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Hemopoietic cell kinase (Hck) and p21-activated kinase 2 (PAK2) are involved in the down-regulation of CD1a lipid antigen presentation by HIV-1 Nef in dendritic cells

Eiji Shinya [a,*], Masumi Shimizu [a], Atsuko Owaki [a], Samantha Paoletti [b], Lucia Mori [b], Gennaro De Libero [b], Hidemi Takahashi [a]

[a] Department of Microbiology and Immunology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo city, Tokyo 113-8602, Japan
[b] Experimental Immunology, Department of Biomedicine, University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland

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ABSTRACT

Dendritic cells (DCs) play a major role in *in vivo* pathogenesis of HIV-1 infection. Therefore, DCs may provide a promising strategy to control and eventually overcome the fatal infection. Especially, immature DCs express all CD1s, the non-MHC lipid antigen -presenting molecules, and HIV-1 Nef down-regulates CD1 expression besides MHC. Moreover, CD1d-restricted $CD4^+$ NKT cells are infected by HIV-1, reducing the number of these cells in HIV-1-infected individuals. To understand the exact role of DCs and CD1-mediated immune response during HIV-1 infection, Nef down-regulation of CD1a-restricted lipid/glycolipid Ag presentation in iDCs was analyzed. We demonstrated the involvement of the association of Nef with hemopoietic cell kinase (Hck) and p21-activated kinase 2 (PAK2), and that Hck, which is expressed strongly in iDCs, augmented this mutual interaction. Hck might be another therapeutic target to preserve the function of HIV-1 infected DCs, which are potential reservoirs of HIV-1 even after antiretroviral therapy.

Introduction

CD1 glycoproteins are non-classical MHC class I-like molecules that present lipid antigen (Ag) to CD1-restricted T cells. Although their role has been mainly studied in mycobacterial infections, several reports have suggested that CD1 molecules may also play important roles in viral infections (Chen et al., 2006; Moll et al., 2010; Raftery et al., 2008; Raftery et al., 2006; Shinya et al., 2004). CD1d-restricted NKT cells respond to infections by HIV-1 (Moll et al., 2009), HSV (Yuan et al., 2006), and influenza virus (De Santo et al., 2008). However, it is still not clear whether the T cell receptor (TCR) recognizes the viral antigens (Ag) presented by CD1 molecules, or the self-lipids induced by cellular metabolism due to viral infection, or a combination of both. It has been speculated that viral Ag could be presented by CD1c molecules as N-terminally acylated lipopeptides, similar in sequence to HIV-1 Nef (Van Rhijn et al., 2009). However, there is no evidence that this happens during viral infection.

The function and size of the human T cell repertoire that recognizes lipid Ag presented by CD1 molecules remains poorly defined. However a recent report showed that a large portion of circulating T cells appeared to be CD1a-autoreactive, and had all the known functional properties of Th22 cells including the expression of skin homing molecules (CCR4, CCR10, and CLA) (de Jong et al., 2010), suggesting the importance of CD1a-restricted T cells in skin immunosurveillance and possibly immunopathology. Moreover, a second, large population of circulating T cells, which remains to be characterized, appears to be restricted to CD1c and can recognize endogenous Ag (de Lalla et al., 2011). Importantly, viruses have evolved a series of mechanisms that directly interfere with the plasma-membrane expression of CD1 molecules, suggesting that CD1-restricted T cells may also participate in protection during viral infections. Indeed, we and others have reported that HIV-1 Nef protein down-regulates CD1a and CD1d surface expression in immature DCs (iDCs) (Cho et al., 2005; Shinya et al., 2004). This could lead to reduced Ag presentation, and represents an evasion mechanism of the pathogen similar to that responsible for the immune-evasion of HIV-1 infected T lymphocytes from cytotoxic T lymphocyte recognition following the down-regulation of peptide Ag presentation by MHC class I molecules (Collins et al., 1998). Of the accessory genes of HIV-1, *nef* is well known as a key factor of immune-evasion. In addition to down-regulating MHC class I, HIV-1 Nef also down-regulates MHC class II surface

* Corresponding author. Tel. +81 3 3822 2131 ext.5547 Fax. +81 3 3827 3381.
E-mail address: eiji@nms.ac.jp(E.Shinya)

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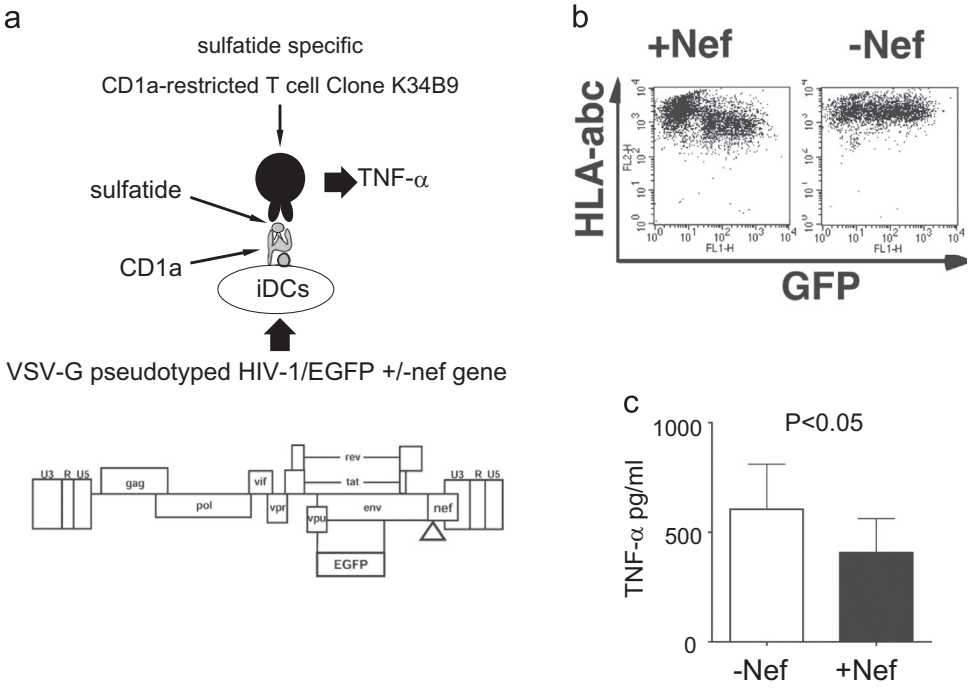

Fig. 1. Down-regulation of CD1a lipid antigen presentation on iDCs infected with VSV-G-pseudotyped recombinant HIV. a) The CD1a lipid antigen presentation assay. PBMC-derived iDCs were used as antigen presenting cells and infected with VSV-G pseudotyped single-cycle recombinant HIV-1/EGFP (with the intact *nef* gene designated as +Nef or with the crippled *nef* gene designated as -Nef). Subsequently, the DCs were incubated with sulfatide to stimulate the T cell clone K34B9.1 and TNF-α released in the supernatants was measured by ELISA. b) and c) The infection of iDCs by VSV-G-pseudotyped HIV-1 with the intact *nef* gene (+Nef) showed no more than 30% of infected (EGFP positive) iDCs, which still showed a significant reduction of TNF-α production compared to the crippled *nef* gene (-Nef) ($P < 0.05$ by the paired *t* test).

expression (Stumptner-Cuvelette et al., 2001). Furthermore, it has recently been reported that HIV-1 Vpu together with Nef inhibits lipid Ag presentation in DCs by CD1d (Moll et al., 2010). However, with regards to CD1a, only the down-regulation of surface expression of CD1a in iDCs has been reported (Shinya et al., 2004).

