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*J Immunol* 2007; 178:2862-2871; doi: 10.4049/jimmunol.178.5.2862
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Involvement of Src and Syk Tyrosine Kinases in HIV-1 Transfer from Dendritic Cells to CD4+ T Lymphocytes

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Dendritic cells (DCs) are considered as key mediators of the early events in HIV-1 infection at mucosal sites. Although several aspects of the complex interactions between DCs and HIV-1 have been elucidated, there are still basic questions that remain to be answered about DCs/HIV-1 interplay. In this study, we examined the contribution of nonreceptor TKs in the known ability of DCs to efficiently transfer HIV-1 to CD4+ T cells in trans. Experiments performed with specific inhibitors of Src and Syk family members indicate that these tyrosine kinases (TKs) are participating to HIV-1 transfer from immature monocyte-derived DCs (IM-MDDCs) to autologous CD4+ T cells. Experiments with IM-MDDCs transfected with small interfering RNAs targeting Lyn and Syk confirmed the importance of these nonreceptor TKs in HIV-1 transmission. The Src- and Syk-mediated effect on virus transfer was linked with infection of IM-MDDCs in cis-as monitored by quantifying integrated viral DNA and de novo virus production. The process of HIV-1 transmission from IM-MDDCs to CD4+ T cells was unaffected following treatment with protein kinase C and protein kinase A inhibitors. These data suggest that Src and Syk TKs play a functional role in productive HIV-1 infection of IM-MDDCs. Additional work is needed to facilitate our comprehension of the various mechanisms underlying the exact contribution of Src and Syk TKs to this phenomenon. 


The involvement of dendritic cells (DCs)3 in the pathogenesis of HIV-1 infection was discovered very soon after the identification of this retrovirus (1). These cells play a pivotal role not only in establishment and dissemination of HIV-1 infection but also in generating a virus-specific immune response because they are recognized as the most potent APCs of the immune system. It is now well accepted that the initial attachment step of HIV-1 to DCs is a complex process modulated by a large variety of interactions between the virus and the target cell surface (reviewed in Refs. 2 and 3). For example, the association between the oligosaccharides found on the external envelope glycoprotein gp120 and mannose C-type lectin receptors, such as the mannose receptor (MR) (CD206), langerin (CD207), and DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), results in the capture and transmission of HIV-1 to CD4+ T cells in an effective trans-infectious mode (4–8). A viral entity that is bound to C-type lectin receptors is rapidly taken up within endolysosomal vacuoles and protected from degradation while remaining in an infectious state for 1–3 days (9), which is approximately the time required for DCs to migrate to lymph nodes (10). Following the contact between DCs and CD4+ T cells, the internalized viruses are concentrated at the virological synapse and are eventually transferred to the latter cell type where a proficient virus production will ensue (11, 12).

Although some basic understanding of the role played by the above-mentioned surface proteins in the interaction between HIV-1 and DCs has been acquired, little is known as yet on the contribution of intracellular signal transducers in virus capture and transfer. The possible implication of nonreceptor tyrosine kinases (nRTKs) in this process deserves some attention considering that such molecules regulate a wide variety of basic cellular functions such as proliferation (13, 14), migration (13), endocytosis (15), antigenic presentation (14, 16), transport of Fcγ-chain to lysosomes (17), phagocytosis (18, 19), production of reactive oxygen species (20), biosynthesis of lipid metabolites (21), intracellular signaling (22, 23), and cellular communication (24). To date, the most studied nRTKs are members of Src and Syk families. Src was the first TK to be identified by Hunter in 1980 (25). This cellular homolog of the Rous sarcoma virus-transforming protein (26) is implicated in intra- and extracellular communication, cellular growth, and embryonic development (24, 27). In addition to Src, this family includes several other members such as Blk, Fgr, Fyn, Lck, Lyn, and Yes, and their roles have been discussed previously (reviewed in Ref. 23). It is now clear that aggregation or engagement of some specific receptors induces a rapid and transient activation of the Src family members, which then initiate several downstream signaling pathways (15, 28–31). Interestingly, similar observations have been made for Syk family members (i.e., Syk and Zap-70). Indeed, activation of Syk mediates several of the responses mentioned above, and Syk also acts as a tumor suppressor in human breast carcinomas (13). Moreover, Syk is involved in maturation of DCs and IL-12 production (32). Zap-70 is found primarily in T lymphocytes, and there is still no report on its presence in myeloid cells, including DCs. In contrast, Syk is more widely expressed because it has been found in hematopoietic cells (33), epithelial cell lines (34), normal human breast tissue (34), and endothelial cells (13).
Activation of Src and Syk TKs in nontubulin myeloid cells such as neutrophils, monocytes, and DCs occurs also by aggregation of cell surface receptors such as MR, Fcγ, complement, dec- 
tin, integrin, and several other receptors lacking intrinsic protein TK activity. In these cells, processes such as endocytosis, phago- 
cytosis, antigenic presentation, and maturation are all regulated by 
activation of both TK families (16, 18, 32, 35–39). It has been 
shown that Lyn and Syk sequentially bind to FcγRII upon phago-
cytosis-mediated signaling events (35, 40), as they do with the 
BCR, the high-affinity FcγR, and the TCR (14, 31, 41, 42). Fusion 
with lysosomes and targeting of Fcγ-chain to lysosomal degrada-
tion have also been attributed to activation of Src and Syk TKs (17, 36). Moreover, these TKs play an important role in lymphocyte 
activation in response to ligation of various receptors such as 
CD16 (43), TCR (41), FcγRII (42), and CD40 (44). Signal trans-
duction events mediated through FcRs, BCRs, or TCRs involve 
recruitment of Syk to their ITAMs, and this process is also re-
quired for certain subsequent cellular responses such as the target-
ing of γ-chains to lysosomes (17, 45).

