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Pandemic HIV-1 Vpu overcomes intrinsic herd immunity mediated by tetherin

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Among the four groups of HIV-1 (M, N, O, and P), HIV-1M alone is pandemic and has rapidly expanded across the world. However, why HIV-1M has caused a devastating pandemic while the other groups remain contained is unclear. Interestingly, only HIV-1M Vpu, a viral protein, can robustly counteract human tetherin, which tethers budding virions. Therefore, we hypothesize that this property of HIV-1M Vpu facilitates human-to-human viral transmission. Adopting a multilayered experimental-mathematical approach, we demonstrate that HIV-1M Vpu confers a 2.38-fold increase in the prevalence of HIV-1 transmission. When Vpu activity is lost, protected human populations emerge (i.e., intrinsic herd immunity develops) through the anti-viral effect of tetherin. We also reveal that all Vpus of transmitted/founder HIV-1M viruses maintain anti-tetherin activity. These findings indicate that tetherin plays the role of a host restriction factor, providing 'intrinsic herd immunity', whereas Vpu has evolved in HIV-1M as a tetherin antagonist.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), emerged around 100 years ago[1] through zoonotic transmission of simian immunodeficiency viruses (SIVs) in chimpanzees (SIVcpz)[2,3] and gorillas (SIVgor)[4]. According to molecular phylogenetic analyses, zoonotic transmission has occurred at least 4 times, leading to diversification of the virus into 4 different groups; namely, HIV-1 M ("major" or "main"), N ("new" or "non-M-non-O"), O ("outlier"), and P[5]. Among these 4 HIV-1 groups, HIV-1M alone is pandemic and currently infects more than 30 million people worldwide. However, why HIV-1M rapidly created a worldwide pandemic while the other groups remained endemic is unclear.

In 2009, Neil *et al.*[6] and Van Damme *et al.*[7] identified a cellular anti-HIV-1 protein called tetherin (also known as bone marrow stromal antigen 2 [BST2], CD317, and HM1.24). Tetherin impairs viral release by tethering bud virions to the surfaces of HIV-1-producing cells. Conversely, tetherin is antagonized by viral protein U (Vpu), an accessory protein encoded by HIV-1[6,7]. In addition, Vpu potently induces the degradation of CD4 molecules in infected cells[8]. Interestingly, HIV-1M Vpu alone possesses both of these

