Structural basis of CD4 downregulation by HIV-1 Nef

Yonghwa Kwon1, Robyn M. Kaake2,3, Ignacia Echeverria4, Marissa Suarez5, Mohammad Karimian Shamsabadi1, Charlotte Stoneham5,6, Peter W. Ramirez6, Jacob Kress1, Rajendra Singh5,6, Andrej Sali4,7, Nevan Krogan8,2,3, John Guatelli5,6 and Xiaofei Jia1✉

The HIV-1 Nef protein suppresses multiple immune surveillance mechanisms to promote viral pathogenesis and is an attractive target for the development of novel therapeutics. A key function of Nef is to remove the CD4 receptor from the cell surface by hijacking clathrin- and adaptor protein complex 2 (AP2)-dependent endocytosis. However, exactly how Nef does this has been elusive. Here, we describe the underlying mechanism as revealed by a 3.0-Å crystal structure of a fusion protein comprising Nef and the cytoplasmic domain of CD4 bound to the tetrameric AP2 complex. An intricate combination of conformational changes occurs in both Nef and AP2 to enable CD4 binding and downregulation. A pocket on Nef previously identified as crucial for recruiting class I MHC is also responsible for recruiting CD4, revealing a potential approach to inhibit two of Nef’s activities and sensitize the virus to immune clearance.

The HIV-1 protein Nef is a critical factor in viral pathogenesis1. Expression of Nef in vivo is required for high viral loads and for progression to AIDS2. Individuals infected with HIV-1 encoding defective nef genes do not develop AIDS for decades3,4. Nef is a peripheral membrane protein that anchors to the lipid membrane via an N-terminal myristoyl group. Abundantly expressed early during the viral replication cycle, Nef modulates the surface levels of many host proteins and thereby interferes with immune processes responsible for detecting and combating the infection1,5,6. Two of the most prominent targets of Nef are MHC-I, which is crucial for antigen presentation and the killing of infected cells by cytotoxic T lymphocytes, and CD4.

CD4 is the entry receptor for HIV-1, but later in the viral replication cycle it is problematic for the virus: it disrupts processing of the viral glycoprotein, Env, inhibiting infectivity7; it interferes with the release of new virions8,9; and it causes vulnerability to superinfection, causing premature cell death and limiting viral productivity10. Furthermore, binding of CD4 to Env exposes otherwise-concealed Env epitopes, rendering infected cells more susceptible to antibody-dependent cellular cytotoxicity and virus particles more susceptible to neutralizing antibodies11–13. HIV-1 has evolved strategies to mitigate these problems. Newly synthesized CD4 is targeted in the endoplasmic reticulum by the viral Vpu protein for proteasomal degradation14. Surface-expressed CD4, in contrast, is targeted by Nef for endocytosis and lysosomal degradation15–18.

Nef’s effect on CD4 involves hijacking of clathrin AP2-dependent endocytosis19,20. Adaptor protein (AP) complexes mediate the clathrin-dependent trafficking of membrane proteins21. Members of this family (AP1–5) share a common heterotetrameric structure, which adopts either a closed or an open conformation. When activated and open, AP complexes reveal their cargo-binders sites, which then associate with specific sorting motifs in the cytoplasmic domains of membrane cargos. Two sorting motifs are commonly recognized by APs. The tyrosine-based motifs (YxxΦ, where Φ is a bulky hydrophobic residue) bind to the μ subunits of APs, while acidic dileucine motifs ([E/D]xxL[1/1]) bind to a pocket formed largely by the σ subunits. To mediate downregulation of CD4 from the cell surface, Nef co-opts the clathrin AP2 complex partly by mimicking the acidic dileucine motif22. Although how Nef associates with a part of the tetrameric AP2 is understood23, to our knowledge, a complete understanding of the interaction, especially how CD4 is sequestered by Nef into a complex with AP2, has remained elusive. Here we describe the mechanism of this key Nef function using a high-resolution crystal structure, cross-linking MS (XL-MS) and structure modeling, along with biochemical and functional validation.

Results

Nef functions as a ‘connector’ between clathrin AP2 and CD4. We pursued a high-resolution structure of Nef in complex with tetrameric AP2 and the cytoplasmic domain of CD4 (CD42T). To facilitate the assembly of the protein complex, we fused CD42T to the C terminus of Nef via a 36-amino-acid-long, flexible linker (Fig. 1a). Instead of using the full-length CD4 tail (394–433), we included only residues 394–419, containing all of the CD4 determinants reportedly required for Nef-mediated downregulation24,25,26. Since Nef residues within the N-terminal amphipathic helix are dispensable for CD4 downregulation26, we truncated 25 amino acids from the Nef N terminus. We engineered the tetrameric AP2 complex by removing the mobile C-terminal domain of the μ2 subunit (136–423), enabling AP2 to adopt an open conformation in which its cargo-binders sites are accessible27. Binding between the Nef-CD42T fusion protein and the modified AP2 construct (AP2μ2-CTS) was confirmed using a GST pulldown assay (Fig. 1b).
We then solved the crystal structure of the Nef-CD4\textsubscript{CD} fusion and AP2\textsubscript{2-CTD} complex to a resolution of 3.0 Å (Fig. 1c and Table 1). All polypeptides are largely resolved except for the flexible linker between Nef and CD4\textsubscript{CD}, which is disordered as expected, and part of the N-terminal region of β2. As revealed by the structure, Nef functions as a ‘connector’ between AP2 and CD4\textsubscript{CD}; CD4\textsubscript{CD} binds Nef but none of the subunits of AP2. As previously reported, Nef’s C-terminal loop interacts with AP2 in part via mimicry of the acidic dileucine motif (Fig. 1d)\textsuperscript{22}. The extensive interface here, involving C-terminal loop interacts with AP2 in part via mimicry of the acidic functions as a ‘connector’ between AP2 and CD4\textsubscript{CD}; CD4\textsubscript{CD} binds β2 of the N-terminal region of 2. As revealed by the structure, Nef interacts with AP2 mainly through its C-terminal loop (148–180). The rest of Nef is not shown for an unblocked view.