Myeloid iDCs are the only peripheral Ag presenting cells (APCs) that are known to express all human CD1 isoforms (CD1a, CD1b, CD1c, CD1d, and CD1e) and initiate lipid Ag processing pathways in response to activating stimuli. Moreover, $CD1a^+$ iDCs, or Langerhans cells, are thought to be the first cells to encounter HIV-1 at mucous membranes, and capture viral particles to allow them productive replication and long-term viral dissemination that are later transferred to $CD4^+$ lymphocytes (Burleigh et al., 2006; Coleman et al., 2011; Dong et al., 2007; Turville et al., 2004; Wang et al., 2007). On the other hand, the C-type lectin DC-SIGN is expressed on the surface of iDCs and enhanced HIV-1 trans-infection (Geijtenbeek et al., 2000). DC-SIGN positive iDCs from human rectal mucosa is known to bind and transfer HIV-1 to CD4+ T cells efficiently and, in human rectal mucosa, DC-SIGN antibodies could block 90% of HIV-1 binding although only 1–5% of total mucosal mononuclear cells (Gurney et al., 2005). Taken together, iDCs seem to be more relevant in establishing an immune response against HIV than mature DCs.

In this study, we used PBMC-derived iDCs to show that HIV-1 Nef significantly down-regulated lipid Ag presentation by CD1a together with its surface expression on iDCs. Furthermore, using a series of mutant *nef* genes, we confirmed the intermolecular interaction of HIV-1 Nef and CD1a together with hemopoietic cell kinase (Hck) and p21-Activated Kinase 2 (PAK2). Hck was highly expressed in iDCs and HIV-1 takes advantage of Hck in iDCs as well as PAK2 for the down-regulation of CD1a lipid Ag presentation and immune-evasion from this lipid Ag recognition system.

Results

CD1a lipid Ag presentation is impaired by Nef in HIV-1 infected iDCs

In this study, we analyzed the influence of HIV-1 Nef on CD1a lipid Ag presentation. As antigen presenting cells (APCs), we used peripheral blood mononuclear cells (PBMC)-derived iDCs to measure CD1a lipid Ag presentation, (Figs. 1 and 2). Since iDCs are resistant to transfection by conventional techniques such as those used with DNA plasmids, we used the VSV-G pseudo-typed single-cycle recombinant HIV-1 vector (Shinya et al., 2003, 2004) to introduce the *nef* gene into iDCs (Fig. 1a). The CD1a-restricted T cell clone K34B9.1, which is both CD1a restricted and sulfatide-specific, was used as a responder cell (Shamshiev et al., 2002).

Despite an efficiency of infection no greater than 30% (Shinya et al., 2004), on infection of PBMC-derived iDCs with the single-cycle reporter HIV-1 pseudotyped with VSV-G (Fig. 1b), there was significant down-regulation of TNF-α secretion by the virus encoding the *nef* gene (+Nef) relative to the virus not expressing Nef due to the *crippled nef* gene (−Nef, Fig. 1c), suggesting that HIV-1 Nef abrogated CD1a lipid Ag presentation.

To obtain higher efficiency expression, *EGFP* mRNA was electroporated into iDCs (Fig. 2a) and showed greater than 90% GFP+ expression in the iDCs (Fig. 2b). Moreover, the mRNA electroporation with the *EGFP* gene did not cause the significant changes in the surface expression of CD1a, HLA-abc, CD83 or DC-SIGN (Fig. 2b).

Mutation in the PXXP SH3 binding motif and R106 abrogated the Nef-mediated impairment of CD1a lipid Ag presentation

A series of mutations were introduced into the *nef* gene (Fig. 3), which was fused in frame to the 5′ end of the *EGFP* gene and the mRNA of the *nef -EGFP* gene capped with the anti-reverse cap

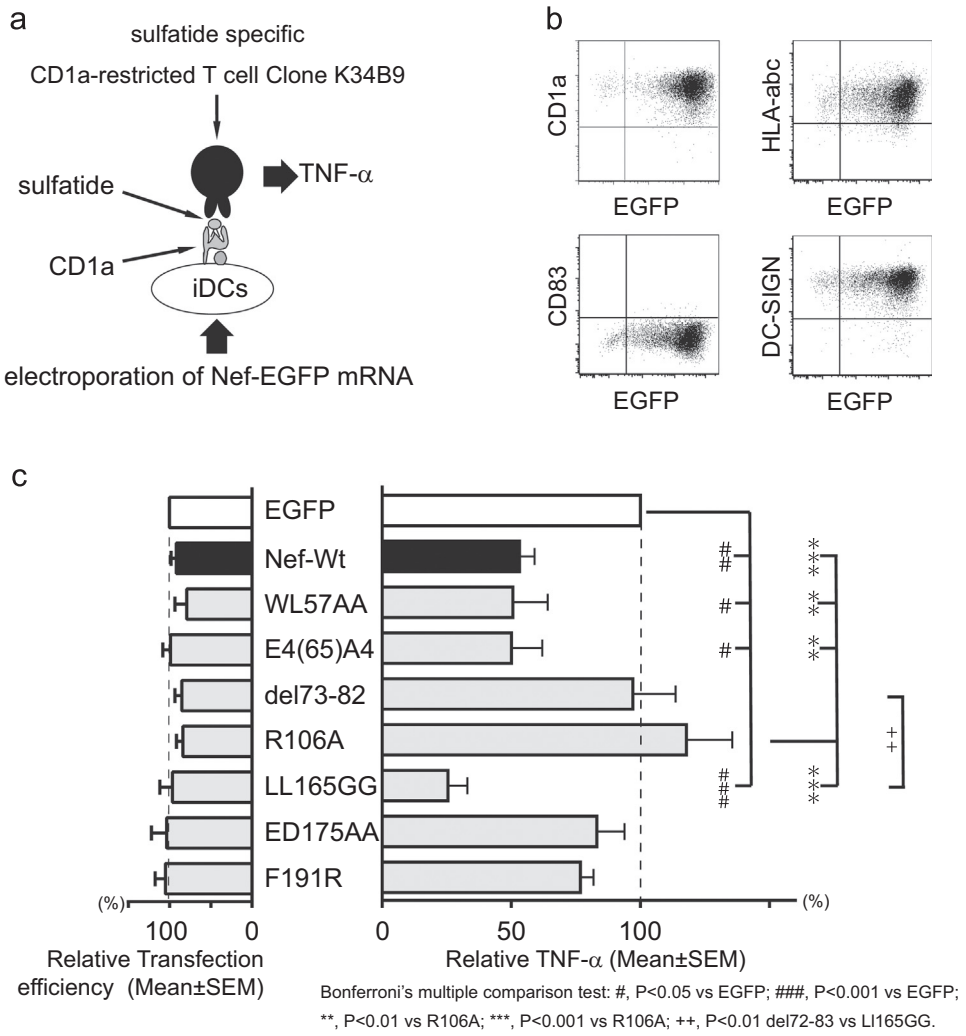

Fig. 2. Down-regulation of CD1a lipid Ag presentation by Nef. a) PBMC-derived iDCs were used as antigen presenting cells and were first transfected by electroporation with mRNA of a series of mutant *nef* genes, together with *DsRed2* mRNA to monitor transfection efficiency. TNF-α levels in the supernatant were subsequently quantified by ELISA. b) mRNA electroporation of the *EGFP* gene resulted in more than 90% of EGFP positive iDCs without changes in CD1a, HLA-abc, CD83, or DC-SIGN surface expression. c) The responses of the sulfatide-specific CD1a-restricted cell clone K34B9.1. Values represent mean+SEM of the relative percentages of TNF-α concentrations to that of iDCs expressing *EGFP* (right panel). Transfection efficiency showed by DsRed2 expression was constant and not significantly different between all mutants, as shown in the left panel.

