Extracellular ATP acts on P2Y2 purinergic receptors to facilitate HIV-1 infection

Claire Séror, Marie-Thérèse Melki, Frédéric Subra, Syed Qasim Raza, Marlène Bras, Hélène Saïdi, Roberta Nardacci, Laurent Voisin, Audrey Paoletti, Frédéric Law, Isabelle Martins, Alessandra Amendola, Ali A. Abdul-Sater, Fabiola Ciccosanti, Olivier Delelis, Florence Niedergang, Sylvain Thierry, Najwane Said-Sadier, Christophe Lamaze, Didier Métivier, Jérome Estaquier, Gian Maria Finia, Laura Falasca, Rita Casetti, Nazanine Modjtabahedi, Jean Kanellopoulos, Jean-François Mouscadet, David M. Ojcius, Mauro Piacentini, Marie-Lise Gougeon, Guido Kroemer, Jean-Luc Perfettini

Extracellular adenosine triphosphate (ATP) can activate purinergic receptors of the plasma membrane and modulate multiple cellular functions. We report that ATP is released from HIV-target cells through pannexin-1 channels upon interaction between the HIV-envelope protein and specific target cell receptors. Extracellular ATP then acts on purinergic receptors, including P2Y2, to activate proline-rich tyrosine kinase 2 (Pyk2) kinase and transient plasma membrane depolarization, which in turn stimulate fusion between Env-expressing membranes and membranes containing CD4 plus appropriate chemokine coreceptors. Inhibition of any of the constituents of this cascade (pannexin-1, ATP, P2Y2, and Pyk2) impairs the replication of HIV-mutant viruses that are resistant to conventional antiretroviral agents. Altogether, our results reveal a novel signaling pathway involved in the early steps of HIV-1 infection that may be targeted with new therapeutic approaches.

HIV-1 infection poses a public health problem that is partially controlled by a combination of specific antiretroviral agents. Nonetheless, the surge of multiresistant HIV-1 strains will require the development of novel antiviral strategies. Targeting early infection by vaccines and microbicides represents the main challenge to end the AIDS epidemic (Haase, 2010; Virgin and Walker, 2010).
ATP release boosts HIV-1 infectivity | Séror et al.

A better understanding of the early steps of HIV-1 infection is critical to achieving this goal. Enveloped viruses must fuse their membranes with host cell membranes to allow productive infection. Major transient changes in the charge and architecture of the host plasma membrane, including extreme curvature, occur during early steps of infection and facilitate viral replication (Miller et al., 1993; Davis et al., 2004). However, little is known about the consequences of these cell membrane alterations on the early signaling pathway required for viral infection.

Recent studies revealed that membrane stress induced by mechanical or chemical stimuli (shear stress [Wan et al., 2008], osmotic swelling [Darby et al., 2003], and membrane shrinking [Corriden and Insel, 2010]) stimulates ATP release. Initially described as a second messenger in the nervous and vascular systems (Schwiebert et al., 2002; Housley et al., 2009), extracellular ATP may also act as a proinflammatory mediator released during acute inflammation upon cell damage or bacterial infection, thus representing a generic marker of damage which can alert the immune system to danger (Gallucci and Matzinger, 2001). In addition, extracellular ATP inhibits infection by intracellular bacterial pathogens (Lammas et al., 1997; Coutinho-Silva et al., 2003) and modulates immune responses by participating in the chemotaxis of immune cells.

Figure 1. ATP release through pannexin-1 hemichannels modulates interactions of HIV-1 with target cells. (A) ATP released by CD4+CXCR4+ cells during HIVNL43 infection (MOI = 1; black circle) or in the absence of infection (white circle) was determined at different time points by ATP-dependent bioluminescence in three independent experiments. One representative experiment is shown (mean ± SEM; three independent experiments; *, P < 0.01). (B) CD4+CXCR4+ cells expressing a Tat-inducible β-galactosidase (β-gal) reporter gene were infected with HIVNL43 (MOI = 1) during 48 h in the presence of different concentrations of apyrase. Then, the medium containing apyrase was removed, replaced by complete medium, and target cell infection was determined 48 h after infection (mean ± SEM; three independent experiments; *, P < 0.05). (C) ATP release was expressed as a fold increase of ATP release during co-culture of HIV-1 target cells with Env+ or Env− cells. Error bars indicate SEM of three independent determinations (*, P < 0.05). (D) Effect of ATP depletion by apyrase on HIV-1 envelope mediated actin polymerization. Actin polymerization in pairs of interacting cells was determined in three independent experiments (means ± SEM; *, P < 0.05) by fluorescence microscopy. The inset illustrates actin polymerization (green) between an Env+ cell (red) and an interacting Env− cell. Error bars indicate SD of three independent experiments (*, P < 0.01). (E) Effect of DIDS or SITS on HIV-1 infection and on p24 antigen release was determined. Error bars indicate SD of three independent experiments (*, P < 0.01; **, P < 0.001). (F) CD4+CXCR4+ cells transfected with siRNA specific for pannexin-1 or control siRNA and then infected with HIVNL43 (MOI = 1) was measured by quantitative real-time PCR. The effect of pannexin-1 depletion on p24 antigen release was determined as described in Materials and methods (means ± SEM; n = 3; *, P < 0.01; **, P < 0.001).
(eosinophils, neutrophils, monocytes/macrophages, and immature DCs; Chen et al., 2006; Kronlage et al., 2010), by activating the NALP3 inflammasome (Mariathasan et al., 2006), or by mediating costimulatory signals for antigenic stimulation (Schenk et al., 2008). Recent studies revealed that ATP can also be released under basal conditions and influences a large array of cellular responses. Thus, ATP appears to act as an inside-out messenger that fine tunes signal transduction pathways (Corriden and Insel, 2010).

