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The inability to disrupt the immunological synapse between infected human T cells and APCs distinguishes HIV-1 from most other primate lentiviruses

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Viruses that infect T cells, including those of the lentivirus genus, such as HIV-1, modulate the responsiveness of infected T cells to stimulation by interacting APCs in a manner that renders the T cells more permissive for viral replication. HIV-1 and other primate lentiviruses use their Nef proteins to manipulate the T cell/APC contact zone, the immunological synapse (IS). It is known that primate lentiviral Nef proteins differ substantially in their ability to modulate cell surface expression of the TCR-CD3 and CD28 receptors critical for the formation and function of the IS. However, the impact of these differences in Nef function on the interaction and communication between virally infected T cells and primary APCs has not been investigated. Here we have used primary human cells to show that Nef proteins encoded by HIV-2 and most SIVs, which downmodulate cell surface expression of TCR-CD3, disrupt formation of the IS between infected T cells and Ag-presenting macrophages or DCs. In contrast, *nef* alleles from HIV-1 and its simian precursor SIVcpz failed to suppress synapse formation and events downstream of TCR signaling. Our data suggest that most primate lentiviruses disrupt communication between virally infected CD4+ Th cells and APCs, whereas HIV-1 and its SIV precursor have largely lost this capability. The resulting differences in the levels of T cell activation and apoptosis may play a role in the pathogenesis of AIDS.

Introduction

The interaction and communication of T cells and APCs via a contact zone known as immunological synapse (IS) is a key event of the adaptive immune response (1, 2). Synapse formation is a highly ordered process initiated by the binding of the TCR-CD3 complex on T cells to the peptide-MHC complex on the surface of APCs, such as DCs or macrophages (3). This interaction leads to the local clustering of various surface receptors, adhesion molecules, signaling factors, and cytoskeleton components into a well-organized supramolecular spatial structure. Besides TCR-CD3/peptide-MHC binding, a functional activating IS also requires the interaction between costimulatory molecules, such as CD28 on CD4+ T cells and CD80 or CD86 on professional APCs (3, 4). The CD4 molecule on T cells, which is the primary receptor for HIV/SIV entry, is involved in the clustering process and may boost recognition of the MHC-peptide ligand by the TCR (5, 6). Deregulation of T cell/APC communication leads to immunological malfunctions, such as autoimmunity or tolerance (7).

Given that the IS is crucial for the initiation and regulation of adaptive immune responses, it is not surprising that various viruses manipulate it to suppress specific antiviral immune responses and hence to facilitate viral immune evasion (8–11). Lymphotropic viruses also modulate the responsiveness of virally infected T cells to stimulation by interacting APCs in a manner that renders them more permissive for viral replication (12–14). HIV-1 and other primate lentiviruses manipulate the function of the IS and T cell activation mainly by means of the accessory multifunctional Nef protein, which also impairs MHC Ag presentation and enhances viral infectivity and replication (14–16). Most importantly, Nef is required for efficient viral replication in vivo and greatly accelerates disease progression in HIV-1–infected individuals (17, 18). The HIV-1 Nef protein perturbs the interaction of virally infected T cells with APCs by downmodulating CD4 and (weakly) CD28 from the cell surface (19–21) and was reported to alter TCR trafficking and signaling, as well as the actin rearrangements induced by TCR triggering (11). It has been suggested that these Nef effects could be advantageous for HIV-1 because they reduce TCR-mediated T cell activation to levels sufficient for effective viral replication, but low enough to prevent exaggerated activation and premature activation–induced death of HIV-1–infected T cells (13, 14). Contrary to this suggestion, however, other studies have shown that Nef substantially enhances the responsiveness of T cells to stimulation and proposed that the hyperactivated phenotype of HIV-1–infected T cells may contribute to the high levels of chronic immune activation and apoptosis that are associated with progression to AIDS (22–26).

While it remains debatable whether HIV-1 Nefs reduce or enhance the responsiveness of virally infected T cells to stimulation, it is clear that the effects of most primate lentiviral Nef proteins on TCR signaling and potentially also on the formation of the IS are much more severe than those of HIV-1 Nef proteins, because they

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also downmodulate TCR-CD3 and are substantially more effective in removing CD28 from the cell surface (20, 21, 27–30). Notably, the ability of Nef to downmodulate TCR-CD3 was specifically lost in the primate lentiviral lineage that gave rise to HIV-1: SIVcpz, its direct precursor from chimpanzees and some closely related SIVs from *Cercopithecus* monkeys (30). HIV-2 or SIV *nef* alleles that downmodulate CD3 block the responsiveness of virally infected primary T cells to TCR-mediated activation, whereas those of HIV-1 and its simian precursors fail to suppress T cell activation and programmed cell death (30). Besides host factors, these differences in Nef function may explain why high levels of chronic immune activation, associated with accelerated T cell turnover rates and apoptosis, are a hallmark of pathogenic HIV-1 infection but are absent in natural nonpathogenic SIV infections (15, 31).

The differential effects of HIV-1 and other primate lentiviruses on TCR-mediated stimulation of virally infected T cells by APCs may play an important role in the infection-associated levels of immune activation that drive the exhaustion of the immune system. Despite the possible importance of these processes in the pathogenesis of AIDS, however, the influence of various primate lentiviral Nef proteins on the formation and function of the IS between primary virally infected T cells and professional APCs has not been investigated. In the present study, we examined the effect of *nef* alleles from HIV-1 and other primate lentiviruses on the interaction of primary virally infected T lymphocytes with DCs — which are professional APCs and especially potent activators of CD4+ helper T cells — and macrophages. We found that HIV-2 and SIV Nefs that downmodulate TCR-CD3 disrupted the formation of T cell/APC conjugates. The few interactions observed did not represent a functional IS. In contrast, Nef proteins of HIV-1 and its simian counterpart, SIVcpz, neither significantly reduced the capacity of virally infected T lymphocytes to form an IS nor prevented T cell activation by APCs. These differences in the ability of Nef to modulate the formation of the IS and TCR signaling may have important consequences for the antiviral immune response and the pathogenic outcome of primate lentiviral infections.