CD4 recruitment and role of the Nef N-terminal loop. CD4 is recruited to a pocket on Nef that is opposite the C-terminal loop. The association is mainly hydrophobic and involves three CD4 residues: Ile\textsubscript{410}, Leu\textsubscript{413} and Leu\textsubscript{414} (Fig. 2a). The dileucine motif of CD4—Leu\textsubscript{143}/144—is within a short helix. Leu\textsubscript{144} and Ile\textsubscript{410} dip into a hydrophobic pocket of Nef, which is formed by Phe\textsubscript{121}, Leu\textsubscript{37}, Asn\textsubscript{52} and Cys\textsubscript{55} (Fig. 2b). Leu\textsubscript{413} of CD4 sits just outside of that pocket and is accommodated by Nef residues Phe\textsubscript{121}, Leu\textsubscript{37}, Asn\textsubscript{52} and Cys\textsubscript{55} (Fig. 2b). Nef residue Asp\textsubscript{123} contributes to CD4 binding by hydrogen-bonding with the backbone nitrogen of CD4 Leu\textsubscript{413} and supports the helix-turn of CD4 (Fig. 2b). These structural findings explain previously observed roles of CD4 residues—the dileucine motif and Ile\textsubscript{410} (refs. \textsuperscript{16,24,27})—as well as Nef residues F121, D123 and Trp\textsubscript{124} (refs. \textsuperscript{28,29}).

CD4 recruitment is secured by the Nef N-terminal loop, which is highly ordered in the structure (Fig. 2c and Extended Data Fig. 1). The part of the loop immediately connected to the rigid core of Nef, residues Phe\textsubscript{68} to Pro\textsubscript{75}, wraps around the core. N-terminally, the part of the loop immediately connected to the rigid core of Nef, residues Cys\textsubscript{55} to Val\textsubscript{33}, forms a wall-like structure to support CD4 binding. Here, contacts are made by several Nef residues with the short helical turn of CD4 and flanking residues. This conformation of the Nef N-terminal loop explains the crucial roles of Trp\textsubscript{57}, Leu\textsubscript{58}, Tyr\textsubscript{115} and Pro\textsubscript{122} in CD4 down-regulation observed in previous studies\textsuperscript{20,21,25,26,32}. It also explains the cooperativity in the three-way binding between CD4\textsubscript{CD}, Nef and the α/σ2 hemicompex of AP2 (ref. \textsuperscript{31}).

We then challenged these structural observations using mutagenesis. First, binding between a fluorescence tag-labeled CD4 cytoplasmic tail (tetramethylrhodamine-CD4\textsubscript{CD}; TMR-CD4\textsubscript{CD}) and the purified Nef–AP2 complex was characterized by an in vitro fluorescence polarization assay. While unbound TMR-CD4\textsubscript{CD} led to
low polarization signal due to its fast tumbling and thus depolarization, binding of TMR-CD4\(_{\text{cyt}}\) to wild-type (WT) Nef and AP2 at higher protein concentrations resulted in drastic increase in molecular size, leading to slower tumbling and thereby high polarization signal (Fig. 2e). In contrast, reduced CD4-binding ability was observed for all Nef mutants tested. These mutations, according to the structure, affect either direct CD4 binding (D123K L37D) or positioning of the Nef N-terminal short helix (W57A L58A L112D) or both (F121D). Second, these same Nef mutations were evaluated in a CD4 surface downregulation assay (Fig. 2f–h).

HeLa cells that stably express CD4 were cotransfected with plasmids expressing GFP (as a transfection marker) and plasmids expressing Nef. Two-color flow cytometry was used to measure cell-surface CD4 as a function of GFP intensity. As expected, WT Nef robustly downregulated CD4. Consistent with the in vitro fluorescence studies, CD4 downregulation was severely impaired for all of the mutants tested (W57A, L58A, L112D, F121D and D123R) (Fig. 2f–h). The L37D mutant was also inactive but poorly expressed (data not shown). Overall, these binding and functional data on the Nef mutants support the structural observations on
NATuRE STRUCTUrAL & MOLECulAR BIOLoGY

**Fig. 3 | Nef binding induces conformational change in the β2 subunit of AP2.** a. Current structure is overlaid, on the α-μ2 half, with the open AP2 structure (PDB 2XΔ7). β2 (green) and μ2 (pink) subunits in the current structure move outward in comparison with β2 (light blue) and μ2 (blue) subunits of the unbound AP2. b. The first helix of β2 binds to the Nef core, while the next three helices become disordered. c. Density for the first helix of β2 (pink mesh, 2F – F, map at 1.0σ) is shown. Residues important for association with the Nef core are labeled.

Nef binding induces a conformational change in the β2-μ2 half of AP2. Overlaying the α and μ2 subunits of the ‘open’ AP2 structure on the current structure reveals that the μ2 subunit and the μ2 N-terminal domain (μ2-NTD) of AP2 in the current structure ‘move’ out as one rigid body, causing a greater ‘opening’ of the tetramer (Fig. 3a). In addition, a large portion of the β2 N terminus, residues 1–88 encompassing the first four helices, is displaced from the rest of β2. If β2 had maintained its original fold, then its N terminus would clash with the Nef core (Extended Data Fig. 2); the observed displacement is likely necessary to accommodate Nef binding. While most of this displaced portion of β2 becomes disordered, the first helix preserves its helical structure and binds to the Nef core through hydrophobic interactions (Fig. 3b,c).