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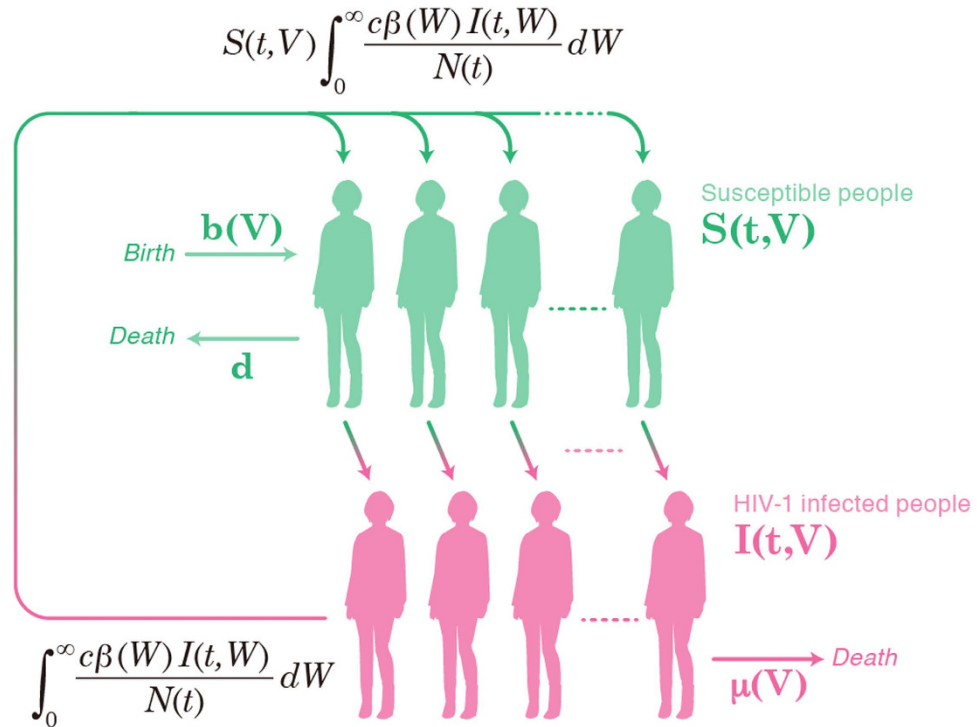
Figure 1. Construct of the mathematical model. The variables $S(t, V)$ and $I(t, V)$ denote the number of susceptible and infected individuals, respectively, and $N(t)$ is the total number of host individuals. All variables are specified at time t. The parameters $b(V)$ and d denote the birth and removal rates of susceptible individuals respectively, and $\mu(V)$ is the death rate of infected individuals. The force of infection is assumed as $\int_0^\infty c\beta(W)I(t, W)/N(t)dW$, where c and $\beta(V)$ denote the partner exchange rate and the transmission probability, respectively. Therefore, the rate of change in the number of susceptible and infected individuals at time t (i.e., the *de novo* transmission) is $S(t, V)\int_0^\infty c\beta(W)I(t, W)/N(t)dW$. Figure 1 was drawn by KS using Illustrator CS5.1 (Adobe).

abilities, while the Vpus of the other HIV-1 groups (i.e., N, O, and P) possess neither or only one ability[9–11]. Regarding anti-tetherin activity, HIV-1N Vpu is a much weaker tetherin antagonist than HIV-1M Vpu[11,12], and the Vpus of HIV-1O and HIV-1P exert no adverse effect on tetherin[10,11]. From these observations, Kirchhoff proposed that the ability of Vpu to counteract tetherin has promoted human-to-human viral transmission[13]. However, because human-to-human spread cannot be experimentally replicated, this hypothesis remains speculative. Therefore, we conducted an experimental-mathematical investigation, and concluded that human tetherin is a potent inducer of intrinsic herd immunity in humans and that HIV-1M Vpu has acquired the ability to overcome this hurdle.

Methods

A structured epidemiological model based on the distribution of set-point viral loads. To investigate the relationship between the distributed set-point viral loads in HIV-1 infected patients and human-to-human viral transmission, we developed a novel mathematical model that accounts for host heterogeneity (i.e., the different set-point viral loads). The novel mathematical model is given by (Fig. 1):

$$\frac{\partial S(t, V)}{\partial t} = b(V) - dS(t, V) - S(t, V)\int_0^\infty \frac{c\beta(W)I(t, W)}{N(t)}dW, \tag{1}$$

$$\frac{\partial I(t, V)}{\partial t} = S(t, V)\int_0^\infty \frac{c\beta(W)I(t, W)}{N(t)}dW - \mu(V)I(t, V), \tag{2}$$

where $S(t, V)$ represents the number of susceptible individuals who will acquire the set-point viral load V if infected by HIV-1 in the future, and $I(t, V)$ is the number of infected individuals with the set-point viral load V at time t. $N(t)$ is the total host population size (i.e., $N(t) = \int_0^\infty S(t, V)dV + \int_0^\infty I(t, V)dV$). Here we assume that the set-point viral load V of each infected individual is determined by host properties such as the genetic background and immune state[14–17]. Host populations are born at rate $b(V)$ and