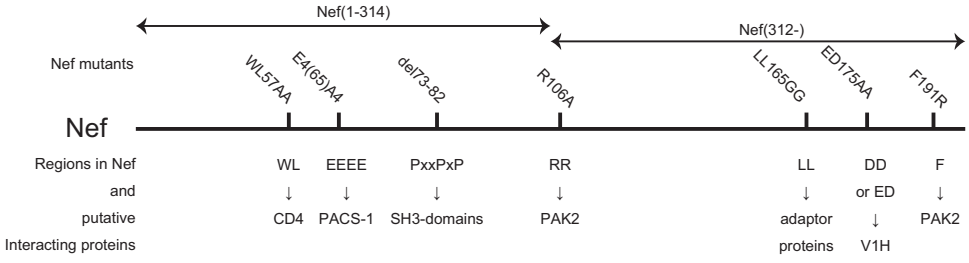

Fig. 3. A series of mutant *nef* genes used in the experiments. Nef(-314) gene encodes amino acids 1–104 of Nef, whereas Nef(312-) gene encodes amino acids 105 to the C-terminal ends. Mutations were introduced into each *nef* gene motif already known to interact with several proteins; The WL motif was replaced with AA in WL57AA, and the EEEE motif in E4(65)A4 with AAAA. The PxxPxP motif was deleted in del73-82, RR in R106A was replaced with RA, LL in LL165GG with GG, ED in ED175AA with AA, and F191 in F191R with R. Interacting proteins already known to each motif are shown at the bottom.

analog (Stepinski et al., 2001) was produced. Each mRNA generated was introduced into iDCs by electroporation. Subsequently, iDCs were pulsed with sulfatide, and analyzed for its presentation to K34B9 T cells. The del73-82, R106A, ED175AA and F191R mutations (5th, 6th, 8th and 9th bars, right panel, Fig. 2c) did not show the down-regulation of CD1a lipid Ag presentation in contrast to Nef-Wt (2nd bar, right panel, Fig. 2c). In the del73-82 mutation (Aldrovandi et al., 1998), the two terminal proline residues were deleted in the 5′ PXXP SH3 binding motif, which binds with high affinity to the SH3 domain of the Src-family tyrosine kinases such as Hck (Saksela et al., 1995). It is well known that Nef disrupts the linker/SH3 interaction, and interacts with the SH3 domains of Src family kinases with different affinities, of which the highest affinity is for Hck (Lee et al., 1995). In R106A, an R to A mutation was introduced in the *nef* gene at R106, which is known to be involved in the interaction with PAK2, as is the case

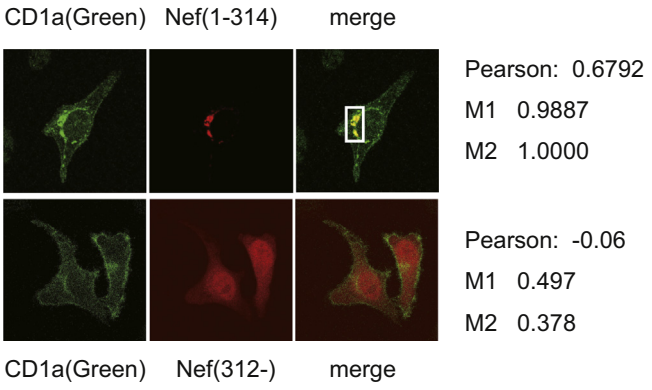

Fig. 4. Analysis with Laser Confocal Microscopy. The interaction between Nef and CD1 is dependent on the N-terminal half of Nef. Either *Nef(1–314)* or *Nef(312-)* gene fused to the GFP gene and CD1a fused to DsRed2 gene were transfected simultaneously into HeLa cells and their subcellular localization was analyzed by Laser Confocal Microscopy. CD1a (upper left) and Nef(1–314) (upper middle) co-localized (upper right), but CD1a (lower left) and Nef(312-) (lower middle) did not (lower right). A representative data was shown. Additional results of the observation are shown in Supplementary 1 and 2. Colocalization analysis was done within the region of interest when indicated with white line.

for F191 (Khan et al., 1998). The 5′ PXXP SH3 binding motif also contains a potential protein kinase C phosphorylation site (threonine 80), and previous reports have shown that the binding of SH3 to HIV-1 Nef is also required for the activation of PAK2 (Manninen et al., 1998). Moreover, F191R is known to abolish Nef association with PAK2 without reducing other Nef functions (Agopian et al., 2006). Taken together, these results suggest the important role of the interaction of Nef and CD1a together with both Hck and PAK2 in the context of CD1a lipid Ag presentation by iDCs.

Of the other Nef mutants tested, two mutants are known to abolish Nef binding to AP-2 (LL165GG and ED175AA, 7th and 8th bar, right panel, Fig. 2c) (Lindwasser et al., 2008). But LL165GG down-regulated the CD1a lipid Ag presentation as Nef-Wt did and, in contrast, ED175AA did not. No effects on CD1a Ag presentation were observed either with the remaining two mutants, namely the WL57AA mutant involved in the down-regulation of CD4 (3rd bar, right panel, Fig. 2c) (Stoddart et al., 2003) or the E4(65)A4 mutant responsible for the down-regulation of MHC class I molecules (4th bar) (Stoddart et al., 2003).

Interaction between HIV-1 Nef and CD1a was dependent upon N-terminal half of Nef

To analyze the interaction between Nef and CD1a, the gene encoding N-terminal half, *Nef (1–314)* or that encoding C-terminal half of Nef, *Nef(312-)* (Fig. 3) was fused to *DsRed2* and transfected into HeLa cells simultaneously with *CD1A* and their subcellular localization was observed by laser confocal microscopy. We showed that Nef(1-314) but not the C-terminal half significantly co-localized with CD1a (Fig. 4, Supplementary 1 and 2), suggesting that the N-terminal half of Nef, which includes the PxxPxP motif may be involved in the Nef–CD1a interaction, although Nef(312-) showed a completely different intracellular localization from that of Nef(1-314).

Since a previous report showed that the interaction between Nef and CD1a was dependent on the cytoplasmic domain of CD1a (CD1a cyt.) (Shinya et al., 2004), we further analyzed the interaction between the CD1a cytoplasmic domain and Nef, Nef (1–314), or Nef (312-) using a yeast two hybrid assay. In this assay, either the intact *nef* gene (Nef), *Nef(1–314)*, or *Nef(312-)* was fused to the *GAL4(1–147)* DNA binding domain gene in the pGBKT7 vector (Matchmaker Two-Hybrid system 3, Clontech, Mountain View, CA, USA) as bait, and *CD1A* cytoplasmic domain gene *(CD1a cyt.)* was fused to the *GAL4(768–881)* transcriptional activator domain gene in the pGADT7 vector (Clontech) as prey (Fig. 5a). *S. cerevisiae* AH109 His- MelI- (Clontech) was transformed with a combination of each of these fusion genes. Nef and Nef(1-314) but not Nef(312-) yielded His+ colonies with CD1a cyt (Fig. 5b) and His+/X-α-galactosidase+ blue colonies on SD/-His/Leu/-Trp/X-α-gal plates (Fig. 5c), suggesting that the interaction between CD1a and Nef was dependent upon N-terminal Nef (1–312), which also supports the results from the laser confocal microscopy analysis (Fig. 4, Supplementary 1 and 2).