Outside of the cell, ATP acts as an autocrine/paracrine signal, modulating a variety of cellular functions by activating purinergic receptors (Corriden and Insel, 2010). These plasma membrane–localized receptors belong to a larger family that can be classified into ionotropic P2X receptor and metabotropic P2Y receptors (Ralevic and Burnstock, 1998). Metabotropic receptors are coupled to intracellular signaling pathways through heterotrimeric G proteins (Abbracchio et al., 2006), whereas ionotropic P2X receptors are associated with pores that open upon ATP binding, allowing Ca2+ influx and K+ efflux (Ralevic and Burnstock, 1998). Seven members of the P2X family (P2X1–7; Ralevic and Burnstock, 1998) and eight P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) have been characterized (Abbracchio et al., 2003). Upon activation, these receptors, which are widely distributed throughout the body, modulate an array of cellular functions like plasma membrane permeabilization, Ca2+ influx, and cell death (Surprenant and North, 2009). Purinergic receptors have been extensively involved in the development of innate and/or adaptive immune responses against pathogens (Lammas et al., 1997; Coutinho-Silva et al., 2003; Chen et al., 2006; Mariathasan et al., 2006; Kronlage et al., 2010) but have also been associated with chemotherapy-driven anticancer immune responses (Ghiringhelli et al., 2009).

In this study, we determined whether ATP and purinergic receptors might modulate HIV-1 infection. We found that the HIV-encoded envelope glycoprotein complex (Env) can stimulate the release of ATP from HIV-1 target cells and that extracellular ATP then stimulates purinergic signals that facilitate HIV-1 infection. We characterized several elements of the signal transduction pathway—that facilitates ATP release from HIV-1 target cells and mediates autocrine ATP effects—that are essential for the early steps of HIV-1 infection.

RESULTS

ATP release from target cells participates in HIV-1 infection

Because membrane stress can induce the release of ATP from mammalian cells (Corriden and Insel, 2010), we speculated that HIV-1 infection might trigger ATP release from host cells. We found that infection of human cells with X4-tropic HIV-1 (HIVNL43), which relies on the expression of CD4 and CXCR4, can stimulate the release of ATP from HIV-1 target cells and that extracellular ATP then stimulates purinergic signals that facilitate HIV-1 infection. We characterized several elements of the signal transduction pathway—which facilitates ATP release from HIV-1 target cells and mediates autocrine ATP effects—that are essential for the early steps of HIV-1 infection.

Figure 2. Purinergic receptors modulate HIV-1 infection. (A–C) Effects of CXCR4 receptor antagonist AMD3100 and the general P2 receptor antagonists suramin, PPADS, and OxATP on p24 release (A), host cell infectivity (B), and hemifusion/fusion (C), as observed during infection of PHA+IL-2–stimulated human PBMCs with HIVNDK and HIVBaL (A), during infection of CD4–CXCR4+ cells with HIVNL43WT and HIVNL43Env (B), or during coinfection of Env+ cells with HIV-1 target cells (C). Columns in A–C show means ± SEM (n = 3; *, P < 0.05). (D and E) Effects of 5 µM AMD3100, 10 U/ml apyrase, 10 µM suramin, 100 µM PPADS, 100 µM OxATP, and 500 nM efavirenz (Efav) on viral RNA expression and on total viral DNA expression of wild-type (HIVNL43WT) or VSVG pseudotyped (HIVNL43Env) HIV-1 in CD4–CXCR4+ cells. Viral RNA was detected by quantitative PCR. Note that the internalization of pseudotyped HIVNL43Env virus was not reduced by these inhibitors (D). Total viral DNA was determined by PCR and results were normalized with respect to GAPDH (E). Efavirenz was used as an internal control of reverse transcription activity inhibition. The results are representative of three independent experiments.
as the cells were engaging in conjugates (Fig. 1 C). Apyrase abolished conjugate formation and the accompanying actin polymerization in juxtaposed cells (Fig. 1 D). The release of ATP from cells constitutes a common response to shear stress and osmotic imbalance (Wang et al., 2005) and can be mediated through mechanosensitive pannexin-1 hemichannels (Bao et al., 2004). Indeed, pannexin-1 was enriched at the contact site between HIV-1–infected lymphoblasts (which express Env on the surface) and uninfected lymphoblasts (Fig. 1 E). Pannexin-1 polarization occurred in conditions in which the levels of pannexin-1 protein remained constant (Fig. S1 G) and was preferentially detected in uninfected cells (Fig. S1, H and I). In addition, pannexin-1 was associated with virological synapses that formed between infected and uninfected cells (Fig. 1 F).

Pharmacological inhibition of pannexin-1 with 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) or 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) protected target cells against HIV-1–induced cell death (Fig. 1 G), reduced p24 antigen release (Fig. 1 G), and inhibited HIV-1 infection and fusion between CD4/CXCR4 and Env-expressing cells (Fig. 1 H; and Fig. S1, J and K). In these 6–11-long experiments, DIDS and SITS inhibited HIV-1 infection at doses that are lower than those required to prevent fusion between HIV Env+ and CD4+CXCR4+ cells, perhaps reflecting cumulative effects over multiple viral cycles and/or distinct avidities (and hence susceptibilities to inhibition) of the interaction of infectable cells with HIV-1 Env present at the surface of cells versus viral particles. siRNA-mediated depletion of pannexin-1 from HIV-1 target cells also decreased p24 antigen release (Fig. 1 I), reduced HIV-1 infection (Fig. 1 J), blocked HIV-1 Env–mediated fusion (Fig. S1, L), and inhibited the release of ATP from host cells during conjugate formation (Fig. S1 L) and HIV-1 infection (Fig. 1 K). Thus, extracellular ATP release plays a major role in Env-dependent interactions between HIV-1 and host cells.