removed at rate d. Because most of the HIV infection occurs during the asymptomatic period and the transmission probability per partnership is correlated with the patients' set-point viral load V, we model this relationship by an increasing Hill function as described elsewhere[18,19]; That is we write $\beta(V) = \beta_{max} V^{k_\beta}/(V^{k_\beta} + V_{\beta 50}{}^{k_\beta})$ where β_{max} is the maximum transmission probability and $V_{\beta 50}$ is the viral load at which the probability is half its maximum. The parameter k_β is the steepness of the transmission probability versus the set-point viral load. Therefore, the force of infection at time t is given by $\int_0^\infty c\beta(V)I(t,V)/N(t)\,dV$ where c is the contact rate. Finally, we assume that the asymptomatic period endures as a decreasing Hill function of the viral load as described elsewhere[18,19]; that is, $1/\mu(V) = D_{max} D_{50}{}^{k_D}/(V^{k_D} + V_{D50}{}^{k_D})$ where D_{max} is the maximum duration of the asymptomatic period in years, V_{D50} is the viral load at which the duration is half its maximum, and k_D is the steepness of the (decreasing) duration versus the set-point viral load. The initial condition is $S(0,V) = b(V)/d$ and $N(0) = \int_0^\infty S(0,V)\,dV$. Similar mathematical models have been proposed in previous studies[18–20].

Cell culture, infection, transfection, Western blotting, TZM-bl assay, flow cytometry.   293T cells, HeLa cells and TZM-bl cells (obtained through NIH AIDS Research and Reference Reagent Program) were maintained in DMEM (Sigma) medium containing FCS and antibiotics. Human PBMCs were maintained in RPMI1640 (Sigma) medium containing FCS and antibiotics. For *in vitro* HIV-1 infection assay (Figure S1), PHA-activated human PBMCs were infected with WT or vpu-deficient HIV-1 (strain NL4-3) at multiplicity of infection 0.1 (i.e., 10,000 TCID50 [50% tissue culture infectious dose] of virus solution was inoculated into 100,000 PHA-activated human PBMCs). The expression plasmids of HA-tagged Vpus of T/F viruses were obtained from GeneArt Gene Synthesis service (Life Technologies). Transfection was performed using Lipofectamine 2000 (Life Technologies). Western blotting, TZM-bl assay, and flow cytometry were performed as previously described[21–23].

Results

HIV-1M Vpu increases the set-point viral load of HIV-1-infected humans.   To examine whether or not the anti-tetherin ability of Vpu is linked to effective human-to-human HIV-1 transmission[13], we performed a virus replication assay using primary human CD4$^+$ T lymphocytes. The experimental data were analyzed by a previously proposed mathematical model[24,25], which verified that Vpu increases the virus production rate (p) (see **Text S1**). We also analyzed 15 datasets provided in previous studies[7,26–31] and found that the average production rate of wild-type HIV-1 was 1.54-fold higher than that of *vpu*-deficient HIV-1. The estimated parameter values are listed in Table S1. Because the virus production rate is known to affect the set-point viral load (V) in infected individuals[32,33], we expected that a typical set-point of 1.0×10^5 copies/ml (for example) would decrease to 0.4×10^5 copies/ml in HIV-1M lacking *vpu* (see **Text S2**). These findings strongly suggest an important connection between Vpu and the within-host dynamics of HIV-1.

Epidemiological impact of set-point viral loads.   Because HIV-1-infected patients with higher viral loads are more infectious[34,35] and have reduced lifespans[34,36,37], the efficacy of human-to-human HIV-1 transmission is determined by the patients' set-point viral load. In addition, the distribution of the set-point plays a critical role in viral spread[35]. We describe the human-to-human spread of HIV-1M by the structured epidemiological model described in **Methods** (Fig. 1). This model includes the transmission rate ($\beta(V)$) and the death rate of the patient ($\mu(V)$) as functions of viral load[34]. Because patients' set-point values are highly variable[34,35], by adopting the set-point distribution approach in our mathematical model we can quantitatively detail the spread of HIV-1. Here we used the distribution of set-point viral loads in 311 untreated heterosexual HIV-1M infected patients in the Zambian transmission study[35] and calculated the basic reproduction number (R_0^W), the expected number of people infected throughout their infectious lifespan[38,39] (see **Text S3**). The estimated value of R_0^W (4.67; see **Text S4**) is consistent with previous estimates[39,40].