The Nef-induced destabilization and structural changes of the β2-μ2 half of AP2 were further characterized by chemical XL-MS and integrative structure modeling (Extended Data Fig. 3). Here, disuccinimidyl sulfoxide (DSSO), an MS-cleavable, bifunctional amine-reactive small molecule, was used to cross-link proximal Lys residues or N termini of the Nef-CD4 complex and fusion and AP2 NTD-CTD complex. Cross-linked proteins separated by SDS–PAGE were trypsin-digested and resulting peptides analyzed by specialized liquid chromatography–MS (LC–MS) experiments for identification of cross-linked residues (Extended Data Fig. 3a). Application of this pipeline to the complex identified intra- and inter-linked peptides corresponding to 90 unique cross-linked residues (Supplementary Table 1 and Extended Data Fig. 3b). Lys residues from the displaced β2 N-terminal domain (1–88) are involved in a total of 24 cross-links (Supplementary Table 1, bold entries). Importantly, most of these cross-links are made by Lys residues flanking the first helix (that is, Lys5, 11, 12, 26 and 27), consistent with this helix binding specifically in the complex. In contrast, Lys residues from the other dislocated β2 N-terminal helices (that is, Lys29, 31, 35, 36, 45, 66, 67 and 78) are rarely observed, consistent with a lack of fixed residence within the complex. The XL-MS data, which capture structural information from a conformationally heterogeneous population of protein complexes as they exist in solution, the crystal structure and other structural information (Methods) were used for integrative modeling to produce a model ensemble that describes the complex in full (Extended Data Fig. 3c). Segments that are disordered in the crystal structure are modeled and represented as helices or flexible strings of beads (Extended Data Fig. 3c). The model ensemble agrees satisfactorily with the observed cross-links (89% of cross-links are satisfied; Extended Data Fig. 3d). Furthermore, it indicates large variability in the positions and orientations of the helices in the partially unfolded β2 segment (Extended Data Fig. 3e–g), consistent with the structural heterogeneity of this region indicated by the crystallographic data.

Nef uses a ‘molecular switch’ to differentially downregulate MHC-I via AP1 or CD4 via AP2. Binding of the first helix of β2 to the Nef core in the current structure closely resembles how the N-terminal amphipathic helix of Nef (deleted from the construct used herein) binds the same location when Nef hijacks AP1 for MHC-I downregulation (Fig. 4a). That interaction, mediated by Nef Trp13 and Met20, is critical for MHC-I downregulation but is dispensable for CD4 downregulation. Our current structure indicates that intramolecular association of this Nef helix with the Nef core would force the Nef N-terminal loop to deviate from the conformation associated with CD4 binding (Extended Data Fig. 4). We suspect that this is an intrinsic plot of the virus: Nef binding forces the N-terminal helices of β2 to destabilize and unfold. By providing a binding site for the first β2 helix on its core, Nef partially compensates for this destabilization. Moreover, the N-terminal β2 helix is now used to compete the Nef amphipathic helix off the core, freeing the N-terminal loop of Nef to adopt the conformation shown in Fig. 2c for CD4 downregulation. A potential caveat to this hypothesis is that the L100A I109A mutation of Nef, which should affect the binding of the β2 helix to the Nef-core (Fig. 4a), minimally impairs the downregulation of CD4 (Fig. 4b). However, since this mutation likely affects the binding of the β2 helix and the Nef N-terminal amphipathic helix similarly (Fig. 4a), it might have minimal influence on the competition between these two helices for the Nef core in vivo. In contrast, and consistent with the competition hypothesis, the same mutation impairs the downregulation of class I MHC (Fig. 4c), presumably because it disrupts the required binding between the N-terminal amphipathic helix of Nef and the Nef core.

Comparing the current structure with our earlier structure of Nef in complex with the μ1 C-terminal domain (μ1-CTD) and the MHC-I cytoplasmic domain reveals versatility and specificity in how the structurally homologous AP1 and AP2 are selectively co-opted to downregulate MHC-I and CD4 (Fig. 4d). For MHC-I downregulation, Nef interacts solely with the μ1 subunit and exploits the conserved Tyr-based motif-binding site on AP1 (ref. 35). For CD4 downregulation, Nef exploits the acidic diacidine-binding site on the α and μ2 subunits of AP2 and contacts all subunits except μ2. Our models show that by allowing the N-terminal Nef helix and the N-terminal β2 helix to bind the same pocket on the core, Nef creates a ‘molecular switch’ that links its use of different AP complexes with the modulation of different targets: binding to AP2 frees the N terminus of Nef from the core to recruit CD4, whereas binding to AP1 leaves the core free to bind the N terminus of Nef and thereby facilitates the recruitment of MHC-I.

Despite these distinct modes of binding, the cytoplasmic domains of MHC-I and CD4 share much of the same binding ‘pocket’ on Nef (Fig. 4e). As evident from the structural overlay, the two cytoplasmic domains partially overlap at this location. Notably, Asp123 is the only Nef residue in this pocket that is crucially involved in both binding mechanisms as well as required for the downregulation of both CD4 (refs. 28,29) (Fig. 2b,e–h) and MHC-I (refs. 28,29,35–37). Nonetheless, several residues at this site on Nef are highly conserved (Extended Data Fig. 5).

**Discussion**

Our data enable us to postulate the sequence of events leading to the sequestration of CD4 by Nef. Nef first binds to AP2 using its C-terminal loop. This binding, involving the acidic diacidine motif-binding site of AP2, is extensive and robust (Fig. 1d). Unlike typical membrane cargos, which use only short cytoplasmic tails to bind AP2, Nef also contains a bulky core domain, which affixes to
the α and σ2 half of AP2. Sterically, the bound Nef core is incompatible with an intact β subunit (Extended Data Fig. 2). Destabilization of β2 then occurs, causing the N-terminal helices to deviate from the bundled state (Fig. 3). The first helix of β2 becomes available to bind the Nef core, competing off the N-terminal helix of Nef and thereby freeing the rest of the Nef’s N-terminal loop (Fig. 4a). This loop then moves over, placing a short helical anchor at the interface between Nef and the α subunit and extending its arm to complete the CD4-binding site (Fig. 2).

