Interactions between HIV accessory proteins and host factors BST2 and CD4: at the interface of virus dissemination and persistence

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To my late mother: for the unconditional love, teachings, words of wisdom, encouragement and leading a life of a hero

ABSTRACT

Tetherin/BST2 and CD4 are increasingly being recognized as chief modulators of human immunodeficiency virus (HIV) dissemination and persistence. BST2 is a type I interferon (IFN)-stimulated restriction factor that inhibits HIV-1 release by tethering nascent Env-containing virions at the surface of infected cells, and is counteracted by HIV-1 Vpu. Vpu also acts in concert with Nef to down-regulate expression of the virus primary receptor, CD4. The interactions between Vpu and the host factors BST2 and CD4 are still ill-defined, and a subject of intense investigation. In the first part of the study, we sought to elucidate determinants within Vpu that govern BST2 antagonism. We identify highly conserved residues adjacent to the functionally important Vpu transmembrane domain (TMD) that regulate the ability of Vpu to bind and co-localize with BST2. Moreover, these residues, particularly a glutamic acid residue positioned immediately following the TMD, are a determinant not only for efficient targeting of BST2, but also for binding and degradation of CD4. Mechanistically, our data suggest a role of these residues in maintenance of the Vpu TMD conformational configuration such that interactions with membrane-associated host targets are favoured.

The second part of the research investigates the roles of BST2 and CD4 in clearance of infected cells via antibody-dependent cell-mediated cytotoxicity (ADCC), an important element of the anti-HIV response. We show that CD4 sensitizes infected cells to ADCC mediated by CD4-induced (CD4i), non-neutralizing antibodies (nNAbs) whose binding depends on CD4-Env interactions. Accumulation of CD4 on infected cells unmasked epitopes on Env that are recognized by these CD4i nNAbs. We further

demonstrate that BST2-mediated virion tethering, which increases the amount of Env epitopes at the cell surface, enhances ADCC mediated by CD4-independent broadly neutralizing antibodies (bNAbs) and, to a lesser extent, by nNAbs. Importantly, HIV uses its Vpu and Nef proteins to target CD4 and BST2 and, in so doing, shields infected cells from lysis. In accordance with this, WT virus-infected cells, which are virtually not susceptible to nNAb-mediated ADCC, can be sensitized via blocking Vpu and Nef. Similarly, while bNAbs can mediate ADCC against WT virus-infected cells, inactivating Vpu and Nef significantly potentiates their killing. Moreover, increasing BST2-mediated virion crosslinking using IFNα treatment enhances ADCC susceptibility. Furthermore, the data reveal that reactivated HIV latent cells are susceptible, albeit poorly, to ADCC lysis. Likewise, their killing could be enhanced by both exogenous IFN administration and removal of the viral accessory proteins.

Altogether, this work emphasizes a novel role of BST2 and CD4 towards elimination of infected cells, as well as a mechanism by which HIV Vpu and Nef function synergistically to protect infected cells from ADCC, thereby promoting viral persistence. As such, strategies aimed at enhancing ADCC activity via treatments with IFN and/or small molecules targeting these accessory proteins represent a promising avenue for protection from HIV acquisition, post-infection control of HIV, as well as enhanced clearance of latent viral reservoirs in “Shock and Kill” cure approaches. To this effect, the study identifies a vulnerable region within Vpu that may represent a feasible target for development of Vpu inhibitors.

RÉSUMÉ

Les protéines Tetherin/BST2 et CD4 sont de plus en plus reconnues comme étant les principaux modulateurs de la diffusion et de la persistance du virus de l'immunodéficience humaine (VIH). BST2 est un facteur de restriction qui est induit par l'interféron (IFN) de type I; elle inhibe la relâche des nouveaux virions détenant des glycoprotéines Env à la surface des cellules infectées, et est contrecarrée par la protéine Vpu du VIH-1. Vpu agit également de concert avec Nef pour réguler négativement l'expression de CD4, le récepteur primaire du virus. Les interactions entre Vpu et les facteurs de l'hôte BST2 et CD4 sont encore mal définies et restent un sujet de recherche intense. Dans la première partie de cette étude, nous avons cherché à élucider les déterminants de Vpu qui gouvernent son activité anti-BST2. Nous identifions des acides aminés hautement conservés adjacents au domaine transmembranaire (TMD) de Vpu qui sont importants pour la liaison et la co-localisation de Vpu avec BST2. De plus, ces acides aminés, en particulier l'acide glutamique positionné immédiatement après le TMD, ont un rôle non seulement dans le ciblage efficace de BST2, mais également dans la dégradation de CD4. D'un point de vue fonctionnel, nos données suggèrent que ces acides aminés contribuent au maintien de la configuration conformationnelle du TMD de Vpu de sorte que les interactions de Vpu avec certaines molécules membranaires de l'hôte sont favorisées.

La deuxième partie de ce travail étudie les rôles de BST2 et de CD4 dans l'élimination des cellules infectées via la cytotoxicité cellulaire dépendante des anticorps (ADCC), un élément important de la réponse anti-VIH. Nous montrons que CD4

sensibilise les cellules infectées à l'ADCC induite par les anticorps non-neutralisants (nNAbs) induits par CD4 (iCD4) dont la liaison dépend des interactions CD4-Env. L'accumulation de CD4 sur des cellules infectées démasque des epitopes de l'Env qui sont reconnus par ces nNAbs CD4i. Nous démontrons en outre que l'ancrage du virion par BST2, qui augmente la quantité d'épitopes Env à la surface cellulaire, améliore l'ADCC médiée par des anticorps neutralisants à large spectre indépendants de CD4 (bNAbs) et, dans une moindre mesure, par les nNAbs. Il est important de noter que le VIH utilise ses protéines Vpu et Nef pour cibler CD4 et BST2 et, ce faisant, protège les cellules infectées de la lyse. Conformément à cela, les cellules infectées par le virus WT, qui ne sont pratiquement pas sensibles à l'ADCC à médiation par nNAb, peuvent être sensibilisées via Vpu et Nef. De même, alors que les bNAbs peuvent servir de médiateur ADCC contre les cellules infectées par le virus WT, l'inactivation de Vpu et Nef potentialise de manière significative leur destruction. De plus, l'augmentation de virions retenus par BST2 suite à l'ajout d'IFNα accentue la susceptibilité à l'ADCC. De plus, les données révèlent que les cellules latentes infectées au VIH qui sont par suite réactivées sont susceptibles, bien que faiblement, à la lyse par ADCC. Leur destruction pourrait être accélérée par l'administration d'IFN exogène et par l'élimination des protéines accessoires virales.

Dans l'ensemble, ce travail met l'accent sur un nouveau rôle de BST2 et de CD4 dans l'élimination des cellules infectées, ainsi que sur un mécanisme par lequel les protéines Vpu et Nef du VIH fonctionnent de manière synergique pour protéger les cellules infectées de l'ADCC, favorisant ainsi la persistance virale. Ainsi, les stratégies visant à améliorer l'activité de l'ADCC par l'intermédiaire de traitements avec de l'IFN et

/ ou de petites molécules ciblant ces protéines accessoires représentent une voie prometteuse pour une protection prophylactique contre le VIH, le contrôle post-infection du VIH ainsi qu'une élimination plus efficace des réservoirs viraux latents par l'approche "Shock and Kill". À cet effet, l'étude identifie une région vulnérable dans Vpu qui peut représenter une cible intéressante pour le développement d'inhibiteurs de Vpu.

PREFACE

The thesis was written in accordance with McGill University's "Guidelines for Preparation of a Thesis." The format of this thesis conforms to the "Manuscript-based thesis" option which states:

As an alternative to the traditional thesis format, the thesis research may be presented as a collection of scholarly papers of which the student is the author or co-author; that is, it can include the text of one or more manuscripts, submitted or to be submitted for publication, and/or published articles reformatted according to the requirements described under "Guidelines for Preparation of a Thesis".

The contributions of co-authors to published and/or submitted research articles appear on the title page of each chapter. As well, the elements of the thesis that are considered original scholarship and distinct contributions to knowledge are indicated on the title page of each chapter. Very briefly, the work presented in chapter 2 identifies for the first time an important regulatory role of the transmembrane-proximal Vpu hinge region residues towards enabling the protein to efficiently interact with its target host proteins BST2 and CD4. In Chapter 3, we uncover an important mechanism whereby HIV Nef and Vpu function synergistically to protect infected cells from Ab-mediated lysis. To this extent, we provide evidence that accumulation of CD4 on infected cells, which unmasks epitopes on Env that are recognized by CD4i nNAbs, sensitizes infected cells to ADCC mediated by these CD4i, nNAbs. We further demonstrate that increasing the amount of Env epitopes at the cell surface *via* BST2-mediated virion tethering

enhances ADCC mediated by such Abs. Lastly, in Chapter 4 we reveal that CD4-independent NAbs are capable of mediating ADCC, and that BST2-mediated virion tethering significantly enhances ADCC mediated by NAbs. Moreover, we show that while bNAbs can mediate ADCC against WT virus-infected cells, either inactivating Vpu and Nef or increasing BST2-mediated virion crosslinking using IFNα treatment significantly potentiates their killing. Furthermore, we uncover that reactivated HIV latent cells are susceptible, albeit poorly, to ADCC lysis. Likewise, their killing can be enhanced by both exogenous IFN administration and removal of the viral accessory proteins.

TABLE OF CONTENTS

List of Figures

List of abbreviations

μL – Microliter

μM – Micromolar

Ab – antibody

ADCC – antibody-dependent cell-mediated cytotoxicity

ADCP – antibody-dependent cell-mediated phagocytosis

ADCVI – antibody-dependent cell-mediated viral inhibition

AIDS – acquired immunodeficiency syndrome

AP1/2 – Adaptor protein complexes 1 and 2

APOBEC – apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like

ART – antiretroviral therapy

bNAb – broadly neutralizing antibody

β-COP – coatomer beta subunit

β-TrCP – beta-transducin repeats-containing protein

BST2 – Bone Marrow Stromal Cell Antigen 2

CA – HIV-1 Capsid

CCR5 – C-C chemokine receptor 5

CCR7 – C-C chemokine receptor-7

CD4bs – CD4 binding site

CD4i – CD4 induced

cDNA – copy DNA

CK-II – Casein kinase II

coR-bs – coreceptor binding site

CPSF – cleavage and polyadenylation specificity factor subunit 6

CRFs – Circulating recombinant Forms

CTL – cytolytic T lymphocyte

CXCR4 – C-X-C chemokine receptor type 4

Cyp A – cyclophylin A

DAPI – 4', 6-diamidino-2-phenylindole

DCAF – DDB1- and CUL4-associated factor 1

DMEM – Dulbecco's Modified Eagle Medium

DNA – deoxyribonucleic acid

DNase – Deoxyribonuclease

DNAM-1 – DNAX accessory molecule-1

dNTP – deoxynucleoside triphosphate

eIF2 – eukaryotic initiation factor 2

Env – HIV-1 envelope protein

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum associated degradation

ESCRT – endosomal sorting complex required for transport

FBS – Fetal Bovine serum

Fc – Fragment, crystallizable

Fc-R – Fc receptor

FDA – food and drug administration

Gag – HIV Group-specific antigen

Gag-Pol – HIV-1 precursor 160 kd polyprotein

GFP – green fluorescent protein

GPI – glycosylphosphatidylinisotol

HDAC – histone deacetylase

HeLa – Henrietta Lacks immortal cell line

HIV-1 – human immunodeficiency virus type 1

IgG – immunoglobulin G

IFN – Interferon

IFN-I – Interferon type I

IκB – I kappa B

IKK – I kappa B (IκB) kinase

ILT7 – immunologlobulin-like transcript 7

IN – HIV-1 integrase protein

IRF – IFN regulatory factor

ISG – interferon-stimulated gene

Kb – Kilobase

kDa – kilodaltons

LRA – Latency reversal agent

LEDGF/p75 – lens epithelium-derived growth factor/transcriptional coactivator 75

LTR – long terminal repeat

MA – HIV-1 matrix protein (p17)

MDDC – monocyte-derived dendritic cell

MDM – monocyte-derived macrophage

MFI – Mean/median fluorescence intensity

MHC – major histocompatibility complex

mL – milliliter

mM – millimolar

MPER – Membrane proximal external region

mRNA – mature RNA

MX2 – human myxovirus resistance 2

MVB – multivesicular body

Nab – neutralizing antibody

nNAb – non-neutralizing antibody

NC – HIV-1 nucleocapsid protein (p7)

Nef – HIV-1 negative factor protein

NFAT – nuclear factor of activated T cells

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – natural killer

NKT – natural killer T

NKG2D – natural-killer group 2, member D ligand

NLS – nuclear localization signal

nM – nanomolar

NMR – nuclear magnetic resonance

NPC – nuclear pore complex

NTB-A – NK-T-B-antigen

Nup – nucleoporin

ORF – open reading frame

PBMC – peripheral blood mononuclear cells

pDC – plasmocytoid dendritic cells

PBS – Tris buffered saline

PCC – Pearson correlation coefficient

PIC – HIV-1 pre-integration complex

PKR – Protein kinase R

PM – plasma membrane

P-TEFb – positive transcription elongation factor b

PVR – Polio virus receptor

Rev – HIV-1 replication viral factor protein

RNA – Ribonucleic acid

RNase H – Ribonuclease hybrid

RRE – rev responsive element

RT – HIV-1 reverse transcriptase protein

RTC – HIV-1 reverse-transcription complex

SAHA – suberoylanilide hydroxamic acid

SAMHD1 – Sterile alpha motif and histidine-aspartic domain (HD) containing protein 1

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SERINC – serine incorporator

siRNA – small interference RNA

SIV – Simian immunodeficiency virus

$(SCF)^{\beta\text{-}TrCP}$ – Skp1-Cullin1-F-Box (SCFβ-TrCP2) E3 ubiquitin ligase

SNAT1 – sodium-coupled neutral amino acid transporter

STAT3 – signal transducer and activator of transcription 3

SU – HIV-1 surface protein (gp120)

TAK1 – transforming growth factor β-activated kinase 1

TAR – HIV-1 trans activating response element

Tat – HIV-1 transactivating regulatory protein

TBS – Tris buffered saline

TBS-T – Tris buffered saline - Tween

TCR – T-cell antigen receptor

TGN – trans-Golgi network

TLR – Toll-like receptor

TM – HIV-1 transmembrane protein (gp41)

TNF – tumor necrosis factor

TNPO3 – Transportin-3

TRAF – TNF-receptor-associated factor

TRIM5α – tripartite motif 5-alpha

UNAIDS – Joint United Nations Programme on HIV/AIDS

UNG-2 – uracil DNA glycosylase-2

UTR – Untranslated region

Vif – HIV-1 virion infectivity factor protein

Vpr – HIV-1 viral protein r

Vpu – HIV-1 viral protein u

vRNA – unspliced viral genomic RNA

VSV-G – vesicular stomatitis virus G glycoprotein

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CHAPTER 1: INTRODUCTION

1.1 HIV Overview

More than three decades following its first isolation and identification as the causative agent of acquired immunodeficiency syndrome (AIDS), human immunodeficiency virus (HIV) continues to pose a serious threat to human health. Two types of the virus have been identified – HIV type 1 (HIV-1) and HIV-2, representing multiple zoonotic transmission events of the 'ancestral' simian immunodeficiency virus (SIV). HIV-1 and HIV-2, both of which can infect humans, were transmitted into humans from chimpanzees and sooty mangabeys, respectively [1]. HIV-1 itself is divided into four major phylogenetic groups: M (major), O (outlier), N (non-M/non-O) and the recently identified group P (pending the identification of further human cases), yet again demonstrating independent transmission events (Figure 1.1A). HIV-1 group M (HIV-1M) is further divided into nine subtypes or clades (A, B, C, D, F, G, H, J, K), with clade C being the most abundant (Figure 1.1B). Several recombinant strains have also been isolated. Since the start of the epidemic, a staggering 78 million people have become infected, 35 million of whom have already succumbed to death from AIDS-related illnesses (UNAIDS, 2016). 1.1 million of those deaths occurred in 2015 alone. An estimated 36.7 million people live with HIV, with 2.1 million people newly infected in 2015 alone (Figure 1.1C) (UNAIDS, 2016). Developing countries have been the hardest hit, experiencing the greatest HIV/AIDS morbidity and mortality (UNAIDS, 2016). More than 95% of these infections are caused by HIV-1 and the rest accounted for by HIV-2. Group M is the main cause of the epidemic (98% of HIV-1 infections) [2]. Of the current infections, group O and N contribute approximately 30,000 and less than 20 cases,

respectively, while only 2 cases of group P infections have been reported [3]. Not surprisingly therefore, strategies aimed at reducing or eliminating HIV infections primarily focus on the M group.

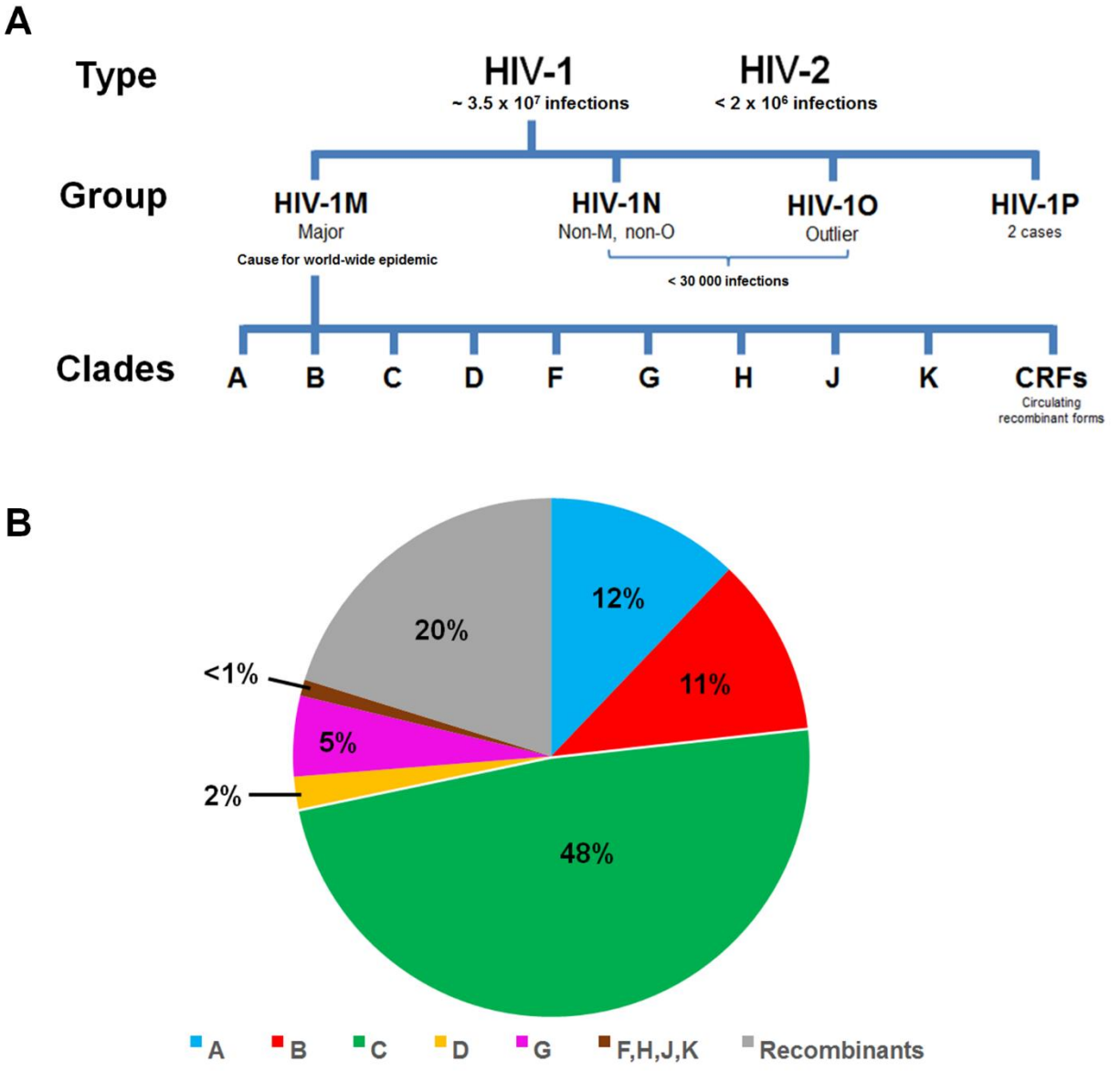


Figure 1.1: HIV classification and overview of the global HIV/AIDS epidemic. A) HIV types, HIV-1 phylogenetic groups and the various clades associated with HIV-1 group M. The indicated values depict

estimates of current infections under each HIV type or group. **B)** Abundance of the subtypes of HIV-1M (as per data reported in [4]). **C)** The global estimates of the HIV/AIDS epidemic as per a 2016 update from the UNAIDS.

1.2 HIV pathogenesis

HIV primarily infects T cells and monocyte/macrophage lineage cells expressing the viral receptor CD4 along with a chemokine receptor CCR5 and/or CXCR4 at their surfaces. Activated $CD4^+$ T cells are infected with greater efficiency relative to quiescent cells, at least in cell cultures [5]. While monocytes and macrophages both express low levels of CD4 and are infectable by macrophage-tropic viruses, monocytes experience a blockage to viral entry and reduced viral DNA synthesis [5, 6]. Within the first few weeks of infection, before the patient is even aware of their HIV status, the virus spreads to proximal lymphoid organs and then broadly disseminates throughout the body. Still within this period, $CD4^+$ T cells start declining, especially within the gut-associated lymphoid tissue. After the first few weeks of infection, the patient developes immune responses (largely in the form of adaptive cytotoxic $CD8^+$ T cell response) against the virus. These responses allow for some control of viral replication and a rebound in $CD4^+$ T cell numbers. Subsequently, however, persistent HIV replication, systemic infection and inflammation together with a decline in $CD4^+$ T cell numbers continue over a clinically asymptomatic phase of ~ 8-10 years. The extent of infection correlates with $CD4^+$ T cell counts. Without any treatment, most infected patients then show signs of

significant immunodeficiency and are vulnerable to fatal opportunistic infections – characteristics of AIDS.

1.2.1 HIV-1 Latency

While HIV-1 efficiently infects metabolically active $CD4^+$ T lymphocytes, some of these infected cells could be in a process of transitioning or reverting to a quiescent or resting, memory state [7]. These cells could allow for post-entry replication steps (detailed in Section 1.3) including reverse transcription, nuclear entry and integration, but not gene expression. In other cases, HIV-1 can infect memory $CD4^+$ T cells in their resting state [8, 9]. In these cells, viral transcription is halted or silenced and no viral proteins are produced as a result. Consequently, these cells are invisible to the immune system, for as long as transcription continues to be silenced. The cells, in which the viral genome has been stably integrated but transcriptionally silent are referred to as latently infected. This transcriptional repression results from several non-exclusive mechanisms including chromatin repression, transcriptional interference and lack of factors necessary for transcriptional elongation [10]. Along this line, the role of histone deacetylases (HDACs) in promoting high-affinity interactions between histones and DNA, which leads to removal or inaccessibility of docking signals essential for binding of activating transcription factors has been well established in transcription inhibition or silencing [11].

Upon reversal of the silenced transcription, viral genes get transcribed and circulating viral loads increase. Thus, latently infected cells represent a formidable hurdle in the fight for an HIV-1 cure as they represent a major source of HIV-1

reservoirs that cannot be targeted by either the immune system or antiretroviral therapy (ART, described below). Indeed, ART interruptions result in rebound of viraemia in infected patients. Current efforts aimed at eradicating HIV-1 are heavily based on the "Shock and Kill" approach, which involves reactivating viral gene expression in the latently infected cells, using latency-reversing agents (LRAs), and then facilitating elimination of the reactivated cells [12]. To date, several LRAs have been identified, including HDAC inhibitors SAHA (suberoylanilide hydroxamic acid or vorinostat [13, 14], panobinostat [15] and romidepsin [16], which have since been tested in clinical trials [11, 17]. HDAC inhibitors are favorable because they induce HIV-1 expression without causing global T cell activation, which has been shown to be toxic, and significantly reduces total $CD4^+$ T cell counts [18]. Of the HDAC inhibitors that result in an increase in plasma HIV RNA, romidepsin is the most potent, and can achieve HIV expression at physiologically relevant concentrations [16-18]. While these LRAs convincingly show that HIV latency can be reversed in vivo, none of them reduces the reservoir size [18], indicating that latency reversal is not sufficient in the quest for an HIV cure. Additional interventions are thus necessary for the clearance of latently infected cells.

1.2.2 Antiretroviral Therapy

A considerable advancement in our understanding of HIV-1 infection led to the introduction of combination antiretroviral therapy (ART) for HIV infected patients. Rightfully considered as one of the best successes of modern biomedical research, ART reduces HIV replication and has significantly improved both the life-expectancy and quality of life of many people living with HIV/AIDS. Encouragingly, an estimated 17

million of people living with HIV were already on ART by the end of 2015 (UNAIDS, 2016). However, while the therapy represents a monumental step in the fight against HIV, it still does not eradicate the virus due to an early establishment of a long-lived HIV reservoir (described above) [19]. Infected individuals therefore have to stay enrolled on ART for the rest of their lives, as any interruption in ART leads to a rapid viral rebound [20-23]. Additionally, while ART is increasingly being used in treatment as prevention [24] and pre-exposure prophylactic strategies [25], there is still no effective preventative vaccine against HIV. Much like with other infectious diseases, an HIV vaccine would be cost-effective, less stigmatizing compared to taking ART drugs as treatment following diagnosis of HIV infection, as well as pave the way for total control of HIV infections, while making it feasible to achieve an HIV-free world. Furthermore, significant concerns over side effects of the life-long therapy and the likelihood of emergence of drug-resistant viral strains remain. As such, the need for new and improved drugs remains a priority. Towards this, the role of basic and translational biomedical research remains enormous. At the core of all these attempts is an understanding of the HIV replication cycle, which offers the opportunity to target multiple, distinct steps of the cycle – an approach that is critical in order to reduce the danger of emergence of drug-resistant strains.

1.3 HIV-1 Genome and Overview of HIV-1 life cycle

HIV-1 contains two copies of RNA that are converted into a proviral genome following reverse transcription. The HIV genome has nine open reading frames flanked

by two long terminal repeats (LTRs, containing the viral promoter). These nine genes encode for both structural and non-structural proteins (Figure 1.2A).

1.3.1 HIV-1 structural and non-structural proteins

Group-specific antigen (Gag), Pol polyprotein, and Envelope (Env) glycoprotein are considered structural, and are common to all retroviruses. Proteolytic cleavage of the polyprotein precursor Gag (p55) by the viral protease produces capsid (CA, p24), matrix (MA, p17), and nucleocapsid (NC, p7) and p6 proteins. Gag proteins play central roles in the replication of the virus – from facilitating viral protein translation to regulating assembly and packaging of essential components that promote infections of new target cells. Synthesis of the Gag protein occasionally (~ 5-10 % of the time) leads to the synthesis of a 160 kDa Gag-Pol polyprotein product, through a process referred to as ribosomal frameshifting. During viral assembly, the Gag-Pol polyprotein is cleaved into reverse transcriptase (RT), protease (PR) and Integrase (IN) enzymes by the viral protease. These enzymes are critical for HIV replication, playing vital roles from facilitating maturation of a budding virion to enabling important steps such as reverse transcription, nuclear entry and integration in a newly infected target cell. Meanwhile, proteolytic cleavage of the precursor Env glycoprotein (gp160) by host furin-like proteases produces a noncovalent complex of gp120 surface (SU) and gp41 transmembrane (TM) proteins, which mediate attachment, receptor interactions as well as fusion to host target cells. Env proteins constitute the major targets for the humoral

immunity. In fact, anti-HIV antibodies (Abs) against Env are frequently found in HIV-infected patients [26, 27].

In addition to the structural proteins, the 'complex' HIV-1 genome encodes for six regulatory proteins: transactivating regulatory protein (Tat), Regulator of expression of virion proteins (Rev); Negative regulatory factor (Nef), Viral infectivity factor (Vif), Viral protein R (Vpr), and Viral protein U (Vpu). Tat, which primarily enhances proviral transcription, and Rev, which mainly exports unspliced or incompletely spliced viral mRNAs from the nucleus to the cytoplasm, are absolutely essential for replication. On the other hand, Vpu, Nef, Vpr and Vif proteins are dispensable for replication *in vitro*, hence considered auxiliary or accessory [28]. However, it has since been established that *in vivo* these so-called accessory proteins play critical roles in counteracting various antiviral cellular factors that inhibit viral replication (termed host restriction factors) and in allowing HIV to escape several types of host immune responses (discussed in section 1.4). Indeed, their significance for HIV infection and dissemination can no longer be overstated.

1.3.2 The HIV-1 virion

Several of the viral proteins form part of the infectious mature virion. With a diameter of ~100nm, the spherically shaped virion has a lipid membrane outer coat that is derived from the host cells during the budding stage of the replication cycle (Figure 1.2B). Importantly, embedded in it are several proteins including 7–14 trimeric complexes or spikes of the Env protein (gp120-gp41 complexes) that are required for

recognizing and ultimately for mediating fusion with host target cells (further described below) [29, 30]. The gp120 protrudes away from the surface of the viral envelope, while gp41 remains embedded within the matrix (Figure 1.2B). Tethered to the inner surface of the viral membrane are viral matrix (p17) proteins that actually associate with gp41 cytoplasmic domains. At the core of the virion is a cone-shaped capsid that is made of Gag protein p24 (CA). Inside the capsid is the HIV RNA genome, consisting of two single strands of HIV RNA molecules of positive polarity. Each viral RNA is about 10 kb, and is associated with NC proteins. Also packaged into the virions are PR, RT, IN, Vif, Vpr, Nef viral proteins as well as other cellular factors important for replication including $tRNA^{Lys}$ that is required, as a primer, during reverse transcription.

1.3.3 Overview of HIV-1 life cycle

HIV-1 replication occurs via multiple steps, generally divided into early and late events. Early events begin with binding of an incoming virion to the target cell and finishes with integration of the viral genome, whereas the late phase comprises events from proviral transcription until the maturation and release of infectious progeny virions. The current ART therapy targets a combination of these steps, an approach that is key to minimizing emergence of drug resistance.

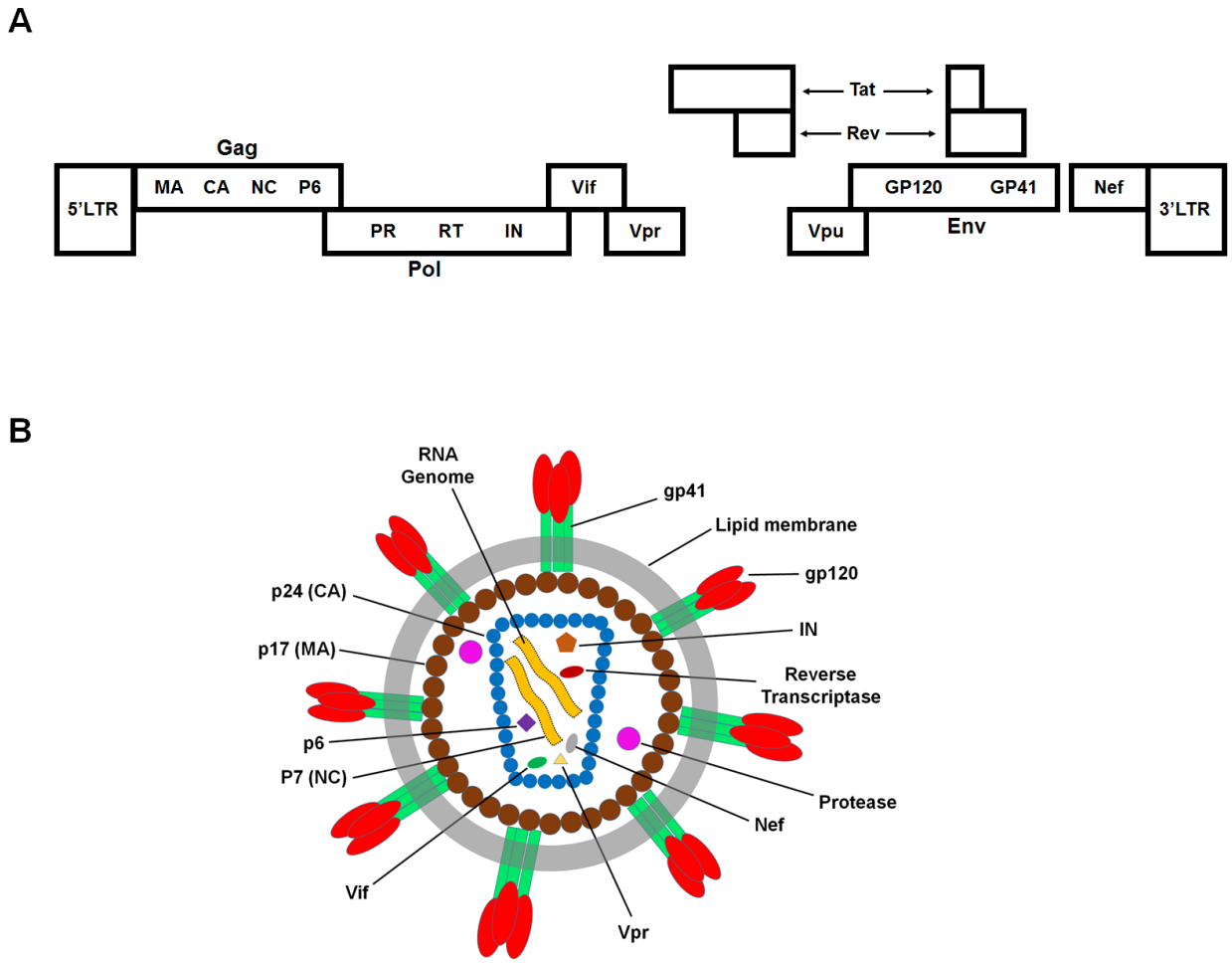


Figure 1.2: The HIV-1 genome and schematic diagram of a mature virion. A) The HIV-1 genome showing the proteins encoded by the ORFs as well as the processed protein products in the case of Gag, pol and Env. **B)** A structural representation of a mature virion, indicating all associated and encapsidated viral proteins

1.3.3.1 Early phase of the HIV life cycle

1.3.3.1.1 Virus attachment and Entry

The requirement for CD4 receptors in order for successful HIV-1 infections of T lymphocytes was quickly realized around the time of HIV-isolation (reviewed in [31]), and led to characterization of the direct interaction between gp120 and the receptor [32, 33]. However, earlier studies investigating HIV-1 infections of $CD4^+$ T lymphocytes and macrophages revealed a virus strain-specific bias in replication efficiencies in these cell types, leading to virus isolates being labeled either M-tropic or T-tropic based on whether they replicate efficiently in monocyte-derived macrophages or $CD4^+$ T cells, respectively. This implied that there were additional cell-specific receptors required for efficient infection of these cells. It was not until the mid-1990s that the C-X-C chemokine receptor type 4 [34] and the C-C chemokine receptor 5 (CCR5) (reviewed in [35]) were identified as coreceptors for infection. Viruses that use CCR5, CXCR4 or both coreceptors are referred to as R5, X4 or R5X4, respectively [36].

HIV-1 entry into target cells is initiated by an attachment of the HIV-1 virion to the membrane of the target cell. Several ‘attachment’ factors have been proposed, involving Env and other cellular proteins incorporated on the incoming virion, as well as surface proteins on the target cell including lectins, poly-glycans, among others [37, 38]. This attachment facilitates binding of the trimeric Env gp120 proteins to their primary receptor CD4 on the target cell (Figure 1.3A). This interaction induces some conformational changes on gp120, leading to unveiling of conserved functional sites necessary for engagement of either the CCR5 or CXCR4 coreceptor [35]. Once the

coreceptor binds, further conformational changes occur that result in insertions of trimeric Env gp41 fusion peptides into the target cell membrane. A subsequent folding of each of the gp41 proteins forms a six-helical bundle that brings or pulls together the virion and target cell lipid membranes. Consequently, the two membranes fuse, and the virion contents are delivered into the target cell through a so-called fusion pore [37]. An alternative endocytic pathway in which the CD4-bound virion is internalized into endosomes, followed by a fusion of the virion and host endosomal membranes to release the virion contents into the cytoplasm has also been proposed [39, 40].

Targeting virus entry constitutes a major approach towards prevention or controlling of HIV-1 infections. Indeed, the cell surface is really a battleground that presents various opportunities to inhibit interactions between Env and the various cellular receptors. The dependence on sequential interactions during entry, whereby engagement of a receptor or co-receptor has to induce conformational changes within Env in order to enable a subsequent phase in the entry process, is a mechanism that allows HIV-1 to hide or conceal highly conserved functional epitopes. These sites are thus protected from anti-Env antibodies.

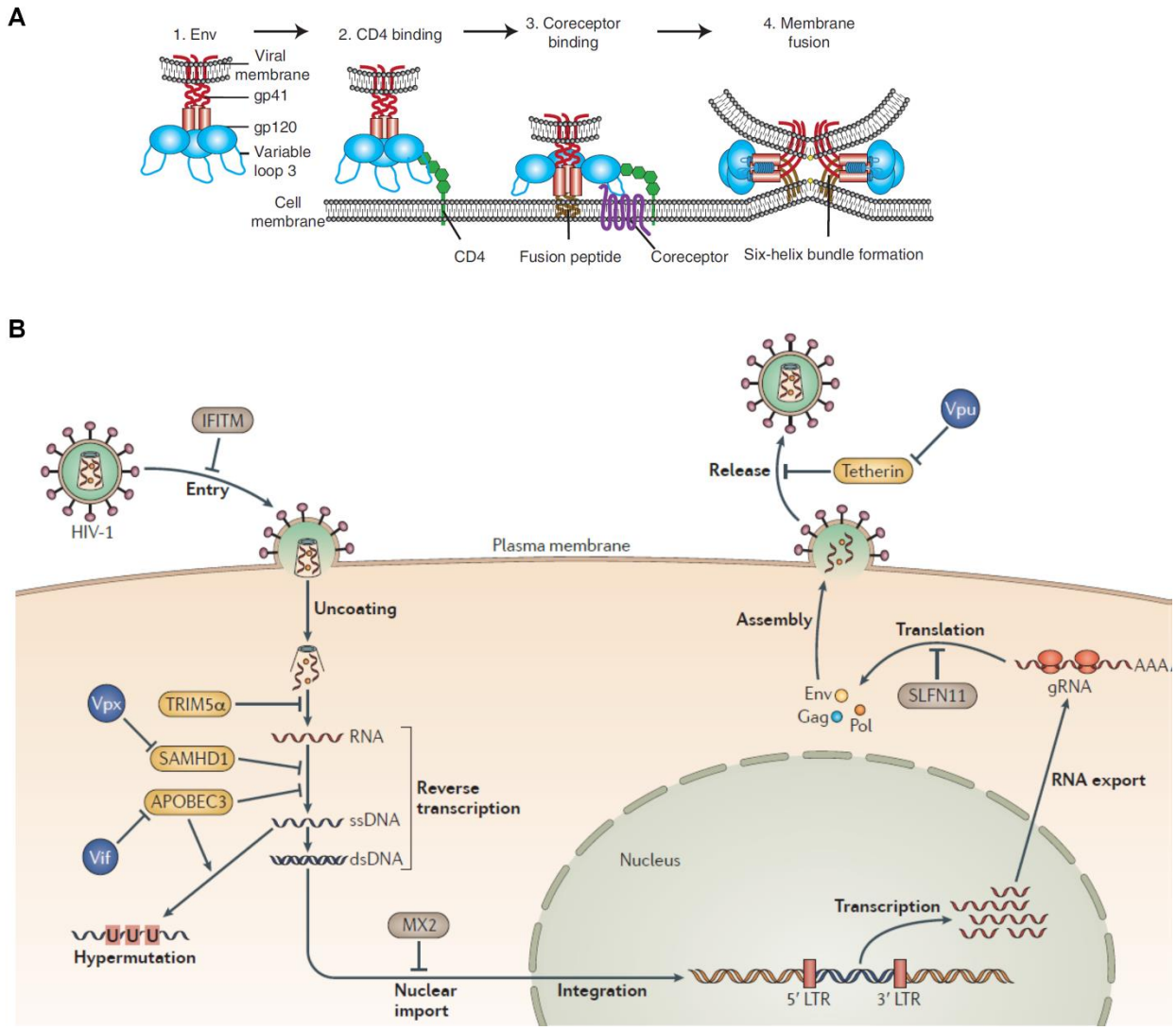


Figure 1.3: HIV replication cycle. A) Steps involved in HIV entry (Adapted from [37], and used with permission from, and copyright to, Cold Spring Harbor Laboratory Press). **B)** Overview of the HIV-1 replication cycle including steps that are targeted by host HIV resistance and restriction factors, as well as viral proteins that counter the restriction factors. Interferon-inducible transmembrane proteins (IFITMs) inhibit viral entry while tripartite motif 5-alpha (TRIM5α) induces pre-mature disassembly of the incoming virions thus blocking viral cDNA synthesis. SAMHD1 depletes dNTP levels in non-dividing cells thereby inhibiting cDNA synthesis and also targets RNA for degradation, and is targeted by HIV-2 Viral protein X (Vpx). APOBEC3, which is counteracted by Vif, hypermutates the viral genome, and also directly inhibits reverse transcription. IFN-inducible human myxovirus resistance 2 (MX2) inhibits nuclear import. Schlafen

(SLFN) proteins inhibit HIV-1 mRNA translation. BST2, which restricts the release of nascent virions from the surface of infected cells, is counteracted by Vpu. Not shown are SERINC 3/5 proteins which decrease infectivity of mature virions, and are counteracted by Nef (Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Macrobiology [41], copyright 2015).

1.3.3.1.2 Reverse transcription

Following delivery of the virion contents, consisting of the dimeric complex of two single-stranded RNAs protected by the nucleocapsid, into the cytoplasm of the host target or infected cell, the viral RNA needs to be reverse-transcribed into linear double-stranded DNA (linear DNA duplex) that is integration-competent. During HIV-1 assembly, all necessities for reverse transcription, including reverse transcriptase (RT) and $tRNA^{Lys3}$ that primes or initiates DNA synthesis, are packaged into the virion. RT possesses both RNA-dependent and DNA-dependent polymerase activities, allowing it to copy either RNA or DNA templates. RTs, however, differ from cellular polymerases in that they possess no proofreading nuclease activity, and misincorporated bases are not removed as efficiently [42]. Consequently, they are error-prone, which partly accounts for the observed low fidelity in HIV replication [42, 43]. Moreover, RT has ribonuclease hybrid (RNase H) activity that allows it to degrade both the tRNA primer and the RNA strand following formation of the RNA/DNA hybrid intermediates. It is these enzymatic activities that catalyze the conversion of viral RNA into double-stranded linear DNA that can then be guided towards nuclear entry.

1.3.3.1.3 Uncoating

While earlier studies had proposed that following fusion of the two membranes the RNA genome in the viral core was immediately released into the cytoplasm of the host cell, recent evidence argues that HIV-1 undergoes an exquisitely regulated dissociation of the capsid from the viral core [44]. Cyclophilin A (Cyp A), which is also incorporated into the virion *via* Gag interactions, helps prevent early uncoating of the CA, and this regulation is vital for a successful viral replication [45]. Accordingly, mutants within the CA that dysregulate the timing of uncoating have been shown to negatively affect viral infectivity. It is presumed that the tight regulation of uncoating ensures that the viral proteins incorporated during assembly including RT, IN, Vpr, MA, NC, remain associated with the virion, in what is then termed a reverse transcription complex (RTC). Even though the precise timing of uncoating and reverse transcription remains unknown, it has become clear that these processes are very closely linked. Partial disassembly of the CA shell is important for successful reverse transcription, presumably to allow for cellular factors to access the RTC [46, 47]. Notwithstanding this, it was recently shown that the structure of the intact CA shell can selectively allow deoxynucleoside triphosphates (dNTPs) passage into the encapsidated viral genome [48]. Interestingly, inhibiting reverse transcription significantly delays uncoating as well [44]. It is becoming clear though that uncoating guides the RTC as it transitions into a pre-integration complex (PIC). Premature or delayed disassembly of the CA affects both reverse transcription and nuclear import of the newly synthesized DNA, hence resulting in abortive infection [49]. As well, uncoating plays an important role in protecting the

viral genome from DNA sensors [50, 51]. Overall, uncoating begins shortly after membrane entry, and is completed at the nuclear membrane [49, 52].

1.3.3.1.4 Nuclear Entry

The ability of HIV-1 to infect nondividing terminally-differentiated cells including macrophages implied the existence of a mechanism to transport the newly synthesized viral DNA into the nucleus where it can access the host cell chromosomal DNA. The PIC, with which the viral DNA remains associated, ultimately enables passage into the nucleus. Given that the PIC complex is actually much larger than the nuclear pore limit for passive diffusion, nuclear entry has to be an active process [53]. Although several viral proteins packaged into virions including Vpr, IN and MA, are believed to play roles in viral nuclear entry, some studies have fallen short in providing support for their contributions in this regard (reviewed in [54]). Indeed, there were no nuclear entry defects observed in HIV-1/MLV chimera-based studies in which both Vpr and MA were absent (MLV is unable to infect nondividing cells such as macrophages, but HIV-1 can) [54]. Conclusive evidence on the role of IN is still lacking, mainly complicated by the fact that any mutations on the residues required for nuclear entry in IN have multiple effects in IN nuclear entry-independent functions.

There is strong evidence for a role of CA, which remains associated with the PIC [44, 55] in nuclear entry, including the fact that mutations in CA impaired nuclear import [55]. As well, several factors have since been identified that are important for the CA-dependent nuclear entry (reviewed in [56, 57]. For instance, the ability of cleavage and polyadenylation specificity factor subunit 6 (CPSF6) to interact with CA, as well as the

nuclear localization signal (NLS) of CPSF6 are important for guiding the PIC for nuclear entry [56]. Other factors such as transportin 3 (TNPO3) and nucleoporins (NUPs)153 and 358 have also been implicated in nuclear entry, mainly through interactions with CA [54, 56, 57].

1.3.3.1.5 Integration

As discussed above, the CA associated with the PIC interacts with nucleoporins and other cellular factors that ultimately allow the PIC to translocate into the nucleus where the viral DNA integrates into the host genome. Central to the integration process is the HIV-1 IN enzyme that, conveniently, is packaged into assembling virions. IN catalyzes two important reactions: 3' end processing of the double-stranded viral DNA ends and processing of the host DNA, which then allow the viral and host DNA to bind. Following this strand transfer by IN, the cellular repair machinery completes the integration process by filling any gaps resulting from the insertion of the viral DNA into the chromosomal DNA strands. The integrated viral DNA, referred to as provirus, becomes a part of the host cell genome – replicated together with the host DNA and, therefore, the cell remains infected for its life span. This provirus serves as the template for synthesis of viral RNAs. HIV-1 generally integrates in actively transcribed genes [58], and this selection is influenced by several host factors including the lens epithelium-derived growth factor/transcriptional coactivator 75 (LEDGF/p75) that increases integration efficiency by interacting and tethering IN to chromatin [59, 60]. Factors that associate with the PIC complex, such as TNPO3 and NUP358, have also been shown to play a role in this regard [61].

1.3.3.2 Late phase of HIV-1 life cycle

1.3.3.2.1 Transcription

Integration of the viral genome marks the end of the early phase of the HIV-1 replication cycle, and beginning of the late phase that focuses on generating viral RNA genomes, expressing viral genes as well as viral assembly and egress. Transcription of viral messenger and genomic RNAs relies on both viral and cellular factors. It is mainly regulated by the HIV-1 5' LTR that harbors, in its U3 region, the viral promoter, binding sites for several host transcription factors such as NF-κB, nuclear factor of activated T cells (NFAT), specificity protein 1 and activator protein 1, as well as other regulatory elements [62]. Recruitment of the RNA polymerase II (RNA pol II) enzyme initiates viral RNA synthesis. The initial rate of transcription, especially of the elongated transcripts, is typically inefficient and requires the Tat regulatory protein for significant enhancement of transcript elongation. Tat binds cyclin T1 and cyclin-dependent kinase 9, thus recruiting the positive transcription elongation factor b (P-TEFb) to the proviral transactivation response (TAR) element [62]. This allows for phosphorylation of the C-terminal domain of RNA pol II, which in turn dramatically stimulates synthesis of full length HIV-1 transcripts.

Transcription of the 'complex' HIV-1 genome produces more than 25 or 30 different viral mRNA species, mainly regulated by several alternative splicing events. The full-length unspliced mRNA, which is ~9 kb, either serves as genomic RNA or encodes for structural and enzymatic proteins (Gag and Gag-Pol precursors). The multiple splice donor and acceptor sites within the viral mRNA results in singly spliced

mRNA (4-5 kb) encoding for Env, Vpu, Vpr and Vif, and fully spliced mRNA (~ 2 kb) that encodes Tat, Rev and Nef [63]. The fully spliced mRNAs, which are first to be transcribed, are readily exported from the nucleus and, in a feedback-like manner, aid the rate of transcription of the remaining transcripts. In this regard, Nef upregulates expression of cellular factors that promote LTR-driven transcription, including NF-κB, NFAT and activator protein 1 [64, 65]. While Tat functions as discussed above, Rev, which shuttles between the nucleus and cytoplasm, facilitates nuclear export of both unspliced and singly spliced viral transcripts. Rev acts as an adaptor that attaches the viral RNAs to the nuclear export machinery by binding to both the rev responsive element (RRE) within the viral mRNA *env* coding region and the cellular export factors such as Exportin 1 [66].

1.3.3.2.2 Translation

Once transcribed, the viral mRNA is capped and polyadenylated, and can serve as mRNA for the synthesis of the HIV-1 proteins or RNA genome that is packaged into assembling virions. Once in the cytoplasm, the viral mRNA (as part of a messenger ribonucleoprotein complex) recruits host translational machinery and viral protein translation proceeds via either cap-dependent or cap-independent mechanisms [67, 68], although the latter remains a subject of debate. Cap-dependent translation initiation involves recruitment of the 40S ribosomal subunit, as part of the 43S pre-initiation complex, to the 5'-cap structure bound eukaryotic initiation factor 4F complex [69, 70]. The pre-initiation complex then scans the 5' untranslated region (UTR) mRNA in the 5' to 3' direction until it encounters an AUG start codon. For cap-independent translation

initiation, on the other hand, an internal ribosomal entry site at the 5' UTR of the mRNA mediates the recruitment of the 40S ribosomal unit-containing PIC to sites closer to the AUG codon [69, 70].

Viral proteins Tat, Rev and Gag modulate viral mRNA translation (reviewed in [71, 72]). Tat is important for relieving the inhibitory effects of the HIV-1 5' UTR on translation. Not only does the HIV-1 5' UTR present a structural complexity that prevents optimal ribosome recruitment, but also has TAR hairpin sequences that activate protein kinase R (PKR), which in turn phosphorylates the eukaryotic initiation factor 2 (eIF2) and inhibits translation initiation [71, 72]. Tat binds to TAR/PKR, which prevents eIF2 phosphorylation and, therefore, allows for translation to proceed [71]. Tat also binds the cellular RNA helicase DDX3 which facilitates both nuclear export and translation of highly structured TAR-hairpin containing 5' UTR of HIV-1 [72]. Rev, on the other hand, is important for both viral RNA export and recruitment of ribosomes, which directly impacts translation. In fact, at low-intermediate concentrations, Rev promotes translation, but at high concentrations it plays an inhibitory role [73]. Gag, just like Rev, enhances translation at low concentrations, but is inhibitory at high concentrations [74].

1.3.3.2.3 Assembly

HIV-1 assembly refers to the highly coordinated process in which the viral proteins Gag, Gag-Pol, Env, Vif and Vpr, together with the viral genome, form a virion or viral particle to be transmitted to a new target cell. This process is heavily reliant on the Gag proteins. In fact, Gag proteins are necessary and sufficient for the formation of virus particles (non-infectious). Following Gag synthesis in the cytoplasm, the MA

domains of Gag and Gag-Pol precursors direct and bind the proteins to the plasma membrane. An N-terminal myristate moiety, together with a highly basic region within the MA domains, promote stable interactions with negative charges on the plasma membrane lipid bilayer [75]. While in the cytoplasm, Gag proteins begin to multimerize through CA-CA domain interactions. These multimerized small Gag complexes then assemble at the cell membrane, where they form even larger complexes. In addition, viral Env (gp160) glycoproteins also interact with the MA domains, making them part of the assembling virion. At the plasma membrane, both Env and Gag proteins preferentially localize to lipid raft microdomains [75]. The NC domains of Gag, which are also highly basic, bind the HIV-1 full-length unspliced genomic RNA through its stem-loop packaging signal, thus recruiting it to the assembly sites [75]. Two strands of genomic viral RNA are encapsidated per virion. The p6 “late domain” of Gag, which is primarily required for virion budding, interacts with Vpr, leading to its incorporation. In addition to Vpr, other proteins such as Vif and cellular CypA are also incorporated. The assembling of large complexes of Gag and viral proteins at the plasma membrane induces the formation of curved crescent-like viral capsids on the inner leaflet of the membrane, and the capsids then form a spherical particle that gets coated by the cellular membrane.

1.3.3.2.4 Budding and Maturation

As the spherical virion traverses the plasma membrane, acquiring the cellular membrane as its envelope coat, it starts pinching off from the host cell membrane, in a process called budding. At this point, the virion is still in its immature, non-infectious

form. The p6 domain of Gag then mediates recruitment of cellular factors, most notably components of the endosomal sorting complex required for transport (ESCRT) that mediate fission of the membrane [75]. Without the p6 domain, budding off of the immature virions is inhibited.

During or shortly after budding, the viral PR gets activated and cleaves both Gag and Gag-Pol precursors into their mature components. This forms part of the maturation process that renders the virions infectious. The structure of the mature virion gets reorganized such that a cone-shaped capsid, made of Gag CA, forms the inner core of the virion, inside which is the viral genome (Figure 1.2B).

1.3.3.2.5 Mature Virion Release

Following budding and maturation, the mature, virus particles are ready to be released in order to initiate infections of new target cells. However, the innate immune system has devised ways through which it can block or restrict the release of mature viral particles. Central to this restriction is the host restriction factor called Tetherin or Bone Marrow Stromal Cell Antigen 2 (BST2, CD317, HM1.24) [76, 77], which is a major focus of this thesis, and discussed in great detail from Section 1.6.

1.4 HIV-1 Accessory proteins modulate viral replication and pathogenesis

As alluded to earlier, the critically important roles of Nef, Vpu, Vif and Vpr are now widely appreciated, even though their targets are generally still under intense

investigation. Their actions are described in detail below, with emphasis on Nef and Vpu since they are a major focus in the thesis.

1.4.1 Nef:

Nef is a 27–35 kDa, ~ 206 residue HIV-1 negative regulatory factor protein, so-called because earlier reports had indicated that its overexpression impaired viral replication in *in vitro* systems [78, 79], even though later works suggested otherwise [80-82]. Nevertheless, the critical necessity of Nef for HIV-1 replication and pathogenesis in humans was demonstrated in studies showing significantly lower viral titers, absence of $CD4^+$ T cell depletion and lack of progression to the disease state (AIDS) in individuals infected with Nef-deficient viruses [83, 84]. Indeed, Nef has been shown to be a key regulator of HIV-1 pathogenicity [85].

Nef is a myristoylated multifunctional protein, playing important roles in both facilitation of infection as well as evasion of immune responses. Indeed, Nef down-modulates major histocompatibility complex (MHC)-I molecules from the surface of infected cells, thereby impairing the ability of these cells to present foreign (viral) antigens to T cells [86]. Thus, the infected cells are protected from immune responses mediated by cytolytic T lymphocytes (CTLs) [87]. As well, Nef has been shown to down-regulate cell surface expression of MHC-II molecules in infected cells [88-90]. Additionally, Nef down-modulates cell surface molecules that mediate natural killer (NK) cell activation, including natural-killer group 2, member D ligand (NKG2D-L) and polio virus receptor (PVR), which are ligands for activating receptors NKG2D and DNAX accessory molecule-1 (DNAM-1), respectively, found on NK cells and $CD8^+$ T cells [91-

93]. Consequently, these CTLs exhibit impaired cytolytic activity in co-culture systems. It must be noted, however, that Barker and colleagues did not observe Nef-mediated PVR downregulation in infected cells [94]. Recently, Nef was shown to antagonize the antiviral host factor proteins serine incorporator (SERINC) 3 and SERINC5 that otherwise decrease infectivity of mature virions [95, 96]. SERINC3/5 block HIV replication during or immediately following the fusion step, but before reverse transcription, in target cells. Nef excludes or prevents incorporation of these restriction factors into an assembling virion [95, 96]. Whether this is the main reason for packaging of Nef into virions remains unknown. Indeed, Nef encapsidation might just be a result of its plasma membrane association, owing to the fact that the protein is myristoylated and has an N-terminal stretch of basic amino acids [97]. Added to all the above, Nef also modulates the HIV-1 life cycle by enhancing the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity in order to initiate proviral transcription [98]. Very importantly as well, Nef also facilitates CD4 down-regulation from the cell surface, a function described in great detail under section 1.6 [99-101]. Overall, Nef plays important roles in HIV infection, and the list for its targets continues to grow [102].

1.4.2 Vpu:

Vpu is a 15-18 kD, 77-86 amino acid type 1 membrane-associated protein produced late during the HIV-1 replication cycle [103, 104]. It is translated from a singly spliced transcript that, in an alternate reading frame, encodes the Env glycoprotein [105]. The *vpu* gene is expressed in genomes of both HIV-1 and the HIV-1 precursor

SIVcpz, but not in HIV-2 or the HIV-2 precursor SIVs that infect sooty mangabeys or rhesus macaques [103, 104].