Tetherin-mediated intrinsic herd immunity and its counteraction by Vpu.   To investigate the impact of Vpu on the human-to-human spread of HIV-1M, we assumed that HIV-1 has lost its Vpu function (i.e., we consider *vpu*-deficient HIV-1) and compared several quantities concerning the epidemiological contribution of Vpu. As explained above, viruses lacking Vpu reproduce more slowly and yield a lower set-point viral load in patients (see **Text S2**). Interestingly, *vpu*-deficient HIV-1 did not establish infection in some patients with lower set-point viral loads ($< 3.19 \times 10^4$ RNA copies/ml) (see **Text S5**). In other words, the anti-viral effect of tetherin confers potential protection against HIV-1 infection among some human populations[41]. Because the distribution of viral loads in the Zambian transmission study is well described by a weighted skew-normal distribution of the logarithm of the viral load (see **Text S4**), we can estimate that 20% of human populations are protected from *vpu*-deficient HIV-1 by tetherin (Fig. 2A) and are prevented from transmitting HIV-1 (this situation is known as herd immunity). In addition, several observed distributions of the set-point are negatively skewed[34,35], suggesting that tetherin-mediated herd immunity plays a critical role in the human-to-human spread of HIV-1M.

In *vpu*-deficient HIV-1, the calculated R_0^M reduced to 3.90, suggesting that human-to-human transmission is 1.17-fold more efficient in wild-type HIV-1 than in *vpu*-deficient HIV-1. However, comparing
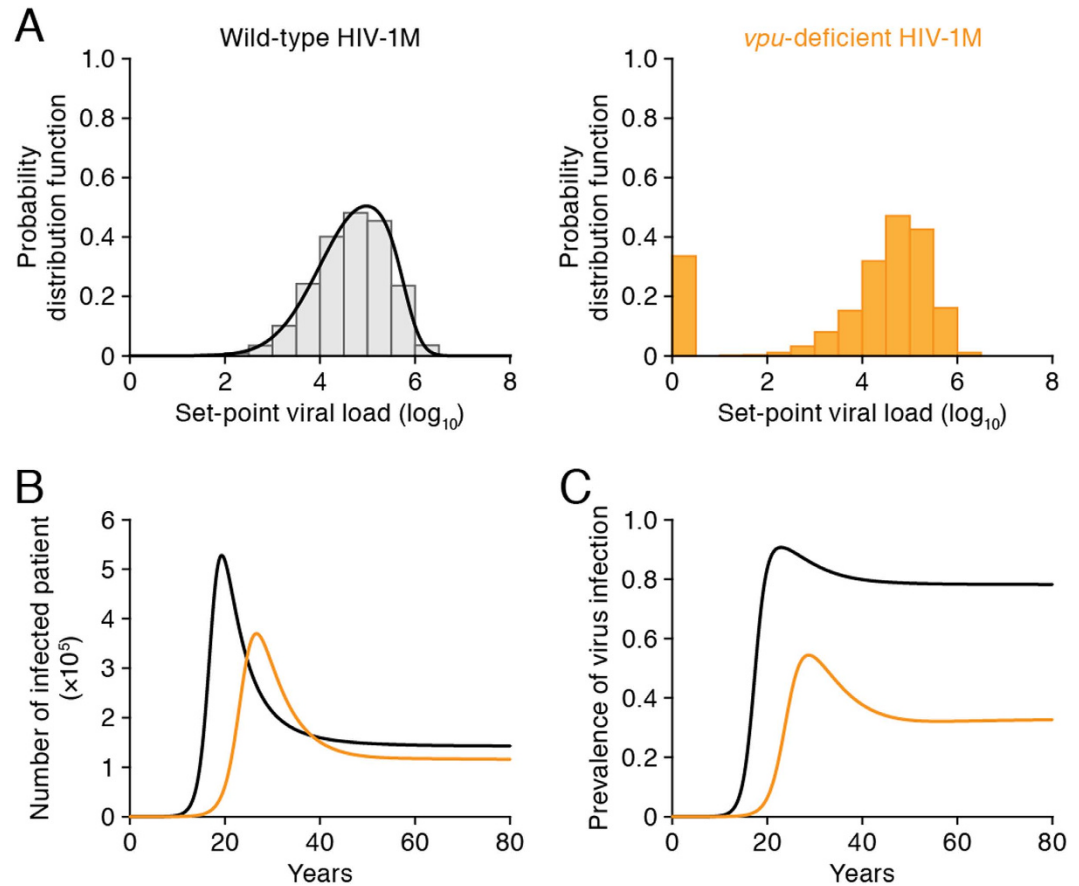
Figure 2. Prediction of tetherin-mediated intrinsic herd immunity. (A) (left) The distribution of set-point viral load in untreated individuals in the Zambian Transmission Study (black bars). The black curve is the distribution predicted by the structured epidemiological model. (right) The predicted distribution of set-point viral load in untreated individuals in the Zambian Transmission Study, assuming non-functional Vpu (orange bars). The bar at "0" shows the fraction of populations protected from *vpu*-deficient HIV-1 infection. **(B)** Simulated spread of wild-type and *vpu*-deficient HIV-1 among a population of 1 million individuals. The black and orange curves describe the number of individuals infected by wild-type and mutant virus, respectively. **(C)** Prevalence of wild-type (black curve) and *vpu*-deficient (orange curve) HIV-1 infection in (B).