It has been reported that the uptake of HIV-1 by DCs and its 
eventual transfer to more susceptible target cells require some de-
efined receptors, the best known of which are MR and DC-SIGN 
(46). Interestingly, it is known that MR activates Src TKs (37). In 
addition, tyrosine residues located in the internalization dileucine 
moiety of the DC-SIGN cytoplasmic tail have been described as 
playing a major role in internalization and transmission of HIV-1 
(47). More recently, Lyn and Syk were both found to be associated 
with DC-SIGN in DCs (48). Moreover, different receptors at the 
surface of DCs can mediate signal transduction through TKs (i.e., 
CD4, FcRs, MR, and integrin). This previous published information 
led us to scrutinize the possible involvement of TKs in the 
intricate interactions between HIV-1 and DCs through the use of 
some pharmacological inhibitors and small interfering RNA 
(siRNA). We demonstrate here that treatment of immature monocyte-
derived DCs (IM-MDDCs) with the Src family inhibitor pyrazolopy-
rimidine (PP2) and the Syk inhibitor piceatannol or transfection with 
siRNAs targeting Lyn and Syk leads to a greater HIV-1 production in 
a coculture system consisting of IM-MDDCs and autologous CD4+ 
T lymphocytes. These findings are not due to an effect on CD4+ 
T cells because HIV-1 replication in this cell type is diminished upon pre-
treatment with PP2 or piceatannol. Additional studies indicate that 
the TK inhibitors affect de novo HIV-1 production in IM-MDDCs. These 
data suggest that Src and Syk TKs play a dominant role in the mul-
tifaceted interplay between DCs and HIV-1.

Materials and Methods

Reagents

3′-Azido-3′-deoxthymidine, PHA-L, LPS, LPS-free DMSO, and trans-
stibene were purchased from Sigma-Aldrich. Piceatannol, PP2, PP3, 889, 
and Ro-318220 were obtained from Calbiochem. IL-2 and Efavirenz 
were obtained through the AIDS Repository Reagent Program. IL-4 and IFN-γ 
were purchased from R&D Systems, whereas GM-CSF was a gift from 
Cangene (Winnipeg, Canada). The culture medium consisted of RPMI 1640 
medium supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin 
(100 U/ml), and glutamine (2 mM), which were all purchased from Wisent.

Antibodies

The monoclonal anti-ICAM-1 Ab RR/J1.1.1 was supplied by R. Rothlein 
(Boehringer Ingelheim; Ridgefield, CT). The anti-CD3 (OKT3, specific for the 
ζ-chain), anti-CD11a (TSI/22.1), anti-CD18 (TSI/18.1), and anti-HLA-DR 
(L243) hybridomas were obtained from the American Type Culture Collec-
tion. The anti-CD86 (BU-63) was supplied by D. L. Hardie (University of 
Birmingham, Birmingham, United Kingdom). The anti-DC-SIGN Ab DC28, 
which recognizes the DC-SIGN repeat region and cross-reacts with DC-
SIGNR (49), was obtained from the AIDS Repository Reagent Program. 
The anti-CD19 (LT19) and anti-CD14 (MEM-18) were obtained from 
EXBIO Praha, whereas the anti-CD83 (HP1SE) was purchased from Re-
search Diagnostics. PE-conjugated goat anti-mouse IgG was purchased 
from Jackson Immunoresearch Laboratories.