Vpu plays important roles in establishment of HIV-1 infection, as well as early dissemination of the virus [106-108]. Similar to Nef, Vpu is a multifunctional protein whose functions and relevance for pathogeneses continue being heavily researched. In infected cells the two best described functions of Vpu are to i) proteasomally degrade CD4 [109, 110] and ii) overcome the host restriction factor Tetherin or BST2 in order to enhance release of progeny virions (both functions are described in detail in later sections) [76, 77, 111, 112]. Vpu and Nef show some redundancy in their functions. For example, both proteins down-modulate surface CD1d, which is an MHC-like molecule that presents lipid antigens to natural killer T (NKT) cells, and, in so doing, Vpu and Nef dampen NKT cell cytolytic activity [113, 114]. As was the case with Nef, some studies have observed a role of Vpu in downregulating PVR [92, 115], whereas others have not [93, 94]. Furthermore, Vpu also down-modulates surface NK-T-B-antigen (NTB-A), a self-ligand coactivating receptor that is present on NK, T and B cells, and has been shown to facilitate killing of HIV-infected cells by NK cells [116-118]. Very recently, Vpu was shown to down-regulate the C-C chemokine receptor-7 (CCR7) and the adhesion molecule L-Selectin (CD62L), both of which promote migration of $CD4^+$ T cells to lymphoid tissues [119, 120]. As well, the recently described role of Vpu in downregulating the sodium-coupled neutral amino acid transporter (SNAT1), an alanine transporter, appears to prevent T cell activation and likely reduces expression of viral proteins as a protective mechanism from the immune response or to favour establishment of latent HIV reservoirs [102, 121]. Added to all of the above, and quite

interesting, while Nef enhances NF-κB activity in order to initiate proviral transcription, Vpu has been shown to inhibit NF-κB activity, a function that likely contributes towards impeding production of interferon (IFN) and interferon-stimulated genes (ISGs) such as BST2, among others [98, 122]. By constitutively interacting with beta-transducin repeats-containing proteins 1 or 2 (β-TrCP1/2) subunit of the Skp, Cullin, F-box-containing (SCF) E3 ubiquitin ligase complex [$(SCF)^{\beta\text{-}TrCP1/2}$], Vpu sequesters the adaptor protein away from its substrates including the NF-κB inhibitors IκB. In consequence, poly-ubiquitination and degradation of IκB is inhibited, which in turn prevents the translocation of NF-κB subunits into the nucleus [122-124]. Interestingly, Vpu exists in a phosphorylated form that can be continuously complexed with β-TrCP, yet not targeted for degradation. Overall, a clearer picture is emerging in which Vpu promotes efficient virus replication and/or production, while shielding infected cell from immune responses.

1.4.3 Vif:

Vif, as the name implies, is a 23kDa essential protein that enhances HIV-1 infectivity by facilitating viral replication and inducing the degradation of an endogenous host antiviral protein that inhibits virus replication - apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G), a DNA cytidine deaminase protein [125]. Relevance of Vif in HIV pathogenesis comes from reports of correlations between mutations in *vif* gene and the absence or reduced progression to the disease state in HIV-infected patients [126-128]. In the absence of Vif, APOBEC3G is packaged into the virion, and exerts its function within the host target or newly infected cell, where it

converts cytidines to uridines during reverse transcription [129]. This cytidine deamination function leads to G-to-A hypermutations of the viral genome as well as degradation of RT products, thus restricting its replication. Vif triggers the degradation of APOBEC3G through a ubiquitin-proteasomal pathway [125, 130, 131].

1.4.4 Vpr:

Vpr is a 14 kDa, 96-amino acid protein that is packaged into budding virions, suggesting it may play a role early in the replication cycle, that is, it may be necessary for establishment of infection in newly infected target cells. It is required for efficient HIV replication *in vivo*, an observation that is further supported by the correlations between specific Vpr mutations and disease progression [132, 133]. A major and well-described function of Vpr is to arrest cells in the G2/M phase of the cell cycle, as reviewed in [134]. Following evidence that an association between Vpr and DDB1- and CUL4-associated factor 1 (DCAF1) E3 ubiquitin ligase complex is required for cell cycle arrest, it has recently been shown that Vpr mediates degradation of a host cellular factor helicase-like transcription factor (HLTF) [135, 136]. Additionally, Vpr facilitates infection of macrophages [137]. Vpr also augments degradation of uracil DNA glycosylase-2 (UNG-2), a function whose biological consequences are yet to be elucidated, but are not associated with the cell cycle arrest function [138, 139]. In addition, Vpr mediates induction of apoptosis, even though it remains unclear whether this is as a result of the cell cycle disruption [139]. Several other roles of Vpr have been reported, including facilitation of reverse transcription, nuclear entry and integration, but these remain controversial (reviewed in [139, 140]). A role of Vpr in transactivating viral gene

expression from the HIV-1 LTR, which bears the viral promoter, has also been proposed [141, 142].

1.5 Host factors modulating virus replication

It has long been apparent that successful HIV infections are dependent on complex interactions between the virus and the host. Indeed, numerous host factors have been identified that are exploited by the virus in order to achieve efficient replication – the so-called HIV-dependency factors. These include the primary HIV receptor CD4; the HIV entry cofactor CCR5; factors facilitating nuclear import such as Nups 153 and 358, TNPO3, CPSF6 [54, 56, 57]; the IN interacting protein LEDGF/p75 [59, 60], ESCRT-I proteins that facilitate release of nascent virions [75], as well as a plethora of others that have been recently identified from genome-wide screening studies. On the flip side, there are several cellular factors expressed by mammalian cells that confer resistance to HIV infection, generally referred to as either HIV resistance and/or restriction factors. Host restriction factors utilize various mechanisms to potently inhibit various stages of the virus life cycle, and are considered important for prevention of cross-species transmission of retroviruses. These factors share several features including that they are; 1) under positive selective pressure influenced by host-pathogen coevolution; 2) constitutively expressed, but are also interferon-inducible; 3) dominantly acting or cell autonomous, and 4) are frequently suppressed by viral accessory proteins. Among the well established restriction factors are: i) tripartite motif 5-alpha (TRIM5α), which induces pre-mature disassembly of the incoming virions thus

blocking viral cDNA synthesis [41]; ii) Sterile alpha motif and histidine-aspartic domain (HD) containing protein 1 (SAMHD1) that depletes dNTP levels in non-dividing cells thereby inhibiting cDNA synthesis [143, 144] and also targets RNA for degradation [145]; iii) APOBEC3 cytidine deaminases that hypermutate the viral genome, introducing G-to-A substitutions that are detrimental to replication of the virus as well as directly inhibiting reverse transcription [125, 129]; iii) SERINC 3/5, which decrease infectivity of mature virions [95, 96] and iv) BST2, which restricts the release of nascent virions from the surface of infected cells [76, 77]. In addition to these restriction factors, several resistance factors have been reported to inhibit virus replication, and it remains unclear if the virus is able to counteract them. Such antiviral factors include: a) the IFN-inducible human myxovirus resistance 2 (MX2, also referred to as MxB) that acts as an antiviral factor, exerting its blockage at nuclear import and/or integration of the viral DNA into the host cell chromosomes [146-148]; b) Interferon-inducible transmembrane proteins (IFITMs), which inhibit viral entry and replication [41]; and c) Schlafen (SLFN) proteins that inhibit HIV-1 mRNA translation [41].

While the HIV-dependency, resistance and restriction factors act at various stages of the viral life cycle, the importance of the plasma membrane for the ability of the virus to initiate infection and, following successful integration and viral protein translation, to bud off from the infected cells to initiate infection in new cells cannot be overstated. Indeed, the cell surface has long been considered the battlefield in the fight against HIV, for it is the very first point of contact between the virus and the host target cell, as well as the site that can mediate involvement of the immune system. Host cellular molecules that play critical roles in regulating viral entry or release of newly

synthesized virions are very attractive as focus-points for development of anti-HIV prophylactics or therapeutics. In this context, the host factors CD4 and the virion-tethering BST2 have received considerable attention.

1.5.1 CD4 plays a double-edged-sword role in HIV-1 replication and dissemination

HIV-1 selectively infects and replicates in $CD4^+$ T cells, mostly due to the role of CD4 as a receptor for the incoming virion. CD4 is a type I transmembrane glycoprotein expressed at the surface of macrophages and T lymphocytes that, together with the T-cell antigen receptor (TCR), associate with MHC-II molecules on professional antigen presenting cells (APCs). As such, CD4 is important for recognition of peptides or antigens presented by these MHC-II molecules, and for the subsequent activation of the immune response. Prior to engagement of MHC-II molecules, the CD4 cytoplasmic tail stably interacts with a tyrosine kinase called Lck [149]. Binding of both CD4 and TCR to MHC-II molecules therefore brings Lck in close proximity with the signalling cascade of the TCR complex, leading to activation of the T cell. Following T cell activation, CD4 dissociates from Lck, is internalized into endosomes, and then recycles to the plasma membrane [101].

While CD4 expression at the plasma membrane is critically important for virus entry, continuous expression of the receptor is detrimental to efficient viral replication and dissemination. First, as discussed above, accumulation of CD4 at the cell surface further enhances T cell activation following presentation of viral antigens on MHC-II molecules and, therefore, activates the immune response [150]. Second, CD4 at the

surface of infected cells can interact with Env proteins on incoming virions, thereby increasing the likelihood of superinfection of the infected cells and, ultimately, their premature death [151-153]. In terms of virus spread, it would presumably be ‘wasteful’ or less efficient to have many virions infect a single cell, versus targeting multiple cells. Third, CD4 accumulation at the surface allows for Env-CD4 interactions and, therefore, promoting either recruitment of CD4 molecules into virions or reduced incorporation of Env, altogether resulting in impairment of viral infectivity [154-156]. Fourth, CD4 at the surface can reduce release of newly formed virus particles. This could be due to CD4 binding to and trapping mature virions at the cell surface [157]. Aside from surface CD4, the expression of the receptor within intracellular compartments also poses a problem for virus replication. The intracellular pool of CD4 can prematurely bind to Env proteins prior to them reaching virion assembly sites [158]. Binding to CD4 intracellularly sequesters Env, thus reducing the amount of Env incorporated into the assembling virion and, consequently, reduces the infectivity of the released virions [159]. Given these, CD4 expression is exquisitely regulated following HIV infection. Perhaps illustrative to the importance of this regulation, HIV-1 has devoted three of its proteins (Nef, Vpu and Env) to regulate CD4 expression, *via* distinct mechanisms as discussed in Section 1.6 [101, 160].

1.5.2 BST2 as a restriction factor and an immune modulator

Shortly after its identification, the role of Vpu in enhancing the release of nascent virus particles became apparent [111, 112, 161, 162]. Several studies reported a

potential role of Vpu towards overcoming a host cellular factor that was inhibiting virus release at the plasma membrane, in a cell type-specific manner [163-165]. The virions 'stuck' at the cell surface were shown to be endocytosed in a clathrin-dependent manner and, again, this endocytosis was only observed in Vpu-defective conditions, meaning it was inhibited by Vpu [165, 166]. Retrospectively, one of the major advances towards identification of the then putative host factor restricting virus release came from a 2006 proteomics study that identified BST2 as a cellular host target for both Vpu and a Kaposi sarcoma-associated herpesvirus K5 protein, showing a reduction in endogenous expression of BST2 following individual transfections of the two viral proteins [167]. Further insights about the mysterious cellular factor came from a study by Neil and colleagues, demonstrating that the said factor was tethering virions to the surface and was IFN-inducible [168]. It was only in 2008 that two seminal reports directly identified BST2 as the previously elusive host restriction factor that tethered virus particles to the cell surface of infected cells, and was counteracted by Vpu [76, 77].

BST2 was originally identified in bone marrow stromal cells, hence the BST2 name, and in several types of cancers (reviewed in [169, 170]). The role of BST2 in these transformed cells remains unknown. Constitutive expression has since been shown in hepatocytes, activated T cells, monocytes, macrophages, plasmacytoid dendritic cells (pDCs), pancreas and kidney cells, and many other cell types. BST2 expression is further enhanced by IFN, with its promoter containing binding sites for several transcription factors including signal transducer and activator of transcription 3 (STAT3), IFN regulatory factors (IRF), NF-κB, NFAT and IFN stimulated gene factor 3

(ISGF3) [171-175]. As well, the immunoregulatory cytokine interleukin-27 also directly stimulates BST2 expression, in a manner independent of IFN [176].

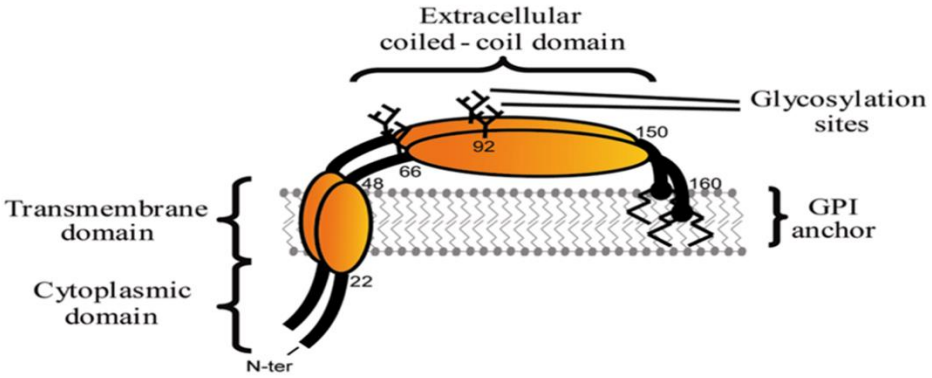
The type II transmembrane BST2 has a rather unique structural topology relative to other mammalian proteins. It consists of a short N-terminal cytosolic domain, an α-helical TMD, followed by a coiled-coil ectodomain and a C-terminal glycosylphosphatidylinisotol (GPI) anchor (Figure 1.4A) [177]. The cytosolic domain contains a highly conserved non-canonical tyrosine-based motif (YxY) that has been reported to interact with AP-2, leading to clathrin-dependent internalization of BST2 from the plasma membrane [178, 179]. AP-2 recruitment is essential for the ability of BST2 to constitutively recycle between the plasma membrane and both endosomal and TGN compartments [179, 180]. Recently, a short isoform of BST2 has been identified (Figure 1.4B). This isoform lacks the YxY endocytic motif and, predictably, would localize mainly at the plasma membrane [181]. Expression of the short isoform results from a weak Kozak consensus at the first methionine, resulting in a leaky ribosomal scanning hence the usage of the methionine at position 13 as the start codon [181].

The ectodomain of the mature form of BST2 (at the surface or within recycling compartments), is glycosylated at two asparagine residues (N65 and N92) [171, 177]. This N-linked glycosylation occurs while the protein transits from the ER to the Golgi *en route* to the plasma membrane, and is important for proper anterograde transport of the protein [182]. In addition, the BST2 ectodomain has three cysteine residues (C53, C63 and C91) that form intermolecular disulfide linkages, which stabilize homodimerization of the protein [177, 183, 184]. There exist additional determinants for BST2 dimerization, as evidenced by the retained ability of a BST2 mutant lacking all cysteine

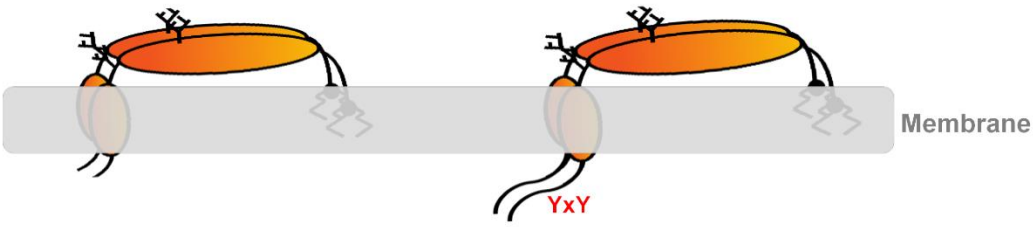
residues to form non-covalent dimers [185]. It has been proposed that the TMDs in the homodimers may be interacting with each other, which may promote or strengthen the dimerization of the protein [186]. As well, a dimer of BST2 can interact with another dimer, forming a BST2 tetramer [187].

Addition of the GPI anchor to BST2 occurs while the protein is still maturing within the ER, following cleavage of the C-terminus end of the protein. GPI addition, which occurs at a serine residue (S161), is important for the ability of the protein to traffic to the plasma membrane, otherwise it would be retained within the ER [182]. At the plasma membrane, the GPI is believed to drive the localisation of BST2 to cholesterol-rich microdomains [177]. Curiously, while the GPI anchor partitions into these lipid rafts, the TMD at the N-terminal end appears to be excluded from these rafts, and in fact the cytosolic tail of BST2 is reported to be a membrane microdomain-exclusion motif [188]. The prerequisites for lipid raft localization are still unclear, and Hammonds and colleagues reported the presence of determinants within the ectodomain that facilitated lipid rafts partitioning [189]. Indeed, other reports have questioned the existence of the GPI anchor, in favour of a second TMD at this C-terminal anchoring position [190, 191].

A


Extracellular
coiled - coil domain
Glycosylation
sites
92
150
66
48
160
Transmembrane
domain
GPI
anchor
Cytoplasmic
domain
22
N-ter

B


Short isoform of BST2
Long isoform of BST2
Membrane
YxY

C

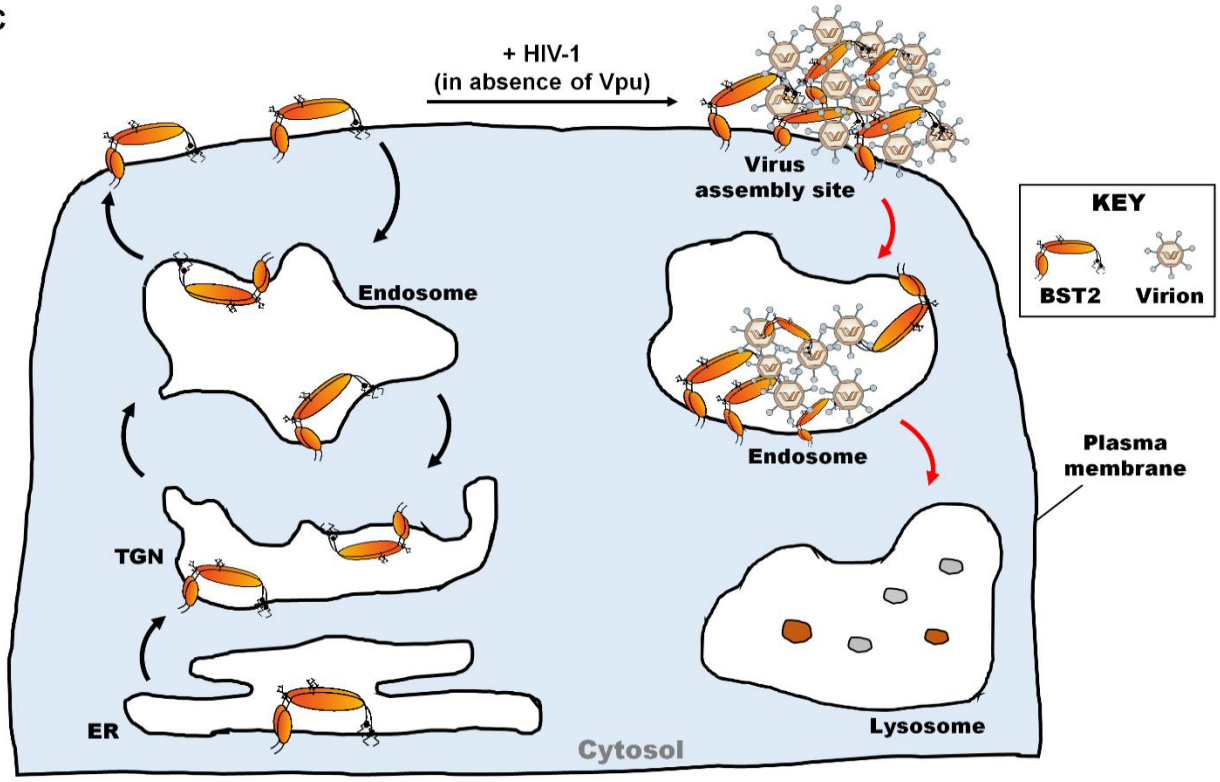

+ HIV-1
(in absence of Vpu)
Virus
assembly site
KEY
BST2
Virion
Endosome
Endosome
Plasma
membrane
TGN
ER
Lysosome
Cytosol

Figure 1.4: BST2 structure and function. A) Topology of BST2 indicating cytoplasmic, transmembrane, extracellular and GPI domains. **B)** Short and long isoforms of BST2. As a result of a leaky ribosomal scanning, the short isoform of BST2 uses the Met at position 13 as a start codon, hence lacks the first 12 residues including a YxY endocytic motif. **C)** BST2 localization and virion tethering function. BST2 constitutively recycles between the plasma membrane, endocytic compartments and back to the plasma membrane via the TGN. At the cell surface, within virus assembly sites, BST2 crosslinks nascent virions thus restricting their release.

1.5.2.1 Role of BST2 in virus pathogenesis

Human BST2 has been associated with inhibition of cross-species transmission of SIVcpz, the immediate precursor of HIV-1, to humans [192]. While SIVcpz uses Nef to antagonize chimpanzee BST2, human BST2 is resistant to Nef, owing to a deletion of five residues within the cytoplasmic tail of human BST2 that would otherwise be targeted by Nef [192-194]. Evidence indicates that the four independent zoonotic transmission events leading to HIV-1 groups M, N, O and P were heavily regulated by human BST2 [192]. HIV-1 group M viruses evolved to efficiently target BST2 using their Vpu proteins, whereas Vpu from groups N, O, P could not optimally target the restriction factor [192]. Vpu from group N, however, is not totally defective for BST2 counteraction, but appears to have lost the ability to target CD4 in the process of acquiring BST2 antagonistic activity [192]. Interestingly, group O viruses evolved to utilize their Nef protein to target BST2, even though Nef-mediated BST2 antagonism is not as efficient as that mediated by Vpu from the M group [195, 196]. Thus, only HIV-1M is associated

with efficient viral infection and spread, leading to the HIV-1 pandemic that has crippled the world.

The role of BST2 in virus pathogenesis has been evidenced in the limited number of mice studies performed to date. In one study, BST2 significantly inhibited Moloney murine leukemia virus (Mo-MLV) replication, and its expression was necessary to realize the antiretroviral activity of IFNα [197]. More relevantly, in humanized mice, Vpu-mediated BST2 antagonism appears important for the establishment of infection, as evidenced by improved replication kinetics of Vpu-expressing viruses relative to Vpu-deficient viruses within the very early stages of infection, but the observed advantage in replication kinetics between the two viruses is lost following multiple rounds of replication events [107, 108].

Additional evidence for a role of BST2 in HIV pathogenesis has come from HIV-infected patients. Pillai and colleagues reported an inverse correlation between HIV-1 viral load and expression of BST2 and APOBEC3G in pegylated IFNα-treated HIV-1 and HCV co-infected individuals [198]. Moreover, preservation of anti-BST2 activity in Vpu is heavily selected for, from acute to long-term chronically infected patients [199-201]. This is in spite of the fact that *vpu* is considered one of the highly variable HIV-1 genes. Furthermore, several reports have emerged correlating polymorphisms within the BST2 promoter regions to both HIV-1 acquisition [202, 203] and progression to AIDS [202, 204].

1.5.2.1.1 Virion tethering

In non-infected cells, BST2 constitutively recycles between the plasma membrane, endocytic compartments, TGN and back to the cell surface (Figure 1.4C). The mechanisms through which BST2 restricts virus release have been a subject of great interest in the field. It is now widely believed that the unique topology of BST2 is critical to the antiviral activity of the protein. In fact, the topology of the restriction factor, rather than its primary sequence, is a determinant for the virion tethering function [182]. In such a configuration, BST2 dimers simultaneously insert one of their membrane anchors into the virion particle while the other remains inserted into the host cell membrane, thus tethering the virions to the surface (Figure 1.4C) [182, 205]. In addition to tethering of virions to the host membrane, electron microscopy revealed that BST2 also tethers virions to each other [76, 206]. Consistent with the GPI anchor partitioning into lipid rafts, as well as the fact that virus assembly occurs within the same microdomains, the GPI end of the protein preferentially inserts into the budding virions [207]. Deletions of either the TMD or GPI anchor of BST2 abrogates its ability to restrict virus release [76, 77, 182]. As was reported prior to identification of BST2, the tethered virions at the surface are subsequently endocytosed and targeted for degradation (Figure 1.4C) [165, 166].

Given the nature of the mechanism through which BST2 prevents virus release, it came as no surprise that BST2 inhibits the release of a wide range of enveloped viruses (reviewed in [208]). The immediate, and obvious, consequence of this restrictive ability of BST2 is that less viral particles are released and, as such, this tethering of virions at the surface was generally believed to be the major benefit to the host. However, in the

grand scheme of things, this mechanism of action only targets the cell-free virus spread, which is believed to be less efficient at promoting virus dissemination compared to cell-to-cell spread [209]. The role of BST2 in cell-to-cell virus propagation among T cells remains controversial, with some studies reporting a BST2 restrictive role [210, 211] while some observed no inhibition of cell-to-cell transmission [212]. Interestingly though, BST2 was shown to restrict both cell-free and cell-to-cell virus transmission in studies investigating virus transfer from monocyte-derived macrophages (MDMs) or monocyte-derived dendritic cells (MDDCs) to $CD4^+$ T cells [213-215]. The basis for the cell-type specificity of the BST2 effect in these *in vitro* assays is currently unclear. While *in vivo* studies are still lacking, a couple of studies have indicated a role of BST2 in replication [107, 108, 197]. Importantly, infection of BST2-expressing or BST2-knockout mice using the MLV retrovirus indicated that BST2 plays a role in controlling the infection (lowering viraemia) in a manner that correlated with induction of immune responses such as NK cell and virus-specific $CD8^+$ T cell responses [216]. Indeed, recent studies have compellingly demonstrated a critical role of surface BST2-mediated virion tethering towards innate sensing and/or immune signaling events that trigger IFN production and generation of an antiviral state, as discussed below.

1.5.2.1.2 Modulation of immune responses by BST2

1.5.2.1.2.1 NF-κB activation

It is interesting that BST2 was originally identified in screens for cellular factors that triggered NF-κB activation [217], which suggests that it could play a role in

induction of NF-κB-dependent proinflammatory responses. Indeed, BST2-mediated virion entrapment induces NF-κB activation, in a manner similar to that obtained from crosslinking surface BST2 molecules using antibodies or overexpression of BST2 by transient transfection [181, 218, 219]. As such, the ability of BST2 to retain particles and then trigger the NF-κB signal transduction pathway allows it to act as an immune sensor of virus assembly at the surface, facilitating detection of the infected cell by the immune system. Vpu-mediated BST2 antagonism therefore serves to promote virus release and to evade induction of immune responses.

Mechanistically, virion retention at the surface clusters BST2 dimers, an event that induces phosphorylation of the cytosolic tyrosine residues in BST2, and the subsequent recruitment of spleen tyrosine kinases (Syk) [220]. As well, complexes associated with the TNF-receptor-associated factor (TRAF) E3 ubiquitin ligases are recruited, through currently unknown mechanisms, leading to ubiquitin-mediated activation of the transforming growth factor β-activated kinase 1 (TAK1) kinase complex [218, 219]. TAK1, in turn, induces I kappa B (IκB) kinase (IKK)-mediated phosphorylation and proteasomal degradation of IκB, thereby enabling NF-κB subunits to translocate into the nucleus and induce gene expression [218, 219, 221, 222] (Figure 1.5A). This NF-κB signalling activity is dissociable from the virus restriction function of BST2. For virus restriction, the cytoplasmic YxY endocytic motif on BST2 is dispensable, whereas it is absolutely required for signalling [181, 218, 219]. In keeping with this, the short isoform of BST2, which lacks this motif, is unable to mediate NF-κB signalling [181]. In fact, the short isoform acts as a dominant inhibitor of signal transduction, indicating that only homodimers of the long isoform are able to activate

NF-κB signalling [220]. As well, the GPI anchor is important for release but not so much for signalling [219]. The relative importance of both the GPI and YxY motifs for virion restriction versus signalling could further account for the preferential insertion of the GPI into the budding virions while the TMD, to which the cytoplasmic YxY motif is connected, remains anchored to the host cell. Interestingly, a very rare BST2 single nucleotide polymorphism (SNP) with a histidine substituting for an arginine at position 19 (R19H) impaired NF-κB signalling, but exerted no effect on restriction [223]. However, other structural configurations, including the coiled-coil ectodomain, are necessary for both restriction and signal transduction functions [218, 219].

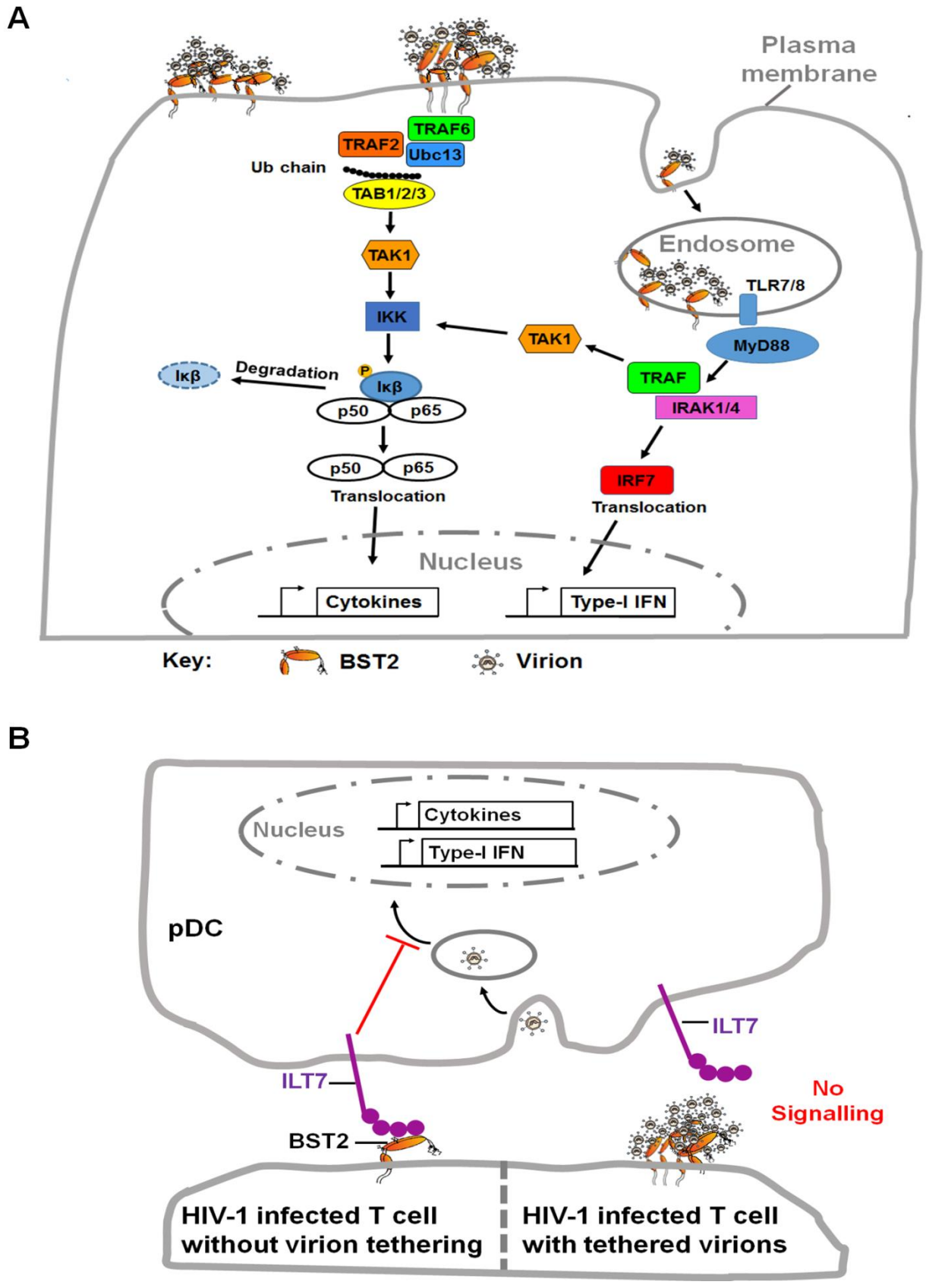
1.5.2.1.2.2 TLR Signalling

Virions trapped at the cell surface get internalized into endosomal compartments *en route* for degradation [76, 165]. Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) may recognize some viral pathogen-associated molecular patterns and then trigger the IRF pathway [218, 224]. In humans, TLR7/8, which senses ssRNA from RNA viruses, is predicted to play a central role in this regard, leading to the recruitment of a signalling complex involving IL-1R-associated kinases (IRAKs), TRAFs, IKK, MyD88 and IRF7 to the TLR [224]. IRF7 gets phosphorylated and translocates into the nucleus where it enhances expression of antiviral genes including IFNs [224] (Figure 1.5A). Noteworthy, complexes such as TRAFs and IKK are also part of the pathway that induces NF-κB translocation into the nucleus, hence they can activate NF-κB signalling as well, leading to production of inflammatory cytokines and BST2. It is worth remembering that the BST2 promoter actually has an IRF binding site [175].

Unlike with NF-κB signalling, both isoforms of BST2 would be predicted to be able to mediate this TLR-dependent signalling.

1.5.2.1.2.3. ILT7 Receptor Signalling

In addition to inducing NF-κB and TLR signalling pathways, BST2-mediated virion crosslinking modulates IFN type 1 (IFN-I) production by pDCs. Even though pDCs constitute less than 1% of the total human peripheral blood mononuclear cells (PBMCs), they are the major source of IFNs and cytokines following viral infections [225]. Upon cell-to-cell contacts with infected T cells, pDCs, which express CD4, CXCR4 and CCR5, endocytose virions leading to activation of TLR7 [226]. As described above, this leads to enhanced transcription or production of IFN-I and pro-inflammatory cytokine genes. Interestingly, BST2 acts as a ligand for the immunologlobulin-like transcript 7 (ILT7, also referred to as LILRA4 and CD85g) inhibitory receptor on pDCs that modulates IFN-I and pro-inflammatory cytokine production [227]. Upon BST2 engagement, ILT7 suppresses activation of TLR7 and TLR9, and therefore dampens IFN-I production (Figure 1.5B) [227, 228]. Accumulation of tethered virions at the surface sterically inhibits this BST2-ILT7 interaction, resulting in higher IFN-I production (Figure 1.5B) [228]. Once again, Vpu-mediated counteraction of virion restriction ensures that there are free or unliganded BST2 molecules at the cell surface that can interact with ILT7, thereby inhibiting the pDC-dependent IFN production [228].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TRAF6
TRAF2
Ubc13
Ub chain
TAB1/2/3
TAK1
IKK
Endosome
TLR7/8
MyD88
TAK1
TRAF
IRAK1/4
Degradation
Iκβ
P
Iκβ
p50
p65
p50
p65
Translocation
IRF7
Translocation
Nucleus
Cytokines
Type-I IFN
Key:
BST2
Virion
B
Nucleus
Cytokines
Type-I IFN
pDC
ILT7
ILT7
No Signalling
BST2
HIV-1 infected T cell without virion tethering
HIV-1 infected T cell with tethered virions

Figure 1.5: Immunomodulatory roles of BST2. A) BST2-mediated virion tethering triggers NF-κB and TLR signalling pathways. NF-κB signalling pathway: Virion retention at the surface clusters BST2 dimers, which initiates a series of phosphorylation/activation events leading to translocation of NF-κB subunits into the nucleus where they induce gene expression. TLR signalling pathway: Virions tethered at the surface are ultimately internalized into endosomal compartments where they are sensed by TLRs, leading to phosphorylation and translocation of IRF7 into the nucleus where it enhances expression of antiviral genes including IFNs. As well, TLR signalling also recruits components of the NF-κB signalling pathway. **B)** BST2-mediated virion crosslinking modulates IFN-I production by pDCs. In the absence of virion tethering, BST2 interacts with ILT7, resulting in inhibition of TLR-mediated signalling that modulates IFN-I production. Virion tethering sterically inhibits the BST2-ILT7 interaction, allowing for TLR-mediated signalling.

1.6 HIV-mediated CD4 depletion

In infected cells, CD4 levels are tightly regulated by a concerted effort of Nef, and Vpu. The structures of Nef and Vpu are depicted in Figure 1.6. Structurally, Nef comprises of two flexible regions (the first or N-terminal 54 residues and residues 150–180) and a globular folded core (residues 55–149 and 181–206) (Figure 1.6A). One study reported that Nef forms dimers, which are necessary for downregulation of cell surface molecules such as CD4 [229]. Several motifs within Nef have been shown to be functionally important in as far as CD4 degradation is concerned, and these include 57-WLE-59 residues important for binding to CD4 [230, 231] and a di-acidic 155-EE-156 motif that interacts with coatomer beta subunit (β-COP) [232]. An acidic di-leucine motif

(160-ExxxLL-165) and a di-acidic motif 174-(D/E)D-175 regulate interactions with clathrin adaptor protein complexes (AP-1 and AP-2) [233-236].

The structure of Vpu consists of a short luminal N-terminal tail, a transmembrane domain (TMD) and a cytoplasmic domain that contains a flexible hinge region (transmembrane-proximal), followed by two helices that are separated by yet another linker region (Figure 1.6B). The helical TMD can mediate formation of oligomers, resulting in formation of a cation channel whose function remains elusive [237, 238]. Vpu possesses several functional motifs including a 59-ExxxLV-64 di-leucine motif that mediates recruitment of AP-1/2 proteins [239, 240]. Moreover, the serine residues of a DSGxxS motif occurring within the linker region between the cytosolic helices serve as phosphorylation sites for the cellular casein kinase-2 (CK-II), which results in recruitment of β-TrCP [110, 241]. Vpu phosphorylation was recently shown to be also important for recruitment of the AP-1/2 proteins [240]. An important theme that stands out from these functional domains within both Nef and Vpu is their implication or relevance in cellular trafficking of their target proteins. Indeed, Nef and Vpu are frequently considered masters in manipulation of host proteins in order to favor virus replication and dissemination.

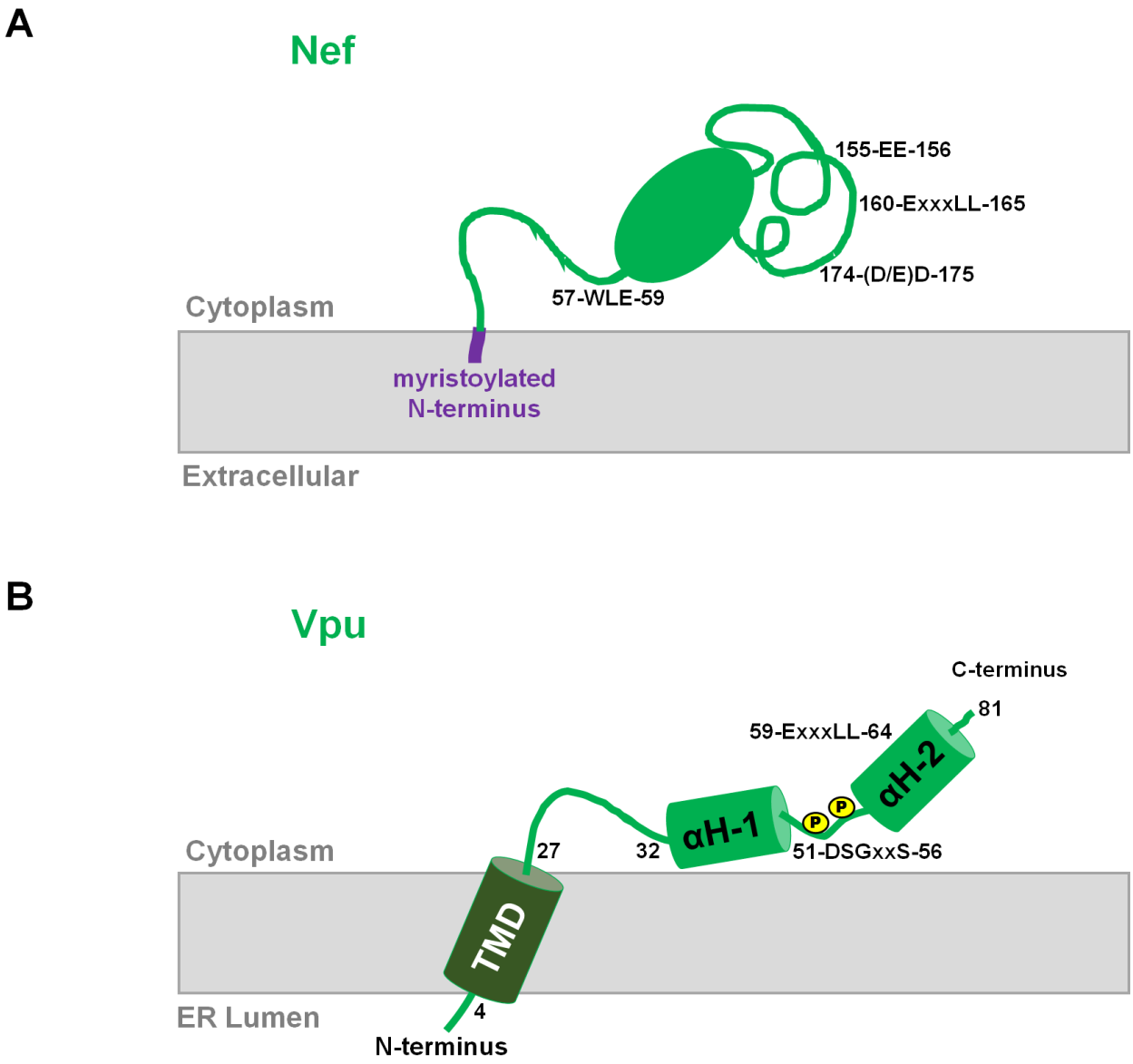


Figure 1.6: Schematic diagrams of Nef and Vpu structures. A) Nef consists of a myristoylated N-terminus, two disordered regions and a globular domain. Shown are several motifs within Nef that regulate CD4 targeting, including 57-WLE-59 residues important for binding to CD4 [230] and a di-acidic 155-EE-156 motif that interacts with β-COP [232]. The 160-ExxxLL-165 acidic di-leucine and 174 (D/E)D di-acidic motifs regulate interactions with clathrin adaptor protein complexes AP-1/2 [233-236]. **B)** Vpu consists of a short luminal N-terminal tail, a transmembrane domain (TMD) and a cytoplasmic domain. A transmembrane-proximal flexible hinge region connects the TMD to the cytoplasmic domain. The two

helices within the cytoplasmic tail are separated by a linker region that contains the DGSxxS β-TrCP recognition motif. Within the helix-2 of Vpu is an acidic di-leucine motif that regulates recruitment of AP complexes.

1.6.1 Nef-mediated CD4 depletion

Nef, which is produced early following infection, targets CD4 already at the cell surface or within the endocytic compartments. Myristoylation of Nef brings it to close proximity with surface CD4, allowing for a direct interaction between the CD4 cytoplasmic tail and several Nef residues including the 57-WLE-59 motif [230, 242]. Importantly, through its flexible loop comprising 160-ExxxLL-165 and 174-(D/E)E-175 motifs, Nef recruits AP-2 that mediates endocytosis of CD4 in a clathrin-dependent manner and, eventually, sorting of the viral receptor to lysosomes for degradation [234, 236, 243-246] (Figure 1.7A). The precise mechanisms of sorting to lysosomes are still under consideration. While it has been reported that Nef hijacks the ESCRT machinery to target the internalized CD4 to multivesicular bodies (MVBs) and eventually to the lysosomes [247, 248], others have proposed that Nef recruits β-COP that ultimately leads the viral receptor to lysosomes [232, 249, 250].

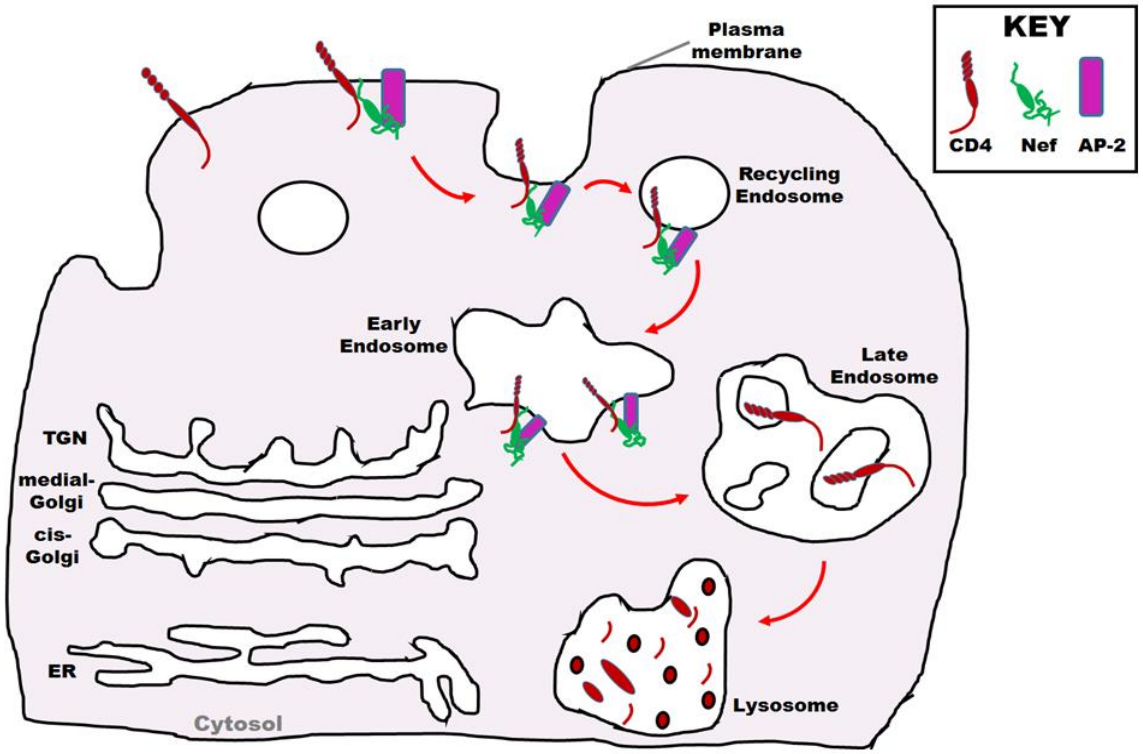
1.6.2 Vpu-mediated CD4 depletion

In addition to the early actions of Nef towards down-regulating CD4 from the surface, both Env and Vpu, produced later in the virus life cycle, target intracellular CD4 to reduce the ‘re-supply’ of surface CD4 from newly synthesized pools. The gp160 Env precursor, with its high affinity for CD4, binds and retains newly synthesized CD4 within

the endoplasmic reticulum (ER) (Figure 1.7B) [251]. Consequently, trafficking of CD4 to the plasma membrane is attenuated. From the virus' standpoint, however, this has an undesirable consequence in that Env gets retained within the ER, and perhaps degraded together with CD4. As such, less Env traffics to the surface where it can be incorporated into virions, an occurrence with greater implications on infectivity of newly formed virions [252, 253]. This, however, is alleviated by Vpu, which induces degradation of the complexed CD4, thereby liberating and allowing Env to transit to the cell surface [109, 158] (Figure 1.7B). Thus, Vpu targets newly synthesized CD4 molecules within the ER, retaining them within these compartments and ultimately delivering them to an ER-associated degradation (ERAD) pathway [254-256]. Vpu is very efficient at mediating this degradation of the viral receptor, reducing its typically 6 h half-life to ~15 min (reviewed in [161]). Towards this, Vpu plays a role of an adaptor – linking the viral receptor to [(SCF)$^{\beta\text{-TrCP1/2}}$ E3 ubiquitin ligase complex via its di-phosphoserine DSGxxS motif [110, 257]. Recruitment of β-TrCP leads to the poly-ubiquitination of lysines and serines/threonines residues within the cytoplasmic tail of CD4, which partly traps CD4 within the ER [254, 255, 258]. Once CD4 is poly-ubiquitinated, a VCP-UFD1L-NPL4 dislocase complex is then recruited, which facilitates the extraction of CD4 molecules from the ER membrane to the cytosol (Figure 1.7B) [255]. The extracted CD4 molecules are then delivered to the proteasome for their degradation [254-256]. Interestingly, while substrates of β-TrCP get degraded, Vpu is able to escape degradation, even though it actually possesses some ubiquitination sites.

Along with recruitment of β-TrCP E3 ubiquitin ligase complex, degradation of CD4 requires a physical interaction between Vpu and CD4. This binding involves cytoplasmic residues of both CD4 (membrane-proximal residues) and residues within both helices of Vpu [259-264]. Moreover, residues within the TMD of both proteins are also important for the interaction [255, 265-267]. Noteworthy, replacement of the CD4 TMD with a vesicular stomatitis virus G glycoprotein (VSV-G) TMD protected the resulting protein from Vpu-mediated degradation [266]. A similar experiment in which the Vpu TMD was replaced with a VSV-G TMD also abolished both the ability of Vpu to retain CD4 molecules within the ER as well as Vpu-mediated CD4 degradation [255]. In fact, under conditions where CD4 degradation was inhibited, interactions through the Vpu TMD were sufficient to mediate CD4 retention within the ER [267]. Indeed, Magadan and Bonifacino have further underscored the importance of several residues within the Vpu TMD towards CD4 degradation, especially highlighting a role of a highly conserved Trp residue in enabling translocation of CD4 from the ER membrane to the cytosol, as well as a contribution of Val and Ser residues towards ER retention via mechanisms that are still unclear [267]. It has been suggested that the attenuation in function emanating from these Vpu TMD residues is likely a result of structural perturbations in the membrane helix of the protein [268]. Indeed, there are non-specific interactions between the TMD domains of the two proteins that are essential for stabilizing the Vpu-CD4 interactions and, therefore, important for Vpu-mediated CD4 degradation [267, 268]. Taken together, maintenance of a proper TMD structural orientation is important for the ability of Vpu to target CD4.

A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CD4
Nef
AP-2
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Recycling Endosome
Early Endosome
Late Endosome
TGN
medial-Golgi
cis-Golgi
ER
Cytosol
Lysosome

B

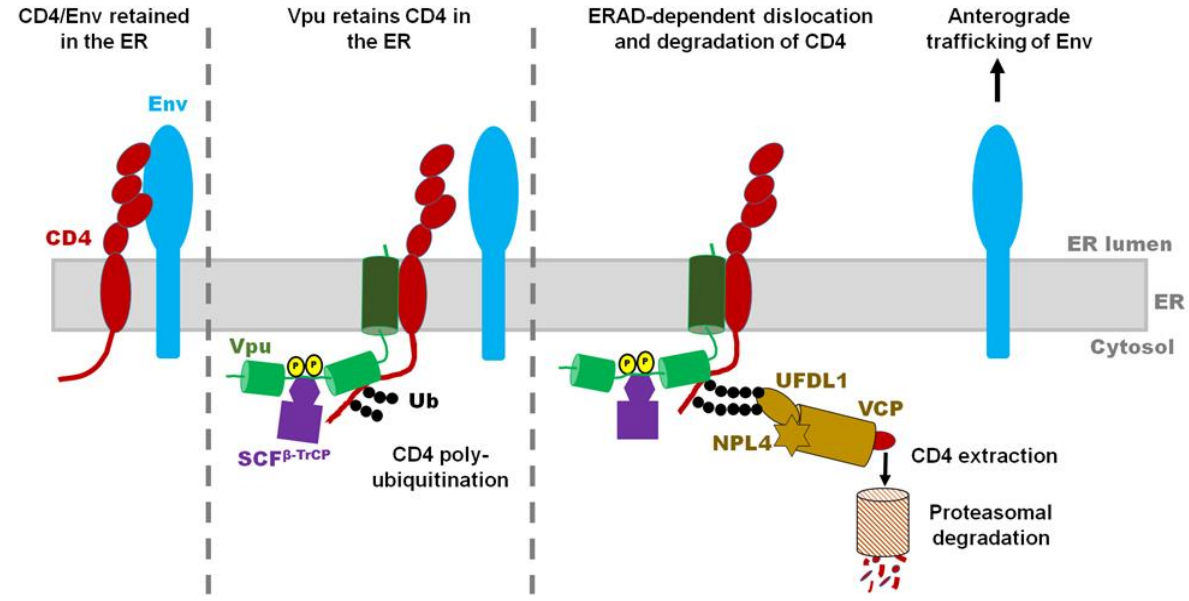
CD4/Env retained in the ER
Vpu retains CD4 in the ER
ERAD-dependent dislocation and degradation of CD4
Anterograde trafficking of Env
Env
CD4
ER lumen
ER
Cytosol
Vpu
Ub
SCF β-TrCP
CD4 poly-ubiquitination
UFDL1
VCP
NPL4
CD4 extraction
Proteasomal degradation

Figure 1.7. HIV-mediated regulation of CD4 expression. A) Nef-mediated depletion of CD4 from the surface. Nef directly interacts with both AP-2 and the CD4 cytoplasmic tail, resulting in endocytosis and targeting of CD4 for lysosomal degradation. **B)** Env and Vpu mediated targeting of intracellular CD4. gp160 (Env precursor), binds and retains newly synthesized CD4 within the ER. In the presence of Vpu, Vpu directly interacts and retains the CD4 molecules within the ER, allowing Env to dissociate from the receptor. Ultimately, Vpu delivers the CD4 molecules to an ERAD pathway for proteasomal degradation.

1.7 Vpu-mediated BST2 antagonism

Despite it being close to a decade since the identification of BST2 as a restriction factor targeted by Vpu, and the great interest in the subject, the exact mechanisms by which Vpu overcomes BST2 are still incompletely understood and a subject of intense investigation. Nonetheless, there have been significant advances in our understanding of how Vpu enhances the release of nascent virions, thereby preventing the antiviral activities of BST2. Overall, Vpu modulates the amount and/or distribution of BST2 at the plasma membrane to ensure the restriction factor is removed from virus assembly sites, where it would otherwise exert its tethering function. In this context, Vpu has been shown to employ BST2 intracellular trapping and sequestration, surface downregulation and displacement mechanisms (Figure 1.8) [76, 77, 269-272].

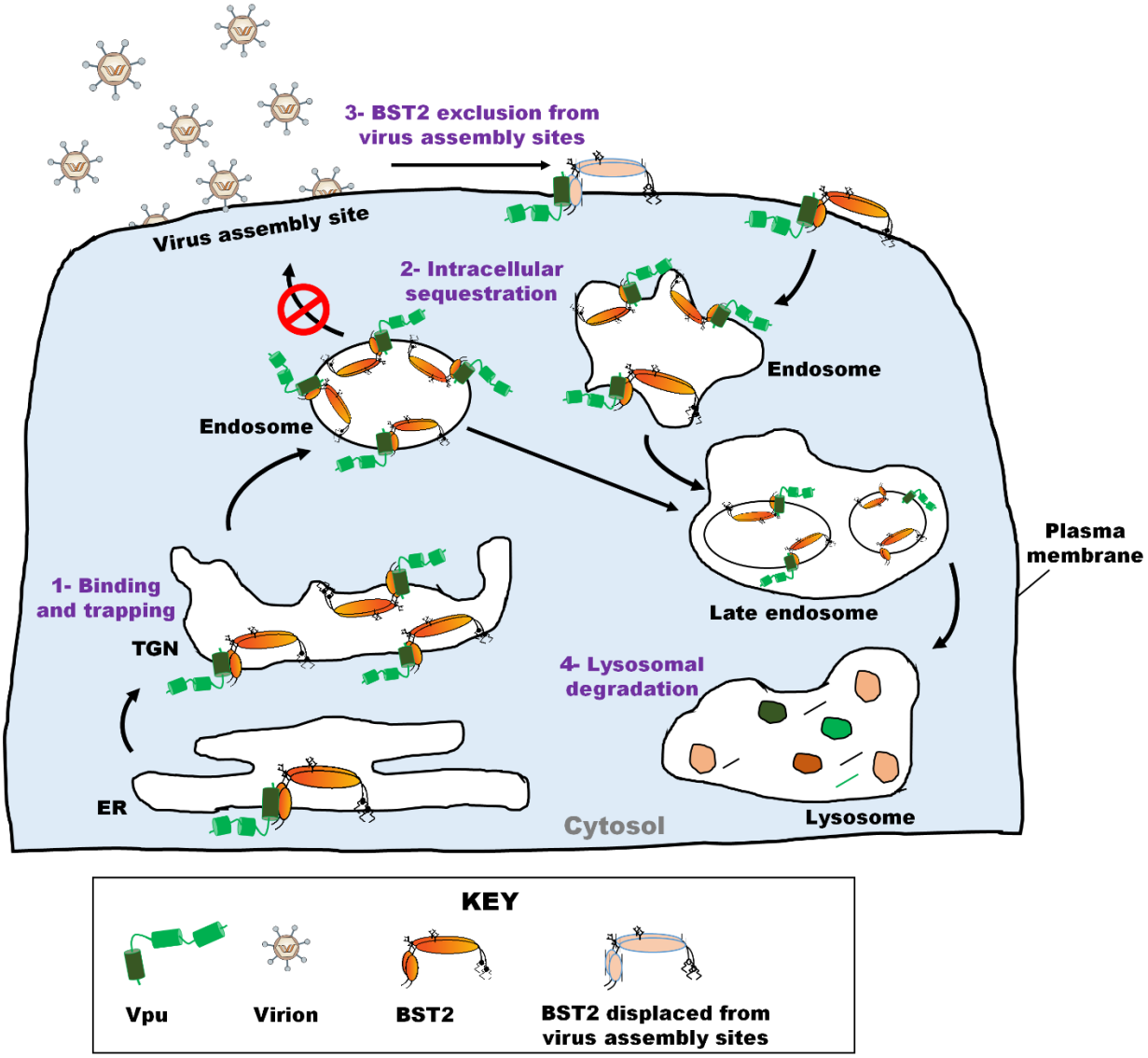

Figure 1.8: Vpu-mediated BST2 antagonism. Vpu binds and traps BST2 within intracellular compartments including the TGN (1). Vpu also mediates recruitment of AP-1 complexes that actively sequester Vpu-BST2 complexes within endosomal compartments (2). At the surface, Vpu recruits AP-2 that displaces BST2 from virus assembly sites (3). Ultimately, the sequestered Vpu-BST2 complexes are targeted for lysosomal degradation (4).

