard N, Walker B, Lewis GK, Kaufmann DE, Brockman MA, Finzi A. 2016. Nef Proteins from HIV-1 Elite Controllers Are Inefficient at Preventing Antibody-Dependent Cellular Cytotoxicity. *J Virol* 90:2993-3002.
45. Schaefer MR, Wonderlich ER, Roeth JF, Leonard JA, Collins KL. 2008. HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells. *PLoS Pathog* 4:e1000131.
46. Dikeakos JD, Atkins KM, Thomas L, Emert-Sedlak L, Byeon IJ, Jung J, Ahn J, Wortman MD, Kukull B, Saito M, Koizumi H, Williamson DM, Hiyoshi M, Barklis E, Takiguchi M, Suzu S, Gronenborn AM, Smithgall TE, Thomas G. 2010. Small molecule inhibition of HIV-1-induced MHC-I down-regulation identifies a temporally regulated switch in Nef action. *Mol Biol Cell* 21:3279-92.
47. Edmonds TG, Ding H, Yuan X, Wei Q, Smith KS, Conway JA, Wiczorek L, Brown B, Polonis V, West JT, Montefiori DC, Kappes JC, Ochsenbauer C. 2010. Replication competent molecular clones of HIV-1 expressing Renilla luciferase facilitate the analysis of antibody inhibition in PBMC. *Virology* 408:1-13.

48. Atkins KM, Thomas L, Youker RT, Harrieff MJ, Pissani F, You H, Thomas G. 2008. HIV-1 Nef binds PACS-2 to assemble a multikinase cascade that triggers major histocompatibility complex class I (MHC-I) down-regulation: analysis using short interfering RNA and knock-out mice. *J Biol Chem* 283:11772-84.
49. Lenassi M, Cagney G, Liao M, Vaupotic T, Bartholomeeusen K, Cheng Y, Krogan NJ, Plemenitas A, Peterlin BM. 2010. HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. *Traffic* 11:110-22.
50. Komoto S, Tsuji S, Ibrahim MS, Li YG, Warachit J, Taniguchi K, Ikuta K. 2003. The vpu protein of human immunodeficiency virus type 1 plays a protective role against virus-induced apoptosis in primary CD4(+) T lymphocytes. *J Virol* 77:10304-13.
51. Miyagi E, Andrew AJ, Kao S, Strebel K. 2009. Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion. *Proc Natl Acad Sci U S A* 106:2868-73.
52. Chu H, Wang JJ, Qi M, Yoon JJ, Chen X, Wen X, Hammonds J, Ding L, Spearman P. 2012. Tetherin/BST-2 is essential for the formation of the intracellular virus-containing compartment in HIV-infected macrophages. *Cell Host Microbe* 12:360-72.
53. Shugars DC, Smith MS, Glueck DH, Nantermet PV, Seillier-Moiseiwitsch F, Swanstrom R. 1993. Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo. *J Virol* 67:4639-50.
54. Alberti MO, Jones JJ, Miglietta R, Ding H, Bakshi RK, Edmonds TG, Kappes JC, Ochsenbauer C. 2015. Optimized Replicating Renilla Luciferase Reporter HIV-1 Utilizing Novel Internal Ribosome Entry Site Elements for Native Nef Expression and Function. *AIDS Res Hum Retroviruses* 31:1278-96.
55. Jacob RA, Edgar CR, Prevost J, Trothen SM, Lurie A, Mumby MJ, Galbraith A, Kirchhoff F, Haeryfar SMM, Finzi A, Dikeakos JD. 2021. The HIV-1 accessory protein Nef increases surface expression of the checkpoint receptor Tim-3 in infected CD4(+) T cells. *J Biol Chem* 297:101042.
56. Pawlak EN, Dirk BS, Jacob RA, Johnson AL, Dikeakos JD. 2018. The HIV-1 accessory proteins Nef and Vpu downregulate total and cell surface CD28 in CD4(+) T cells. *Retrovirology* 15:6.
57. Cohen EA, Terwilliger EF, Sodroski JG, Haseltine WA. 1988. Identification of a protein encoded by the vpu gene of HIV-1. *Nature* 334:532-4.
58. Heigele A, Kmiec D, Regensburger K, Langer S, Peiffer L, Sturzel CM, Sauter D, Peeters M, Pizzato M, Learn GH, Hahn BH, Kirchhoff F. 2016. The Potency of Nef-Mediated SERINC5 Antagonism Correlates with the Prevalence of Primate Lentiviruses in the Wild. *Cell Host Microbe* 20:381-91.
59. Kmiec D, Iyer SS, Sturzel CM, Sauter D, Hahn BH, Kirchhoff F. 2016. Vpu-Mediated Counteraction of Tetherin Is a Major Determinant of HIV-1 Interferon Resistance. *MBio* 7.
60. Bolduan S, Hubel P, Reif T, Lodermeier V, Hohne K, Fritz JV, Sauter D, Kirchhoff F, Fackler OT, Schindler M, Schubert U. 2013. HIV-1 Vpu affects the anterograde transport and the glycosylation pattern of NTB-A. *Virology* 440:190-203.
61. Bolduan S, Reif T, Schindler M, Schubert U. 2014. HIV-1 Vpu mediated downregulation of CD155 requires alanine residues 10, 14 and 18 of the transmembrane domain. *Virology* 464-465:375-384.
62. Vassena L, Giuliani E, Koppensteiner H, Bolduan S, Schindler M, Doria M. 2015. HIV-1 Nef and Vpu Interfere with L-Selectin (CD62L) Cell Surface Expression To Inhibit Adhesion and Signaling in Infected CD4+ T Lymphocytes. *J Virol* 89:5687-700.
63. Prevost J, Pickering S, Mumby MJ, Medjahed H, Gendron-Lepage G, Delgado GG, Dirk BS, Dikeakos JD, Sturzel CM, Sauter D, Kirchhoff F, Bibollet-Ruche F, Hahn BH, Dube M, Kaufmann DE, Neil SJD, Finzi A, Richard J. 2019. Upregulation of BST-2 by Type I