Purinergic receptors contribute to viral uptake

Extracellular ATP constitutes a common danger signal that is sensed by distinct purinergic receptors (Khakh and North, 2006). General inhibitors of purinergic receptors, including suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), and oxidized ATP (OxATP), blocked the replication of both X4-tropic HIV (e.g., HIVΔvif) and R5-tropic HIV (e.g., HIVΔvpr) in activated T lymphoblasts (Fig. 2 A and Fig. S2 A). Purinergic receptor antagonists also blocked the replication of R5-tropic HIV-1 in macrophages (Fig. S2, B and C) or DCs (Fig. S2, D and E) and that of clinical HIV-1 isolates in C8166 T leukemia cells (Fig. S2, F–H).

Cervical epithelial (HeLa) cells engineered to express CD4/CXCR4, as well as a Tat-inducible β-galactosidase (β-Gal) reporter, expressed β-Gal upon infection with a lymphotropic HIV-1 (HIVNL4.3Δenv). Such cells also expressed β-Gal upon infection by an isogenic virus in which the endogenous Env gene had been replaced by that of vesicular stomatitis virus (VSV; HIVNL4.3Δenv) allowing the virus to infect cells which lack CD4/CXCR4 (Reiser et al., 1996). Purinergic receptor antagonists were more efficient in inhibiting β-Gal induction by Env-expressing than that by VSV-pseudotyped HIV-1 (Fig. 2 B and Fig. S3 A), indicating that they mainly affect the receptor-dependent facet of HIV-1 infection. Purinergic receptor inhibition also reduced the hemifusion (which causes the exchange of fluorescent lipids between interacting cells) and cell-to-cell fusion (syncytium formation with mixture of the cytoplasm) between CD4+CXCR4+ cells and Env-expressing cells (Fig. 2 C and Fig. S3 B). Finally, purinergic receptor antagonists prevented the entry of HIV-1 into cells, as revealed by their inhibitory effects on the detection of HIV genomes at the RNA (Fig. 2 D) and DNA (Fig. 2 E) levels within host cells. In contrast, purinergic receptor inhibition did not affect the replication of VSV-pseudotyped
HIV-1 (Fig. 2, D and E). These results demonstrate that purinergic receptors are critical for the Env-mediated internalization of HIV-1 into suitable target cells.

Purinergic receptor P2Y2 modulates HIV-1 infection

To identify the principal purinergic receptors involved in HIV-1 internalization, we determined purinergic receptor expression on HIV-1 target cells (Fig. S4, A–N) and we selectively depleted the mRNAs coding for P2X1, P2X4, P2X7, P2Y1, P2Y2, and P2Y6 by siRNAs (Fig. S5, A–F). Depletion of P2Y2 consistently resulted in the strongest reduction in Env-triggered syncytium formation (Fig. S5 G). Immuno-reactive P2Y2 was detectable at higher levels in lymphoid tissues (Fig. 3, A and C), in the frontal cortex (Fig. 3, B and D), and in circulating leukocytes (Fig. 3, E and F) of untreated HIV-1 carriers, as compared with uninfected carriers (Fig. 3, A, B, and E) and samples stained with isotype control antibody (Fig. S5, H and I). The levels of P2Y2 protein rapidly increased during in vitro HIV-1 infection of human PBMCs (Fig. 3 G), or CD4+CXCR4+ HeLa cells (Fig. S5 J), as well as after stimulation of human PBMCs with PHA and IL-2 (Fig. 3 H). Both HIV-1–infected and uninfected cells over-expressed P2Y2 (Fig. S6 A). Immunofluorescence microscopy
revealed the polarization of P2Y2 at the contact site between HIV-1–infected and uninfected lymphoblasts (Fig. 4, A–F; and Fig. S6, B–D). P2Y2 distributed to ring-like structures (Fig. 4, B–E; and Fig. S6 E) that also contained the HIV-1 glycoprotein gp41 (Fig. 4 E) and the co-receptor CD4 (not depicted), indicating that P2Y2 accumulated at the virological synapse that is formed between HIV-1–infected and uninfected lymphoblasts. This P2Y2 polarization was observed preferentially in uninfected cells (Fig. 4 F and Fig. S6 E). Pharmacological P2Y2 inhibition with kaempferol (Kaulich et al., 2003; Fig. 4, G–I; and Fig. S6, F–H) or knockdown of P2Y2 (Fig. 4, J–M; and Fig. S6 I) reduced HIV-1–associated cell death (Fig. 4 G), HIV-1 infection (Fig. 4, G–L), and Env-mediated hemifusion and fusion (Fig. 4, I and M; and Fig. S6, G and H), suggesting that P2Y2 controls HIV-1 infection. Indeed, the transfection–overexpression of P2Y2 increased the fusion between Env + and CD4 +CXCR4 + cells (Fig. 4 N). In addition, the fusion–inhibitory effect of a P2Y2–specific siRNA was overcome by transfection with a noninterferable P2Y2 cDNA but not with a nonfunctional P2Y2 mutant, P2Y2/4A (Seye et al., 2004), underscoring the contribution of this purinergic receptor to HIV-1 infection (Fig. 4 N).

P2Y2 triggers plasma membrane depolarization to allow HIV-1 infection

Infection with HIV-1 is associated with a transient depolarization of the plasma membrane (Melikyan, 2008; Fig. 5 A). Transient abolition of the plasma membrane K + gradient by culturing cells in a medium containing 70 mM KCl for 3 h strongly induced membrane depolarization (Fig. 5, B and C) and enhanced the fusion of Env + and CD4 +CXCR4 + cells that was reduced in the presence of the CXCR4 antagonist AMD3100 (Fig. 5 D), demonstrating that plasma membrane depolarization of HIV-1 target cells is required for Env–dependent fusion events. Plasma membrane depolarization induced by HIV-1 infection was abolished by addition of AMD3100, ATP-depleting apyrase, or several purinergic receptor antagonists (Fig. 5 E). To assess the involvement of P2Y2 in this event, we transfected CD4 +CXCR4 + with a nonmutated P2Y2 cDNA (or as a control with the nonfunctional P2Y2/4A mutant) and observed that overexpression of functional P2Y2 increased the plasma membrane depolarization of CD4 +CXCR4 + cells co-cultured with Env + cells (Fig. 5 F). Conversely, depletion of P2Y2 reduced HIV-1 Env–mediated plasma membrane depolarization (Fig. 5 F). These results demonstrate that the Src homology domain 3 binding sites (PxxP) of P2Y2 (which is mutated in the carboxy-terminal tail of P2Y2/4A) is required for plasma membrane depolarization and contributes to Env–dependent fusion events.