1.7.1 Binding and cellular localization

The importance of the Vpu TMD for enhancement of virus release was reported long before BST2 was identified [237, 265, 273]. We now know that a direct interaction between Vpu and BST2, which occurs via their respective helical TMDs, is critical for BST2 antagonism [269, 272, 274, 275]. Nuclear magnetic resonance (NMR) and immunoprecipitation studies indicate that the interaction requires a Vpu $A_{10}xxxA_{14}xxxA_{18}xxxW_{22}$ hydrophobic TMD interface, believed to be the primary determinant for interaction, hence antagonism. Several other residues within the Vpu TMD, including I4, V6, A7, V20, V21, S23, V25, I26, I27, together with the extracellular N-terminal residues Q2 and P3, have been shown to be involved to a certain extent, even though their functional importance remains to be tested [272, 275]. From the BST2 side, residues including L23, L24, G25, G27, I28, V30, I33, I34, I36, L37, P40, L41 and T45 confer sensitivity to Vpu [272, 275-279]. A recent crosslinking study has identified L22, L29, I43, F44 and I46 as relevant for binding to Vpu, but it remains unknown whether the contacts they mediate have any functional significance [272]. In addition to the identity of the residues, the overall structural configuration of the TMDs is important for function. To ensure access to the BST2 TMD, the Vpu TMD $A_{10}xxxA_{14}xxxA_{18}xxxW_{22}$ interacting interface orients such that it aligns with the bulky residues on the BST2 TMD, which likely fit between the alanine-based interface of the Vpu TMD [275, 280]. Consistently, the Vpu TMD interface faces away from the cytoplasmic domain of the protein, ensuring accessibility to its binding partners [280]. Moreover, the TMDs of both proteins have to adopt optimal tilt angles that are permissive to a direct interaction [275] [281]. Along this line, correlations between the extent of Vpu binding and tilt angles

have since been reported in Vpu TMD mutants [281]. Similarly, and quite remarkably, mimicking the TMD sequences of nonhuman orthologs of BST2 that are insensitive to Vpu demonstrated that their unresponsiveness is a consequence of altered tilt angles of the BST2 TMD, and not an intrinsic inability to interact with Vpu [275]. Indeed, NMR evidence demonstrated that such BST2 TMD mutants were unable to interact with Vpu when confined within lipid bilayers, yet they efficiently interacted with Vpu in solution [275]. Altogether, an intricately regulated orientation or positioning of the TMDs, together with the direct contacts between Vpu-BST2 TMD residues are critically important for the ability of Vpu to antagonize BST2.

The exact cellular compartment(s) in which Vpu encounters and binds BST2 have not been precisely defined. The interaction is believed to occur in the ER and/or the TGN. Vpu is capable of interacting with its membrane associated partners within the ER, as described for CD4. However, BST2 antagonism is mediated post-ER, consistent with the distinctiveness in mechanisms through which Vpu targets the two host proteins [268]. Vpu from HIV-1 clade B (VpuB) primarily accumulates in the TGN and, to an extent, endosomal compartments [282, 283]. Intriguingly, HIV-1 clade C Vpu (VpuC) localizes both at the plasma membrane and within the TGN, likely due to the variability in the cytoplasmic domain sequence, including the presence and/or positioning of the putative di-leucine [(D/E)xxxL(L/V/I/M)] as well as tyrosine-based [Yxxϕ, where ϕ is a bulky hydrophobic residue] motifs (Figure 1.9) [284]. Nevertheless, localization within the TGN allows Vpu to intercept both newly synthesized and recycling pools of BST2. Indeed, BST2 antagonism positively correlates with the accumulation of VpuB within the TGN [283]. In keeping with this, BST2 counteraction was attenuated following mutations

of the intervening arginine and lysine residues in VpuB Yxxϕ (YRKI), which reduced Vpu localization within the TGN even though these residues would have been predicted to be inconsequential for Vpu trafficking [239, 283]. All considered, the propensity of Vpu to localize within the TGN allows it to intercept and trap BST2 molecules within these compartments, and may then re-route or mediate trafficking of Vpu-BST2 complexes to compartments that are not compatible with transit to the cell surface, as outlined below.

1.7.2 Vpu subverts Vpu-BST2 complex trafficking to the cell surface

The current consensus on Vpu-mediated BST2 antagonism centers around a role of Vpu in usurping/hijacking the host clathrin-dependent trafficking machinery in order to potently block surface trafficking of both newly synthesized and recycling BST2. Clathrin-dependent sorting of proteins generally necessitates interactions between the cargo and components of the clathrin coats or vesicles such as the heterotetrameric family of AP complexes [285, 286]. Among the currently five members of the AP family, AP-1 and AP-2 have been implicated in Vpu-BST2 trafficking [240, 287]. Whereas AP-1 mediates cargo sorting between the TGN and the endosomes, AP-2 functions at the plasma membrane where it facilitates sorting into clathrin-rich domains and endocytosis of cargo [285, 286].

An acidic di-leucine sorting motif (ExxxLV) within the second helix of most Vpu variants, except those from clades C and F (Figure 1.9), has been demonstrated to facilitate recruitment of the clathrin AP complexes [240, 271, 272, 287]. In addition to the sorting signal, the interaction between Vpu and BST2 is absolutely required for the formation of ternary AP-Vpu-BST2 complexes [240]. Rather unexpectedly, Kueck and

colleagues further demonstrated a requirement for Vpu phosphorylation at the conserved serine residues within the DSGxxS motif, which likely induces conformational changes that facilitate efficient recruitment of the AP complexes [240]. Moreover, the Vpu first helix, which forms non-canonical contacts with AP complexes, and the tyrosine-based motif within the cytoplasmic tail of BST2 are also necessary [240, 287]. The dileucine motif commits BST2 to a clathrin-rich endosomal compartment that is yet to be precisely defined [271]. This intracellular sequestration mechanism, which is conceivably targeting both *de novo* synthesized and recycling pools of BST2, results in the depletion of BST2 levels at the cell surface. Moreover, by virtue of mediating recruitment of the AP-2 complex that functions exclusively at the plasma membrane, the motif facilitates exclusion or lateral displacement of BST2 from viral assembly sites to clathrin-rich domains along the plasma membrane [240, 272]. This ability of Vpu to sort BST2 molecules away from viral assembly sites along the plasma membrane likely accounts for earlier reports to the effect that enhancement of virus release could be observed in the absence of surface BST2 downregulation [288]. Engagement of AP-2, which mediates cargo endocytosis from the plasma membrane, could also explain the minor effect of Vpu on the rate of BST2 endocytosis [270, 289, 290].

The actively sequestered Vpu-BST2 complexes are ultimately targeted for ESCRT-mediated endo-lysosomal degradation following recruitment of β-TrCP2 to the Vpu DSGxxS motif (Figure 1.8) [271, 291-294]. It is important to note, however, that even though BST2 degradation is dependent on the presence of both the acidic di-leucine sorting signal and the 51-DSGxxS-56 motif, it is generally dissociable from enhancement of virion release, especially in conditions of low BST2 expression levels.

Indeed, CK-II mediated phosphorylation of Vpu S52/56, but not β-TrCP recruitment, was critical for AP recruitment and enhancement of virus release [240]. As such, clathrin-based sorting appears to be the essential first step in BST2 antagonism, with BST2 degradation as a latter event in the process. Thus, the 51-DSGxxS-56 motif plays two independent roles in BST2 antagonism: regulating formation of ternary AP-Vpu-BST2 complexes as well as recruiting β-TrCP to facilitate BST2 ubiquitination and targeting to lysosomes for degradation [240].

Interestingly, the short isoform of BST2 is insensitive to downregulation and degradation mediated by Vpu. The lack of an endocytic tyrosine-based motif together with the lack of a putative serine-threonine (STS) motif shown to be important for BST2 ubiquitination and downregulation from the cell surface may account for this [181, 295, 296]. The reasons as to why Vpu has evolved to efficiently target (downregulate and degrade) only the long isoform of tetherin remain a mystery. It is tempting to speculate that there may be some replication or pathogenesis advantages associated with leaving a pool of BST2 at the surface. Could it be due to the fact that the short isoform dominantly blocks BST2-related signal transduction? It can also be argued of course that the virus is able to efficiently overcome any residual antiviral activities mediated by the short isoform, albeit via different mechanisms. Indeed, it has recently been shown that HIV-1 effectively displaces the short BST2 from virus assembly sites, thereby freeing and allowing BST2 to interact with ILT7, which blocks IFN production by pDCs [228]. Very interestingly then, by differentially and exquisitely modulating the surface levels or distribution of the two BST2 isoforms, Vpu enhances virus release while inhibiting both NF-κB/TLR-mediated signalling and IFN-I production by pDCs.

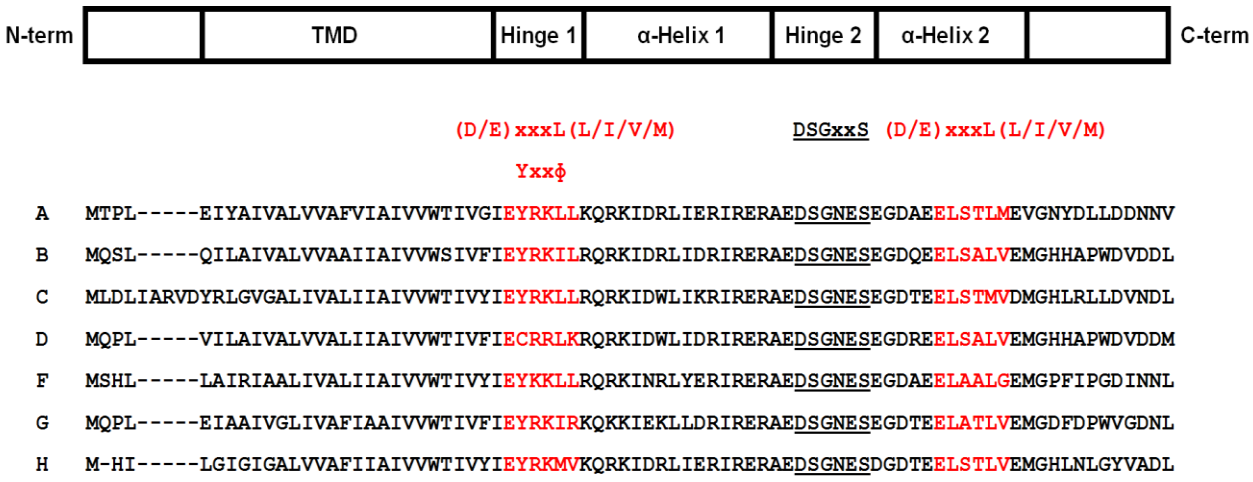

Figure 1.9: Vpu consensus sequences. Schematic representation of the Vpu structural domains. Vpu consensus sequences of HIV-1M Clades A, B, C, D, F, G, H derived from the Los Alamos HIV sequence database. [(D/E)xxxL(L/V/I/M), where x represents any residue] and [Yxxϕ, where ϕ is a bulky hydrophobic residue and x can be any amino acid] denote acid-based di-leucine and tyrosine-based motifs, respectively. Within the hinge 2 region is a DSGxxS motif that harbors two Ser residues that are phosphorylated by CK-II.

1.8 A potential role of BST2 and CD4 in enhancing antibody-mediated control of HIV infection

1.8.1 Antibodies against HIV-1

Hardly two years following HIV-1 isolation, it was already reported that sera from infected patients generate antibodies (Abs) as means to control the infection [297, 298]. Still in 1985, these anti-HIV antibodies were demonstrated to target antigens on the viral Env glycoproteins, which are expressed on the surfaces of virions in infected cells [26]. To date, the viral Env antigens continue to be the major and only functionally relevant targets of Abs. While having the 'target in sight' was perhaps good news, it was quickly realized that HIV-1 Env has an enormous diversity, displaying up to 35% variation between viruses from different subtypes [299]. Additionally, analyses of anti-Env Abs at various stages post-infection, including during acute and chronic phases of infection, indicate that Ab responses are rather sophisticated and evolve over time [300, 301]. The anti-Env Abs can be categorised into two major classes: neutralizing and non-neutralizing Abs, based on their ability to neutralize virus isolates. Neutralization in this context defines the loss of infectivity resulting from an interaction between an Ab and an Env functional spike of a cell-free virion.

1.8.1.1 Neutralizing Abs

Neutralizing Abs (NAbs) interact with their conserved epitopes on Env, and prevent infection of target cells. NAbs are either strain-specific or broadly neutralizing. Strain-specific NAbs recognize epitopes on the Env functional spike of the infecting or

autologous virus strain, but fail to recognize and neutralize other viral strains. Given they are generated relatively early (from about three months) following infection, they would be considered of interest in HIV control [302]. However, variations in their epitopes easily result in neutralization escape, rendering them ineffective, especially considering the high mutation rate associated with HIV [303]. Indeed, rapid escape of HIV from NAb response has been well documented in many longitudinal studies (reviewed in [299]). Through multiple cycles of viral escape and Ab affinity maturation, these NAbs can evolve into broadly NAbs (bNAbs) [304]. Unlike the strain-specific, bNAbs have a much improved breadth of coverage, neutralizing a wide spectrum of different HIV strains. bNAbs generally remain potent in spite of the variability on the Env trimer, likely because they may target epitopes whose amino acids or sequence show a conserved character. Generation of bNAbs has indeed been considered the 'holy grail' in the Ab-mediated approaches against HIV-1. Unfortunately, these bNAbs take years to develop and, even then, only up to ~30% of infected individuals actually end up developing such broadly acting Abs [301, 305-307].

Thus far, there have been at least five mapped bNAb epitope clusters, located at different sites on the Env trimer (Figure 1.10).

i) *CD4 binding site (CD4-bs) on gp120:* The CD4-bs is an interesting target because the virus uses it for virus entry and, therefore, has to be conserved and cannot be occluded or hidden by conformational changes or the glycan shield. Abs targeting this region include 3BNC117 [308, 309]. In a phase 1 clinical trial, 3BNC117 infusion reduced the viral load by up to a 2.5 log, and viraemia

remained suppressed for 4 weeks [310]. Very recently, 3BNC117 was reported to accelerate, albeit *via* unknown mechanisms, the emergence of bNAbs, leading to protection even against neutralization-resistant (Tier 2) strains [311].

ii) *Quaternary first and second variable domains (V1/V2 Apex) on gp120:* This is an apex resulting from a convergence of V1/V2 conserved domains as well as conserved regions of the V3 loop. This site is usually protected by densely packed glycans, especially N156 and N160, as well as hypervariable V1/V2 loops. Antibodies binding to this side, such as PG9 [312], require unusually long (26-39 residues) anionic heavy chain complementarity determining region loop three (CDR-H3) [312, 313]. PG9 preferentially binds to the quaternary trimer, and very poorly to the monomeric gp120 [314].

iii) *Glycan patch centered around the third variable domain (V3) base on gp120*: Similar to the V1/V2 apex, the V3 epitopes are protected by glycans and bNAbs require long CDR-H3s in order to access the residue peptide epitope. Several Abs targeting this site have been described, and include PGT126 [314], PGT121 [314, 315] and 10-1074 [309]. A majority of these Abs typically show a high dependency on N332 glycans, even though other glycans may also be implicated. PGT126 recognizes an epitope that consists of *N*-glycans associated with N331 and N332, together with amino acid residues within the V3 loop [315]. The clonally related PGT121 and 10-1074 bind to glycans attached to both N332 and a V1/V2 region, together with residues that are at the base of the V3 loop

[316]. Interestingly, a single infusion of PGT121 in rhesus monkeys chronically infected with a pathogenic SHIV virus rapidly reduced (up to a 3.1 log) plasma viraemia to undetectable levels, and further reduced proviral DNA in peripheral blood and tissues without evidence of viral escape [317]. In a different study, the 10-1074 Ab also rapidly reduced virus loads to undetectable levels for up to a week [309]. Unfortunately, virus rebound was then observed, accompanied by neutralization resistant variants [309]. A rather different bNAb, 2G12, does not penetrate the glycan shield, hence makes no peptide contact [318, 319]. Instead, 2G12 binds to the tips or terminals of a cluster of high-mannose glycans associated with N295, N332, N339 and N392. Thus, whereas 2G12 recognizes quaternary epitopes, PGT121, PGT126 and 10-1074 Abs can bind monomeric gp120.

iv) *gp120 and gp41 interface:* Epitopes within the gp120-gp41 interface have recently been shown to be targets of bNAbs, including 8ANC195 [320] and 35022 [321, 322]. Binding of 8ANC195 is dependent on glycans at positions N234 and N276 on gp120, while the Fab light chain of the bNAb interacts with the gp41 [320]. The epitope for 35022 bNAb lies within a conserved region between gp120 and gp41, close, yet distinct, to the N234 used by 8ANC195. Binding of 35022 is strengthened by CD4 attachment, which induces some rearrangements that creates sufficient room for the Ab as it approaches the binding site [321]. The epitope of 35022 is highly dependent on the quaternary Env structure, unlike that of 8ANC195. As such, 35022 binding is limited to the

trimeric (closed and pre-fusion) form of Env, and is dependent on a few gp120 glycans attached to N88 (at the gp120-gp41 interface), N230 and N241 [321].

v) *Membrane proximal external region (MPER) on gp41:* A conserved stretch of hydrophobic residues linking the TMD and the ectodomain of gp41 present linear epitopes that are targeted by Abs including 7H6 and 10E8 [321, 322]. These Abs are believed to target the conformation of the gp41 fusion intermediate that brings together the viral and cellular membranes. 10E8 and 7H6 are somatic variants of the same immunoglobulin G (IgG) clone (IgG1 Abs), sharing the same heavy chains and a difference of only two residues in their light chains [322]. They are the most potent of the anti-MPER bNAbs described thus far, with 10E8 neutralizing more than >98% of viruses tested [322]. In fact, one study reported that 10E8 conferred complete protection following mucosal challenge of rhesus macaques with simian HIV (SHIV) [323].

1.8.1.2 Non-neutralizing Abs

Non-neutralizing Abs (nNAbs), on the other hand, target conserved HIV-1 Env epitopes that are occluded on the native Env trimer spike, hence these Abs are unable to bind and prevent entry of virions into susceptible cells. The epitopes are only revealed during sequential interactions between the viral Env and the host cell surface receptor CD4 and coreceptor CCR5 or CXCR4. To illustrate, Env binding to CD4 results in conformational changes within the trimer that then uncovers conserved functional

sites necessary for coreceptor engagement which, in turn, allows for further Env remodelling events leading to viral fusion. This highly regulated, multi-stage successive Env binding events is a key mechanism through which HIV-1 masks critical functional sites from the human Ab response. Nevertheless, it is during these Env rearrangement events that Ab binding sites within the inner domains of gp120 and those overlapping the coreceptor binding sites (coR-bs), among others, are transitionally exposed. Several nNAbs have been isolated from HIV-1 infected patients, including monoclonal Abs A32 and 17b. A32 is a CD4-dependent or CD4-induced (CD4i) Ab that recognizes a conformational epitope in the first constant (C1) and variable V1/V2 regions of gp120 [324, 325]. This epitope, overlapping the inner domains of gp120, is typically buried in the Env trimer, and only becomes exposed following an Env-CD4 interaction during entry [326, 327]. 17b, on the other hand, binds to a CD4i discontinuous epitope on gp120 that overlaps with the coR-bs [328-330]. As would be predicted, gp120 mutations at positions D368, E370 (both within C3 region), W427 or D457 (C4 region), which disrupt Env-CD4 binding, attenuate binding of these CD4i Abs [328].

Even though they do not neutralize viruses, at least based on *in vitro* assays, nNAbs are very important in the control of HIV infection, especially in combination with cellular effector functions such as killing of infected cells, as discussed below. nNAbs are especially interesting because they arise very early (within days or weeks) during infection [331, 332], and harnessing their potential may therefore significantly impact viral pathogenesis. Indeed, nNAbs have been shown to offer protection from virus infection as well as negatively impact disease progression [333-339].

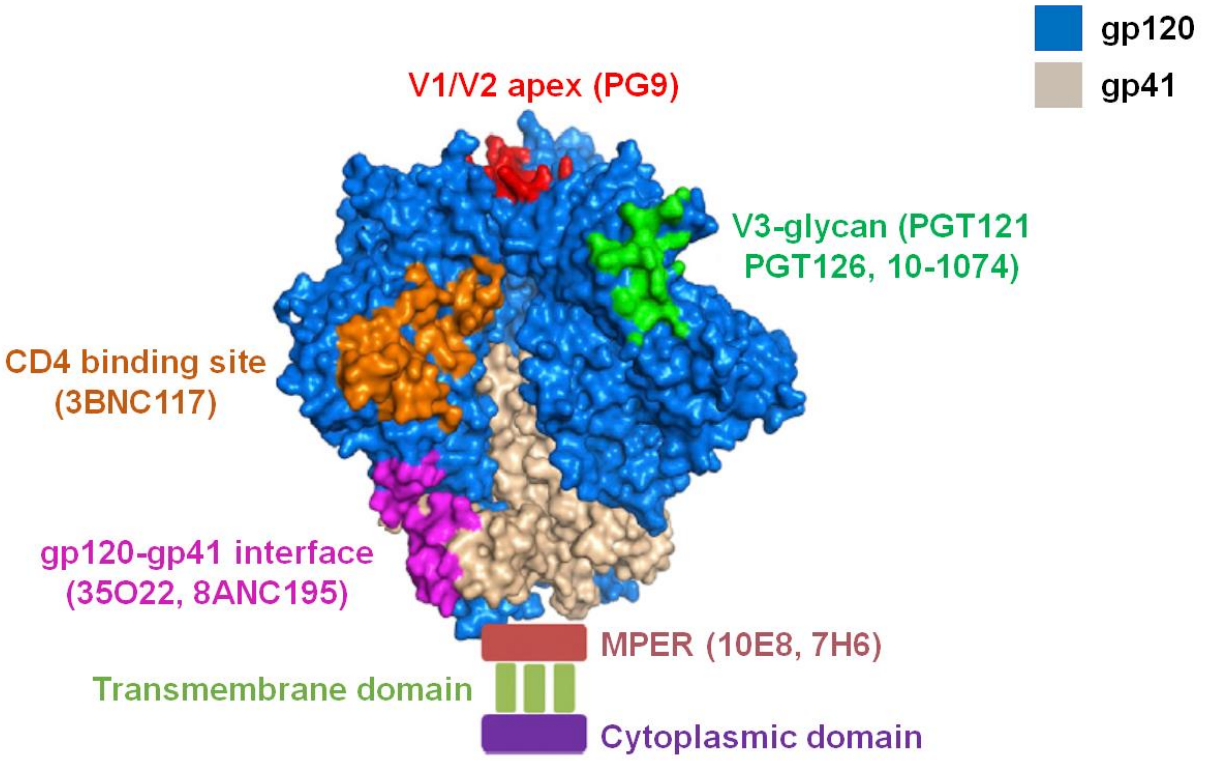

Figure 1.10: bNAb epitope clusters on Env. Antibodies recognizing the CD4 binding site, V1/V2 apex, V3 base glycans, gp120-gp41 interface and MPER epitopes are also indicated. The indicated Abs are used in our studies. Not shown in the figure is 2G12, a bNAb that recognizes quaternary epitopes (N295, N332, N339, N392 glycan tips). Binding sites for the CD4i Abs 17b (CoR-bs) and A32 (V1/V2 & C1) are occluded on the Env, and are only revealed following engagement of CD4. Figure adapted from [340].

1.8.2 Fc receptor-mediated Ab function in HIV infection

Abs can inhibit HIV-1 infections through neutralizing the virus and/or mediating the destruction of infected cells through mechanisms including antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and antibody-dependent cell-mediated viral inhibition (ADCVI). Killing of infected cells is dependent on Fc receptors (FcRs) expressed on immune cells, which bind the Fc (Fragment, crystallizable) region of the Abs, leading to exertion of function. Eloquent studies have indeed demonstrated that monoclonal Abs that were defective for FcR engagement were remarkably less protective against SHIV challenges compared to those that efficiently engaged FcRs, demonstrating both the neutralizing and FcR-driven effector functions [341, 342]. Several other studies have corroborated the importance of the effector cell mechanisms for prevention of infection and post-infection viral inhibition [335, 336, 339, 343-345], among others.

1.8.2.1. ADCC

ADCC is a cell lysis mechanism in which specialized Fc receptors (FcγRIIIa, also referred to as CD16) on innate immune effector cells recognize and bind the Fc portion of Abs that coat the surface of antigen-expressing (infected) cells and mediate killing of the infected cells (Figure 1.11). Among the effector cells of the immune system such as NK cells, monocytes and neutrophils, NK cells play a predominant role in mediating ADCC of HIV-infected cells [346-349]. Indeed, NK cells express high levels of only the CD16 receptor [350], the expression of which directly correlates with the potency of

ADCC responses [351]. Crosslinking or aggregation of Ab-bound CD16, which is necessary for HIV-specific ADCC, activates NK cells, leading to degranulation and secretion of cytotoxic granules such as perforin and granzyme B, and may also lead to secretion of cytokines such as chemokines, and tumor necrosis factor (TNF) [352, 353] .

The role of ADCC in HIV infections has long been acknowledged, with first reported evidence in 1987 (reviewed in [354]). Since then, numerous studies conducted in HIV-infected humanized mice and SIV-infected monkeys, as well as in HIV-infected patients have correlated ADCC activity with protection from HIV/SIV infection, lower viral loads, high $CD4^+$ T cell counts, and slower disease progression (reviewed in [355, 356]). Additionally, there is now preliminary evidence indicating that the presence of anti-HIV nNAbs in breast milk reduces the risk of vertical transmission even in cases when the plasma viraemia in the mother is high [357]. Furthermore, Milligan and colleagues reported a survival benefit of passively acquired ADCC activity in HIV-infected infants [358].

While most vaccine studies had been unquestionably successful in interfering with either viral susceptibility or progression of viral infection in non-human primate models, efforts in human vaccine trials had been frustrating as there was no efficacy or significant protection observed. The vaccine studies in animal models provided evidence for a role of Ab-mediated immunity, primarily ADCC activity, in the observed viral protection or post-infection viral control. Interestingly, even though there was no efficacy, analyses of a Vax004 Phase III vaccine trial in humans [359] revealed an inverse correlation between HIV infection rate and activities of vaccine-induced Abs [360]. These Ab activities were later shown to mediate ADCVI, but not virus

neutralization, which raised the possibility of an ADCC contribution [344]. After several disappointing human trials, renewed optimism came following the modestly successful RV144 Phase III vaccine trial that showed a statistically significant 31.2% efficacy or protective immunity after 42 months following vaccination [333]. In fact, further analyses of the RV144 trial indicated that protection against HIV acquisition had been up to 60.5% by the end of the first 12 months [361].

Importantly, follow up analyses of the RV144 trial indicated that it elicited Abs against various Env epitopes that are known to be targets for ADCC, re-kindling the interest in the role of ADCC in protective immunity [334, 361]. These vaccine-induced Abs, which were later shown to be non-neutralizing, targeted epitopes within the C1 and V1/V2 regions of Env [334, 362]. Intriguingly, these epitopes are also recognized by a highly competent ADCC-inducing, CD4i nNAb A32 isolated from an HIV-infected patient more than two decades ago [324-326]. As such, the RV144 vaccine trial induced A32-like nNAbs whose activities correlated with a reduction in HIV infection and, possibly, a post-infection control of viraemia [334, 335, 339]. Further analyses of the vaccine-induced Abs associated with protection from infection revealed that they were mainly of the IgG1 and IgG3 isotypes [339, 363, 364]. Interestingly, the RV144 trial also elicited IgA Abs that competed with the IgG Abs for the same epitopes, resulting in a block in ADCC mediated by the IgG anti-Env Abs and, consequently, reduced protection from HIV acquisition [335, 339].

Taken together, the correlations between ADCC activity and both HIV protection and post-infection control unambiguously demonstrate the importance of FcR-dependent effector functions in the case of nNAbs, which have no or minimal

neutralization functions. On the other hand, the roles of NAbs towards controlling HIV infection have remained somewhat unclear. Their neutralization function, however, has been demonstrated to confer protection against viral infections [365, 366]. Nevertheless, convincing evidence also support a significant contribution of NAb FcR-mediated effector functions towards prevention of viral infections [341, 342]. The neutralization-independent mechanisms through which these FcR-dependent effector functions inhibit viral replication are yet to be fully elucidated.

1.8.2.2 Other (non ADCC) Fc receptor-mediated Ab function in HIV infection

a) *ADCP*: ADCP is a mechanism whereby Fc-FcR engagement leads to phagocytosis of both opsonized virions and opsonized infected cells. Consequently, free virions and infected cells may be eliminated from circulation, allowing for the control of infection. Cells capable of acting as phagocytes include; macrophages, monocytes, neutrophils, dendritic cells and mast cells, all of which express FcγRIIa (CD32a), a widely expressed FcγR implicated in phagocytosis [367]. Support for involvement of ADCP in HIV infection has mainly been through studies correlating CD32a polymorphisms with both HIV protection and disease progression [368, 369] as well as correlations between reduction in CD32a expression levels and progressive infection, which was shown to be due to an impairment in ADCP activity [370]. Very recently, it was reported that a vaccine that elicited nNAbs capable of mediating phagocytosis led to protection against low-dose SHIV challenges in monkey models [371].

b) *ADCVI:* ADCVI is an overall measure of multiple inhibitory effects of Abs on HIV infection, including neutralization and ADCC. In ADCVI, an infected target cell, expressing the Ab-coated foreign antigen, interacts with an effector cell that expresses at least one FcγR, leading to cell lysis (via mechanisms such as ADCC) and creation of an antiviral state via production of cytokines/chemokines, altogether leading to viral inhibition. Given the lack of distinction in these Fc-mediated functions, ADCVI assays are usually not preferable in mechanistic studies aimed at dissecting Ab contributions towards viral neutralization and/or ADCC. Nonetheless, several studies have demonstrated a positive role of ADCVI in viral inhibition [344, 345, 372-374].

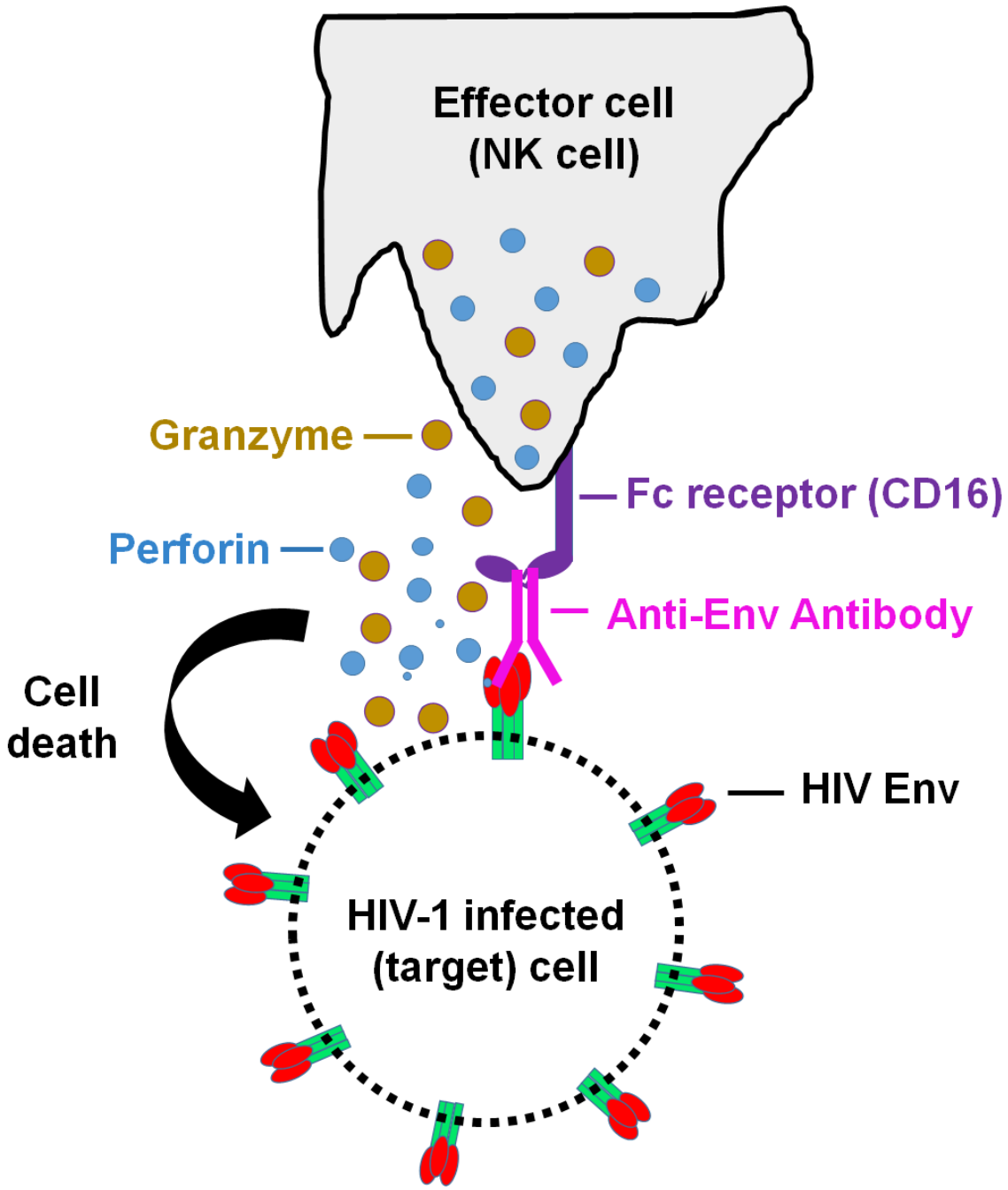

Figure 1.11: ADCC principle in the context of HIV-1 infection. In ADCC, CD16 Fc receptors on innate immune effector cells such as NK cells recognize and bind the Fc portion of Abs that coat the surface of infected (target, antigen-expressing) cells and mediate killing of the target cells. Binding of the Ab to CD16 activates the NK cells, leading to degranulation and secretion of cytotoxic granules such as perforin and granzyme B that mediate cell lysis.

1.9 Rationale and objectives

1.9.1 Determinants governing Vpu functions

Several regions within VpuB have been demonstrated to govern the ability of the protein to counteract BST2. Among others, these include the Vpu TMD that is absolutely critical for the direct interactions between the two proteins, and the helix-2 di-leucine trafficking signal (ExxxLV) that mediates recruitment of clathrin complexes AP-1 and AP-2, which commit Vpu-BST2 complexes into clathrin-rich endosomal compartments that preclude surface trafficking of the complexes or mediate lateral displacement of BST2 away from virus budding sites [271, 272, 274, 275, 375]. Noteworthy, Vpu possesses other highly conserved regions including a transmembrane-proximal hinge region whose functional relevance has not been elucidated. Interestingly, within this transmembrane-proximal hinge region are residues that resemble an acidic di-leucine motif (ExxxIL), which have not yet been associated with any function. Of note, the membrane-proximal VpuB ExxxIL and membrane-distal ExxxLV mirror the MHC II-associated invariant chain membrane-proximal ExxxML and membrane-distal DxxxLI acidic di-leucine-based signals that are independently sufficient to recruit AP cofactors and target the transmembrane protein to endo-lysosomal compartments [376, 377]. Given the functional relevance of Vpu cellular localization, AP recruitment and trafficking of Vpu-BST2 complexes for BST2 counteraction, this raises the possibility that the hinge region could be important for the BST2 antagonistic activity of the protein. As such, we sought to investigate the roles of

these highly conserved hinge region residues, encompassing this putative di-leucine sorting motif, towards the ability of Vpu to target BST2.

1.9.2 BST2 and CD4 play critical roles in sensitizing infected cells to ADCC

The study above aimed to identify vulnerable regions within Vpu that can be targeted in order to enable BST2 to efficiently mediate virus restriction. Noteworthy, aside from reducing amounts of free virions released, BST2-mediated virion tethering is increasingly being recognized for its roles in modulating immune responses to HIV infection [218, 219, 228]. These roles, including evasion of immune sensing, are currently of great interest in approaches towards controlling HIV infection. HIV-infected patients produce Abs that primarily target the Env proteins, and some of these Abs (e.g. CD4i A32) can mediate killing of infected cells via ADCC [334]. Immune correlate studies from the modestly successful RV144 vaccine trial [333-335, 339] showed a positive correlation between protective immunity and the presence of nNAbs capable of mediating ADCC. The ADCC epitopes on Env were similar to those targeted by A32. Given A32 recognition is dependent on Env-CD4 interaction, we investigated the role of CD4 in sensitizing infected cells to ADCC. Considering that despite the presence of ADCC-mediating Abs, HIV infection continues to spread within and between individuals, we hypothesized that HIV has evolved to shield infected cells from such killing. In this context, we predicted that HIV uses its Vpu and Nef proteins to down-modulate CD4 molecules and, as such, interfere with unveiling of ADCC-competent epitopes on Env.

In addition, given the role of BST2 in crosslinking Env-containing nascent virions to the surface of infected cells, we hypothesized that this physical tethering of virus

particles enhances the quantity of Env epitopes at the surface and therefore augments killing of infected cells via ADCC. In this context, by antagonizing BST2, we further hypothesized, Vpu shields infected cells from such killing.

Based on the results from the above study showing a role of both BST2 and CD4 in enhancing ADCC, and a contribution of Vpu to ADCC in a CD4-independent manner, we asked if CD4-independent Abs (bNAbs) were also capable of mediating ADCC. Indeed, the ADCC potential of bNAbs had not been characterized, yet about 10-30% of HIV-infected patients can develop anti-HIV Abs capable of broadly neutralizing viruses [301, 305-307]. Earlier studies demonstrated a role of bNAb Fc-mediated effector functions, but the mechanism through which the Fc receptors conferred protection against HIV remained unclear [341]. We therefore investigated whether such neutralizing Abs can mediate ADCC, and whether tethering of Env-containing virions at the cell surface would influence such a response. Lastly, we tested whether the antagonistic activity of Vpu modulates the susceptibility of infected cells to such lysis.

1.10 Significance of research

More than three decades since the isolation and identification of HIV as the etiological agent for AIDS, there is still neither a cure nor an effective vaccine. HIV is particularly intelligent at usurping host cell machineries and processes to aid its replication and dissemination. As such, it is able to efficiently replicate while evading the humoral immune response. One of the most potent host factors, BST2, restricts virus replication by tethering nascent virions to the surface of infected cells, thereby preventing their release. Additionally, recent studies have revealed important immunomodulatory roles of BST2 that likely influence or interfere with HIV pathogenesis [218, 219, 228]. Indeed, BST2 modulates various signaling events that essentially govern the induction of IFN production, which in turn regulates generation of an antiviral state following HIV infection. As with most other restriction factors, HIV has devised extensive mechanisms through which it can remove BST2 from the sites of virus assembly, thereby enabling newly formed virions to bud off unhindered. These mechanisms, centered around the actions of the HIV Vpu protein, are currently not fully understood. While some regions within Vpu have been identified as important for the interaction with BST2, these regions are not obvious drug targets because they either occur within TMD regions, or are not conserved across Vpu variants from different HIV clades. The importance and urgency of studies like the one presented in this thesis, which seek to identify conserved determinants allowing Vpu to efficiently counteract BST2 cannot be overstated. An understanding of such determinants would allow for the development of small molecules aimed at inhibiting the role of Vpu, which would then

enable BST2 to perform its antiviral functions. Furthermore, Vpu acts in conjunction with Nef to target the host cell factor and viral receptor CD4, whose continuous expression is detrimental to virus replication. CD4 expression following virus entry reduces both infectivity and rate of release of newly formed virions. An improved understanding of the determinants governing the Vpu-CD4 interaction is therefore of great relevance in our approaches to thwart HIV-1 replication and spread.

The second part of the thesis addresses the immune roles of the above-mentioned host proteins BST2 and CD4 towards controlling viral infections, as well as the countermeasures by the virus in its efforts to promote spread. HIV-infected patients produce Abs that primarily target the Env proteins, and some of these Abs can mediate killing of infected cells via ADCC, a mechanism that has long been implicated in the anti-HIV immune response. More importantly, ADCC research is indeed very timely given very recent results from immune correlates studies from the modestly successful RV144 vaccine trial that showed a positive correlation between protective immunity and the presence of nNAbs capable of mediating ADCC. In the absence of both a preventative vaccine and rapid development of bNAbs, ADCC likely represents a critically important mechanism to control HIV infections. Furthermore, ADCC has the potential of eliminating reactivated latently infected cells, an enticing possibility given the frustrated efforts towards reducing the persistent reservoir that presents major challenges in approaches aimed at finding an HIV cure. However, thus far very little is known regarding ADCC in HIV infection. Therefore, the thesis specifically addresses the contributions of BST2 and CD4 in mediating elimination of infected cells via ADCC. Remarkably, HIV-1 still persists despite the presence of these ADCC-competent

antibodies, suggesting that HIV-1 may have evolved to evade such responses, much like it has with host restriction factors. Hence, building on the roles of BST2 and CD4 in ADCC, the thesis further investigates mechanisms *via* which HIV evades lysis of the infected cells. Additionally, and very importantly, the study investigates the potency of different classes of Abs towards mediating ADCC, from nNAbs to bNAbs. While there is weak evidence of a role of nNAbs in ADCC, it is unknown whether bNAbs can mediate such killing. Moreover, the thesis characterizes the potential role of ADCC in eliminating latently infected cells. All these have tremendous implications as they allude to the possibility of harnessing both preventative and therapeutic activities of the Abs, which could be key in realizing the elusive goal of controlling HIV infections. Corroborating on the scholarly significance and potential impact in the HIV field of the work presented herein, many groups are now working around the clock to define the roles of host factors in ADCC, as well as approaches that can sensitize infected cells to Ab-mediated killing.

CHAPTER 2: DETERMINANTS GOVERNING VPU INTERACTIONS WITH BST2 AND CD4

Preface

Author contributions: The work presented in this chapter is part of a manuscript [378] published in the journal “Retrovirology” entitled “Conserved residues within the HIV-1 Vpu transmembrane-proximal hinge region modulate BST2 binding and antagonism” by **Lukhele S (SL)** and Cohen EA (EAC). **SL** and EAC conceived and designed experiments. **SL** performed all experiments. **SL** and EAC analyzed the data and wrote the manuscript.

***Original scholarship and distinct contributions to knowledge*:** The work presented in this chapter identifies for the first time an important regulatory role of the transmembrane-proximal Vpu hinge region residues towards enabling the protein to efficiently interact with its target host proteins BST2 and CD4. Given that this cytosolic Vpu hinge region is highly conserved across HIV clades, it may represent an attractive target for the development of anti-Vpu inhibitors.

Conserved residues within the HIV-1 Vpu transmembrane-proximal hinge region modulate BST2 binding and antagonism

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Running Title: HIV-1 Vpu hinge region modulates Vpu functions

2.1 Abstract

Background: BST2 inhibits HIV-1 release by tethering nascent virions to the surface of infected cells. HIV-1 Vpu overcomes this restriction by removing BST2 from viral budding sites via BST2 intracellular trapping and sequestration, surface downregulation and/or displacement mechanisms. Vpu is composed of a short luminal tail, a transmembrane domain (TMD) and a cytoplasmic hinge region that is followed by two helices. BST2 counteraction relies on the ability of Vpu to physically bind BST2 through TMD interactions and recruit the clathrin-dependent trafficking machinery via a canonical acidic di-leucine signalling motif within the helix-2 of Vpu. The highly conserved Vpu transmembrane-proximal hinge region encompasses residues that resemble an acidic leucine-based trafficking motif, whose functional roles are currently ill-defined. In this study, we investigated the contribution of these residues towards Vpu-mediated BST2 antagonism.

Results: We show that while these conserved residues have no intrinsic activity on the cellular distribution of Vpu in the absence of BST2, they regulate the ability of Vpu to bind to BST2 and, consequently, govern both BST2-dependent trafficking properties of the protein as well as its co-localization with BST2. Moreover, these residues, particularly a glutamic acid residue positioned immediately following the TMD, are a determinant not only for efficient targeting of BST2, but also binding and degradation of CD4, another host membrane protein targeted by Vpu. Mechanistically, our data are consistent with a role of these residues in the maintenance of the Vpu TMD

conformational configuration such that interactions with membrane-associated host targets are favoured.

Conclusions: Altogether, this work demonstrates an important regulatory role of the transmembrane-proximal Vpu hinge region residues towards enabling the protein to efficiently engage its target host proteins. Thus, this highly conserved, cytosolic Vpu hinge region may represent an attractive target for the development of anti-Vpu inhibitors.

Keywords: HIV Vpu, BST2 antagonism, Vpu-BST2 interaction, Vpu-BST2 trafficking, Vpu-CD4 interaction

2.2 Background

BST2 (also referred to as Tetherin, CD317 or HM1.24) is a type I interferon (IFN-I)-induced membrane-associated restriction factor that inhibits HIV-1 release by directly cross-linking nascent virions to the surface of infected cells [76, 77]. Structurally, BST2 consists of a short N-terminal cytosolic domain, a transmembrane domain (TMD), followed by a coiled-coil ectodomain and a C-terminal glycosylphosphatidylinisotol anchor (GPI). Tethering of virions results from insertion of one of the membrane anchors, preferentially the GPI anchor, into a budding virus particle while the other remains inserted into the host cell membrane [207]. In order to counteract BST2-mediated virion tethering, HIV-1 utilizes viral protein U (Vpu), a small membrane associated accessory protein that is also present in other related SIVs, but not in HIV-2

[103, 104]. Structurally, Vpu comprises a short luminal N-terminal tail, a TMD and a cytoplasmic domain that contains two helices separated by a linker region bearing two casein kinase II serine target sites. Phosphorylation of these serine residues (S52, S56) mediate recruitment of the β-TrCP2 subunit of the Skp1-Cullin1-F-Box ($SCF^{\beta\text{-}TrCP2}$) E3 ubiquitin ligase [110, 241]. A short, flexible hinge region connects the TMD and the cytoplasmic domain. Maintenance of proper structural elements of Vpu, such as the TMD, is important for the ability of the protein to target BST2 as well as an array of other host factors, including CD4, NK-T-B-antigen (NTB-A), Polio virus receptor (PVR), sodium-coupled neutral amino acid transporter (SNAT1) and the C-C chemokine receptor-7 (CCR7) [92, 102, 118, 119, 255, 274].

Vpu counteracts BST2 antiviral activity by mediating removal of BST2 from virus budding sites via intracellular trapping and sequestration, surface downregulation and/or displacement mechanisms [76, 77, 270, 272, 379]. A direct interaction between Vpu and BST2, which occurs via their respective TMDs, is critical for BST2 antagonism [272, 274, 275]. The interaction requires a Vpu $A_{10}xxxA_{14}xxxA_{18}xxxW_{22}$ hydrophobic TMD interface, as well as several other residues within the TMD [272, 274, 275]. This physical association, which is believed to occur in the endoplasmic reticulum (ER) and/or the *trans*-Golgi network (TGN), traps both newly synthesized and recycling BST2 within intracellular compartments including the TGN [270, 271, 283, 289, 379].

BST2 antagonism is closely linked to cellular distribution of Vpu, and is reliant on hijacking of the host clathrin-dependent trafficking machinery [240, 283, 287]. A canonical acidic di-leucine (ExxxLV) sorting motif within the second helix of Vpu from Clade B (VpuB) of the M group of HIV-1 (HIV-1M) facilitates recruitment of clathrin

adaptor protein complexes (AP-1 and AP-2), leading to formation of ternary Vpu-BST2-AP complexes [240, 272, 287]. In addition to the sorting signal, recruitment of AP cofactors requires the interaction between Vpu and BST2, as well as phosphorylation of Vpu, both of which are believed to modulate conformational changes that promote interaction with these cofactors. Moreover, a dual tyrosine-based motif within the cytoplasmic tail of BST2 as well as the Vpu first helix, which forms non-canonical contacts with AP complexes, are also necessary [240, 287]. The Vpu di-leucine sorting motif targets BST2 to a yet-to-be-specifically defined endosomal compartment that is incompatible with transit of Vpu-BST2 complexes to the cell surface, and is also required for exclusion or lateral displacement of BST2 from viral assembly sites [271, 272, 375]. These actively sequestered Vpu-BST2 complexes are ultimately targeted for ESCRT-mediated endo-lysosomal degradation following BST2 polyubiquitination by $SCF^{\beta\text{-}TrCP2}$ [291, 292, 380]. BST2 degradation, however, is generally dissociable from enhancement of virion release, especially in conditions of low BST2 expression levels [240].

Interestingly, even though the VpuB helix-2 di-leucine sorting signal drives the first step of BST2 counteraction, it is not conserved across all Vpu variants, as is most notable in Vpu variants from HIV-1M clades C (VpuC) and F. In these variants, curiously, an equivalent putative acidic di-leucine motif occurs within the transmembrane-proximal hinge region of the protein. Intriguingly, in addition to the VpuB helix-2 di-leucine signal, there exists a putative acidic di-leucine motif (ExxxIL) within the transmembrane-proximal hinge region. Therefore, based on the canonical sequence of acidic di-leucine motifs (D/ExxxLL/I/V/M) [239], VpuB has an optimal helix-

2 signal while the hinge region putative motif would be predicted sub-optimal. Noteworthy, the membrane-proximal VpuB ExxxIL and membrane-distal ExxxLV mirror the major histocompatibility complex class II-associated invariant chain (CD74) membrane-proximal ExxxML and membrane-distal DxxxLI acidic di-leucine-based signals that are independently sufficient to recruit AP cofactors and target the transmembrane protein to endo-lysosomal compartments [376, 377].

Even though not optimal, there are positions within the VpuB transmembrane-proximal hinge region putative motif that are highly conserved, raising the possibility that the hinge region contributes to BST2 antagonism either by modulating Vpu trafficking, BST2 sequestration or through some other mechanism. To date, it is unclear whether this putative di-leucine motif plays any functional role. As well, the overall roles of the transmembrane-proximal hinge region within which the putative motif occurs are still ill-elucidated. As such, we set out to investigate the role of the VpuB hinge region residues, encompassing the putative sorting signal, towards Vpu-mediated BST2 antagonism. Additionally, considering the importance of the structural configuration of Vpu for targeting both BST2 and its other host protein target CD4, as well as the roles of disordered regions in modulating structural orientation or affording structural plasticity that facilitates interaction with target proteins, we further examined whether these conserved hinge region residues orient Vpu in such a way that binding to BST2 and CD4 is favoured.

In this study, we show that while the putative sorting motif within the transmembrane-proximal hinge region of Vpu does not influence the cellular localization of the protein in the absence of BST2, mutations of highly conserved residues within

this region affect the subcellular distribution of Vpu and its co-localization with BST2 in BST2-expressing cells. We further show that these residues allow not only for optimal BST2 binding and counteraction but also for efficient CD4 interaction and degradation, suggesting a role of the transmembrane-proximal hinge region residues in properly positioning the Vpu TMD to efficiently engage its target host proteins. By demonstrating an important regulatory role of the transmembrane-proximal Vpu hinge region residues in BST2 binding, our study underscores the critical link between efficient binding of Vpu to BST2 and optimal trafficking of Vpu-BST2 complexes to endo-lysosomal compartments for sequestration and ultimately degradation.

2.3 Results

2.3.1 Highly conserved transmembrane-proximal hinge region residues influence Vpu subcellular distribution in a BST2-dependent manner. In order to investigate the involvement of Vpu membrane-proximal hinge region residues (28**E**YRKI**L**33) in Vpu-mediated BST2 antagonism, we first evaluated their roles in governing Vpu cellular localization. Considering that in active di-leucine sorting motifs the glutamic acid (E) and leucine (L) residue positions are critical for activity, we generated provirus-based Vpu mutants that would allow for an assessment of whether the hinge region E28 and L33 residues modulate Vpu trafficking. These included mutants bearing mutations within the hinge region (28AYRKIA33, E28A/L33A), the second helix sorting signal (59KLSAFV63, E59K/L63F) while preserving the Env sequence, or both regions (E28A/L33A-E59K/L63F) (Figure 2.1A). A Vpu A10L/A14L/A18L (Vpu-AAA) mutant, which is

defective for BST2 interaction [274] was also included. Given the functional importance of Vpu accumulation within TGN compartments [271, 283, 379], we analysed the TGN distribution of the mutants in HeLa cells transfected with provirus plasmids expressing either wildtype (WT) Vpu or the various mutants. As previously reported [271, 274, 283], our confocal microscopy data revealed a preferential accumulation of WT Vpu in the TGN, as determined by the co-staining of Vpu with the TGN marker TGN46 (Figure 2.1 B-D). Importantly, the localization of the E28A/L33A mutant within the TGN was significantly reduced (Pearson correlation coefficient (PCC) = 0.54) relative to WT Vpu (PCC = 0.65) in these BST2-expressing cells. A corresponding two-fold increase in the percentage of Vpu distributing beyond the TGN was observed (16% for WT Vpu versus 35% for E28A/L33A) (Figure 2.1D). Indeed, a redistribution of the mutant protein, albeit to varying extents, could be detected in punctuate structures beyond the TGN (see representative panels 1 and 2, Figure 2.1B). An even greater reduction in TGN distribution was observed for both the E59K/L63F and E28A/L33A-E59K/L63F mutants (PCC = 0.43), in agreement with the increases in percentages of Vpu occurring beyond the TGN (52% and 54%, respectively) (Figure 2.1 B-D). Phenotypically, the E59K/L63F and E28A/L33A-E59K/L63F mutants redistributed towards the periphery in a comparable manner (Figure 2.1 B-D), indicating that mutation of the putative sorting motif in the hinge region did not augment the Vpu localization defect. Noteworthy, the accumulation of the Vpu-AAA mutant within TGN46-positive perinuclear compartments was comparable to WT Vpu (Figure 2.1 B-D). Of particular importance, none of the mutants showed any intrinsic defects in their TGN abundance following shRNA-mediated depletion of BST2 in HeLa cells (Figure 2.2 A-B and Additional file 2.1: Figure

S2.1), emphasizing that their differential cellular distribution is BST2-dependent. The fact that the BST2-binding defective Vpu mutant, Vpu-AAA, remains localized within the TGN as well as WT Vpu in the presence of BST2 (Figure 2.1 A-B) suggests that the Vpu redistributed outside the TGN likely represents Vpu whose trafficking properties are BST2-dependent.

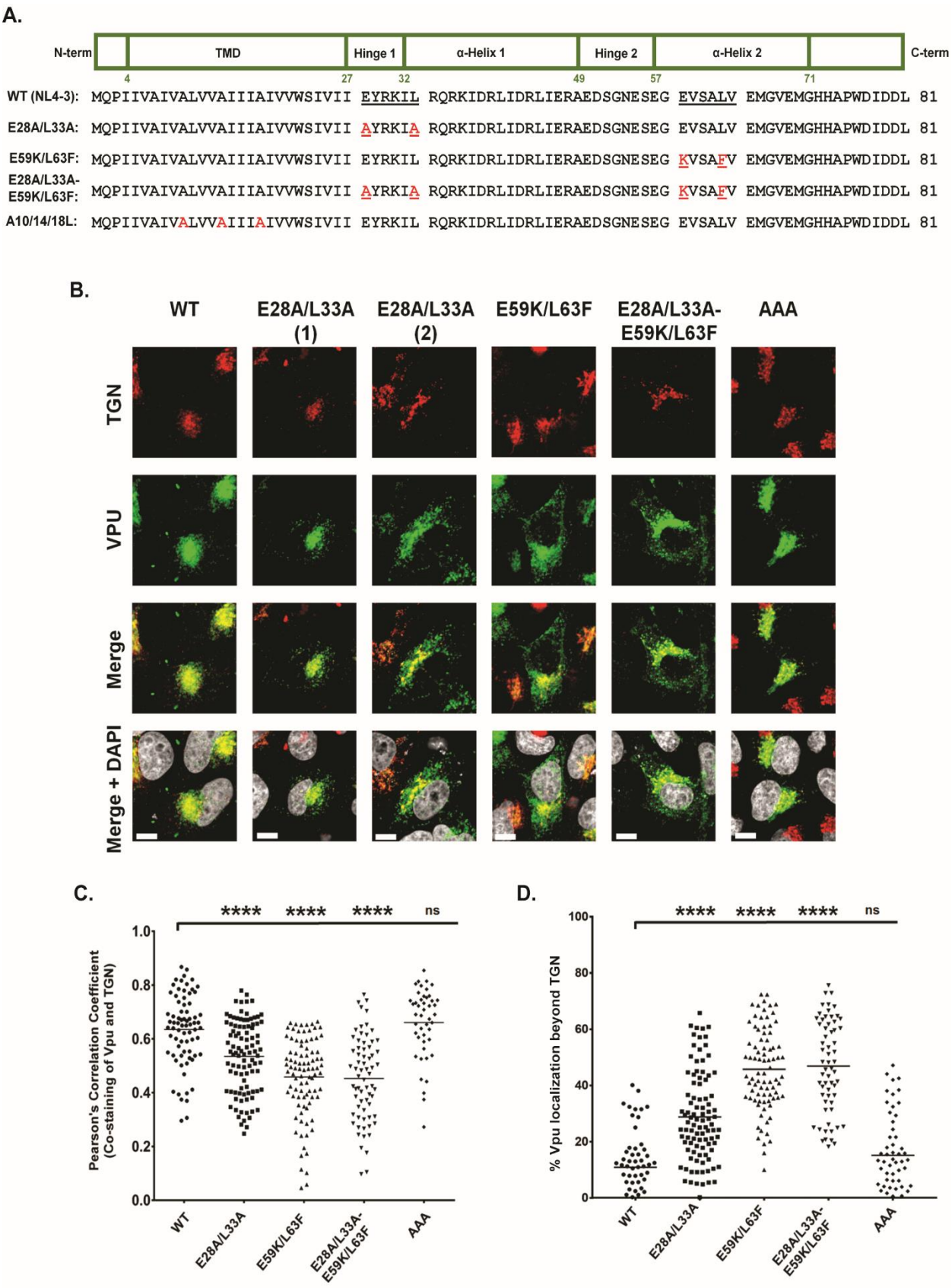
A.
N-term
TMD
Hinge 1
α-Helix 1
Hinge 2
α-Helix 2
C-term
4
27
32
49
57
71
WT (NL4-3): MQPIIVAIVALVVAIIIAIVVWSIVII EYRKIL RQRKIDRLIDRLIERAEDSGNESEG EVSALV EMGVEMGHHAPWDIDDL 81
E28A/L33A: MQPIIVAIVALVVAIIIAIVVWSIVII AYRKIA RQRKIDRLIDRLIERAEDSGNESEG EVSALV EMGVEMGHHAPWDIDDL 81
E59K/L63F: MQPIIVAIVALVVAIIIAIVVWSIVII EYRKIL RQRKIDRLIDRLIERAEDSGNESEG KVSAFV EMGVEMGHHAPWDIDDL 81
E28A/L33A-E59K/L63F: MQPIIVAIVALVVAIIIAIVVWSIVII AYRKIA RQRKIDRLIDRLIERAEDSGNESEG KVSAFV EMGVEMGHHAPWDIDDL 81
A10/14/18L: MQPIIVAIVALVVAIIIAIVVWSIVII EYRKIL RQRKIDRLIDRLIERAEDSGNESEG EVSALV EMGVEMGHHAPWDIDDL 81
B.
WT
E28A/L33A (1)
E28A/L33A (2)
E59K/L63F
E28A/L33A-E59K/L63F
AAA
TGN
VPU
Merge
Merge + DAPI
C.
Pearson's Correlation Coefficient (Co-staining of Vpu and TGN)
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
ns
WT
E28A/L33A
E59K/L63F
E28A/L33A-E59K/L63F
AAA
D.
% Vpu localization beyond TGN
100
80
60
40
20
0
****
****
****
ns
WT
E28A/L33A
E59K/L63F
E28A/L33A-E59K/L63F
AAA

Figure 2.1: Membrane-proximal, hinge region E28/L33 residues are important for Vpu localization in the TGN. A) Schematic representation of the structural domains and the sequence of the prototypical HIV-1 clade B NL4-3 Vpu (NL4-3, WT). Shown also are sequences of various Vpu mutants used in the study. **B)** Intracellular localization of the Vpu mutants. HeLa cells were transfected with proviral plasmids encoding either WT Vpu or one of E28A/L33A, E59K/L63F, E28A/L33A-E59K/L63F and A10L/A14L/A18L Vpu mutants and were co-stained with anti-TGN46 (red, for TGN) and anti-Vpu (green) Abs as well as with DAPI (grey, for nucleus). Shown are representative confocal microscopy pictures for each of the Vpu mutants, with two prototypical patterns (1&2) of localization observed with the E28A/L33A mutant. **(C-D)** Quantification of the co-staining of anti-Vpu and anti-TGN46 Abs obtained from at least 50 distinct transfected cells per mutant. Shown are Pearson correlation coefficients (PCC) for each mutant **(C)** as well as Vpu distribution beyond the TGN **(D)**. The percentage of Vpu distributing beyond the TGN was determined by calculating the ratio of the intensity of Vpu not co-staining with TGN versus total Vpu intensity in transfected cells. The white bars in B represent a distance of 10µm and the horizontal lines (C and D) represent mean values of the PCC (C) and percentage of Vpu distributing beyond the TGN (D). Statistical analyses were performed using Mann-Whitney test.

