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HIV-infected cells are major inducers of plasmacytoid dendritic cell interferon production, maturation, and migration

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Abstract

Plasmacytoid dendritic cells (PDC), natural type-1 interferon (IFN) producing cells, could play a role in the innate anti-HIV immune response. Previous reports indicated that PDC IFN production is induced by HIV. Our results show a more robust IFN induction when purified PDC (>95%) were exposed to HIV-infected cells. This effect was not observed with non-viable cells, DNA, and RNA extracted from infected cells, and viral proteins. The response was blocked by anti-CD4 and neutralizing anti-gp120 antibodies as well as soluble CD4. IFN induction by HIV-infected cells was also prevented by low-dose chloroquine, which inhibits endosomal acidification. PDC IFN release resulted in reduced HIV production by infected CD4+ cells, supporting an anti-HIV activity of PDC. Stimulated CD4+ cells induced PDC activation and maturation; markers for PDC migration (CCR7) were enhanced by HIV-infected CD4+ cells only. This latter finding could explain the decline in circulating PDC in HIV-infected individuals.
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Keywords: HIV; Plasmacytoid dendritic cells; Interferon induction; TLR-9; CD4+ cells

Introduction

During hematopoiesis, stem cells give rise to two major types of dendritic cell (DC) precursors: myeloid (MDC, pre-DC1) and lymphoid or the plasmacytoid dendritic cells (PDC, pre-DC2) (Liu, 2001). The latter cells, also known as natural interferon (IFN)-α producing cells (Fitzgerald-Bocarsly, 1993), have been identified as the major source of type-1 IFN (Cella et al., 1999; Fitzgerald-Bocarsly, 2002; Siegal et al., 1999). Upon exposure to CD40 ligand (CD40L), PDC differentiate into dendritic cells (DC-2) (Grouard et al., 1997), promoting naive CD4+ T lymphocytes to produce IL-4, IL-5, and IL-10, and directing the immune response into a T-helper (T_H)-2 phenotype (Kadowaki et al., 2000; Rissoan et al., 1999). Upon response to viral pathogens, PDC maturation drives a potent T_H-1 polarization (Cella et al., 2000). These observations link PDC to innate and adaptive immunity (Kadowaki et al., 2000; Rissoan et al., 1999).

A reduction in PDC numbers has been reported in HIV primary infection (Pacanowski et al., 2001) as well as in advanced stages of disease (Almeida et al., 2005; Chehimi et al., 2002; Donaghy et al., 2001; Feldman et al., 2001; Finke et al., 2004; Soumelis et al., 2001). PDC numbers have also been shown to correlate directly with CD4+ cell numbers and inversely with plasma HIV viral load (Barron et al., 2003; Soumelis et al., 2001). These observations suggest an important role of PDC in controlling HIV replication in infected individuals.

It is still unclear what mechanisms contribute to the decline of circulating PDC in HIV infection. PDC, which express high levels of surface CD4 as well as the chemokine receptors CCR5 and CXCR4, can be infected by HIV (Donaghy et al., 2003; Fong et al., 2002; Patterson et al., 2001; Schmidt et al., 2004b; Yonezawa et al., 2003), but their viability is not affected (Schmidt et al., 2004b; Yonezawa et al., 2003). Their decline could be explained by enhanced migration of these cells to secondary lymphatic tissue (Cyster, 1999). Evidence for this hypothesis comes from the observation that PDC mature and show an upregulation of the cell migration marker, CCR7

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(Fonteneau et al., 2004) concomitant with the release of IFN-α after exposure to high concentrations of HIV (Fong et al., 2002; Yonezawa et al., 2003).

The present study was conducted to characterize the mechanism of IFN induction by HIV. In addition to the findings by others (Fong et al., 2002, 2004; Yonezawa et al., 2003), we have observed that virus-infected CD4+ cells induce a more robust IFN production and maturation of PDC than virus alone. Virus production by the infected CD4+ cells is subsequently reduced. This induction is specially regulated since antibodies to CD4 and gp120 as well as soluble CD4, but not antibodies to CXCR4, block these effects on PDC. A similar result was observed with antibodies to BDCA-2, and with chloroquine, suggesting that endocytosis is required for PDC type 1 IFN induction by HIV-infected cells. Moreover, the infected CD4+ cells readily induced high CCR7 expression in PDC. These findings could explain changes in the levels of circulating PDC in infected individuals and reflect the potential function of PDC in controlling HIV replication.