the basic reproduction numbers may be of limited applicability when infected individuals are rare in a population. To further assess the impact of Vpu on the spread of HIV-1M, we simulated wild-type and *vpu*-deficient HIV-1 transmission among 1 million individuals (Fig. 2B). The parameter values used in these simulations are summarized in Table S2. Our simulations show that Vpu shortens the time between initial and peak HIV-1 infection and increases the steady-state number of infected individuals (Fig. 2B). Interestingly, the prevalence (i.e., the number of infected individuals divided by the number of total individuals) of wild-type and *vpu*-deficient HIV-1 infection were calculated as 78.2% and 32.8%, respectively (Fig. 2C). Thus, the Vpu of HIV-1M increases the viral prevalence by 2.38-fold, indicating that Vpu effectively overcomes tetherin mediated herd immunity.

Conserved anti-tetherin ability of Vpu in transmitted/founder (T/F) viruses. Reportedly, the antagonistic effect of Vpu against tetherin is conferred by 3 amino acids in the transmembrane domain of Vpu ([14]AxxxAxxxW[22] in strain NL4-3). In addition, 2 amino acids in the cytoplasmic domain of Vpu ([52]SxxxS[56] in strain NL4-3) are essential for down-regulating CD4[42]. To further validate the importance of Vpu on human-to-human viral transmission, we analyzed the *vpu* sequences of transmitted/founder (T/F) viruses, the viruses that are transferred and efficiently established in new individuals[43,44], and recovered from patients at each Fiebig stage (III–V). As shown in Fig. 3A, both of the motifs important for CD4 down-regulation and tetherin antagonism are highly conserved, suggesting that the ability of Vpu to down-regulate tetherin and CD4 is important for establishing HIV-1 infection in new patients.