**P2Y2–dependent activation of proline–rich tyrosine kinase 2 (Pyk2) is required for HIV-1 infection**

P2Y2 (but not its mutant P2Y2/4A, see previous section) participates in the formation of a polyprotein complex that activates Pyk2 (Seye et al., 2004). We found that the activating phosphorylation or autoprophosphorylation on tyrosine residue 402 of Pyk2 (Pyk2Y402*) increased after P2Y2 overexpression during infection of human PBMCs with HIV-1 (Fig. 6 A) and during the formation of conjugates between Env + and CD4 +CXCR4 + HeLa cells (Fig. S7 A). Pyk2Y402* overexpression was mainly detected in HIV-1–infected cells (Fig. S7 B). Moreover, Pyk2Y402* was enriched in ring-like structures at the contact site between such conjugates (Fig. 6, B and D) and between HIV-1–infected lymphoblasts and uninfected lymphoblasts (Fig. 6, C and E). In addition, Pyk2Y402* phosphorylation was preferentially observed in uninfected lymphoblasts (Fig. S7 C). The phosphorylation of Pyk2 was also detectable in lymph nodes from untreated HIV-1 carriers (Fig. 6, F and H), in the frontal cortex from patients with HIV–associated encephalitis (HAE; Fig. 6, G and I), and in circulating leukocytes from HIV-1 carriers, correlating with viral load (Fig. 6 J). Approximately 40% of CD3 +CD4 +, 60% of CD4 +, and 15% of CD19 + cells obtained from PBMCs revealed the activating phosphorylation of Pyk2 after in vitro
HIV-1 infection (Fig. S7 D). In addition, the vast majority of syncytia detected in lymph node (H) and frontal cortex biopsies (I) was quantified. Error bars represent means ± SEM (*, P < 0.01; n = 5). (J) Correlation between Pyk2Y402* detected on PBMCs and viremia obtained from untreated HIV-1 carriers. The p-value corresponds to the correlation coefficient. (K) Effect of HAART on Pyk2Y402* detected on PBMCs obtained from treated patients, as compared with untreated HIV-1–infected donors (mean ± SEM; *, P = 0.0009). (L and M) Effects of P2Y2 knockdown on Pyk2Y402* polarization at the contact site between conjugates during co-culture of HIV Env+ cells and CD4+CXCR4+ cells (L) or co-culture of HIV-1–infected lymphoblasts and uninfected lymphoblasts (M) was determined by confocal microscopy in three independent experiments (mean ± SEM; *, P < 0.01). (N and O) Effects of Pyk2 depletion on intracellular p24 (N and O) and p24 antigen release (O), as determined by immunofluorescence staining and ELISA, respectively. The indicated parameters were measured after HIVNDK infection of CEM clones transduced with shRNAs specific for Pyk2, 6 d after infection. Representative FACS profiles of three independent experiments are shown in N and quantitative results (mean ± SD; n = 3; *, P < 0.01) are shown in O. (P) Effects of Pyk2 knockdowns on HIVNL4.3WT infection, hemifusion, and fusion induced by HIV-1 envelope (mean ± SEM; n = 3; *, P < 0.01). The knockdown of Pyk2 by two nonoverlapping siRNAs was confirmed by Western blot (inset).
Similarly, purinergic receptor antagonists were able to inhibit infection by genetically engineered therapy-resistant HIV-1 mutants (Fig. 7 B) and tritherapy-selected viruses (Fig. 7 C), consistent with the possibility that inhibition of the pan- nexin-1→ATP→P2Y2→Pyk2 cascade can mediate antiretroviral effects under conditions in which conventional therapy fails. In addition, purinergic receptor antagonists reduced the death of activated CD4+ T lymphoblasts infected by HIV-1 (Fig. 7 D), suggesting that interruption of P2Y2 signaling confers immunoprotective effects.

**DISCUSSION**

Although many early steps of infectious processes are associated with host membrane alterations, there are no studies that evaluate whether extracellular nucleotides might impact on the viral life cycle. In this paper, we show that ATP is released into the extracellular milieu during the early steps of HIV-1 infection and that this extracellular ATP is required for efficient viral uptake. During the initial stages of the infectious process, ATP release may determine viral clearance or the persistence of the infection by effects on the immune system. Massive ATP release during infection reportedly can deliver a danger signal to immune effectors. Thus, extracellular ATP may activate the inflammasome, which participates in the caspase-1–dependent maturation and secretion of the proinflammatory cytokines IL-1β, IL-18, and IL-33 (Schroder and Tschopp, 2010) or stimulate neutrophil migration and phagocytosis (Chen et al., 2006). Moreover, extracellular ATP can favor the killing of some intracellular pathogens (Lammas et al., 1997; Coutinho-Silva et al., 2003) or, on the contrary, promote the survival of other infectious agents (Coriden and Insel, 2010). Thus, a recent study revealed that OppA, the substrate-binding domain of Mycoplasma hominis permease increases the microbe’s extracellular survival by inducing release of ATP from host cells (Hopf and Henrich, 2008). In addition, treatment of Mycobacterium avium–infected cells with a pyrrole decreased intracellular survival of the pathogens (Woo et al., 2007). Our results differ from these studies in that they demonstrate that extracellular ATP released by HIV-1 host cells favors the initial steps of HIV-1 infection. Thus, ATP that is rapidly released during the infectious process from HIV-1 target cells does not only act as a danger signal but also contributes to viral uptake.