Our results weigh against the notion that dimerization of Nef is required for CD4 downregulation. The dependence of CD4 downregulation on Asp123 has been previously attributed to its role in mediating Nef dimerization34. In addition, a quadruple Nef mutant, L100A I109A L164A L165A (ILYF), which cannot dimerize, is unable to downregulate CD4 (refs. 32,38). However, as revealed here, a single Nef molecule is sufficient to recruit CD4<sub>CD</sub> into AP2. Moreover, the residues involved in dimerization are directly involved in critical elements of the model here. For example, Asp123 is in direct contact with CD4 (Fig. 2a). Three of the four residues involved in the quadruple mutant, Leu112, Tyr115 and Phe121, interact directly with either CD4 or Nef Trp57/Leu58 (Fig. 2b,d).

Finally, the ILYF-dependent dimerization of Nef, which occurs when Nef is in complex with the Src-family kinase Hck<sup>39</sup>, is incompatible with the conformation of the Nef N-terminal loop shown here as important for CD4 downregulation (Extended data Fig. 6).

Our results also weigh against the notion that the helix binding site on the Nef core is a promiscuous acceptor for the cytoplasmic domains of target proteins<sup>40</sup>. This hypothesis derived from the observation that this hydrophobic crevice is occupied by the acidic leucine motif from an adjacent Nef molecule in a crystal structure of a simian immunodeficiency virus (SIV) Nef<sup>40</sup>. Portions of the cytoplasmic domains of CD4 and the CD3 ζ-chain could also be modeled into this site<sup>40</sup>. Our data and model reveal a very different scenario: this hydrophobic crevice on the Nef core binds two distinct helices: a helix from the β2 N terminus when in complex with CD4 and AP2, or a helix from the Nef N terminus when in complex with MHC-I and AP1 (Fig. 4a). As detailed above, this helix binding site on the Nef core is the basis for an elegant molecular switch mechanism that links the use of specific AP complexes with the modulation of different cellular targets.

A key feature of the β2 subunit revealed here is that it is structurally labile and, upon Nef binding, can deviate from its original

---

**Fig. 4** The downregulations of MHC-I and CD4 are distinct both mechanistically and structurally, yet their cytoplasmic domains share a common binding site on Nef. a, Nef-μ1-MHC-I<sub>μ2</sub> (PDB 4EMZ) and Nef-AP2-CD4<sub>ε2</sub> (current structure) are overlaid on Nef (cyan). The Nef N-terminal helix (gray) and the first helix of β2 (green, current structure) occupy the same site on the Nef core. Trp13 and Met20 residues in the Nef N-terminal helix important for the intramolecular helix-core association are shown. Important residues on β2 (I15, L18 and L22) are also shown. Nef L100 and I109 make direct contact with the bound helices. b–c, CD4 (b) and MHC-I (c) downregulation by Nef mutants was measured using cell-surface staining and flow cytometry. GFP is a transfection marker; GFP-only control is indicated (−). Nef L164A L165A and M20A mutants were used as negative controls for CD4 and MHC-I downregulation, respectively. CD4-positive HeLa cells were used for the CD4 assays, and HeK293 cells, which express HLA-A2, were used for the MHC-I assays. Each experiment was done at least twice (independent transfections). Cellular expression of Nef proteins was compared by western blot; the cellular control is β-actin. Uncropped blot images are shown in Supplementary Fig. 1.
fold. Nef-induced destabilization of the β2 subunit has also been observed in a recently published structure describing how Nef from an SIV hijacks AP2 to recruit rhesus BST2 for surface down-regulation\(^3\). There, the structural changes at the N terminus of β2 occur in a completely different manner than here. Part of its first helix (14–17) loses its helical fold and instead refolds into a β-sheet together with a preceding β2 segment (6–12) and a segment of Nef’s C-terminal loop. This β-sheet then becomes part of the binding pocket for the recruitment of the rhesus BST2 tail. Whether such a conversion into a β-sheet occurs when Nef, of either HIV or SIV, targets other cellular surface proteins via AP2-dependent endocytosis is unknown. Similarly, whether the conformational change in β2 observed in the present study (Fig. 3) is involved in other downregulation events mediated by Nef remains to be shown. Nonetheless, Nef clearly takes advantage of the destabilization that it causes in β2 to induce distinct β2 conformations that facilitate recruitment of specific target proteins into clathrin-coated vesicles.

Antiretroviral drugs targeting Nef have the potential to unleash immunologic surveillance mechanisms. Our finding that the structural basis of the downregulation of MHC-I and CD4 involves a common and conserved binding site suggests that both activities could be inhibited by a single agent. Such a Nef inhibitor could facilitate the clearance of infected cells and contribute to HIV cure strategies\(^3\).

### Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41594-020-0463-z.

Received: 21 January 2020; Accepted: 16 June 2020; Published online: 27 July 2020

---

### References

1. Kirchhoff, F., Schindler, M., Specht, A., Arhel, N. & Munch, J. Role of Nef in primate lentiviral immunopathogenesis. *Cell. Mol. Life Sci.* **65**, 2621–2636 (2008).

2. Kestler, H. W. III et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**, 651–662 (1991).

3. Kirchhoff, F., Gre nou th, T. C., Brettler, D. B., Sullivan, J. L. & Des ro siers, R. C. Brief report: absence of intact Nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N. Engl. J. Med.* **322**, 228–232 (1995).

4. Deacon, N. J. et al. Genomic structure of an attenuated quasi-species of HIV-1 and p56\(^{\text{orf}}\) protein-typ e I cytoplasmic domain. *J. Virol.* **73**, 1964–1973 (1999).

5. Pereira, E. A. & daSilva, L. L. Human immunodeficiency virus type 1 Nef protein targets CD4 to the multivesicular body pathway. *J. Virol.* **83**, 6578–6590 (2009).