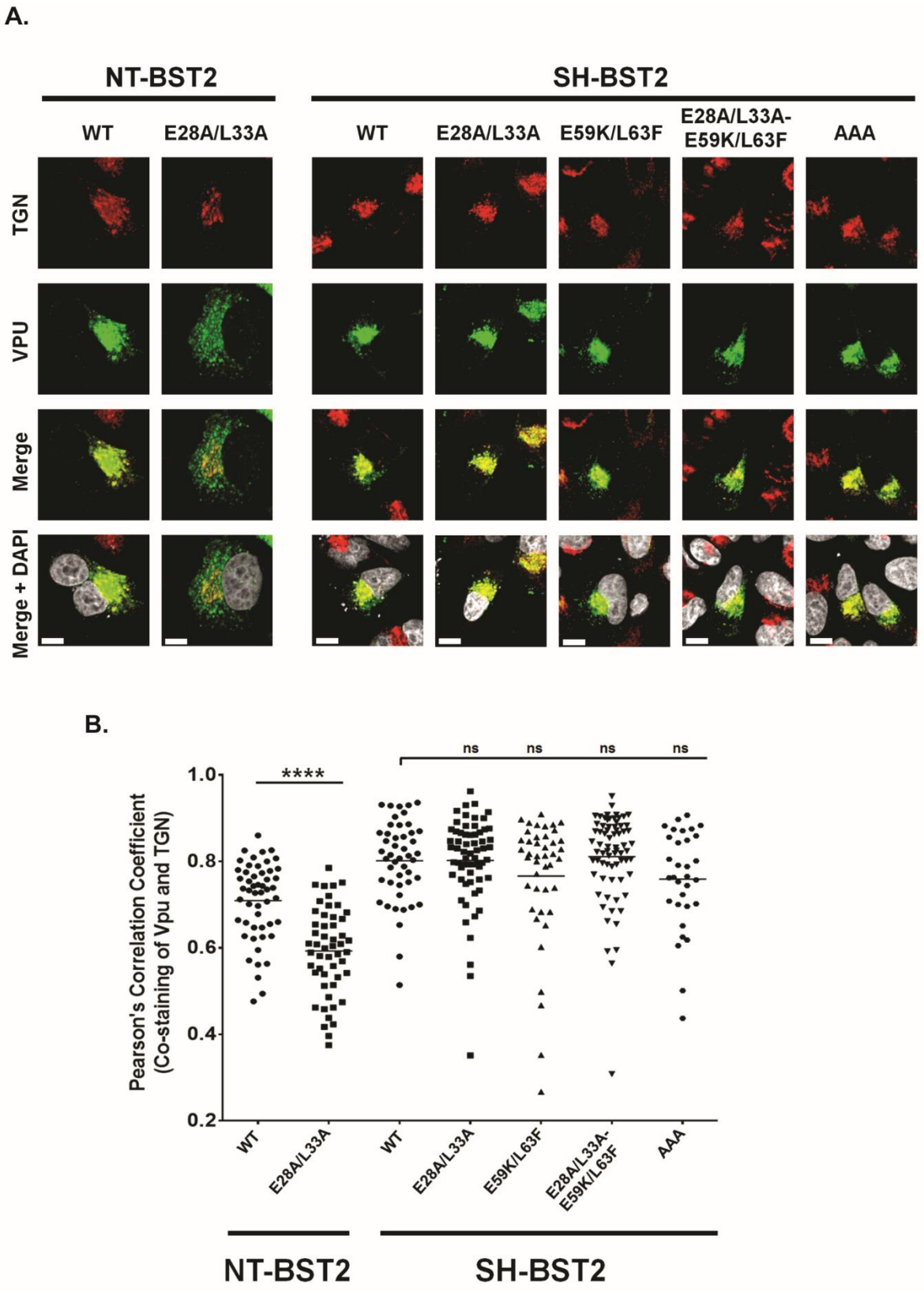
A.
NT-BST2
SH-BST2
WT
E28A/L33A
WT
E28A/L33A
E59K/L63F
E28A/L33A-
E59K/L63F
AAA
TGN
VPU
Merge
Merge + DAPI
B.
Pearson's Correlation Coefficient
(Co-staining of Vpu and TGN)
1.0
0.8
0.6
0.4
0.2
****
ns
ns
ns
ns
WT
E28A/L33A
WT
E28A/L33A
E59K/L63F
E28A/L33A-
E59K/L63F
AAA
NT-BST2
SH-BST2

Figure 2.2: E28/L33 residues have no intrinsic activity on Vpu cellular distribution in the absence of BST2. A) Intracellular localization of Vpu mutants in HeLa cells depleted of BST2 (SH-BST2, treated with shRNA against BST2) or not (NT-BST2, treated with non-targeting shRNA). Transfected cells were co-stained with anti-TGN46 (red, for TGN) and anti-Vpu (green) Abs as well as with DAPI (grey, for nucleus). Shown are representative confocal microscopy pictures for each of the Vpu mutants. The white bars represent a distance of 10µm. **B)** Quantification of the co-staining of anti-Vpu and anti-TGN46 Abs obtained from at least 50 distinct transfected cells per mutant. Shown are PCC values from each mutant. The horizontal lines represent the mean PCC. Statistical analyses were performed using Mann-Whitney test.

Given the BST2-dependent cellular distribution of the mutants, we next assessed the extent of their co-localization with BST2. Our data indicate that WT Vpu, which can efficiently bind, sequester and mediate degradation of BST2, co-localizes extensively with the restriction factor essentially within a perinuclear region. Interestingly, in the presence of the BST2 binding impaired Vpu-AAA mutant, BST2 subcellular distribution was altered with an increased localization outside the perinuclear region where co-localization with Vpu-AAA was minimal, highlighting that BST2 trafficking is influenced by formation of BST2 complexes (Figure 2.3 A-B). Given the reduced BST2 binding capacity of the Vpu-AAA mutant, its perinuclear co-localization with BST2 likely represents a mere overlap in staining resulting from the primary localization of both proteins in the TGN (Figure 2.1A). Interestingly, the E28A/L33A mutant showed a statistically significant reduction in the extent of co-localization with BST2 compared to WT Vpu, in a large part because of a reduced co-localization outside the perinuclear

region where most of the co-staining was detected (Figure 2.3 A-B). Relative to WT Vpu, the E59K/L63F mutant revealed an overall stronger co-localization with BST2 both outside and in a perinuclear region, most likely due to lack of degradation of Vpu-BST2 complexes in the case of this mutant (Additional file 2.1: Figure S2.1). Importantly, the extent of BST2 co-localization of E28A/L33A-E59K/L63F was lower compared to the E59K/L63F mutant, even though both E59K/L63F and E28A/L33A-E59K/L63F do not mediate BST2 degradation (Additional file 2.1: Figure S2.1; compare the levels of BST2 in the presence of Vpu E59K/L63F or E28A/L33A-E59K/L63F with those in the presence of the Vpu S52/56D mutant, which is unable to degrade BST2).

Taken together, our immuno-localization data indicate that the Vpu hinge region residues E28 and L33, which are part of a putative acidic di-leucine sorting motif, influence the BST2-dependent cellular distribution of Vpu beyond the TGN as well as the efficient co-localization of the protein with BST2. Moreover, the fact that mutations in the membrane-proximal hinge region motif did not augment the Vpu localization defect resulting from alterations of the second helix di-leucine motif, suggests a potential functional dependence between these two motifs.

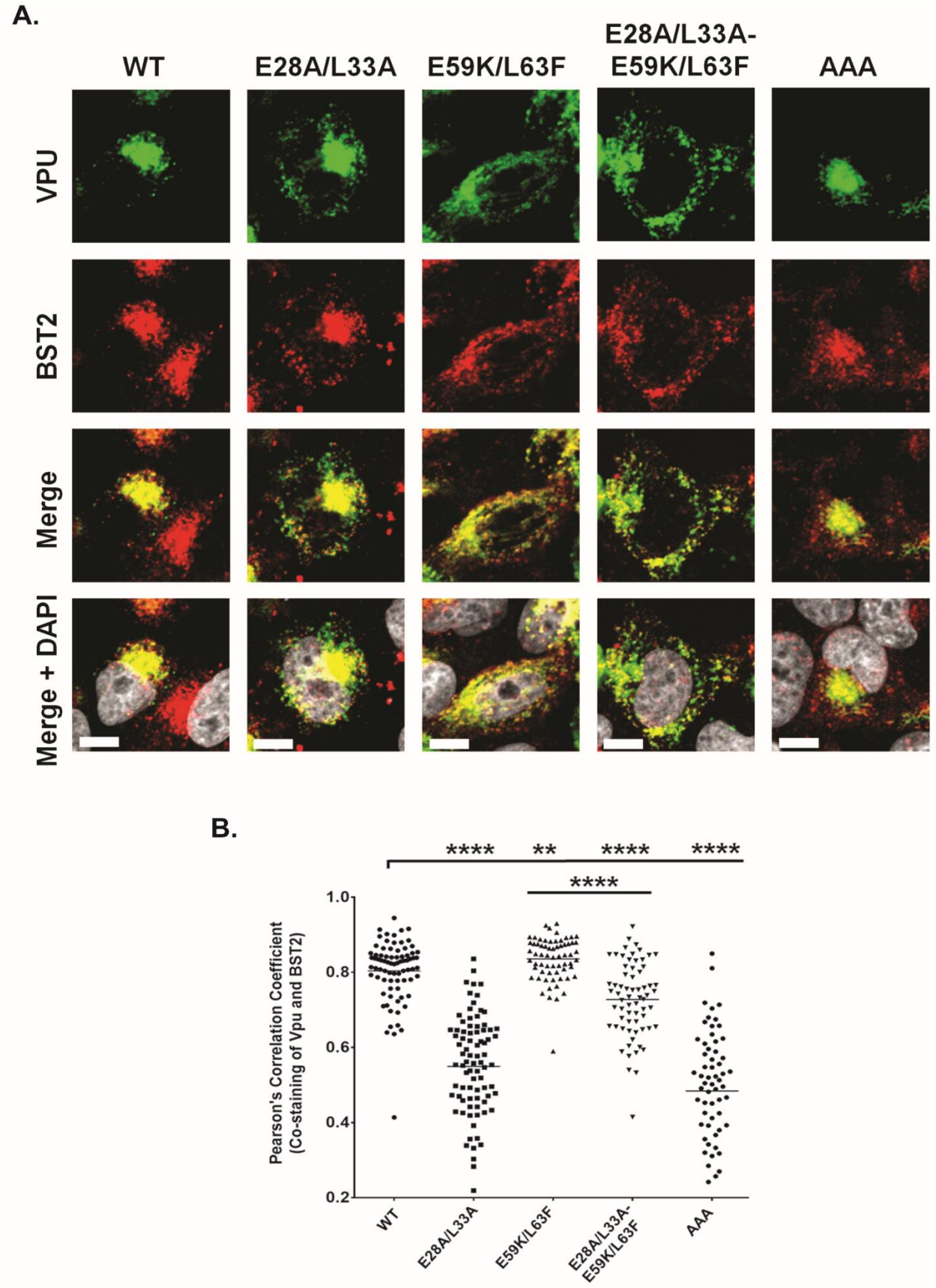
A.
WT
E28A/L33A
E59K/L63F
E28A/L33A-
E59K/L63F
AAA
VPU
BST2
Merge
Merge + DAPI
B.
****
**
****
****
****
Pearson's Correlation Coefficient
(Co-staining of Vpu and BST2)
1.0
0.8
0.6
0.4
0.2
WT
E28A/L33A
E59K/L63F
E28A/L33A-
E59K/L63F
AAA

Figure 2.3: Hinge region E28/L33 residues are important for Vpu co-localization with BST2. A) Representative pictures showing extent of co-localization of Vpu mutants with endogenous BST2 in HeLa cells as determined by the co-staining of anti-Vpu (green) and anti-BST2 (red) Abs following intracellular staining for both Vpu and BST2. **B)** PCC values obtained from quantification of co-stainings of anti-Vpu and anti-BST2 Abs from at least 40 distinct transfected cells. The white bars in panel A represent a distance of 10µm and the horizontal lines in panel B represent the mean PCC. Statistical analyses were performed using Mann-Whitney test.

2.3.2 Vpu hinge region E28/L33 residues modulate the ability of Vpu to antagonize BST2.

To test if the differential cellular distribution of the Vpu mutants has any functional consequences, we assessed their capabilities to downregulate surface BST2 as well as to enhance release of viral particles from transfected HeLa cells. Compared to WT provirus, a Vpu-deficient (dU) provirus was impaired in its surface BST2 downregulation function, as previously reported [77, 291, 379] (Figure 2.4A). Meanwhile, the E28A/L33A mutant was moderately, albeit significantly, attenuated for BST2 downregulation (Figure 2.4 A-B). In line with earlier reports [271], the E59K/L63F mutation drastically reduced the extent of Vpu-mediated BST2 cell surface downregulation. Of particular note, simultaneously mutating both regions (E28A/L33A-E59K/L63F) yielded a cumulative defect on surface BST2 downregulation. In terms of BST2 antagonism, as measured by the ability of Vpu to enhance release of nascent virions, the dU mutant was unable to counteract BST2 (Figure 2.4 C-D). Most importantly, the ability of the E28A/L33A mutant to promote virus release was

significantly attenuated. Consistent with the BST2 downregulation phenotype, the E59K/L63F mutant gave an even more pronounced attenuation. Yet again, the E28A/L33A-E59K/L63F mutant displayed a cumulative effect of the two mutated regions, giving a phenotype that is slightly higher to that of dU. It is worth mentioning that the E28A/L33A mutant was still able to mediate some BST2 degradation, albeit to a lesser extent than the WT Vpu (Additional file 2.1: Figure S2.1), unlike the E59K/L63F, E28A/L33A-E59K/L63F and β-TrCP binding-deficient S52/56D mutants that were totally defective.

Altogether, our data highlight the significance of the conserved hinge region E28/L33 residues towards overcoming BST2 restriction. Of note, their contribution to BST2 antagonism is additive with that of the second helix di-leucine sorting signal, suggesting that they act at different stages of the counteraction.

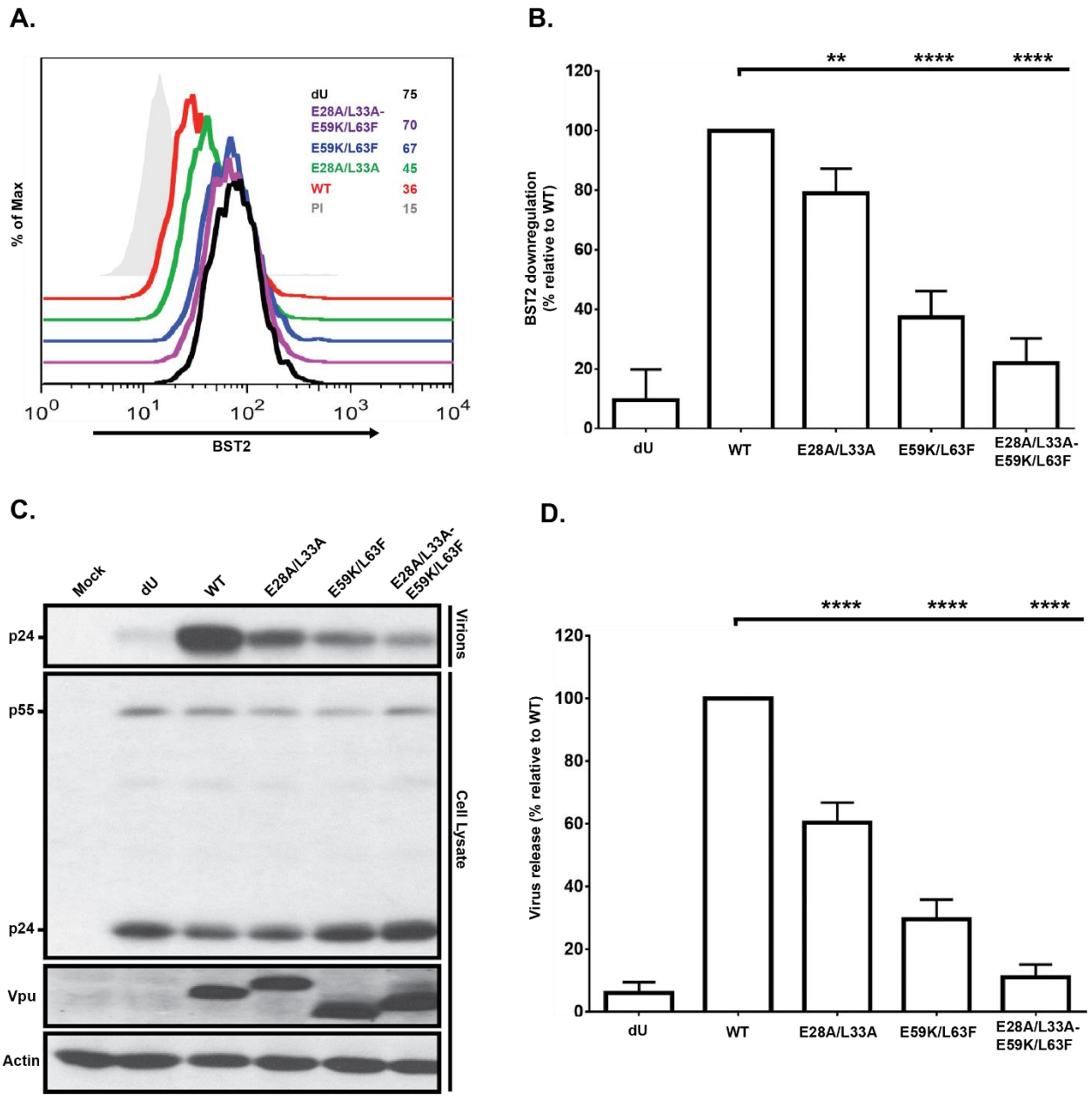

Figure 2.4: Conserved residues within the membrane-proximal hinge region of Vpu are important for BST2 counteraction. **A)** A flow cytometry representative overlay showing the amount of surface BST2 following transfections of HeLa cells with either WT Vpu or the indicated Vpu mutants. Mean fluorescence intensity (MFI) values are indicated on the right. B) A compilation of four independent experiments showing the extent of surface BST2 downregulation as determined by subtracting BST2 MFI values obtained from transfected cells (GFP+) from those in non-transfected cells (GFP-), and expressed as a percentage relative to the efficiency of BST2 downregulation obtained from WT Vpu, which in turn

was arbitrarily set at 100%. **(C-D)** Efficiency of virus particle release following transfection of HeLa cells with provirus plasmids encoding WT Vpu and the indicated mutants. **C)** A representative Western blot showing the amount of virion-associated p24 released into the supernatant (virion) and Gag products (p24 and p55) in the cell lysate. **D)** A summary of quantifications of released virus particles by densitometric analyses of the intensity of Gag-related band signals from the Western blots from four different experiments. The efficiency of virus release was determined by calculating the ratio of virion-associated p24 released into the supernatant versus total Gag (cell- and virus-associated), and is expressed as a percentage of the release efficiency of the WT provirus, which in turn was set at 100%. For both (B) and (D), the error bars represent standard deviation (SD). Statistical analyses were performed using a two-way ANOVA, Tukey's multiple comparison test.

2.3.3 Vpu E28/L33 residues are important for binding to BST2. Having shown the defect of these hinge region residues on BST2 antagonism and co-localization, we next evaluated the ability of the E28A/L33A mutant to interact with BST2. Towards this, we performed co-immunoprecipitation (Co-IP) assays in which we co-transfected proviral plasmids expressing the appropriate Vpu mutants together with a BST2 expressor into HEK293T cells, and pulled down Vpu using anti-BST2 antibodies (Abs). As shown in Figure 5A, whereas WT Vpu and S52/56D were efficiently pulled down (lanes 3 and 7), there was no enrichment for a Vpu-AAA mutant known to be defective for BST2 interaction (lane 8), in accordance with the importance of the TMD for binding to BST2 [274]. Importantly, while the E59K/L63F mutant was efficiently pulled down (compared to the WT) in these assays (lanes 3 and 5), the E28A/L33A mutant was significantly

attenuated for binding to BST2 (compare lane 4 with lane 3). A largely comparable binding defect was observed with the E28A/L33A-E59K/L63F mutant (compare lane 6 with lanes 3 and 5). The binding defect of the E28A/L33A mutant was independent of the degradation of the Vpu-BST2 complexes as it could not be rescued when the assay was performed using a BST2 expressor encoding for a short isoform of BST2 that contains the TMD involved in Vpu binding but is insensitive to Vpu-mediated downregulation and degradation (Figure 2.5B) [181, 296]. Thus, our data indicate that the hinge region E28/L33 residues are important for the ability of Vpu to optimally interact with BST2, a condition that is necessary for Vpu-mediated BST2 counteraction. These results further suggest that the attenuated binding of E28A/L33A to BST2 could be responsible for the observed cellular localization and BST2 co-localization defects (Figures 2.1 and 2.3). In order to test this, we introduced mutations in Vpu TMD residues (A14L, A18L and A10L/A18L) that are relevant for binding to BST2 and correlated their binding strength (or lack thereof) to their cellular distribution. While the Vpu A14L was severely impaired for both BST2 binding and counteraction (resembling the Vpu-AAA mutant), the A18L and A10L/A18L gave intermediate BST2 binding and BST2 antagonistic phenotypes (Additional File 2.2: Figure S2.2 and Figure 2.6A). In fact, their BST2 binding and functional profiles were comparable to the E28A/L33A mutant. Very importantly, the Vpu A18L and A10L/A18L mutants distributed beyond the TGN in a manner mirroring that obtained with the E28A/L33A mutant (Figure 2.6 B-D). Thus, the Vpu-BST2 binding affinity modulates the cellular distribution of Vpu. Taken together, the E28/L33 residues are a determinant for interaction with BST2, which in turn regulates both the cellular distribution and functional properties of the protein.

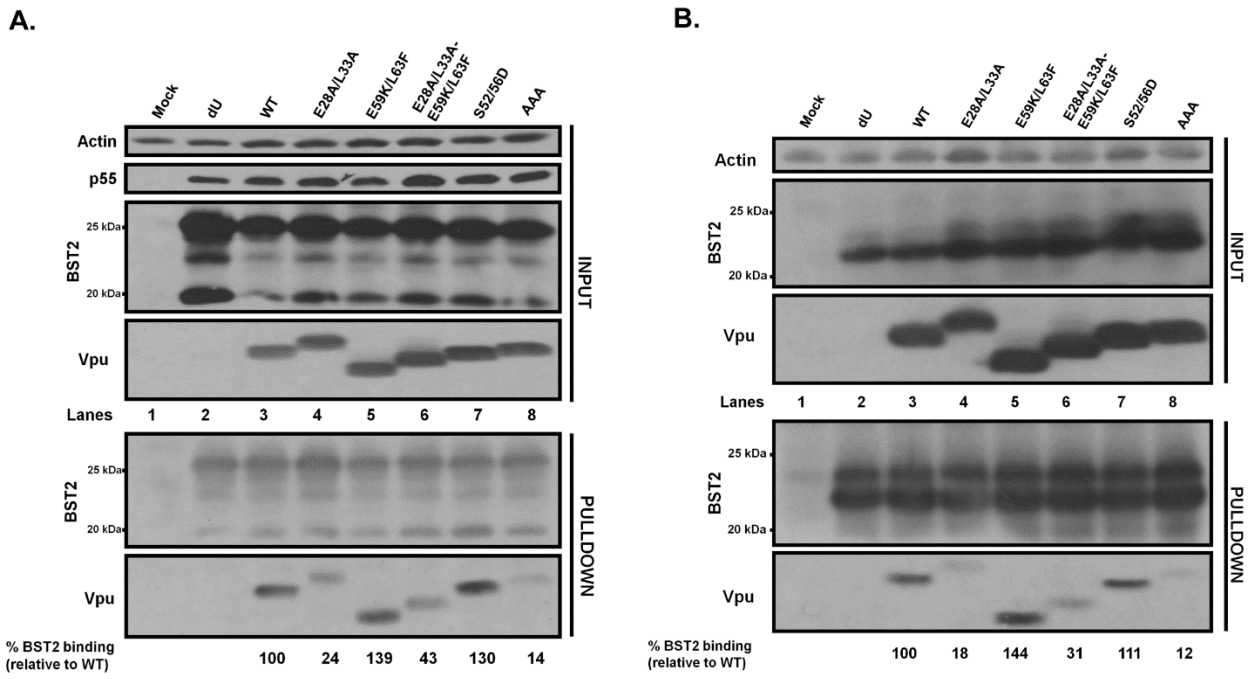

Figure 2.5: E28/L33 residues are important for binding of Vpu to BST2. **A)** HEK293T cells were co-transfected with a proviral construct encoding WT Vpu or the indicated Vpu mutants and a BST2 expressor. Co-IP assays were then performed using an anti-BST2 Ab to pull down Vpu. Shown is a representative Western blot indicating the expression levels of proteins of interest in both the input lysate (actin (loading control), p55 (transfection control), BST2 and Vpu) and the immunoprecipitated fraction (BST2 and Vpu). **B)** Co-IP following co-transfection of HEK293T cells with a proviral construct encoding WT Vpu or the indicated Vpu mutant and an expressor encoding for the short isoform of BST2 that contains the TMD involved in Vpu binding but is insensitive to Vpu-mediated degradation. Below each blot is the extent of BST2 binding of each Vpu mutant, relative to WT Vpu (set at 100%). For each condition, BST2 binding efficiency was determined from the ratio obtained from densitometric analyses of the intensities of Vpu- and BST2-related band signals in the immunoprecipitated fractions.

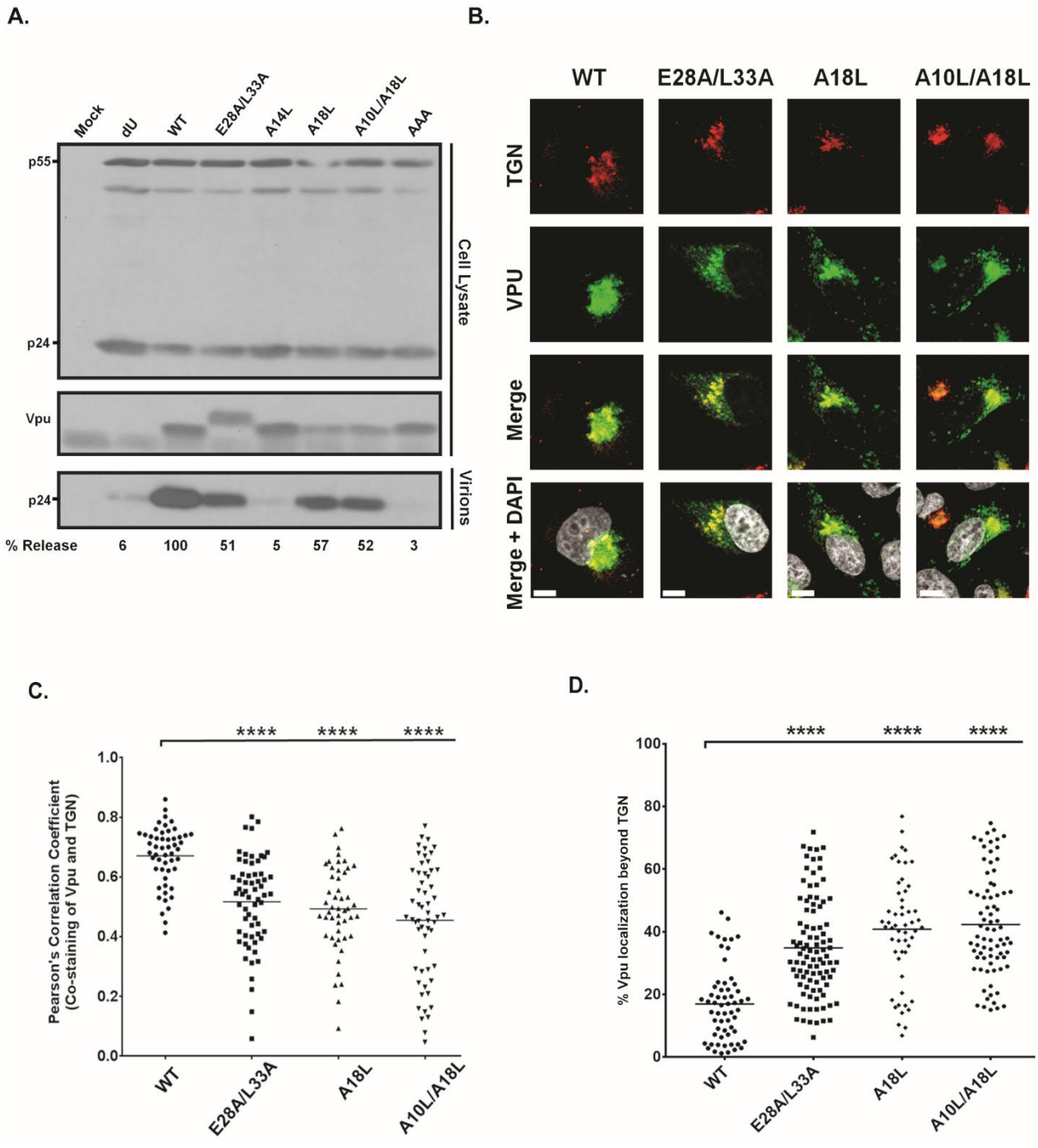

Figure 2.6: Impaired BST2-binding Vpu TMD mutants display defects in cellular distribution. A) Enhancement of virus release by Vpu TMD and E28A/L33A mutants. A representative Western blot showing the amount of virion-associated p24 released into the supernatant (virion) and Gag products (p24 and p55) in the cell lysate. Below the blot are values indicating the efficiency of virus release (quantified and expressed as mentioned in Figure 2.4 legend). **(B-D)** Vpu TMD mutants are defective for

TGN localization. **B)** HeLa cells transfected with either WT, E28AL/L33A, A18L or A10L/A18L Vpu mutants were co-stained with anti-TGN46 (red, for TGN) and anti-Vpu (green) Abs as well as with DAPI (grey, for nucleus). Shown are representative confocal microscopy pictures for each of the Vpu mutants. **(C-D)** Quantification of the co-staining of anti-Vpu and anti-TGN46 Abs obtained from at least 50 distinct transfected cells per mutant. Shown are Pearson correlation coefficients (PCC) for each mutant **(C)** as well as Vpu distribution beyond the TGN **(D)**. The percentage of Vpu distributing beyond the TGN was determined by calculating the ratio of the intensity of Vpu not co-staining with TGN versus total Vpu intensity in transfected cells. The white bars in B represent a distance of 10µm and the horizontal lines (C and D) represent mean values of the PCC (C) and percentage of Vpu distributing beyond the TGN (D). Statistical analyses were performed using Mann-Whitney test.

2.3.4 The E28/L33 residues are important for CD4 binding and degradation.

Efficient interaction between Vpu and BST2 is dependent on both the identities of the amino acids and the proper structures of their respective TMD helices [274, 275, 280]. To ensure access to the BST2 TMD, the Vpu TMD $A_{10}xxxA_{14}xxxA_{18}xxxW_{22}$ interacting interface orients such that it aligns with the bulky residues on the BST2 TMD, facing the opposite direction of the Vpu cytoplasmic domain [275, 280]. Moreover, the TMDs of both proteins have to adopt optimal tilt angles that are permissive to a direct interaction [275, 281]. Along this line, correlations between the extent of Vpu binding and tilt angles have since been reported [281]. As such, our data raise the possibility that the E28A/L33A mutation perturbs the structural orientation of the Vpu TMD. To test this, we evaluated the ability of the E28A/L33A mutant to interact with another host membrane

target protein of Vpu, the CD4 viral receptor, which is intercepted in the endoplasmic reticulum (ER) prior to trafficking to more distal cellular compartments and that requires an intact Vpu TMD. Vpu mediates the degradation of CD4 by connecting the viral receptor to components of the ER-associated protein degradation (ERAD) pathway through a process that requires Vpu-CD4 binding via both the TMD and first helix of Vpu, as well as recruitment of the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex [110, 255, 257, 260, 265].

First, to assess the ability of the Vpu mutants to degrade CD4, HEK293T cells were co-transfected with a CD4 expressor and proviruses expressing WT Vpu or Vpu mutants (dU, E28A/L33A or S52/56D), and CD4 steady-state levels were evaluated by Western blotting. While WT Vpu was efficient at mediating CD4 degradation relative to dU, the S52/56D mutant was significantly impaired for this function, owing to the fact that it is defective for β-TrCP recruitment (Figure 2.7 A-C). Importantly, the E28A/L33A mutant was significantly attenuated for CD4 degradation, yet retained the ability to efficiently interact with β-TrCP (Figure 2.7 A-C). We then asked whether the E28A/L33A mutant was still able to bind CD4. Towards this, we co-transfected HEK293T cells with a CD4 expressor and the proviral Vpu mutants, and performed Co-IP assays using anti-CD4 Abs to pull down Vpu. Whereas WT Vpu and S52/56D were efficiently pulled down, the E28A/L33A mutant was not, indicating that the E28/L33 residues contribute to the interaction of Vpu with CD4 (Figure 2.7D).

Taken together with the BST2 binding data, the results demonstrate that the E28/L33 residues are essential for optimal binding to both BST2 and CD4.

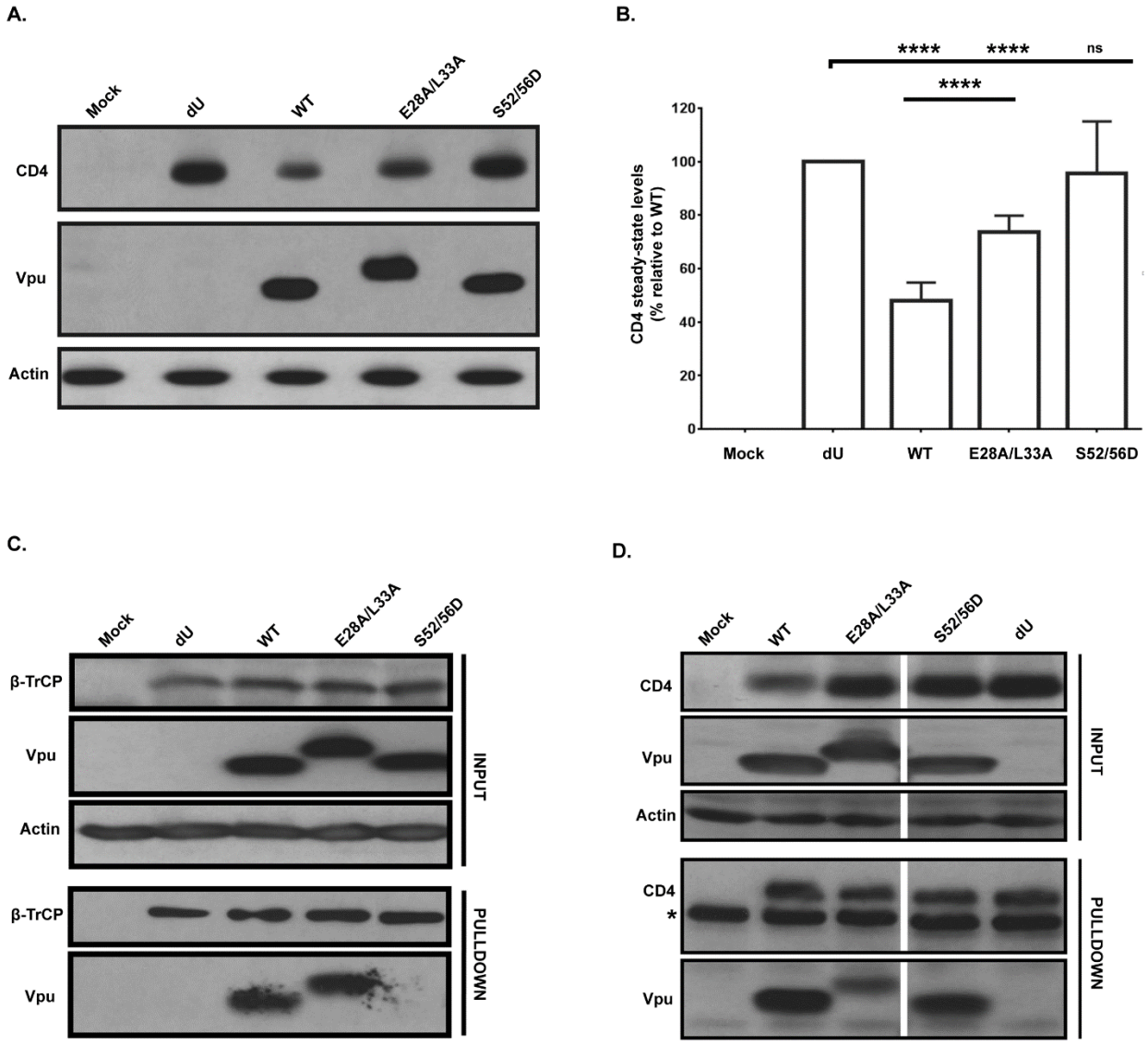

Figure 2.7: E28A/L33A mutation affects the ability of Vpu to target CD4. (A-B) E28A/L33A is attenuated for CD4 degradation. HEK293T cells were co-transfected with a proviral construct encoding WT Vpu or the indicated Vpu mutant proviruses and a CD4 expressor, and probed for the steady state levels of CD4. **A)** Shown is a representative Western blot. **B)** A summary of the densitometric quantifications of the steady-state CD4 levels from independent experiments, together with the SD (n=7). Statistical analysis was performed using a two-way ANOVA, Tukey's multiple comparison test. **C)** E28A/L33A is efficient at recruiting β-TrCP. HEK293T cells were co-transfected with the indicated proviral constructs and a myc-tagged β-TrCP2 expressor. A Co-IP assay was performed using anti-myc Abs to

pull down Vpu. **D)** E28A/L33A is attenuated for CD4 binding. HEK293T cells were co-transfected as in (A), and a Co-IP assay performed using anti-CD4 Abs to pull down Vpu. Shown is a representative Western blot indicating the amount of Vpu in the lysate and pulled down fractions in each case. The asterisk denotes an Ab-related band.

2.3.5 E28 is a determinant for both Vpu-mediated CD4 degradation and BST2 antagonism. We next wanted to delineate the contributions of the E28/L33 residues towards the observed functional and binding phenotypes. Considering the proximity of the E28 residue to the functionally important TMD, as well as the fact that it is highly conserved across Vpu variants (Figure 2.8A), we hypothesized that it is primarily the glutamate that acts as a membrane anchor for this TMD region. Focusing on the glutamate, we introduced E28A, E28D and E28Q point mutations in order to discern whether the charge, polarity and/or intrinsic structural elements are essential for targeting CD4 and/or BST2. Noteworthy, the Gln is also found in other variants of Vpu, especially those from HIV-1M G subtype [Los Alamos HIV sequence database, http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html, accessed June 2015]. In terms of mediating CD4 degradation, our results indicate that whereas both E28D and E28Q mutants were comparable to WT Vpu, the E28A mutant was significantly defective, suggesting that the E28 position can accommodate a polar or semi-conservative residue (Figure 2.8 B-C). In fact, the E28A mutation was sufficient to account for the difference or attenuated phenotype observed with the E28A/L33A mutant. For BST2 antagonism, however, it was particularly interesting that all the

mutants showed a modest, yet statistically significant attenuation compared to WT Vpu (Figure 2.8 D-E). Noteworthy, while the L33 position was previously reported to be dispensable for virus release [283], mutating it in the context of the E28A was necessary in order to give the defective phenotype observed with the E28A/L33A mutant. Similar to their BST2 antagonistic activities, the single mutants were all attenuated for Vpu-mediated BST2 degradation (Additional File 2.3: Figure S2.3). As such, the data argue that the glutamate residue offers some unique intrinsic properties that are important for targeting BST2. In line with these observations, E28A also displays a reduced binding to BST2 (Figure 2.9). Interestingly, this attenuation in BST2 binding appears slightly less than that of the E28A/L33A mutant, suggesting that the intermediate ability of Vpu E28A to counteract BST2 correlates with its extent of binding to the restriction factor. Altogether, the above observations are consistent with a role of the glutamic acid residue in allowing for optimal counteraction of BST2 as well as efficient targeting and degradation of CD4.

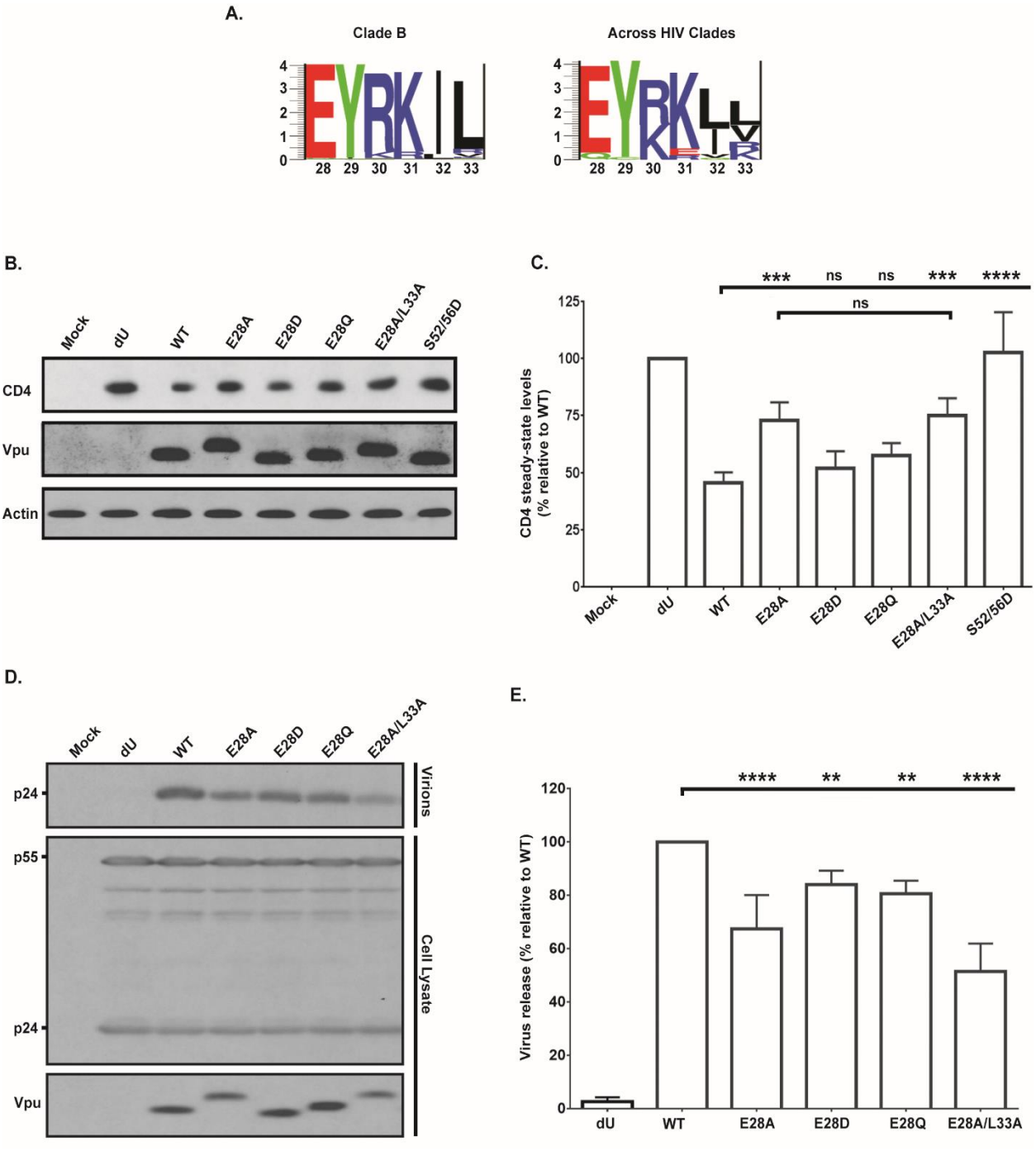

Figure 2.8: The conserved glutamic acid residue within the hinge region of Vpu modulates the protein functions. **A)** Logo plots demonstrating the amino acid conservation at each position within the 28EYRKIL33 transmembrane-proximal hinge region of Vpu isolates from HIV-1 Clade B (left panel) as well as from all HIV-1 clades (right panel) [Los Alamos HIV sequence database,

http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html, accessed June 2015]. **(B-C)** CD4 degradation mediated by E28 single-point mutants. **B)** A representative Western Blot indicating steady state levels of CD4 following co-transfections of HEK293T cells with a CD4 expressor and the indicated proviral constructs. **C)** Quantitative densitometric analyses of efficiency of Vpu mutants to degrade CD4 from a compilation of five independent experiments. **(D-E)** E28 is important for efficient enhancement of virus release in HeLa cells transfected with proviral constructs encoding Vpu single-point mutations. **D)** A representative Western blot showing the amount of virion-associated p24 released into the supernatant (virion) and Gag products (p24 and p55) in the cell lysate. **E)** A summary of the efficiency of virus release (quantified and expressed as mentioned in Figure 2.4 legend) from five different experiments. For both (C) and (E), the error bars represent SD. Statistical analyses were performed using a two-way ANOVA, Tukey's multiple comparison test.

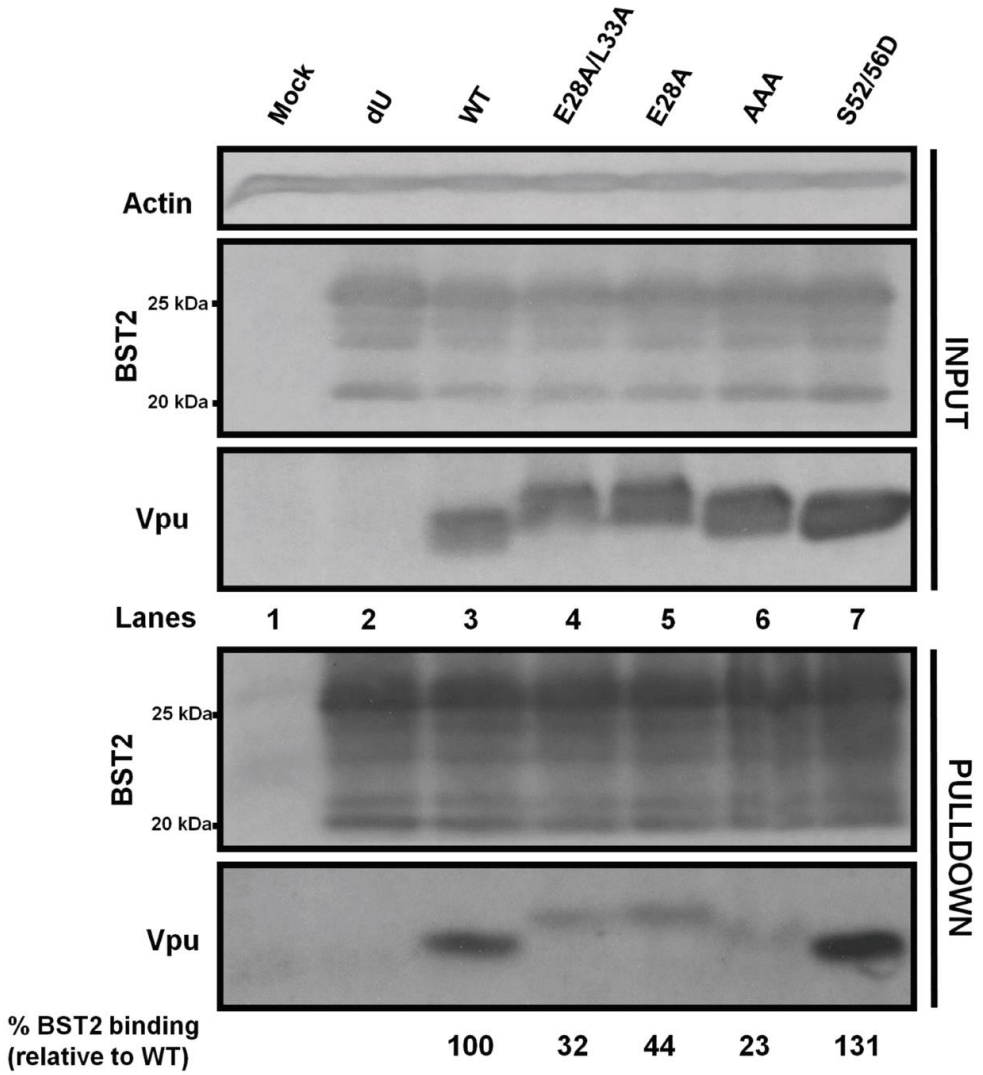

Figure 2.9: E28 residue is a key modulator of binding of Vpu to BST2. Co-IP following co-transfection of HEK293T cells with a proviral construct encoding WT Vpu, E28A/L33A or E28A Vpu mutants and a BST2 expressor. Below the blot is the extent of BST2 binding of each Vpu mutant, relative to WT Vpu (set at 100%). BST2 binding efficiency was determined as indicated in Figure 2.5 legend.

2.4 Discussion

In this study, we investigated the contribution of the highly conserved transmembrane-proximal hinge region (28EYRKIL33) towards Vpu-mediated BST2 antagonism. We show that while residues in this region have no intrinsic activity on the cellular distribution of Vpu in the absence of BST2, they regulate the ability of Vpu to bind BST2 and, consequently, govern both BST2-dependent trafficking properties of the protein as well as co-localization with BST2. We further show that these conserved residues are important not only for BST2 counteraction but also for the capacity of Vpu to efficiently target the CD4 viral receptor for degradation in the ER. Ultimately, our results reveal that these conserved hinge region residues are important for the ability of Vpu to optimally counteract BST2 and degrade CD4. While mutagenesis analysis of conserved residues revealed subtle differences regarding the relative importance of these residues for BST2 or CD4 targeting, evidence points towards a potential role of these residues in the maintenance of the Vpu TMD conformational configuration such that interactions with membrane-associated host targets, via association between their respective TMDs, are favoured.

Optimal binding of Vpu to BST2 is a critically important pre-requisite for antagonism. To date, this extensively characterized direct binding has been shown to involve residues within the transmembrane regions of both proteins, with no evidence implicating the Vpu hinge region residues in said interaction [272, 274, 275]. Our finding that the hinge region residues also regulate this interaction was therefore unexpected, and highlights a previously unappreciated important determinant for BST2 binding that

occurs beyond the reported TMD residues. This binding modulation is likely due to a regulatory effect on the conformational integrity of the Vpu TMD, as evidenced by the defects in Vpu-mediated functions that are dependent on interactions involving the TMD. Accordingly, these residues are important for targeting BST2 and CD4, and could conceivably play a role in the interaction with other membrane-associated host targets of Vpu including NTB-A, PVR, SNAT1 and CCR7. Preservation of both secondary and tertiary structures, as well as positioning of the TMD likely enable interactions with these target proteins. The conformational integrity of the Vpu TMD is indeed essential for optimal biological activity, as recently demonstrated by the positive correlation between alterations in tilt angles and attenuation in BST2 antagonism observed in Vpu TMD mutants [281]. Similarly, altered tilt angles of BST2 TMD mutants confined within lipid bilayers desensitized them to the antagonistic activity of Vpu, yet they retained an intrinsic ability to interact with Vpu in solution [275]. Furthermore, nuclear magnetic resonance data on the direct helix-helix interaction between Vpu and BST2 TMDs demonstrate the need for appropriate alignment of the Vpu interacting interface in order to directly contact the bulky BST2 TMD residues that likely fit between the alanine-based interface of the Vpu TMD [275]. Consistently, the Vpu TMD interface faces away from the cytoplasmic domain of the protein, ensuring accessibility to its binding partners [280].

The evidence presented herein implying a contribution of the Vpu hinge region residues, especially the glutamate at the TMD-cytoplasmic interface, towards anchoring the TMD is in accordance with previous findings demonstrating a modulatory function of a similarly positioned glutamate residue in the conformational positioning of a model

poly-Leu TMD helix [381]. Mutating the glutamate residue is believed to abrogate its role of linking the TMD hydrophobic segment to the aqueous phase, resulting in a mismatch between the hydrophobic helix relative to the length of phospholipids in the bilayer and, ultimately, altered TMD tilt angles [382, 383]. Interestingly, glutamate residues flanking TMDs of bacteriorhodopsin were also shown to modulate the conformational configurations of the TMDs, including the mobility of Ala and Val TMD residues [384, 385]. More interesting still, and corroborating our findings herein, these studies also document the uniqueness of the glutamate intrinsic properties, as it could not be substituted even with a semi-conservative glutamine residue, [384, 385]. This likely reflects the superior capability of the Vpu Glu to form stabilizing interactions with the positive residues R30/K31/R34 via hydrogen bonds or salt bridges [386, 387].

The interaction of Vpu and BST2 mediates the recruitment of clathrin AP complexes that govern BST2 intracellular sequestration, degradation, endocytosis and displacement from viral assembly sites [240, 287]. Thus, by disrupting Vpu interaction with BST2, mutations of conserved residues of the hinge region are likely to affect the recruitment of AP via the di-leucine trafficking motif in the second helix of Vpu [240]. Indeed, the aberrant BST2-dependent cellular distribution of the E28A/L33A mutant is likely a consequence of such a defect, in line with the notion that mutations that affect AP recruitment lead to defects in cellular localization only in the context of the Vpu-BST2 complex [240, 271]. In fact, the extent of BST2 counteraction by the E28A/L33A mutant appears to correlate with both its significantly compromised binding phenotype and its slightly altered cellular localization in the presence of BST2. Interestingly, the altered Vpu localization triggered by the E28A/L33A mutant was not found to be additive

with the localization alteration induced by the second helix di-leucine mutant, consistent with the requirement of a Vpu-BST2 physical interaction for recruitment of AP proteins [240, 271]. Thus, our data support previous results emphasizing the strong correlation between Vpu-mediated BST2 antagonism and both Vpu-BST2 interaction and Vpu-BST2 complex cellular localization.

The functional contribution of the glutamate residue is distinct from that mediated by the other hinge region residues (28EYRKIL33). While our previous findings showed that Y29 was dispensable for BST2 antagonism, the R30/K31 residues were found important, yet were dispensable for both binding to BST2 and CD4 degradation [283]. Mechanistically, unlike the E28/L33 residues, the R30/K31 residues appear important for the intrinsic cellular distribution of Vpu within the TGN [283], implying that they are unable to efficiently intercept recycling and/or newly synthesized BST2. Altogether, therefore, these E28/R30/K31/L33 flexible hinge region residues play unique, multifaceted functional roles, much like the other membrane-distal flexible linker occurring between the two alpha helices of Vpu that regulates binding to both AP proteins and β-TrCP [110, 240, 241].

Considering the functional importance of the transmembrane-proximal hinge region glutamate, it is not surprising that this residue is highly conserved across HIV-1M Vpu variants. Consistent with our findings herein, a recent study reported that only two out of a pool of 304 patient-derived Vpu alleles had a lysine substitution at this position, and were significantly impaired in their ability to mediate both BST2 antagonism and surface CD4 downregulation [199]. Intriguingly, in the case of Vpu variants with a canonical di-leucine motif within the second helix, such as in VpuB, the hinge region

residues offer some plasticity in that only a single amino acid substitution would be sufficient to generate an optimal trafficking motif (ExxxIL vs ExxxLL). Along this line, analysis of the Los Alamos HIV database reveals that 13% of VpuB variants (18) with mutations at critical positions within their helix-2 di-leucine motifs (136 out of a total of 1648 Vpu variants) have optimal putative transmembrane-proximal hinge region trafficking signals [Los Alamos HIV sequence database, http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html, accessed June 2015]. Indeed, while the influence of the hinge region residues on BST2 binding, and hence Vpu (NL4.3) cellular distribution, is consequential, we cannot formally rule out that these residues, which are part of a putative acidic di-leucine trafficking motif, also contribute directly to Vpu-BST2 complex trafficking. Future studies aimed at characterizing the exact compartments into which the mislocalized Vpu (NL4.3) is distributed would be essential in discerning whether these residues play any role in modulating trafficking of the protein in the presence of BST2. Nevertheless, there appears to be a selective pressure to maintain the glutamate residue at this position in order to ensure optimal activity of the TMD as shown herein. Of note, in the case of VpuC, the hinge region residues do meet the canonical sequence requirements for a di-leucine motif (ExxxLL), whereas the second helix possesses a putatively sub-optimal di-leucine motif (ExxxMV). It remains to be elucidated whether the hinge region glutamate residue in VpuC contributes towards binding to BST2 and/or CD4, and whether it is involved as a part of an active trafficking signal.

2.5 Conclusions

Overall, this work demonstrates an important regulatory role of the Vpu transmembrane-proximal hinge region for the ability of the protein to optimally interact with its target host factors. As such, it is an important determinant for optimal Vpu-mediated BST2 antagonism and CD4 degradation, both of which are key for viral pathogenesis. Furthermore, considering that this domain is highly conserved, occurs within the cytosolic region of the protein, and modulates binding to various host targets, it may represent an attractive target for the development of anti-Vpu inhibitors, unlike the TMD, which by virtue of its association to the membrane is less accessible.