Intermolecular interaction between HIV-1 Nef, CD1a, PAK2 and Hck: protein fragment complementation assay

To confirm the intermolecular interactions between HIV-1 Nef, CD1a, PAK2, and Hck, a protein fragment complementation assay was performed using the monomeric Kusabira-Green (mKG) reporter protein gene (Fig. 6) (Ueyama et al., 2008). In this assay, the *mKG* gene was divided into two fragments (*mKGN and mKGC*), and fused to the *nef* (*mKGN-nef*), *PAK2 (mKGC-PAK2), or Hck* genes *(mKGC-Hck*). Should the two expressed target proteins interact, the divided mKG fragments would spatially approach each other and increase the local effective concentration. As a result, mKG fragments formed the same steric structure as the undivided mKG does and emitted fluorescence from the chromophore (Fig. 6a). The simultaneous transfection of *mKGC-PAK2* and *mKGN-nef* (Fig. 6b and e), *mKGC-Hck* and *mKGN-nef* (Fig. 6b and d) or *mKGC-CD1a* and *mKGN-nef* (Fig. 6c) genes were performed in HCT116 cells. The reconstituted green fluorescence of the mKG protein was then analyzed by flow cytometry, and was observed in a significant number of cells in each combination. The interaction between HIV-Nef and Hck was stronger than that of HIV-Nef and PAK2 (Fig. 6b), therefore confirming the intermolecular interaction of HIV-1 Nef with both Hck and PAK2 and also suggesting that the interaction of Nef with Hck is significantly stronger than that with PAK2.

Next, the interaction between Nef and CD1a with/without HCK was examined in HCT116 cells (Fig. 6c) by the simultaneous transfection of *mKGC-CD1a* and *mKGN-nef* together with *Hck-PLUM* fusion gene or *PLUM* gene. However, without Hck (left panel, Fig. 6c), the interaction was fairly weak and there were no significant differences within the series of *nef* mutants. Since *Hck* expression has been reported in cells of macrophage/monocyte lineage in a tissue-specific manner (Greenway et al., 2003) and the catalytic activity of Hck was dramatically up-regulated when its SH3 domain was bound by Nef while inhibited in other Src kinases, Lck and Fyn, we analyzed the expression of *Hck* in several cell lines including PBMC-derived iDCs (Fig. 7). Furthermore, it is known that, while PAK2 does not contain a SH3 domain like that in Hck, the association of Nef and PAK2 requires the Nef polyproline (PxxP) motif. Taken together, we hypothesized that Hck that has a SH3 domain might augment the interaction between Nef and PAK2 to interfere with the interaction of HIV-1 Nef and CD1a.

As shown using quantitative PCR analysis (Fig. 7a), there was a high level of tissue specific *Hck* expression in iDCs among the investigated cell types, whereas *Hck* was weakly expressed in the THP-1 cell line (derived from the peripheral blood of a male with acute monocytic leukemia), and showed much weaker but nevertheless significant expression in C1R (an EBV-transformed B lymphoblastoid cell line), and primary T cells. No significant expression of *Hck* was detected in Jurkat cells (a T cell line), K34B9.1T cells, or HTLV-1 transformed macrophages (Takeuchi et al., 2010) (indicated as MF in Fig.7). Notably, we did not detect any significant *Hck* expression either in the HCT116 cells and HeLa cells used in this

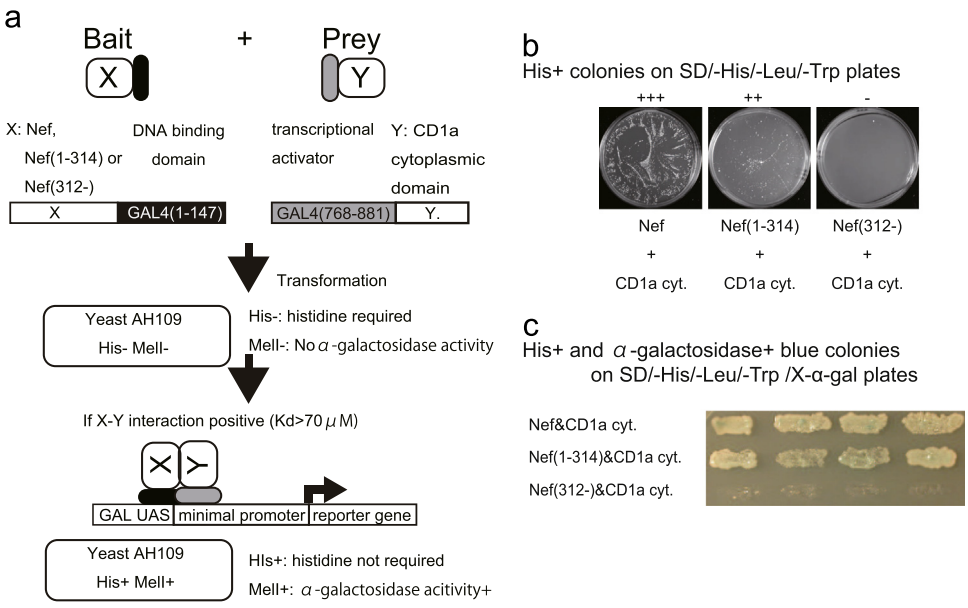

Fig. 5. A yeast two hybrid assay shows that the interaction between Nef and CD1a depends on the N-terminal half of Nef and the CD1a cytoplasmic domain. a) Either intact *nef* gene (Nef), *Nef(1-314)* or *Nef(312-)* was fused to the GAL4(1-147) DNA binding domain in pGBKT7 (Matchmaker Two-Hybrid system 3, Clontech) as "bait", and CD1a cytoplasmic domain was fused to GAL4(768-881) transcriptional activator domain in pGADT7 (Clontech) as "prey". *S. cerevisiae* AH109 His- MelI- (Clontech) was transformed with each combination of the fusion genes. Neither His+ colonies on SC/-His/-Leu/-Trp plates (b) nor His+/X-α-galactosidase+ blue colonies on SD/-His/Leu/-Trp/X-α-gal plates (c) were yielded by the combination of CD1a cyt.&Nerf(312-) but by the other combination, CD1a cyt.&Nef or CD1a cyt.&Nef(1-314).

study (Fig. 7a). Immunoblot analysis of the Hck also confirmed the high level of Hck expression in iDCs compared to the other cell types, supporting the results of quantitative PCR analysis (Fig. 7b and c). Accordingly, the significant role of Hck could be expected in iDCs, in which the expression of CD1a is also specifically high, towards the down-regulation of CD1a lipid Ag presentation.

Therefore, the effect of Hck on the interaction between CD1a and Nef was analyzed (right panel, Fig. 6c) by the simultaneous transfection of *mKGC-CD1a* and *mKGN-nef* together with *Hck-PLUM* fusion gene. FACS analysis of the reconstituted mKG positive- PLUM positive cells showed that Hck augmented the interaction of CD1a with all the Nef mutants except del73-82 mutant (left vs. right 4th bars, Fig. 6c). Furthermore, with Hck, the interaction of Nef and CD1a was significantly down-regulated by the del73-82 mutation (1st vs. 4th bar, right panel, Fig. 6c).

Subsequently, the interaction between Nef and Hck was analyzed, showing that not only the polyproline (PxxP) motif (4th bar, Fig. 6d), but also F191 (8th bar) were involved which was unexpected.

Finally, Nef–PAK2 interaction was studied with or without Hck, which showed that Hck significantly augments Nef–PAK2 interaction (left and right bars, Fig. 6e), and E4(65)A4, del73-82 and F191R mutations significantly abolished the Nef–PAK2 interaction regardless of Hck. This confirms that del73-82 and F191R showed reduced binding capacity to PAK2 protein (Khan et al., 1998; Schindler et al., 2007). In contrast, it is controversial whether E4(65)A4 is involved in the Nef–PAK2 interaction (Baugh et al., 2008; Piguet et al., 2000), but our results correspond with the results of Baugh et al., showing that E4 (65)A4 mutation is not specific for MHC-I down-regulation but also defective for the interaction with PAK2.

It has been reported that the majority of Nef alleles fail to activate PAK2 and only interact with the activated PAK2 (Pulkkinen et al., 2004), whereas a SH3 domain binding to HIV-1 Nef is required for PAK2 activation (Manninen et al., 1998). Consistent with this, our results present new evidence that Hck, whose SH3 domain interacts with the N-terminal PXXP SH3-binding motif of Nef, may be required for the Nef–PAK2 interaction, which is most likely in iDCs in which Hck is highly expressed.