Results

Nef alleles from HIV-2 and SIVmac — but not those of HIV-1 and SIVcpz — prevent the formation of conjugates between virally infected T cells and APCs. Our previous analysis of a large number of primate lentiviral Nefs showed that they can be divided into those that are unable and those that are able to downmodulate TCR-CD3 (groups 1 and 2, respectively; ref. 30). To examine possible differences in their effect on the IS, we selected 3 representative *nef* alleles from each group (see Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI38994DS1). The alleles of group 1 were the HIV-1 NA7 *nef* allele, derived from an AIDS patient (32), the *nef* gene of the NL4-3 molecular clone, and the SIVcpz GAB2 *nef*, obtained from a wild-captured chimpanzee of the *Pan troglodytes troglodytes* subspecies in Gabon (33). The group 2 *nef*s were derived from HIV-2 BEN, the SIVmac239 molecular clone, and SIVblu from a Blue monkey (*Cercopithecus mitis*). All 6 *nef* alleles have been characterized in previous studies (29, 30, 34, 35) and were chosen because they are functionally active and represent the 2 human AIDS viruses (HIV-1 and HIV-2), their simian counterparts (SIVcpz and SIVmac), and a naturally SIV-infected small monkey species (SIVblu). The SIVmac239 and SIVblu Nefs were also selected because of the availability of specific mutants that are selectively active (mac tNef) or defective (SIVblu RR-AA; also referred to as bluRR) in TCR-CD3 modulation (30, 36).

To examine the effect on the IS, we cocultivated primary PBLs infected with proviral HIV-1 constructs coexpressing eGFP and Nef from bicistronic RNAs (30, 34) with autologous monocyte-derived macrophages (MDMs) or DCs pulsed with the superantigen (sAg) SEE and labeled with the aliphatic red dye PKH26. Notably, the proviral HIV-1 constructs differ only in their *nef* coding region and coexpress Nef and eGFP at correlating levels (30, 34). We used replication-competent VSV-G–pseudotyped viruses for infection because this allowed us to bypass the effect of Nef on virion infectivity (37) and excluded the possibility that lack of Env expression may bias the results. Prior to all interaction studies, we verified that comparably high numbers of cells were infected by all HIV-1 constructs (example shown in Supplemental Figure 1). The differential capability of the HIV-1 and SIV *nef* alleles to downmodulate TCR-CD3 was verified for virally infected CD4+ T cells (Supplemental Table 2) as well as PBLs, and the phenotype of the DC and MDM preparations was verified by flow cytometric analysis (example shown in Supplemental Figure 2).

First, we assessed the proportion of HIV-1–infected (eGFP+) PBLs contacting APCs by standard fluorescence microscopy (Figure 1A). Examination of eGFP+ PBLs from randomly selected areas of the cocultures demonstrated that the HIV-1 NA7 *nef* allele (33) did not significantly reduce the frequency of contacts with Ag-presenting MDMs or DCs compared with the *nef*-defective control (Figure 1A). In both cases, the number of T cell/APC conjugates was similar to that observed between noninfected CFSE-labeled PBLs and autologous SEE-pulsed APCs (data not shown). Similar results were obtained with the NL4-3 and GAB2 Nefs (Figure 1A). In contrast, expression of the SIVmac239, SIVblu, and HIV-2 BEN Nefs reduced the number of conjugates between virally infected lymphocytes and APCs about 4-fold (Figure 1A), down to the background levels obtained with medium-pulsed APCs (data not shown). To verify this finding, we also determined the frequency of HIV-1–infected PBLs engaged in contacts with APCs by flow cytometry for cells in the MDM and DC gate that were positive for both eGFP and PKH26 (Figure 1B). In agreement with the results of the microscopic examination, HIV-2 and SIVmac Nefs, but not HIV-1 and SIVcpz Nefs, significantly impaired the capability of virally infected T cells to interact with APCs (Figure 1B). These data demonstrated that, in strict contrast to group 1 Nefs, those of group 2 viruses did not disrupt the formation of conjugates between primary T cells and APCs (summarized in Supplemental Table 2).

Nef-mediated downmodulation of TCR-CD3 plays a key role in the disruption of the IS. To assess a possible causal relationship between Nef-mediated TCR-CD3 downmodulation and the disruption of the IS, we used previously described mutant Nefs that are selectively active or disrupted in this function (Supplemental Table 1 and ref. 30). Microscopic examination and flow cytometric analysis showed that a truncated form of the SIVmac239 *nef* gene, tNef, which downmodulates TCR-CD3 but is otherwise defective (30, 36), was still capable of reducing the number of contacts between virally infected lymphocytes and Ag-presenting MDMs and DCs (Figure 1). Conversely, 2 point mutations of RR/AA in the SIVblu Nef that specifically abrogate downmodulation of TCR-CD3 also reduced its ability to prevent the interaction between HIV-1–infected PBLs and Ag-presenting MDMs or DCs (Figure 1). However, the potency