2.6 Methods

2.6.1 Antibodies and reagents: Rabbit sera for pre-immune and anti-Vpu, mouse anti-p24 monoclonal and anti-CD4 (OKT4) Abs were described previously [283]. Rabbit serum for anti-BST2 and mouse anti-myc (clone 9E10) Abs used for co-immunoprecipitation were also described previously [379]. Polyclonal sheep anti-TGN46 and mouse anti-BST2 used in confocal microscopy immunostainings were obtained from Serotec and Abnova, respectively. Rabbit Abs directed against CD4 (Santa Cruz Biotechnology), Myc (Sigma) and actin (Sigma) used for Western blot analyses were obtained from commercial sources as indicated. All secondary Alexa-conjugated immunoglobulin G (IgG) Abs used for flow cytometry and confocal microscopy were obtained from Life Technologies. Paraformaldehyde (PFA) was obtained from Sigma.

2.6.2 Plasmids, cell lines and transfections:

Plasmids: WT HIV-1 NL4.3 was obtained through the NIH AIDS Reagent Program and a dU provirus plasmid was kindly provided by Dr. Klaus Strebel. All Vpu mutants described were in the parental NL4.3 provirus genome background, and were generated by PCR-based Quick-change site-directed mutagenesis according to standard protocols using cloned Pfu (Agilent). The oligonucleotides used are (only the sense strand is shown): ***E28A*** 5'-CATAGTAATCATAGCATATAGGAAAATATTAAGA-3'; ***E28D*** 5'-CCATAGTAATCATAGATTATAGGAAAATATTAAGAC-3'; ***E28Q*** 5'- CCATAG-TAATCATACAATATAGGAAAATATTAAGAC-3'; ***E28A/L33A*** 5'-GTCCATAGTAATCAT-AGCATATAGGAAAATAGCAAGACAAAGAAAAATAGACAG-3'; ***E59K/L63F*** 5'-CATAGAATATAGGAAATTATTAAGACAAAGAAAAATAGACAGG-3'; ***S52/56D*** 5'-GAAAGAGCAGAAGACGATGGCAATGAGGATGAAGGAGAAGTATCAGCA-3'; ***A14L*** 5'-GTAGCATTAGTAGTATTAATAATAATAGCAATAGTTGTGTGGTCC-3'; ***A18L*** 5'-GCATTAGTAGTAGCAATAATAATATTAATAGTTGTGTGGTCCATAG-3'; ***A10L/A18L*** 5'-CCTATAATAGTAGCAATAGTACTATTAGTAGTAGCAATAATAATACTAATAGTTGT-GTGGTCC-3'; ***A10L/A14L/A18L*** 5'-CCTATAATAGTAGCAATAGTATTATTAGTAGTA-TTAATAATAATATTAATAGTTGTGTGGTCC-3'. For the E28A/L33A-E59K/L63F mutant, sequential mutagenesis was performed using the E59K/L63F oligonucletides on the E28A/L33A template plasmid. The provirus Vpu-AAA mutant was also described previously [228], except that the provirus backbone used here did not encode GFP. All plasmid constructs were confirmed by DNA sequencing. The expression plasmids pCR3.1, encoding the short isoform of BST2 [228], SVCMV-CD4 [254], and pSVCMV VSV-G encoding the vesicular stomatitis virus glycoprotein G (VSV-G) [379] were

described previously as indicated. The expression plasmid for pCR3.1 HA-BST2 gene was kindly provided by Dr. Bieniasz [388] while the pcDNA/Myc-His-β-TrCP plasmid was obtained from Dr. Richard Benarous [110].

Cell lines: HEK293T and HeLa cells were obtained from the American Type Culture Collection. Both cell types were maintained in Dulbecco's Modified Eagle Medium (DMEM, Wisent) supplemented with 10% fetal bovine serum (FBS) and a combination of Penicillin-Streptomycin antibiotics. HeLa cells depleted of BST2 were generated by transducing lentiviral vector particles encoding shRNA targeting BST2 [389] or, as a control, a non-targeting shRNA. The HeLa-TZM-bl cells were obtained through the NIH AIDS Reagent Program.

Transfections: HEK293T and HeLa cells were seeded overnight and transfected using the calcium-phosphate method and lipofectamine 2000™ (Invitrogen), respectively.

2.6.3 Virus particle release assay: Viral particle release was analyzed by Western blot as described previously [379]. The intensity of Gag signal was measured by scanning densitometry analyses using ImageJ software (NIH). The ratio of virion-associated Gag (p24) signal to cell-associated Gag (p24 and p55) was indicative of the viral particle release efficiency. Histograms indicate Vpu-specific virus release efficiencies following subtraction of any background or non-Vpu specific effect as determined by the dU control. Vpu-mediated virus release efficiency was normalized to the value obtained from HIV-1 WT Vpu cultures, which was arbitrarily set at 100%.

2.6.4 BST2 surface staining and flow cytometry: BST2 cell-surface staining was performed on HeLa cells co-transfected with proviral constructs expressing the indicated mutants together with a GFP expressor plasmid for gating purposes. Preparation of cells and flow cytometry analysis were described previously [379]. Surface BST2 down-regulation efficiency was determined by subtracting BST2 geometric mean fluorescence intensity (MFI) values obtained from GFP-expressing (transfected) cells from BST2 MFI values obtained from GFP non-expressing cells. For histograms, the efficiency of BST2 downregulation was expressed relative to that obtained from HIV-1 WT Vpu cultures, which was arbitrarily set at 100%.

2.6.5 Production of VSV-G pseudotyped HIV-1 viruses: HEK293T cells were co-transfected with NL4.3 (or appropriate mutants) proviral constructs and pSVCMV VSV-G as described previously [270]. Forty-eight hours post-transfection, supernatants of transfected cells were clarified by centrifugation, filtered through a 45-µm filter, and pelleted by ultracentrifugation onto a 20% sucrose-PBS cushion for two hours at 112 000 × g at 4°C. Concentrated viruses were resuspended in DMEM supplemented with 10% FBS. Viruses were titrated using a standard MAGI assay as previously described [379].

2.6.6 Protein steady-state levels:

BST2 degradation: HeLa cells were infected with VSV-G-pseudotyped HIV-1 proviruses expressing WT, dU or Vpu mutants at an MOI of 1.5 in presence of polybrene. The infection medium was replaced four hours post-infection. Forty-eight

hours post-infection, cell lysates were harvested and lysed in RIPA-DOC buffer. BST2 steady state levels were analyzed by Western blotting as described [379].

CD4 depletion: HEK293T cells were co-transfected with a CD4 expressor and the appropriate proviral plasmids. Following lysis at forty-eight hours post-transfection, lysates were subjected to Western blotting and probed for steady-state levels of CD4.

2.6.7 Cellular localization and confocal microscopy: Cover-slip seeded BST2-expressing or BST2-depleted HeLa cells were transfected with NL4.3 proviral plasmids expressing either WT Vpu or the indicated Vpu mutants. Twenty-four hours post-transfection, cells were fixed for 30 min in 4% PFA and then permeabilized in 0.2% Triton for 5 min. Following washes, permeabilized cells were incubated for 2 hours at 37°C in 5% milk-PBS containing rabbit anti-Vpu, sheep anti-TGN46 and/or mouse anti-BST2 Abs as appropriate. The cells were then washed and incubated with the appropriate Alexa Fluor-coupled secondary Abs for 45 min at room temperature followed by an incubation with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min also at room temperature. All analyses were acquired using a 63× Plan Apochromat oil immersion objective on an LSM710 Observer Z1 laser scanning confocal microscope (Zeiss). Quantitative analyses were performed using the Volocity software (PerkinElmer Inc.), using automated signal thresholding.

2.6.8 Co-IP binding assays:

Vpu and BST2 interaction: HEK293T cells were co-transfected with a BST2 expressor plasmid (WT or a short isoform of BST2) together with proviral plasmids

expressing either WT Vpu or indicated mutants. Forty-eight hours post-transfection, cells were harvested in PBS/EDTA, centrifuged and lysed in CHAPS buffer (50 mM Tris, 100 mM NaCl, 0.5% CHAPS, pH 7.2) supplemented with a cocktail of protease inhibitors (Protease Inhibitors Complete, with EDTA). An aliquot of 10% of cell lysate was preserved for Western blot loading as “Input” control. Cell lysates were then pre-cleared with protein A sepharose beads coated with pre-immune rabbit serum for 1 h at 4°C. Following pre-clearing, the lysates were incubated with rabbit anti-BST2 specific serum for 3 hours at 4°C, and then precipitated using protein A sepharose beads in a further 3-hour incubation at 4°C. Western blotting was then used to probe for presence of Vpu and BST2 in immunoprecipitated fractions.

Vpu and CD4 interaction: HEK293T cells were co-transfected with a CD4 expressing plasmid and the appropriate proviral plasmids, and similar steps were followed as above, except pre-immune mouse serum was used for pre-clearing, and mouse anti-CD4 Abs were used to pull down Vpu.

Vpu and β-TrCP binding: HEK293T cells were co-transfected with a myc-tagged β-TrCP2 expressing plasmid and the appropriate proviral plasmids, and similar Co-IP steps were followed as above, except pre-immune mouse serum was used for pre-clearing, and mouse anti-myc Abs were used to pull down Vpu.

2.6.9 Statistical analysis: Statistical analyses for confocal microscopy data were performed using an unpaired, Mann-Whitney test. All other analyses were performed using two-way ANOVA, with Tukey’s multiple comparison test. Values were considered statistically significant at ‘p’ values of <0.05. For all statistical analyses, ****, ***, **, *

and ‘ns’ denote $p<0.0001$, $p<0.001$, $p<0.01$, $p<0.05$ and $p>0.05$, respectively (ns = not significant).

2.7 Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: All data generated during this study are included in this published article and its additional files. The HIV sequence datasets analysed during the current study are available in the Los Alamos HIV sequence database, http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html, accessed June 2015.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was supported by grants from the Canadian Institutes of Health Research (CIHR: PJT-148686) to E.A.C. E.A.C. is a recipient of the IRCM-Université de Montréal Chair of Excellence in HIV Research. S.L. is a recipient of a ‘hommage à la découverte’ studentship from the Institut de Recherches Cliniques de Montréal. The funding body played no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions: SL and EAC conceived and designed experiments. SL performed the experiments. SL and EAC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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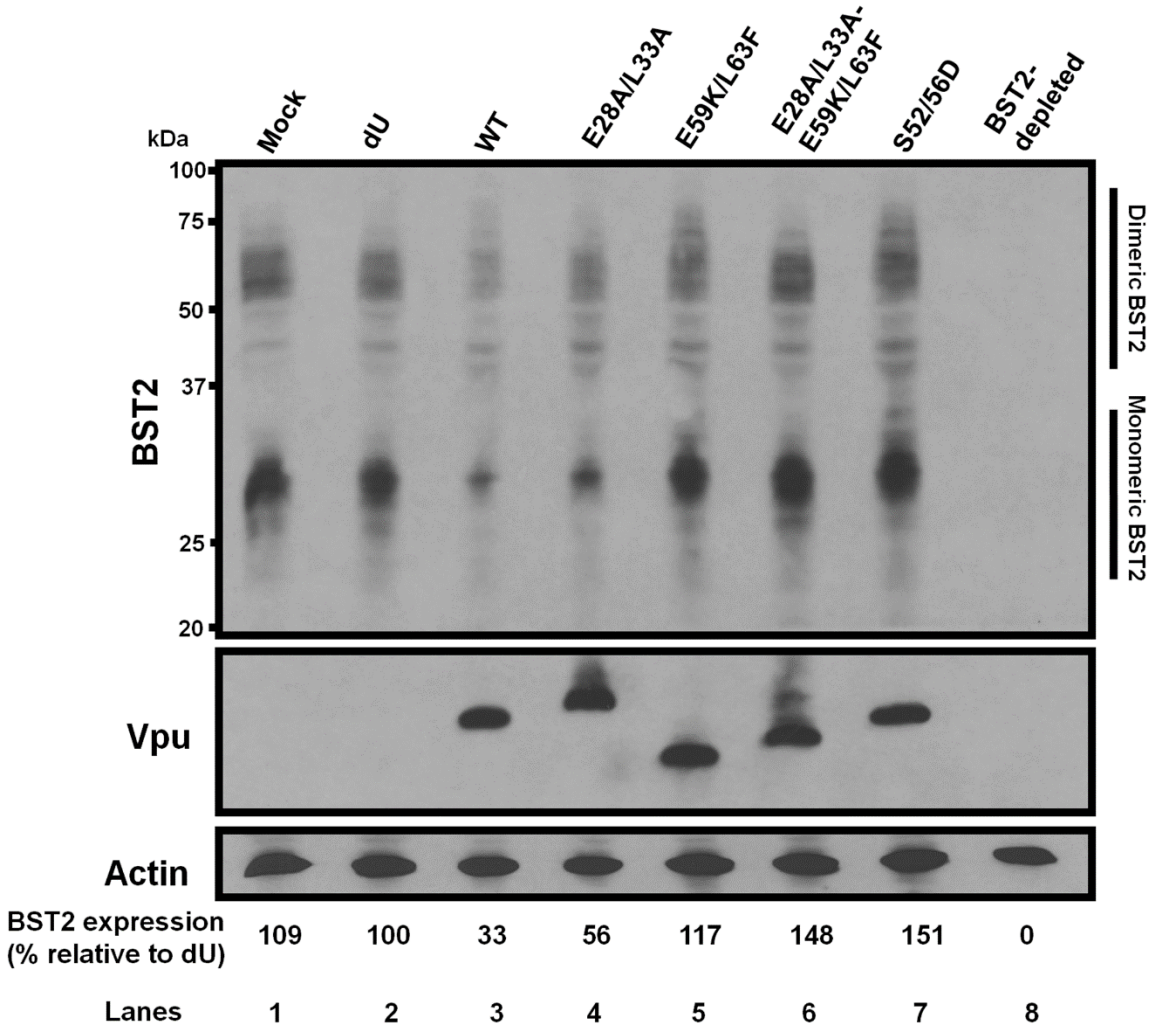
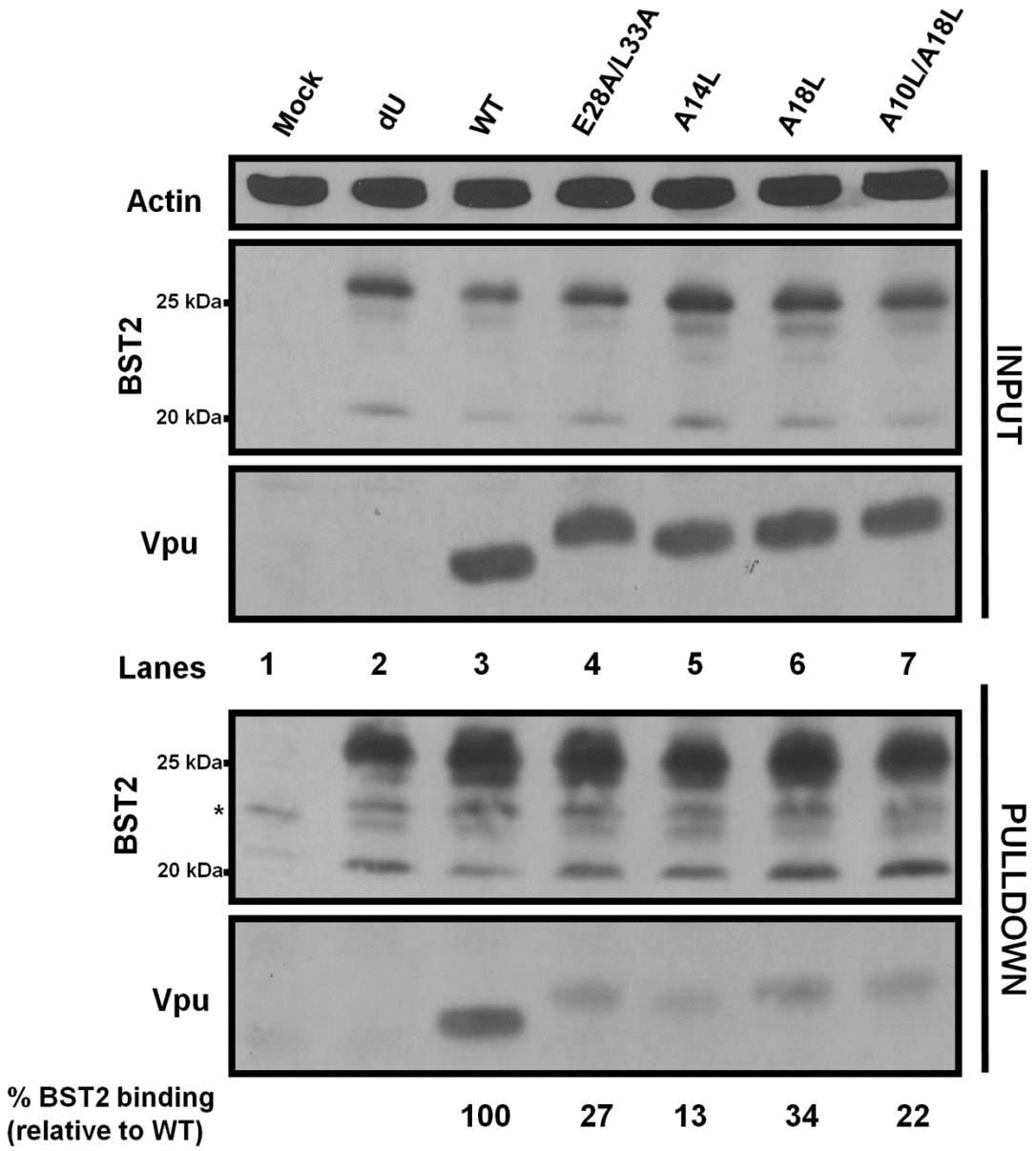
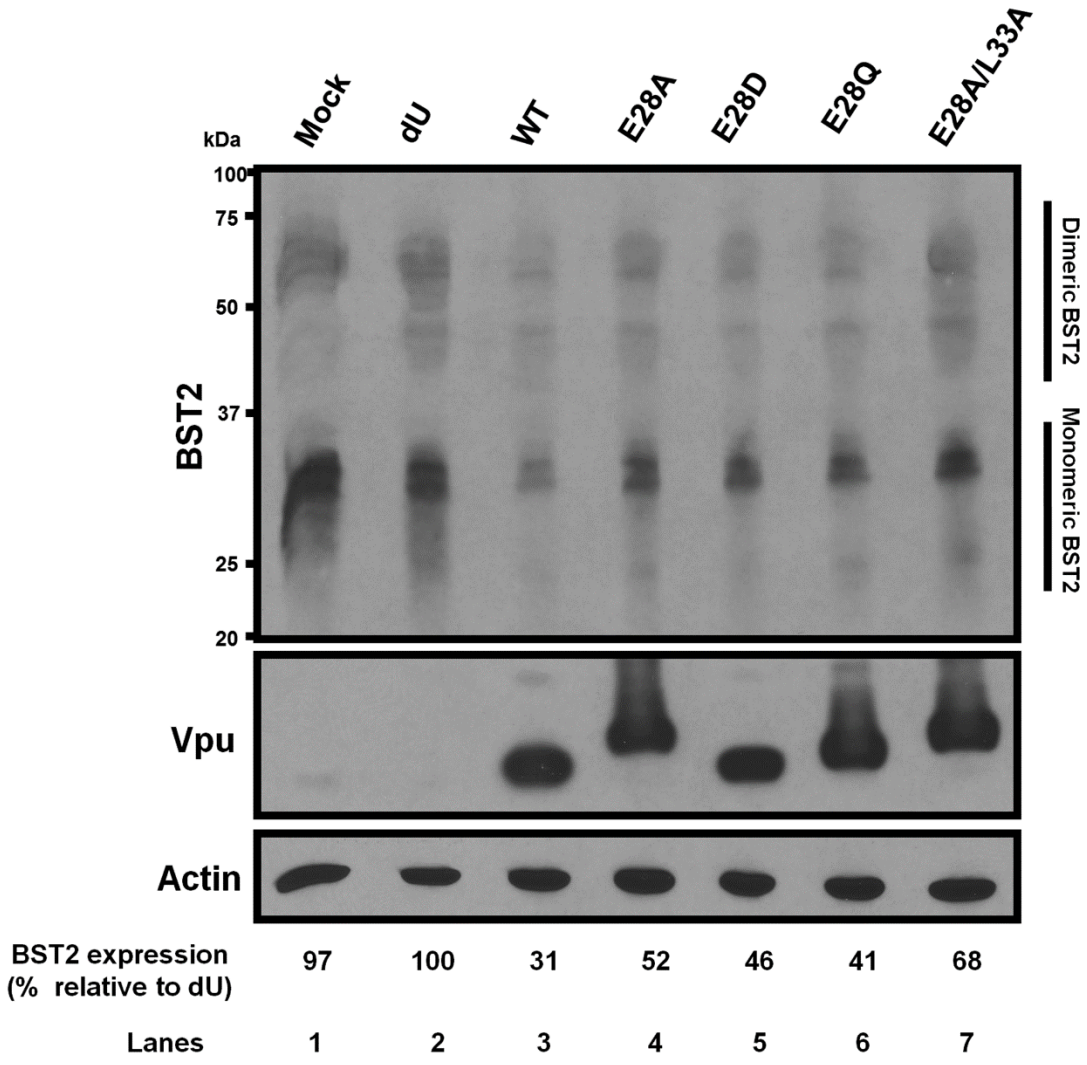
2.8 Additional Files



Additional File 2.1: Figure S2.1: Ability of Vpu mutants to mediate BST2 degradation. Shown is a representative Western blot indicating the steady state levels of BST2 in mock-infected HeLa cells (lane 1) as well as in Hela cells following infections with VSV-G-pseudotyped HIV-1 viruses encoding WT Vpu and the indicated Vpu mutants (lanes 2-7). Analysis of BST2 expression in BST2-depleted HeLa cells is also shown in lane 8. Below the blot is the extent of BST2 expression based on densitometric analyses of the intensities of BST2-related band signals obtained from each Vpu mutant, relative to dU (set at 100%).



Additional File 2.2: Figure S2.2: **BST2 binding capacity of Vpu TMD mutants**. Co-IP following co-transfection of HEK293T cells with a proviral construct encoding WT Vpu, E28A/L33A or the indicated Vpu TMD mutants and a BST2 expressor. Below each blot is the extent of BST2 binding of each Vpu mutant, relative to WT Vpu (set at 100%). For each condition, BST2 binding efficiency was determined from the ratio obtained from densitometric analyses of the intensities of Vpu- and BST2-related band signals in the immunoprecipitated fractions. The asterisk denotes an Ab-related band.



Additional File 2.3: Figure S2.3: **E28 is important for efficient degradation of BST2.** Shown is a representative Western blot indicating the steady state levels of BST2 in mock-infected HeLa cells (lane 1) as well as in Hela cells following infections with VSV-G-pseudotyped HIV-1 viruses encoding WT Vpu and the indicated Vpu mutants (lanes 2-7). Below the blot is the extent of BST2 expression based on densitometric analyses of the intensities of BST2-related band signals obtained from each Vpu mutant, relative to dU (set at 100%).

Chapter 2 – Chapter 3 bridging text

Chapter 2 demonstrates an important role of the Vpu transmembrane-proximal hinge region for the ability of the protein to optimally interact with BST2 and CD4. Thus, this cytosolic hinge region of Vpu may represent a feasible target for development of anti-Vpu inhibitors, a long-term quest in the field. Such inhibitors would prevent Vpu-mediated CD4 degradation, thereby increasing both intracellular and surface levels of CD4. Consequently, intracellular CD4 may sequester Env thus reducing amounts of Env molecules reaching virus assembly sites and incorporated into virions. Moreover, CD4 at the cell surface can interact with Env, resulting in incorporation of Env-CD4 complexes into virions. Importantly, Env-CD4 interactions uncover epitopes on Env that are targeted by CD4-induced anti-HIV Abs (such as A32), potentially leading to killing of the infected cells through ADCC. With this in mind, Chapter 3 investigates whether Vpu (together with its CD4 co-targeting partner Nef) modulates ADCC activity mediated by CD4i nNAbs. Essentially, we are testing if enhancing CD4 expression through inactivating or depleting Vpu and Nef promotes Env-CD4 interactions at the cell surface that ultimately facilitate cell lysis.

In addition to preventing CD4 degradation, Vpu inhibitors or inactivating Vpu would promote BST2-mediated virion tethering at the cell surface. Importantly, this BST2-mediated virion crosslinking at the cell surface increases the quantity of Env molecules that can interact with CD4, yet again leading to ADCC. As such, we also investigated whether BST2-mediated virion tethering augments cell lysis.

CHAPTER 3: BST2 AND CD4 AUGMENT OR FACILITATE KILLING OF INFECTED T CELLS *VIA* ADCC

Preface

Author Contributions: The work presented in this chapter is part of an article [389] published in Retrovirology (2014) entitled “Nef and Vpu protect HIV-infected $CD4^+$ T cells from antibody-mediated cell lysis through down-modulation of CD4 and BST2” by Pham TN (TNQP), **Lukhele S (SL)**, Hajjar F (FH), Routy JP (JPR) and Cohen ÉA (EAC). TNQP, **SL** and EAC conceived and designed experiments. TNQP, **SL** and FH performed the experiments. TNQP developed and performed the ADCC assays. TNQP and **SL** performed flow cytometry stainings, established and characterized BST2-depleted $CD4^+$ T cell lines, characterized effects of Vpu and Nef on CD4 expression as well as BST2 antagonism. **SL** and FH produced viruses. JPR provided patient plasmas. TNQP, **SL** and EAC analyzed the data. TNQP and EAC wrote the manuscript. As such, in addition to data not presented in the manuscript, SL contributed to Figures 3.1; 3.4 and 3.5, as well as Additional file 3.1: Figure S3.1 and Additional file 3.2: Figure S3.2.

Original scholarship and distinct contributions to knowledge: We provide evidence that accumulation of CD4 on infected cells, which unmasks epitopes on Env that are recognized by CD4i nNAbs, sensitizes infected cells to ADCC mediated by

these CD4i, nNAbs. We further demonstrate that increasing the amount of Env epitopes at the cell surface *via* BST2-mediated virion tethering enhances ADCC mediated by such Abs. Overall, we uncover an important mechanism whereby HIV Nef and Vpu function synergistically to protect infected cells from Ab-mediated lysis. As such, the study provides insights into the factors promoting viral persistence, demonstrating that WT virus-infected cells are insensitive to ADCC.

HIV Nef and Vpu Protect HIV-Infected CD4$^+$ T Cells from Antibody-Mediated Cell Lysis through Down-Modulation of CD4 and BST2

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Running title: HIV Nef and Vpu spare infected T cells from ADCC

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3.1 Abstract

Background: HIV proteins Nef and Vpu down-modulate various host factors to evade immune defenses. Indeed, the CD4 receptor is down-regulated by Nef and Vpu, whereas virion-tethering BST2 is depleted by Vpu. Antibody-dependent cell-mediated cytotoxicity (ADCC) is increasingly recognized as a potentially powerful anti-HIV response. Given that epitopes which are specific for ADCC-competent anti-HIV antibodies are transitionally exposed upon CD4-mediated HIV entry, we investigated whether by depleting CD4 and BST2, HIV could negatively affect ADCC function.

Results: Using anti-envelope (Env) Abs A32 and 2G12 to trigger ADCC activity, we find that interactions between CD4 and Env within infected cells expose ADCC-targeted epitopes on cell-surface Env molecules, marking infected T cells for lysis by immune cells. We also provide evidence to show that by cross-linking nascent virions at the plasma membrane, hence increasing cell-surface Env density, BST2 further enhances the efficiency of this antiviral process. The heightened susceptibility of T cells infected with a virus lacking Nef and Vpu to ADCC was recapitulated when plasmas from HIV-infected patients were used as an alternative source of Abs.

Conclusions: Our data unveil a mechanism by which HIV Nef and Vpu function synergistically to protect infected cells from ADCC and promote viral persistence. These findings also renew the potential practical relevance of ADCC function in vivo.

Keywords: HIV accessory proteins Nef and Vpu, BST2, CD4-Env interactions, ADCC

3.2 Background

The human immunodeficiency virus (HIV)-type 1 gains access to its target cells, primarily $CD4^+$ T cells and macrophages, through the cellular receptor CD4 and co-receptor CCR5 or CXCR4. The HIV-1 RNA genome encodes many proteins including the structural envelope (Env) and accessory proteins Vpu and Nef. Over the years, studies have been conducted to decipher how HIV-1 exploits its viral elements, especially the accessory proteins, to modulate the host's fundamental cellular machineries and responses in order to perpetuate its existence. Aside from their involvement in altering the expression of other host factors, Vpu and Nef, along with Env, can down-regulate CD4, albeit through distinct mechanisms [101, 390]. Indeed, Env and Vpu, which are expressed late during viral replication, target newly synthesized CD4 molecules in the endoplasmic reticulum (ER), while the early expressed Nef focuses on CD4 already at the plasma membrane (PM). CD4 down-modulation by Env [391, 392] is mediated through the formation of CD4-Env complexes in the ER, thus preventing CD4 trafficking to the cell surface [251], whereas that by Vpu occurs through CD4 ubiquitination and degradation via an ER-associated protein degradation (ERAD)-like mechanism [254, 255]. Nef induces endocytosis of cell-surface CD4 molecules, targeting them for degradation in the lysosomes [243, 393]. This functional redundancy leading to CD4 depletion is likely to be beneficial to the virus since it is thought to allow efficient Env trafficking to viral assembly sites while at the same time preventing superinfection and premature cell death [101, 153]. In addition, Vpu also down-

modulates BST2, a type 1 interferon-induced host factor that cross-links nascent virions at the cell surface, thus restricting their release from infected cells [76, 77].

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a type of humoral immune response mediated by effector cells of the innate immune system including natural killer (NK) cells and monocytes/macrophages [347]. In ADCC, binding of antibodies (Abs) to antigens present on target cells occurs through the Fab portion of the Ab, and leads to target-effector cell engagement via the Fc portion of the Ab and the Fcγ receptor (FcR) on effector cells. Binding of antigen-coated IgG to the FcR presumably causes a release of cytolytic granules and subsequent lysis of target cells.

Over the past decade, Fc-mediated effector functions including ADCC have been increasingly recognized as a potentially powerful host response against HIV-1 infection and dissemination. Indeed, findings from studies with HIV-infected patients and simian immunodeficiency virus (SIV)-infected macaques have implicated ADCC as an immune correlate of viral selection pressure, dampened viral replication, delayed disease progression, and even infection immunity [394-397]. Along this line, non-neutralizing Abs that can mediate FcR-dependent effector functions against HIV-1 [324, 334, 335] are thought to have contributed to protection in the Thai RV1144 vaccine trial [333]. Furthermore, passive transfer of Abs to macaques established the critical importance of the Fc portion of IgGs in preventing infection [341, 342]. In summary, these studies underscore the often underappreciated importance of Fc-mediated effector responses driven by non-neutralizing Abs in the overall Ab-mediated protection against HIV-1.

The strength of ADCC function can be influenced by various host and viral factors. Among these are: (1) FcR expression levels on effector cells, (2) specificity of

the Fab region, (3) abundance of viral antigens, and (4) accessibility of Abs to their cognate epitopes. In this context, recent studies focused on advancing our understanding of the nature of Abs that are capable of directing lysis of infected T cells [324, 327, 334]. As such, much of the research has been centered on Abs against the HIV-1 Env, since this protein represents a major viral antigen targeted by the host's immune responses. Env is expressed on the surface of infected cells and is incorporated into virions during viral assembly. The functional spike of the virion is a trimeric complex consisting of gp120 and the non-covalently bound transmembrane gp41 subunit [37]. Env gp120 is exposed on the virion surface and binds to the CD4 receptor, whereas gp41 is normally buried within the viral envelope. Upon binding to CD4, gp120 undergoes sequential conformational changes that allow interactions with one of the primary co-receptors, CXCR4 or CCR5, which subsequently trigger exposure of the fusogenic gp41 ectodomain within the Env trimer [38]. Ultimately, the transition of the gp41 ectodomain configuration into a six-helix bundle results in fusion of the virus to target cells [38].

Most recently, it has been suggested that the face of gp120 occluded in the trimeric Env by gp41 is a potent ADCC target [327]. Indeed, analyses of human monoclonal Abs that recognize transitional epitopes exposed during Env-CD4 interactions revealed a strong bias of ADCC-competent Abs for Cluster A epitopes contained within this region of gp120 [327]. Among such anti-Env Abs is A32, which recognizes a discontinuous epitope on the inner domain of gp120, and has been documented to be capable of mediating ADCC [324, 334]. The A32 epitope, which is expressed on $CD4^+$ T cells infected in vitro with transmitted/founder viruses, could

trigger efficient ADCC activity on both virally infected and gp120-coated $CD4^+$ T cells [324]. More importantly, the A32 Fab fragment could block the majority of ADCC activity in plasma of HIV-1 infected patients, suggesting that if efficiently accessible, the A32 epitope is highly recognizable by Abs produced during HIV infection [324].

In light of the data discussed above, we asked whether HIV might exploit its natural propensity to down-modulate CD4 and BST2 to conceal ADCC-targeted epitopes and shield infected cells from destruction through ADCC. Here-in, using an in vitro infection system whereby primary $CD4^+$ T cells are infected with isogenic viruses deficient of Nef and/or Vpu accessory proteins, we delineate the synergistic contributions of these two HIV proteins to the removal of CD4 and BST2 from the cell surface, thereby shielding infected T cells from ADCC. With these results, our study unveils a potential mechanism by which HIV evades the host's immune defenses to promote persistence.

3.3 Results

3.3.1 Enhanced binding of anti-Env antibodies on $CD4^+$ T cells infected with viruses deficient of HIV Nef and/or Vpu. To assess the recognition of Env by anti-Env Abs on infected T cells, $CD4^+$ T cells were infected with CCR5-tropic NL4-3.ADA.IRES.GFP WT virus or its derivatives lacking Vpu (ΔVpu or U-), Nef (ΔNef or N-) or both (ΔNefΔVpu or N-U-) and evaluated for Env expression. For a comparative analysis with A32, we used neutralizing Ab 2G12, which recognizes a discontinuous,

glycan-dependent epitope on the gp120 outer domain and, as such, is distinct from other neutralizing Abs that recognize CD4-induced epitopes [318]. To this end, Env staining by A32 was about 2 to 2.5-fold higher on CEM.NKR $CD4^+$ T cells infected with the ΔNef or ΔVpu virus and intriguingly, nearly 8-fold higher on those infected with the ΔNefΔVpu virus ($P < 0.005$) (Figure 3.1A). Notably, the Env staining profile by 2G12 was different with the ΔVpu virus relative to the ΔNef in that the former displayed a significantly higher increase in epitope recognition ($P < 0.005$), suggesting a potential contribution of BST2 to this enhancement. Similar to A32, 2G12 staining was significantly higher with the ΔNefΔVpu virus ($P < 0.0005$) (Figure 3.1B). The Env staining patterns by A32 and 2G12 were largely similar for primary $CD4^+$ T cells infected with the same viruses, indicating that the data were not unique to cell lines (Figure 3.1C and D).

3.3.2 Development and validation of a FACS-based ADCC assay. As shown in Figure 2A, enhanced A32 binding on infected T cells could be blocked by pre-incubating target cells with the A32 Fab fragment prior to A32 exposure, demonstrating its specificity. Subsequently, we examined whether augmented binding of A32 on T cells would promote their lysis by ADCC.

Our FACS-based ADCC assay makes use of the fact that infected target cells were GFP-marked, while effector cells were labelled with a dye, allowing for subsequent gating of GFP-positive/dye-negative cells at the end of the assay (Figure 3.2B). The % cell lysis was determined by [(proportion of GFP+ cells in the absence of effector cells – proportion of GFP+ cells in the presence of effector cells and test Ab)/proportion of

GFP+ cells in the absence of effector cells] × 100. For the example shown in Figure 2C, % cell lysis in the presence of A32 was computed to be [(24.8-15.7)/24.8] × 100 = 36.7%. Similarly, % cell lysis in the presence of control IgG was 16.5%. Importantly, in all evaluations, % cell lysis mediated by control IgG was determined simultaneously with test Abs, and used as background killing to calculate the net Ab (e.g., A32)-specific cell lysis. For Figure 3.2C example, the net A32-mediated lysis was calculated to be 36.7%-16.5% = 20.2%. By the same formulas, the net A32-mediated lysis in the presence of the A32 Fab was 6.1% (22.6%-16.5%). The attenuated ADCC activity, as a result of target cell pre-treatment with the A32 Fab, was replicated over many analyses using PBMC from different donors as effectors (Figure 3.2D). In addition, Figure 3.2D also revealed a differential ability by different PBMC donors to induce ADCC. The observed variations, which are not uncommon in analyses involving primary cells, could be due to inter-individual differences in FcR expression levels or the activation status of effector cells at the time of PBMC acquisition.

To further validate the assay, we examined the extent of target cell lysis using different effector:target (ET) ratios, and found that the magnitude of ADCC was ET-dependent (Figure 3.2E). Lastly, to determine which immune cell subsets might be responsible for inducing cell death, we used, as effector cells, total PBMC or PBMC that had been depleted of NK cells or monocytes/macrophages. As shown in Figure 3.2F, both cell subsets could elicit ADCC, although NK cells were apparently more efficient.

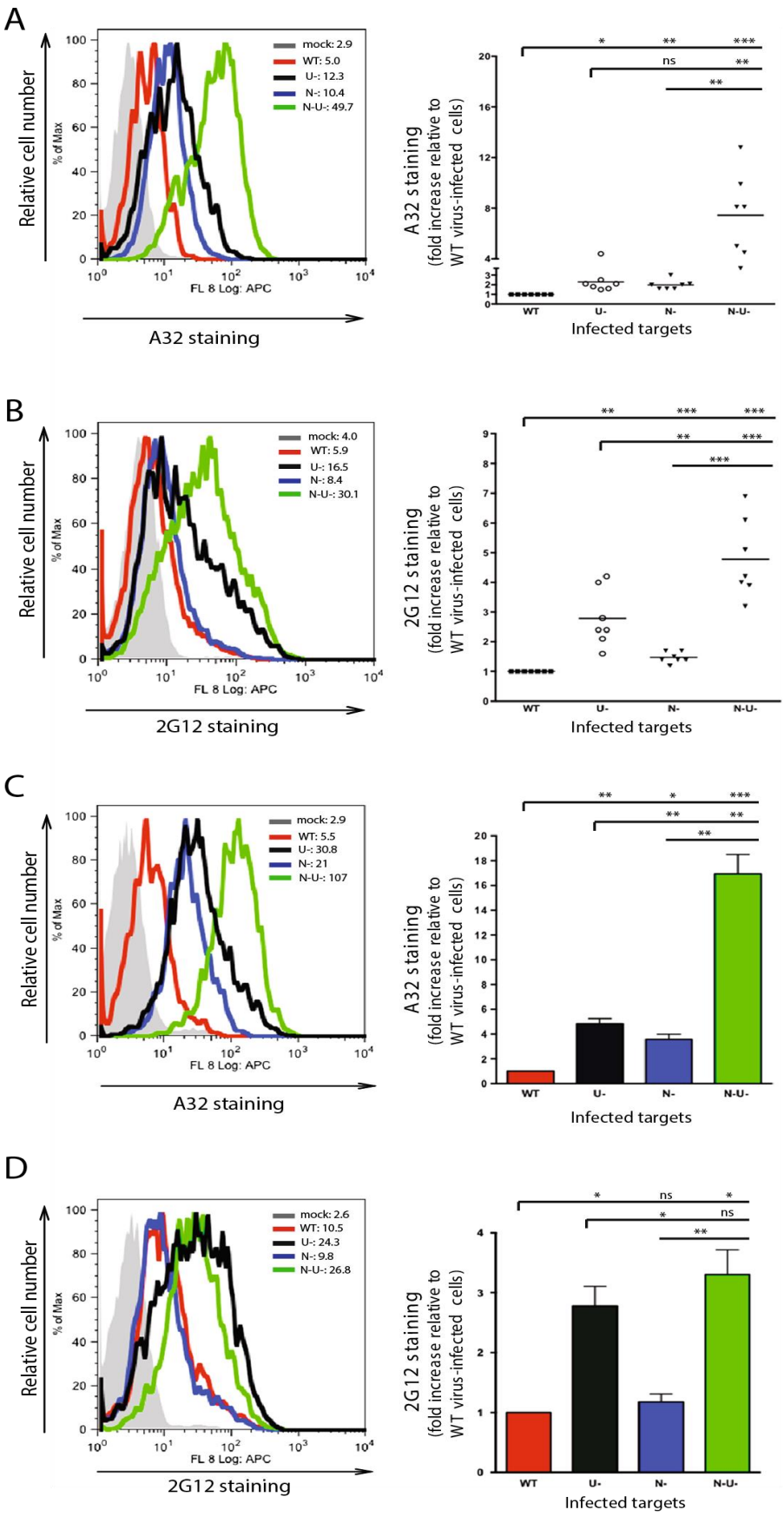
A
Relative cell number
% of Max
mock: 2.9
WT: 5.0
U-: 12.3
N-: 10.4
N-U-: 49.7
FL 8 Log: APC
A32 staining
A32 staining
(fold increase relative to WT virus-infected cells)
Infected targets
WT
U-
N-
N-U-
B
Relative cell number
% of Max
mock: 4.0
WT: 5.9
U-: 16.5
N-: 8.4
N-U-: 30.1
FL 8 Log: APC
2G12 staining
2G12 staining
(fold increase relative to WT virus-infected cells)
Infected targets
C
Relative cell number
% of Max
mock: 2.9
WT: 5.5
U-: 30.8
N-: 21
N-U-: 107
FL 8 Log: APC
A32 staining
A32 staining
(fold increase relative to WT virus-infected cells)
Infected targets
D
Relative cell number
% of Max
mock: 2.6
WT: 10.5
U-: 24.3
N-: 9.8
N-U-: 26.8
FL 8 Log: APC
2G12 staining
2G12 staining
(fold increase relative to WT virus-infected cells)
Infected targets

Figure 3.1: HIV-1 envelope expression profiles on infected CD4$^+$ T cells. CEM.NKR **(A and B)** or activated primary CD4$^+$ T cells **(C and D)** were infected with CCR5-tropic NL4.3.ADA.IRES.GFP wild-type (WT) virus or derivatives lacking Vpu (U-), Nef (N-) or both (N-U-) as detailed in Methods. Infected cells were analyzed by flow cytometry for Env expression using anti-Env A32 and 2G12 mAbs. **(A-D)** The left panels depict the extent of Env staining shown as geo-mean fluorescence intensity (MFI) on gated GFP+ cells from a representative infection. The right panels summarize the results of **(A and B)** seven experiments with each dot representing an analysis, and of **(C and D)** data obtained with primary CD4$^+$ T cells from four donors. Shown are average fold increase (+/- SEM) in Env staining relative to WT virus-infected cells. Fold increase was determined as the ratio of MFIs of GFP$^+$ cells infected with different mutants over that for the WT virus. Statistical analysis of data was done using paired Student's t-tests.

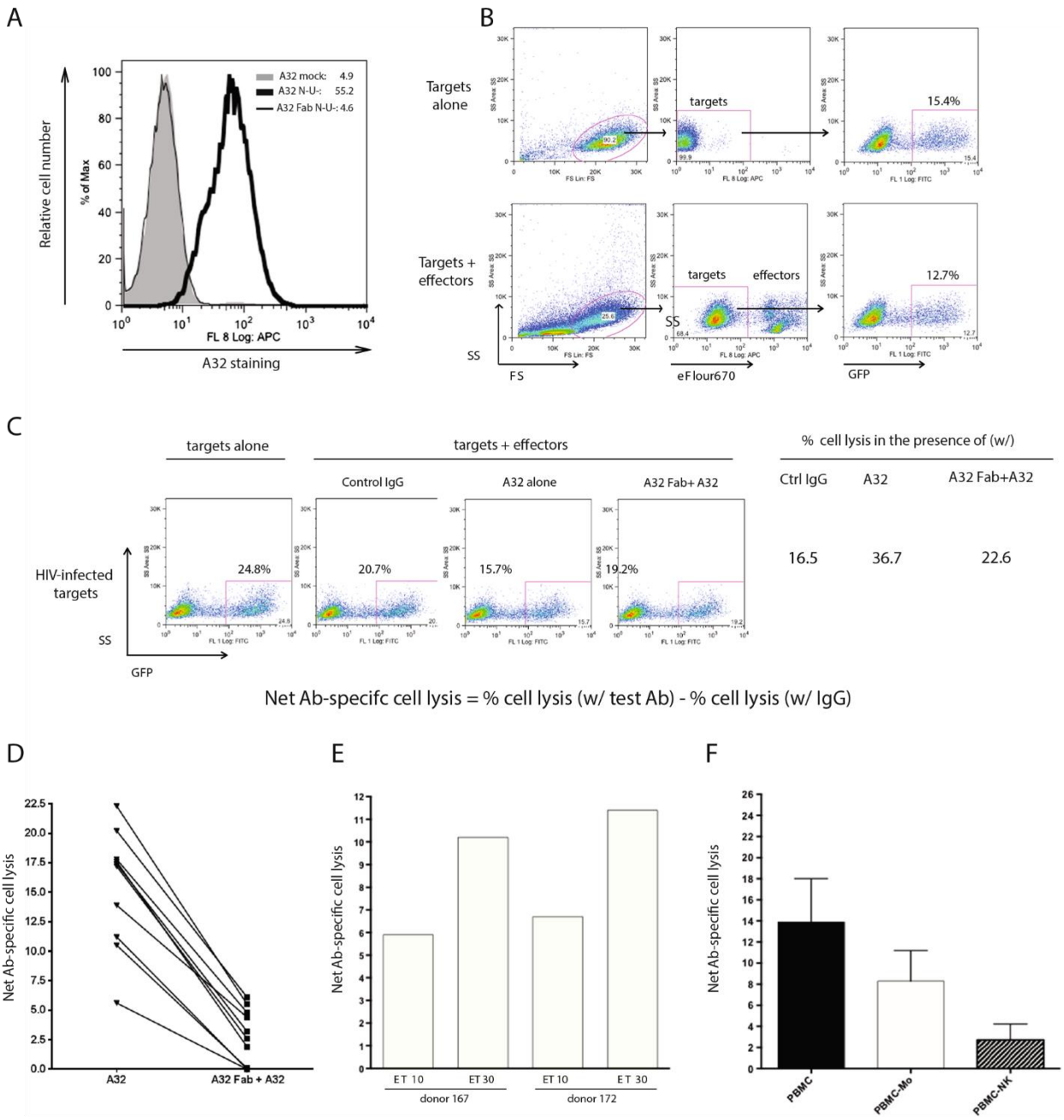

Figure 3.2: Antibody-dependent cellular cytotoxicity assay: development and validation. CEM.NKR cells were infected for 48 h with CCR5-tropic NL4.3.ADA.IRES.GFP ΔNefΔVpu and analyzed for their susceptibility to ADCC by PBMC under different testing parameters. Target cells were exposed to either control IgG, A32 or, alternatively, pre-exposed to the A32 Fab prior to the A32 exposure. Env staining was done as described in Figure 3.1 legend and ADCC was performed as described in Methods. **(A)** Evaluation of A32 staining specificity using the A32 Fab. **(B)** Gating strategy to select

eFlour670-negative, GFP + target cells by flow cytometry. **(C)** Determination of net Ab-specific cell lysis using gating strategy depicted in **(B)**. The number shown inside dot plot depicts % of GFP^+ cells remaining at the end of assay. Percent of cell lysis was calculated as [(% GFP^+ cells in the absence of effectors -% GFP^+ cells in the presence of effectors plus control IgG, A32 or A32 Fab + A32)/% GFP^+ cells in the absence of effectors] × 100. **(D)** Cell lysis in the absence (A32) or presence of the A32 Fab fragment (A32 Fab + A32). Net Ab-specific cell lysis was obtained following subtraction of % cell lysis in the presence of IgG from that by either A32 or A32 Fab + A32. Each line represents PBMC-mediated lysis from a donor. **(E)** Effect of varying ET ratios (10 or 30) on net Ab-specific cell lysis using A32 as test Ab and IgG as control. Shown are results from two representative donors. **(F)** Contributions of different cell subsets to induction of ADCC. Total PBMC or PBMC depleted of monocytes/macrophages (PBMC-Mo) or NK cells (PBMC-NK) were used as effector cells. A32 was used as test Ab and IgG as control. Histograms represent average net A32-specific lysis +/- SD of four experiments with five donors.

3.3.3 Heightened susceptibility of $CD4^+$ T cells infected with Nef- and Vpu-deficient HIV to A32-mediated ADCC.

Using the established ADCC assay, we performed a comparative analysis of the susceptibility of T cells infected with the WT virus or its derivatives to ADCC. Prior to each analysis, as a qualitative control for the infection, we performed in parallel Env staining of target cells using appropriate test Ab(s). Shown in Figure 3.3A is an example of A32 staining for the ADCC analysis illustrated in Figure 3.3B. Although A32 recognized its epitope comparably well on T cells infected with ΔNef or ΔVpu virus, those infected with the ΔNef appeared more susceptible to lysis than their ΔVpu counterparts (Figure 3.3B). Intriguingly, T cells

infected with the ΔNefΔVpu virus were consistently much more prone to lysis than all other target cells combined (Figure 3.3C and D). Also, the ADCC activity against the ΔNefΔVpu virus-infected targets was clearly not additive of that against ΔNef and ΔVpu virus. T cells infected with the WT virus were poorly susceptible to ADCC.

Given that ΔNefΔVpu virus-infected cells were invariably most prone to ADCC, we next compared the ability of A32 and 2G12 in activating killing of these target cells. For the example given in Figure 3.3E, the net Ab-specific cell lysis for A32 (60.2%-45.0% = 15.2%) was about 4-fold higher than that for 2G12 (48.7%-45.0% = 3.7%). Similar observations were made with PBMC from different donors (Figure 3.3F).

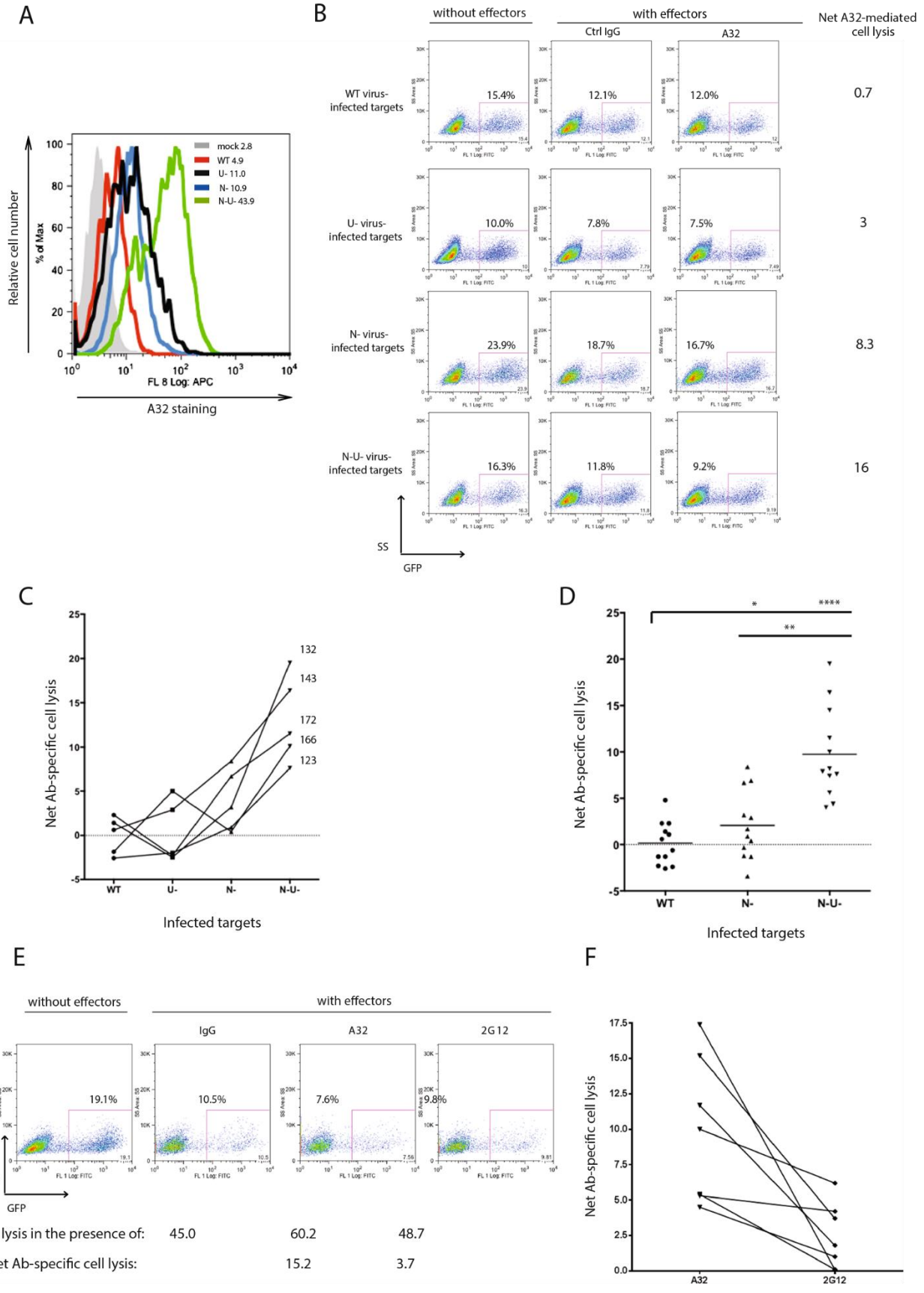
A
Relative cell number
% of Max
mock 2.8
WT 4.9
U- 11.0
N- 10.9
N-U- 43.9
FL 8 Log: APC
A32 staining
B
without effectors
with effectors
Ctrl IgG
A32
Net A32-mediated cell lysis
WT virus-infected targets
15.4%
12.1%
12.0%
0.7
U- virus-infected targets
10.0%
7.8%
7.5%
3
N- virus-infected targets
23.9%
18.7%
16.7%
8.3
N-U- virus-infected targets
16.3%
11.8%
9.2%
16
SS
GFP
C
Net Ab-specific cell lysis
132
143
172
166
123
WT
U-
N-
N-U-
Infected targets
D
*
**
****
Net Ab-specific cell lysis
WT
N-
N-U-
Infected targets
E
without effectors
with effectors
IgG
A32
2G12
19.1%
10.5%
7.6%
9.8%
SS
GFP
% lysis in the presence of: 45.0 60.2 48.7
Net Ab-specific cell lysis: 15.2 3.7
F
Net Ab-specific cell lysis
A32
2G12

Figure 3.3: Nef and Vpu reduce susceptibility of infected CD4$^+$ T cells to A32-mediated ADCC. CEM.NKR cells were infected with CCR5-tropic NL4.3.ADA. IRES.GFP viruses as mentioned in Figure 3.1 legend. Target cells were incubated with control IgG, A32 or 2G12 and analyzed for susceptibility to ADCC mediated by PBMC effector cells. Net Ab-specific cell lysis was computed as described in Figure 3.2 legend. As a qualitative control for infection, prior to the ADCC evaluation, target cells were also stained for Env expression using anti-Env Abs. **(A)** Env staining of target cells using A32. **(B)** An example of net Ab-specific cell lysis (using A32 as test Ab) from a representative donor (donor 143 in Panel **C**). **(C)** Comparative analysis of susceptibility of different target cells to A32-mediated ADCC. The five sets of numbers indicated on the right hand side of the lines represent five donors. **(D)** Summary of net A32-mediated cell lysis from six infections using PBMC from twelve donors as effector cells (each dot represents an individual). **(E and F)** Susceptibility of ΔNefΔVpu virus-infected cells to ADCC induced by A32 compared to that by 2G12. Shown in **(E)** is the result of a representative donor and in **(F)** is a summary of three experiments with seven donors, with each line representing data from a given individual. Statistical analysis of data was done using paired Student's *t*-tests.

3.3.4 A32-mediated ADCC activity is dependent on cell-surface CD4 expression and requires CD4-Env interaction.

At this point, our data have demonstrated a correlation between enhanced A32 binding and heightened ADCC susceptibility. This was most evident in T cells infected with the ΔNefΔVpu virus (Figures 3.1 and 3.3A-D). Given that the A32 epitope becomes transitionally exposed upon CD4-Env engagement during viral entry, and that both Nef and Vpu down-modulate CD4 within infected cells, we asked if the strength of ADCC function was related to CD4 expression. Indeed, CD4 down-modulation was greatest on WT-virus infected cells (about 75 and 90% on

CEM.NKR [Figure 3.4A] and primary $CD4^+$ T [Figure 3.4B] cells, respectively). Similarly, CD4 expression on ΔNef and ΔNefΔVpu virus-infected cells was reduced by 50-60% and 25-35%, respectively, highlighting the individual contributions of Nef and Vpu to CD4 depletion. Nonetheless, the role of Vpu in CD4 down-regulation was less evident in the presence of Nef, owing presumably to the distinct mechanisms utilized by the two proteins to decrease CD4 expression [101, 390]. Interestingly, the use of the ΔNefΔVpu-D368A Env virus, which harbors a mutation within the CD4-binding site of gp120 [398], completely prevented CD4 down-regulation, reaffirming the role of Env in interacting with CD4 and contributing to its depletion [251, 391, 392].

Interestingly, A32 staining on ΔNefΔVpu-D368A Env virus-infected T cells was significantly less ($P < 0.005$) compared to that on their ΔNefΔVpu virus counterparts (Figure 3.4C), highlighting the necessity of CD4-Env interactions within infected cells for unmasking the A32 epitope at the cell surface. In sharp contrast, 2G12 staining was not affected by the presence of the D368A mutation, suggesting that CD4-Env interactions are dispensable for 2G12 recognition (Figure 3.4D). Functionally, the reduction in A32 staining on ΔNefΔVpu-D368A Env virus-infected T cells was correlated with a statistically significant ($P < 0.005$) decrease in ADCC activity compared to that with the ΔNefΔVpu virus (Figure 3.4E).