6. Aiken, C., Konner, J., Landau, N. R., Lenburg, M. E. & Trono, D. Nef induces CD4 endocytosis—requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* **76**, 835–864 (1994).

7. Garcia, J. V. & Miller, A. D. Serine phosphorylation-independent down-regulation of cell-surface CD4 by nef. *Nature* **350**, 508–511 (1991).

8. Guay, B. et al. HIV-1 Nef targets CD4 to the multivesicular body pathway. *J. Virol.* **70**, 2661–2670 (1996).

9. Chaudhuri, R., Lundwasser, O. W., Smith, W. J., Hurley, J. H. & Bonifacino, S. J. Downregulation of CD4 by human immunodeficiency virus type 1 Nef is dependent on clathrin and involves direct interaction of Nef with the AP2 clathrin adaptor. *J. Virol.* **81**, 3877–3890 (2007).

10. Benson, R. E., Sanfridson, A., Ottinger, J. S., Doyle, C. & Cullen, B. R. Inhibition of HIV-1 progeny virion release by the AP2 chaperone complex 2. *Proc. Natl Acad. Sci. USA* **92**, 435–439 (1995).

11. Preusser, A., Briese, L., Baur, A. S. & Willbold, D. Direct in vitro binding of the Nef domain of the HIV-1 Vpu protein regulates the formation of clathrin-coated vesicles. *J. Virol.* **73**, 349–353 (1999).

12. Mangasarian, A., Piguet, V., Wang, J. K., Chen, Y. L. & Trono, D. Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. *J. Virol.* **73**, 1683–1693 (1999).

13. Jackson, L. P. et al. A large-scale conformational change couples membrane recruitment to cargo binding in the AP2 clathrin adaptor complex. *Cell* **141**, 1220–U213 (2010).

14. Aiken, C., Krause, L., Chen, Y. L. & Trono, D. Mutation analysis of HIV-1 Nef: identification of two mutants that are temperature-sensitive for CD4 downregulation. *Virology* **217**, 293–300 (1996).

15. Liu, L. X. et al. Mutation of a conserved residue (D123) required for oligomerization of human immunodeficiency virus type 1 Nef protein abolishes interaction with human thioesterase and results in impairment of Nef biological functions. *J. Virol.* **74**, 5310–5319 (2000).

16. Aiken, C., Krause, L., Chen, Y. L. & Trono, D. Mutational analysis of HIV-1 Nef: identification of two mutants that are temperature-sensitive for CD4 downregulation. *Virology* **217**, 293–300 (1996).

17. Poe, J. A. & Smithgall, T. E. Human immunodeficiency virus type 1 Vpu protein recruits the clathrin adaptor complex 2. *J. Biol. Chem.* **275**, 701–706 (2000).

18. Hua, J., Blair, W., Truant, R. & Cullen, B. R. Identification of regions in HIV-1 Nef required for efficient downregulation of cell surface CD4. *Virology* **231**, 231–238 (1997).

19. Baugh, L. L., Garcia, J. V. & Foster, J. L. Functional characterization of the human immunodeficiency virus type 1 Nef cytoplasmic domain. *J. Virol.* **82**, 9657–9667 (2008).

20. Ross, T. M., Oran, A. E. & Cullen, B. R. Inhibition of HIV-1 glycoprotein release by cell-surface CD4 is relieved by expression of the viral Nef protein. *Curr. Biol.* **9**, 613–621 (1999).

21. Lama, J., Mangasarian, A. & Trono, D. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. *Curr. Biol.* **9**, 622–631 (1999).

22. Benson, R. E., Sanfridson, A., Ottinger, J. S., Doyle, C. & Cullen, B. R. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med.* **177**, 1561–1566 (1993).

23. Vezzette, M. et al. Interaction with cellular CD4 exposes HIV-1 envelope epitopes targeted by antibody-dependent cell-mediated cytotoxicity. *J. Virol.* **88**, 2633–2644 (2014).

24. Pham, T. N., Lukhee, S., Hajjar, F., Routy, J. P. & Cohen, E. A. HIV Nef and Vpu protect HIV-infected CD4\(^+\) T cells from antibody-mediated cell lysis through down-modulation of CD4 and BST2. *Retrovirology* **11**, 15 (2014).
39. Alvarado, J. J., Tarařar, S., Yeh, J. I. & Smithgall, T. E. Interaction with the Src homology (SH3-SH2) region of the Src-family kinase Hck structures the HIV-1 Nef dimer for kinase activation and effector recruitment. J. Biol. Chem. 289, 28539–28553 (2014).

40. Manrique, S. et al. Endocytic sorting motif interactions involved in Nef-mediated downmodulation of CD4 and CD3. Nat. Commun. 8, 442 (2017).

41. Buffalo, C. Z. et al. Structural basis for Tetherin Antagonism as a Barrier to Zoonotic Lentiviral Transmission. Cell Host Microbe 26, 359–368.e8 (2019).

42. Deeks, S. G. HIV: shock and kill. Nature 487, 439–440 (2012).

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2020
Cloning, expression and purification of proteins. The Nef-CD4CD complex was constructed by fusing C4D (394–419) to the C terminus of HIV-1 Nef (26–206), NL4.3) via a flexible linker of 36 amino acids (GVDGSDEASELACPTPKDELGLQQQTNLRGLGSGE). The encoded gene was cloned into a pMAT9 expression vector. The fusion protein was over-expressed in Escherichia coli. Nef-CD4CD fusion chimera were mixed at 1:5 molar ratio to a final concentration of 6.5, 5 mM KCl, 2 mM dithiothreitol. A stock protein solution was then prepared by molecular replacement using PHASER in Phenix. Only one molecule exists in the asymmetric unit. The Protein Data Bank (PDB) identity of the open AP2 complex (2XAZ) was divided into two search models: the α and σ2 hexameric and the β2 and μ2-NTD hexameric. Together with the Nef structure (1EJM), the two models were used successfully to successfully model the two alternative rounds of model building in COOT and refinement in Phenix were carried out. The final model has an R_e/R_m of 0.241/0.277. A Ramachandran plot showed that 96.4% of the residues are in the favored region, together with 3.5% in the allowed region and 0.1% as outliers. The refinement statistics are summarized in Table 1.