Cells

The human DCs were generated from monocytes (i.e., CD14+ 
cells). Briefly, peripheral blood was obtained from normal healthy donors, and 
PBMCs were prepared by centrifugation on a Ficoll-Hypaque density gra-
dient as we described previously (50, 51). Next, CD14+ cells were isolated 
by using a monocyte-positive selection kit according to the manufacturer’s 
instructions (MACS CD14 microbeads from StemCell Technologies). 
CD16+ cells were cultured in 6-well plates at a density of 106 cells/ml. To 
generate IM-MDDCs, purified monocytes were cultured in complete cul-
ture medium that was supplemented every other day with GM-CSF (1000 
U/ml) and IL-4 (200 U/ml) for 7 days. The maturation of IM-MDDCs was 
defined on the fifth day by culturing them for 46 h with the above-de-
scribed cytokines supplemented with IFN-γ (1000 U/ml) and LPS (100 
g/ml). The final phenotype of IM-MDDCs and mature monocyte-derived 
DCs (M-MDDCs) was monitored by flow cytometry (data not shown).

ImM-MDDCs express HLA-DR, CD86, DC-SIGN, and low levels of CD14, 
whereas M-MDDCs express CD83 and high amounts of ICAM-1, HLA-
DR, and CD86 but lower amounts of DC-SIGN and CD14 than IM-
MDDCs. Expression of CD3 and CD19 was measured to assess contami-
nation with T and B cells, respectively. Autologous CD4+ T cells were isolated 
using a negative selection kit according to the manufacturer’s in-
structions (StemCell Technologies). These cells were activated with 
PHA-L (1 μg/ml) and maintained in complete culture medium supple-
mented with IL-2 (30 U/ml) at a density of 2 × 106 cells/ml. Experiments 
were performed with cell preparations that were devoid of contamination 
(i.e., DCs: purity, 95%; CD4+ T cells: purity, 98%).

Production of virus stocks

Virions were produced by transient transfection in human embryonic kid-
dney 293T cells as described previously (52). Plasmids used included 
the pNL4-3bal (R5-tropic), pNL4-3balaov (R5-tropic), and pNL4-3 
(X4-tropic). Progeny viruses were also produced upon acute infection of 
PBMCs for 7 days with various laboratory and clinical HIV-1 isolates (i.e., JR-CSF, 
NL-43-balaov, 93TH054/R5-tropic, 91US056/R5-tropic, 92TH026/R5-
tropic, 93US151/R5-tropic, and 92HT599/X4-tropic). The pNL4-3-balaov 
vector (provided by R. Pomerantz, Thomas Jefferson University, Philadel-
phia, PA) was generated by replacing the env gene of the Tropic HIV-1 
strain, NL-43, with that of the macrophage-tropic HIV-1 Bal strain, thus 
resulting in an infectious molecular clone with macrophage-tropic proper-
ties (53). The other molecular constructs and HIV-1 strains were obtained 
from the AIDS Repository Reagent Program. The virus-containing super-
nants were filtered through a 0.22-μm cellulose acetate syringe filter 
and normalized for virion content using an in-house-sensitive double-Ab 
sandwich ELISA specific for the viral p24gag protein (54).

Transfer studies

DCs (105 cells in 100 μl) were either left untreated or treated with the 
tested pharmacological inhibitors before being pulsed with virus prepara-
tions (2 or 10 ng of p24gag) for 60 min at 37°C. Next, the virus-cell mixture 
was washed three times with PBS to remove untrapped virions. DCs were 
cocultured with autologous activated CD4+ T lymphocytes (ratio 1:3) in 
complete RPMI 1640 medium supplemented with IL-2 (30 U/ml) in 96-
well plates in a final volume of 200 μl. Every 2 days, half of the medium 
was removed and kept frozen at −20°C, and fresh medium was added to 
the culture. Virus production was estimated by measuring p24gag levels in 
culture supernatants.

Gene silencing of Lyn or Syk with siRNAs

siRNAs targeting Lyn or Syk as well as control siRNAs (containing scrum-
bled sequences) were obtained from Dharmacon Research and dissolved in 
an appropriate buffer. IM-MDDCs were washed with OptiMEM (Inviro-
gen Life Technologies) without serum and antibiotics. The tested siRNAs 
were transfected at a final concentration of 200 pmol/well using Oligo-
fectamine according to the manufacturer’s instructions (Invitrogen Life 
Technologies). Control cells were treated either with Oligofectamine alone 
or Oligofectamine plus scrambled sequences. Forty hours following trans-
feciton, a coculture made of IM-MDDCs and autologous CD4+ T cells 
was initiated as described above. Silencing efficiency of Lyn or Syk was mon-
tored by western blot analysis as described previously (55).