- Interferons Reduces the Capacity of Vpu To Protect HIV-1-Infected Cells from NK Cell Responses. *mBio* 10.
64. Bour S, Schubert U, Strebel K. 1995. The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. *J Virol* 69:1510-20.
65. Chen MY, Maldarelli F, Karczewski MK, Willey RL, Strebel K. 1993. Human immunodeficiency virus type 1 Vpu protein induces degradation of CD4 in vitro: the cytoplasmic domain of CD4 contributes to Vpu sensitivity. *J Virol* 67:3877-84.
66. Lenburg ME, Landau NR. 1993. Vpu-induced degradation of CD4: requirement for specific amino acid residues in the cytoplasmic domain of CD4. *J Virol* 67:7238-45.
67. Vincent MJ, Raja NU, Jabbar MA. 1993. Human immunodeficiency virus type 1 Vpu protein induces degradation of chimeric envelope glycoproteins bearing the cytoplasmic and anchor domains of CD4: role of the cytoplasmic domain in Vpu-induced degradation in the endoplasmic reticulum. *J Virol* 67:5538-49.
68. Margottin F, Benichou S, Durand H, Richard V, Liu LX, Gomas E, Benarous R. 1996. Interaction between the cytoplasmic domains of HIV-1 Vpu and CD4: role of Vpu residues involved in CD4 interaction and in vitro CD4 degradation. *Virology* 223:381-6.
69. Prevost J, Edgar CR, Richard J, Trothen SM, Jacob RA, Mumby MJ, Pickering S, Dube M, Kaufmann DE, Kirchhoff F, Neil SJD, Finzi A, Dikeakos JD. 2020. HIV-1 Vpu Downregulates Tim-3 from the Surface of Infected CD4(+) T Cells. *J Virol* 94.
70. Binette J, Dube M, Mercier J, Halawani D, Latterich M, Cohen EA. 2007. Requirements for the selective degradation of CD4 receptor molecules by the human immunodeficiency virus type 1 Vpu protein in the endoplasmic reticulum. *Retrovirology* 4:75.
71. Mitchell RS, Katsura C, Skasko MA, Fitzpatrick K, Lau D, Ruiz A, Stephens EB, Margottin-Goguet F, Benarous R, Guatelli JC. 2009. Vpu antagonizes BST-2-mediated restriction of HIV-1 release via beta-TrCP and endo-lysosomal trafficking. *PLoS Pathog* 5:e1000450.
72. Gustin JK, Douglas JL, Bai Y, Moses AV. 2012. Ubiquitination of BST-2 protein by HIV-1 Vpu protein does not require lysine, serine, or threonine residues within the BST-2 cytoplasmic domain. *J Biol Chem* 287:14837-50.
73. Belaidouni N, Marchal C, Benarous R, Besnard-Guerin C. 2007. Involvement of the betaTrCP in the ubiquitination and stability of the HIV-1 Vpu protein. *Biochem Biophys Res Commun* 357:688-93.
74. Kueck T, Neil SJ. 2012. A cytoplasmic tail determinant in HIV-1 Vpu mediates targeting of tetherin for endosomal degradation and counteracts interferon-induced restriction. *PLoS Pathog* 8:e1002609.
75. Kueck T, Foster TL, Weinelt J, Sumner JC, Pickering S, Neil SJ. 2015. Serine Phosphorylation of HIV-1 Vpu and Its Binding to Tetherin Regulates Interaction with Clathrin Adaptors. *PLoS Pathog* 11:e1005141.
76. Mangeat B, Gers-Huber G, Lehmann M, Zufferey M, Luban J, Piguet V. 2009. HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *PLoS Pathog* 5:e1000574.
77. Arias JF, Heyer LN, von Bredow B, Weisgrau KL, Moldt B, Burton DR, Rakasz EG, Evans DT. 2014. Tetherin antagonism by Vpu protects HIV-infected cells from antibody-dependent cell-mediated cytotoxicity. *Proc Natl Acad Sci U S A* 111:6425-30.
78. Alvarez RA, Hamlin RE, Monroe A, Moldt B, Hotta MT, Rodriguez Caprio G, Fierer DS, Simon V, Chen BK. 2014. HIV-1 Vpu antagonism of tetherin inhibits antibody-dependent cellular cytotoxic responses by natural killer cells. *J Virol* 88:6031-46.