To demonstrate the link between Vpu activity and establishment of HIV-1 infection, we constructed expression plasmids for the Vpus of T/F viruses and evaluated their ability to infect cell lines. Intriguingly, most of the transfected Vpus down-regulated tetherin but did not efficiently down-regulate CD4
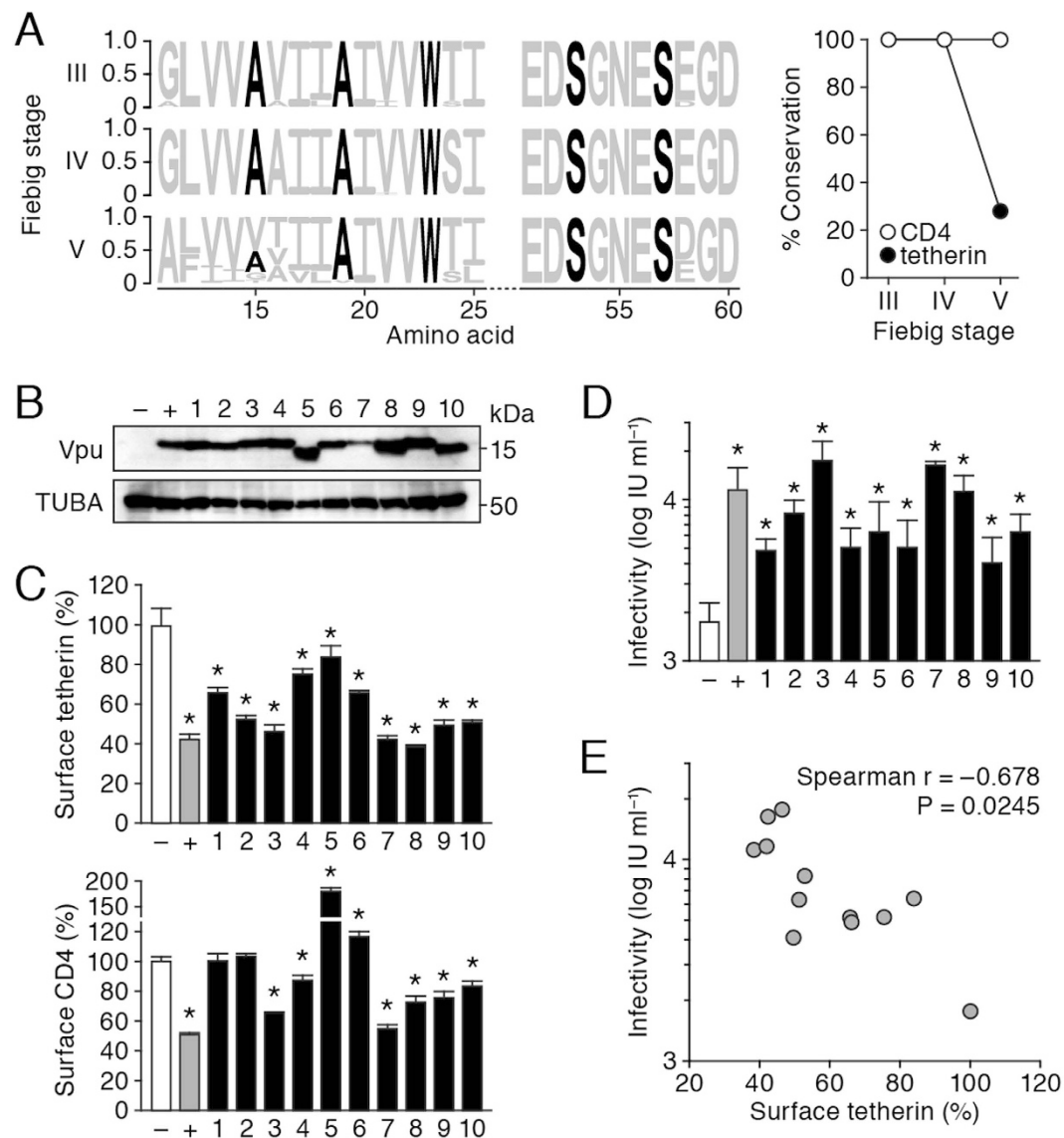
Figure 3. Conservation of Vpu motifs and the ability of T/F virus Vpus. (A) Conservation of the motifs in HIV-1M Vpus that counteract tetherin and down-regulate CD4 at each stage of viral infection. (left) Logo plot of the Vpu amino acid sequences at Fiebig stages III (n = 183), IV (n = 29), and V (n = 119). The crucial Vpu motifs for tetherin counteraction (AxxxAxxxA motif) and CD4 down-regulation (SxxxS motif) are indicated in black. (right) The percentage conservation of these motifs at each Fiebig stage. **(B–E)** Infection ability of the Vpus T/F viruses. The HA-tagged Vpu-expressing plasmids were co-transfected with pNL4-3$\Delta vpu\Delta nef$ (B, D, E) or without pNL4-3$\Delta vpu\Delta nef$ (C) into HeLa cells (B, D, E) or TZM-bl cells (C). Labels denote: –, empty vector; +, NL4-3 Vpu; 1-10, T/F virus Vpus: 1, THRO_TF1 (Genbank accession no. JN944930); 2, SUMA_TF1, (JN944928); 3, RHPA_TF1 (JN944917); 4, TRJO_TF1 (JN944936); 5, CH040_TF1 (JN944905); 6, REJO_TF1 (JN944911); 7, CH077_TF1 (JN944909); 8, CH058_TF1 (JN944907); 9, WITO_TF1 (JN944938); 10, CH106_TF1 (JN944897). **(B)** Representative result of Western blotting (kDa = kilodalton). **(C)** Surface expression levels of tetherin (top) and CD4 (bottom) on cells transfected with Vpu-expressing plasmids. **(D)** The infectivity of released virus was determined by TZM-bl assay. **(E)** The correlation between the level of surface tetherin (shown in panel c, top) and virus infectivity (shown in panel d). The data are expressed as averages and the standard deviations of triplicate assays. In Panels C and D, statistical significance was determined by Student's t test ($P < 0.05$). In Panel E, the correlation between the level of surface tetherin (x-axis) and virus infectivity (y-axis) was determined by Spearman's rank test.