In this paper, we report the identification of proteins involved in HIV-1–induced ATP release and in ATP-mediated signaling events that contribute to viral infection. Thus, we identified several novel proteins that are required for efficient HIV-1 infection of CD4+ host cells and showed that HIV-1 can stimulate ATP release into the extracellular milieu through pannexin-1 hemichannels, perhaps as a result of mechanical stress of the host cell membrane (Bao et al., 2004). ATP then acts in an autocrine and paracrine fashion to activate purinergic receptors, in particular P2Y2, a seven-transmembrane–anchored G protein–coupled receptor that can induce the phosphorylation/recruitment of the proline/tyrosine kinase Pyk2 (Liu et al., 2004). Indeed, pannexin–1, P2Y2, and Pyk2 were all recruited to the contact site between Env–containing and CD4/CXCR4-containing membranes, suggesting that they act within the virological synapse to transduce signals that enhance membrane-to-membrane fusion, participate in the infectious process, and facilitate viral cell-to-cell transmission (Fig. 7 E; Jolly et al., 2007; Hübner et al., 2009).
At present, the exact mechanisms by which P2Y2 modulates HIV-1 infection remain elusive. However, we found that after the interaction between HIV-1 envelope and its co-receptors, P2Y2 was activated and could directly interact with filamin A, a modulator of the actin cytoskeleton involved in the formation of stable receptor–co-receptor complexes (Jiménez-Baranda et al., 2007). P2Y2 can also indirectly, through its SH3 binding (PxxP) site, control the phosphorylation of Pyk2 on tyrosine 402 (Liu et al., 2004), and this event might induce its phosphorylation/relocation to the virological synapse. Pyk2 regulates multiple signaling events that participate in the reorganization of the actin cytoskeleton and contribute to macrophage polarization and migration in response to chemokine stimulation (Okigaki et al., 2003). Pyk2 can control HIV-1 Env–mediated fusion (Harmon and Ratner, 2008). During HIV-1 infection, Pyk2 is phosphorylated after interactions between distinct Env variants (from X4− and R5−tropic strains) and Env receptors on HIV-1 target cells (CD4 and chemokine receptors; Davis et al., 1997). The activating phosphorylation or autophosphorylation of Pyk2 (Pyk2Y402*) that we detected during HIV-1 infection in vitro and in vivo might be induced in response to chemokine stimulation or elevations of intracellular Ca2+ concentrations (Lev et al., 1995). Activated Pyk2 then may modulate the formation of a signaling complex that contains Src family kinases and actin interacting proteins (Block et al., 2010). In this paper, we identified the ATP–mediated stimulation of the purinergic receptor P2Y2 as an upstream event required for the phosphorylation of Pyk2 (Pyk2Y402*). P2Y2 activation is known to modulate Ca2+ influx after nucleotide binding (Ralevic and Burnstock, 1998) and may also regulate Pyk2 activities by recruiting it into a polyprotein complex that also contains the tyrosine kinase Src (Seye et al., 2004). Although we demonstrated that P2Y2 activation induced Pyk2Y402*, the precise mechanisms linking P2Y2 activation and polarization/phosphorylation of Pyk2 remain elusive. Moreover, future investigations must explore how plasma membrane depolarization, which is essential for HIV-1 infection, results from the activation of P2Y2 and Pyk2.

Inhibition of any of the constituents of the molecular cascade that we delineated in this study (pannexin-1→ATP→P2Y2→Pyk2) could interrupt the HIV-1 life cycle at the level of viral entry. Thus, inhibition of pannexin hemichannels, enzymatic depletion of extracellular ATP, antagonizing P2Y2 receptors, or suppression of Pyk2 kinase activity could constitute effective strategies for the blockade of HIV-1 infection, be it locally (for instance by application of topical gels) or systemically (ideally with orally available drugs). The toxicology of some putative therapeutic agents acting on this cascade (such as apyrase) is satisfactory (Marcus et al., 2005), whereas that of others (such as suramin) is not (Grossman et al., 2001), underlining the need for the development of highly specific inhibitors against pannexin-1, P2Y2, and Pyk2. Beyond these clinical considerations, it will be important to investigate whether any or all of the constituents of the pannexin-1→ATP→P2Y2→Pyk2 pathway may participate in host cell infection by HIV-1–unrelated viruses.

**MATERIALS AND METHODS**

**Cells and culture conditions.** PBMCs were isolated from the blood of normal volunteers (Établissement Français du Sang [EFS] Cabanel, Paris, France) over a Ficoll-Hypaque density gradient. Blood was obtained through the EFS in the setting of EFS-Institut Pasteur Convention. A written informed consent was obtained for each donor to use the cells for clinical research in compliance with French Law and the Biomedical Research Committee Board from Institut Pasteur (Paris, France). CD14+ monocytes were isolated from PBMC by positive selection using anti-CD14 beads (Miltenyi Biotec). To generate monocyte–derived macrophages (MDMs), purified monocytes were incubated in RPMI 1640 medium supplemented with 100 µg/ml penicillin–100 µg/ml streptomycin (Invitrogen) and 10% FCS in the presence of 20 ng/ml recombinant human (rh) M-CSF (PeproTech). After 6 d of culture, adherent cells corresponding to the macrophage–enriched fraction were harvested, washed, and used for subsequent experiments. To generate monocyte–derived DCs (MDDCs), purified monocytes were cultured in RPMI 1640 medium supplemented with 10% antibiotics and FCS. Cultures were maintained for 6 d in 24-well trays (106 cells/well) supplemented with 10 ng/ml rhGM-CSF (PeproTech) and rhIL-4 (PeproTech). The medium was replaced every 2 d. After 6 d of culture, MDDCs were semi-adherent cells and expressed high levels of DC-SIGN but not monocyte/macrophage markers such as CD14 and CD16. Flow cytometry analysis (CellQuest software; BD) demonstrated that MDMs and MDDCs were >90% pure. T cells were subsequently prepared from the monocyte–depleted cell fractions. PBLS were cultured for 48 h in fresh medium supplemented with 2.5 µg/ml PHA (Sigma-Aldrich) and 1 µg/ml rIL-2 (PeproTech). PBLS were then washed and cultured in growth medium containing 1 µg/ml rIL-2 for 24 h. These cells were infected with HIV-1 during 3 d with a multiplicity of infection (MOI) of 1, as previously described (Saiidi et al., 2008; Melki et al., 2010). HeLa cells stably transfected with the Env gene of HIV-1 LAI/IIIB (HeLa Env) and HeLa cells transfected with CD4 (HeLa CD4) were cultured alone or together at a 1:1 ratio in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen) in the absence or in presence of tested molecules. CEM T lymphocytes were maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen).