79. Prevost J, Richard J, Medjahed H, Alexander A, Jones J, Kappes JC, Ochsenbauer C, Finzi A. 2018. Incomplete Downregulation of CD4 Expression Affects HIV-1 Env Conformation and Antibody-Dependent Cellular Cytotoxicity Responses. *J Virol* 92.
80. Ding S, Veillette M, Coutu M, Prevost J, Scharf L, Bjorkman PJ, Ferrari G, Robinson JE, Sturzel C, Hahn BH, Sauter D, Kirchhoff F, Lewis GK, Pazgier M, Finzi A. 2016. A Highly Conserved Residue of the HIV-1 gp120 Inner Domain Is Important for Antibody-Dependent Cellular Cytotoxicity Responses Mediated by Anti-cluster A Antibodies. *J Virol* 90:2127-34.
81. Ferrari G, Pollara J, Kozink D, Harms T, Drinker M, Freel S, Moody MA, Alam SM, Tomaras GD, Ochsenbauer C, Kappes JC, Shaw GM, Hoxie JA, Robinson JE, Haynes BF. 2011. An HIV-1 gp120 envelope human monoclonal antibody that recognizes a C1 conformational epitope mediates potent antibody-dependent cellular cytotoxicity (ADCC) activity and defines a common ADCC epitope in human HIV-1 serum. *J Virol* 85:7029-36.
82. Tomaras GD, Ferrari G, Shen X, Alam SM, Liao HX, Pollara J, Bonsignori M, Moody MA, Fong Y, Chen X, Poling B, Nicholson CO, Zhang R, Lu X, Parks R, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Gilbert PB, Kim JH, Michael NL, Montefiori DC, Haynes BF. 2013. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. *Proc Natl Acad Sci U S A* doi:10.1073/pnas.1301456110.
83. Pollara J, Bonsignori M, Moody MA, Liu P, Alam SM, Hwang KK, Gurley TC, Kozink DM, Armand LC, Marshall DJ, Whitesides JF, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Robb ML, O'Connell RJ, Kim JH, Michael NL, Montefiori DC, Tomaras GD, Liao HX, Haynes BF, Ferrari G. 2014. HIV-1 vaccine-induced C1 and V2 Env-specific antibodies synergize for increased antiviral activities. *J Virol* 88:7715-26.
84. Santra S, Tomaras GD, Warriar R, Nicely NI, Liao HX, Pollara J, Liu P, Alam SM, Zhang R, Cocklin SL, Shen X, Duffy R, Xia SM, Schutte RJ, Pemble Iv CW, Dennison SM, Li H, Chao A, Vidnovic K, Evans A, Klein K, Kumar A, Robinson J, Landucci G, Forthal DN, Montefiori DC, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Robb ML, Michael NL, Kim JH, Soderberg KA, Giorgi EE, Blair L, Korber BT, Moog C, Shattock RJ, Letvin NL, Schmitz JE, Moody MA, Gao F, Ferrari G, Shaw GM, Haynes BF. 2015. Human Non-neutralizing HIV-1 Envelope Monoclonal Antibodies Limit the Number of Founder Viruses during SHIV Mucosal Infection in Rhesus Macaques. *PLoS Pathog* 11:e1005042.
85. Huang Y, Ferrari G, Alter G, Forthal DN, Kappes JC, Lewis GK, Love JC, Borate B, Harris L, Greene K, Gao H, Phan TB, Landucci G, Goods BA, Dowell KG, Cheng HD, Bailey-Kellogg C, Montefiori DC, Ackerman ME. 2016. Diversity of Antiviral IgG Effector Activities Observed in HIV-Infected and Vaccinated Subjects. *J Immunol* 197:4603-4612.
86. Costa MR, Pollara J, Edwards RW, Seaman MS, Gorny MK, Montefiori DC, Liao HX, Ferrari G, Lu S, Wang S. 2016. Fc Receptor-Mediated Activities of Env-Specific Human Monoclonal Antibodies Generated from Volunteers Receiving the DNA Prime-Protein Boost HIV Vaccine DP6-001. *J Virol* 90:10362-10378.
87. Bradley T, Pollara J, Santra S, Vandergrift N, Pittala S, Bailey-Kellogg C, Shen X, Parks R, Goodman D, Eaton A, Balachandran H, Mach LV, Saunders KO, Weiner JA, Searce R, Sutherland LL, Phogat S, Tartaglia J, Reed SG, Hu SL, Theis JF, Pinter A, Montefiori DC, Kepler TB, Peachman KK, Rao M, Michael NL, Suscovich TJ, Alter G, Ackerman ME, Moody MA, Liao HX, Tomaras G, Ferrari G, Korber BT, Haynes BF. 2017. Pentavalent HIV-1 vaccine protects against simian-human immunodeficiency virus challenge. *Nat Commun* 8:15711.
88. Meyerhoff RR, Searce RM, Ogburn DF, Lockwood B, Pickeral J, Kuraoka M, Anasti K, Eudailey J, Eaton A, Cooper M, Wiehe K, Montefiori DC, Tomaras G, Ferrari G, Alam