Results

Relative induction of PDC IFN production by HIV and HIV-infected cells

PDC have been demonstrated to produce type 1 interferons in response to HIV (Fong et al., 2002; Yonezawa et al., 2003). With purified PDC (>95%) in over 20 experiments, we noted a limited release of IFN-α (up to 400 pg/ml) when 10^4 PDC were exposed to HIV alone for 24 h and only when a very high input of the X4-tropic SF33 isolate of HIV-1 (e.g. 10,000 $TCID_{50}$) was used. In contrast, a robust IFN response (mean, 1561.4 pg/ml, 95% confidence level, 1174.6–1948.2 pg/ml) was readily observed in over 30 separate assays with different PDC donors when PDC (10^4 cells) were cocultured with CD4+ cells acutely infected with HIV-1 at a low MOI, 2 days prior to coculture (data summarized from Schmidt et al., 2004a). This response of PDC was shown to be directly dependent on the extent of HIV-replication in the CD4+ cells (Fig. 1a). Similarly, variations in the interferon induction by 2d-infected CD4+ cells were noted and depended on the extent of viral replication in these cells.

The interferon production was not detected before 12 h of coculture of the PDC with infected CD4+ cells, indicating a time period needed for activating the interferon release (data not shown). This effect on PDC was not a function of cell death since the mock-infected cultures showed the same limited extent of apoptosis as the infected cells as measured by annexin V staining. Moreover, similar results were found using CD4+ cells infected with HIV-1 R5 and X4 isolates, as well as cytopathic and noncytopathic HIV-2 isolates (e.g. UC-1) (Evans et al., 1988) (data not shown).

Evaluating further this quantitative difference of free virus and virus-infected cells on IFN production, we tested supernatants of infected CD4+ cells and pelleted virus from these fluids. IFN-α was only induced when a high viral input was used (Fig. 1b). Because virus preparations can have cellular contaminants that could influence the results (Trubey et al., 2003), we filtered the HIV stock prior to inoculation onto PDC. After this procedure, the extent of IFN induction by high-titered virus was reduced by 70% (Fig. 1c). The amount of IFN-α released by PDC in response to virus preparations was substantially increased by using 40,000 instead of 10,000 PDC as well as removal of the supernatant after 48 h instead of 24 h (Fig. 1d). Moreover, using an AT-2 inactivated virus, substantial interferon induction (up to 4000 pg/ml) was observed when 3×10^4 PDC, but not 1×10^4 cells, were exposed to a large quantity of this virus (10^9 RNA molecules/ml). However, HIV-infected CD4+ cells induced substantially higher IFN levels under the same conditions. Finally, separation of PDC and HIV-infected CD4+ cells by a transwell device prevented the induction of IFN release (Fig. 1e).

We also determined directly the relative extent of IFN release by PDC after exposure to free virus compared to virus-infected cells. In these studies, CD4+ cells inoculated with a high multiplicity of HIV infection (MOI 0.5) for only 6 h induced a much greater IFN response than high-titered virus alone (Table 1). These results were observed even though the levels of virus particles in the cell culture fluids, measured immediately before and 24 h after PDC coculture, were very low (RT activity, 10×10^3 cpm/ml) compared to the SF33 virus stock added directly to the PDC (5418×10^3 cpm/ml). Because the CD4+ cells were trypsinized 1 h after the initial virus inoculation, the IFN induction by the infected cells could not be attributed to adherent virus (Tang and Levy, 1991). All of these findings indicate that the HIV-infected cell, more than free virus, is a major inducer of PDC production.

Effect of non-viable cells, nucleic acids, and viral proteins on interferon production

In evaluating the importance of viable cells in IFN induction, we subjected PHA-stimulated, uninfected, or HIV-infected CD4+ cells to repeated cycles of freezing and thawing prior to coculture with PDC. In these experiments, non-viable cells, infected or uninfected, did not induce IFN release by PDC obtained from three different donors. Similarly, no IFN induction was observed with uninfected CD4+ cells irradiated with 700 to 3000 rad, resulting in up to 50% apoptotic cells. Irradiation of HIV acutely infected CD4+ cells completely abolished IFN induction, as confirmed in four separate experiments. Similarly, IFN was not induced when PDC from two different donors were challenged with DNA or RNA extracted from infected or uninfected CD4+ cells. Finally, in three separate experiments, viral components such as recombinant Env (monomeric gp120 from different HIV-1 isolates and trimeric gp120 from HIV-1_{SF162}), even when added in combination with CD40L, as well as Gag (p24 and p55) and Tat proteins elicited no type 1 IFN release by PDC (see Methods for description of components).