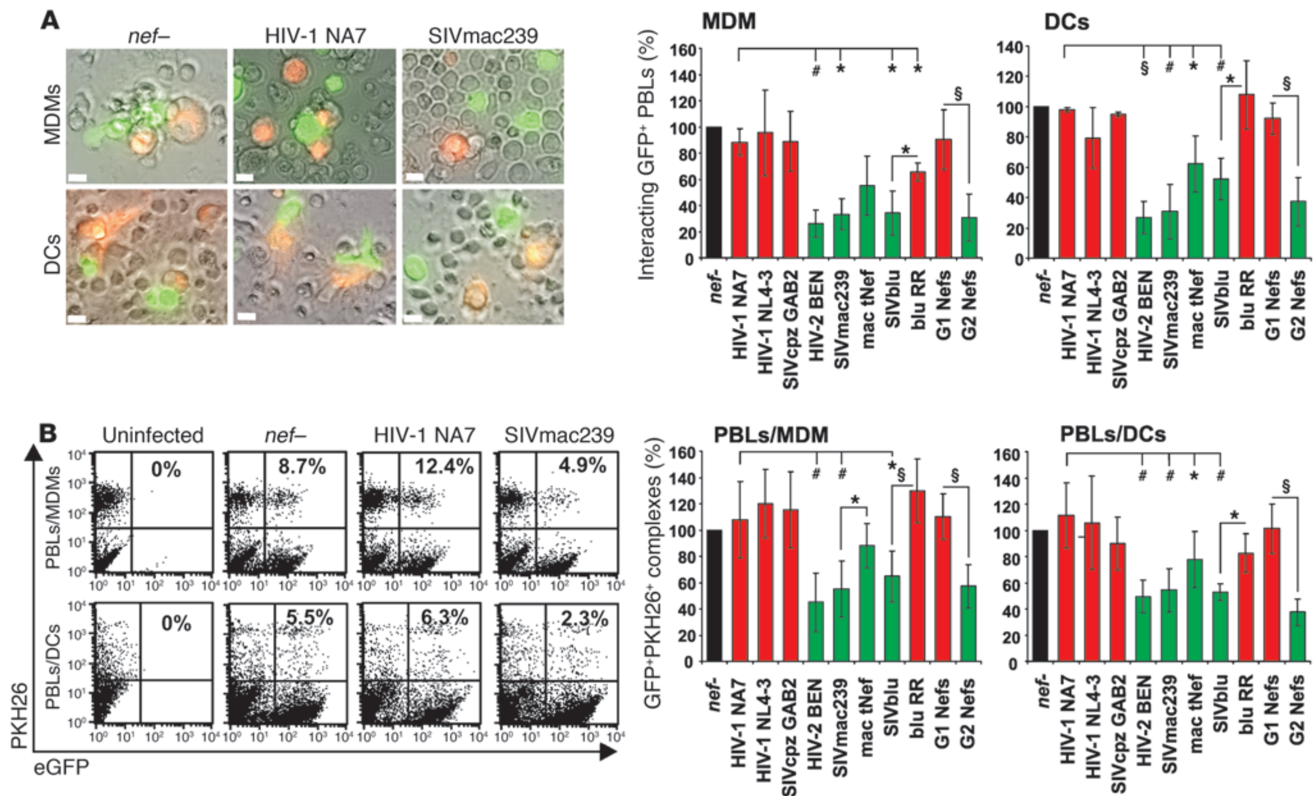

Figure 1

Nef alleles that downmodulate TCR-CD3 impair the ability of virally infected primary T cells to form complexes with APCs. (**A** and **B**) PBLs were infected with NL4-3–IRES–eGFP viruses encoding different Nefs, and complex formation with autologous SEE-pulsed DCs and MDMs was assessed 4 days after infection by fluorescence microscopy (**A**) and flow cytometry (**B**). (**A**) Representative acquisition frames of cocultures between infected PBLs and MDMs or DCs, with phase-contrast (Nomarski), eGFP, and PKH26 superimposed. Scale bars: 10 μm. The proportion of infected (eGFP⁺) cells contacting labeled autologous MDMs or DCs was scored randomly by single-blind method with $n = 60$ per sample per experiment. Results are mean ± SD of 4–5 independent experiments. In all experiments, 25%–55% of PBLs infected with the *nef*-defective control HIV-1 construct (*nef*–) formed complexes with autologous APCs; *nef*– values are set as 100%. (**B**) Representative flow cytometry acquisitions for complex formation between infected PBLs and MDMs or DCs. Percentages denote the number of eGFP⁺ cells in the DC gating that are PKH26⁺ (as a percentage of all eGFP⁺ cells). Graphs show mean ± SD of 3 and 4 experiments in DCs and MDMs, respectively. In all experiments, 3%–13% of PBLs infected with *nef*– formed complexes with autologous APCs; *nef*– values are set as 100%. Red and green bars denote *nef* alleles of group 1 and group 2, respectively. Combined results for group 1 (G1) and group 2 (G2) Nefs represent average values ± SD. $^{*}P < 0.05$; $^{\#}P < 0.01$; $^{\S}P < 0.001$.

of the tNef in disrupting the IS was slightly reduced compared with the wild-type SIVmac239 Nef, and the SIVblu RR-AA Nef frequently showed a phenotype intermediate between the HIV-1 and the parental SIVblu Nefs. This observation may be explained by the fact that, in contrast to wild-type mac239 Nef, the truncated tNef did not downmodulate CD28, and that SIVblu RR-AA Nef was more active than HIV-1 NA7 Nef in removing this costimulatory factor from the cell surface (Supplemental Table 1). Although CD28 is not required to initiate the contact between T cells and APCs, its presence at the cell surface may affect the strength and duration of this interaction (3, 4). Correlation analyses showed that all Nefs had similar disruptive effects on the interaction of infected PBLs with MDMs or DCs and verified that microscopic examination and flow cytometric analyses yielded similar results (Supplemental Figure 3). The reduction of conjugate formation between virally infected PBLs and APCs correlated with the efficiency of Nef-mediated downmodulation of TCR-CD3 (Supplemental Figure 4). Together, these data showed that Nef-induced TCR-CD3 downmodulation was required and sufficient to disrupt synapse formation, but further suggested that the effect of Nef on CD28 also affects the functional interaction between virally infected T cells and APCs.

Only Nef alleles that do not downmodulate TCR-CD3 allow the formation of actual ISs. The formation of an intact IS is associated with the clustering of surface receptors into focal points of supramolecular interacting signaling platforms, referred to as SMACs (1–4). Some molecules — such as TCR-CD3 and the tyrosine kinase Lck, which becomes transiently activated after TCR engagement — are recruited to the central SMAC, whereas others — like the adhesion factor LFA-1 and the cytoskeletal protein talin — are enriched in the peripheral SMAC. Finally, some factors, such as the large sialoprotein CD43, are actively excluded from a functional IS (38). To assess whether the conjugates between HIV-1–infected PBLs expressing various *nef* alleles and APCs represent real ISs, we labeled cocultures of purified CD4⁺ T cells infected with various HIV-1 IRES-eGFP *nef* constructs and autologous sAg-pulsed DCs with CD3, Lck, talin, or LFA-1 and examined them by scanning confocal laser microscopy. These analyses confirmed that T cells expressing the HIV-2 BEN,

Figure 2
Effect of primate lentiviral Nefs on TCR-CD3 cell surface expression and clustering at the IS. Primary CD4+ T cells were not infected (n.i.) or were infected with HIV-1 constructs expressing the indicated *nef* alleles. At 3 days after infection, the T cells were incubated with autologous sAg-pulsed DCs, fixed, and stained with anti-CD3. Images show representative confocal acquisitions from cocultures between infected CD4+ T cells and autologous sAg-pulsed DCs labeled with DAPI. Shown are CD3 Ab labeling alone and merged images of eGFP, DAPI, and CD3. Arrowheads point to zones of close contact between infected T cells and DCs. Close contact was rarely observed in T cell/APC cultures infected with viral constructs expressing group 1 Nefs, and these cells did not express CD3 at their surface. Scale bars: 5 μm.

SIVmac239, tNef, and SIVblu Nefs did not express CD3 at the cell surface or at the IS (Figure 2). In comparison, we observed the typical clustering of CD3 at the contact zones between virally infected T cells and APCs for Nefs unable to downmodulate TCR-CD3 (Figure 2). Furthermore, only PBLs expressing HIV-1 or SIVcpz *nef* alleles showed normal frequencies of Lck polarization at the zone of contact with APCs (Figure 3). Thus, the group 2 Nefs affected the targeting of Lck to the IS much more severely than did those of group 1, although we noted that cells infected with virus constructs expressing HIV-1 or SIVcpz Nefs showed a moderate decrease of Lck at the IS and higher quantities of Lck in intracellular compartments than uninfected cells (Figure 3A). Next, we examined the effect of the various Nef alleles on the localization of talin, a large cytoskeletal adaptor protein that is a ubiquitously expressed and thought to be critical for the activation of integrins (39). We found that only *nef* alleles that downmodulate TCR-CD3 prevented the polarization of talin at the zone of contact between virally infected T cells and APCs (Figure 4). The differences in the effect of group 1 and group 2 Nefs on Lck or talin recruitment to the synapse were highly significant (Figure 3B and Figure 4B). Notably, none of the *nef* alleles had a marked impact on the number of T cell/APC conjugates showing polarization of LFA-1 (Supplemental Figure 5). LFA-1 functions as an adhesion molecule by binding to ICAM-1 on APCs (40) and may not be affected because it is involved in mediating the initial contact rather than the formation of a mature IS. Finally, we found that only conjugates between T cells infected with HIV-1 constructs incapable of downmodulating CD3 and autologous DCs showed CD43 exclusion (Figure 5). These analyses showed that HIV-1–infected PBLs expressing *nef* alleles unable to downmodulate TCR-CD3 were generally capable of forming ISs with APCs, as demonstrated by CD3, Lck, and talin polarization to the zone of contract, as well as by CD43 exclusion. In striking