- SM, Liao HX, Korber B, Gao F, Haynes BF. 2017. HIV-1 Consensus Envelope-Induced Broadly Binding Antibodies. *AIDS Res Hum Retroviruses* 33:859-868.
89. Sung JA, Pickeral J, Liu L, Stanfield-Oakley SA, Lam CK, Garrido C, Pollara J, LaBranche C, Bonsignori M, Moody MA, Yang Y, Parks R, Archin N, Allard B, Kirchherr J, Kuruc JD, Gay CL, Cohen MS, Ochsenbauer C, Soderberg K, Liao HX, Montefiori D, Moore P, Johnson S, Koenig S, Haynes BF, Nordstrom JL, Margolis DM, Ferrari G. 2015. Dual-Affinity Re-Targeting proteins direct T cell-mediated cytolysis of latently HIV-infected cells. *J Clin Invest* doi:10.1172/JCI82314.
90. Tuyishime M, Garrido C, Jha S, Moeser M, Mielke D, LaBranche C, Montefiori D, Haynes BF, Joseph S, Margolis DM, Ferrari G. 2020. Improved killing of HIV-infected cells using three neutralizing and non-neutralizing antibodies. *J Clin Invest* 130:5157-5170.
91. Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X, Hwang KK, Gilbert PB, Huang Y, Gurley TC, Kozink DM, Marshall DJ, Whitesides JF, Tsao CY, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Kim JH, Michael NL, Tomaras GD, Montefiori DC, Lewis GK, Devico A, Evans DT, Ferrari G, Liao HX, Haynes BF. 2012. Antibody-Dependent Cellular Cytotoxicity-Mediating Antibodies from an HIV-1 Vaccine Efficacy Trial Target Multiple Epitopes and Preferentially Use the VH1 Gene Family. *J Virol* 86:11521-32.
92. Cheng HD, Dowell KG, Bailey-Kellogg C, Goods BA, Love JC, Ferrari G, Alter G, Gach J, Forthal DN, Lewis GK, Greene K, Gao H, Montefiori DC, Ackerman ME. 2021. Diverse antiviral IgG effector activities are predicted by unique biophysical antibody features. *Retrovirology* 18:35.
93. Pollara J, Jones DI, Huffman T, Edwards RW, Dennis M, Li SH, Jha S, Goodman D, Kumar A, LaBranche CC, Montefiori DC, Fouda GG, Hope TJ, Tomaras GD, Staats HF, Ferrari G, Permar SR. 2019. Bridging Vaccine-Induced HIV-1 Neutralizing and Effector Antibody Responses in Rabbit and Rhesus Macaque Animal Models. *J Virol* 93.
94. Fisher L, Zinter M, Stanfield-Oakley S, Carpp LN, Edwards RW, Denny T, Moodie Z, Laher F, Bekker LG, McElrath MJ, Gilbert PB, Corey L, Tomaras G, Pollara J, Ferrari G. 2019. Vaccine-Induced Antibodies Mediate Higher Antibody-Dependent Cellular Cytotoxicity After Interleukin-15 Pretreatment of Natural Killer Effector Cells. *Front Immunol* 10:2741.
95. Lewis GK, Ackerman ME, Scarlatti G, Moog C, Robert-Guroff M, Kent SJ, Overbaugh J, Reeves RK, Ferrari G, Thyagarajan B. 2019. Knowns and Unknowns of Assaying Antibody-Dependent Cell-Mediated Cytotoxicity Against HIV-1. *Front Immunol* 10:1025.
96. Easterhoff D, Pollara J, Luo K, Tolbert WD, Young B, Mielke D, Jha S, O'Connell RJ, Vasan S, Kim J, Michael NL, Excler JL, Robb ML, Rerks-Ngarm S, Kaewkungwal J, Pitisuttithum P, Nitayaphan S, Sinangil F, Tartaglia J, Phogat S, Kepler TB, Alam SM, Wiehe K, Saunders KO, Montefiori DC, Tomaras GD, Moody MA, Pazgier M, Haynes BF, Ferrari G. 2020. Boosting with AIDSVAX B/E Enhances Env Constant Region 1 and 2 Antibody-Dependent Cellular Cytotoxicity Breadth and Potency. *J Virol* 94.
97. Tolbert WD, Van V, Sherburn R, Tuyishime M, Yan F, Nguyen DN, Stanfield-Oakley S, Easterhoff D, Bonsignori M, Haynes BF, Moody MA, Ray K, Ferrari G, Lewis GK, Pazgier M. 2020. Recognition Patterns of the C1/C2 Epitopes Involved in Fc-Mediated Response in HIV-1 Natural Infection and the RV114 Vaccine Trial. *mBio* 11.
98. Madani N, Princiotto AM, Mach L, Ding S, Prevost J, Richard J, Hora B, Sutherland L, Zhao CA, Conn BP, Bradley T, Moody MA, Melillo B, Finzi A, Haynes BF, Smith AB, III, Santra S, Sodroski J. 2018. A CD4-mimetic compound enhances vaccine efficacy against stringent immunodeficiency virus challenge. *Nat Commun* 9:2363.
99. Strebel K. 2013. HIV accessory proteins versus host restriction factors. *Curr Opin Virol* 3:692-9.