Effect of interferon production by PDC on HIV-infected cells

Since the HIV-infected CD4+ cells induce IFN production by PDC, we examined the antiviral effect of the released IFN on

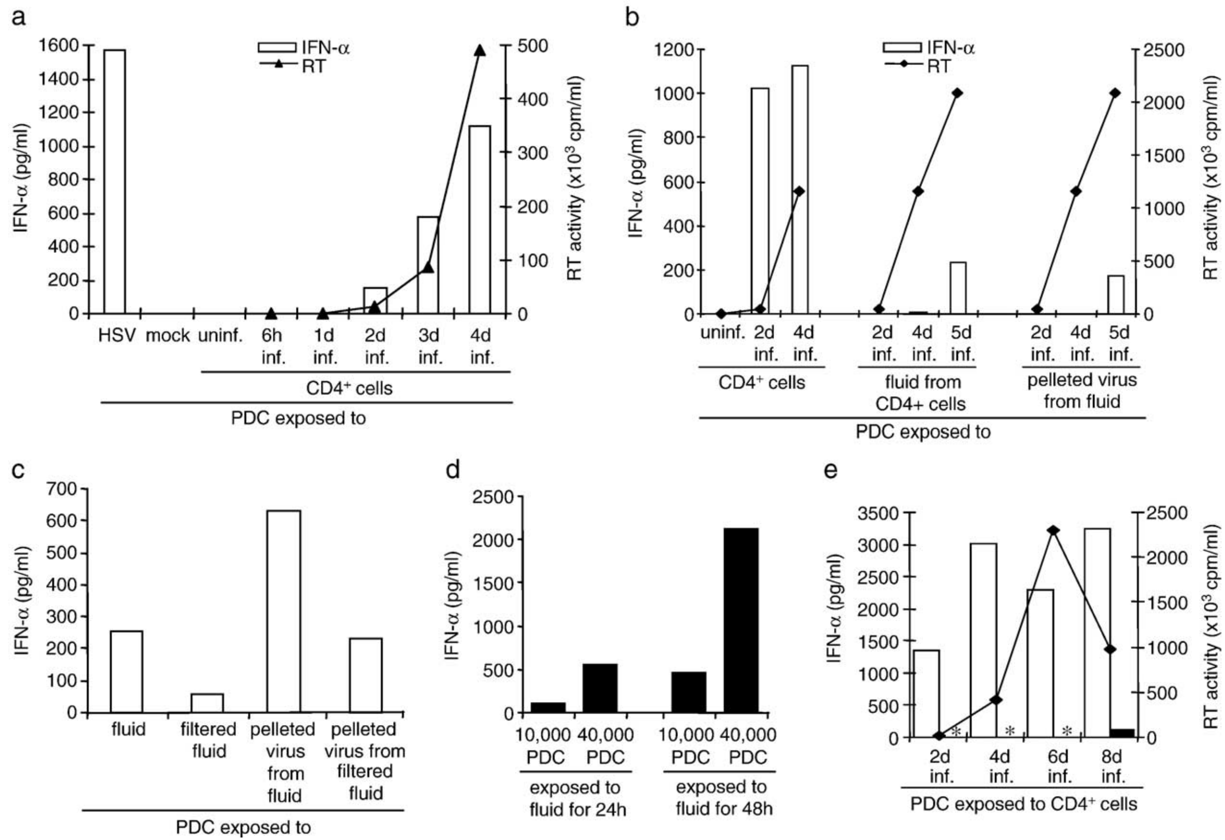

Fig. 1. Comparison of interferon (IFN) release by plasmacytoid dendritic cells (PDC) after exposure to HIV-1 or HIV-1-infected cells. (a) IFN-α release by PDC after exposure to herpes simplex virus (HSV) or PHA-stimulated CD4+ cells uninfected (uninf.) or infected (inf.) with HIV-1_{SF33} for 6 h to 4 days prior to coculture with PDC. To observe a dose-dependent effect, CD4+ cells were infected at a 10-fold lower MOI than usual. The reverse transcriptase (RT) activity was determined in the fluids of the infected CD4+ cells (Hoffman et al., 1985) prior to coculture with PDC. Supernatants for IFN-α activity were harvested 24 h after exposure of PDC to the different stimuli. This experiment is representative of three independent studies using different PDC donors. (b) Exposure of PDC to HIV-infected CD4+ cells as well as the HIV-containing supernatants from these CD4+ cells and virus pelleted from the respective supernatants. Virus content was determined by measuring particle-associated RT activity in the culture fluids of CD4+ T cells prior to PDC exposure. (c) IFN-α release by inoculation of PDC with fluid containing 10,000 $TCID_{50}$ of HIV-1_{SF33} pelleted from unfiltered and filtered fluids. Data represent three donors whose PDC responded to high-titered HIV-1_{SF33} with a limited IFN release. (d) IFN-α induction by 10,000 or 40,000 PDC exposed to fluids containing 10,000 $TCID_{50}$ of HIV-1_{SF33}, with supernatants removed after 24 h and 48 h of exposure. These data are representative of three separate experiments. (e) IFN release by PDC cultured with CD4+ cells infected (inf.) with HIV-1_{SF33} for 2–8 days without (w/o) (□) or with (w/t) (■) a transwell insert. Supernatants for IFN-α activity were harvested 24 h after PDC exposure. Wells with no measurable IFN-α activity are labeled with an asterisk (*).