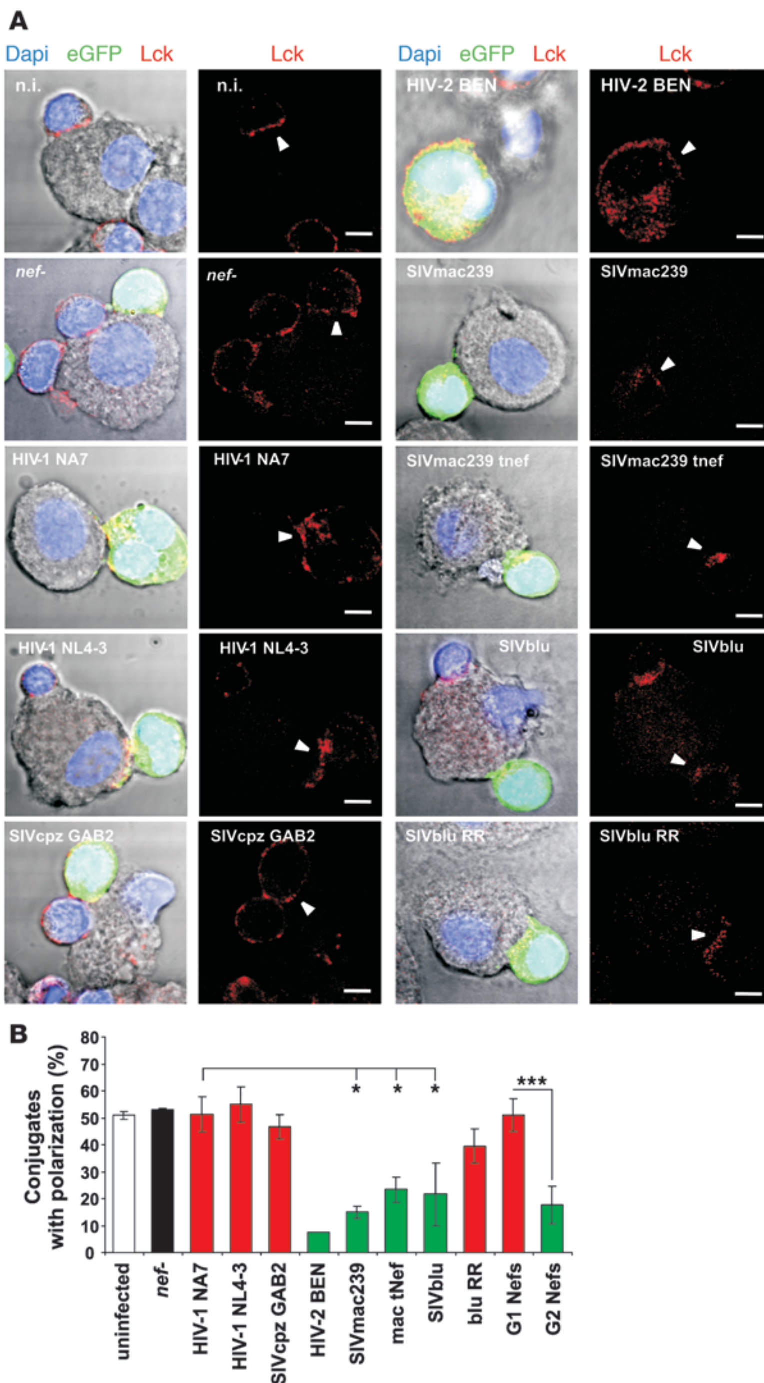

Figure 3
Nef-mediated downmodulation of TCR-CD3 prevents Lck polarization at the IS. (**A**) CD4+ T cells infected with HIV-1 constructs expressing the indicated *nef* alleles were incubated with autologous sAg-pulsed DCs, fixed, and stained with anti-Lck. Images show representative confocal acquisitions from cocultures between infected CD4+ T cells and autologous sAg-pulsed DCs labeled with DAPI. Shown are Lck Ab labeling alone and merged images of eGFP, DAPI, and Lck. Arrowheads point to zones of close contact between infected T cells and DCs. Scale bars: 5 μm. (**B**) Number of HIV-1–infected eGFP+ T cells engaged in complex formation with APCs, showing accumulation of Lck at the IS. Results are mean ± SD of 2 independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$.

contrast, conjugates between T cells infected with viral constructs capable of downmodulating CD3 and autologous DCs did not usually show an accumulation of synapse markers at the site of contact. Thus, even the few T cell/APC conjugates that were detected by microscopy and FACS analysis (Figure 1) did not represent real ISs. Our finding that tNef was sufficient to impair the clustering of Lck and talin or the exclusion of CD43 at the T cell/APC contact zone and that the RR-AA mutation severely reduced the disruptive effect of the SIVblu Nef, as well as correlation analyses (Supplemental Figure 4), support a key role of Nef-mediated downmodulation of CD3 in the disruption of ISs.

The HIV-1 Nef lost its ability to block early TCR-CD3 signaling events. It was previously reported that the primary target cells of HIV and SIV infection in vivo are memory CD4+ T cells that are phenotypically resting but represent a recently activated cell population (41, 42). To mimic this phenotype in vitro, we infected PHA-stimulated PBLs with HIV-1 Nef-IRES-eGFP constructs and incubated them in the absence of exogenous stimuli until they expressed low levels of activation markers and eGFP (data not shown). Thereafter, we exposed the cells for 15 minutes to anti-CD3/CD28 beads, which crosslink the TCR with CD28 (43) and hence resemble stimulation by APCs. To assess the ability of virally infected T cells to respond to stimulation, we measured the phosphorylation of the signaling molecule ZAP-70, which occurs within seconds to minutes of T cell/APC interaction and TCR triggering (44). We used an antibody that recognizes ZAP-70 phosphorylated at position Tyr493, previously reported to represent the primary site of TCR-induced tyrosine phosphorylation by Lck (45). Flow cytometric analysis showed that ZAP-70 phosphorylation was prevented by HIV-2, SIVmac, and SIVblu Nef proteins, whereas PBLs infected with viral constructs expressing HIV-1 or SIVcpz Nefs showed levels of ZAP-70 phosphorylation similar to those infected with the *nef*-defective control HIV-1 construct (Figure 6A). The SIVmac tNef and SIVblu RR-AA Nef alleles were usually both poorly active in inhibiting the induction of ZAP-70 phosphorylation. Thus, Nef-mediated downmodulation of both TCR-CD3 and CD28 was required to efficiently disrupt early TCR signaling events, at least upon high-affinity TCR ligation by anti-CD3/CD28 beads. Kinetic analyses showed that Nef alleles that downmodulate CD3 did not exhibit delayed ZAP-70 phosphorylation (Figure 6B). These results support that the group 2 Nefs prevent, not just delay, early TCR-CD3 signaling events.