Infection of CD4+ T cells

Purified CD4+ T cells (3 × 105 cells) were either left untreated or treated 
with the tested pharmacological inhibitors before infection with HIV-1

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(15 ng p24<sub>ag</sub>) (JR-CSF, NL4-3, or NL4-3). Cells were exposed to HIV-1 particles for 2 h at 37°C and then washed three times with PBS. The cells were maintained in complete RPMI containing IL-2 (30 U/ml) for 3 days and virus production was measured by assessing p24<sub>ag</sub> levels in the culture supernatants.

**Real-time PCR test**

The amount of integrated viral DNA was measured with a real-time PCR approach as described by Suzuki and coworkers (56). Briefly, the DNA in IM-MDDCs was extracted at day 12 postinfection with the Qiagen DNeasy tissue kit according to the manufacturer’s instructions. Briefly, a first round of PCR amplification was conducted using 100 ng of DNA and TaqDNA polymerase (Promega). An Ala-specific sense primer (5′-TCC CTA CTC GGG AGG CTG AGG-3′) was used in combination with an antisense HIV-1 specific primer (M661) (5′-CCT GCG TCG AGA GAT CTC CTC TG-3′, 673–695). Cycling conditions included an initial denaturation step (94°C for 3 min), followed by 22 denaturation cycles (94°C for 30 s), annealing (66°C for 30 s), and extension (70°C for 10 min) followed by a final extension (72°C for 10 min). The first PCR products were diluted 5-fold and subjected to a real-time PCR assay targeting the HIV-1 R/U5 region. The specific sense primer (M667) (5′-GGC TAA GGG AAC CCA CTG C-3′, 496–517) coupled to an antisense primer (AA55) (5′-CTG CTA GAG ATT TTC CAC ACT GAC-3′, 612–635) were used with the fluorogenic probe TaqMan 5′-AM (5′-AM TAC GAC TAC GAC-3′) (Biosearch Technologies). Finally, PCR was performed using the Rotor-Gene 3000 four-channel multiplexing system (Corbett Research). Cycling conditions included 40 denaturation cycles (95°C for 20 s) and extension (60°C for 1 min). NL4-3 DNA was used for the standard curve (i.e., from 469 to 30,000 copies). HIV-1 standards contained 1 ng of DNA from uninfected cells as a carrier.

**Statistical analysis**

Statistical analyses were conducted according to the methods outlined in Zar (57). Means were compared using either the Student’s t test or a single factor ANOVA followed by Dunnett’s multiple comparison when more than two means were considered. When a same test had to be performed five times or more for a same experiment, a sequential Bonferroni correction was applied to minimize the probability of type I errors (58). Values of \( p < 0.05 \) were deemed statistically significant. For all figures, an asterisk (*) denotes a \( p \) value of <0.05, whereas two asterisks (**) denotes a \( p \) value of <0.01. Calculations were made with the GraphPad Prism software.

**Results**

Src and Syk TKs modulate HIV-1 transfer by IM-MDDCs

Our initial series of investigations were focused on Src family kinases because they are cytoplasmic protein TKs known to be essential for many cell functions. To test the relative contribution of Src TKs in the process of HIV-1 transmission from DCs to CD<sub>4</sub> T cells, IM-MDDCs as well as M-MDDCs were first treated with the selective Src family inhibitor PP2 for 10 min before pulsing such cells with a R5-tropic HIV-1 strain (i.e., JR-CSF). The cell-virus mixture was next cocultured with autologous CD4<sup>+</sup> T lymphocytes that were used as receptor cells. Transmission of HIV-1 was found to be slightly more rapid and efficient when using M-MDDCs as compared with IM-MDDCs (Fig. 1), which is in agreement with the data found in the literature (50, 59). Interestingly, the process of trans-infection was increased upon treatment of IM-MDDCs with PP2 (Fig. 1A). The Src inhibitor had no such comparable effect on HIV-1 transfer by M-MDDCs (Fig. 1B). It should be noted that similar observations were made in transfer experiments when using IM-MDDCs pulsed with various R5-tropic clinical HIV-1 isolates amplified in primary human cells (i.e., 93TH054, 91US056, 92TH026, and 93US151) (data not shown). Virus transfer was not affected when X4-tropic laboratory and clinical HIV-1 variants (NL4-3 and 92HsT599, respectively) were used to pulse IM-MDDCs similarly treated with PP2 (data not shown). Therefore, subsequent experiments were performed exclusively with IM-MDDCs in combination with R5 viruses. The use of IM-MDDCs is also prompted by the concept that immature DCs will be most likely the first cell type to come
in contact with the virus because they reside in mucosal tissues. The next virus transfer experiments were also performed in the presence of IL-2 to assure a constant virus production at earlier time points following initiation of coculture and to eliminate the possibility that several rounds of virus replication in autologous CD4+ T lymphocytes could mask an initial difference in transfer by IM-MDDCs. The specificity of the observed effects was tested by performing transfer studies with PP3, which is an inactive analog of PP2. As illustrated in Fig. 1C, transmission of HIV-1 from IM-MDDCs to autologous CD4+ T cells was still augmented upon treatment of DCs with PP2 but was not affected by a similar treatment with the inactive analog PP3, thereby confirming the validity of our results. The PP2-induced modulatory effect was rapid because an increase was already detected as early as 2 days following coculture of virus-pulsed IM-MDDCs with autologous CD4+ T cells (Fig. 1C, small inset).