(Fig. 3B–D). Furthermore, the surface expression level of tetherin was significantly negatively correlated with the level of virus release (Fig. 3E; r = −0.678, $P = 0.0245$ by Spearman rank correlation coefficient), suggesting that the ability of Vpu to antagonize tetherin is well correlated with efficient virus release.

Collectively, these findings suggest that the potential of Vpu to antagonize tetherin is more important than its anti-CD4 activity, and is well conserved in T/F viruses.

Discussion

Tetherin suppresses viral release in single-round replication assays in cultured cells such as HeLa cells, and is a recognized inhibitory factor of HIV-1 replication[6,7]. However, the anti-viral effect of tetherin seems enigmatic because *vpu*-deficient HIV-1, which cannot counteract tetherin, can replicate in cultures of tetherin-expressing cells such as Jurkat cells and human primary peripheral mononuclear cells (PBMCs). Thus, whether tetherin exerts a lesser effect on HIV-1 replication *in vivo* is a pertinent question. In fact, previous studies by our group and others have demonstrated that *vpu*-deficient HIV-1 efficiently replicates in humanized mouse models[45,46], indicating that Vpu is dispensable for viral propagation *in vivo*. However, Vpu significantly enhances the efficiency of HIV-1 infection in a humanized mouse model[46]. Especially interesting is that only the Vpus of the pandemic virus HIV-1M counteract both tetherin and CD4. The Vpus of the other endemic HIV-1 groups (i.e., N, O, P) antagonize at most one of these protective agents[9–11]. From these observations, we hypothesize that the ability of Vpu to antagonize both tetherin and CD4 increases the infective efficacy of HIV-1M, enabling extensive human-to-human viral transmission. Furthermore, there had been only a dozen of HIV-1N-infected individuals in Cameroon[47–49], and the antagonistic activity of HIV-1N against tetherin has been shown to be intrinsically weak[11]. Sauter *et al.* revealed that the Vpu of N1Fr2011[50], a recently designated HIV-1N strain, has acquired anti-tetherin activity after the virus was introduced to Europe from Cameroon[12]. These findings further support a close association between anti-tetherin ability of Vpu and viral epidemics. However, because the efficacy of human-to-human virus transmission cannot be evaluated from experimental studies alone, we supplemented our experimental techniques with a mathematical investigation. Comparing the infectivity of wild-type and *vpu*-deficient HIV-1, we demonstrated that active Vpu confers a 2.38-fold advantage in prevalence of infection (Fig. 2C). This suggests that tetherin plays a "herd immunity" role against HIV-1 in human populations, and that HIV-1M has retaliated by expressing Vpu. Indeed, tetherin impairs the release of a broad spectrum of enveloped viruses[51]. Therefore, the tetherin-mediated herd immunity in human populations may affect the spread of diverse viruses.