the infected cells. The CD4+ T cells, infected by HIV-1_{SF33} (Fig. 2a), HIV-1_{SF162} (Fig. 2b), or HIV-1_{SF2} (Fig. 2c) for 2 days and then cocultured with PDC readily induced IFN production and these CD4+ cells showed a marked decrease in HIV replication. This effect appeared to be a function of the extent of virus replication in the CD4+ cells at the time of the assay. Virus production in cells infected for only 6 h showed the highest sensitivity to interferon release by PDC (80% suppression) (data not shown). In support of this effect of IFN on HIV replication, increasing amounts of recombinant IFN-α showed a dose-

Table 1
Induction of interferon (IFN) production after exposure of plasmacytoid dendritic cells (PDC) to CD4+ cells infected with HIV-1 for 6 h prior to coculture or to supernatant containing high-titered HIV-1

Parameters	Exposure of PDC to					
	CD4+ cells infected with HIV-1_{SF33} for only 6 h prior to coculture					Supernatant containing HIV-1_{SF33}
Viral $TCID_{50}$ used for infection of 60,000 CD4+ cells	60,000	6000	600	60	6	n.a.
Viral $TCID_{50}$ added directly	n.a.	n.a.	n.a.	n.a.	n.a.	10,000
RT prior to addition to PDC	5.3	3.5	2.1	2.1	1.6	5418
RT 24 h after addition to PDC	10.3	8.0	7.3	7.8	7	n.a.
IFN-α, 24 h after addition to PDC	1203.6	423.6	109.5	7.2	0	256.3

CD4+ cells were trypsinized 1 h post-infection to remove virus attached to the cell surface (Tang and Levy, 1991).
10^4 PDC were exposed to HIV-1_{SF33}-infected CD4+ cells at a ratio of 1:6 (PDC/CD4) in 96-well flat-bottom plates.
RT, viral reverse transcriptase activity (cpm × 10^3/ml); $TCID_{50}$, tissue culture infectious dose 50%; n.a., not applicable.