Subsequently, we studied the possible implication of Syk because this TK acts more downstream of Src family members in several endocytosis/phagocytosis-signaling pathways and DC-SIGN-mediated signal transduction events (16, 18, 32, 35, 36, 40, 48). To this end, IM-MDDCs were first treated with the Syk inhibitor 3,4,3',5'-tetrahydroxy-trans-stilbene (piceatannol) before virus exposure, followed by a coculture with autologous CD4+ T lymphocytes. Pretreatment of IM-MDDCs with such a Syk-selective TK inhibitor resulted in a significant increase in HIV-1 transfer (Fig. 2, small inset). The specificity of the piceatannol-dependent effect was confirmed by the inability of the inactive analog trans-stilbene to induce a similar enhancement of virus transmission.

Our next series of investigations were performed with increasing doses of the specific Src and Syk inhibitors and their appropriate inactive analogs using a highly infectious R5-tropic virus isolates (i.e., NL4-3balenv). Data shown in Fig. 3 indicate that there is a dose-dependent PP2- and piceatannol-mediated increase in virus transfer with a peak reached in both instances at a 10 μM concentration. The inactive analogs (i.e., PP3 and trans-stilbene) were still unable to affect HIV-1 transmission from IM-MDDCs to autologous CD4+ T cells (data not shown). It should be noted that all concentrations tested were found to be noncytotoxic using a colorimetric assay (data not shown). Importantly, HIV-1 transmission was again increased upon treatment with both PP2 and piceatannol when using this time a R5-tropic clinical isolate of HIV-1 (i.e., 91US056) (Fig. 4), which provides physiological significance.
p24gag after 60 min of pulsing under our experimental conditions, 4% of the IM-MDDCs were found to be positive for intracellular virus. An average of 60 ± SD of triplicate samples and are representative of four independent experiments. Means were compared using single factor ANOVAs followed by Dunnett’s multiple comparisons.

Next, we have used the siRNA technology to confirm the implication of Src and Syk TKs in the observed phenomenon and also to circumvent nonspecific effect(s) mediated by chemical inhibitors. More specifically, siRNAs targeting Lyn and Syk were introduced within IM-MDDCs before exposure to HIV-1 and initiation of the coculture. As depicted in Fig. 5, a more important HIV-1 transfer from IM-MDDCs to autologous CD4+ T cells was seen when protein expression of Lyn or Syk was diminished through the use of siRNAs. A reduced expression of Lyn and Syk was confirmed by western blot analysis (Fig. 5, small inset) and intracellular flow cytometry (data not shown).

**HIV-1 infection in cis of IM-MDDCs is increased upon inhibition of Src and Syk TKs**

To shed light on the putative mechanism(s) by which Src and Syk kinases can regulate the ability of IM-MDDCs to transfer HIV-1 particles to CD4+ T cells in trans, we measured the effect of Src- and Syk-selective inhibitors on the initial interactions between DCs and HIV-1. Flow cytometry analyses demonstrated that cell surface expression of DC-SIGN and MR was not modulated upon treatment with PP2 or piceatannol (data not shown). Moreover, results from virus internalization studies revealed that similar amounts of viruses were found inside IM-MDDCs that were either left untreated or pretreated with PP2 or piceatannol. A reduced expression of Lyn and Syk was confirmed by western blot analysis (Fig. 5, small inset) and intracellular flow cytometry (data not shown).