- 953 100. Ramirez PW, Famiglietti M, Sowrirajan B, DePaula-Silva AB, Rodesch C, Barker E,
954 Bosque A, Planelles V. 2014. Downmodulation of CCR7 by HIV-1 Vpu results in
955 impaired migration and chemotactic signaling within CD4(+) T cells. *Cell Rep* 7:2019-30.
- 956 101. Usami Y, Wu Y, Gottlinger HG. 2015. SERINC3 and SERINC5 restrict HIV-1 infectivity
957 and are counteracted by Nef. *Nature* 526:218-23.
- 958 102. Rosa A, Chande A, Ziglio S, De Sanctis V, Bertorelli R, Goh SL, McCauley SM,
959 Nowosielska A, Antonarakis SE, Luban J, Santoni FA, Pizzato M. 2015. HIV-1 Nef
960 promotes infection by excluding SERINC5 from virion incorporation. *Nature* 526:212-7.
- 961 103. Liu Y, Fu Y, Wang Q, Li M, Zhou Z, Dabbagh D, Fu C, Zhang H, Li S, Zhang T, Gong J,
962 Kong X, Zhai W, Su J, Sun J, Zhang Y, Yu XF, Shao Z, Zhou F, Wu Y, Tan X. 2019.
963 Proteomic profiling of HIV-1 infection of human CD4(+) T cells identifies PSGL-1 as an
964 HIV restriction factor. *Nat Microbiol* 4:813-825.
- 965 104. Fu Y, He S, Waheed AA, Dabbagh D, Zhou Z, Trinite B, Wang Z, Yu J, Wang D, Li F,
966 Levy DN, Shang H, Freed EO, Wu Y. 2020. PSGL-1 restricts HIV-1 infectivity by
967 blocking virus particle attachment to target cells. *Proc Natl Acad Sci U S A* 117:9537-
968 9545.
- 969 105. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. 1996. Endocytosis of major
970 histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat*
971 *Med* 2:338-42.
- 972 106. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. 1998. HIV-1 Nef protein
973 protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature*
974 391:397-401.
- 975 107. Cerboni C, Neri F, Casartelli N, Zingoni A, Cosman D, Rossi P, Santoni A, Doria M.
976 2007. Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the
977 activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. *J Gen*
978 *Virol* 88:242-50.
- 979 108. Fausther-Bovendo H, Sol-Foulon N, Candotti D, Agut H, Schwartz O, Debre P, Vieillard
980 V. 2009. HIV escape from natural killer cytotoxicity: nef inhibits NKp44L expression on
981 CD4+ T cells. *AIDS* 23:1077-87.
- 982 109. Shah AH, Sowrirajan B, Davis ZB, Ward JP, Campbell EM, Planelles V, Barker E. 2010.
983 Degranulation of natural killer cells following interaction with HIV-1-infected cells is
984 hindered by downmodulation of NTB-A by Vpu. *Cell Host Microbe* 8:397-409.
- 985 110. Matusali G, Potesta M, Santoni A, Cerboni C, Doria M. 2012. The human
986 immunodeficiency virus type 1 Nef and Vpu proteins downregulate the natural killer cell-
987 activating ligand PVR. *J Virol* 86:4496-504.
- 988 111. Apps R, Del Prete GQ, Chatterjee P, Lara A, Brumme ZL, Brockman MA, Neil S,
989 Pickering S, Schneider DK, Piechocka-Trocha A, Walker BD, Thomas R, Shaw GM,
990 Hahn BH, Keele BF, Lifson JD, Carrington M. 2016. HIV-1 Vpu Mediates HLA-C
991 Downregulation. *Cell Host Microbe* 19:686-95.
- 992 112. Moll M, Andersson SK, Smed-Sorensen A, Sandberg JK. 2010. Inhibition of lipid antigen
993 presentation in dendritic cells by HIV-1 Vpu interference with CD1d recycling from
994 endosomal compartments. *Blood* 116:1876-84.
- 995 113. Chen N, McCarthy C, Drakesmith H, Li D, Cerundolo V, McMichael AJ, Screaton GR, Xu
996 XN. 2006. HIV-1 down-regulates the expression of CD1d via Nef. *Eur J Immunol* 36:278-
997 86.
- 998 114. Pacyniak E, Gomez ML, Gomez LM, Mulcahy ER, Jackson M, Hout DR, Wisdom BJ,
999 Stephens EB. 2005. Identification of a region within the cytoplasmic domain of the
1000 subtype B Vpu protein of human immunodeficiency virus type 1 (HIV-1) that is
1001 responsible for retention in the golgi complex and its absence in the Vpu protein from a
1002 subtype C HIV-1. *AIDS Res Hum Retroviruses* 21:379-94.