Efficient Nef-mediated downmodulation of TCR-CD3 and CD28 suppresses late TCR-CD3 signaling events. The activating signaling cascades initiated by IS formation ultimately lead to the activation of transcription factors that drive cytokine expression. To test the effect of various Nef alleles on these late signaling events, we cocultivated HIV-1–infected T cells with autologous primary DCs

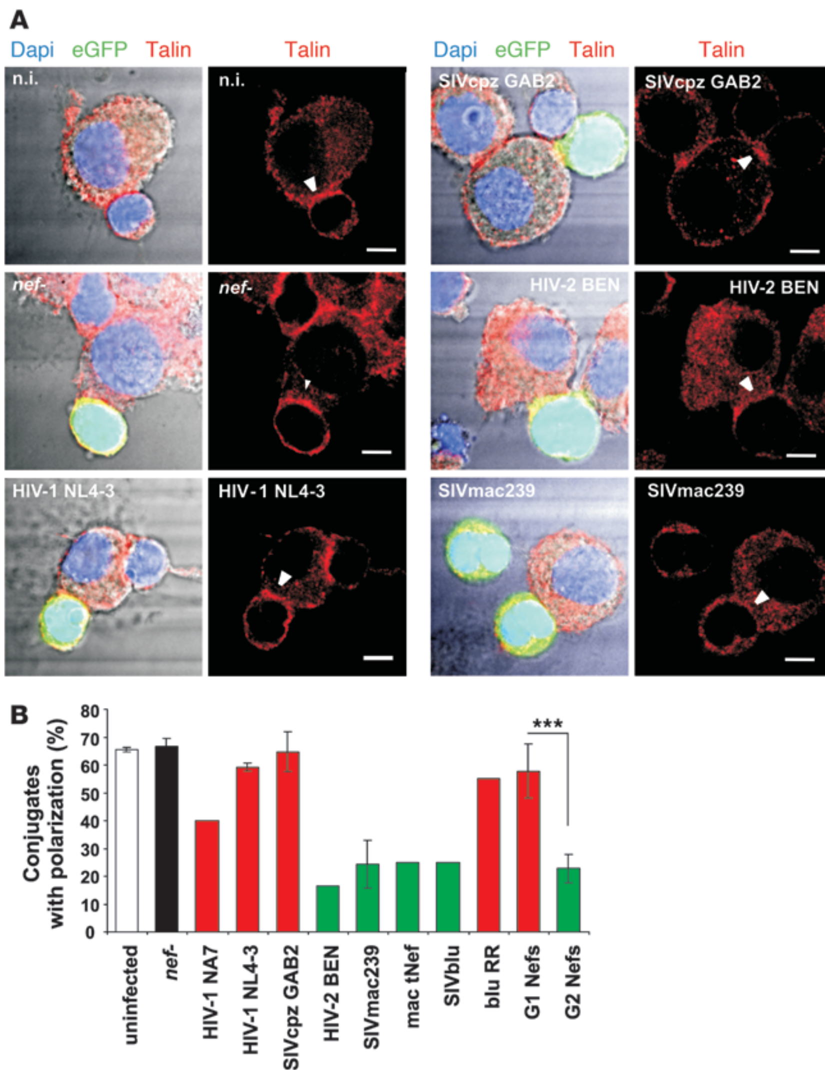

Figure 4

Nef alleles that downmodulate CD3 inhibit talin clustering at the IS. (**A**) CD4+ T cells infected with HIV-1 constructs expressing the indicated *nef* alleles were incubated with autologous sAg-pulsed DCs, fixed, and stained with anti-talin. Images show representative confocal acquisitions from cocultures between infected CD4+ T cells and autologous sAg-pulsed DCs labeled with DAPI. Shown are talin Ab labeling alone and merged images of eGFP, DAPI, and talin. Arrowheads point to zones of close contact between infected T cells and DCs. Scale bars: 5 μm. (**B**) Number of HIV-1–infected eGFP+ T cells engaged in complex formation with APCs, showing polarization of talin at the zone of contact. Results are mean ± SD of 2 independent experiments (except for NA7, tNef, and SIVblu Nefs, which were derived from a single experiment). ***$P < 0.001$.

or MDMs and measured the frequency of virally infected T cells that subsequently showed increased levels of intracellular IL-2 by flow cytometric analysis. We focused on IL-2 because it is one of the best-characterized cytokines and known to play a crucial role in regulating immune activation and homeostasis (46). We found that HIV-1 and SIVcpz Nef expression resulted in unaltered or slightly increased numbers of IL-2–expressing HIV-1–infected T cells after cocultivation with DCs or MDMs (Figure 7). In contrast, expression of HIV-2 and SIVmac239 Nefs was associated with decreased numbers of IL-2+ virally infected T cells compared with the *nef*-defective control. The difference between group 1 and group 2 Nefs was highly significant in both PBL/DC and PBL/MDM cocultures (Figure 7). Our set of *nef* alleles also allowed us to assess the role of Nef-mediated downmodulation of TCR-CD3 and CD28 in late TCR signaling events. The tNef, which downmodulates CD3 but not CD28 (Supplemental Table 1), suppressed the induction of IL-2 production as efficiently as the parental SIVmac239 Nef (Figure 7). The SIVblu RR-AA Nef, which shows essentially the opposite functional properties because it does not affect CD3 but efficiently removes CD28 from the cell surface, also suppressed IL-2 expression (Figure 7). This was expected, because costimulatory signaling through CD28 is known to play an essential role in TCR-mediated IL-2 production through activation of NF-κB (47, 48). The disruptive effect of the SIVblu RR-AA Nef was particularly pronounced in PBL/DC cocultures, possibly because IS of T lymphocytes with DCs and macrophages differ in their dynamics and signaling intensity (49). Notably, the efficient induction of IL-2 in virally infected T cells expressing HIV-1 and SIVcpz Nefs shows that the relatively modest downmodulation of CD28 by these Nef alleles is insufficient to suppress late TCR-signaling events. Taken together, our results showed that efficient Nef-mediated downmodulation of both TCR-CD3 and CD28 was sufficient to impair late TCR-signaling events. The differential levels of induction and secretion of IL-2 and other cytokines may impact uninfected bystander cells and thus the overall levels of infection-associated immune activation.