Previous reports have shown that HIV-1 is transferred from DCs to CD4+ T cells via a process involving two distinct phases (9, 60). An initial transfer phase occurs whereby the virus located within endosomal compartments in IM-MDDCs is transported to the DCs-T cell synapse (i.e., early transfer). This event is followed by a second kinetic phase that is dependent on productive infection of IM-MDDCs and eventual transfer of progeny virus to CD4+ T cells (i.e., late transfer). To define whether the Src- and Syk-mediated action on virus transfer is affecting the early and/or late transfer phase, IM-MDDCs were either left untreated or treated with the antiretroviral drug Efavirenz before initiation of the coculture. This treatment blocks productive virus infection of IM-MDDCs and allows only the early transfer phase to occur. A significant diminution in HIV-1 is detected when the late transfer phase is abolished (Fig. 6A). More importantly, under such experimental conditions, no increase in virus transfer was observed upon treatment with PP2 or piceatannol, thus suggesting that the early phase transfer is not affected upon treatment with the tested TK inhibitors. Similar observations were made when the antiviral agent 3′-azido-3′-deoxythymidine was used under comparable experimental conditions (data not shown). To confirm that the Src and Syk TKs are affecting primarily the late phase transfer, IM-MDDCs were either left untreated or treated with the studied chemical drugs before acute infection with a highly infectious R5-tropic viral strain (i.e., NL4-3Balenv). Virus production was evaluated at day 12 postinfection, a time lapse sufficient enough to measure the release of viruses from HIV-1-infected IM-MDDCs. Results depicted in Fig. 6B indicate that de novo virus production in IM-MDDCs is increased by a pretreatment with PP2 or piceatannol, thereby corroborating the idea that Src and Syk TKs influence the late transfer phase (i.e., productive infection of IM-MDDCs). As expected, HIV-1 infection in cis of IM-MDDCs was unaffected upon a pretreatment with the inactive analogs (data not shown). Moreover, cellular DNA was extracted from such virus-infected IM-MDDCs at day 12 postinfection. Thereafter, the amount of integrated viral DNA was quantified using a real-time PCR test that provides a measure of viral integration into the host cell chromatin. This technical strategy uses primer sets designed to encompass both cellular (i.e., Alu) and HIV-1-specific DNA sequences. The number of integrated viral DNA copies was higher in IM-MDDCs that were pretreated with PP2 or piceatannol as compared with untreated cells (Fig. 6C). The amounts of integrated HIV-1 DNA copies were not affected by the inactive analogs (data not shown).

**Effect of other kinases on HIV-1 transfer by IM-MDDCs**

We next tested whether HIV-1 transmission by IM-MDDCs could be affected by other signal transducers such as the serine/threonine kinases protein kinase C (PKC) and protein kinase A (PKA) because they are known to play essential roles in the fine-tuning of signaling cascades of several cellular process, including Ag uptake.
and formation of the immunological synapse (61, 62). Pharmacological inhibition of PKC and PKA was achieved through the use of the selective inhibitors Ro-318220 and H89, respectively. In contrast to what is seen with Src and Syk inhibitors, the process of HIV-1 transfer from IM-MDDCs to autologous CD4\(^+\)/H11001 T cells was unaltered by both Ro-318220 and H89 (Fig. 7A). Virus infection of IM-MDDCs, which corresponds to de novo virus production, was also unaffected upon pretreatment with Ro-318220 and H89 (Fig. 7B). These findings were corroborated when estimating integrated viral DNA copies in either untreated, Ro-318220-treated, or H89-treated IM-MDDCs (Fig. 7C). In this set of experiments, piceatannol was used as a positive control. Altogether, these results indicate that PKC and PKA do not play a functional role in HIV-1 transfer from IM-MDDC to CD4\(^+\) T cells.

**Effect of Src and Syk inhibitors on HIV-1 infection of CD4\(^+\) T cells**

Experiments were subsequently performed to estimate whether part of the observed enhancement of HIV-1 transfer might be due to a transport of the tested drugs from DCs, which would next act at the level of autologous CD4\(^+\) T lymphocytes. This scenario was addressed by pretreating CD4\(^+\) T cells with the Src- and Syk-selective inhibitors before infection with HIV-1. In sharp contrast to what is seen when measuring infection *in trans* from
IM-MDDCs to autologous CD4+ T lymphocytes, data shown in Fig. 8 demonstrate that replication of R5-tropic JR-CSF (Fig. 8A) and NL4-3-balenv (Fig. 8B) was significantly reduced upon pretreatment of CD4+ T lymphocytes with PP2 and piceatannol, respectively. These findings were confirmed when using X4-tropic isolates of HIV-1 produced in PBMCs (i.e., NL4-3 and 92HT599) (data not shown). A colorimetric assay revealed that the studied compounds were not cytotoxic at the concentration tested (data not shown).