115. Sharma S, Jafari M, Bangar A, William K, Guatelli J, Lewinski MK. 2019. The C-Terminal End of HIV-1 Vpu Has a Clade-Specific Determinant That Antagonizes BST-2 and Facilitates Virion Release. *J Virol* 93.
116. Pickering S, Hue S, Kim EY, Reddy S, Wolinsky SM, Neil SJ. 2014. Preservation of Tetherin and CD4 Counter-Activities in Circulating Vpu Alleles despite Extensive Sequence Variation within HIV-1 Infected Individuals. *PLoS Pathog* 10:e1003895.
117. Iwami S, Sato K, Morita S, Inaba H, Kobayashi T, Takeuchi JS, Kimura Y, Misawa N, Ren F, Iwasa Y, Aihara K, Koyanagi Y. 2015. Pandemic HIV-1 Vpu overcomes intrinsic herd immunity mediated by tetherin. *Sci Rep* 5:12256.
118. Chen BK, Gandhi RT, Baltimore D. 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. *J Virol* 70:6044-53.
119. Richard J, Veillette M, Brassard N, Iyer SS, Roger M, Martin L, Pazgier M, Schon A, Freire E, Routy JP, Smith AB, III, Park J, Jones DM, Courter JR, Melillo BN, Kaufmann DE, Hahn BH, Permar SR, Haynes BF, Madani N, Sodroski JG, Finzi A. 2015. CD4 mimetics sensitize HIV-1-infected cells to ADCC. *Proc Natl Acad Sci U S A* 112:E2687-94.
120. Prevost J, Zoubchenok D, Richard J, Veillette M, Pacheco B, Coutu M, Brassard N, Parsons MS, Ruxrungtham K, Bunupuradah T, Tovanabutra S, Hwang KK, Moody MA, Haynes BF, Bonsignori M, Sodroski J, Kaufmann DE, Shaw GM, Chenine AL, Finzi A. 2017. Influence of the Envelope gp120 Phe 43 Cavity on HIV-1 Sensitivity to Antibody-Dependent Cell-Mediated Cytotoxicity Responses. *J Virol* 91.
121. Prevost J, Richard J, Ding S, Pacheco B, Charlebois R, Hahn BH, Kaufmann DE, Finzi A. 2018. Envelope glycoproteins sampling states 2/3 are susceptible to ADCC by sera from HIV-1-infected individuals. *Virology* 515:38-45.
122. Richard J, Prevost J, von Bredow B, Ding S, Brassard N, Medjahed H, Coutu M, Melillo B, Bibollet-Ruche F, Hahn BH, Kaufmann DE, Smith AB, 3rd, Sodroski J, Sauter D, Kirchhoff F, Gee K, Neil SJ, Evans DT, Finzi A. 2017. BST-2 Expression Modulates Small CD4-Mimetic Sensitization of HIV-1-Infected Cells to Antibody-Dependent Cellular Cytotoxicity. *J Virol* 91.
123. Mielke D, Stanfield-Oakley S, Borate B, Fisher LH, Faircloth K, Tuyishime M, Greene K, Gao H, Williamson C, Morris L, Ochsenbauer C, Tomaras G, Haynes BF, Montefiori D, Pollara J, deCamp AC, Ferrari G. 2021. Selection of HIV Envelope strains for standardized assessments of vaccine-elicited antibody-dependent cellular cytotoxicity (ADCC)-mediating antibodies. *J Virol* doi:10.1128/JVI.01643-21:JVI0164321.
124. Prevost J, Tolbert WD, Medjahed H, Sherburn RT, Madani N, Zoubchenok D, Gendron-Lepage G, Gaffney AE, Grenier MC, Kirk S, Vergara N, Han C, Mann BT, Chenine AL, Ahmed A, Chaiken I, Kirchhoff F, Hahn BH, Haim H, Abrams CF, Smith AB, III, Sodroski J, Pazgier M, Finzi A. 2020. The HIV-1 Env gp120 Inner Domain Shapes the Phe43 Cavity and the CD4 Binding Site. *mBio* 11.
125. Richard J, Prevost J, Alsaifi N, Ding S, Finzi A. 2018. Impact of HIV-1 Envelope Conformation on ADCC Responses. *Trends Microbiol* 26:253-265.
126. Alpert MD, Heyer LN, Williams DE, Harvey JD, Greenough T, Allhorn M, Evans DT. 2012. A novel assay for antibody-dependent cell-mediated cytotoxicity against HIV-1- or SIV-infected cells reveals incomplete overlap with antibodies measured by neutralization and binding assays. *J Virol* 86:12039-52.
127. Fontaine J, Chagnon-Choquet J, Valcke HS, Poudrier J, Roger M. 2011. High expression levels of B lymphocyte stimulator (BLyS) by dendritic cells correlate with HIV-related B-cell disease progression in humans. *Blood* 117:145-55.