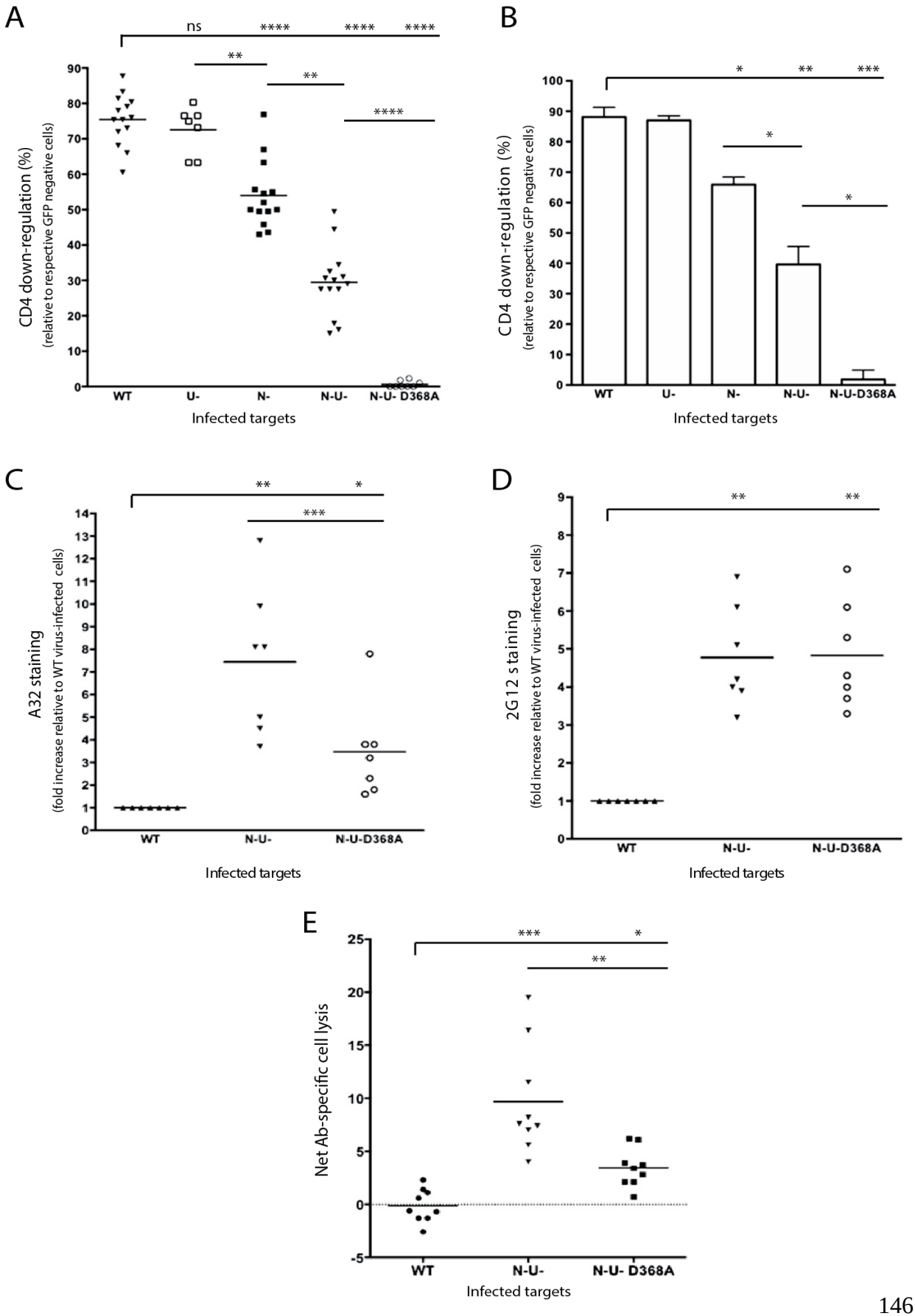
A
ns
****
****
****
**
**
****
CD4 down-regulation (%)
(relative to respective GFP negative cells)
WT
U-
N-
N-U-
N-U- D368A
Infected targets
B
*
**
***
*
*
CD4 down-regulation (%)
(relative to respective GFP negative cells)
WT
U-
N-
N-U-
N-U-D368A
Infected targets
C
**
*
***
A32 staining
(fold increase relative to WT virus-infected cells)
WT
N-U-
N-U-D368A
Infected targets
D
**
**
2G12 staining
(fold increase relative to WT virus-infected cells)
WT
N-U-
N-U-D368A
Infected targets
E
***
*
**
Net Ab-specific cell lysis
WT
N-U-
N-U- D368A
Infected targets

Figure 3.4: Heightened susceptibility to A32-mediated ADCC is intimately dependent on CD4 expression and CD4-Env interactions on target cells. CEM.NKR cells or primary $CD4^+$ T cells were infected with CCR5-tropic NL4.3.ADA.IRES.GFP viruses as mentioned in Figure 3.1 legend. The ΔNefΔVpu-D368A Env (N-U-D368A) virus harbours a mutation within the CD4-binding site of Env protein leading to defective CD4-Env interactions. **(A-B)** CEM.NKR cells **(A)** and primary $CD4^+$ T cells **(B)** were examined by flow cytometry for CD4 expression. The latter was determined based upon MFI values obtained for gated GFP^+ cells. % CD4 down-regulation was calculated as: (MFI of infected cells / MFI of GFP^- (uninfected) cells) × 100. Shown are average % of CD4 down-regulation of **(A)** a series of experiments (each dot represents an analysis), or **(B)** five evaluations with five donors. Error bars indicate SEM. **(C-D)** CEM.NKR T cells infected with WT, ΔNefΔVpu or ΔNefΔVpu-D368A virus were evaluated for Env expression using A32 **(C)** and 2G12 **(D)** Abs as detailed in Figure 3.1 legend. Env staining was determined based upon the MFI values obtained for gated GFP^+ cells. Calculations of fold increase in Env staining were as described in Figure 3.1 legend. **(E)** CEM.NKR T cells were evaluated for their susceptibility to A32-mediated ADCC as described in Figure 3.2 legend. Shown are average net cell lyses from four infections. Cell killing was done using PBMC from nine donors as effectors, with each dot representing an individual. Statistical analysis of data was done using paired Student's *t*-tests.

3.3.5 BST2 partially contributes to enhanced ADCC activity on infected T cells. At this point, we clearly demonstrated the necessity of cell-surface CD4 accumulation and CD4-Env interactions for the A32 epitope to be exposed. However, the residual A32 staining and ADCC activity observed with the ΔNefΔVpu-D368A Env mutant, which were still higher than that seen with the WT virus (Figure 3.4C and E), implied a potential involvement of a CD4-independent factor. We thus hypothesized that the remaining 3-fold increase in A32 epitope recognition and ADCC activity was due to the

accumulation of Env-containing virions at the cell surface arising from the absence of Vpu-mediated BST2 antagonism. To test this, we depleted BST2 from CEM.NKR T cells using a lentivirus-based vector that contains non-targeting (NT) or BST2-targeting (SH) shRNA. Using this system, BST2 expression was depleted in at least 95% of the cells (MFI 9.1 on SH cells vs. 80.3 on NT cells, Figure 3.5A upper panel), leaving about 5% still displaying BST2 at levels comparable to those on NT cells. Having said that, BST2 depletion in the 95% of cells was not complete since low-level BST2 was still detectable (compare staining of SH cells with pre-immune serum to that with BST2 Ab: MFI 2.7 vs. 9.1, respectively in Figure 3.5A). Of importance, the CD4 level was comparable between BST2-expressing (i.e., NT) and BST2-depleted (i.e., SH) cells. To confirm that BST2 depletion would mediate a change in virus particle release, the cells were infected with WT or ΔVpu, (U-) virus, and evaluated for virus particle release (Figure 3.5B). As expected, the level of viral release by ΔVpu virus-infected T cells in the NT cell line was about 25% of that from cells infected with the WT virus, reaffirming the role of BST2 in tethering virions at the cell surface (note the accumulation of mature p24 in cell lysates of ΔVpu virus-infected T cells in the upper left panel of Figure 3.5B). In contrast, in BST2-depleted (SH) cells, the release of the Vpu-defective virus was restored to levels that were largely comparable to that of the WT virus (right panels, Figure 3.5B; in this context, cell-associated p24 levels were similar between WT and ΔVpu virus-infected T cells). The observed partial restriction in viral particle release is likely the result of the incomplete BST2 depletion achieved with this cell line (Figure 3.5A).

Upon BST2 depletion, we found that Env recognition by the A32 and 2G12 Abs was differentially reduced in cells infected with the different viruses (Figure 3.5C and

Additional file 3.1: Figure S3.1). Indeed, A32 staining on ΔNefΔVpu virus-infected T cells of the SH line was decreased by ~30-60% depending on experiments, while that on the ΔNefΔVpu-D368A Env counterparts, by ~50%. However, for both NT and SH cells, A32 binding on ΔNefΔVpu virus-infected T cells remained higher than that on cells infected with the ΔNefΔVpu-D368A Env virus, underscoring the independent contributions of CD4 and BST2 on A32 epitope exposure (Figure 3.5C, upper panels). In contrast, while 2G12 recognition was generally reduced upon BST2 depletion, the staining patterns between ΔNefΔVpu and ΔNefΔVpu-D368A Env virus-infected cells were similar for both cell lines. This finding reaffirmed the data shown in Figure 3.4D in that Env staining by 2G12 does not require CD4-Env interactions. Interestingly, in conditions where BST2 was depleted (Additional file 3.1: Figure S3.1) or naturally absent (Additional file 3.2: Figure S3.2), the patterns of A32 and 2G12 staining on ΔVpu virus-infected cells were comparable to those on cells infected with the WT virus, implying that the enhanced Env recognition on BST2-expressing cells was likely attributed by the virion-tethering effect of BST2. On this note, analysis of Jurkat T cell lines expressing varying levels of CD4 and/or BST2 clearly revealed the relative contributions of CD4 and BST2 expression to the increase in Env staining by A32 and 2G12 in the context of WT and ΔNef and/or ΔVpu HIV infections (Additional file 3.2: Figure S3.2).

Functionally, we observed no significant decrease in ADCC activity against NT and SH cells infected with the ΔNefΔVpu virus (Figure 3.5D). However, the reduced A32 epitope exposure on ΔNefΔVpu-D368A Env virus-infected cells in the SH line abolished the “residual” ADCC activity, suggesting that BST2 most likely contributed to the ADCC function in ΔNefΔVpu-D368A Env virus-infected NT cells.

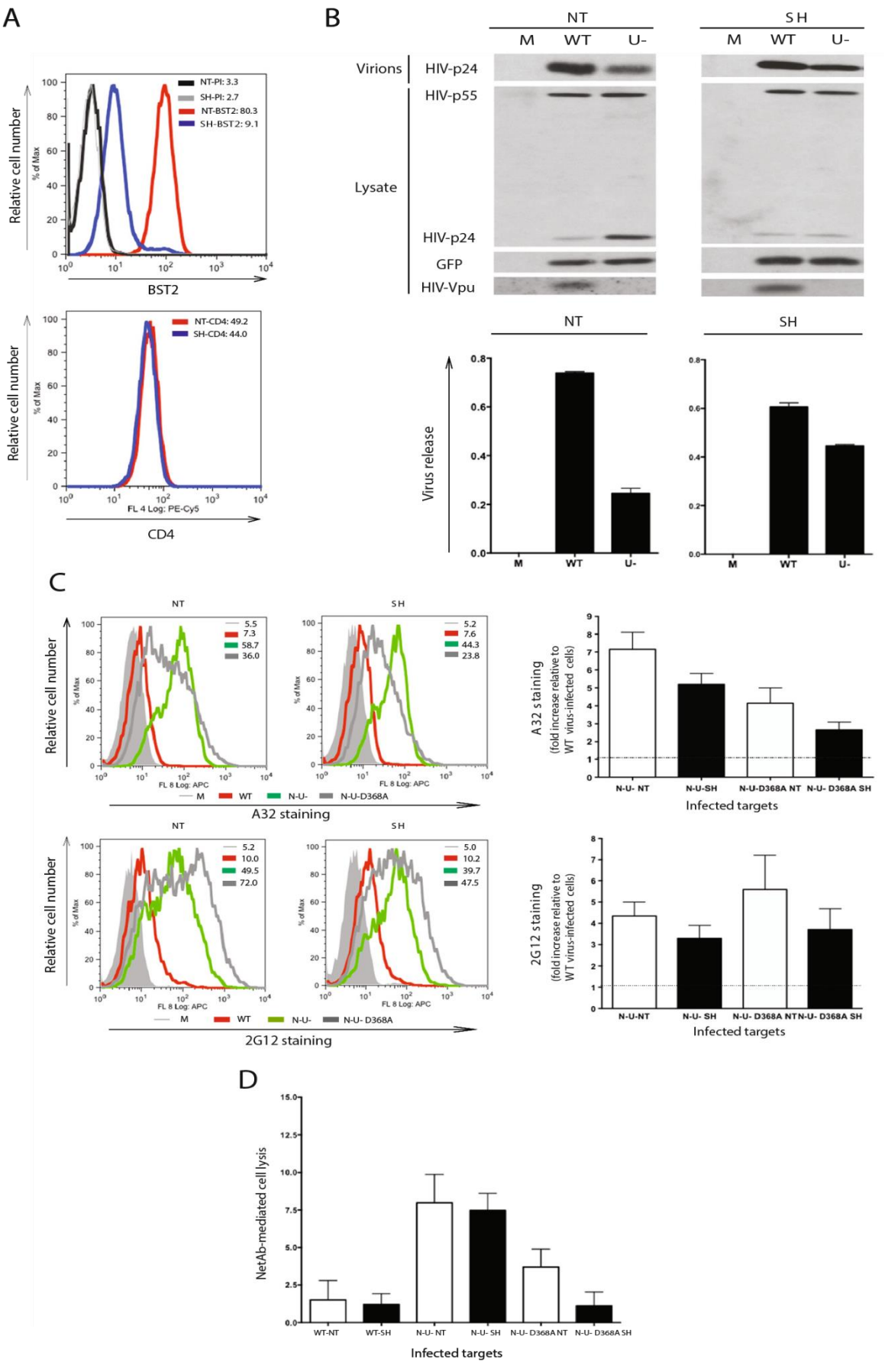
A
NT-PI: 3.3
SH-PI: 2.7
NT-BST2: 80.3
SH-BST2: 9.1
Relative cell number
% of Max
BST2
NT-CD4: 49.2
SH-CD4: 44.0
FL 4 Log: PE-Cy5
CD4
B
NT
SH
M
WT
U-
Virions
HIV-p24
HIV-p55
Lysate
HIV-p24
GFP
HIV-Vpu
Virus release
C
5.5
7.3
58.7
36.0
5.2
7.6
44.3
23.8
FL 8 Log: APC
M
WT
N-U-
N-U-D368A
A32 staining
A32 staining (fold increase relative to WT virus-infected cells)
N-U- NT
N-U-SH
N-U-D368A NT
N-U- D368A SH
Infected targets
5.2
10.0
49.5
72.0
5.0
10.2
39.7
47.5
2G12 staining
2G12 staining (fold increase relative to WT virus-infected cells)
D
NetAb-mediated cell lysis
WT-NT
WT-SH
N-U- NT
N-U- SH
N-U- D368A NT
N-U- D368A SH
Infected targets

Figure 3.5: Dissecting the potential involvement of BST2 in enhancing target cell lysis via ADCC. CEM.NKR $CD4^+$ T cells were transduced with a lentivirus containing a non-targeting (NT) or a BST2-targeting (SH) shRNA as described in Methods. **(A)** BST2 and CD4 expression on NT and SH cells as examined by flow cytometry. Parallel staining with a rabbit PI was used as a control for BST2 staining. Shown next to the overlays are expression levels in MFI obtained for gated GFP^+ infected cells from a representative analysis. **(B)** NT and SH T cells were infected with WT or ΔVpu (U-) virus and assessed for HIV-1 viral release efficiency by Western blotting. Mock (M)-infected cells were used as control. Parallel virions and cell lysates were analyzed for total Gag proteins, GFP and Vpu. The histograms underneath the Western blots depict the average quantifications of the densitometric signals from two experiments. Virus release was determined to be the ratio of the virion-associated Gag signal (corresponding to the mature p24) over all cell-associated Gag signals (corresponding to p24 and precursor p55). Within the NT and SH cells, viral release by WT virus was considered to be 100% and that by ΔVpu virus counterparts was expressed as % of the WT. **(C-D)** NT and SH $CD4^+$ T cells were infected with WT, ΔNefΔVpu (N-U-), or ΔNefΔVpu-D368A Env (N-U-D368A) virus. Env staining by A32 and 2G12 was analyzed by flow cytometry **(C)**. The histograms depict average fold increase (+/- SD) in Env recognition relative to WT virus-infected cells in three experiments. In parallel, target cells were evaluated for their susceptibility to A32-mediated cell lysis by ADCC as detailed in Figure 3.2 legend **(D)**. Shown are average (+/-SD) net cell lyses by PBMC from six donors. See also Additional file 3.1: Figure S3.1 and Additional file 3.2: Figure S3.2.

3.3.6 Plasmas from HIV-infected individuals induced robust ADCC activity against T cells infected with the ΔNef and ΔVpu viruses.

To further validate the role of CD4 and BST2 in promoting ADCC in an in vivo relevant setting, we examined whether plasmas from HIV-infected individuals could mediate lysis of infected targets in a

manner similar to A32. To this end, we found that the Env recognition patterns by patient plasmas nearly mirrored those by A32 (Figure 3.6A): the ΔNefΔVpu-virus-infected targets were most predominantly stained. As with A32, the use of the ΔNefΔVpu-D368A Env virus reduced the level of Env recognition, albeit to varying extent (1.5 to 5-fold) depending upon plasmas. In line with the A32 data, these infected plasmas elicited the most robust ADCC activity in ΔNefΔVpu-virus-infected targets (Figure 3.6B and C). Intriguingly, we found that for certain plasmas, the difference in ADCC activity against ΔNefΔVpu D368A Env and ΔNefΔVpu virus-infected targets was not statistically significant (compare N-U- to N-U-D368A of Figures 3.6B and 3.6C). Coincidentally, in the cases where this difference was not achieved (plasmas 1 and 4 shown in Figure 3.6C) we also observed a remarkably higher ADCC activity for the ΔVpu relative to the ΔNef virus implying perhaps a greater abundance of Abs behaving like 2G12 in these plasmas. It should however be mentioned that these characteristics of the plasmas were established post-analysis.

Aside from the differences in the types of anti-Env Abs that are potentially present, variations in host factors such as FcR expression levels and phenotype of the receptor may have also contributed to the kind of ADCC activity observed (compare plasma 4, donor 142 in Figure 3.6B to plasma 4, donors 135 and 137 in Figure 3.6C). This being said, these results clearly indicate that Vpu and Nef protect HIV-infected cells from ADCC-mediated by antibodies present in plasmas of HIV-infected individuals.

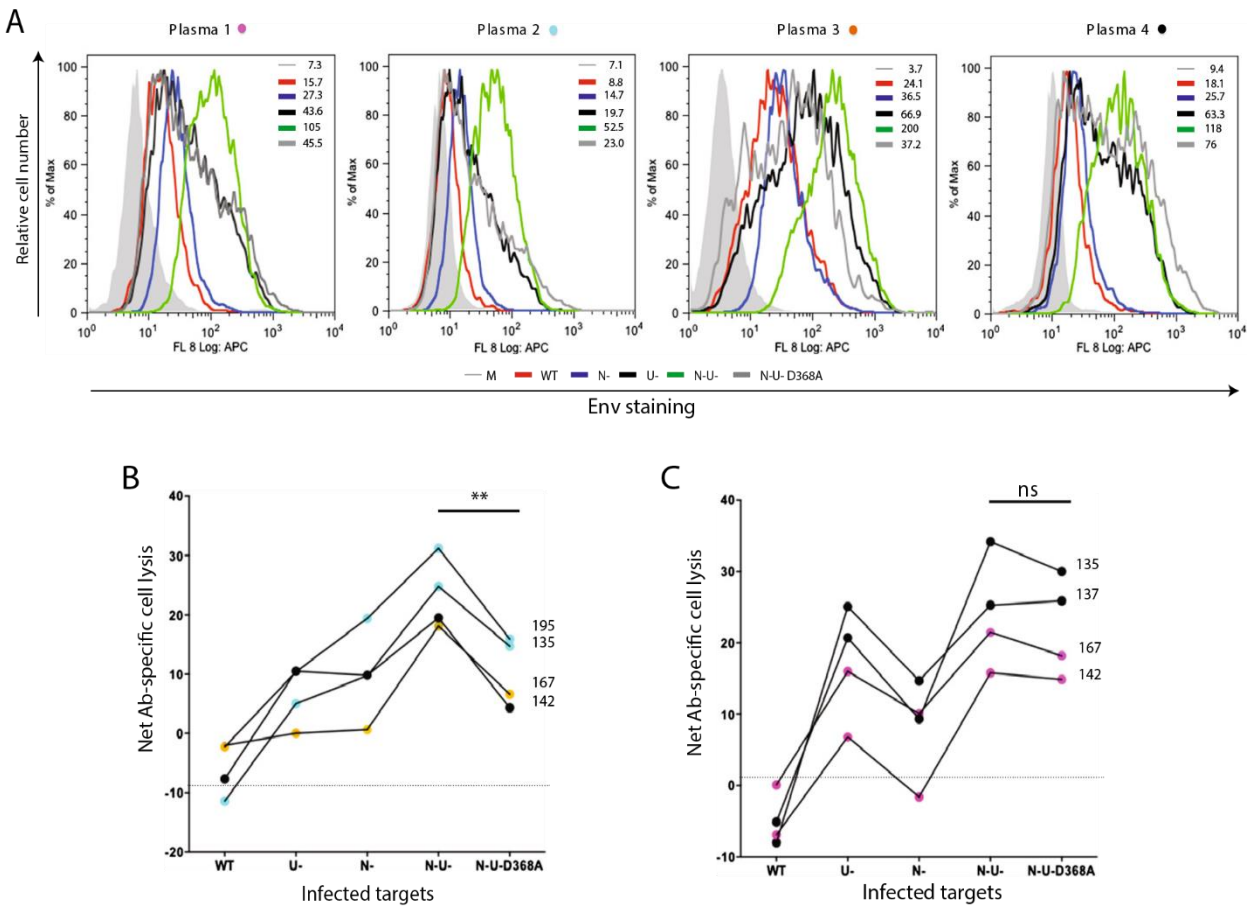

Figure 3.6: The presence of Nef and Vpu efficiently shields HIV-infected T cells from ADCC mediated by plasma from HIV-infected individuals. CEM.NKR cells were infected with viruses as mentioned in Figure 3.1 legend. **(A)** Target cells were stained with plasma samples from an HIV seronegative person (164HH) or from HIV-infected individuals (different plasmas were color coded). Env expression was determined as described in Figure 3.1 legend. **(B-C)** Target cells incubated with plasmas as described in **(A)** were analyzed for their susceptibility to ADCC using PBMC as effector cells. Net Ab-specific cell lysis was computed as described in Figure 3.2 and obtained following subtraction of background killing of the same targets induced by the 164 HH plasma. Panel **B** depicts ADCC activity mediated by patient plasmas, which appeared enriched with A32-like anti-Env Abs. Panel **C** illustrates ADCC activity induced by patient plasmas containing A32-like as well as other ADCC-competent anti-Env Abs. These characteristics of the patient plasmas were established post-analysis. The four sets of numbers indicated on the right hand side of the lines represent four donors. Data shown are

representative of 3 analyses using plasmas from 6 infected individuals and PBMCs from 8 donors. Statistical analysis of data was done using paired Student's *t*-tests.

3.4 Discussion

Recent studies have offered insights on the characteristics of the epitopes on HIV-1 gp120 that are recognized by ADCC-mediating Abs such as A32 [324, 327]. With these results, there emerges a renewed interest in understanding not only how Abs targeting CD4-exposed epitopes could contribute to the overall HIV-induced ADCC response, but also how the virus might evolve to circumvent this mode of defence. Our data presented here-in demonstrate that enhanced exposure of the A32 epitope is intimately correlated with augmented ADCC activity against infected T cells. Importantly, the magnitude of epitope recognition by A32 is invariably dependent on both cell-surface expression of CD4 and Env and CD4-Env interactions on infected cells. Lastly, by removing CD4 and BST2 from the cell surface, HIV Nef and Vpu function individually yet synergistically to dampen infected cell susceptibility to ADCC. We postulate that it is these actions by the two accessory proteins that could help protect infected T cells from ADCC, revealing yet another mechanism of immune evasion exploited by HIV-1. In fact, the evidence reported here-in demonstrates that both Vpu and Nef contribute to the protection of HIV-infected cells from ADCC mediated by Abs that are present in plasmas of HIV-infected individuals. This underlies the in vivo relevance of our findings.

When $CD4^+$ T cells were infected with the ΔNef or the ΔVpu virus, we observed a moderate increase of 2–3 fold in A32 binding compared to that on WT virus-infected T cells. Surprisingly, the A32 epitope recognition was significantly higher (8–16 fold) on T cells infected with the ΔNefΔVpu virus, suggesting a synergistic effect by Nef and Vpu. Functionally, this enhanced exposure of the A32 epitope led to heightened ADCC-mediated lysis of target cells, with those infected with the ΔNefΔVpu virus being most susceptible, and those infected with the ΔNef virus moderately prone to ADCC. Subsequent analysis of CD4 expression at the surface of infected cells (WT, ΔNef, ΔVpu and ΔNefΔVpu) revealed a compelling inverse correlation between the extent of CD4 down-modulation and the degree of cell susceptibility to ADCC. Indeed, the most potently lysed ΔNefΔVpu virus-infected cells had only 30% of their CD4 down-regulated, while those moderately susceptible to ADCC (e.g., cells infected with the ΔNef virus) had their CD4 expression reduced by 50%. In contrast, when the vast majority of CD4 was depleted from the cell surface, as in the case of WT or apparently ΔVpu virus-infected cells, cells were poorly susceptible to ADCC. Having said that, the effect of Vpu on CD4 down-regulation may have been masked by that of Nef since Vpu's activity was only evident in the context of the double mutant virus in primary $CD4^+$ T cells and CEM NKR T cells. On this note, given the spatially separated and temporally distinct mechanisms exploited by Nef and Vpu to degrade CD4 [101], it is likely that in the presence of Nef, the effect of Vpu cannot be adequately quantified during the 48-hour infection. In turn, this may result in the seemingly lower susceptibility of ΔVpu virus-infected targets to A32-mediated ADCC. One possible approach towards further elucidating the contribution of Nef-mediated down-regulation of cell-surface CD4 to

ADCC would be to use Nef mutants that are incapable of mediating CD4 endocytosis. These evaluations, which are currently underway, would help corroborate our ADCC data with the ΔNef virus. Since ADCC is mediated mainly by NK cells through the Fcγ receptor, it would seem unlikely that the Vpu-mediated downregulation of NK cell receptor SLAMF6/NTBA [118] may have contributed to the lower susceptibility of ΔVpu virus-infected to ADCC.

The accumulation of CD4 at the cell surface was not sufficient to trigger cell lysis. We found that Env and CD4 interactions were necessary to unmask the A32 epitope since the use of the ΔNefΔVpu-D368A Env virus led to significantly reduced A32 binding and decreased ADCC function. In this context, similar findings were also recently reported by others [399]. On the notion of CD4-Env interactions, a recent analysis of cryo-EM structures of virion-associated Env trimer predicted the occlusion of the ADCC-competent JR4 epitope by gp41, in the presence of soluble CD4 (N. Gohain et al., 2013, Keystone Symposia: HIV vaccines, abstract). The cited study also revealed that JR4, together with other ADCC-competent Abs recognizing the so-called Cluster A epitopes [327], bound poorly to surface Env trimers in the presence of soluble CD4 but reacted efficiently with surface-bound virions, lending further support to the requirement of Env and cell-surface CD4 binding for the exposure of A32-like epitopes. Taken together, these findings suggest that CD4 molecules accumulated at the surface of infected cells would engage cell-surface Env molecules in a way similar to that when an incoming virion interacts with the CD4 receptor, through its Env, to enter a target cell.

Aside from showing the necessity of CD4-Env interactions, our analysis with the ΔNefΔVpu-D368A Env virus also implicated the involvement of BST2 in Env recognition,

most likely through the retention of virus particles at the cell surface. Indeed, when BST2 was depleted at the plasma membrane, the residual ADCC activity against T cells infected with the ΔNefΔVpu-D368A Env was essentially abolished. As for the ΔNefΔVpu virus, although the depletion of BST2 led to a moderate reduction in A32 binding without significantly changing the ADCC function, we think this could have been due to the incomplete removal of BST2 from the CEM cells by the shRNA. BST2 contribution to the enhancement of ADCC function was strengthened by our findings with plasmas from HIV-infected individuals. They clearly show that BST2 can contribute significantly to this process. We are currently pursuing additional studies using Vpu mutants that are defective for BST2 binding to further strengthen this argument. These investigations would complement the analyses with the Nef mutants that were mentioned in the previous section and, as such, should provide key insights into the relative contributions of BST2 and CD4 to CD4-induced and non CD4-induced epitope-sensitization. At this juncture, however, the existing data strongly support the notion that CD4 is a more predominant player in potentially inducing a conformational change in the Env and, as such, a more pronounced ADCC function. On a relevant note, a recent work has postulated a role for ADCC in the complete protection of macaques previously vaccinated with a unique live attenuated SIV [397]. They revealed that animals inoculated with a persistent SIVΔNef strain mounted potent ADCC activity, and that it was this Ab-mediated effector response that afforded the apparent sterilizing protection against SIVmac251 challenge. In this context, our findings that CD4 and BST2 contributed to ADCC enhancement warrant further investigations since in SIV, Nef down-regulates both BST2 and CD4 [193, 194].

Since 2G12 recognizes mannose residues on the exterior domain of gp120 [318], the residual binding of this Ab on BST2-depleted cells infected with the ΔNefΔVpu-D368A Env virus could be an accumulative consequence of carbohydrate modulations by Vpu and/or Nef and of virus particle retention by the residual BST2 that remained at the cell surface. In any case, regardless of how the 2G12 epitope might be recognized on T cells infected with the viruses used in our study, the key message is that 2G12 binding is not dependent on CD4-Env interactions, and that despite its efficient binding on Env, 2G12 is still significantly less potent than A32 at inducing ADCC. This observation was consistent with those reported earlier using CEM.NKRCCR5 T cells coated with recombinant Env or infected with HIV [324].

3.5 Conclusions

In summary, our study provides a further insight as to how epitopes recognized by ADCC-mediating Abs can be most efficiently accessible on target cells. This work also unveils yet another mechanism by which HIV, through its accessory proteins Nef and Vpu, can evade the host's immune defenses. Hence, by allowing for efficient release of progeny virus particles and preventing CD4 accumulation at the cell surface, HIV-1 ensures that Env epitopes targeted by ADCC remain unexposed. Therefore, inhibition of Vpu and Nef, could represent a promising therapeutic avenue to render infected cells susceptible to ADCC.

3.6 Methods

3.6.1 Reagents and antibodies: Phytohemagglutinin-L was purchased from Sigma-Aldrich (St. Louis, MO, USA). The eFlour670 dye was from eBioscience. Human recombinant interleukin-2 (IL-2) [400] was obtained through the NIH AIDS Research and Reference Reagent Program. Anti-GFP was acquired from Invitrogen. Mouse anti-p24 mAb (Cat. # HB9725) was isolated from the culture supernatant of hybridoma cells from the American Type Culture Collection. PerCP-Cy 5.5-conjugated anti-human CD4, allophycocyanin (APC)-conjugated anti-human Fc Ab, and AF647-conjugated anti-IgG secondary Ab were from Biolegend. Anti-HIV gp120 mAbs A32 and 2G12 were obtained through the NIH AIDS Research and Reference Reagent Program from Dr. James E. Robinson [325] and Dr. Hermann Katinger [318], respectively. The A32 Fab was obtained from Dr. Guido Ferrari and Dr. Barton Haynes (Center for HIV-AIDS Vaccine Immunology, Duke University). Anti-Vpu and anti-BST2 rabbit sera were described previously [379]. HIV-infected plasmas were obtained through the Montreal Primary Infection cohort that is part of the Fonds de Recherche du Québec-Santé (FRQ-S) AIDS Network. Plasmas from HIV- and HCV- seronegative donors were obtained through a cohort of healthy volunteers maintained at the Institut de recherches cliniques de Montréal (IRCM). Research protocols were approved by the research ethics review board at the IRCM.

3.6.2 Plasmid and proviral DNA constructs: The vesicular stomatitis virus (VSV) glycoprotein G-expressing plasmid, pSVCMVin-VSV-G, was previously described [379].

The infectious CCR5-tropic NL4.3.ADA.IRES.GFP wild-type (WT), which contains all accessory proteins, and its Vpu-deficient derivative (ΔVpu, U-) were generated as described [107]. The WT construct was used to generate various isogenic proviruses using standard molecular biology techniques. The NL4.3.ADA.IRES.GFPΔNef (N-) was created by introducing a frame-shift mutation at the unique Xho1 site within the Nef coding region, thus, generating a truncated inactive Nef protein of 38 amino-acid residues. The isogenic NL4.3.ADA.IRES.GFP ΔNefΔVpu (N-U-) combined the two mutations present in the ΔVpu and ΔNef proviral constructs, while the NL4.3.ADA.IRES.GFP ΔNefΔVpu-D368A Env (N-U-D368A) was generated by introducing a substitution mutation (D/A) at position 368 of Env using Quickchange mutagenesis (Stratagene).

3.6.3 Production of VSVg-pseudotyped lentiviral vectors and HIV-1 viruses: For lentiviral vector production, HEK293 T cells were transfected with plasmid pLKO.1 (puromycin-resistant) expressing shRNA targeting BST2 (Clone ID: TRCN0000107018, from OpenBiosystem) or control shRNA together with the packaging construct psPAX2 (a gift from Dr. D. Trono at Swiss Institute of Technology) and VSV-G expressing plasmid pSVCMVin-VSV-G using a calcium phosphate precipitation method. Vectors were purified by ultracentrifugation 48 h later [401].

For virus production, HEK 293 T cells were transfected with appropriate proviruses and pSVCMVin-VSV-G using the calcium phosphate method [379]. Viruses were harvested 48 h later [401] and titrated by MAGI assay as described [154].

3.6.4 Preparation of CEM.NKR CD4$^+$ T cell line depleted of BST2: CEM.NKR cells were transduced by spin-inoculation [402] using lentiviral vector particles containing shRNA targeting BST2 or control shRNA. Forty-eight hours later, puromycin was added and puromycin-resistant cells were selected after 10 days. BST2 expression was determined by flow cytometry. CEM.NKR T cells expressing or depleted of BST2 were characterized functionally for viral release 48 h post-infection with the CCR5-tropic NL4-3.ADA.IRES.GFP WT or NL4-3.ADA.IRES.GFPΔVpu virus.

3.6.5 Jurkat T cell lines: phenotype, infection and assessment of surface molecule expression by flow cytometry: Four different Jurkat T cell lines were used in this study. Their phenotype for CD4 and BST2 expression as determined by flow cytometry is as follows. The first line, derived from a Jurkat line that stably expresses the SV40 large T antigen [403], is negative for both CD4 and BST2 [404] and shall be referred to in the paper as CD4-/BST2-. The second line, a derivative of the first one, expresses high levels of CD4 upon stable transfection with a SV40 origin-containing CD4 expressor. This line is referred to in the paper as CD4hi/BST2-. The third and fourth lines of Jurkat are derivatives of the E6.1 clone from the ATCC. The former expresses minimally CD4 and positive for BST2 (CD4lo/BST2), while the latter expresses CD4 and BST2 at relatively comparable levels (CD4$^+$ /BST2$^+$).

3.6.6 Preparation of primary effector/target cells and infection of CD4$^+$ T cells: Peripheral blood mononuclear cells (PBMC) were prepared from whole blood of HIV- and HCV- seronegative donors as described [402]. In certain experiments where

effector cells were destined to have no monocytes/macrophages, PBMC were cultured in serum-free RPMI 1640 (Wisent) for 2 h to remove monocytes/macrophages by plastic-adherence. Non-adherent cells were recovered to be used as effector cells. When effector cells were to contain no NK cells, PBMC were depleted of NK cells using CD56 microbeads (Miltenyi Biotec). Cell purity was confirmed by flow-cytometry using anti-human CD14 or anti-human CD56 Abs, respectively. In all cases, effector cells to be used in ADCC assays were cultured overnight in complete RPMI 1640 medium (10% FBS supplemented with L-Glutamine, Pennicilin-streptomycin, and 100 U/mL IL-2).

For HIV-infection of $CD4^+$ T cells, activated primary $CD4^+$ T cells were spin-infected [402] as well as T cell lines (CEM.NKR and Jurkat) with CCR5-tropic NL4.3.ADA.IRES.GFP viruses at multiplicity of infection of 0.5-1 depending on cell types. Forty-eight hours post-infection, T cells were analyzed by flow cytometry, when appropriate, for CD4, BST2 and Env expression or for ADCC activity.

3.6.7 Flow cytometry: CD4 staining was done as per manufacturer's protocols. For Env staining, infected cells were stained with anti-human Env primary Abs (A32 or 2G12) or control IgG for 30 min at 4°C, and then exposed to APC-conjugated anti-human Fc secondary Ab (Biolegend). For Env staining analyses using human plasmas, target cells were stained with diluted plasma (1:250 to 1:1000 dilutions) from HIV-infected individuals or as control, with plasma from healthy donors. Staining conditions were as described for A32. Fluorescence signals were revealed using AF647-conjugated anti-IgG secondary Ab. In certain experiments where A32 binding specificity was being evaluated, cells were pre-exposed to the A32 Fab (3.8 µg/mL) for 30 min at

room temperature (RT) prior to the A32 incubation step. BST2 staining was done as described using anti-rabbit BST2 Ab or as a control, a rabbit pre-immune (PI) serum [379].

3.6.8 Antibody-dependent cytotoxicity assay (ADCC): 50,000 target (T) cells, plated in 96-well V-bottom plates, were exposed to A32 (1.4 μg/mL), 2G12 (1.4 μg/mL) or human control IgG Ab for 30 min at RT. In certain experiments, target cells were pre-incubated with the A32 Fab for 30 min before adding A32. As an alternative source of Abs, target cells were incubated with empirically-determined concentrations of plasmas (1:250–1:1000 dilutions) from HIV-infected patients or from healthy donors (as control). Effector cells, that had been labelled with eFlour670 dye were mixed with target cells at effector:target ratios of 10:1 to 30:1. The cell mixtures were spun for 3 min at 400 × g and cultured for 4–4.5 h at 37°C. Subsequently, medium was removed by centrifugation, cells were fixed in 1% PFA and analyzed on a CyAn ADP analyzer for GFP expression. Percent of cell lysis was determined as [(% GFP+ in the absence of effectors -% GFP+ in the presence of effector cells and test Ab)/% GFP+ in the absence of effectors] × 100. Depending on analysis, test Ab could be plasma (uninfected or infected), IgG, 2G12, A32 or A32 Fab + A32.

3.6.9 Viral particle release assay: BST2-expressing or BST2-depleted CEM.NKR $CD4^+$ T cells were infected for 48 h with CCR5-tropic NL4-3.ADA.IRES.GFP WT or ΔVpu virus. Virions were purified by ultracentrifugation [379]. Virions and cells were lysed in RIPA-DOC buffer (10 mM Tris pH 7.2, 140 mM NaCl, 8 mM Na2HPO4, 2 mM

NaH2PO4, 1% Nonidet-P40, 0.5% sodium dodecyl sulfate, 1.2 mM deoxycholate), and Western blotting was performed [379] using Abs specific for Vpu, GFP or Gag (the anti-Gag Ab recognizes the precursor p55 and the processed forms of Gag, including p24).

3.6.10 Statistical analyses: Unless otherwise stated, data are expressed as average ± SEM. Statistical analyses of the data were done using two-tailed, paired (when appropriate) Student's t-tests. P values of ≤ 0.05 were considered statistically significant: * ≤ 0.05; ** ≤ 0.005; ***≤0.0005; ****≤0.00005; and ns, not significant.

3.7 Declarations

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: TNQP, SL and EAC conceived and designed experiments. TNQP, SL and FH performed the experiments. J-PR provided patient plasmas. TNQP, SL and EAC analyzed the data. TNQP and EAC wrote the manuscript. All authors read and approved the final manuscript.

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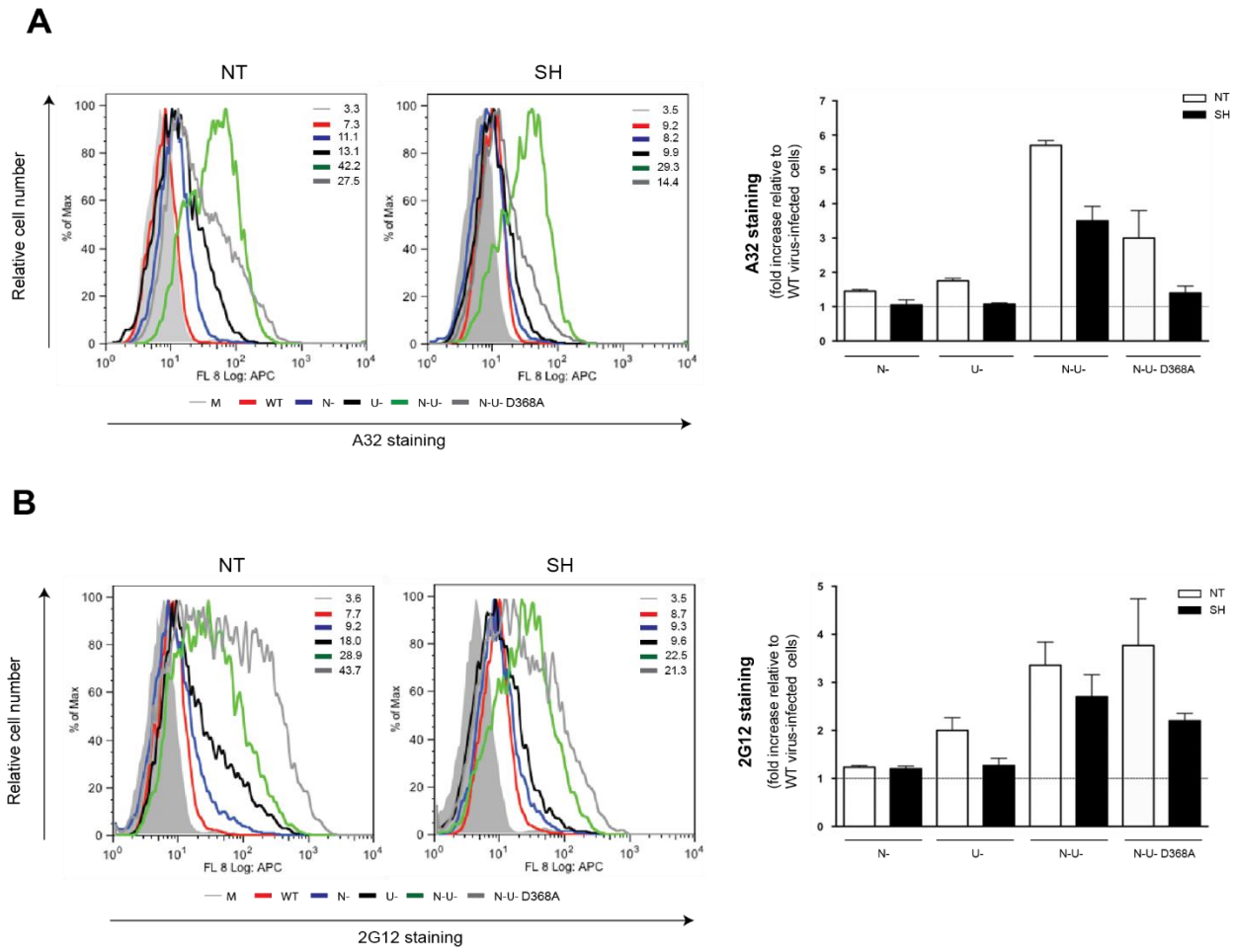
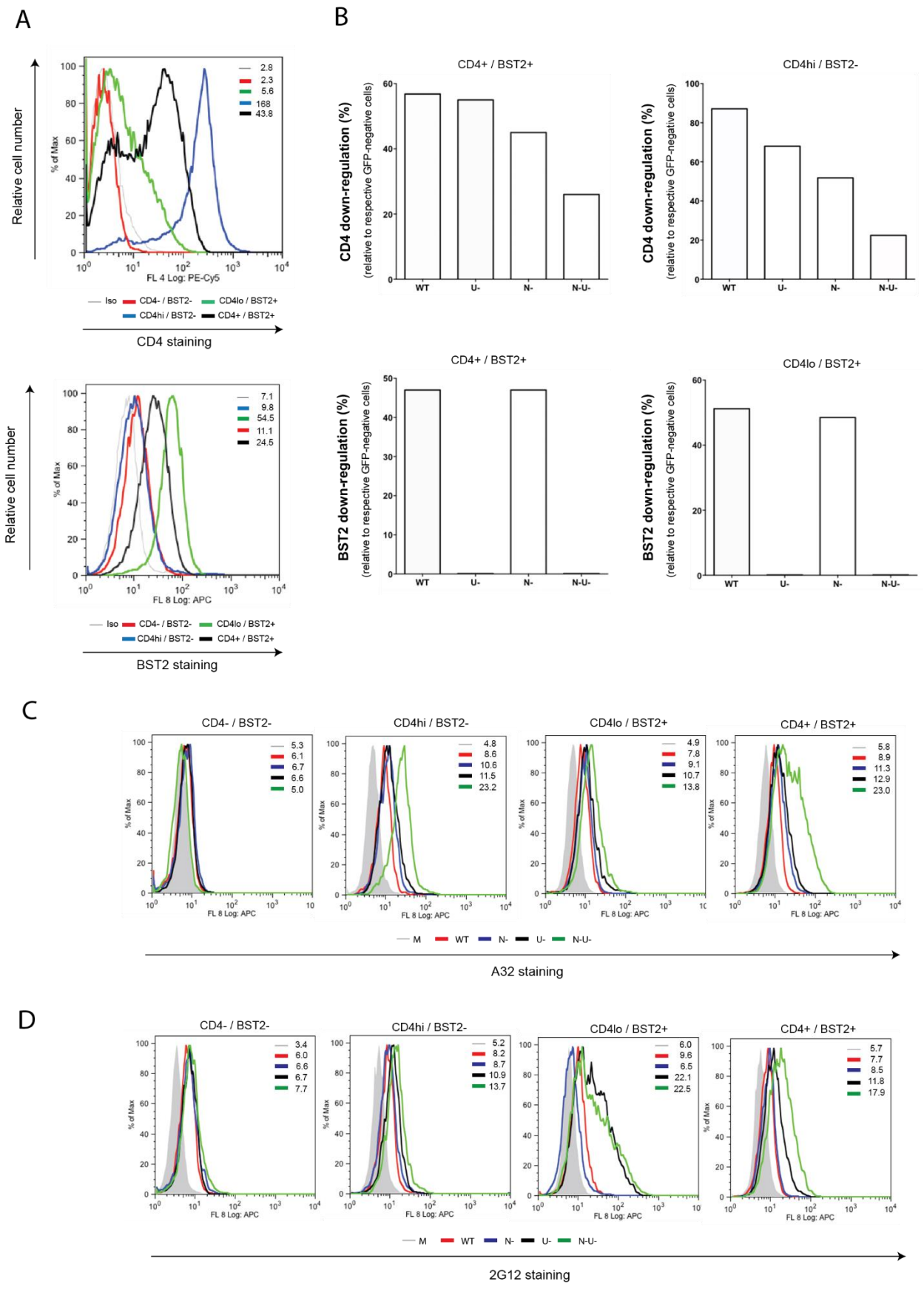
NL4.3.ADA.IRES.GFP WT and NL4.3.ADA.IRES.GFP ΔU constructs. We also thank Mario Legault of the FRQ-S AIDS Network for coordinating the acquisition of plasma samples from HIV-infected persons. We thank Mariana Bego and Vibhuti Dave for helpful discussions; Eric Massicotte and Julie Lord-Grignon for assistance with flow cytometry; Dr. Pierre Larochelle, the IRCM clinic staff, and all healthy volunteers for providing blood samples. The anti-HIV gp120 mAbs A32 and 2G12 were obtained through the NIH AIDS Research and Reference Reagent Program from James E Robinson and Hermann Katinger, respectively. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) (MOP 111226) to E.A.C, CIHR and Canadian HIV Trials Network (CTN 247, MOP 103230) to J-P.R. and from the FRQ-S AIDS Network to E.A.C and J-P.R. J-P.R is the holder of the Louis Lowenstein Chair in Hematology & Oncology. E.A.C is a recipient of the Canada Research Chair in Human Retrovirology.

3.8 Additional Files



Additional file 3.1: Figure S3.1: Effect of BST2 depletion on HIV-1 envelope expression profiles on infected CD4⁺ T cells. BST2 was depleted from CEM.NKR CD4⁺ T cells as described in Methods. BST2-expressing (NT) and BST2-depleted (SH) CEM.NKR T cells were infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus or derivatives lacking Vpu (U-), Nef (N-) or both (N-U-). The N-U- D368A viral construct contains a mutation at residue 368 of Env which prevents CD4-Env interactions. Forty-eight hours post-infection, cells were stained with A32 (A) or 2G12 (B) Abs and analyzed for Env expression by flow cytometry. Mock (M)-infected cells stained in parallel were used as control. Shown next to the overlays are Env levels in MFI obtained for gated GFP + infected cells from a representative analysis. The histograms shown depict the average fold increase (+/- SD) in Env staining relative to WT virus-infected cells in two experiments.


A
B
Relative cell number
% of Max
FL 4 Log: PE-Cy5
2.8
2.3
5.6
168
43.8
Iso
CD4- / BST2-
CD4lo / BST2+
CD4hi / BST2-
CD4+ / BST2+
CD4 staining
CD4+ / BST2+
CD4hi / BST2-
CD4 down-regulation (%)
(relative to respective GFP-negative cells)
WT
U-
N-
N-U-
7.1
9.8
54.5
11.1
24.5
FL 8 Log: APC
BST2 staining
CD4lo / BST2+
BST2 down-regulation (%)
C
CD4- / BST2-
CD4hi / BST2-
CD4lo / BST2+
CD4+ / BST2+
5.3
6.1
6.7
6.6
5.0
4.8
8.6
10.6
11.5
23.2
4.9
7.8
9.1
10.7
13.8
5.8
8.9
11.3
12.9
23.0
M
WT
N-
U-
N-U-
A32 staining
D
3.4
6.0
6.6
6.7
7.7
5.2
8.2
8.7
10.9
13.7
6.0
9.6
6.5
22.1
22.5
5.7
7.7
8.5
11.8
17.9
2G12 staining

Additional file 3.2: Figure S3.2: Examining the relative contributions of CD4 and BST2 in promoting Env staining by A32 or 2G12 Abs. Jurkat T cell lines which vary in their expression of CD4 and BST2 (A) were infected with NL4.3.ADA.IRES.GFP WT virus or derivatives lacking Vpu (U-), Nef (N-) or both (N-U-) as described in Methods. Forty-eight hours later, cells were stained for (B) CD4 and BST2 and for Env using (C) A32 or (D) 2G12 Abs, and analyzed for their expression by flow cytometry. Mock (M)- infected cells stained in parallel were used as control. Indicated next to the overlays (A, C and D) were expression levels shown in MFI for infected T cells (GFP^+) from a representative analysis. The histograms (B) depict the percentage of CD4 or BST2 down-regulation in GFP-positive cells relative to respective GFP-negative cells.

Chapter 3 – Chapter 4 bridging text

In Chapter 3, we identified a significant contribution of CD4 towards sensitizing cells to ADCC mediated by a non-neutralizing, CD4-i Ab, with BST2 playing a relatively minor role. Interestingly, ADCC mediated by Abs present in some plasmas of HIV-infected individuals suggested a significant contribution of CD4-independent Abs, with Vpu playing a much more prominent role in facilitating ADCC. Given that some HIV-infected patients can develop CD4-independent anti-HIV Abs capable of broadly neutralizing viruses, in Chapter 4 we investigated whether such NAbs can mediate ADCC. Moreover, we sought to test whether tethering of Env-containing virions at the cell surface would influence such a response. In addition, considering the challenge that latent cells present in HIV cure efforts, we also examined whether ADCC could be a viable strategy in approaches to eliminate reactivated cells.

CHAPTER 4: BST2-MEDIATED VIRION TETHERING ENHANCES ADCC BY NEUTRALIZING ANTIBODIES

Preface

Author Contributions: The work presented in this chapter is part of a manuscript [405] that was published (17 November 2016) in the journal *Scientific Reports* entitled “Enhancing Virion Tethering by BST2 Sensitizes Productively and Latently HIV-infected T cells to ADCC Mediated by Broadly Neutralizing Antibodies” by Tram N.Q. Pham (TNQP), **Sabelo Lukhele (SL)**, Frédéric Dallaire (FD), Gabrielle Perron (GP) and Éric A. Cohen (EAC). TNQP, **SL** and EAC conceived and designed experiments. TNQP, **SL**, FD and GP performed experiments. TNQP performed ADCC assays. TNQP and FD performed neutralization assays. TNQP and GP generated latently infected T cell lines. TNQP and **SL** established and characterized BST2-depleted CEM T cell line. TNQP and **SL** performed flow cytometry assessment of BST2 expression. **SL** characterized contribution of accessory proteins and Env mutants to BST2-mediated virion tethering. **SL** characterized effects of IFN on BST2 antagonism. TNQP, **SL** and EAC analyzed data. TNQP, **SL** and EAC wrote the manuscript. As such, in addition to data not presented in the manuscript, SL contributed to Figures 4.2; 4.3; 4.4; and 4.5, Additional file 4.2: Figure S4.2 and Additional file 4.3: Figure S4.3, as well as contributed to analyses of all figures.

Original scholarship and distinct contributions to knowledge: We reveal that CD4-independent NAbs are capable of mediating ADCC. Importantly, BST2-mediated

virion tethering, which increases the amount of Env epitopes at the cell surface, significantly enhances ADCC mediated by NAbs. Similarly, while bNAbs can mediate ADCC against WT virus-infected cells, inactivating Vpu and Nef significantly potentiates their killing. Moreover, increasing BST2-mediated virion crosslinking using IFNα treatment enhances ADCC susceptibility to WT virus-infected cells. Furthermore, we uncover that reactivated HIV latent cells are susceptible, albeit poorly, to ADCC lysis. Likewise, their killing can be enhanced by both exogenous IFN administration and removal of the viral accessory proteins. Overall, the study proposes that strategies aimed at enhancing ADCC activity via treatments with IFN and/or small molecules targeting Vpu represent a promising avenue for protection from HIV acquisition, post-infection control of HIV, as well as enhanced clearance of latent viral reservoirs in “Shock and Kill” cure approaches.

Enhancing Virion Tethering by BST2 Sensitizes Productively and Latently HIV-infected T cells to ADCC Mediated by Broadly Neutralizing Antibodies

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4.1 Abstract

Binding of anti-HIV antibodies (Abs) to envelope (Env) glycoproteins on infected cells can mark them for elimination via antibody-dependent cell-mediated cytotoxicity (ADCC). BST2, a type I interferon (IFN)-stimulated restriction factor that anchors nascent Env-containing virions at the surface of infected cells has been shown to enhance ADCC functions. In a comprehensive analysis of ADCC potency by neutralizing anti-HIV Abs (NAbs), we show in this study that NAbs are capable of mediating ADCC against HIV-infected T cells with 3BNC117, PGT126 and PG9 being most efficient. We demonstrate that HIV-induced BST2 antagonism effectively attenuates Ab binding and ADCC responses mediated by all classes of NAbs that were tested. Interestingly, IFNα treatment can reverse this effect in a BST2-dependent manner. Importantly, while reactivated latent T cell lines display some susceptibility to ADCC mediated by broadly NAbs, inactivating BST2 viral countermeasures and/or exogenous IFNα augment their elimination. Overall, our findings support the notion that NAbs can induce ADCC. They highlight that while BST2 antagonism by HIV promotes ADCC evasion, strategies aimed at restoring BST2 restriction could improve anti-HIV responses and potentially provide a means to eliminate reactivated cells in latent reservoirs.

4.2 Introduction

Human immunodeficiency virus (HIV)-type 1 enters target cells, primarily $CD4^+$ T cells and macrophages, through sequential interactions between viral envelope (Env), composed of a trimer of gp120 and gp41 heterodimers, and cell surface receptors CD4 and CCR5 (or CXCR4) [35]. Each interaction causes conformational changes in Env, and in turn enable a subsequent phase of the entry process. Binding of gp120 to receptor CD4 causes the trimer to assume a structure (CD4-induced or CD4i) that allows gp120 to bind co-receptor CCR5 or CXCR4. Co-receptor engagement triggers additional remodeling within the gp41 transmembrane subunits, rearranging them into a stable six-helix bundle that facilitates fusion between viral and cellular membranes. This multi-stage mechanism of entry allows HIV-1 to mask conserved functional sites from humoral immunity [160, 406].

HIV infection triggers production of antibodies (Abs) against Env gp120 and gp41 subunits, some of which can bind free virus and prevent new infection. While Abs capable of neutralizing the infecting virus maybe readily produced, only 20-30 % of patients make Abs that can neutralize a broad spectrum of viruses, and typically after several years [301, 305, 306, 321]. These so-called broadly neutralizing Abs (bNAbs) target the CD4-binding site (CD4-bs) on gp120, glycans on the V1/V2 apex of gp120, V3-glycans on gp120, the membrane proximal external region (MPER) on gp41 as well as the gp120-gp41 interface. Passive transfers of bNAbs have been shown to protect macaques and humanized mice from challenges with simian-HIVs or HIV-1, respectively [341, 407-409], and to interfere with establishment of reservoirs in

humanized mice [410]. In human studies, a single infusion of CD4-bs Ab 3BNC117 reduced viral load by up to 2.5 log [310]. Although it was implied that the protective effects of Abs required Fc - Fc receptor engagement [337, 341, 407, 410, 411], the involvement of antibody-dependent cell-mediated cytotoxicity (ADCC) was only directly addressed in some studies [337, 410].

HIV-1 infection downregulates CD4 [390] and BST2 [76, 77] from the surface of infected cells and such modulation correlates with reduced ADCC activity [399, 412-414]. BST2 is a type I interferon (IFN-I)-upregulated restriction factor that tethers nascent virions at the surface of infected cells, thereby preventing their efficient release [76, 77]. HIV-1 Vpu-mediated antagonism of BST2 [76, 77] conceivably leads to reduced levels of tethered Env-containing virions and less efficient recognition of infected cells by ADCC-mediating Abs. In addition, decreasing CD4 expression by Nef and Vpu [390] presumably prevents Env from engaging CD4, a step that is necessary to uncover certain CD4i, ADCC-promoting epitopes on Env. An example of such epitopes is that recognized by the non-neutralizing A32 Abs. It is currently not understood whether different classes of bNAbs are subjected to ADCC evasion by Nef and Vpu. Nor is it entirely defined whether reactivated HIV latent cells are susceptible to ADCC [415, 416], and if modulating activities of Nef and Vpu would alter susceptibility of latent cells to ADCC by bNAbs.