Discussion

It has been well established that nrTKs are involved in regulating many key cellular responses and particularly the uptake and/or endocytosis of some specific ligands. Considering that the capture of HIV-1 by DCs and its eventual transmission to more susceptible target cells such as CD4+ T cells involve endocytosis, we have investigated the participation of nrTKs in virus transfer. Here, we present evidence that Src and Syk TKs can limit HIV-1 transmission from IM-MDDCs to autologous CD4+ T lymphocytes. Purified CD4+ T cells (3 × 10^6 cells) were either left untreated or preincubated with PP2 or piceatannol (10 µM) for 10 min before pulsing with JR-CSF (A) or NL4-3-balenn (B) (10 ng of p24\textsuperscript{ng/ml}) for 60 min at 37°C. After three washes with PBS, CD4+ T cells were cultured in complete culture RPMI 1640 medium supplemented with IL-2. Cell-free supernatants were collected at day 6 following virus infection and assayed for p24\textsuperscript{ng/ml}. Data shown correspond to the means ± SD of triplicate samples and are representative of three independent experiments. Means were compared using single factor ANOVAs followed by Dunnett’s multiple comparisons.

Quantitative measurements of internalized virions in IM-MDDCs indicated that HIV-1 entry is not affected upon a treatment with the studied Src- and Syk-specific inhibitors. For example, an average of 4% of the IM-MDDCs that were either left untreated or treated with the studied chemical compounds were found to be positive for p24\textsuperscript{ng/ml} following virus exposure. Such a low percentage of cells capturing HIV-1 particles compared with previously published studies can be explained by differences in experimental methodologies. For example, a virus input corresponding to 2–3 µg of p24\textsuperscript{ng/ml} per 10^6 DCs was used in the work described by Turville et al. (9) compared with a virus input of 100 ng of p24 per 10^6 DCs in the present study. This also helps to explain why we have performed quantitative analyses of proviral DNA in IM-MDDCs at 12 days postinfection compared with 3 days postinfection in the study by Turville et al. (9). Additionally, we have used a real-time PCR test that permits to estimate the amount of integrated proviral DNA copies while Turville et al. (9) have performed proviral DNA quantification using a real-time quantitative PCR test that does not allow to discriminate between unintegrated and integrated proviral DNA copies.

Three distinct and not mutually exclusive hypotheses can be proposed to explain the augmentation of productive HIV-1 infection in IM-MDDCs and enhancement of virus transfer that are seen following treatment with Src- and Syk-specific chemical compounds (Fig. 9). First, Src and Syk TKs could alter the virus entry route by favoring endocytosis of the incoming virions. This is based on the demonstrated capacity of Src and Syk TKs to promote internalization of ligands and possibly viruses by a direct action on phagocytosis/endocytosis pathways (16, 18, 32, 35, 36, 40). It is now recognized that the HIV-1 entry mode into myeloid cells can result either in cytosolic delivery that results in productive infection (65–67), preservation in intracellular vesicles in an infectious state for a subsequent transmission through the virological synapse (7, 12, 68, 69), or degradation by lysosomal enzymes in the endosomal apparatus (9). In view of this, we propose that virions can remain for a longer time period on the surface of IM-MDDCs without being internalized when Src and Syk TKs are inhibited. This process will favor interactions between gp120 and a complex made of CD4 and an appropriate coreceptor (e.g., CCR5) and the release of the viral material in the cytosol as a consequence of a pH-independent fusion of viral and cellular membranes. Interestingly, it is now known that this mechanism of HIV-1 entry into target cells results in productive infection (65–67). This postulate is supported by the ability of CCR5 antagonists to inhibit HIV-1 infection in cis of IM-MDDCs (data not shown). Second, it can be proposed that Src and Syk TKs promote the degradation of virions in the endosomal apparatus. This scenario is validated by the previous observation that members of Src and Syk families target several cell surface receptors for degradation inside the lysosome (17). Interestingly, it is now clear that a significant lysosomal degradation occurs following uptake of HIV-1 by IM-MDDCs (9). Third, based on previous studies by Sedlik et al. (32) and Wilflingseder et al. (70), it can also be postulated that Src and Syk TKs might be involved in maturation of DCs. Indeed, treatment with Src- and Syk-specific inhibitors could block the maturation process of IM-MDDCs, thus resulting in a more efficient infection in cis of DCs based on the idea that M-MDDCs do not support active HIV-1 replication as it is the case for IM-MDDCs (59, 64). However, this postulate is unlikely because uninfected IM-MDDCs that were treated with the tested TK inhibitors were not frozen in an immature state (as monitored by flow cytometry...
through the use of anti-CD83, anti-CD86, anti-DC-SIGN, and anti-HLA-DR Abs (data not shown).