Intermolecular interaction between HIV-1 Nef, PAK2 and Hck: combination of microscopic analysis and protein fragment complementation Assay

The *HIV-1 nef -EGFP* fusion gene and *PAK2-PLUM* fusion gene were transfected simultaneously into HeLa cells and their intracellular distributions were investigated using confocal laser scanning microscopy. In a representative cell shown in Fig. 8a, Nef and PAK2 (Fig. 8a i) and ii), respectively) localized to the plasma membrane (Fig. 8a iii). With the auto threshold calculation by Coloc2 plugin with Fiji/ImageJ, Pearson's R was 0.82, Manders M1 and M2 were 0.932 and 0.999, respectively and Costes *P*-value was 1.00, indicating a significant co-localization of the two proteins in transfected HeLa cells. Observing several cells (Supplementary 3), Manders M1 and M2 were always no less than 0.8 with positive value of Pearson's *R*, indicating significant co-localization of Nef and PAK2, although their localization to the plasma membrane was not always observed. These results are in agreement with the previous reports that showed Nef–PAK2 association at cellular membranes, where both proteins were selectively partitioned in the detergent-resistant micro domains of plasma membranes (Pulkkinen et al., 2004).

To observe the interaction between the three Nef, PAK2 and Hck proteins, a combination of protein fragment complementation assay and observation with laser microscopy was performed. Briefly, HeLa cells were transfected with *mKGN-Nef*, *mKGC-PAK2* and *PLUM-Hck* genes. The positive interaction between Nef and PAK2 resulted in the reconstituted mKG (Fig. 8b, i) that co-localized significantly with Hck (Fig. 8b, ii and iii). In the reconstituted mKG and Hck, Pearson's R value above threshold was 0.65, Manders M1 and M2 were 1.000 and 0.976, respectively and Costes *P*-value was 1.000, thus showing the significant co-localization of mKG and Hck. Observation of multiple cells also support the significant co-localization of the three proteins, Nef, PAK2 and Hck (Supplementary 4), which supports the presence of an interaction between Nef, PAK2 and Hck.

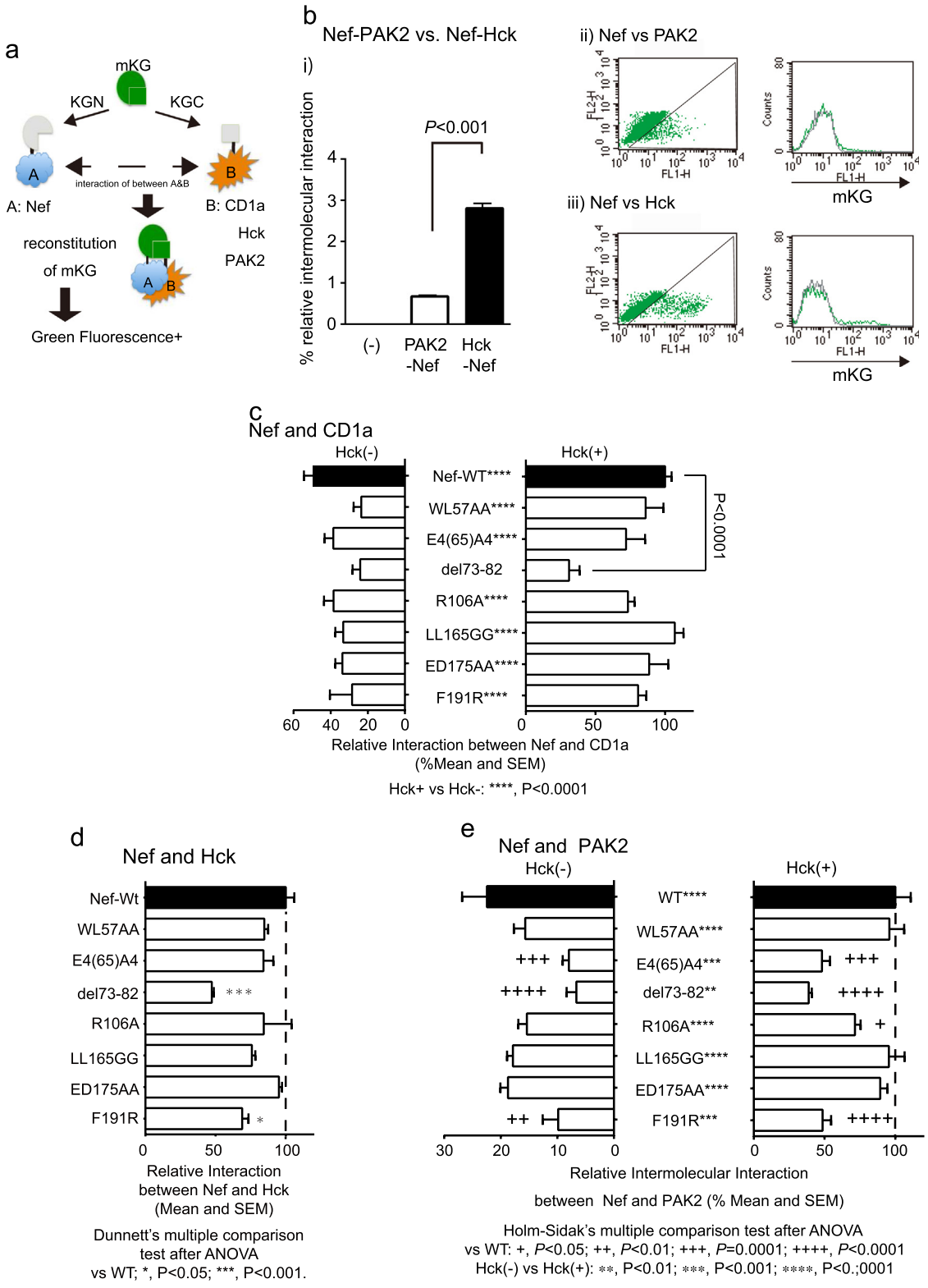

Fig. 6. Protein fragment complementation assay. a) Interaction between Nef, CD1a, Hck, or PAK2 was further analyzed. The Monomeric Kusabira Green (*mKG*) gene was genetically divided into *KGN* and *KGC*. Two of the genes for the proteins (A and B) to be analyzed were fused to *KGN* or *KGC* and transfected simultaneously to HCT116 cells where the expression of *Hck* is not detected by PCR. Subsequently, transfected HCT116 cells were analyzed by FACS for the green fluorescence of reconstituted mKG indicating positive interaction between two proteins. b) i) Nef was shown to have positive interaction with both Hck and PAK2, although the interaction with Hck was significantly stronger than that with PAK2. A representative result of the FACS analysis was shown in ii) and iii). c) Nef vs. CD1a: In the absence of Hck, Nef-CD1a showed positive interaction but no significant difference was seen between Nef mutants (left panel). Addition of Hck (right panel) significantly augmented the Nef–CD1a interaction except del73–82 mutant (4th bar, right panel). d) Nef vs. Hck: Significant down-regulation of Nef–Hck interaction was shown by del73–82 (4th bar) and by F191R (bottom bar). e) Nef vs. PAK2: In the absence of Hck (left panel), a relatively weak but significant interaction was observed, which was down-regulated with E4(65)A4 (3rd bar), del73–82 (4th bar) or F191R mutation (bottom bar). With Hck (right panel), the Nef–PAK2 interaction was always augmented even with del73–82 mutation (comparison of left and right bar with each Nef mutant), with which the interaction was somehow down-regulated. With HCK, the down-regulation of the Nef–PAK2 interaction by R106A was significant and that by F191R more prominent.