Discussion

In the present study, we demonstrate that HIV-2, SIVmac, and SIVblu Nefs, but not those of HIV-1 and its simian precursor SIVcpz, strongly disrupted the formation and function of the IS. The availability of Nef mutants that are selectively active or defective in TCR-CD3 downmodulation revealed that this Nef function was sufficient and required to eliminate the capability of virally

Figure 5
Nef alleles that downmodulate TCR-CD3 impair CD43 exclusion at the IS. Images show representative confocal acquisitions from cocultures between infected PBLs and autologous SEE-pulsed DCs. Shown are CD43 Ab labeling alone and merged images of eGFP, PKH26, and CD43. Scale bars: 5 μm. Close contact was rarely observed in PBL/APC cultures infected with viral constructs expressing HIV-2 BEN or SIVblu Nefs, and occasional cell-cell complexes did not show CD43 exclusion, as determined by microscopic examination and analysis of the CD43 signal intensity along the plane of the cell-cell interface. The *x* axis represents distance along the contact zone; the *y* axis denotes pixel intensity. The location of 3–4 representative points per construct, and their corresponding intensities, are shown by the numbered arrowheads.

infected T lymphocytes to form functional synapses with APCs. It has been shown previously that Nef-mediated downmodulation of TCR-CD3 function was specifically lost in the primate lentiviral lineage that gave rise to HIV-1 (30). Taken together, these results clearly suggest that the inability to suppress TCR-mediated activation and programmed death of virally infected T cells by disrupting their interaction with APCs distinguishes HIV-1 and its simian precursors from most other primate lentiviruses.

It remains to be clarified why the primate lentiviral lineage that gave rise to HIV-1 evolved to become much less effective in disrupting the communication between infected helper $CD4^+$ T cells and APCs. Of note, primate lentiviruses containing *nef* alleles that are unable to downmodulate TCR-CD3 usually contain a *vpu* gene: HIV-1; its simian precursor, SIVcpz; and some closely related *Cercopithecus* viruses, whose ancestor recombined with that of SIVrcm in chimpanzees to become SIVcpz (30). The fact that CD3 downmodulation by Nef was actually lost twice during primate lentiviral evolution when the virus acquired a *vpu* gene (30) suggests that Vpu reduces the selective pressure for effective disruption of the IS and hence suppression of TCR-mediated T cell activation. Recently, it was shown that Vpu counteracts a host restriction factor, tetherin, that tethers nascent virions at the cell surface and is induced by IFN-α (50). Thus, we previously proposed that HIV-1 and its simian precursors could possibly afford to cause higher levels of T cell activation, and hence more effective transcription of the proviral genome, because the acquisition of *vpu* allowed efficient viral spread in a more inflammatory environment (51). Notably, recent data show that some SIVs that do not contain a *vpu* gene use Nef to antagonize tetherin (52, 53).

The observed disruption of interactions between infected T cells and APCs is concordant with the previously reported downmodulation of surface CD3 and CD28 by HIV-2 and SIV (27–30). Still, although HIV-1 Nef does not reduce TCR-CD3 surface expression (27–30) and is only poorly effective in downmodulating CD28 (20, 21), a previous study suggested that it also impairs IS formation (11). Thoulouze and coworkers reported that the HIV-1 Nef causes a 2- to 3-fold reduction of conjugate formation between HIV-1–infected T lymphocytes and sAg-pulsed Raji B cells, resulting in reduced TCR signaling (11). In comparison, we found that HIV-1 Nefs did not impair the

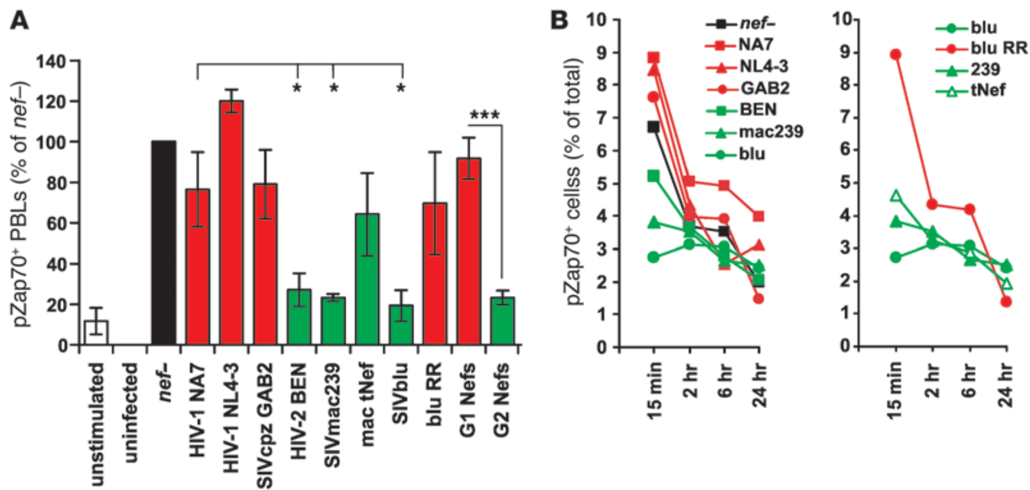

Figure 6
Downmodulation of IS formation perturbs early TCR-CD3 signaling events after coculture with APCs. PBLs were allowed to return to a less-activated state after infection by removal of all cytokines in the growth medium for 3 days. PBLs were stimulated with CD3/CD28 beads for the indicated times or left unstimulated, and then labeled for intracellular phospho–ZAP-70 kinase. (**A**) Percentage of pZAP-70+ infected cells, shown as mean ± SD of 3 independent experiments. In all experiments, 5%–20% of PBLs infected with *nef*– were pZAP-70+ after 15 minutes' stimulation with CD3/CD28 beads; *nef*– values are set as 100%. *$P < 0.05$; ***$P < 0.001$. (**B**) Kinetic of ZAP-70 phosphorylation in PBL cultures infected with HIV-1 constructs expressing the indicated *nef* alleles. Values represent percent ZAP-70+ cells and are representative of 2 independent experiments performed.

ability of virally infected T cells to form functional ISs with primary sAg-pulsed APCs. In part, this discrepancy may result from the use of different types of APCs: Raji B cells in the previous study (11) compared with primary DCs and macrophages in the present study. Although HIV-1 Nefs do not affect synapse formation, they certainly deregulate the communication between virally infected T cells and APCs by changing TCR-induced actin dynamics (13, 14) and by modulating downstream TCR signaling pathways (54, 55). Notably, we confirmed the previous finding (11) that HIV-1 Nef expression increased Lck accumulation in intracellular compartments (Figure 3). Whether HIV-1 Nefs reduce or increase the responsiveness of virally infected T cells to stimulation is a matter of debate (11–15, 22–26). We found that HIV-1 Nef expression in infected PBLs did not alter or even slightly enhance late signaling events, as measured by IL-2 production upon stimulation. Notably, we assessed this effect for the first time to our knowledge in the cell types that are most relevant for viral replication and Ag presentation in vivo and found that Nef proteins of other primate lentiviruses efficiently blocked the responsiveness of virally infected T cells to stimulation.