**Viral and pseudo-viral constructs.** Viral stocks of wild-type X4, R5, and resistant HIV-1 were obtained after transfection of 293T with virus encoding plasmids as previously described (Perfettini et al., 2008; Delelis et al., 2009). The standardization of HIV-1 infection was achieved by means of the All-in-One HIV-1 P24 antigen ELISA assay (PerkinElmer) and infection of P4 cells followed by fixation and determination of β-galactosidase activity. Viral RNA and DNA and viral production were analyzed, as previously described (Delelis et al., 2009).

**Pharmacological inhibitions.** Primary human lymphoblasts, MDMs, MDDCs, lymphoctic CEM cells, or CD4 CXCR4+ target cells were seeded into 96-well culture plates (1–2 × 104 cells/well) and incubated 30 min with increasing concentrations of apyrase, CXCR4 antagonist (AMD3100), the P2 receptor antagonist suramin, the P2X–selective antagonist OxaATP, the P2Y-selective antagonist PPADS, carbamoloxolone (CBX), DIDS, and SITS. Cells were incubated with wild-type HIV-1 VSVD-pseudotyped HIV-1, therapy-resistant HIV-1 mutated viruses (1 ng of p24 antigen), or clinical HIV-1 isolates (15 ng of p24 antigen) for 3 h at 37°C in a 5% CO2 atmosphere. After washing out unabsorbed virus, cells were cultured for the indicated times in the presence of tested molecules. The absolute number of total remaining viable cells and cell death were analyzed, as previously described (Lecoere et al., 2002; Melki et al., 2010). Apyrase, AMD3100, ATP, ATP–γS, suramin, PPADS, OxaATP, CBX, DIDS, and SITS were purchased from Sigma-Aldrich.

**RNA interference.** siRNAs specific for pannexin-1 (siRNA-1, 5′-GCUC-CAAGGUAUGAACAU-3′; siRNA-2, 5′-ACGAAUUGGACCUC-UACCA-3′), CD4 (siRNA, 5′-ACUGAGGAGCUUCUAUC-3′),
P2X7 (siRNA, 5′-GGAAAACUCCCUCUUGAGCC-3′, P2X8 (siRNA, 5′-GCUUUGGC-3′, P2Y11 (siRNA, 5′-GCAGGCACTGGGACCTG-3′), P2Y2 (siRNA-1, 5′-GGGCTGGCTGATGGACTG-3′), P2Y2 (siRNA-2, 5′-GGCTGGAACCCAGAGC-3′, and control siRNA (5′-GGCGUUAACUAAUCAGAAA-3′), P2Y4 (siRNA-1, 5′-GGGCUAAGCCGCAUUGAA-3′, siRNA-2, 5′-GGCCUGUAACUCAUUCAAAA-3′), P2Y6 (siRNA, 5′-GGUCCACAAAACAUAGCU-3′), were transfected 48 h before HIV-1 infection or cell fusion using Oligofectamine (Invitrogen), according to the manufacturer's instructions. For short hairpin RNA (shRNA) lentiviral particle transduction, the pRS shRNA expression retrovector vector coding for each target gene was purchased from Origene. Oncorvirus vector particles were generated by cotransfection of three plasmids coding for the gag-pol genes from moloney mouse leukemia virus (pUMVC3-gag-pol, University of Michigan), for the vector genome carrying your shRNA of interest (pRS shRNA), and for the plasmid coding for an envelope of VSV (pMDG; Tronolab). Cotransfection was effected into 293T cells (human embryonic kidney cell) using superFect transfection protocol. On day 2 after transfection, supernatants were filtered (0.45-µm cellulose acetate filters; Sartorius Stedim), aliquoted, and frozen at −80°C. For transduction, oncovirus aliquots were added to T lymphocytes: CEM-T4 cell line, and 24 h after infection the medium was replaced with fresh growth medium containing 1 µg/ml puromycin (Cayla). Clonal populations were obtained by transferring single cells into U-bottom 96-well plates and by maintaining selection pressure for 3 wk. Silencing of proteins was determined by quantitative RT-PCR and/or by Western blotting.