CD4 downregulation assays. HeLa cells expressing CD4 (TZM-bl, obtained from Dr. John Kappes via the National Institutes of Health AIDS Reagent Program) were transfected with Lipofectamine2000 (Thermo Fisher Scientific) with pcGFP (a gift from Dr. Jack Grakowski) and pcI-NL, a pcI-neo-based plasmid (Promega) expressing Nef, or the indicated Nef mutants. In each transfection, 1.6 μg of each plasmid DNA was cotransfected with 0.8 μg of pcGFP or derivative mutants. One of the replicates experiments was done using Fugene 6 (Promega) as the transfection reagent, 0.3 μg of pcGFP and 0.9 μg of pcI-NL or derivative mutants. After 1 d, half of the cells were stained for surface CD4 (anti-human CD4, BioLegend, conjugated directly to APC), fixed in formaldehyde, then analyzed by two-color flow cytometry using an Accuri 6 flow cytometer (BD). ‘Live cell’ gates were set using untransfected cells; gates for GFP were set using cells transfected only with pcI-neo; and gates for CD4 were set using cells stained with an APC-conjugated antibody isotype control. Two-color contour plots were prepared using FlowJo Software (v.10.6.1). The other half of the cells were lysed and the proteins were resolved on an SDS–PAGE gel before transfer to PVDF membranes. Nef was detected using a polyclonal antiserum raised to NL4-3 Nef in sheep (a gift from Dr. Celsa Spina, University of California, San Diego). β-actin was detected using a murine monoclonal antibody (Sigma-Aldrich). Species-specific secondary antibodies conjugated to HRP were visualized using Western Clarity detection reagent (Bio-Rad). Chemiluminescence was recorded using a ChemiDoc XRS Imaging System (Bio-Rad). The cells were tested for mycoplasma using a PCR assay and were negative.

Class I MHC downregulation assays. HEK293 cells, which naturally express HLA-A2, were transfected with Lipofectamine2000 with pcGFP (0.4 μg) and pcI- NL, the indicated Nef mutants or the empty vector pcI-neo (1.2 μg). After 1 d, half of the cells were stained for surface HLA-A2 (BioLegend, anti-HLA-A2 conjugated directly to APC), fixed in formaldehyde, then analyzed by two-color flow cytometry using an Accuri 6 flow cytometer. ‘Live cell’ gates were set using untransfected cells; gates for GFP were set using cells transfected only with pcI-neo; and gates for HLA-A2 were set using cells stained with an APC-conjugated antibody isotype control. Two-color contour plots were prepared using FlowJo Software (v.10.6.1). The other half of the cells were processed for western blot as described in the CD4 downregulation assays. The cells were tested for mycoplasma using a PCR assay and were negative.

DSSO-based XL-MS analysis. Individual preparations of Nef-CD4CD Δ2-CTD complex (2.8 mg ml−1 and 0.7 mg ml−1 at 1:5 Nef-CD4CD molar excess) were cross-linked using increasing molar ratios of DSSO (Thermo Fisher Scientific), for 5, 10 or 30 min at 4 or 37 °C. Cross-linked proteins were separated on 4–20% TGX gradient SDS–PAGE gels (Bio-Rad) and stained with MS-safe AcquaStain (Budapest Bio). Species-specific secondary antibodies conjugated to HRP were visualized using Western Clarity detection reagent (Bio-Rad), Chemiluminescence was recorded using a ChemiDoc XRS Imaging System (Bio-Rad). The cells were tested for mycoplasma using a PCR assay and were negative.
Integrative modeling of the Nef-CD4−AP2−CTD complex.

We applied an integrative structural modeling approach to characterize the structure of the Nef-CD4−AP2−CTD complex in solution, based on the crystal structure and the 90 DSSO cross-links. Integrative structure determination proceeded through the standard four stages: (1) gathering data; (2) representing subunits and translating data into spatial restraints; (3) configurational sampling to produce an ensemble of structures that satisfies the restraints; and (4) analyzing and validating the ensemble structures and data.

(1) Gathering data: Modeling was based on the crystal structure, a comparative model of the β2 subunit 24–89 region built on the AP2 structure using MODELLER and the 90 DSSO cross links.

(2) Representing subunits and translating data into spatial restraints: To maximize computational efficiency while avoiding too coarse a representation, we used the coarse-grained one-residue-per-bead representation. The regions absent from refinement may be more flexible and thus should be less constrained by the input data; (2) representing subunits and translating data into spatial restraints; (3) configurational sampling to produce an ensemble of structures that satisfies the restraints; and (4) analyzing and validating the ensemble structures and data; the integrative structure modeling protocol is, stages 2, 3, and 4 was scripted using the Python Modeling Interface package, a library for modeling macromolecular complexes based on our open-source Integrative Modeling Platform package, v.2.8 (https://integrativemodeling.org). Files containing the input data, scripts and output results are available at https://github.com/integrativemodeling/Nef_CD4_AP2.

(i) Gathering data: Modeling was based on the crystal structure, a comparative model of the β2 subunit 24–89 region built on the AP2 structure using MODELLER and the 90 DSSO cross links.

(ii) Estimation of sampling precision: The precision at which sampling sampling can be quantified by the Cramer's V value, a measure of association between two nominal variables. Third, the non-parametric Kolmogorov–Smirnov two-sample test (two sided) indicated that the difference between the two score distributions was statistically insignificant (P = 0.02) was quantified by the Cramer's V value 44, Xue, X. Y. et al. Structures of two coronavirus main proteases: A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 (2010).