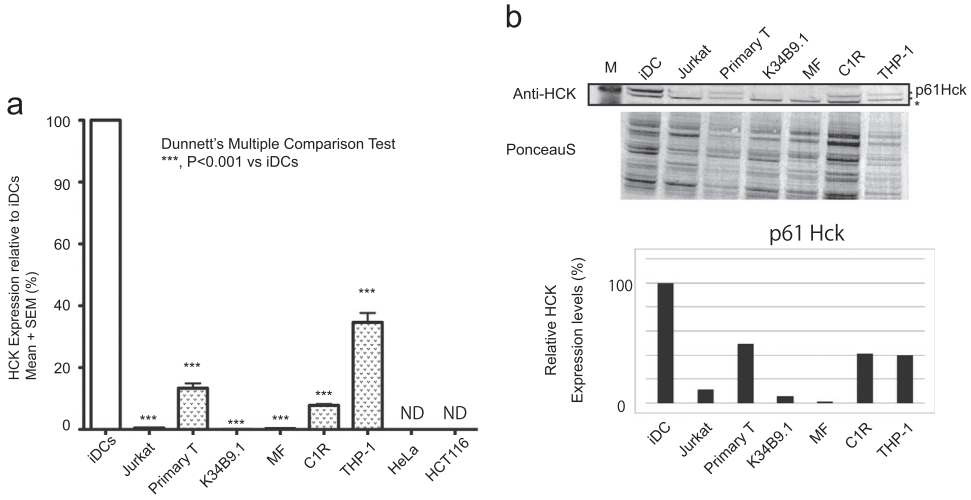

Fig. 7. Strong *Hck gene* expression in immature DCs. The expression of the *Hck* gene was analyzed by real-time PCR and immunoblotting. a) Total RNA was extracted from PBMC-derived iDCs, Jurkat cells, Primary T cells, K34B9.1, the CD1a-restricted CTL line, HTLV-I transformed macrophages (MF), C1R (a B cell line) and THP-1 cells. *Hck* expression is shown relative to iDCs. Significant results as per Dunnett's multiple comparison test are shown (***, $P < 0.001$ vs. iDCs). b) Immunoblot with anti-Hck antibody. After protein transfer, the membrane was stained with Ponceau S, quickly de-stained and imaged for total protein quantification. After de-staining in TBST, immunoblot was performed with anti-Hck antibody. Relative p61 Hck expression levels adjusted to total protein transfer (Ponceau S staining) was determined by densitometry using the image analysis program ImageJ. The asterisk indicates a nonspecific band.

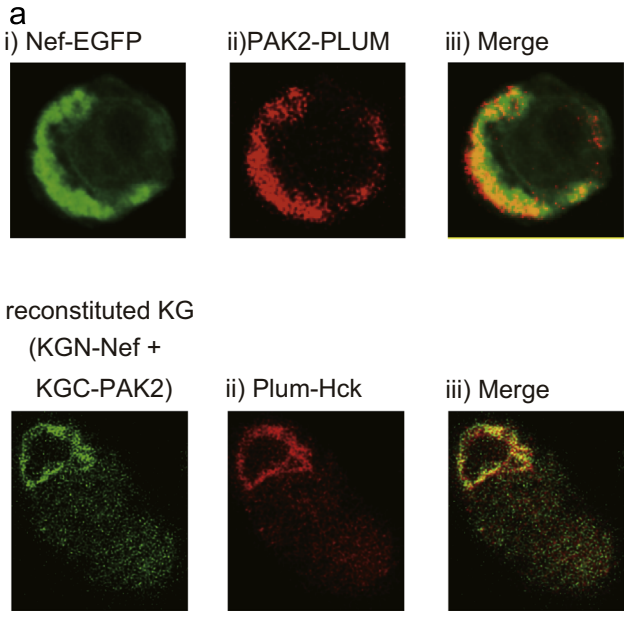

Fig. 8. Analysis of the interaction of Nef with PAK2 and Hck. a) The Nef-EGFP and PAK2-PLUM genes were transfected into HeLa cells and showed that Nef (i) and PAK2 (ii) co-localized significantly (iii). The observation of the additional cells are shown in Supplementary 3, which also indicates the positive interaction between Nef and PAK2. b) To analyze the interaction between the three molecules, Nef, PAK2 and Hck, *KGN-Nef*, *KGC-PAK2* and PLUM-Hck genes were transfected simultaneously. The green fluorescein of reconstituted mKG was observed with KGN-Nef and KGC-PAK2 (i) showing the positive interaction of Nef and PAK2. Furthermore, the reconstituted mKG by Nef and PAK2 (i) also co-localized with Hck (ii, iii) indicating the positive interaction of the three proteins, Nef, PAK2, and Hck. In Supplementary 4, additional data are shown. Colocalization analysis was done within the region of interest when indicated with white line.

Discussion

In this study, we have shown the down-regulation of CD1a lipid Ag presentation by HIV-1 Nef in iDCs, in which Hck and PAK2 might be involved, while it was already reported that HIV-1 Nef together with HIV-1 Vpu down-regulate lipid Ag presentation by CD1d (Moll et al., 2010), which presents lipid Ag delivered to the endocytic compartments.

CD1 Ag-presenting molecules consist of group 1 CD1 isoforms (CD1a, b, c) and group 2 CD1 (CD1d). Group 1 CD1 isoforms differ from CD1d in the extent of its ability to enter the early, intermediate, or late compartments of the endosomal network (Briken et al., 2000). Especially, CD1a molecules encounter Ag in the secretory pathway and early endosomes and recycle them back to the cell surface, but do not efficiently enter late endosomes and lysosomes (Briken et al., 2000). In contrast, CD1d efficiently enters the late endosomes/lysosomes where several mechanisms facilitate Ag loading and recognition by T cells such as pH-mediated changes in CD1 conformation to facilitate Ag access to CD1 grooves (Cheng et al., 2006), pH-activated lipid transfer proteins (Zhou et al., 2004), and pH-activated glycosidases that process the oligosaccharidic components of glycolipid Ag (Prigozy et al., 2001). These results imply that Nef down-regulates CD1a and CD1d lipid Ag presentation by different mechanisms.

To further explore the molecular basis of the down-regulation of CD1a lipid Ag presentation by HIV-1 Nef, we analyzed the effects of wild-type Nef and a series of Nef mutants, to show that R106A, del73-82, and F191R significantly interfere with CD1a lipid Ag presentation in iDCs. Since R106A and F191R mutations are important in the interaction of Nef and PAK2, and the del73-82 mutation is involved in the interaction of Nef with Hck, we can potentially infer that both PAK2 and Hck are involved in the down-regulation of CD1a lipid Ag presentation by HIV-1 Nef. Moreover, both confocal laser scanning microscopy and the protein fragment complementation assay revealed that Nef and PAK2 significantly co-localized (Fig. 8a, Supplementary 3), and that their intermolecular interaction depended on both R106 and F191 (Fig. 6e). Both R106 and F191 are widely recognized to be involved in the intermolecular interaction between HIV-1 Nef and PAK2. On the other hand, some reports showed that Nef residue F191 is specifically involved in PAK2 binding (Agopian et al., 2006), whereas being critical for the accurate Nef core structure, R106A impaired the multiple functions of Nef (O'Neill et al., 2006). Our results are in line with these reports and indicate that F191 has an essential role.