Quantitative real-time RT-PCR. miRNA was isolated from cells after the indicated treatments using the RNeasy kit (QIAGEN) according to the manufacturer’s instructions, and total RNA was converted into cDNA by standard reverse transcription with the TaqMan kit (Applied Biosystems). Quantitative PCR were performed with the cDNA preparation (1:50) in the Mx3000P system (Agilent Technologies) in a 25-µl final volume with Brilliant QPCR Master Mix (Agilent Technologies). The primers for human Panx-1 were 5′-GGTTGAGACCAAAGCGCAGC-3′ (forward) and 5′-GGCCATGGACCTCACCACT-3′ (reverse) (ThermoFisher). The primers for human P2X7 were 5′-GTTAGGCGCTGAGTGCTG-3′ (forward) and 5′-ATGAGGCCGCTCGAGGTCTG-3′ (reverse). The primers for human P2X4 were 5′-TTGATAAAGTCTCTGCGAGGAAAAC-3′ (forward) and 5′-GATCATCAATAATGACGACGTTAG-3′ (reverse). The primers for human P2X7 were 5′-GATGAAAGATCTTCTGAGTCTG-3′ (forward) and 5′-TTGGACAAATCTGTTGAAGTCCAT-3′ (reverse). The primers for human P2X1 were 5′-GCTGTTGCTGAATAGAAAGGTTG-3′ (forward) and 5′-ATGAGGCCGCTCGAGGTCTG-3′ (reverse). The primers for human P2X4 were 5′-GTTCTCGTCGAGGCCATCTGAG-3′ (forward) and 5′-CTCATGCTGCCCCAACACATC-3′ (reverse). The primers for human P2Y4 were 5′-GCCTGCGCCACCTTCTAC-3′ (forward) and 5′-CCCGAGTTGTTGCTAGTGCCAGAC-3′ (reverse). The primers for human P2Y5 were 5′-TTGGTACTGCTTCCACACACCT-3′ (forward) and 5′-CCTACTGCTGCCCCAACACATC-3′ (reverse). The primers for human P2Y6 were 5′-AACCTGGTCTGGCTGAGACTG-3′ (forward) and 5′-CTACTGCTGCCCACACTGAC-3′ (reverse). The primers for human P2Y6 were 5′-ATGGGCTGGTTGTCAGCTG-3′ (forward) and 5′-GCCAGACACTGGTGTGGTG-3′ (reverse). The primers for human P2Y8 were 5′-GTCTCGTCGACCCATTGAGG-3′ (forward) and 5′-GAGGCTGCTGGAGGCTGAC-3′ (reverse). The primers for human P2Y10 were 5′-GTGGTCTCAGTGGTCCGCTG-3′ (forward) and 5′-GATGGGCGATGGAAAGTGGC-3′ (reverse). The primers for human P2Y11 were 5′-GAGGCTGCTGGAGGCTGAC-3′ (forward) and 5′-ACGTGTGACCCCGAGAGTAG-3′ (reverse). The primers for human P2Y12 were 5′-ACCGGCTCAGTGGAAACGAC-3′ (forward) and 5′-GGACGAATGTTGGGACCTTCCAG-3′ (reverse). The primers for human P2Y13 were 5′-GGGAAGACACCATCAGTCGTC-3′ (forward) and 5′-GACGAGGTGGTGTTGGAGG-3′ (reverse) and for human GAPDH were 5′-CAATGTCAATGGCCCAAGCAG-3′ (forward) and 5′-GTCAATGGAGGAATGCGCAC-3′ (reverse). Real-time PCR included initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and one cycle at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. The CT values were normalized to the housekeeping gene GAPDH.

Immunoblots, immunofluorescence, and flow cytometry. Total cellular proteins were extracted in 250 mM NaCl-containing lysis buffer (250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10 mM Na2VO4, 10 mM NaF, 5 mM DTT, 3 mM Na4P2O7, and the protease inhibitor cocktail [EDTA-free protease inhibitor tablets; Roche]). 50 µg of protein extracts were run on 4–12% SDS-PAGE and transferred at 4°C onto a nitrocellulose membrane. After blocking, membranes were incubated with primary antibody at room temperature overnight. Horseradish peroxidase–conjugated goat anti-mouse or anti–rabbit (SouthernBiotech) antibodies were then incubated for 1 h and revealed with the enhanced ECL detection system. After 3 d of infection with HIVNDK (MOL = 1), PHA/IL-2–stimulated peripheral blood lymphocytes were stained with 10 µM 5-chloromethylfluorescein diacetate (Cell Tracker green; CMFDA; Invitrogen) and cocultured with unlabeled lymphocytes. Polyclonal rabbit antibodies against pannexin-1 were obtained from Abcam and Millipore. The polyclonal rabbit antibodies used for the detection of Pyk2 and the phosphorylated form of Pyk2 on tyrosine 402 (Pyk2Y402*) were obtained from Cell Signaling Technology. The monoclonal mouse antibody against HIV-1 gp41 (50–69) was obtained from National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). Anti sera were used for immunodetection in PBS containing 1 mg/ml BSA, and revealed with goat anti–rabbit IgG conjugated to Alexa Fluor 546 fluorescence (Milenyi Biotec). Phenotypic analyses were realized by flow cytometry as previously described (Mekli et al., 2010), and we used the monoclonal mouse antibody against HIV-1 p24 (KC57) conjugated to FITC (Beckman Coulter), the monoclonal mouse antibodies against CD3 or CD4 conjugated to FITC or to peridinin chlorophyll protein complex (PerCP, BD), and the monoclonal mouse antibody against CD19 conjugated to allophycocyanin (Milenyi Biotec).

Measurement of extracellular ATP. ATP release during co-cultures or infection with HIV-1 were determined using the Enlihen ATP assay system (Promeza) as described by the manufacturer. The luminescence was measured by integration over a 3-s time interval using the luminometer Fluostar OPTIMA (BMG Labtech).

Hemifusion and cell-cell fusion assays. HeLa Env+ and HeLa CD4+CXCR4+ cells were, respectively, stably transduced with hemifusion analysis with 1 µM 1,1'-dioctadecyldiamino-3,3,3'-tetramethylindocarbocyanine peroxide (DiIC1(3), Invitrogen) and 10 µM 5-chloromethylfluorescein diacetate (Cell Tracker green; CMFDA) or for cell–cell fusion analysis, respectively, with 10 µM 5-chloromethylfluorescein diacetate (Cell Tracker green; CMFDA) and 10 µM 5,6,4-chloromethylbenzoxyl aminotetramethyl rhodamine (Cell Tracker orange; CMTMR, Invitrogen). Cells were then cultured alone or together for 24 h, at a 1:1 ratio in RPMI 1,000-modified Eagle 9s supplemented with 10% FCS, 2 mM l-glutamine, and penicillin/streptomycin (Invitrogen) in the absence or presence of the indicated concentrations of purinergic receptor inhibitors. The effects of these inhibitors on hemifusion and cell–cell fusion mediated by the HIV-1 envelope were evaluated by fluorescence microscopy.