Because HIV/SIV gene expression and replication are dependent on the activation state of the infected T cells (56), our finding that most primate lentiviruses may prevent IS formation and TCR-mediated activation is at first surprising. Indeed, some primate lentiviruses that suppress rather than promote T cell activation may show lower replication rates in vivo. For instance, HIV-2–infected people show lower plasma viral RNA levels, but similar proviral loads, compared with those infected with HIV-1 (57). However, SIVagm and SIVsmm, which downmodulate TCR-CD3 and CD28 (30), achieve high viral loads and efficient replication in their natural monkey hosts (58), which indicates that the infected T cells express the factors required for effective proviral transcription. Thus, these viruses are capable of inducing levels of T cell activation that render infected host cells permissive for viral repli-

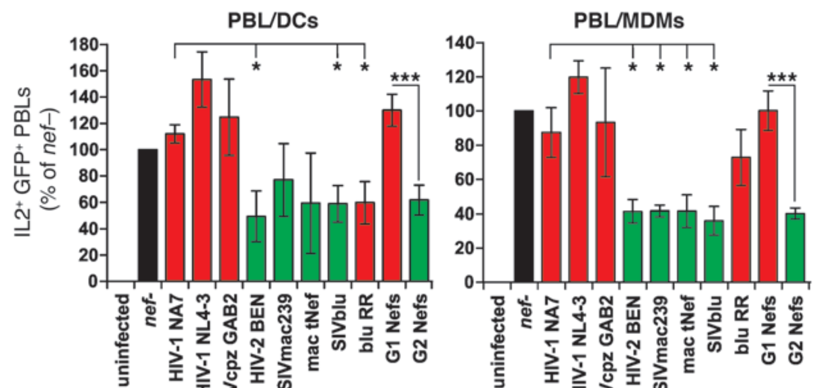

Figure 7
TCR-CD3 downmodulation leads to decreased IL-2 production after dynamic synapse interaction with primary APCs. Infected PBLs were cocultured with autologous SEE-pulsed DCs or MDMs for 16 hours and then labeled for intracellular IL-2. Shown is the percentage of IL-2+ cells in the infected PBL population; results are mean ± SD of 3 independent experiments. In all experiments, 2%–7% of PBLs infected with *nef*– stained positive for intracellular IL-2; *nef*– values are set as 100%. *$P < 0.05$; ***$P < 0.001$.

cation, independently of their interaction with APCs. Our current knowledge suggests several mechanisms by which SIVsmm and SIVagm may achieve this. First, these viruses replicate mainly in CCR5+ memory CD4+ T cells, which have had previous encounters with APCs and are thus at least partly activated. Second, Nef sensitizes infected T cells to stimulation by cytokines, such as IL-2 (59, 60). Thirdly, HIV and SIV Nefs themselves can trigger T cell signaling pathways reminiscent of TCR-mediated T cell activation (61, 62). Furthermore, other viral factors may stimulate key transcription factors; for example, it was previously shown that the viral transactivator Tat induces the expression of NF-κB, which binds to the core enhancer element in the HIV and SIV LTRs and plays an important role in viral gene expression (23). We also observed that Nef-mediated downmodulation of TCR-CD3 did not affect the levels of NF-κB expression in virally infected T cells (data not shown). Thus, uncoupling the stimulation of T cells from their interaction with APCs may allow some primate lentiviruses to more specifically activate the viral promoter. Finally, the disruption of the IS may be advantageous for the virus because it dampens the antiviral immune response and prolongs the period of virus production by delaying activation-induced death of infected T cells (14).

Some of the predicted consequences of IS disruption by primate lentiviruses — such as decelerated death of infected CD4+ T cells, reduced expression of cytokines and death receptors, and muted immune response — should not only be advantageous for the virus, but may also help the infected host to prevent the escalation of immune activation to harmfully high levels. In agreement with this hypothesis, SIVagm and SIVsmm, which downmodulate TCR-CD3 and CD28 (30), replicate efficiently in their natural monkey hosts without causing high-level immune activation or disease (31, 58). In contrast, SIVcpz, which has lost the ability to modulate CD3 (30) and to disrupt the IS, causes an AIDS-like immunopathology in naturally infected chimpanzees (63). It is noteworthy that inefficient CD3 downmodulation by Nef is associated with loss of CD4+ T cells even in naturally SIVsmm-infected sooty mangabeys (64). Thus, accumulating evidence supports that Nef-mediated downmodulation of surface TCR-CD3 and thus the impairment of IS formation contributes to the nonpathogenic phenotype of natural SIV infections and the attenuated pathogenicity of HIV-2. However, Nef-mediated suppression of T cell activation is clearly only one of several factors affecting the clinical outcome of infection. This is most obvious from the fact that effective downmodulation of TCR-CD3 and CD28 by Nef is insufficient to prevent disease in experimentally infected macaques that react with much higher levels of immune activation and T cell activation to SIV infection than the natural sooty mangabey host (65, 66).

In summary, our data show that primate lentiviruses exhibit striking differences in their capability to interfere with the formation and function of the IS and to alter the responsiveness of the infected T cells to TCR stimulation. Further studies are required to elucidate the importance of these distinct viral properties for the overall levels of immune activation and the associated high rates of T cell apoptosis and turnover that drive the progression to AIDS. Notably, most Nef functions that interfere with the formation and function of the IS — such as downmodulation of CXCR4, to inhibit the migration of T cells to APCs (67); of CD3, to prevent ligation and signaling (30); of CD28, to prevent costimulatory signals (20, 21); and of CD4, to limit signal transduction following TCR ligation (5, 6) — are all genetically separable. This separability may not only allow viruses to fine-tune their interaction with the host immune system, but also provide a means to study the role of these Nef activities in viral immune evasion and pathogenicity in appropriate animal models.