References
43. Xue, X. Y. et al. Production of authentic SARS-CoV M-pro with enhanced activity: application as a novel tag-cleavage endopeptidase for protein overproduction. J. Mol. Biol. 366, 965–975 (2007).
44. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
47. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 (2010).

Data availability
The coordinates and structure factors for the crystal structure have been deposited at the Protein Data Bank (PDB) with the accession code 6URI. The proteomics XI-MS data have been deposited at the ProteomeXchange database with the accession code PXD019338. The integrative structural model has been deposited at PDB-Dev with the accession code PDBDev_00000050. Source data are provided with this paper.

Code availability
Files containing the input data, scripts and output results for the integrative structure modeling of the Nef-CD4−AP2−CTD complex are available at https://github.com/integrativemodeling/Nef_CD4_AP2.
48. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).

49. Afonine, P. V. et al. Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr. D Biol. Crystallogr.* **68**, 352–367 (2012).

50. Kaake, R. M. et al. A new in vivo cross-linking mass spectrometry platform to define protein–protein interactions in living cells. *Mol. Cell. Proteom.* **13**, 3533–3543 (2014).

51. Kessner, D., Chambers, M., Burke, R., Agus, D. & Mallick, P. ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics* **24**, 2534–2536 (2008).

52. Gutierrez, C. B. et al. Developing an acidic residue reactive and sulfoxide-containing MS-cleavable homobifunctional cross-linker for probing protein–protein interactions. *Anal. Chem.* **88**, 8315–8322 (2016).

53. Kim, S. J. et al. Integrative structure and functional anatomy of a nuclear pore complex. *Nature* **555**, 475–482 (2018).

54. Russel, D. et al. Putting the pieces together: integrative modeling platform software for structure determination of macromolecular assemblies. *PLoS Biol.* **10**, e1001244 (2012).

55. Ward, A. B., Sali, A. & Wilson, I. A. Integrative structural biology. *Science* **339**, 913–915 (2013).

56. Alber, F. et al. Determining the architectures of macromolecular assemblies. *Nature* **450**, 683–694 (2007).

57. Lasker, K. et al. Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. *Proc. Natl Acad. Sci. USA* **109**, 1380–1387 (2012).

58. Sali, A. et al. Outcome of the first wwPDB Hybrid/Integrative Methods Task Force Workshop. *Structure* **23**, 1156–1167 (2015).

59. Schneidman-Duhovny, D., Pellarin, R. & Sali, A. Uncertainty in integrative structural modeling. *Curr. Opin. Struct. Biol.* **28**, 96–104 (2014).

60. Sali, A. & Blundell, T. L. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815 (1993).

61. Webb, B. & Sali, A. Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci.* **54**, 5.6.1–5.6.37 (2016).

62. Erzberger, J. P. et al. Molecular architecture of the 40S·eIF1·eIF3 translation initiation complex. *Cell* **159**, 1227–1228 (2014).

63. Shi, Y. et al. Structural characterization by cross-linking reveals the detailed architecture of a coatamer-related heptameric module from the nuclear pore complex. *Mol. Cell. Proteom.* **13**, 2927–2943 (2014).

64. Shen, M. Y. & Sali, A. Statistical potential for assessment and prediction of protein structures. *Protein Sci.* **15**, 2507–2524 (2006).

65. Swendsen, R. H. & Wang, J. S. Replica Monte Carlo simulation of spin glasses. *Phys. Rev. Lett.* **57**, 2607–2609 (1986).

66. Viswanath, S., Chemmama, I. E., Cimermancic, P. & Sali, A. Assessing exhaustiveness of stochastic sampling for integrative modeling of macromolecular structures. *Biophys. J.* **113**, 2344–2353 (2017).

67. Chodera, J. D. A simple method for automated equilibration detection in molecular simulations. *J. Chem. Theory Comput.* **12**, 1799–1805 (2016).

68. Merkley, E. D. et al. Distance restraints from crosslinking mass spectrometry: mining a molecular dynamics simulation database to evaluate lysine–lysine distances. *Protein Sci.* **23**, 747–759 (2014).

69. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).

Acknowledgements

We thank Y. Xiong (Yale University) for helpful discussions and valuable input. We thank the beamline staff at the Advanced Photon Source beamline 24-ID and the National Synchrotron Light Source beamline 17-ID. We thank J. Bonifacino (National Institutes of Health (NIH)) for providing the gene of rat αadaptin. This work was supported by the University of Massachusetts Dartmouth startup fund (X.J.) and US NIH grants no. AI102778 and no. AI129706 (J.G.). R.M.K., I.E., A.S. and N.K. were supported by NIH grant no. P50AI150476. R.M.K. was also supported by NIH fellowship grant no. F32AI127291. A.S. was also supported by NIH grants no. U19AI135990, no. R01GM083960, no. P41GM109824 and no. S10OD021596. N.K. was also supported by NIH grants no. P50GM082250 and no. U19AI135990.

Author contributions

Y.K. performed protein expression, purification, binding assays and crystallization. Y.K. and X.J. performed data collection, structure determination, model building and refinement. M.S., C.S. and P.W.R. performed CD4 and MHC-I downregulation assays and mutagenesis. R.M.K. performed XL-MS. I.E. performed integrative modeling. Y.K. and M.K.S. performed in vitro mutagenesis and fluorescence polarization assays. J.G. and R.S. contributed to protein expression and purification. Y.K., R.M.K., I.E., A.S., N.K., J.G. and X.J. designed the experiments. All contributed to data analysis. J.G. and X.J. supervised the project. Y.K., J.G. and X.J. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41594-020-0463-z.

Supplementary information is available for this paper at https://doi.org/10.1038/s41594-020-0463-z.

Correspondence and requests for materials should be addressed to X.J.