Several molecular clones of non-*vpu* encoding HIV-1M strains have been isolated from chronically infected patients. In these strains, designated HXB2[18], BH8[52], MAL[53], and Zr6[54], the initiation triplet AUG of *vpu* has been deleted[18,52–54] or a frameshift mutation has occurred in the *vpu* ORF[55]. Conversely, all documented T/F viruses that have invaded new individuals encode *vpu*[44,56]. Therefore, *vpu* may be essential for infecting new individuals but may be dispensable or less important in viremia maintenance and disease progression. In fact, we have previously revealed that Vpu boosts the number of cell-free viruses and promotes the establishment of viruses in a humanized mouse model[46]. We also found that in earlier pathogenic stages (particularly Fiebig stages III–IV), the motif for tetherin down-regulation (AxxxAxxxW) is conserved and that tetherin is effectively counteracted by the Vpus of T/F viruses (Fig. 3). In contrast, although the motif for CD4 down-regulation (SxxxS) is similarly conserved, CD4 levels may be maintained in the presence of Vpu-producing T/F viruses (Fig. 3). These findings suggest that anti-tetherin activity is more important than CD4 down-regulation, particularly in T/F viruses. However, according to a recent study by Pickering *et al.*[57], Vpu preserves the ability to downregulate CD4 in infected individuals, and also counteracts tetherin despite the extensive sequence variation of Vpu. They attributed both tetherin antagonism and CD4 down-regulation of strain NL4-3 to at least 4 amino acids, [19]A, [23]W, [53]S, and [56]S. Therefore, Vpu may facilitate viral propagation during the human-to-human transmission phase.

The experimental-mathematical approach adopted here has quantitatively revealed the replication dynamics of retroviruses[24,58,59] and enteroviruses[25] in cell culture systems. To our knowledge, we present the first estimate of the anti-viral effect of tetherin in human populations by combining experimental and epidemiological data with a structured mathematical model. The data-driven mathematical approach can elucidate viral infection dynamics in ways that are impossible by conventional experimental strategies alone. Especially, the mathematical model incorporates virological information of infected individuals such as the viral load, a crucial parameter for investigating infectious disease spread. As discussed herein, tetherin-mediated herd immunity is revealed only when the set-point viral load is considered as a distribution. If modeled only by the average viral load, the herd immunity effect might be obscured in the population dynamics and the anti-viral effect of tetherin might be underestimated. Thus, quantitative epidemiological details can only be revealed in a multi-scaled modeling approach.

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Author Contributions

Conceived and designed the study: S.I., K.S., Y.I., K.A. and Y.K. Analyzed the data: S.I., S.M., H.I., Y.K. and F.R. Carried out the experiments: K.S., T.K., J.S.T., Y.K., N.M. and Y.K. Wrote the paper: S.I., K.S., S.M., H.I., Y.I., K.A. and Y.K. All authors read and approved the final manuscript.

Additional Information

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