Methods

Virus stocks and transductions. To generate viral stocks, 293T cells were cotransfected with pBR–NL4-3–IRES–eGFP plasmids encoding various Nef alleles and a plasmid expressing the VSG-G, as described previously (30). The latter was used to achieve comparably high infection levels for functional analysis independently of Nef (37). Viral supernatants were collected 48 hours after transduction and used either immediately or within 24 hours of storage at 4°C to infect PBLs, or concentrated by ultracentrifugation through 20% (w/v) sucrose and stored at –80°C. For transduction, 1×10^6 prestimulated PBLs were infected with virus stocks containing 50 ng p24 capsid Ag, quantified using a capture assay provided by the NIH AIDS Research and Reference Reagent Program. After 2 or 3 days, infected cells were fixed, and the frequency and phenotype of HIV-1–infected GFP+ cells was determined by flow cytometry following staining with PE-conjugated CD3, PE-conjugated CD8, allophycocyanin-conjugated CD2, and PE-conjugated CD21 antibodies (all from BD Biosciences — Pharmingen) or with allophycocyanin-conjugated anti-CD4 (13B8.2, Immunotech).

Primary cell preparation. Citrate human blood was obtained from healthy donors at the Blood Donation Center of the University of Ulm. The study was reviewed and approved by the University of Ulm Institutional Review Board, and individuals provided informed consent prior to donating blood. Monocytes and PBLs were separated using adherence on plastic (45 minutes) after Ficoll gradient. PBLs were activated for 3 days with 1 μg/ml PHA and 10 ng/ml IL-2 prior to infection. Monocytes were differentiated to immature DCs by incubation for 5–7 days with 50 ng/ml recombinant human GM-CSF and 20 ng/ml IL-4 (Immunotools). Alternatively, monocytes were differentiated to macrophages by incubation for 7–10 days with 50 ng/ml GM-CSF alone. Phenotypic characterization prior to infection was carried out by flow cytometry. DCs were CD14– CD1a+CD80loCD86lo, and macrophages were CD14+CD1alo. For analysis of CD3, Lck, talin, and LFA-1 localization, buffy coats were obtained according to institutional guidelines of the ethical committee of the University of Geneva. Monocytes were purified after Ficoll gradient separation with CD14 MicroBeads (Miltenyi Biotec), and autologous CD4+ T lymphocytes were purified by negative selection with CD4+ T Cell Isolation kit II (Miltenyi Biotec). Purified autologous CD4+ T lymphocytes were greater than 95% CD3+ and greater than 95% CD4+. Before infection, medium was replaced by RPMI without PHA and IL-2. All primary cells used in the present study were of human origin.

Complex formation between PBLs and APCs. PBLs were infected with NL4-3–IRES–eGFP viruses encoding different *nef* alleles. At 4 days after infection, autologous macrophages or DCs were labeled with the red lipophilic dye PKH26 (Sigma-Aldrich), according to the manufacturer's instructions. Labeled cells were then pulsed with 10 μg/ml SEE sAg (Toxin Technology) or medium for 15 minutes at 37°C. For microscopy studies, APCs were then seeded onto poly-L-lysine–treated (Sigma-Aldrich) culture wells or coverslips for 15 minutes at 37°C. Infected PBLs were added onto adherent APCs at a 1:1 ratio, and cocultures were fixed after 40 minutes by adding an equal volume of 2% PFA. Noninfected PBLs were stained with CFSE (Invitrogen) according to the manufacturer's guidelines. Complexes were observed either by standard epifluorescence microscopy using a Zeiss Axiovert microscope (×40 objective) or by confocal fluorescence microscopy using a Leica TCS 4Pi scanning confocal laser microscope (×100 objective).

Polarization of CD3, Lck, talin, and LFA-1. For microscopy studies, immature DCs were pulsed with a sAg cocktail (SEE, SEA, SED, SEC3, and TSST, all 1 μg/ml; Toxin Technology) for 15 minutes, washed, and mixed with

infected CD4+ T cells at a 1:1 ratio and seeded onto poly-L-lysine–treated (Sigma-Aldrich) coverslips at 37°C. Cells were fixed after 30–45 minutes by adding an equal volume of 3% PFA. Fixed cells were stained for CD3 (UCHT1; Chemicon) or LFA-1 (HI111; BD Biosciences) under nonpermeabilizing conditions, or for Lck (3A5; Santa Cruz Biotechnology Inc.) or talin (8D4; Sigma-Aldrich) in the presence of 0.05% saponin, followed by secondary antibodies Alexa Fluor 568–conjugated goat anti-mouse (Invitrogen) or rhodamine-conjugated donkey anti-mouse (Jackson Immunoresearch). Nuclei were labeled by DAPI (Roche). All confocal laser scanning microscopy was performed with a LSM 510 Meta microscope using a ×60 Plan Apochromat NA 1.4 DIC objective (Zeiss). Single section images were acquired as 512 pixels by 512 pixels and then processed using NIH ImageJ software (http://rsbweb.nih.gov/ij/). Polarization of Lck, LFA-1, and talin was defined as an immediately obvious enrichment (greater than 50% total staining) of IS markers at the immature DC/T cell contact. Quantification of polarization was carried out on a total of 20–60 conjugates for each condition for 2 different donors.

CD43 exclusion. For studies of CD43 exclusion from the IS, fixed cells were stained for CD43 (mouse anti-CD43; BD Biosciences — Pharmingen; and Alexa Fluor 647–conjugated anti-mouse; Invitrogen) prior to observation. The CD43 signal intensity along the plane of the cell-cell interface was quantified using ImageJ Plot Profile analysis, which displays the intensities of pixels along a line as a 2-dimensional graph.

Effector pathways of IS formation. Virally infected PBLs were treated with PHA and IL-2 as described above and subsequently washed and resuspended in RPMI without cytokines. A resting phenotype (as assessed by morphology, e.g., loss of cell aggregation and small cell size), lack of expression of activation markers, and loss of eGFP expression were observed after a further 3–4 days. For early IS signaling pathways, PBLs were then restimulated with CD3/CD28 beads (Invitrogen) at a 1:1 ratio for 15 minutes, 2 hours, 6 hours, or 24 hours or were left unstimulated. At the given time points, cells were fixed in 2% PFA, permeabilized, and immediately labeled with rabbit anti-pZap70 Tyr493 (0.5 μg/sample; Santa Cruz Biotechnology Inc.) and Alexa Fluor 647–conjugated anti-rabbit (0.5 μg/sample; Invitrogen). Alternatively, for late IS signaling, PBLs were stimulated with autologous MDMs or DCs for 16–24 hours and labeled for intracellular IL-2 using an allophycocyanin-conjugated IL-2 antibody (BD Biosciences — Pharmingen).

Statistics. All statistical calculations were performed with a 2-tailed unpaired Student's *t* test using Graph Pad Prism version 5.0. *P* values less than 0.05 were considered significant. Correlations were calculated with the linear regression module.

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