In contrast to the situation prevailing in IM-MDDCs, Src- and Syk-specific inhibitors resulted in a significant diminution of virus production in CD4+ T cells. Until now, there was a paucity of data concerning the susceptibility of primary human CD4+ T lymphocytes to HIV-1 infection in the presence of TK inhibitors. The significant inhibition of virus production in this cell type might be linked to the previously described properties of Src family members. For example, PP2 could block p56lck activity, a process of prime importance in CD4-mediated signaling (71). It should be noted that HIV-1 infection is affected when the natural association between CD4 and p56lck is prevented (72). Additionally, PP2 might affect the interaction between Hck and Nef (73), a virus-encoded regulatory protein that has a positive effect on viral infection and replication (74). Last, it is also possible that inhibition of Fyn activity might contribute to the observed effect due to its role in the M phase of the cell cycle (75). Regarding the implication of Syk/Zap-70 in the marked inhibition of virus production in CD4+ T cells, it can be proposed that such TKs might favor the recruitment of the CD4/p56lck complex near the TCR as shown previously (76). Piceatannol might also modify this process and thereby affect virus-mediated signaling events, which in turn might influence HIV-1 replication. Alternatively, although piceatannol does not affect HIV-1 entry inside CD4+ T cells (77), it can modulate virus gene expression by its ability to modulate NF-κB induction by various inflammatory agents through inhibition of both IκBα kinase and p65 phosphorylation (78).

Different technical approaches can be undertaken to study the involvement of signal transducers in a definite process. Small-molecular inhibitors have emerged as indispensable tools for studying signal transduction. We have therefore deliberately selected an experimental strategy that consists of treating cells with specific pharmacological inhibitors to study the implication of nrTKs in the complex interplay occurring between DCs and HIV-1. Experiments performed with increasing doses of the tested inhibitors and appropriate inactive analogues permit to validate findings made with chemical tools. We are aware that one cannot completely rule out that pharmacological compounds can alter other known or unknown targets at the cellular level. Therefore, experiments were also conducted with siRNAs targeting Lyn and Syk to exclude indirect effects exerted by the studied chemical compounds. This experimental strategy corroborated the implication of Src, and more precisely of Lyn, and Syk in the control of HIV-1 replication in IM-MDDCs.

We report that Src and Syk family members influence HIV-1 infection of IM-MDDCs (infection in cis) and virus transfer to autologous CD4+ T cells (infection in trans). A better understanding of the precise details on how this is achieved is essential because it might bear consequence for the loading of virus Ags and the mounting of a specific immune response. Indeed, DCs are considered as professional Ag-capturing and -presenting cells able to trigger strong and effective immune responses both in vitro and in vivo (79, 80). There is accumulating evidence that viral pathogens such as HIV-1 can exploit DCs to subvert the immune response and establish a persistent infection in the host by mechanisms that are still unresolved. For example, the uptake of HIV-1 induces, without replication, a specific cytotoxic T lymphocyte activity (81, 82). On the other hand, viral Ags located onto infected DCs and cell-free virions captured and processed by DCs can activate virus-specific CD4+ and CD8+ T cell activities (81, 83, 84).

In summary, this work provides new insights into the complex interconnections between HIV-1 and DCs. Altogether, our results reveal that members of Src and Syk families limit HIV-1 replication in IM-MDDCs. This is in sharp contrast to what is seen when infection is allowed to proceed in primary human CD4+ T cells pretreated with specific Src and Syk inhibitors. Thus, the intricate relationships that are established between HIV-1 and host cells will vary depending on the cell type. Further studies are needed to elucidate how Src and Syk family members can modulate the susceptibility of IM-MDDCs to HIV-1 infection in cis.

Acknowledgments

We express our gratitude to Lahlou Hadji, Chantal Burelout, and Philippe Desaulniers for their critical and constructive comments for this study. We thank Sylvie Méthot and Lahlou Hadji for their excellent technical assistance in writing this manuscript and also Marc Bergeron for his expertise in statistics. We are grateful to Michael Imbeault and Mélanie Tardif for their
assistance with the real-time PCR assay. We appreciate the excellent technical contributions of Maurice Dufour, Odette Simard, and Caroline Côté.

Disclosures

The authors have no financial conflict of interest.

References