In this study, F191R also down-regulated the interaction between Nef and Hck (Fig. 6d), which has not previously been shown. One possible speculation might be that the protein fragment complementation assay was so sensitive, being more

sensitive than the other methods, that it could detect a subtle effect of F191R mutation on the interaction of Nef and Hck. Another possible speculation would be that PAK2 might augment the interaction between Nef and HCK via F191R. Anyway, further analysis is necessary. In addition, the replacement of an acidic cluster (EEEE motif) with AAAA in Nef (E4(65)A4) abolished the interaction between Nef and PAK2 (Fig. 6e) without change in the CD1a Ag presentation (Fig. 2c) or in the interaction between Nef and CD1a (Fig. 6c). Since E4(65)A4 mutation prevents MHC-I down-regulation, E4 acidic cluster in Nef has been known to be required for binding to phosphofurin acidic cluster sorting protein-1 (PACS-1) (Piguet et al., 2000) and PACS-2 (Atkins et al., 2008). On the contrary, it is also reported that the down-regulation of MHC-I by E4(65)A4 is not specific and that the four glutamates merely function as a flexible loop instead of a highly specific protein–protein interface (Baugh et al., 2008). For example, in addition to the MHC-I down-regulation, Nef with E4(65)A4 mutation is also defective both for PAK2 activation and for enhancement of viral infectivity whereas this mutation does not result in CD4 down-regulation. Accordingly, the down-regulation of Nef-PAK2 interaction might be another example of the "non-specific" effects of E4(65)A4 mutation.

Since interference with PAK2 leads to reduce CD1a Ag presentation, PAK2 may be important for organizing the early recycling pathway of CD1a and involved in CD1a Ag presentation. Whether PAK2 also plays an important role in Ag presentation through CD1c, a second CD1 molecule that also recycles through early endosomal compartments, remains to be investigated with CD1c specific T cell line (Shamshiev et al., 2002).

The interaction of the HIV-1 Nef protein and PAK2 has been reported to play a role in T-cell activation (Lu et al., 1996), viral replication (Olivieri et al., 2011), apoptosis (Wolf et al., 2001), T-cell development (Van Nuffel et al., 2013) and progression to AIDS (Pacheco and Chernoff, 2010). However, it has also been reported that Nef has no major role in T-cell activation, viral replication, nor apoptosis of T cells (Schindler et al., 2007). According to our results, the interaction of the Nef protein with PAK2 appears to be significant in iDCs because Hck would interfere with Ag presentation involving early endosomal compartments, such as that by CD1a. These findings support the conclusion that Nef may display unique effects on the different cell types in which it is expressed.

Mutational analyses supports the previous reports, such as that Nef binding to the SH3 domain was significant for PAK2 activation (Manninen et al., 1998) and that the only observed Nef binding to SH3 involved Hck (Karkkainen et al., 2006), since Hck augmented the weak Nef-PAK2 association in cells not expressing Hck (Fig. 6e). Our study demonstrated that Hck is expressed at high levels in PBMC-derived iDCs. We also found that Hck is present in cells of granulocyte and monocytic lineages, especially in macrophages and DCs, while c-Src and Lyn exhibited broader expression patterns including all HIV target cell types. In contrast, T cells do not have an Hck-like high-affinity SH3 ligand for Nef. Thus, the reason for the important role of the interaction between HIV-1 Nef and PAK2 in iDCs, but not in T cells (Schindler et al., 2007), may be explained by the tissue specificity and high expression of Hck in cells of granulocyte and monocytic lineages, including iDCs.

The requirement of Nef in SIV-induced AIDS in non-human primates and the observation of frequent Nef-defective HIV-1 in long-term non-progressors (Deacon et al., 1995; Kirchhoff et al., 1995) support the essential role of Nef in HIV infection pathogenesis. Moreover, expression of Nef alone is sufficient for the development of an AIDS-like syndrome in transgenic mice (Hanna et al., 1998), suggesting a major role for Nef in HIV-1 pathogenesis. The important role of Nef both in DC-mediated transmission of HIV-1 to activated $CD4^+$ T cells and in the activation and proliferation of resting $CD4^+$T cells was also reported, which seems contribute to viral pathogenesis (St Gelais et al., 2012). It is possible for lipid Ag presentation by CD1 molecules of DCs to be involved in the activation of $CD4^+$ T cells, which could result in the modification of the DC-mediated HIV-1 transmission to $CD4^+$T cells by HIV-1 Nef, which remains to be studied.

A series of investigations has also indicated the important role of Hck in mediating the effects on Nef in different cellular functions. For example, in brain-derived microglial cells, HIV-1 infection induced Nef-dependent Hck phosphorylation and an increase in HIV-1 transcription, while the suppression of Nef–Hck interaction inhibited HIV replication (Kim et al., 2006). In addition, Nef-induced AIDS-like disease was delayed in Hck-null mice and completely reversed in mice expressing a Nef mutant unable to bind to Hck (Hanna et al., 1998, 2001). These results suggest the importance of Hck in AIDS pathogenesis, and are in line with the essential role of Hck in promoting the interaction of Nef with PAK2 in cells of the granulocyte and monocytic lineages including iDCs.

Recently, a single-domain antibody (sdAb) was shown to bind HIV-1 Nef with high affinity and inhibit the association of Nef to PAK2, and Nef-induced CD4 down-regulation, and also counteracted Nef-dependent enhancement in virion infectivity (Bouchet et al., 2011). Expressed intracellularly, this antibody inhibited several biological functions of Nef both *in vitro* and *in vivo* in CD4C/HIV-1^{Nef} Tg mice, suggesting the important role of the Nef–PAK2 interaction in AIDS pathogenesis. Furthermore, this anti-Nef sdAb was fused to modified SH3 domains to disrupt the interactions of Nef with both AP complexes and Hck, thus showing the efficient neutralization of all key activities of Nef in both T lymphocytes and macrophages (Bouchet et al., 2012). Should HIV-1 hidden in the iDCs continuously destroy the internal immune system through down-regulation of various Ag-presenting molecules such as MHC and CD1s by Nef, breaking the intermolecular interaction among Nef, PAK2 and Hck will offer another strategy to regulate iDCs and re-constitute the immune system. Therefore, such agents may provide new therapies for treating HIV-1-infected innate iDCs.

Conclusions

In summary, we showed that HIV-1 Nef down-regulated CD1a lipid Ag presentation in iDCs, and involved both Hck and PAK2. Hck was strongly expressed in iDCs, where it promoted Nef–PAK2 interaction. Since CD1a-restricted T cells play an important role in skin immunology, the Nef–PAK2–Hck–CD1a interaction may represent a novel target for potential therapeutic strategies to restore normal T cell immunity during HIV-1 infection.

Methods

Cells, medium, glycolipid Ag, and recombinant virus

Sulfatide-specific CD1a-restricted T cell line K34B9.1 was obtained as previously described (Shamshiev et al., 1999). Sulfatide was obtained from Matreya (Pleasant Gap, PA, USA).

Immature DCs were obtained from PBMCs as previously described (Takeuchi et al., 2003). In brief, PBMCs were freshly isolated with Ficoll-paque (Amersham-Pharmacia, Uppsala, Sweden) from peripheral blood of healthy volunteers, and $CD14^+$ monocytes were immediately separated by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs and a magnetic cell separator (MACS, Miltenyi Biotec) according to the manufacturer's instructions, routinely resulting in $>90\%$ purity of $CD14^+$ cells. Cells were

cultured in 24-well culture plates for 6–7 days in complete medium supplemented with 50 ng/ml GM-CSF (from either PeproTech, Rocky Hill NJ or a conditioned culture medium of 293 FT cells (Invitrogen, Carlsbad, CA, USA) transfected with the *hGM-CSF* gene (Shinya et al., 2009)), and 20 ng/ml IL-4 (Biosource Intl., Camarillo, CA, USA) to obtain iDCs. At days 2 and 4, fresh medium supplemented with the abovementioned cytokines was added. On day 7, a fraction of the cultured cells (1×10^4) were stained with anti-CD1a, CD80, CD83, CD86 and analyzed with FACScan or FACScanto (BD Biosciences, Franklin Lakes, NJ, USA). Immature DCs are defined as DC-SIGN$^+$, CD1a$^+$, CD80$^+$,CD83$^-$. HCT116 cells (ATCC CCL-247) and HeLa cells (ATCC CCL-2) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Moregate, Queensland, Australia), penicillin (50 units/ml), and streptomycin (50 μg/ml) (Invitrogen). C1R cells (CRL-1993) and THP-1 cells (TIB-202) were obtained from ATCC (VA, USA). Primary T cells were obtained from healthy donors using Lympho-Kwik T Cell Isolation reagent (LK-50-T, One Lambda, Canoga Park, CA, USA).