- 1052 128. Fontaine J, Coutlee F, Tremblay C, Routy JP, Poudrier J, Roger M. 2009. HIV infection
1053 affects blood myeloid dendritic cells after successful therapy and despite nonprogressing
1054 clinical disease. *J Infect Dis* 199:1007-18.
- 1055 129. International HIVCS, Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI, Walker BD,
1056 Ripke S, Brumme CJ, Pulit SL, Carrington M, Kadie CM, Carlson JM, Heckerman D,
1057 Graham RR, Plenge RM, Deeks SG, Gianniny L, Crawford G, Sullivan J, Gonzalez E,
1058 Davies L, Camargo A, Moore JM, Beattie N, Gupta S, Crenshaw A, Burt NP, Guiducci
1059 C, Gupta N, Gao X, Qi Y, Yuki Y, Piechocka-Trocha A, Cutrell E, Rosenberg R, Moss
1060 KL, Lemay P, O'Leary J, Schaefer T, Verma P, Toth I, Block B, Baker B, Rothchild A,
1061 Lian J, Proudfoot J, Alvino DM, Vine S, Addo MM, et al. 2010. The major genetic
1062 determinants of HIV-1 control affect HLA class I peptide presentation. *Science*
1063 330:1551-7.
- 1064 130. Kamya P, Boulet S, Tsoukas CM, Routy JP, Thomas R, Cote P, Boulassel MR, Baril JG,
1065 Kovacs C, Migueles SA, Connors M, Suscovich TJ, Brander C, Tremblay CL, Bernard N.
1066 2011. Receptor-ligand requirements for increased NK cell polyfunctional potential in slow
1067 progressors infected with HIV-1 coexpressing KIR3DL1**h*/**y* and HLA-B*57. *J Virol*
1068 85:5949-60.
- 1069 131. Peretz Y, Ndongala ML, Boulet S, Boulassel MR, Rouleau D, Cote P, Longpre D, Routy
1070 JP, Falutz J, Tremblay C, Tsoukas CM, Sekaly RP, Bernard NF. 2007. Functional T cell
1071 subsets contribute differentially to HIV peptide-specific responses within infected
1072 individuals: correlation of these functional T cell subsets with markers of disease
1073 progression. *Clin Immunol* 124:57-68.
- 1074 132. Finzi A, Xiang SH, Pacheco B, Wang L, Haight J, Kassa A, Danek B, Pancera M, Kwong
1075 PD, Sodroski J. 2010. Topological layers in the HIV-1 gp120 inner domain regulate gp41
1076 interaction and CD4-triggered conformational transitions. *Mol Cell* 37:656-67.
- 1077 133. Emi N, Friedmann T, Yee JK. 1991. Pseudotype formation of murine leukemia virus with
1078 the G protein of vesicular stomatitis virus. *J Virol* 65:1202-7.
- 1079 134. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM,
1080 Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL,
1081 Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA,
1082 Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF,
1083 Korber BT, Hahn BH, Shaw GM. 2009. Genetic identity, biological phenotype, and
1084 evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection.
1085 *J Exp Med* 206:1273-89.
- 1086 135. Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, Salazar-
1087 Gonzalez JF, Shattock R, Haynes BF, Shaw GM, Hahn BH, Kappes JC. 2012.
1088 Generation of Transmitted/Founder HIV-1 Infectious Molecular Clones and
1089 Characterization of Their Replication Capacity in CD4 T Lymphocytes and Monocyte-
1090 Derived Macrophages. *J Virol* 86:2715-28.
- 1091 136. Parrish NF, Wilen CB, Banks LB, Iyer SS, Pfaff JM, Salazar-Gonzalez JF, Salazar MG,
1092 Decker JM, Parrish EH, Berg A, Hopper J, Hora B, Kumar A, Mahlokozera T, Yuan S,
1093 Coleman C, Vermeulen M, Ding H, Ochsenbauer C, Tilton JC, Permar SR, Kappes JC,
1094 Betts MR, Busch MP, Gao F, Montefiori D, Haynes BF, Shaw GM, Hahn BH, Doms RW.
1095 2012. Transmitted/founder and chronic subtype C HIV-1 use CD4 and CCR5 receptors
1096 with equal efficiency and are not inhibited by blocking the integrin alpha4beta7. *PLoS*
1097 *Pathog* 8:e1002686.
- 1098 137. Parrish NF, Gao F, Li H, Giorgi EE, Barbian HJ, Parrish EH, Zajic L, Iyer SS, Decker JM,
1099 Kumar A, Hora B, Berg A, Cai F, Hopper J, Denny TN, Ding H, Ochsenbauer C, Kappes
1100 JC, Galimidi RP, West AP, Jr., Bjorkman PJ, Wilen CB, Doms RW, O'Brien M, Bhardwaj
1101 N, Borrow P, Haynes BF, Muldoon M, Theiler JP, Korber B, Shaw GM, Hahn BH. 2013.