Here, we surveyed a panel of anti-Env Abs, that target all known vulnerable regions of Env, for their ability to mount ADCC response against infected T cells. We show that bNAbs mediate ADCC with varying efficiencies. We further demonstrate that Vpu and Nef differentially modulate ADCC activities. Moreover, inactivating BST2

antagonism by HIV-1 enhances Env recognition and, consequently, ADCC activities mediated by all classes of NAbs. Similarly, exogenous IFNα treatment heightens ADCC response against productively infected $CD4^+$ T cells in a BST2-dependent manner. Lastly, we reveal that this approach could sensitize reactivated latent cells to ADCC. Overall, our study suggests that strategies aimed at improving ADCC function using IFNα and/or small molecule inhibitors of BST2 antagonists represent a promising avenue to promote a more effective elimination of productively infected cells and clearance of latent viral reservoirs in "Shock and Kill" HIV cure approaches.

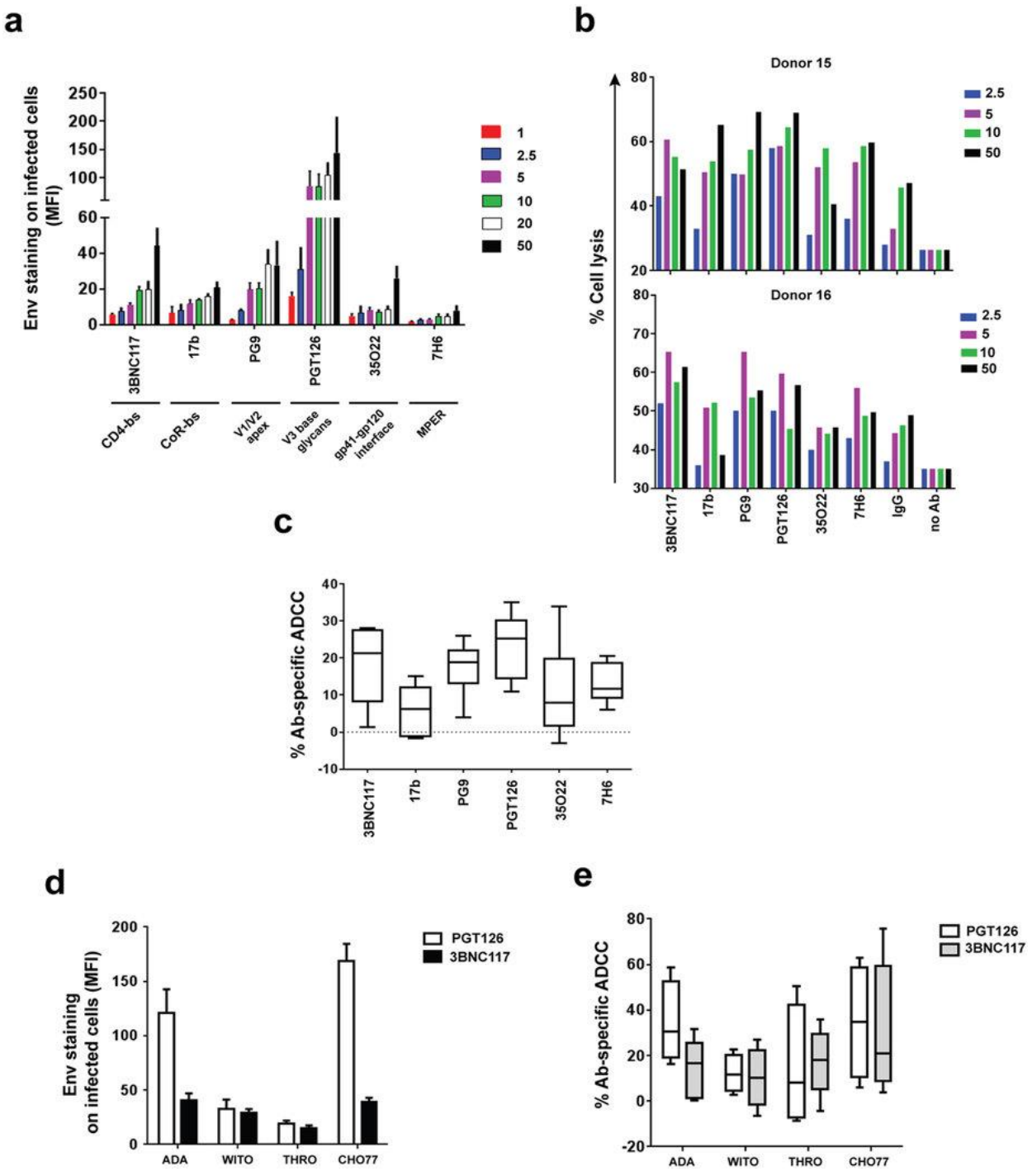
4.3 Results

4.3.1 Neutralizing Abs mediate efficient ADCC against CEM $CD4^+$ T cells infected with different HIV-1 primary isolates. First, we examined how efficient neutralizing Abs recognize their epitopes on T cells infected with prototypic wild-type HIV-1 expressing the ADA Env. While most Abs in the series were broadly neutralizing, 17b (CoR-bs) was included in the analysis to have representative Abs recognizing all known regions on Env. Under our experimental conditions (staining performed at 4 ^oC), the Abs bound Env with varying efficiencies and, unsurprisingly, saturated at different concentrations (2.5 to 10 μg/ml). At a given dose, Abs which target the N332-V3 glycan (PGT126) bound most strongly (several fold higher) than the others (Figure 4.1A). In contrast, Abs which recognize the MPER (7H6) or gp120-gp41 interface (35O22) bound least strongly on infected cells, even at the highest concentration tested. Screening of additional Abs showed similar results (see Supplementary Figure S4.1 online). At near

saturation concentration (5 µg/ml), PGT126 was most potent at mediating ADCC, followed by PG9 (targeting V1/V2 glycans) and 3BNC117 (Figure 4.1B and 1C). For 35O22 and 17b, which showed minimal ADCC activity at 2.5 µg/ml (see Supplementary Figure S4.1 online), increasing Ab concentration to 10 µg/ml improved killing of infected cells (Figure 4.1C; see also Supplementary Figure S4.1 online). Nevertheless, for more potent Abs (i.e., PGT126, PG9 and 3BNC117), such increases were not observed, presumably because of saturation. Although 7H6 recognized the ADA Env most weakly, its median ADCC activity was higher than those of 17b and 35O22, both of which bound Env more efficiently than 7H6. Such a lack of correlation between Env binding and ADCC response could be due to differential stabilities of Ab-Env complexes at 37 ^{o}C (ADCC assay) versus at 4 ^{o}C (Env binding assay), as has also been observed by others [416]. Additionally, at an exceedingly high concentration of 50 µg/ml of antibodies, although there was an increase in Env recognition for a couple of antibodies, this did not necessarily translate to more efficient antibody-specific ADCC (Figure 4.1A and 1B, see also Supplementary Figure S4.1 online).

In assessing if ADCC activity was correlated with neutralization capability, we found that ADCC-potent PGT126, PG9 and 3BNC117 were also effective at neutralization, with IC_{50} ranging from 0.04 to 0.07 µg/ml (see Supplementary Figure S4.1 online). Likewise, 7H6, 17b and 35O22 which were less capable of mediating ADCC were also not as potent at neutralizing (IC_{50}: 0.26, 1.3 and 7.5 µg/ml, respectively). The results with 17b, and especially 35O22, likely reflect poor Ab binding when Env is in a closed, unliganded conformation [321, 417], at least in the context of this virus.

Based on findings with the prototypic virus, PGT126 and 3BNC117 were selected for further analyses with CEM $CD4^+$ T cells infected with transmitted/founder (T/F) viruses. First, PGT126 recognized its epitope much more efficiently than 3BNC117 on CHO77 virus-infected T cells. However, epitope recognition on T cells infected with WITO or THRO isolates was comparable between the two Abs, albeit markedly lower than that of the other two viruses (Figure 4.1D). It is less likely that these observations were due to unequal levels or extents of infection since the proportion of $p24^+$ cells was similar among the different strains, and the observations were independent of the intensity of p24 staining per cell. Importantly, the extent of ADCC activity largely mirrored that of Env recognition except for THRO virus where despite minimal binding, 3BNC117 could still induce significant cell lysis (Figure 4.1E). Overall, our data indicate that NAbs can trigger elimination of infected T cells expressing Env proteins from different primary virus isolates. The variable ADCC responses between virus isolates might be related to surface stability of NAbs and/or differential exposure of relevant epitopes.



4.1: Neutralizing Abs can mediate efficient ADCC against T cells infected with different HIV isolates. (**A-E**) CEM CD4$^+$ T cells were infected with prototypic CCR5-tropic NL4.3.ADA.IRES.GFP wild-type (WT) or (**D** and **E**) the indicated T/F viruses and analyzed by flow cytometry for Env expression (**A** and **D**) and susceptibility to bioluminescence-based ADCC (**B, C** and **E**) using indicated anti-HIV NAbs (in

μg/ml). (**A**) Extent of Env staining (in median fluorescence intensity (MFI) units) on GFP^+ T cells (n= 3 to 4). (**B, C** and **E**) PBMCs from healthy donors were used as effector cells. Percentages of Ab-specific ADCC were determined following subtraction of cell lysis mediated by control IgG from that by test antibody (5 μg/ml). (**B**) ADCC responses by two donors as examples and (**C**) Compilation of median ADCC activity from at least 8 different donors. (**D**) Median Env staining on $p24^+$ T cells from 3 experiments. (**E**) Boxes and whiskers graph showing median ADCC response from 4 donors. ND, not done; MPER, membrane proximal external region.

4.3.2 HIV-1 accessory proteins Vpu and Nef modulate Env recognition by NAbs on infected T cells. The variation in the ability of the same antibody to recognize Env, hence mediate ADCC in T cells infected with different isolates points to differential capabilities of these viruses to shield infected cells. Given previous findings demonstrating that HIV exploits Nef and Vpu to modulate Env recognition and evade ADCC [399, 412-414], CEM $CD4^+$ T cells were infected with prototypic WT strain or its variants lacking Vpu (U-, ΔU), Nef (N-, ΔN) or both proteins (N-U-, ΔNΔU). The ΔNΔU D368A virus was included in the analysis to delineate the importance of CD4-Env engagement in the recognition of target cells by NAbs. Substitutions at residue 368 within the CD4-bs of Env disrupt CD4-Env interactions [398]. To this end, we observed several Env recognition profiles with these viruses.

First, relative to WT virus, 3BNC117 binding was enhanced by two-fold with ΔU but moderately decreased with ΔN (Figure 4.2A, see also Supplementary Figure S4.2 online). The absence of Nef and Vpu did not alter the extent of Env recognition. Although 3BNC117 competes with CD4 for Env binding, the two-fold increase with ΔU virus seemed to argue for a likely contribution from BST2-mediated tethering of virions

and not from CD4 abundance since cell-surface CD4 levels were comparable between WT and ΔU virus-infected cells (see Supplementary Figure S4.2 online). Nonetheless, Env recognition with ΔNΔU was not higher than ΔN (or WT), suggesting that perhaps when CD4 accumulated at the surface engages Env, fewer epitopes are available for 3BNC117 binding, despite the increase in Env-containing virions mediated by BST2. Having said that, the fact that Env recognition was not meaningfully increased when comparing ΔNΔU to ΔNΔU D368A was consistent with previous findings showing that binding of CD4-bs Abs such as VRC01 to Env was impaired when there was a mutation at this residue [308].

Second, while there was a slight increase in 17b binding with ΔU or ΔN virus, the enhancement was highly synergistic with ΔNΔU (~ 6-fold increase), mirroring our previous finding with CD4-induced A32 [412], and highlighting the importance of CD4 accumulation (see Supplementary Figure S4.2 online) for effectively unmasking the CD4i 17b epitope. However, the fact that the presence of the D368A mutation almost abolished such enhancement differs from what had been observed with A32 [412] and indicates a rather significant necessity of CD4-Env engagement in the exposure of epitope, at least at the level of Env recognition. Interestingly, the overall profile of 7H6 binding was very similar to that of 17b except that the decrease in Env staining with ΔNΔU D368A compared to ΔNΔU appeared statistically insignificant.

Third, for PG9, PGT126 and 35O22 a meaningful increase of 1.5-3 fold in binding across all ΔU viruses was observed, and the presence of the D368A mutation did not negatively affect Env recognition. Further, while there was an increase in Env recognition when comparing ΔNΔU to ΔU for PG9 and PGT126, the difference was

significant only for PGT126. At this point, we do not have an explanation for this observation with respect to a role of Nef. Noteworthy, we detected a slight Nef-mediated down-regulation of BST2 on CEM $CD4^+$ T cells (see Supplementary Figure S4.2 online). While this effect of Nef on BST2 could have contributed to reduced Env recognition observed with ΔU, the finding might also be an indirect consequence of Nef having an effect on cellular factor(s) that could be involved in the exposition of these epitopes. Overall, these results suggest that differences in activities of Nef and Vpu variants may modulate the extent of Env recognition and, as such, may partially explain the highly variable levels of Env exposure and ADCC observed between isolates (Figure 4.1D and 1E).

Importantly, similar analyses with primary $CD4^+$ T cells showed comparable patterns of Env recognition by most antibodies, with two exceptions. Firstly, a more profound enhancement in Env staining by PG9 and PGT126 was seen with ΔU virus-infected primary cells. It is possible that the lack of Nef-induced down-regulation of BST2 expression on primary cells could lead to more efficient virion tethering by BST2 and thus better Env recognition by PG9 and PGT126. Secondly, unlike in CEM cells (Figure 4.2A), PGT126 recognized its epitope on ΔNΔU virus-infected primary cells with significantly less efficiency. At this juncture, it is not clear why there is this difference. Despite enhanced recognition in the absence of Vpu-mediated BST2 antagonism, the absence of Nef appears to reduce recognition of PG9 and PGT126 epitopes. Whether this difference is linked to Nef targets SERINC 3 and SERINC 5, which are expressed in primary $CD4^+$ T cells but not in CEM cells [95, 96] is a possibility that requires further investigation.

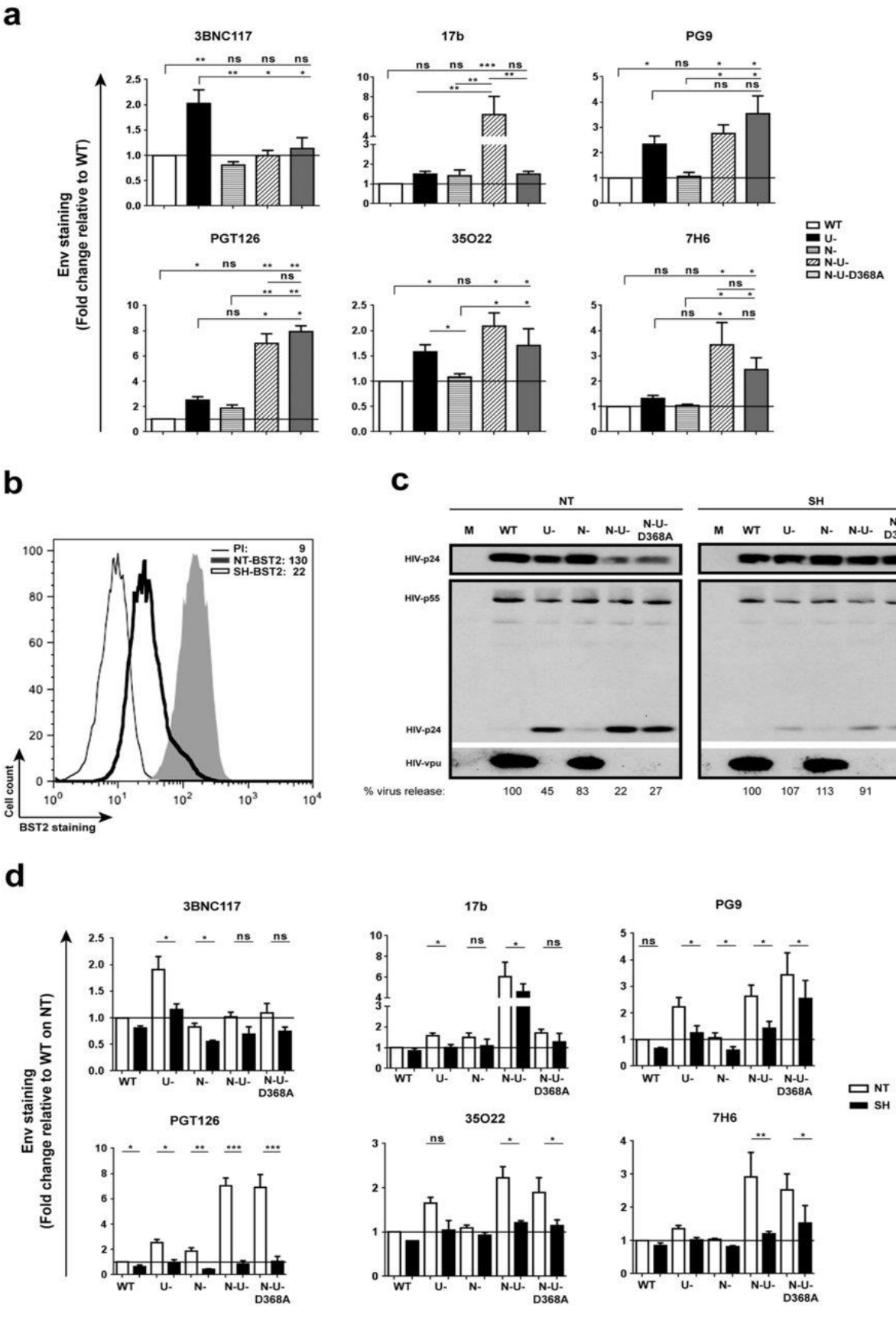

a
3BNC117
17b
PG9
PGT126
35O22
7H6
Env staining
(Fold change relative to WT)
WT
U-
N-
N-U-
N-U-D368A
b
PI: 9
NT-BST2: 130
SH-BST2: 22
Cell count
BST2 staining
c
NT
SH
M
WT
U-
N-
N-U-
N-U-D368A
HIV-p24
HIV-p55
HIV-vpu
Virions
Cells
% virus release:
100 45 83 22 27
100 107 113 91 97
d
3BNC117
17b
PG9
PGT126
35O22
7H6
Env staining
(Fold change relative to WT on NT)
NT
SH

Figure 4.2: Tethering of HIV virions by BST2 enhances Env recognition by all classes of NAbs. (**A**) Parental CEM CD4$^+$ T cells were infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus or derivatives lacking Vpu (U-), Nef (N-) or both proteins (N-U-) and examined for Env recognition by NAbs (5 µg/ml for PGT126 and 10 µg/ml for others). The N-U-D368A virus harbours a mutation within the CD4-binding site of Env causing defective CD4-Env engagement. (**B-D**) CEM cells expressing (NT) or depleted of (SH) BST2 (**B**) were infected with the viruses as in (**A**) and assessed for HIV particle release by Western blotting (**C**) and for Env recognition as in Panel A (**D**). (**A**) Env staining on GFP$^+$ parental CEM T cells. MFI levels on WT-virus infected cells were set at 1 (reference). Shown is a mean fold change of each condition relative to this reference (n = 3 to 4). In this Panel, two-way Anova analysis of variance with Dunn's *post hoc* tests were used to compare Env recognition levels with different viruses. (**B**) BST2 staining on CEM T cells expressing or depleted of BST2 using anti-BST2 Ab or a rabbit pre-immune (PI) serum as control. Indicated inside the graph are MFI values for GFP$^+$ cells from a representative analysis. (**C**) An example of viral particle release with mock (M)-infected cells used as control. Parallel virions and cells were analyzed for total Gag proteins and Vpu. Underneath the immunoblots are quantifications of the densitometry signals. Percentages of virus release were determined as a ratio of virion-associated Gag signals (corresponding to mature p24) over all cell-associated Gag signals (corresponding to p24 and precursor p55) x 100. Virus release by WT virus-infected cells of each cell line (NT or SH) was set at 100. (**D**) Env staining on GFP$^+$ cells (n = 3 to 4). MFI levels on BST2-expressing (NT), WT-virus infected cells were set at 1 (reference). Shown is a mean fold change of each condition relative to this reference. In this Panel, paired, two-tailed Student's *t*-tests were used to compare the effect of BST2 depletion on Env recognition.

4.3.3 BST2-mediated virion tethering enhances Env recognition and ADCC activity by NAbs. We next asked if BST2 depletion [412] from CEM $CD4^+$ T cells (Figure 4.2B) would affect Env recognition and ADCC activities by NAbs through modulation of virion tethering at the cell surface. We first confirmed that BST2 depletion mediated a change in virus particle release. Indeed, when BST2 was expressed (NT), virus release from ΔU virus-infected cells was about 45 % of the WT (note the accumulation of p24 in ΔU virus-infected cells compared to WT) (Figure 4.2C). A minor effect of Nef on particle release was also observed (compare lanes ΔU to ΔNΔU or WT to ΔN in NT cells), consistent with its slight ability to down-regulate BST2 on infected CEM T cells (see Supplementary Figure S4.2 online). In contrast, when BST2 was depleted (SH), viral particle release was comparable between the viruses, confirming the predominant role of BST2 in virion tethering at the cell surface.

Figure 4.2D shows how BST2 depletion affects Env recognition by different NAbs. First, binding of 17b to infected cells was evidently decreased in BST2-depleted cells. Although the difference was statistically significant for ΔU and ΔNΔU viruses ($P < 0.05$, paired Student's *t*-test), the extent of attenuation was not as pronounced as that observed with the other Abs. Indeed, the effect was most striking with PGT126 where Env recognition was not only meaningfully decreased but a difference of as much as 6-fold was also noted for ΔNΔU and ΔNΔU D368A viruses. The fact that Ab binding was reduced with WT and ΔN viruses was not unexpected given the incomplete depletion of BST2 expression in "SH" CEM T cells (Figure 4.2B). In consequence, the observation was likely attributed to a loss of BST2 molecules at virion-assembly sites that would have normally been present on BST2-expressing cells during infections with WT or ΔN

viruses [76, 77]. Taken together, the data further highlight the importance of BST2-mediated virion-tethering in Env recognition by NAbs, although the effect of BST2 depletion varied depending on the class.

To determine whether such changes in Env binding (Figure 4.2D) would accordingly affect ADCC, we assessed susceptibility of CEM $CD4^+$ T cells infected with WT, ΔNΔU or ΔNΔU D368A viruses to ADCC mediated by CD4i 17b and, as an example, "CD4 non-induced" PGT126. First, when BST2 was present (NT), T cells infected with the ΔNΔU virus were considerably more susceptible than their WT counterparts: an average of about 2-fold higher for PGT126 (Figure 4.3A) and 3-fold for 17b (Figure 4.3B). Compared to ΔNΔU virus, the near complete loss of 17b-mediated ADCC observed with ΔNΔU D368A was in line with the data on Env recognition (Figure 4.2A). Second, in the context of BST2 depletion, we observed statistically significant reduction in PGT126-mediated ADCC to levels that were comparable across the different viruses ($P < 0.005$, paired Student's *t*-test). The attenuated killing observed with WT virus in BST2-depleted cells (compare NT to SH of WT; Figure 4.3A) was consistent with decreased Env binding (Figure 4.2D) and could be attributed to the loss of residual BST2 as discussed above. In contrast, the effect of BST2 depletion on 17b-induced ADCC was observed with ΔNΔU virus-infected cells but not with their WT counterparts (Figure 4.3B). The latter finding was expected since it was cell-surface CD4 accumulation that contributed to optimal binding of 17b, and CD4 abundance was meaningfully less on WT virus-infected cells relative to their ΔNΔU virus counterparts (see Supplementary Figure S4.2 online). Interestingly, while ADCC mediated by 17b against ΔNΔU D368A virus-infected cells was statistically comparable to that of their WT counterparts, reduced killing of

ΔNΔU D368A virus-infected cells was also observed when BST2 was depleted, suggesting that when CD4-Env engagement is impaired, BST2-mediated virion tethering is likely the sole contributor to ADCC enhancement. It is worthy to mention that BST2 depletion seemed to have a more pronounced effect on ADCC than Env recognition (Figure 4.2). Overall, the data underline the relevance of BST2-mediated virion tethering in ADCC function elicited by both CD4-non-induced and CD4i NAbs.

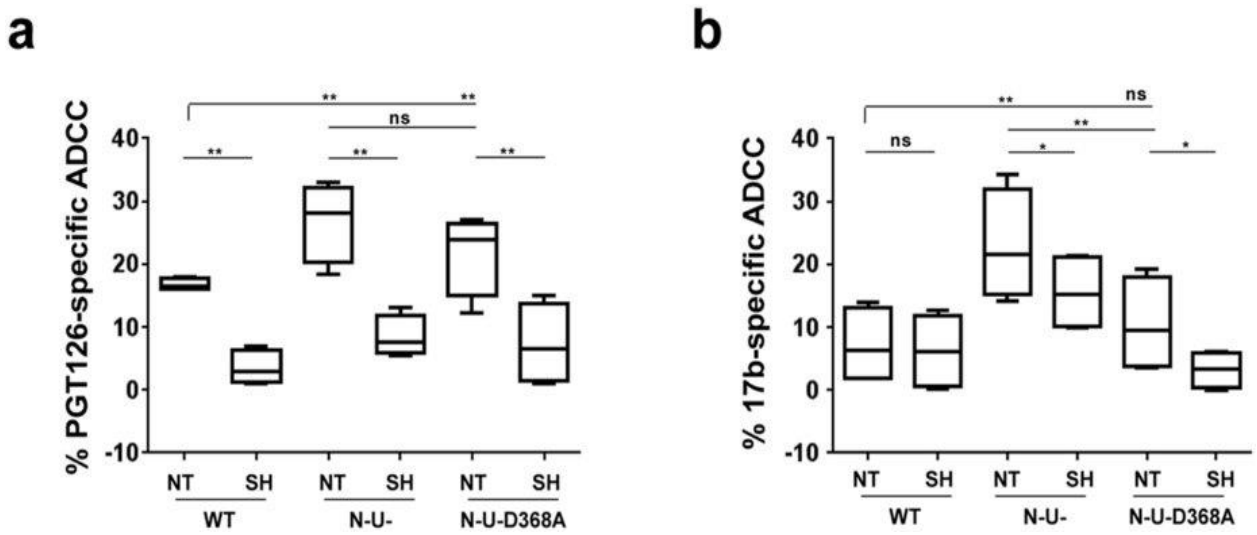


Figure 4.3: BST2 depletion attenuates ADCC activities mediated by both CD4-induced and CD4-non-induced NAbs. CEM CD4$^+$ T cells expressing (NT) or depleted (SH) of BST2 were infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT, N-U- or N-U-D368A viruses as indicated in Figure 4.2 legend and examined for ADCC susceptibility mediated by (**A)** CD4-non-induced PGT126 or (**B**) CD4i 17b Abs as described in Figure 4.1 legend. Panels A and B summarize results from at least 4 donors. In both Panels, paired, two-tailed Student's *t*-tests, were used to compare the effect of BST2 depletion on ADCC activity with each virus.

4.3.4 IFNα treatment enhances Env recognition and ADCC by bNAbs in a BST2-dependent manner. Our findings obtained thus far have demonstrated a close association between virion tethering by BST2 and ADCC enhancement for all classes of NAbs. Given that BST2 is an IFN-I-upregulated restriction factor, we asked if exogenous IFNα would augment ADCC function in a BST2-dependent manner. To this end, we found that IFNα raised BST2 levels by ~ 2-fold on both mock- and WT HIV-infected T cells. The increase was observed despite the presence of Vpu (Figure 4.4A). Further, this upregulation was associated with a ~ 2-fold enhancement in Env recognition (Figure 4.4B) not only by Abs which recognize well the ADA Env like PGT126 and 3BNC117 but also by those that do not such as 35O22 (Figure 4.1A). Importantly, in accordance with the IFNα-induced increase in Env binding, target cells became more susceptible to ADCC (Figure 4.4C). Given the robustness of PGT126-mediated ADCC response over multiple donors (Figure 4.4D), this Ab was chosen for further analyses.

To confirm that such IFNα-mediated augmentation of ADCC was related to enhancement of virion tethering by BST2, CEM $CD4^+$ T cells expressing (NT) or depleted (SH) of BST2 (Figure 4.2B) were infected with the same CCR5-tropic WT virus as in Figure 4.4 and exposed to IFNα. As shown in Figure 4.5A, the treatment led to a 2-fold increase in BST2 expression on NT but not on SH cells and correlated accordingly with a statistically significant increase ($P < 0.005$, Mann-Whitney test) in PGT126 binding on BST2-expressing cells but not their BST2-depleted counterparts (Figure 4.5B). In consequence, we observed greater ADCC activity in BST2-expressing target cells but not in those depleted of BST2 (Figure 4.5C), although the difference

between untreated (UT) and IFNα-treated NT cells was less than that for parental CEM $CD4^+$ T cells under the same experimental conditions (Figure 4.4D). This could be due to inherently higher baseline levels (i.e., IFNα-untreated) of BST2 on uninfected NT cells (Figure 4.2B) compared to parental cells (Figure 4.4A), which could in turn make the "NT" cells less sensitive to IFNα stimulation than parental cells. Mechanistically, the IFNα-induced increase in ADCC seemed to be due to greater retention of budding virions on infected cells, as evidenced by accumulation of cell-associated p24, and a simultaneous 2-fold decrease in particle release in IFNα-treated, WT virus-infected, BST2-expressing (NT) cells (Figure 4.5D). Of importance, this difference was not observed in BST2-depleted CEM $CD4^+$ T cells under the same experimental conditions, strongly indicating that IFNα treatment potentiates susceptibility of infected cells to ADCC in a BST2-dependent manner.

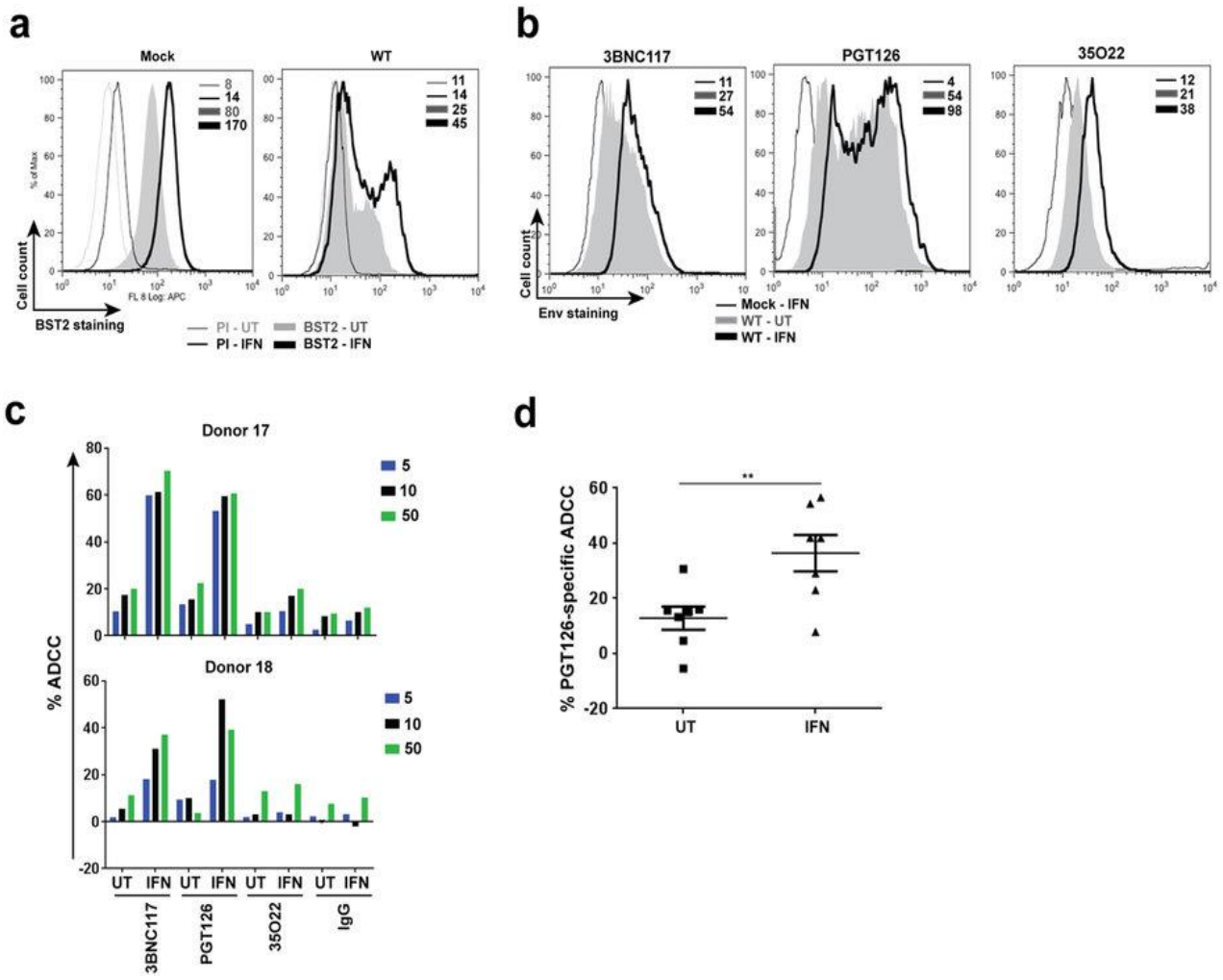


Figure 4.4: Exogenous IFNα enhances susceptibility of productively infected T cells to ADCC mediated by bNAbs. Parental CEM CD4$^+$ T cells were infected or not (mock) with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus for 20h and then exposed (IFN) or not (UT) to IFNα for about 48h. Infected cells were analyzed for BST2 or Env expression using (**A**) antiBST2 or (**B**) the indicated anti-Env Abs, respectively. (**C** and **D)** Cell susceptibility to ADCC was examined as described in Figure 4.1 legend. Panel C shows ADCC activities of 3 donors as examples. Panel D summarizes mean target cell killing mediated PGT126 from multiple donors. Each dot represents one PBMC donor. In this Panel, Mann-Whitney *U*-tests were used to compare ranks between UT and IFN-treated groups.

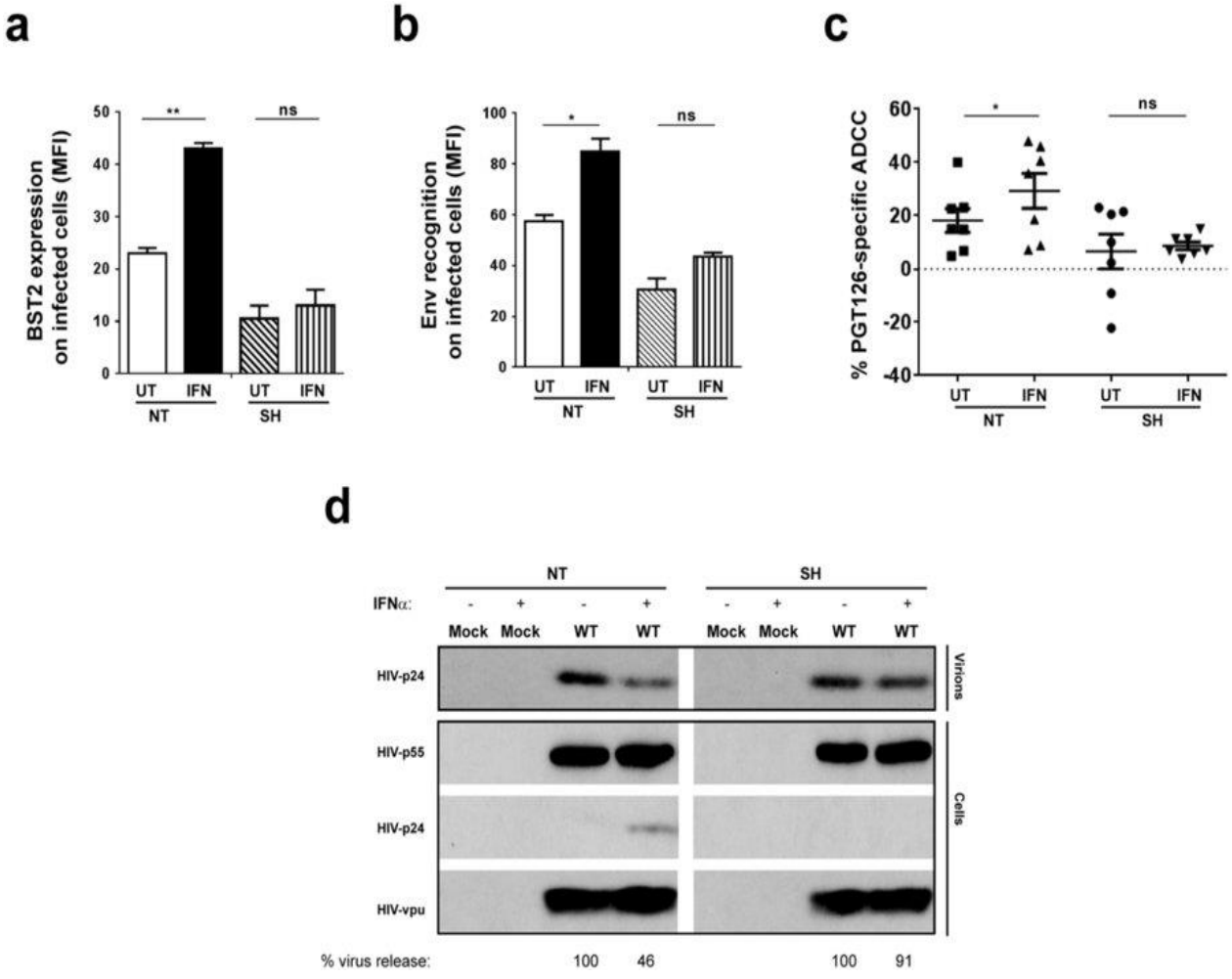


Figure 4.5: Enhancing physical tethering of HIV virions augments ADCC mediated by bNAbs in a BST2-dependent manner. CEM CD4$^+$ T cells expressing (NT) or depleted (SH) of BST2 were infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus and treated with IFNα. Infected cells were analyzed for (**A**) BST2 and (**B**) Env expression, ADCC susceptibility to (**C**) PGT126 and (**D**) HIV particle release as described in Figure 4.2 legend. Methodology and data analysis are as indicated in the legends for Figures 4.1 and 4.2. Each dot in Panel C represents one PBMC donor. Also in Panel C, Mann-Whitney *U*-tests were used to compare ranks between UT and IFN-treated groups for each cell type (NT or SH).

4.3.5 Jurkat-derived T cells latently infected with wild-type HIV are susceptible to ADCC upon reactivation. Having established that productively infected T cells could be eliminated by ADCC, we next investigated whether the same would also be true for reactivated latent T cells. Towards this, a latent cell line, initially infected with VSV-G pseudotyped CCR5-tropic NL4-3.ADA.IRES.GFP WT virus, was established (Figure 4.6A). This viral strain was used to prevent potential complications with *de novo* secondary re-infections. A 24-hour treatment with TNFα (2 ng/ml) was routinely used to reactivate up to 10 % latent cells. However, since prolonged exposure to a more physiologically relevant latency-reversing agent (LRA) such as romidepsin (RMD) [16, 17] was toxic to Jurkat cells, TNFα treatment was kept to 9 h when analyzed with RMD. As expected, HIV latent cells could be reactivated with both LRAs, although TNFα was more effective (Figure 4.6B). Importantly, both CD4 and BST2 were down-regulated on reactivated, WT virus-infected latent cells, reflecting *de novo* synthesis of viral proteins such as Nef and Vpu (Figure 4.6C). In addition, Env proteins were also expressed at the surface of reactivated cells, as evidenced by PGT121 binding (Figure 4.6C). Similar to PGT126, PGT121 binds to V3 base glycans and is sensitive to N332 substitution or removal of the glycan at this residue [314, 316]. Taken together, these markers indicated that GFP expression was an appropriate marker to measure reactivation. Importantly, reactivated latent cells were susceptible to ADCC regardless of the LRAs used (Figure 4.6D). Overall, the data indicate that latently infected T cells could be eliminated via ADCC although their killing remains modest with WT virus.

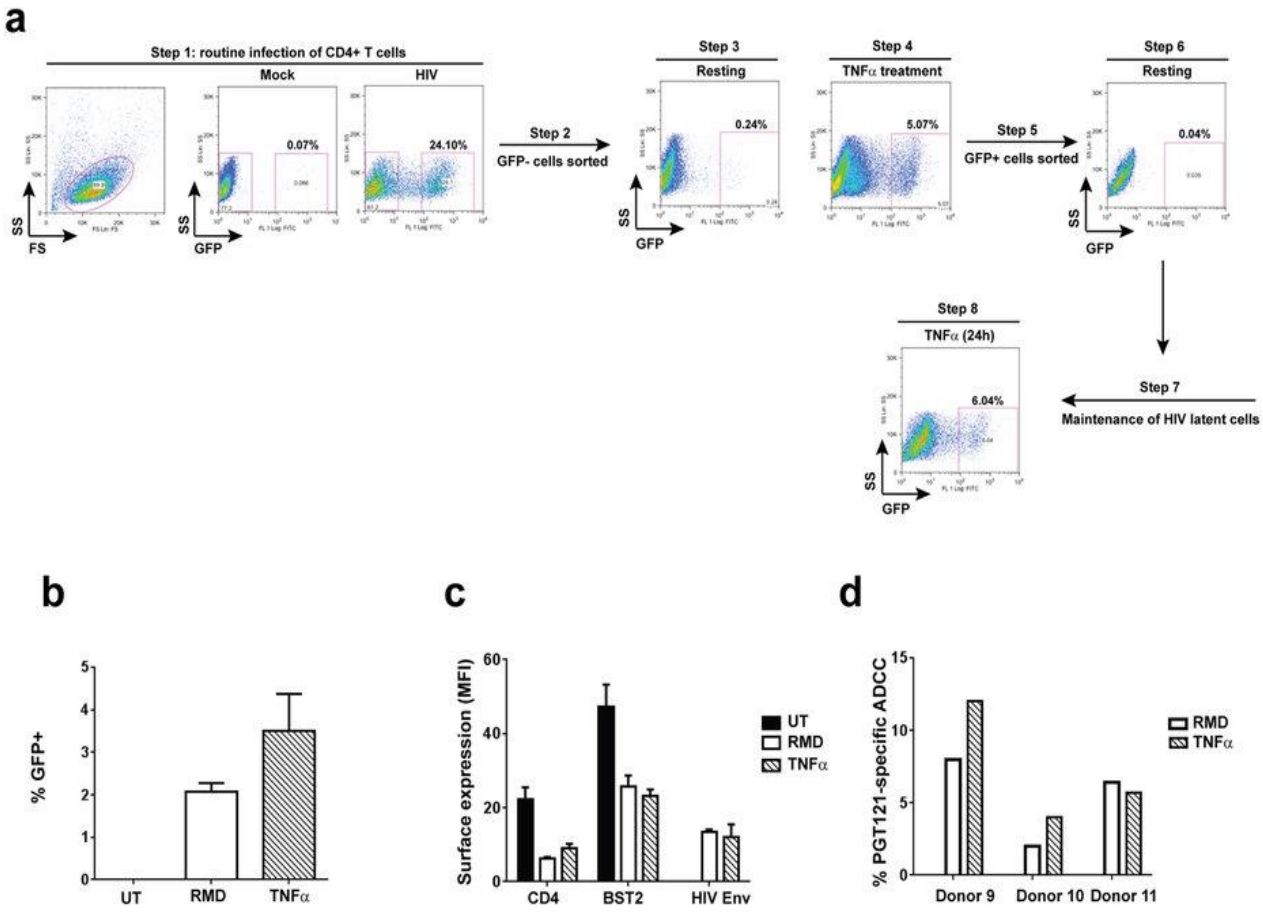


Figure 4.6: Reactivated latent cells are targets of ADCC. (**A**) Jurkat-derived CD4$^+$ T cells were infected with VSV-G pseudotyped, CCR5-tropic NL4.3.ADA.IRES.GFP WT virus and sorted for GFP$^-$ cells by flow cytometry. Following a 7-day recovery, GFP$^-$ cells were treated with TNFα for 24h and GFP$^+$ cells were sorted and maintained in the absence of TNFα as latent cells. These cells could be reactivated with TNFα or other latency reversing agents. (**B-D**) Resting latent cells from Panel A (Step 7) were exposed to romidepsin (RMD) or TNFα for 9h and examined by flow cytometry for reappearance of GFP (**B**) and for cell-surface CD4 and BST2 expression, as well as Env recognition by PGT121 (**C**). Reactivated latent cells were assessed for their susceptibility to FACS-based ADCC (**D**). Shown in Panel d are ADCC activities from 3 donors as examples. Methodology and data analysis are as described for Figure 4.1.

4.3.6 Potentiating susceptibility of HIV latent cells to ADCC through enhancement of BST2-mediated virion tethering. Our data thus far have shown that enhancing BST2-mediated virion tethering by IFNα heightened susceptibility of productively infected cells to ADCC (Figure 4.5), and that reactivated latent cells were targets of ADCC (Figure 4.6). We then asked which conditions would facilitate most efficient elimination of latent cells. In the context of productive HIV infections, we found that target cells infected with the ΔNΔU virus were most susceptible to ADCC compared to those infected with WT or a single mutant virus. Therefore, a T cell line latently infected with a ΔNΔU virus was also established, along with those infected with either ΔN or ΔU virus.

IFNα by itself did not trigger HIV reactivation nor did it augment the effect of RMD (Figure 4.7A). Interestingly, virus reactivation by RMD was about 3-fold more efficient in ΔNΔU virus-infected latent cells compared to WT virus. Consistent with findings shown in Fig. 2, Env recognition was markedly pronounced on ΔNΔU virus-infected cells compared to their WT virus counterparts (Figure 4.7B). In addition, IFNα treatment led to a 2-fold increase in PGT121 binding on both WT and ΔNΔU virus-infected cells (Figure 4.7B) and consequently, a statistically significant ($P < 0.05$, Mann-Whitney *U*-test) increase in ADCC activity (Figure 4.7C and 7D). Consistent with previous data regarding productive HIV infections, Env recognition and ADCC responses were more pronounced in a latent T cell line carrying the ΔNΔU virus. Taken together, our data clearly indicate that enhancing physical tethering of virions by IFNα treatment and by inactivating BST2 antagonism can accumulatively potentiate latent cells to ADCC induced by broadly NAbs. Indeed, the fact that reactivated latent T cells carrying a ΔU

or ΔNΔU virus were comparably more susceptible to PGT121-induced ADCC relative to their WT and ΔN counterparts clearly strengthens this underlying message (see Supplementary Figure S4.4 online).

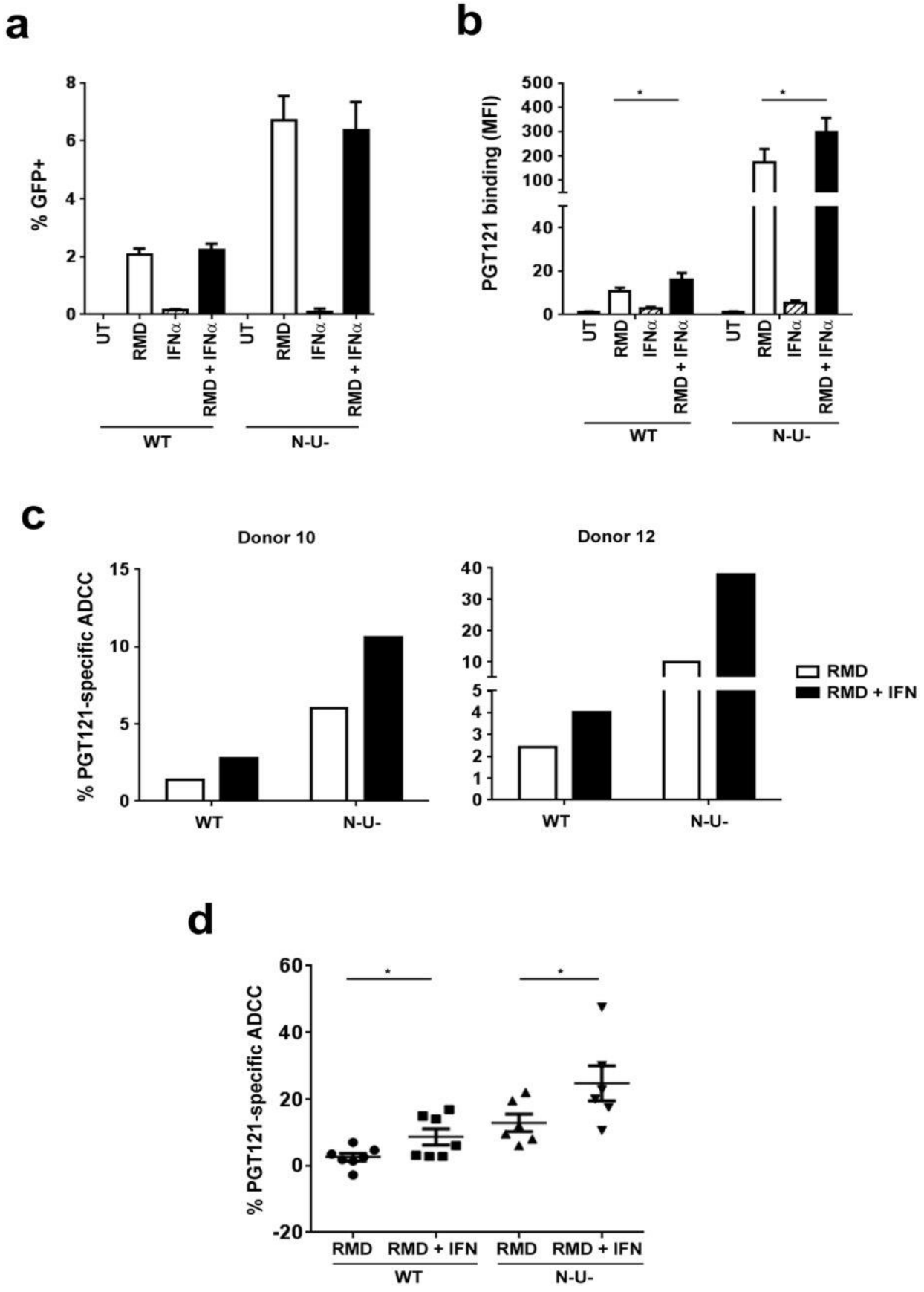

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Figure 4.7: Exogenous IFNα treatment and inactivation of Nef and Vpu enhance ADCC activity against HIV latent cells. Jurkat-based T cells latently infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT or ΔNΔU (N-U-) virus were treated with RMD for 9 h and then with or without IFNα for 15 h. Latent cells were examined by flow cytometry for reappearance of (**A**) GFP, (**B**) Env expression and (**C** and **D**) ADCC susceptibility using PGT121. Panel C indicates examples of ADCC response from 2 donors as examples and Panel D shows averaged Ab-specific ADCC (± s.d.) from 6-7 donors. Each dot represents 1 donor. In Panels B and D, Mann-Whitney *U*-tests were used to compare ranks between RMD and RMD + IFN-treated groups with each virus.

4.4 Discussion

In this study, we show that NAbs mediate ADCC on T cells infected with different primary virus isolates, although susceptibility of these cells varies with infecting strains. Abs directed against glycans in the V1/V2 apex or V3 base are most efficient at inducing ADCC. Additionally, we provide evidence that Vpu and Nef differentially modulate Env recognition and ADCC activity according to the class of NAbs. Our results highlight that BST2-mediated tethering of nascent virions at the surface of infected cells is instrumental for optimal ADCC function by all classes of NAbs. Indeed, exogenous IFNα treatment potentiates killing of infected cells in a BST2-dependent manner. Importantly, we demonstrate that reactivated latently-infected T cell lines are targets of ADCC although the overall killing remains inefficient. However, as with productively infected cells, inhibiting the ability of HIV to antagonize BST2 and/or IFNα

treatment heightened elimination of reactivated cells. Thus, for the first time, we illustrate that this approach could be a new avenue to improve clearance of latent cells.

In our analyses, NAbs that recognize glycan epitopes on V1/V2 or V3 were found to mount a more efficient killing of WT virus-infected T cells compared to those targeting MPER or the gp120-gp41 interface. In many cases, the Abs which effectively blocked HIV entry ($IC_{50} \leq 0.7$ μg/ml) were also potent at triggering ADCC, implying their potential relevance in both prophylactic and therapeutic settings of infection. Nevertheless, for some Abs that were less efficient at either or both functions (e.g., 17b and 35O22), the neutralizing capacity was not always predictive of the ADCC potential, and *vice versa*. In fact, findings analogous to ours in this context were recently reported by von Bredow and colleagues [418]. Further, Env recognition and ADCC induced by neutralizing Abs differed among primary isolates but, importantly, these variations were maintained regardless of the frequency of infected cells (*i.e.*, % $p24^+$) or extent of infection (*i.e.*, fluorescence intensity of p24 signal per cell from FACS analyses). In this regard, our data are consistent with recent findings by Bruel and colleagues [416], suggesting not only heterogeneity at the level of epitopes among viral strains but also potential influence of respective viral proteins on cellular factors that might govern how Env epitopes are displayed at the surface of infected cells. Indeed, functional analyses of Vpu variants from chronic and T/F viruses revealed variations in their ability to down-regulate CD4 and BST2, as well as to enhance virus particle release [192, 199, 200]. Additionally, differences in Vpu-mediated targeting of CD4 modulate the amount of sequestered Env and, consequently, incorporation of the glycoprotein into virions [159]. Similarly, patient-derived Nef variants also differ in their ability to down-modulate CD4

[419, 420], with potential consequences on the levels of Env at the cell surface as well as exposition of CD4i epitopes. Having said that, variations in the percentage of effector cells such as NK cells, as well as their make-up, in different donors could also contribute to variable ADCC responses as clearly shown in this study.

We previously showed that whereas CD4 played a major role in the enhancement of ADCC triggered by a non-neutralizing, CD4i A32 Ab, BST2 had a minor involvement [412]. However, in the context of NAbs as shown here, the contribution of BST2 to ADCC is highly significant. In fact, depletion of BST2 markedly reduces Env binding and ADCC activity mediated by all classes of neutralizing Abs tested. The greatest effect is observed with PGT126. Such a reduction could simply be due to a decrease in Env density otherwise provided by BST2-mediated virion or reduced avidity through fewer cross-linking of epitopes on trapped viral Env spikes to Abs such as PGT126 [315].

In our latency model, a Jurkat-based T cell line infected with the ΔNΔU virus could be reactivated more efficiently than WT virus-infected latent cells, regardless of the type of latency-reversing agent used. Although Nef can boost HIV transcription through NF-κB activation, Vpu seems to be superior at inhibiting this pathway [98]. Thus, it is possible that the absence of Vpu in the ΔNΔU virus provides a cellular environment which enables effective NF-κB-dependent transcription, resulting in better reactivation of latent cells. However, even then, only a small fraction of latent cells could be reactivated at any given time in our model system, echoing the global challenge of reactivating latently infected cells. Although our data show that reactivated latent cells are susceptible to ADCC, the killing remains rather inefficient, especially for those

infected with WT or ΔN virus. The fact that the two latent T cell lines carrying the ΔNΔU or ΔU virus were more susceptible to PGT121-mediated ADCC further strengthens the need to employ alternative approaches, such as one that reverts BST2 antagonism by Vpu, that could potentially enhance cell killing. Nonetheless, studies with T cell lines have their own limitations in that they may not necessarily recapitulate what is happening in vivo. One such limitation would be the level of Env epitopes expressed at the surface of infected cells. Therefore, further studies are needed to evaluate whether our findings obtained with latent cell lines could be extended to primary cells.

Since ΔNΔU virus-infected T cells are significantly more prone to ADCC than their WT counterparts, the use of small molecules aimed at negating the effects of Vpu and Nef on BST2 and/or CD4 down-regulation would conceivably augment ADCC function. While small molecules such as CD4 mimetics may increase killing of infected cells by only CD4i Abs [415, 421], collective data shown here and elsewhere [413, 414] demonstrate that enhancing physical tethering of virions by upregulating BST2 expression can improve ADCC functions by both CD4i and CD4-non induced Abs. Indeed, we provide evidence to support that exogenous IFNα sensitizes infected T cells (productive and latent) to ADCC through a process that involves upregulating BST2 expression and enhancing BST2-mediated virion tethering at the surface of infected cells. On this note, our study not only extends previous findings [414], but also mechanistically links them to virion tethering mediated by BST2. It is worth mentioning that the dose of IFNα used in our study was previously shown to upregulate BST2 to levels comparable to the elevated IFN levels found in HIV-1 infected patients during the chronic phase of infection [422]. Of additional significance, we identify that ADCC

potency of different classes of NAbs could be enhanced using IFNα treatment approach. Lastly, given that these findings together with those reported by others [416] have indicated a heterogeneous display of Env epitopes at the surface of infected cells, cocktails of ADCC-mediating Abs [410, 416] will likely be needed to improve ADCC function.

In summary, our study highlights the role of HIV accessory proteins Nef and especially Vpu in the evasion of ADCC by NAbs. It underscores how physical retention of virions at the cell surface affects susceptibility of infected cells to ADCC, regardless of epitope specificity. The fact that ADCC responses against both productively and latently infected cells could be heightened by enhancing the extent of virion tethering paves a way towards future development of approaches aimed at restoring BST2 restriction in order to facilitate clearance of latent viral reservoirs.

4.5 Methods

4.5.1 Ethics Statement: This study and experiments were approved by the Research Ethics Review Board of the Institut de Recherches Cliniques de Montréal in accordance with the Declaration of Helsinki. All methods were carried out in accordance with the approved guidelines. Blood samples were obtained from HIV- and HCV- seronegative adults who had given written informed consent.

4.5.2 Chemicals, antibodies and proviral DNA constructs: Romidepsin (RMD) and phytohaemagglutinin-P were purchased from Sigma Aldrich. Proliferation dye eFlour670 was from Affymetrix. Interferon-α 2a (IFNα) was obtained from PBL Assay Science, tumor necrosis factor-alpha (TNFα) was from BioLegend and human recombinant interleukin-2 (IL-2) [400] was available through the NIH AIDS Research and Reference Reagent Program. Luciferase Assay System including lysis buffer were from Promega. Rabbit anti-Vpu and pre-immune sera were generated as previously described [379]. Mouse anti-p24 mAb (Cat #: HB9725) was isolated from culture supernatant of hybridoma cells from the American Type Culture Collection (ATCC). PE-conjugated anti-p24 Ab (KC57-RD1) was obtained from Beckman Coulter. PerCP-Cy 5.5-conjugated anti-human CD4 antibody was from BioLegend. Rabbit anti-BST2 Ab was described elsewhere [379]. Purified human serum IgG (Cat #: I4506) was obtained from Sigma Aldrich. AF633-conjugated anti-rabbit IgG and AF647-conjugated anti-human IgG secondary Abs were from Life Technologies.