VSV-G pseudotyped single-cycle recombinant HIV-1 was obtained and infection of iDCs was performed as previously described. (Shinya et al., 2003, 2004).

DNA constructions and in vitro synthesis of mRNA

Mutated *nef* genes were fused to the 5′ end of the *EGFP* gene and subcloned into pcDNA3.1+ (Invitrogen). After plasmid linearization, mRNA of each mutated nef-EGFP fusion gene, capped with the anti-reverse cap analog (Stepinski et al., 2001) with a poly (A) tail, was obtained using the mMESSAGE mMACHINE® T7 Kit (Ambion, Austin, TX, USA).

mRNA electroporation of iDCs

Immature DCs (1×10^6/250 μl) were resuspended in Ingenio solution (Mirus Bio LLC, Madison, WI, USA) in a cuvette with a 4-mm gap at 0 °C. 10 μg of mRNA was added and transfection was performed using Gene Pulsar II (Bio-Rad, Hercules, CA, USA) (250 V, 950 μF) following the manufacturer's protocol.

CD1a lipid Ag presentation assay

Twenty four hours after mRNA electroporation, the lipid Ag presentation assay was performed using previously described procedures (Shamshiev et al., 2002). In short, iDC (6×10^5/50 μl/well in a 96-well plate) cultured in RPMI-1640 medium without FCS were pre-incubated at 37 °C with 2 μg of sonicated sulfatide for 2 h followed by the addition of sulfatide-specific CD1a-restricted T cells K34B9.1 (10^5/100 μl of medium with 20% FCS/well). After 48 h of incubation, the supernatants were harvested and the released TNF-α was detected using ELISA kits (R&D systems, Minneapolis, MN, USA)

Antibodies for cell staining

The mouse monoclonal antibodies (mAbs) HI149 (anti-human CD1a), FITC conjugated mouse anti- human mAbs, G46-2.6 (anti-HLA-ABC), G46-6 (anti-HLA-DR), and phycoerythrin (PE)-conjugated mouse mAb HB15e (anti-CD83) were all obtained from BD Pharmingen (San Diego, CA, USA). PE-conjugated anti human DC-SIGN antibody (FAB161P) was from R&D systems (Minneapolis, MN, USA) and PE-conjugated goat F(ab)$_2$ antibody to mouse IgG (IM0855) was from Beckman Coulter (Fullerton, CA, USA).

Confocal laser scanning microscopy and protein fragment complementation assay

Total RNA of HCT116 cells or iDCs was obtained using the RNAeasy mini kit (QIAGEN GmbH, Hilden, Germany) and was reverse transcribed into cDNA. The *PAK2 gene* was amplified with the primers (underline indicates the XhoI site):
ccgccgCTCGAGatcatgtctgataacggagaactggaagataagcctccagc
and (underline indicates the BamHI site)
cgcGGATCCttaacggttactcttcattgcttctttagctgccatgatcag.
The *Hck* gene was amplified with the primers (underline indicates XhoI site):
ctgcagaaCTCGAGatcatggggtgcatgaagtccaagttcctccag
and (underline indicates the BamHI site)
aaggaaaaaaGCGGCCGCttatggctgctgttgg-
tactggctctctgtggccg.

Each gene was subcloned into the 5′ side of the *mPlum* gene in the pmPlum plasmid (Clontech) and termed as PAK2-PLUM. *Hck-PLUM or PAK2-PLUM,* and *nef-EGFP* expression plasmids were simultaneously transfected into HeLa cells using the HeLa Monster transfection kit (Mirus) and their expression was observed using a LSM710 confocal laser scanning microscope (Carl Zeiss, Iena, Germany) equipped with ZEN 2009 software. A Plan-Apochromat 63x/1.30 oil DIC M27 Zeiss lens was used for imaging. The obtained images were deconvolved with Huygens Essential software (Scientific Volume Imaging, Hilversum, The Netherlands) and the co-localizations were further analyzed by Fiji/ImageJ software (Abramoff et al., 2004) with the Coloc 2 plugin (http://pacific.mpi-cbg.de/wiki/index.php/Colocalization_Analysis).

For the protein fragment complementation assay, *PAK2* and *Hck* genes were subcloned into pKGC-MC (CoralHue® Fluo-chase Kit, MBL, Tokyo, Japan) to be termed *mKGC-PAK2* and *mKGC-Hck* respectively. A series of mutated *nef genes* were subcloned into *mKGN-MC* to be *mKGN-Nef*. HCT116 cells were transfected simultaneously with *mKGC-PAK2* and *mKGN-Nef* with/without *Hck-PLUM*, as described previously (Shinya et al., 2003), and the interaction between PAK2 and HIV-1 Nef and the effect of Hck on their interaction was detected by reconstituted mKG (monomeric Kusabira Green) fluorescence (Ueyama et al., 2008), which was analyzed by flow cytometry.

Yeast two-hybrid assay

The Matchmaker two-hybrid system 3 (Clontech) was used to analyze the interaction between proteins according to the manufacturer's instruction.

Real time PCR analysis

Total RNA was obtained using the RNAeasy mini kit (QIAGEN) and reverse transcribed with a random hexamer. The *Hck* gene was quantified using the THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan) and 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with a pair of primers:
aaagtgatgagggcagcaag and ttacacaccagggatgcaga.

Immunoblot

The cells were lysed in triple-detergent lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, with cOmplete Mini protease inhibitor cocktail (Roche). Bradford assays were used to determine lysate concentration (Bio-Rad, Hercules, CA, USA). Subsequently, protein expression was assessed by Western blot. 20 μg protein lysates were loaded into a NuPAGE Novex 12% Bis-Tris Gel (Invitrogen). The gel was run under reducing conditions and the proteins were transferred onto Invitrolon polyvinylidene fluoride

0.45-μm membrane (Invitrogen). The membrane was stained with Ponceau S (Beacle, Kyoto, Japan) for 2 min and quickly destained. The membrane was then imaged and total protein was quantified using Fiji/ImageJ 2.00-rc-38/1.60b. After destaining in 0.1 M NaOH, the membrane was washed in dH_2O. Subsequently, immunoblot was performed with primary antibody (1:1000, anti-HCK antibody, N-30, Santa Cruz Biotechnology, Texas, USA) and secondary antibody (1:15000, Goat Anti-Rabbit IgG H&L (HRP) (ab97051), Abcam, Cambridge, UK). 3,3 V,5,5 V-affect tetramethylbenzidine (TMB) substrate kit for peroxidase (VECTOR lab., Burlingame, CA, USA) was used to image the membrane. Relative protein expression levels adjusted to total protein transfer (Ponceau S staining) were determined by densitometry using the image analysis program Fiji/ImageJ 2.00-rc-38/1.60b).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software, La Jolla, Ca, USA). All DNA constructions were confirmed by sequencing.

Authors' contributions

E.S. conceived the research, participated in all the procedures, performed the statistical analysis, and drafted the manuscript. M.S. carried out the immunoassays and FACS analysis. A.O. participated in the molecular biological studies including DNA constructions, transfections, sequence analysis, protein fragment complementation assay and FACS analysis. S.P., L.M. and G.D. contributed the CD1a-restricted T cell line K34B9. G.D. also drafted the paper. H.T. supervised the research and participated in its design and coordination and drafted the manuscript. The final manuscript was read and approved by all authors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.10.023.

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