- 1102 Phenotypic properties of transmitted founder HIV-1. *Proc Natl Acad Sci U S A* 110:6626-
1103 33.
- 1104 138. Fenton-May AE, Dibben O, Emmerich T, Ding H, Pfafferoth K, Aasa-Chapman MM,
1105 Pellegrino P, Williams I, Cohen MS, Gao F, Shaw GM, Hahn BH, Ochsenbauer C,
1106 Kappes JC, Borrow P. 2013. Relative resistance of HIV-1 founder viruses to control by
1107 interferon-alpha. *Retrovirology* 10:146.
- 1108 139. Liao HX, Lynch R, Zhou T, Gao F, Alam SM, Boyd SD, Fire AZ, Roskin KM, Schramm
1109 CA, Zhang Z, Zhu J, Shapiro L, Mullikin JC, Gnanakaran S, Hraber P, Wiehe K, Kelsoe
1110 G, Yang G, Xia SM, Montefiori DC, Parks R, Lloyd KE, Searce RM, Soderberg KA,
1111 Cohen M, Kamanga G, Louder MK, Tran LM, Chen Y, Cai F, Chen S, Moquin S, Du X,
1112 Joyce MG, Srivatsan S, Zhang B, Zheng A, Shaw GM, Hahn BH, Kepler TB, Korber BT,
1113 Kwong PD, Mascola JR, Haynes BF. 2013. Co-evolution of a broadly neutralizing HIV-1
1114 antibody and founder virus. *Nature* 496:469-76.
- 1115 140. Gao F, Bonsignori M, Liao HX, Kumar A, Xia SM, Lu X, Cai F, Hwang KK, Song H, Zhou
1116 T, Lynch RM, Alam SM, Moody MA, Ferrari G, Berrong M, Kelsoe G, Shaw GM, Hahn
1117 BH, Montefiori DC, Kamanga G, Cohen MS, Hraber P, Kwong PD, Korber BT, Mascola
1118 JR, Kepler TB, Haynes BF. 2014. Cooperation of B cell lineages in induction of HIV-1-
1119 broadly neutralizing antibodies. *Cell* 158:481-91.
- 1120 141. Salminen MO, Ehrenberg PK, Mascola JR, Dayhoff DE, Merling R, Blake B, Louder M,
1121 Hegerich S, Polonis VR, Birx DL, Robb ML, McCutchan FE, Michael NL. 2000.
1122 Construction and biological characterization of infectious molecular clones of HIV-1
1123 subtypes B and E (CRF01_AE) generated by the polymerase chain reaction. *Virology*
1124 278:103-10.
- 1125 142. Chenine AL, Merbah M, Wiczorek L, Molnar S, Mann B, Lee J, O'Sullivan AM, Bose M,
1126 Sanders-Buell E, Kijak GH, Herrera C, McLinden R, O'Connell RJ, Michael NL, Robb
1127 ML, Kim JH, Polonis VR, Tovanabutra S. 2018. Neutralization Sensitivity of a Novel HIV-
1128 1 CRF01_AE Panel of Infectious Molecular Clones. *J Acquir Immune Defic Syndr*
1129 78:348-355.
- 1130 143. Peachman KK, Karasavvas N, Chenine AL, McLinden R, Rerks-Ngarm S, Jaranit K,
1131 Nitayaphan S, Pitisuttithum P, Tovanabutra S, Zolla-Pazner S, Michael NL, Kim JH,
1132 Alving CR, Rao M. 2015. Identification of New Regions in HIV-1 gp120 Variable 2 and 3
1133 Loops that Bind to alpha4beta7 Integrin Receptor. *PLoS One* 10:e0143895.
- 1134 144. O'Brien WA, Koyanagi Y, Namazie A, Zhao JQ, Diagne A, Idler K, Zack JA, Chen IS.
1135 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of
1136 gp120 outside the CD4-binding domain. *Nature* 348:69-73.
- 1137 145. Li Y, Kappes JC, Conway JA, Price RW, Shaw GM, Hahn BH. 1991. Molecular
1138 characterization of human immunodeficiency virus type 1 cloned directly from uncultured
1139 human brain tissue: identification of replication-competent and -defective viral genomes.
1140 *J Virol* 65:3973-85.
- 1141 146. Theodore TS, Englund G, Buckler-White A, Buckler CE, Martin MA, Peden KW. 1996.
1142 Construction and characterization of a stable full-length macrophage-tropic HIV type 1
1143 molecular clone that directs the production of high titers of progeny virions. *AIDS Res*
1144 *Hum Retroviruses* 12:191-4.
- 1145 147. Krapp C, Hotter D, Gawanbacht A, McLaren PJ, Kluge SF, Sturzel CM, Mack K, Reith E,
1146 Engelhart S, Ciuffi A, Hornung V, Sauter D, Telenti A, Kirchhoff F. 2016. Guanylate
1147 Binding Protein (GBP) 5 Is an Interferon-Inducible Inhibitor of HIV-1 Infectivity. *Cell Host*
1148 *Microbe* 19:504-14.
- 1149 148. Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen IS. 1987. Dual infection
1150 of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science*
1151 236:819-22.

- 1152 149. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA. 1986.
1153 Production of acquired immunodeficiency syndrome-associated retrovirus in human and
1154 nonhuman cells transfected with an infectious molecular clone. *J Virol* 59:284-91.
- 1155 150. Mack K, Starz K, Sauter D, Langer S, Bibollet-Ruche F, Learn GH, Sturzel CM, Leoz M,
1156 Plantier JC, Geyer M, Hahn BH, Kirchhoff F. 2017. Efficient Vpu-Mediated Tetherin
1157 Antagonism by an HIV-1 Group O Strain. *J Virol* 91.
- 1158 151. Takehisa J, Kraus MH, Decker JM, Li Y, Keele BF, Bibollet-Ruche F, Zammit KP, Weng
1159 Z, Santiago ML, Kamenya S, Wilson ML, Pusey AE, Bailes E, Sharp PM, Shaw GM,
1160 Hahn BH. 2007. Generation of infectious molecular clones of simian immunodeficiency
1161 virus from fecal consensus sequences of wild chimpanzees. *J Virol* 81:7463-75.
- 1162 152. Shingai M, Donau OK, Schmidt SD, Gautam R, Plishka RJ, Buckler-White A,
1163 Sadjadpour R, Lee WR, LaBranche CC, Montefiori DC, Mascola JR, Nishimura Y, Martin
1164 MA. 2012. Most rhesus macaques infected with the CCR5-tropic SHIV(AD8) generate
1165 cross-reactive antibodies that neutralize multiple HIV-1 strains. *Proc Natl Acad Sci U S A*
1166 109:19769-74.
- 1167 153. Li H, Wang S, Lee FH, Roark RS, Murphy AI, Smith J, Zhao C, Rando J, Chohan N,
1168 Ding Y, Kim E, Lindemuth E, Bar KJ, Pandrea I, Apetrei C, Keele BF, Lifson JD, Lewis
1169 MG, Denny TN, Haynes BF, Hahn BH, Shaw GM. 2021. New SHIVs and Improved
1170 Design Strategy for Modeling HIV-1 Transmission, Immunopathogenesis, Prevention
1171 and Cure. *J Virol* doi:10.1128/JVI.00071-21.
- 1172 154. R Core Team. 2013. R: A language and environment for statistical computing, R
1173 Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- 1174 155. RStudio team. 2015. RStudio: Integrated Development for R, RStudio, Inc., Boston, MA.
1175 <http://www.rstudio.com/>.

1176

● mock ● CH058 ● JR-FL

A

B

C

D

E

F

A

● mock ● CH058 ● YU-2

B **C** **D** **E** **F** **G**

● WT ● Vpu- ○ Env-IMC
● Nef- ● Nef-Vpu- ○ Env-IMC-LucR.T2A

A

B

C