Measurement of target cell infectability. HeLa cells stably transfected with CD4, as well as the LacZ gene under the control of the HIV-1LTR (HeLa-CD4), were selected in medium containing 500 µg/ml G418. Target
cell infectability was evaluated using HeLa-CD4+CXCR4+ cells. After 2 d of infection, cells were lysed and β-galactosidase activity was measured using the enhanced β-galactosidase assay kit (CPRG; Gene Therapy Systems).

**Plasma membrane depolarization detection.** PHA/IL-2–stimulated human lymphoblasts were stained with 300 nM DiBac4(3) (Invitrogen) for 20 min at 37°C. Cells were then infected with HIVNDk (at an MOI of 1) for 1 h. We then measured the depolarization-associated increase of DiBac4(3) fluorescence intensity by flow cytometry. Propidium iodide uptake was used to exclude necrotic cells from the analysis of DiBac4(3) fluorescence. Using the same procedure, we analyzed plasma membrane depolarization induced by the HIV-1 envelope.

**Patients.** Peripheral blood samples were obtained from healthy and HIV–1–infected individuals (all males, mean age 36 yr) in accordance with the Italian and EU legislations, after approval by the Institutional Review Board of the National Institute for Infectious Disease Lazzaro Spallanzani. Patients were naive for highly active antiretroviral therapy (HAART) with a plasma viral load >20,000 copies/ml or were receiving HAART with a viral load <5,000 copies/ml. Plasma HIV-1 RNA levels were determined by the Abbott Real-time HIV-1 assay according to the manufacturer’s instructions (Abbott Molecular). PBMCs of HIV–1–infected patients were obtained from residual samples intended to HIV RNA quantification for routine clinical management and were isolated from Ficoll/Hypaque (GE Healthcare) centrifugation of heparinized blood from either healthy donors or HIV-seropositive individuals and fixed with 4% paraformaldehyde in PBS, pH 7.2. PBMCs from HAART-treated patients that had undetectable virus in the plasma were used for ex vivo experiments. The amounts of endogenous virus produced and used during these ex vivo experiments were monitored by using Vironostika HIV-1 Antigen assay according to the manufacturer’s instructions (BioMerieux).

**Immunohistochemical analysis.** Biopsies from axillary lymph nodes and from postmortem frontal cortex were obtained in accordance with the Italian and EU legislations, after approval by the Institutional Review Board of the National Institute for Infectious Disease Lazzaro Spallanzani. Human tissue sections in paraffin were deparaffinized, rehydrated, and subjected to high-temperature antigen retrieval in 10 mM sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked by 3% H2O2. Rabbit antibody against P2Y2 (Alomone laboratories) or Pyk2-Y202* (Abcam) and biotinylated goat anti-rabbit IgG were incubated with tissue sections. Immunoreaction product obtained with a preformed horseradish peroxidase–conjugated goat anti–rabbit IgG were incubated with tissue sections. Immunoreaction product obtained with a preformed horseradish peroxidase–conjugated streptavidin (Biogenex) was revealed using aminoethylcarbazole as chromogen substrates and 0.01% H2O2 (Biogenex). Sections were then counterstained in Mayer’s acid hemalum. The lymph node and brain cortex sections obtained from HIV–infected patients were evaluated by three independent observers using a light microscope. For each slide, a minimum of 10 fields was examined at 40× magnification.

**Statistical analysis.** To determine statistical significance, Student’s t test was used for calculation of p-values.

**Online supplemental material.** Fig. S1 demonstrates pannexin–1-dependent ATP release during HIV–1 infection and during HIV–1 Env–mediated fusion. Fig. S2 shows the impact of P2 inhibitors on cell viability, viral production, host cell infection, and hemifusion/fusion during HIV–1 infection. Fig. S3 shows effects of P2 inhibitors on co–cultured Env+ and CD4+CXCR4+ HeLa cells. Fig. S4 reveals purinergic receptor and pannexin–1 expression on human PBMCs and on PHA/IL–2–stimulated PBMC. Fig. S5 shows the identification of purinergic receptors involved in HIV–1 Env–mediated fusion and controls for immunohistochemical analyses. Fig. S6 shows the detection of polarized P2Y2 at the virological synapse mediated by HIVNDk. Fig. S7 shows that Pyk2 is activated during HIV–1 infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101805/DC1.

We thank the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD) and Laurie Erb (Bond Life Sciences Center, USA) for reagents, and A. Jaill (Institut National de la Santé et de la Recherche Médicale U753, France) and O. Duc (Institut Gustave Roussy, France) for technical help.

This work has been supported by a grant from Sidaction (to J.-L. Perfettini), as well as grants from Agence Nationale des Recherches sur le Sida et sur les hépatites virales, La Ligue Nationale contre le Cancer, Sidaction, and the European Commission (RIGHT, ACTIVE P3), Apsys, ArtForce; to G. Kroemer and Istituto Superiore di Sanità (no. 4060, Ricerca Corrente and Finalizzate “Ministero della Salute”, COFIN from Ministero dell’Istruzione, dell’Università e della Ricerca and Associazione Italiana per la Ricerca sul Cancro).

The authors declare no conflicting financial interests.

Submitted: 31 August 2010
Accepted: 27 July 2011

**REFERENCES**


Accepted: 27 July 2011
published August 22, 2011