Peer review information Peer reviewer reports are available. Inês Chen was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | Electron density map for the N-terminal loop of Nef. 2Fo-Fc map (1σ level with B factor sharpened by \(-50 \, \text{Å}^2\)) for Nef residues 34–40 and 47-75 is shown as black mesh. Nef residues 41-46 could not be built due to the lack of density. Density for Nef 34-40 is less defined, although sidechain density for Leu37 is clear.
Extended Data Fig. 2 | β2 subunit, if intact, would clash with the bound Nef. Overlay of the intact β2 subunit (dark red, PDB 2XA7) with β2 in the current structure (green) indicates that clashing would take place between Nef and N-terminus of the intact β2, specifically residues Asn10, Lys11, Lys12, and Gly13 (spheres).
Extended Data Fig. 3 | Crosslinking mass spectrometry and integrative structure modeling of the Nef-CD4Δμ2-CTD complex. a, Overview of the DSSO XL-MS³ analysis method. b, EX-Circos linkage map of all Nef-CD4Δμ2-AP2apos;Δ2-CTD interlinks. c, Integrative structure of the Nef-CD4Δμ2-AP2apos;Δ2-CTD complex. The localization probability density of the ensemble of structures is shown with representative (centroid) structure from the ensemble embedded within it. Regions present in the crystal structure are shown as ribbons and segments not present in the crystal structure are shown as beads. d, Histogram showing the distribution of the cross-linked Cα–Cα distances in the integrative structure. The structural ensemble satisfies 89% of the XLs used to compute it. e, RMSD between rigid-bodies in the model ensemble. The vertical axis corresponds to the rigid body used as reference for superimposition and the horizontal axis are the rigid bodies for which the average RMSD was computed. f, Detail of crosslinks mapped to Nef. Satisfied and violated crosslinks shown in green and pink, respectively. g, Positioning of the unfolded β2 segment.
Extended Data Fig. 4 | Binding of Nef N-terminal helix to the Nef core is incompatible with CD4 downregulation. N-terminal helix of Nef (8-23) is modeled into the current structure. Red dotted line represents the would-be distance between residues 23 and 34, which cannot be covered by ten residues (Nef 24-33).
Extended Data Fig. 5 | Nef residues at the CD4-binding pocket are highly conserved. Nef sequences from HIV sequence compendium 2017 were analyzed through multiple sequence alignment (HIV sequence database, www.hiv.lanl.gov). Alignment was done in HXB2 convention (bottom) and important residues are additionally labeled using the NL4.3 convention on top. D123, shown in red text, is important for both CD4 and MHC-I downregulation. Other residues important for CD4 downregulation are in cyan and black texts. Black texts refer to residues, in addition to D123, that surround CD4. Other residues important for MHC-I downregulation are in orange. The logo representation, with the height of each letter proportional to the observed frequency of the corresponding amino acid residue, was generated by WebLogo70.

70. Crooks, G. E., Hon, G., Chandonia, J. M. & Brenner, S. E. WebLogo: a sequence logo generator. Genome Res. 14, 1188–1190 (2004).
Extended Data Fig. 6 | The unique conformation of Nef N-terminal loop observed in the current structure is incompatible with Nef dimerization. Nef in current conformation (cyan, cartoon) is overlaid with the SH2-SH3-dependent Nef dimer\(^{39}\) (dark blue and red envelopes, PDB 4U5W). While majority of Nef in the current structure overlays well with the Nef protomer shown as the dark blue envelope, the N-terminal region of Nef (circled) intrudes severely into the volume of the other Nef protomer (red envelope).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a
- Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, χ²) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Provide a description of all commercial, open source and custom code used to collect the data in this study, specifying the version used OR state that no software was used.

Data analysis
Provide a description of all commercial, open source and custom code used to analyse the data in this study, specifying the version used OR state that no software was used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The coordinates and structural factors for the crystal structure have been deposited at the Protein Data Bank (PDB) with the accession code 6URI.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- Sample size: n/a
- Data exclusions: n/a
- Replication: n/a
- Randomization: n/a
- Blinding: n/a

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

- n/a Involved in the study
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

### Methods

- n/a Involved in the study
- ChiP seq
- Flow cytometry
- MRI-based neuroimaging

### Antibodies

- Antibodies used: anti-human CD4 (Biolegend, San Diego CA, USA); anti-HLA-A2 (Biolegend, San Diego, CA, USA).
- Validation: per manufacturer

### Eukaryotic cell lines

- Cell line source(s): HeLa-TZM-bl (Dr. John Kappes via the NIH AIDS Reagent Program) and HEK293 (Dr. Celso Spina, UCSD originally from Dr. Joseph Nevins, Duke University).
- Authentication: not authenticated
- Mycoplasma contamination: excluded by PCR assay
- Commonly misidentified lines (See [IGAC register](http://igac.org)): Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

### Flow Cytometry

**Plots**

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.
### Methodology

**Sample preparation**
Cells (HEK293 or HeLa-TZM-bi) were removed from the plates and incubated with APC-conjugated antibody to HLA-A2 (HEK293) or APC-conjugated antibody to CD4 (HeLa-TZM-bi) or APC-conjugated antibody isotype controls. After washing to remove unbound antibody, the cells were fixed with 1% formaldehyde before analysis.

**Instrument**
Accuri A6 benchtop cytometer.

**Software**
CFlow Plus.

**Cell population abundance**
The "live cell gate" was determined empirically based on forward scatter and side-scatter characteristics using cells that were not transfected but were fixed with 1% formaldehyde. 10,000 events (cells) in the live cell gate were used to generate the two-color data presented (CD4-APC or HLA-A2-APC vs. GFP).

**Gating strategy**
The live cell gates were determined using non-transfected but formaldehyde-fixed fixed cells and were applied to all the transfected cell populations. The boundaries for GFP-positive cells were set using cells not transfected to express GFP. The boundaries for CD4-positive or HLA A2-positive cells were set using APC-conjugated antibody isotype controls.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.