Broadly neutralizing antibodies including PG9 (IgG1) [312], PGT121 [314, 315] and PGT126 [314] were obtained through the NIH AIDS Research and Reference Reagent Program. In addition, 3BNC117 (IgG1) and 10-1074 (IgG1) were from Dr. Michel C. Nussenzweig [309]; 8ANC195 (IgG1) was from Dr. Pamela Bjorkman [320]; 35O22, 7H6 and 10E8 (all were IgG1) were from Drs. Jinghe Huang and Mark Connors [321, 322]; and 17b (IgG1) was from Dr. James. E. Robinson [325, 328, 329, 423-425]. Proviral construct CCR5-tropic NL4.3.ADA.IRES.GFP wild-type (WT), which encodes all accessory proteins, and its isogenic derivatives: Vpu-deficient (U- or ΔU), Nef-deficient (N- or ΔN), Nef- and Vpu-deficient (N-U- or ΔNΔU) and ΔNΔU D368A (N-U-D368A)

were generated using standard molecular biology techniques as described [412]. ΔNΔU D368A construct contains a mutation within the CD4-binding site of Env at position 368, resulting in impaired CD4-Env interactions.

Infectious molecular clones of transmitted/founder (T/F) viruses: p.WITO.c/2474 (Cat #: 11739), p.CHO77.t/2627 (Cat #: 11742) and p.THRO.c/2626 (Cat #: 11745) were obtained through the NIH AIDS Research and Reference Reagent Program from Drs. John Kappes and Ochsenbauer [426-430].

4.5.3 Primary cells, T cell lines and infection of T cells: Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood of healthy adults using Ficoll-Hypaque Plus (GE Healthcare). PBMC were cultured overnight in RPMI-1640 media [10 % fetal bovine serum (FBS) supplemented with L-Glutamine, Penicillin-streptomycin] and 100 U/mL IL-2. Primary $CD4^+$ T cells, isolated by negative selection using the human $CD4^+$ T cell isolation kit from Miltenyi Biotec, were activated with 5 µg/ml phytohaemagglutinin-P for 48 h and cultured in the presence of 100 U/mL IL-2. HEK 293T cells were obtained from ATCC and maintained in complete DMEM media (10 % FBS supplemented with Penicillin-streptomycin). Hela TZM-bl and all other T cell lines: CEM.CCR5 $CD4^+$, JLTRG-R5 [431] and 1G5 were obtained from the NIH AIDS Research and Reference Reagent Program. Whereas Hela TZM-bl were cultured incomplete DMEM media, T cell lines were in complete RPMI-1640 media. CEM.CCR5 $CD4^+$ T cells were depleted of BST2 using lentiviral vector particles containing shRNA targeting BST2 (referred to as “SH” cells in text and figures) or control shRNA (referred to as “NT” cells in text and figures) as previously described [412].

Activated primary $CD4^+$ T cells or CEM.CCR5 $CD4^+$ T cells were infected, as appropriate, with VSV-G pseudotyped CCR5-tropic NL4.3.ADA.IRES.GFP viruses or T/F viruses to have about 15-30 % infected cells at 48 h post infection (for CEM T cells). Primary $CD4^+$ T cells were analyzed at 72 h post infection and the infection was in the range of 2-10 % GFP^+ cells depending on donors. In certain experiments, T cells were infected with WT virus for 15 to 20 h and then treated with IFNα (1,000 U/mL) for about 48 h before harvesting for analysis.

4.5.4 Production of VSV-G-pseudotyped lentiviral vectors and HIV-1 viruses: For virus production, HEK 293T cells were co-transfected with an HIV proviral construct and pSVCMV-VSV-G using calcium phosphate precipitation method and harvested at 48h post-transfection [389]. Titration was done by flow cytometry-based analysis of GFP expression in parental CEM.CCR5 $CD4^+$ T cells (referred to as CEM in text and legends) for NL4.3.ADA-based viruses or in JLTRG5 for T/F viruses.

4.5.5 Generation of HIV latent T cells and their reactivation with latency-reversing agents: HIV latent T cell lines were established using a protocol similar to that described by Jordan and colleagues [432]. Briefly, Jurkat-based 1G5 T cells were infected with VSV-G pseudotyped, CCR5-tropic NL4.3.ADA.IRES.GFP WT or its derivatives ΔN (N-), ΔU (U-) and ΔNΔU (N-U-) virus for 4 to 5 days to have about 20-40 % infection. GFP^- population, which contained uninfected or latently infected cells, were sorted by flow cytometry (purity was approximately 99 %). After 7 days of resting, GFP^- cells were treated for 24 h with 2 ng/ml TNFα. GFP^+ cells (about 5-15 %) were again

sorted and rested in the absence of TNFα, which returned them to quiescent state. Latency status was confirmed by analyzing surface expression of HIV Env, CD4 and BST2 on GFP^+ cells.

To reactivate the virus, latent 1G5 cells were routinely treated with RMD (5 nM) or as a control, TNFα (2 ng/mL) for 9 h. Twenty-four hours later, cells were analyzed for GFP expression, Env recognition as well as ADCC susceptibility. For experiments involving IFNα treatment, after the 9-hour treatment with LRAs, cells were washed, fresh media containing IFNα (1,000 U/mL) were added and cells were analyzed at 24 h post LRA treatment.

4.5.6 Flow cytometry: CD4 staining was done as per manufacturer's protocols. BST2 staining was done as described using anti-rabbit BST2 Ab or as a control, a rabbit pre-immune (PI) serum [412]. For Env staining, at 2 days post-infection, cells were stained with human anti-Env Abs (1-50 µg/ml) for 45 min at 4 ^oC. Fluorescence signals were revealed using an AF647-conjugated anti-human Ab. Mock-infected cells were used as control. Cells were analyzed on a CyAn ADP analyzer. Voltage settings on the CyAn were set as appropriate when analyzing samples from experiments that included mutant viruses (versus those with WT virus alone) to allow for appropriate capture of fluorescence signals.

For intracellular p24 staining, T cell cultures were fixed and permeabilized using the BD Cytofix/Cytoperm kit (Cat # 554714) as per manufacturer's instructions. Following a 15-min incubation at 4 ^oC with a blocking buffer (1X BD Perm/Wash buffer containing 0.5 mg/ml human IgG and 5 % FBS), cells were stained with PE-conjugated

anti-p24 antibody for 10 min at 4 ^{o}C and then washed 2 times with BD Perm/Wash buffer (1X). Mock-infected cells were used as control. Cells were analyzed on a CyAn ADP analyzer.

4.5.7 Neutralization assay: Two to five-fold serial dilutions of Abs (50 µl) were incubated in a 96-well plate with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus (50 µl) for 90 min at 37 ^{o}C. Hela TZM-bl cells which express an HIV LTR-driven luciferase gene, were added to the Ab and virus mixture at 10,000 cells in 100 µl. Assays were done in three replicates except for negative (cells alone) and positive (cells with virus but no Ab) controls which had five replicates. At 48 h post infection, supernatant was removed and cells were lysed using 150 µl 1x luciferase lysis buffer. Clarified cell lysates were read on a luminometer for luciferase activity (relative light units, RLUs). Neutralization efficacy was calculated as: 100 x [(RLUs positive control - RLUs test sample) / (RLUs positive control - RLUs negative control)]. Data were analyzed using Prism and IC_{50} values were calculated by the software using Log inhibitor *vs.* Response (Variable slope) equation. IC_{50} (µg/ml) was the concentration at which 50 % of infection was blocked.

4.5.8 Antibody-dependent cell-mediated cytotoxicity assay (ADCC): The protocol is a modified version of that published previously [412]. For bioluminescence-based assay by luciferase, 50,000 CEM $CD4^{+}$ target (T) cells plated in a 96-well V-bottom plate were exposed to NAbs (up to 10 µg/ml) or human control IgG Ab for 30 min at room temperature and then incubated with PBMCs (effectors) at a ratio of 10 effectors per 1

target for 6 h at 37 ^{o}C. For each condition, the assay was done in biological duplicates or triplicates. Subsequently, cells were spun at 400 x *g*, supernatant completely removed and cell pellet lysed for luciferase expression as described for "Neutralization assay". Clarified cell lysates were read on a luminometer for luciferase activity (RLUs). Percent of cell lysis was determined as indicated below. For FACS-based ADCC assay with latent cells as targets, effector cells were labelled with eFlour670 proliferation dye and incubated with target cells at E:T ratios of 0.25 to 5. After 4 h of incubation, cells were collected by centrifugation, fixed in 1 % paraformaldehyde and analyzed for GFP expression. Percentage of Ab-specific ADCC was determined as 100 x [(% GFP^{+} or RLUs) $_{\text{effectors + control Ab}}$ – (% GFP^{+} or RLUs) $_{\text{effectors + test Ab}}$] / [(% GFP^{+} or RLUs) $_{\text{effectors + control Ab}}$]. In certain experiments where cell killing mediated in the absence or presence of (control or test) antibody needs to be separately conveyed, the following formula is used to determine percentage of cell lysis: 100 x [(% GFP^{+} or RLUs) $_{\text{no effectors}}$ – (% GFP^{+} or RLUs) $_{\text{effectors + test agent}}$] / [(% GFP^{+} or RLUs) $_{\text{no effectors}}$]. "Test agent" in the second formula could be in the absence of Ab (to yield antibody-unrelated killing), or presence of Ab (to yield standard ADCC). Standard ADCC by test Ab must be subtracted from that of control IgG to obtain the percentage of Ab-specific ADCC.

4.5.9 Viral particle release assay: CEM $CD4^{+}$ T cells were infected for 48 h with CCR5-tropic NL4-3.ADA. IRES.GFP WT strain or its derivatives deficient of Nef and/or Vpu. In certain experiments, cells were treated with IFNα (1,000 U/mL) at 15-20 h post infection and viral particle release assays were performed at 48 h post infection as

described before [389]. Western blotting was performed using Abs specific for Vpu or Gag.

4.5.10 Statistical analyses: Data were analyzed using GraphPad Prism. Descriptive measures (mean, median, min/max range and percent) were used to summarize the data. Unless otherwise stated, data were expressed as average ± sem. Two-way Anova analysis of variance with Dunn's *post hoc* tests were used to compare levels of Env recognition by different Abs on T cells infected with different viruses. Paired, two-tailed Student's *t*-tests, were used to compare the effect of BST2 depletion on Env recognition. Two-tailed Mann-Whitney *U*-tests were used to compare ranks between two treatment groups. P values of ≤ 0.05 were considered statistically significant: * ≤ 0.05; ** ≤ 0.005; *** ≤0.0005; and ns, not significant.

4.6 Declarations

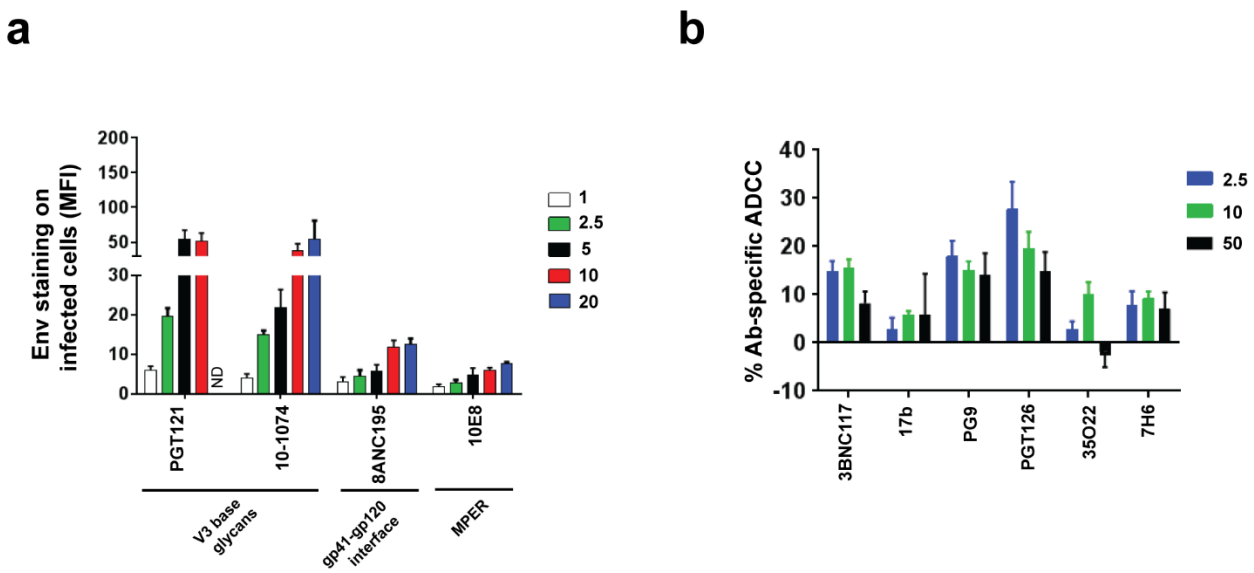
Acknowledgements: We thank Fadi Hajjar for technical assistance, Eric Massicotte and Julie Lord-Grignon for assistance with flow cytometry, Dr. Pierre Larochelle and the IRCM clinic staff for acquisition of blood samples and all blood donors for providing the samples. Except 8ANC195, all other monoclonal anti-Env Abs were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. 8ANC195 was from Dr. Pamela Bjorkman. 3BNC117 and 10-1074 were from Dr. Michel C. Nussenzweig. 35O22, 7H6 and 10E8 were from Drs. Jinghe Huang and Mark Connors. 17b was from Dr. James. E. Robinson. Also provided through the NIH AIDS

Research and Reference Reagent Program were rIL-2 and infectious molecular clones of T/F viruses p.WITO.c/2474 (Cat #: 11739), p.CHO77.t/2627 (Cat #: 11742) and p.THRO.c/2626 (Cat #: 11745). rIL-2 was from Dr. Maurice Gately, Hoffmann – La Roche Inc and the T/F virus molecular clones were provided by Drs. John Kappes and Ochsenbauer. This work was supported by a Canadian Institutes of Health Research (CIHR) grant MOP-111226 and by the Canadian HIV Cure Enterprise Grant HIG-133050 from the CIHR partnership with CANFAR and IAS to EAC. EAC is the recipient of the Université de Montréal-IRCM Chair of excellence in HIV research.

Author contributions: Conceived and designed experiments: TNQP, SL and EAC. Performed experiments: TNQP, SL, FD and GP. Analyzed data: TNQP, SL and EAC. Wrote manuscript: TNQP, SL and EAC. All authors reviewed the manuscript.

Competing financial interests: The authors declare no competing financial interests.

4.7 Additional Files

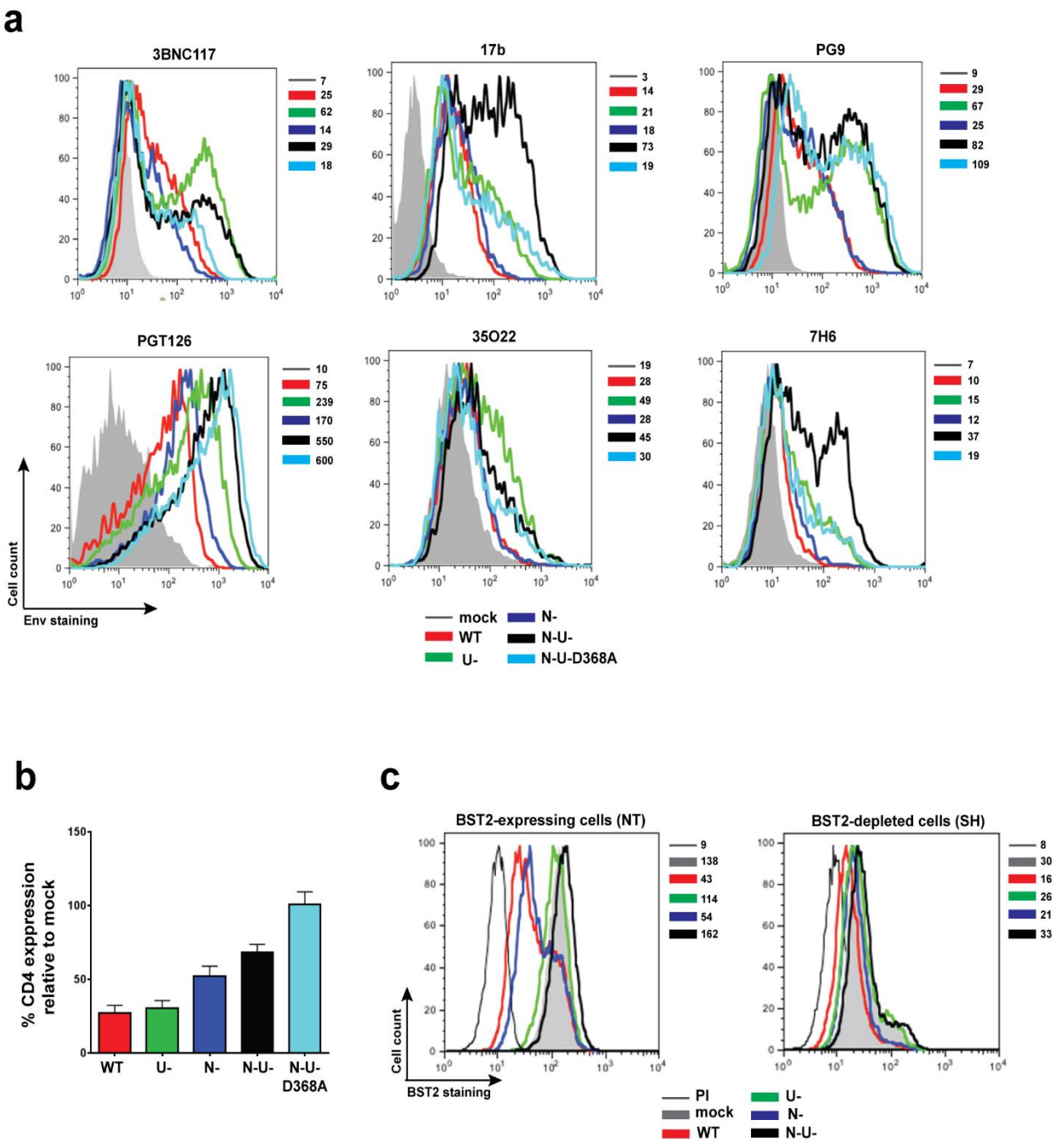


c

Antibody	Epitope (Env region)	IC_{50} ± S.D. (µg/ml)
3BNC117	CD4-bs (gp120)	0.07 ± 0.03
17b	CoR-bs (gp120)	1.30 ± 2.08
PG9	V1/V2 apex (gp120)	0.07 ± 0.02
PGT121	V3 base glycans (gp120)	0.04 ± 0.02
PGT126	V3 base glycans (gp120)	0.04 ± 0.01
35O22	gp41-gp120 interface	17.78 ± 9.50
7H6	MPER (gp41)	0.26 ± 0.10

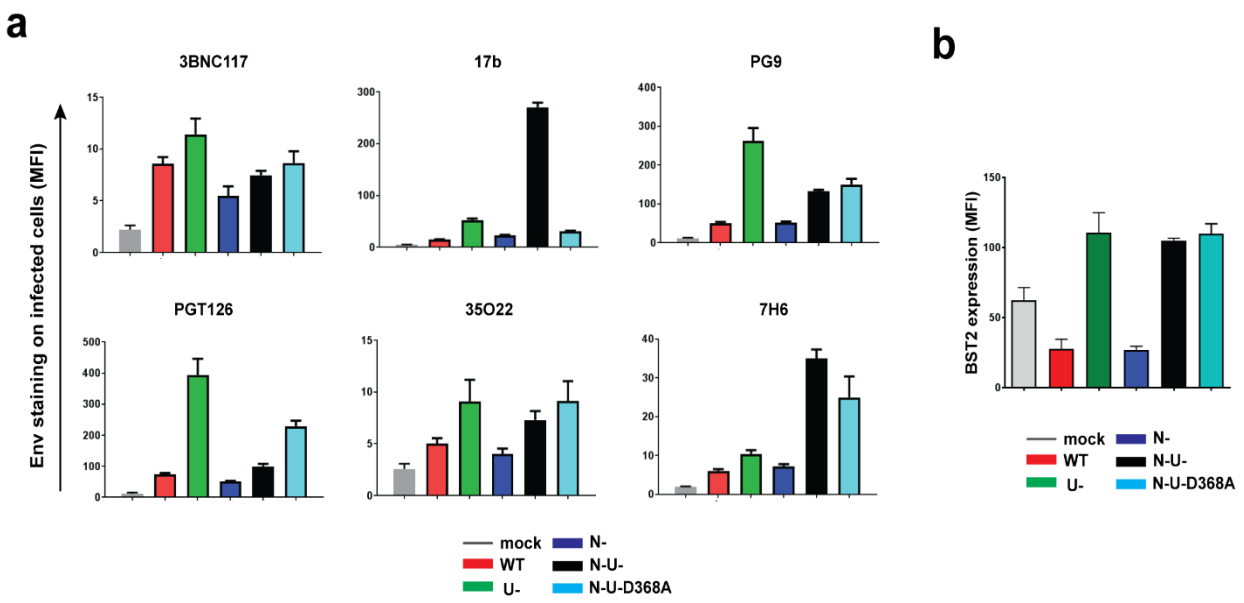
Additional File 4.1: Figure S4.1. Neutralization potency and ADCC potential of anti-HIV antibodies. CEM $CD4^+$ T cells were infected with prototypic virus CCR5-tropic NL4.3.ADA.IRES.GFP WT strain and at 2 dpi infected cells were examined for (**A**) cell-surface Env expression and (**B**) susceptibility to ADCC using different concentrations of NAbs (in µg/ml). (**C**) Neutralizing antibodies were evaluated for their ability to neutralize cell-free virus. Extent of Env staining on GFP^+ T cells over a range of concentrations was expressed as averaged median fluorescence intensity (MFI) units from 3 to 4 experiments. PBMCs from healthy donors were used as effector cells. Percentages of Ab-specific ADCC were determined following subtraction of cell lysis mediated by control IgG from that by test antibody. Mean ADCC activity from at least 4-7 donors is indicated. Serial dilutions of known concentrations of NAbs (or as control, PBS) were pre-incubated with prototypic CCR5-tropic NL4.3.ADA.IRES.GFP WT virus for 45 min at 37 ºC. Luciferase-expressing HeLa TZMbl reporter cells were subsequently added to Ab-virus complexes.

Forty-eight hours later, infected cells were lysed and relative light units enumerated on a luminometer. Data were analyzed using GraphPad Prism. Shown are mean IC50 from 2 to 3 experiments; ND, not done.

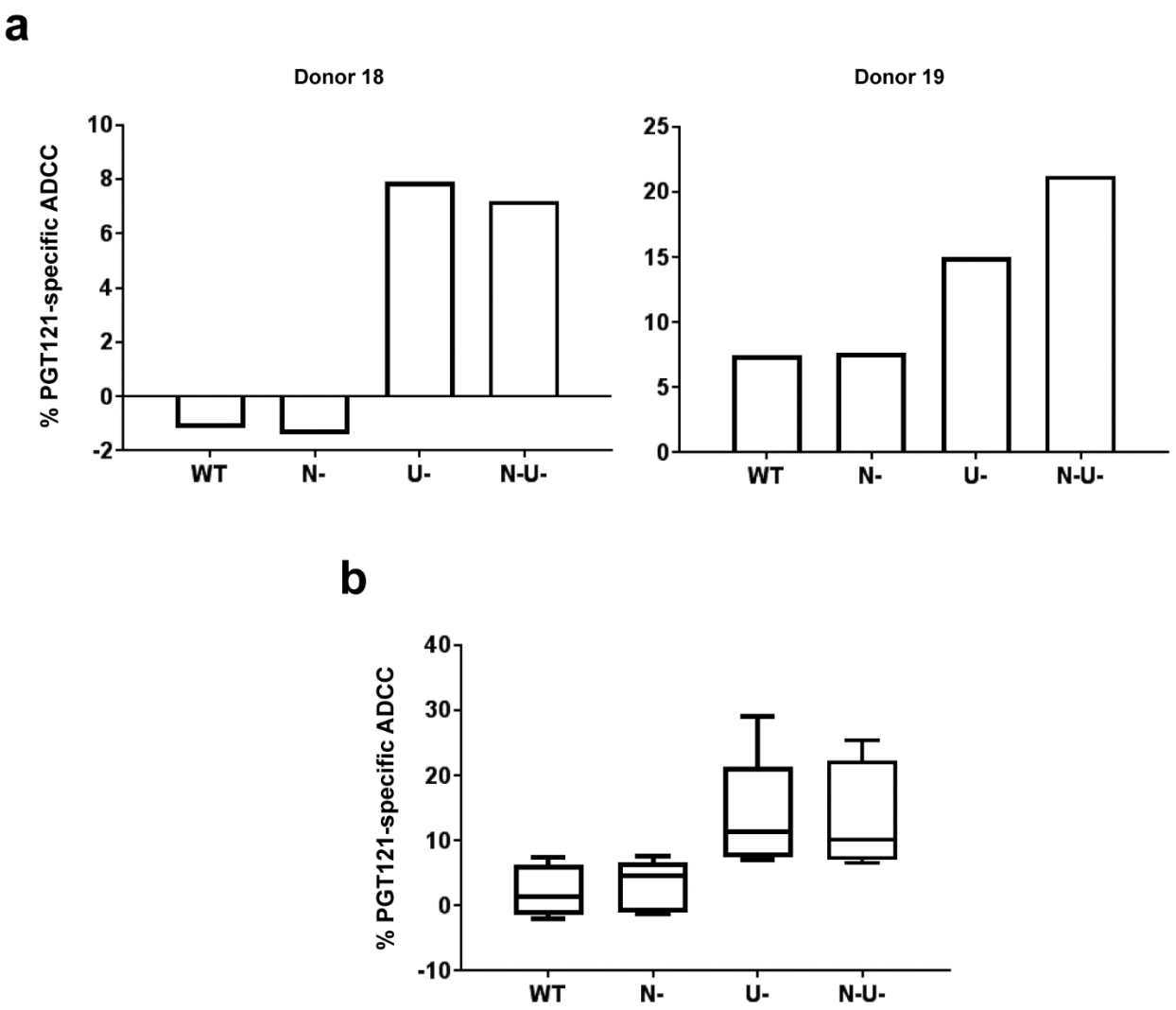


Additional File 4.2: Figure S4.2. Cell-surface envelope, CD4 and BST2 expression profiles on HIV-infected CEM CD4⁺ T cells. Parental CEM CD4⁺ T cells were infected or mock-infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus or derivatives lacking Vpu (U-), Nef (N-) or both (N-U-). N-U-D368A virus has a mutation within the CD4-binding site of Env causing defective CD4-Env interactions. At 2 dpi, infected cells were analyzed by flow cytometry for (**A**) Env using indicated NAbs and (**B**) CD4

using anti-CD4 Ab. (**C**) Alternatively, CEM $CD4^+$ T cells expressing (NT) or depleted (SH) of BST2 were infected with the indicated viruses as in Panel A and analyzed for cell-surface BST2 using anti-BST2 Ab or pre-immune serum (PI) as control. Shown in Panels A and C are expression levels on GFP+ cells presented as MFI units from a representative experiment. Panel B indicates mean CD4 expression from 3 experiments.



Additional File 4.3: Figure S4.3. Cell-surface envelope and BST2 expression profiles on HIV-infected primary CD4⁺ T cells. Activated CD4⁺ T cells were infected or mock-infected with CCR5-tropic NL4.3.ADA.IRES.GFP WT virus or derivatives lacking Vpu (U-), Nef (N-) or both (N-U-). The N-U-D368A virus has a mutation within the CD4-binding site of Env causing defective CD4-Env interactions. At 3 dpi, infected GFP⁺ cells were analyzed by flow cytometry for (**A**) Env using indicated NAbs or (**B**) BST2 using anti-BST2 Ab. Shown in both Panels A and B are mean expression levels on GFP⁺ cells presented as MFI units from 3 different donors.



Additional File 4.4: Figure S4.4. Reactivated latent T cell lines are targets of ADCC and Vpu dampens PGT121-mediated ADCC response. Jurkat-based T cells latently infected with CCR5- tropic NL4.3.ADA.IRES.GFP WT or its mutant derivatives lacking Vpu (U-), Nef (N-) or both (N-U-) viruses were treated with TNFα and examined for their susceptibility to ADCC mediated by PGT121. PBMCs from healthy donors were used as effector cells. (**A**) Examples of ADCC response from 2 donors. (**B**) Compilation of PGT121-specific ADCC. Boxes and whiskers graph shows median ADCC from 4 donors.

CHAPTER 5: GENERAL DISCUSSION

Given that the data presented herein have been discussed within their respective chapters, the focus of this chapter is on the overall perspective and contextual relevance of the thesis work.

5.1 Functional importance of the Vpu hinge region

5.1.1 Roles of the E28/L33 residues relative to other Vpu residues

Human BST2 plays important roles in HIV-1 infection and pathogenesis. Beyond the proposed role in blocking zoonotic transmission of viruses to humans [192], BST2 is associated with protection from HIV-1 acquisition, control of viraemia, as well as reduced progression to AIDS in infected patients [198, 202-204]. BST2 does not only promote restriction of virus release, but also leads to the development of an antiviral state through NF-κB, TLR and ILT7 signalling events that trigger or mediate production of IFN and ISGs, as discussed in Section 1.5 above. HIV-1 group M viruses have evolved to utilize Vpu to effectively counter these antiviral activities of BST2. While the *in vivo* roles of Vpu are still being elucidated, it has already been shown that Vpu facilitates the establishment of infection in humanized mouse models [107, 108]. The importance of Vpu-mediated BST2 antagonism is further emphasized by the strong preservation of this function throughout the infection period - from newly to chronically infected individuals, even though *vpu* itself is a variable gene [199-201]. Not surprisingly therefore, Vpu activities are closely associated with pandemic HIV strains [192]. In light of all these, identification of determinants governing this Vpu-mediated BST2 antagonism has emerged as a crucial approach towards realization of the antiviral effects of BST2.

Vpu targets BST2 *via* several mechanisms including intracellular trapping and sequestration, surface downregulation and displacement mechanisms, all of which serve to ensure that BST2 is removed from virus assembly sites, where it would

otherwise tether virions thereby preventing their release from the infected cell [76, 77, 270-272, 379]. Towards this, the data presented in this thesis directly address the functional importance of highly conserved residues within the transmembrane-proximal hinge region of Vpu, as well as provide mechanistic insights into their contribution to BST2 antagonism. We show that while these hinge region residues, resembling an acidic di-leucine motif (ExxxIL), have no intrinsic activity on the cellular distribution of Vpu in the absence of BST2, but are essential for the ability of Vpu to optimally bind to BST2. Consequently, these residues govern both BST2-dependent trafficking properties of the protein as well as its co-localization with the restriction factor, unlike the helix-2 di-leucine motif, which does not attenuate Vpu colocalization with BST2. It is very interesting that Vpu-mediated BST2 targeting specifically requires the Glu (E28 in ExxxIL), and does not accommodate a conserved Asp substitution for the Glu (DxxxIL). Our data are consistent with the notion that efficient binding of Vpu to BST2 and optimal trafficking of Vpu-BST2 complexes are important and determine the extent of antagonism, as earlier reported [271, 272, 274]. Functionally, the hinge region residues and the helix-2 di-leucine motif were additive, consistent with the fact that they act at different stages of the counteraction. It is interesting to note that even though they are functionally additive, their influence on the BST2-dependent cellular localization of Vpu was not additive, arguing that Vpu cellular distribution, in this context, is not strictly correlated with the extent of antagonism.

Our work provides the first evidence of a role of the hinge region residues towards optimal binding of Vpu to BST2, a critically important pre-requisite for antagonism. Thus, the residues likely attenuate subsequent antagonism steps including

passively trapping BST2 within intracellular compartments as well as recruitment of clathrin AP complexes that sequester BST2 within endosomal compartments, altogether acting to preclude BST2 from trafficking to the cell surface [271]. Our data, pointing to a role of the hinge region in anchoring the Vpu TMD conformation in a manner that is compatible with interactions with target proteins, emphasize the significance of Vpu structural elements for function. While direct experimental evidence evaluating the relationship between Vpu TMD conformational configuration and biological activity are largely lacking, positive correlations between alterations in Vpu TMD tilt angles and attenuation in BST2 antagonism have been reported [281]. As such, approaches aimed at perturbing the Vpu structure may be effective in inhibiting the antagonistic activity of the protein. In this context, further experiments are necessary to experimentally probe the effects of the hinge region residues on the TMD tilt angles, as well as how they modulate interactions with BST2. To this end, NMR and molecular dynamic studies would be essential in providing atomic-detail structural and dynamic properties of the Vpu TMD. In addition, for completeness sake, characterization of the ability of the mutants to interact with AP complexes would yield further mechanistic insights into the contribution of these conserved residues. In terms of therapeutic intervention, interfering with AP recruitment via abrogating the Vpu-BST2 interaction would be preferable compared to targeting the Vpu helix-2 di-leucine (AP-recognition) motif. This is especially because it likely would not affect critical constitutive cellular roles of AP proteins, in addition to the fact that Vpu-BST2 interaction is an early prerequisite for any antagonistic activity mediated by Vpu. Indeed, disrupting Vpu-BST2 binding would likely prevent the ‘passive’ trapping of BST2 molecules within intracellular compartments,

even prior to engagement of AP complexes that mediate the active sequestration of BST2 within endosomal compartments.

While it significantly impedes BST2 counteraction, mutation of the E28/L33 determinant does not fully abrogate BST2 counteraction, necessitating mutations of other Vpu regions. Interestingly, occurring within the same hinge region of Vpu are functionally important residues (R30/K31) that are mechanistically distinct in their contributions to BST2 antagonism [283]. Whereas E28/L33 are important for Vpu-BST2 interaction, R30/K31 regulate the intrinsic accumulation of Vpu within the TGN, which enables Vpu to target both recycling and *de novo* synthesized BST2. Conceivably, therefore, simultaneously targeting both sets of determinants would likely yield cumulative effects on BST2 counteraction, much like the combinatorial substitutions of E28/L33 and E59/L63 residues that almost completely inhibited BST2 antagonism. It would be important to experimentally test this hypothesis by investigating both the functional contribution and mechanism(s) of action of a Vpu mutant harboring E28/R30/K31/L33 mutations.

In addition to modulating virus release, the hinge region is important for other TMD-mediated Vpu interactions, consistent with a generic or common role in maintaining appropriate structural orientation of the Vpu TMD. Indeed, the conserved hinge region residues modulate interactions and, consequently, targeting of the membrane-associated CD4. As discussed in the introduction, regulating the ability of Vpu to target CD4 has several implications including attenuation of virion infectivity, through modulating both Env trafficking to the cell surface and Env or CD4-Env incorporation into virions, as well as in the activation of the immune response, through

activation of T cells [101, 150, 154-156, 159]. Furthermore, by affecting the conformation of the Vpu TMD, the hinge region residues would, by extension, also affect other TMD-dependent Vpu interactions including targeting of NTBA, PVR and SNAT1 [92, 102, 118]. Consequently, a poorly anchored Vpu TMD can allow for NTBA- and/or PVR-dependent activation of NK cells and, potentially lysis of infected cells [92, 118]. Likewise, an attenuation in targeting SNAT1 would allow for a SNAT-dependent supply of metabolites such as alanine that are necessary for activation of T cells, which in turn could activate the immune response and inhibit virus replication or spread [102, 121, 433]. The potential effect of the hinge region on these host factors warrants further investigation. Overall, our work indicates that the hinge region is important for enhancement of virus release. Whether this effect on BST2 antagonism will have any consequences in BST2-mediated NF-κB, TLR and ILT7 signalling events, which mediate production of IFN and ISGs, remains to be tested.

5.1.2 The hinge region as a potential target for Vpu inactivation using small compounds

All considered, the Vpu transmembrane-proximal hinge region determinant is consequential in HIV pathogenesis, and presents a previously unknown region that may be feasible for targeting with small compounds. Targeting HIV proteins is a very complicated undertaking, especially because the virus primarily usurps host machineries to facilitate infection and virus dissemination. In the case of Vpu, several conserved functionally important sites have been identified, including the DSGxxS and helix-2 di-leucine motifs that serve as β-TrCP and clathrin AP complex recognition

signals, respectively. These viral regions, however, cannot be targeted with small molecular compounds because the actions of such inhibitors would not be Vpu specific and, as such, will likely disrupt essential cellular processes mediated by the implicated host proteins. With this in mind, there is no evidence suggesting that the novel determinant identified in here (E28/L33), as well as the R30/K31 residues are targeted by cellular proteins. Furthermore, these residues occur within the cytosolic portion of Vpu, unlike the membrane-embedded TMD that is a tough challenge for inhibition with small molecules. In addition, this new determinant is highly conserved across Vpu variants from different HIV-1M clades, implying that any inhibitors targeting this region would be broadly effective against various pandemic strains. Additionally, the absolute requirement for the Glu residue in BST2 antagonism suggests that the likelihood for development of resistance to drugs targeting this region may be minimal, since acquiring resistance would inadvertently compromise function. In addition, and as alluded to earlier, such inhibitors could potentially be instrumental in preventing potential future pandemics such as those that can emanate from other non-M HIV-1 groups. Considering the association between BST2 and virus transmission and spread, it is concerning that Vpu proteins from such non-pandemic strains (HIV-1N) can evolve to acquire cytoplasmic motifs enabling for efficient BST2 antagonism in a manner comparable to the pandemic HIV-1 group M Vpu proteins [434]. Analyses of the Los Alamos HIV database reveal that Vpu variants from HIV-1N, which are weak BST2 antagonists, have an intact/conserved Glu (E28), whereas those from groups O and P which are unable to antagonize BST2, lack this BST2 antagonism determinant. It is interesting that in the absence of Vpu-mediated BST2 antagonism, group O viruses use

their Nef proteins to target BST2 and enhance virus release [195, 196]. In fact, Nef proteins from some group M isolates have recently been shown to be able to antagonize BST2, especially in conditions where Vpu function was defective [435]. This compensatory mechanism and the overall plasticity of the various HIV-1 groups in overcoming the antiviral activities of BST2 suggest that efforts to thwart the pandemic should consider targeting both accessory proteins.

To date, four small molecular compounds have been proposed to target Vpu. Results from Wilkinson and colleagues proposed that BIT225, which inhibits the ion channel formed by Vpu, blocks HIV-1 release only in MDMs and MDDCs [436, 437]. However, in addition to the cell type limitation, the compound is not effective against inhibiting Vpu-mediated BST2 counteraction [438]. Therefore, it can not promote the essential antiviral activity of BST2, including virion restriction and the associated immune modulatory roles. A related compound, SM111, belonging to the same acylguanidine class as BIT225 was recently reported to impair virus release and reduce virion infectivity *via* currently unknown mechanisms [439]. While acknowledging the low efficacy of the compound, the authors noted that SM111 also directly down-regulates BST2 even in bystander cells and selects for virus strains with mutations within the TMD of Vpu. Indeed, the authors further postulated that SM111 may function like BIT225 and, consequently, BST2 may not be critically important in the compound's antiviral activity. In addition to these two compounds, Cen and colleagues recently reported that two small compounds lapachol [440] and 2-thio-6-azauridine [441] inhibit Vpu-mediated BST2 degradation, without affecting either Vpu-BST2 or Vpu-β-TrCP interactions. Their precise mechanisms of action remain unknown. The effects of these compounds on

Vpu-mediated virus release are unclear, consistent with the fact that BST2 degradation *per se* is not required for enhancement of virus release [240]. As such, development of novel Vpu inhibitors remains a priority.

Testing the roles of the Vpu transmembrane-proximal hinge region (E28/R30/K31/L33) in relevant settings including in primary $CD4^+$ T cells and eventually in humanized mouse models would further validate targeting this region in efforts aimed at developing Vpu inhibitors. Elucidating the contributions of this region in other BST2-related functions, such as IFN production, as well as in interactions with other Vpu targets including NTBA, PVR and SNAT1 would be important in providing a holistic understanding of its potential impact *in vivo*. Along this line, there is a need for appropriate *in vivo* models that will allow for evaluation of both virus release and propagation, as well as the immune response following infection. Nonetheless, previous studies in mice infected with retroviruses such as HIV [107, 108] or MLV [197, 216] have already implicated BST2 in conferring infection control, and one study further reported a role of BST2 in inducing immune responses such as NK cell and virus-specific $CD8^+$ T cell responses [216].

It is intriguing that the functionally important helix-2 acidic di-leucine sorting motif is not conserved in VpuC and VpuF variants. In these variants, curiously, a putative acidic di-leucine signal actually occurs within the transmembrane-proximal hinge region of the protein. It remains to be tested whether this putative motif, which overlaps with the residues implicated in BST2 binding (in VpuB), is active or functional, as well as whether it also governs Vpu interactions. Indeed, it is currently unknown whether the precise position of the trafficking signal within the Vpu cytoplasmic tail is consequential.

Moreover, given the roles of the 51-DSGxxS-56 motif in AP recruitment, the ability of these variants to efficiently recruit AP proteins remains a curiosity. It is worth mentioning, however, that the VpuC or VpuF helix-2 residues corresponding to the VpuB acidic di-leucine motif have not been functionally tested. As such, it remains unknown if mutation of the critical Leu residue has any functional consequences. A comprehensive analysis of the interplay between the helix-2 and hinge region putative sorting signals in these variants would be of interest, and further validate whether the hinge region residues represent a tangible target for development of Vpu inhibitors.

5.2 Importance of BST2 and CD4 in controlling HIV infections

5.2.1 Contributions of CD4 and BST2 to ADCC mediated by nNAbs

The mechanistic studies above suggest that targeting Vpu may restore both the antiviral activities of BST2 and the roles of CD4 in dampening HIV replication. Along this line, recent studies have underscored the notion that BST2-mediated virion tethering serves a broader role, beyond just the simplistic prevention of release of cell-free virion particles. As discussed earlier, it is becoming clear that virion tethering is also a means through which BST2 triggers signalling cascades leading to the production of IFN and ISGs, including BST2 itself, which ultimately modulate the type of immune response to the infection. In here, we reveal a novel role of BST2 in promoting lysis of infected cells *via* ADCC, as well as mechanisms through which HIV evades such killing. ADCC has re-emerged as a powerful means allowing for both prevention of HIV-1 acquisition and

post-infection viral control, especially following its association with significant viral protection in the RV144 Thai vaccine trial [334, 361]. Analyses of the CD4i nNAbs isolated from vaccinees from the trial suggested a role of CD4-dependent ADCC in the protection. In accordance, our work provides experimental evidence emphasizing an important role of CD4 in sensitizing infected cells to ADCC. Accumulation of CD4 on infected cells unmasked epitopes on Env that are recognized by these CD4i nNAbs, hence augment ADCC activity.

5.2.1.1 Vpu and Nef proteins protect infected cells from nNAb-mediated ADCC

However, despite effective mechanisms such as ADCC, HIV continues to spread at alarming rates and, in infected individuals, progresses to a disease state quicker in the absence of ART. This is in line with the notion that HIV has evolved to evade such immune responses, much like it has with the above-mentioned BST2-dependent antiviral events. Indeed, our study demonstrates that cells infected with WT HIV-1 are protected from ADCC mediated by CD4-induced, nNAbs [389]. Significantly, we eloquently reveal that HIV uses its two accessory proteins Vpu and Nef to shield infected cells from such lysis. Consistent with their overlapping roles in CD4 counteraction, sensitizing cells to ADCC is synergistically enhanced in the absence of both viral proteins. Conceivably, the Nef and Vpu protective effect occurs through reducing the amounts of CD4 available to interact with Env. While Nef targets cell surface CD4, Vpu degrades intracellular CD4 leading to less CD4 reaching the surface. Importantly as well, absence of Vpu allows for high levels of CD4 and Env along the anterograde trafficking pathway and, given their high affinity for each other, Env-CD4

complexes will traffic to the surface and be incorporated into the budding virion. At the cell surface, these Env-CD4 complexes will unveil otherwise hidden functional sites including coR-bs as well as other epitopes on Env that can be targeted by ADCC competent CD4i Abs. Indeed, ADCC-competent nNAbs recognizing CD4i epitopes that either overlap with the coR-bs (17b) or those overlapping with the inner domains of gp120 spanning the C1 and V1/V2 regions (A32) yielded similar results. Of particular note, and in accordance with the relevance of ADCC activity at the virus budding stage, our studies further show for the first time that in addition to CD4, virion tethering by BST2 also sensitizes infected cells to CD4i nNAb-mediated ADCC. In this context, the dependency on BST2 is less pronounced than that of CD4, and is fully appreciated in the absence of Env-CD4 interactions. The limited dependency on BST2 is most likely due to the fact that epitope exposure is still largely dependent on the Env-CD4 interactions. Nonetheless, this shows yet again, the importance of virion tethering at the surface, beyond just affecting the quantities of viral particles release into circulation.

5.2.1.2 Inactivating activities of Vpu and Nef proteins as a strategy to enhance nNAb-mediated ADCC

Overall, the roles of CD4 and BST2 towards enhancing susceptibility of WT virus-infected cells to CD4i nNAb-mediated ADCC reveal a potential interventional approach based on targeting the activities of Nef and Vpu. Along this line, small molecular compounds blocking the downregulation and degradation of CD4 by Nef and Vpu, as well as blocking the antagonistic activity of Vpu on BST2 would be of great utility in sensitizing infected cells to lysis. In this context, targeting the Vpu hinge region residues

as discussed above could be an important approach as it attenuates both CD4 and BST2 targeting activities of the protein. However, the potential role of the Vpu hinge region residues (E28/R30/K31/L33) is yet to be directly validated in the context of ADCC. While effective CD4-targeting Nef antagonists are currently not available, a lead compound called B9 (diphenylpyrazolo), which reportedly abrogates Nef dimerization and CD4 downregulation, appears promising [442]. Another interesting avenue involves the use of small molecular CD4-mimetic compounds to trigger exposure of the otherwise hidden ADCC epitopes. Intriguingly, by occupying CD4 binding sites on Env, thereby inducing the conformational arrangements necessary for nNAb engagement, such compounds would in fact not only facilitate ADCC activity, but will also allow the nNAbs to neutralize virions [421, 443]. However, while these CD4-mimetics appear attractive, it remains unknown how they would affect endogenous CD4 functions in both infected and non-infected cells. It is indeed conceivable that, by triggering conformational changes within Env, they may actually facilitate coreceptor engagement hence promote virus entry.

5.2.1.3 Potential contributions of nNAbs towards controlling HIV infections

Moving forward, a case can be made for considering nNAbs in vaccine strategies given that they are among the first Abs to be produced following infection and are effective in their ADCC activity, ADCC results that we have corroborated in our studies. However, they are not effective against the WT virus, unless supplemented with Vpu/Nef antagonists and/or CD4-mimetic small molecules, as discussed above. It would be interesting to test whether the presence of Vpu and Nef, in addition to epitope

escape, contributes to the lack of nNAb-mediated protection observed in some studies in monkey models [444, 445]. Nonetheless, there is tangible evidence supporting a role of nNAbs in post-infection viral control, and this could be strengthened by inhibiting these accessory proteins [335, 338, 339, 343, 345, 446]. Additionally, the short-lived nature of vaccine-induced nNAbs and the protective immune responses they mediate, as was seen during the RV144 trial, would need to be carefully considered in attempts to develop an effective, durable vaccine [361]. Importantly, while there is evidence supporting a role of nNAb-mediated ADCC in controlling viraemia, there exists evidence for contributions by other mechanisms including ADCP, ADCVI and transcytosis inhibition that have not been tested in our studies [338, 345, 371, 445, 447, 448].

5.2.2 bNAbs mediate lysis of infected cells via ADCC

It is interesting that while some ADCC activities mediated by plasmas from HIV-infected persons were A32-like, others were highly dependent on Vpu, implying a role of CD4-independent Abs. This led us to investigate whether NAbs or bNAbs are ADCC competent. bNAbs are of special interest because of their breadth of action, hence relevance in controlling HIV infection *in vivo*. Importantly, we uncovered a previously unappreciated role of bNAbs in that they are capable of mediating ADCC. Moreover, we revealed that their actions are heavily reliant on BST2-mediated virion tethering at the cell surface. As such, and once again, the BST2 antagonistic activity of Vpu has significant consequences in bNAb-mediated cell lysis. Of particular note, and unlike CD4i nNAb-mediated ADCC, cells infected with the WT virus were susceptible to ADCC, a finding that is intriguing given that it is the WT virus that is responsible for the

pandemic. This novel function of bNAbs we identified herein may explain recent observations implicating the role of the FcγR in elimination of infected $CD4^+$ T cells in humanized mouse models [411]. Of significance, sensitivity to ADCC was not only limited to viruses isolated during the chronic stage, as our data also indicated that bNAbs are capable of inducing ADCC in T/F viruses, albeit to various extents. This suggests that administration of bNAbs upon the earliest confirmation of infection may be an effective strategy in controlling HIV infection. In fact, such a strategy may also reduce the potential development of bNAb-resistant quasi-species. It is tempting to consider that the ability of bNAbs to mediate killing of both T/F and chronic virus-infected cells could compensate for the attenuated viral neutralization potential observed in Abs present in clade-matched infected sera from newly infected (pre-seroconversion) compared to post sero-conversion [449].

5.2.2.1 Enhancing BST2-mediated virion tethering augments susceptibility of infected cells to ADCC mediated by bNAbs

Importantly, despite the presence of Vpu, increasing BST2 expression via exogenous IFN enhanced the BST2 virion-tethering function and, consequently, significantly heightened ADCC activity. Therefore, and consistently, strategies aimed at preserving or restoring the action of BST2 in crosslinking virus particles at the surface are likely to be instrumental in post-infection HIV control. While we use IFN in our study, IFN is risky as a therapeutic approach because of its contradictory roles in HIV pathogenesis. Whereas IFN is beneficial during the acute infection phase, partly through induction of antiviral ISGs, it also causes immune activation resulting in an

increase in infection-susceptible (permissive) target $CD4^+$ T cells [41]. Moreover, continuous treatment with IFN into the chronic stages of infection dysregulate IFN signalling resulting in higher viral loads and accelerated decline in $CD4^+$ T cell counts [450, 451]. Additionally, IFN increases overall BST2 expression in both infected and uninfected cells. This could have unintended effects as the *in vivo* roles of BST2 are still ill-defined, especially in cancer. To illustrate, BST2 is elevated in various cancers, correlates with disease pathology and may reduce responsiveness to pro-apoptic drug (reviewed in [452]). Along this line, anti-BST2 Abs have actually been used in ADCC approaches aimed at eliminating cancerous cells (reviewed in [452]). All these argue that focusing on enhancing BST2 antiviral activity using Vpu antagonists maybe a safer approach. Significantly, this would direct and restrict ADCC activity, as well as the other antiviral activities dependent on virion tethering, to infected cells.

5.2.2.2 Potential roles of bNAbs in pre-exposure prophylactic and post-infection control approaches

The ADCC mediating action of bNAbs that we have uncovered in here is especially interesting considering that it generally correlated with the extent of neutralization mediated by these bNAbs, a finding that was also reported by Bruel and colleagues [416]. As such, bNAbs are uniquely positioned to potently protect people from HIV acquisition, *via* virion neutralization and ADCC, as well as mediate post-infection control of HIV through effector functions such as ADCC. They therefore represent a potentially revolutionary resource through which the pandemic can be halted. Indeed, both the neutralization and ADCC functions of bNAbs may account for

the suppression of viraemia observed in both humanized mouse [453, 454] and monkey models [309, 317], as well as in humans [310, 455]. A drawback in this approach, however, is that less than a third of infected individuals actually evolve to generate such bNAbs [301, 305-307]. Even then, bNAbs generally take years to develop, and by the time they do, the virus would have already disseminated, advanced the infection, and generated a broad base of quasi-species exhibiting diverse Env that may affect the effectiveness or potency of the bNAbs. Along this line, a recent study reported a correlation between HIV-1 diversification and neutralization resistance [449]. It is for this reason that great emphasis should focus on inducing an early generation or passive immunization with such Abs. Of significance, one seminal study reported that bNAbs may influence the immune response to accelerate emergence of more bNAbs that can protect against neutralization-resistant strains [311]. This requires further exploration in order to elucidate both the mechanism through which this may be happening, as well as whether it is a general feature of bNAbs and not restricted to the 3BNC117 tested in the study. Altogether, our work and the studies referenced above suggest that bNAbs can be used in both pre-exposure prophylactic and post-infection control approaches. It is very encouraging that bNAbs VRC01 and 3BNC117, both targeting the CD4-bs, have already fared well in terms of clinical safety in phase I dose-escalation studies [310, 456].

An emerging picture argues that enhancing Ab-dependent functions represents a robust approach to developing vaccines that would prevent HIV acquisition and inhibit the virus post-infection. In addition to the possibility of enhancing bNAb-mediated ADCC using Vpu antagonistic small molecules, our findings indicating that all classes of bNAbs

can induce ADCC suggest that bNAbs can actually be combined to achieve an even greater viral strain breadth and potency. Targeting distinct epitopes on Env does not only augment killing of infected cells, but also further improves the neutralization capabilities of the Abs, as recently demonstrated [309, 416, 453, 454, 457]. To illustrate, a single injection of four bNAbs conferred protection to monkey models against 23 weekly SHIV challenges [458]. Additionally, much like ART, combining bNAbs would also effectively reduce HIV-1 escape from Ab monotherapy as has been observed with some bNAbs [309, 394, 454, 459]. Recent developments on this front have led to the generation of bispecific bNAbs (bi-bNAbs) targeting distinct regions of Env. These bi-bNAbs yield markedly broad and potent synergistic viral neutralization in vitro, as well as improved reduction of viral loads in infected humanized mouse models [460]. It remains to be tested if ADCC activity mediated by these bi-bNAbs would also result in a synergistic effect of the two bNAbs.

5.2.2.3 bNAbs mediate elimination of reactivated latent HIV-1 infected cells via ADCC

A major hurdle in the complete elimination of infected cells is the presence of HIV-1 latent reservoirs. ART intensification strategies and latency reversal have failed to reduce the reservoir size, necessitating novel interventions [461]. It was recently shown that bNAbs not only effectively neutralize viruses isolated from latent reservoirs of infected patients that are receiving ART [462], but also delay viral rebound in infected patients following ART interruption [463]. In here, we find that reactivated cells are susceptible to ADCC, but their bNAb-mediated killing is inefficient. In aggrement with our findings, a recent study reported that reactivated cells were indeed a challenge to

be purged *via* ADCC given the heterogeneity and low levels of Env at the cell surface [416]. Most importantly, however, their killing can be significantly potentiated by enhancing BST2-mediated virion trapping, which then allows for efficient engagement of bNAbs. As was the case with productively infected cells, HIV uses its accessory proteins to shield the reactivated cells from ADCC. As such, we put forth that therapies targeting the BST2 antagonistic activity of Vpu as well as passive transfer of bNAbs could have favourable consequences in reducing the latent reservoir pool, in the presence of ART. In a global context, aside from enhancing susceptibility of the reactivated cells to ADCC, the increase in the amount of viral proteins expressed at the surface as a result of inhibiting Vpu function could potentially trigger CTL responses, availing yet another mechanism through which the reservoir can be depleted. In as far as bNAbs and latency studies *in vivo* are concerned, results from a phase IIa clinical trial showed that 3BNC117 efficiently suppresses viral rebound in chronically infected humans following treatment interruption [463]. However, resistant viruses did emerge, and it remains to be tested whether combining bNAbs or using bi-bNAbs would reduce the pool of resistant strains in this context. Earlier studies in humanized mouse models suggested that combining bNAbs could indeed be a plausible strategy [410]. Interestingly, these studies further demonstrated that bNAb-mediated suppression of viral rebound required engagement of the FcR, suggesting effector cell functions are important for establishment of the latent reservoir [410, 453]. Whether ADCC is directly involved at this stage remains unknown.

5.2.2.4 bNAbs usher optimism towards HIV-1 cure and vaccine development

Overall, the multi-functionality of bNAbs positions them as a viable strategy towards controlling HIV infections. Their ability to potently target a broad spectrum of virus strains, as well as the feasibility of fusing multiple bNAbs into a single molecule (generation of bi-bNAbs) has rightfully made them the 'holy grail' in vaccine development efforts. The newly identified role in ADCC that we uncovered in our studies makes them both efficient virus 'neutralizers' as well as potent 'killers' of cells already infected, underscoring the notion that they can be used in both prophylactic and therapeutic efforts. Added to this, they can mediate lysis of reactivated latently-infected cells that have been a major source of frustration in efforts towards curing HIV. The contributions of bNAbs towards other functionally important mechanisms such as ADCP and transcytosis inhibition are yet to be directly investigated [338, 345, 371, 445, 447, 448]. Indeed, a holistic evaluation of the roles of not only bNAbs but also nNAbs may offer essential insights into further modifications or means through which we can enhance their overall potency. Undeniably, we currently do not know if BST2 and CD4 play any significant roles in non-ADCC cell lysis mechanisms, and to what extent Vpu and Nef influence their function. Moreover, Vpu and Nef both target molecules that are essential for NK cell activation and, ultimately, NK cell-mediated cytotoxic functions. Indeed, NTB-A and the binding partners for PVR and NKG2D-L, DNAM-1 and NKG2D, respectively, have already been shown to modulate NK cell-mediated lysis of infected cells in a manner dependent on the accessory proteins [92, 118, 464-466]. Therefore, Nef and Vpu likely play even more roles in protecting infected cells from lysis *in vivo*.

Moving forward, we eagerly await results from several ongoing and proposed clinical trials evaluating roles of bNAbs in the contexts of LRAs, ART, and in combination with other bNAbs in both infected and uninfected individuals. Thus far, the bNAbs' safety profiles, capability to suppress viral rebound following ART interruption, as well as the ADCC competencies we and others have uncovered, all usher continued optimism towards eventually developing an effective HIV vaccine. Along this, significant efforts should also focus on development of small molecules to inhibit Vpu and Nef so as to augment the ADCC potential of bNAbs. In fact, inhibiting these accessory proteins would also enhance the potency of nNAbs, which can then be integrated as part of the strategy against this formidable challenge. In addition, the ability of infused Abs to penetrate into anatomical sites where sufficient amounts of ART cannot reach further makes them an enticing interventional approach towards targeting such 'safe havens' for the virus ([467] and reviewed in [461]). Regrettably, however, they are still poor at distributing into the brain area, which necessitates additional strategies to prevent or control infections in such compartments (reviewed in [461]) .

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