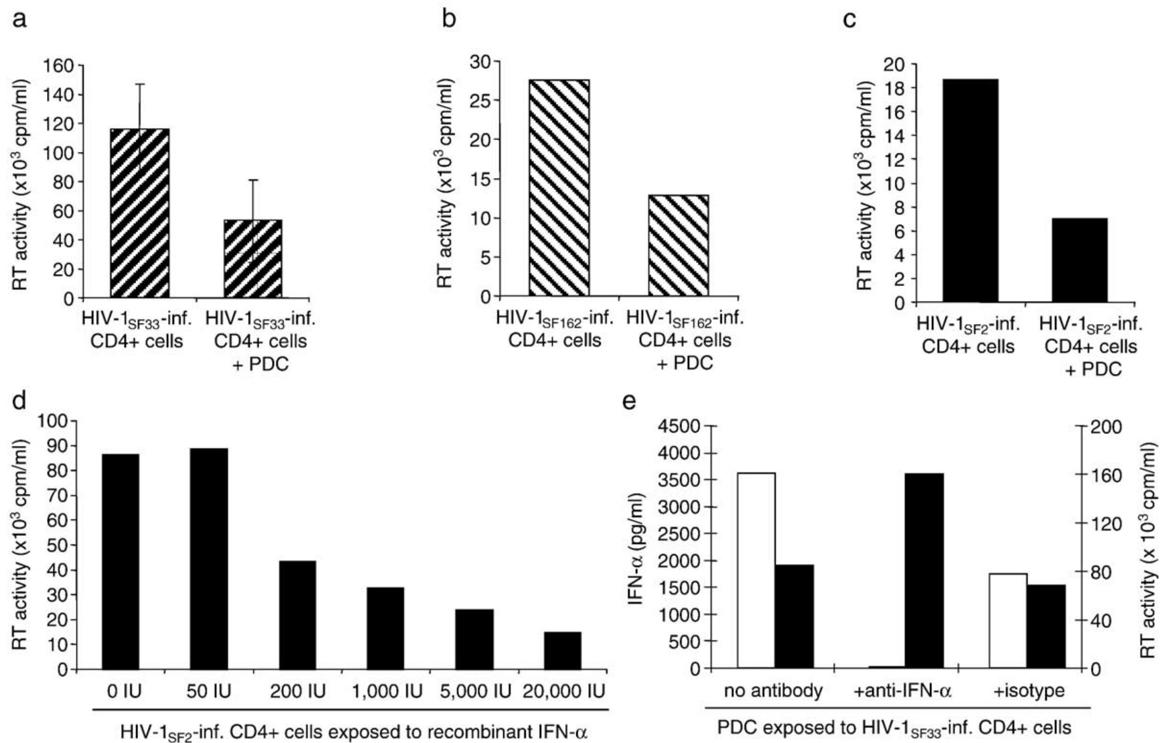

Fig. 2. The effect of IFN release by PDC on HIV replication in the HIV-infected CD4+ T cells. CD4+ cells were infected (inf.) with (a) HIV-1_{SF33}, (b) HIV-1_{SF162}, and (c) HIV-2_{SF2} for 2 days and then exposed to PDC. Supernatants were harvested after another 24 h and assayed for virus replication (measured by reverse transcriptase (RT) activity). The data for HIV-1_{SF33} were obtained from three independent experiments. The experiments with HIV-1_{SF162} and HIV-1_{SF2} are each representative of two separate studies. (d) Virus replication by PHA-stimulated CD4+ cells infected (inf.) with HIV-1_{SF2} for 2 days in the presence of increasing amounts of recombinant IFN-α. (e) IFN-α production (□) and virus replication measured by RT activity (■) after coculture of PDC with 2d HIV-1_{SF33}-infected CD4+ cells in the presence of neutralizing antibodies to IFN-α or the corresponding isotype control antibody. This experiment is representative of three independent studies with X4 and R5 viruses.

dependent reduction in virus production by HIV-1_{SF2}-infected CD4+ cells (Fig. 2d). Importantly, virus replication was increased by adding IFN-α neutralizing antibodies, but not the isotype control antibody, to cocultures of PDC and HIV-infected CD4+ cells (Fig. 2e). These results demonstrate that the IFN-α response of PDC to HIV-infected CD4+ cells can lead to suppression of virus replication within the infected cells.

Features of cell:cell interaction involved in IFN release by PDC

The mechanism by which HIV-infected CD4+ cells induce IFN production was evaluated by a variety of procedures. HIV-infected CD4+ T cells and PDC were cultured together with anti-CD4 antibodies for 24 h. A dose-dependent reduction in IFN induction was observed (Fig. 3a). As controls, the antibodies to CD4 showed no effect on IFN induction by HSV, CpG-A, and the synthetic Toll-like receptor (TLR)-7 agonist S-27609 (Doxsee et al., 2003). A similar effect in preventing IFN production by PDC was noted when soluble CD4 was added to the coculture of PDC with infected CD4+ cells (Fig. 3b). In contrast, HSV-induced IFN production was not affected by soluble CD4 (data not shown). The soluble CD4 and the anti-CD4 antibodies both reduced HIV-1_{SF33} infection of CD4+ cells by >95% (data not shown).

To determine if the CD4 receptor on the HIV-infected CD4+ cells or on the PDC is involved in IFN induction, the PDC were cocultured with pNL4-3-transfected 293 cells that lack CD4 expression. The NL4-3 producing cells consistently induced high levels of PDC IFN production, which could be blocked by anti-CD4 and soluble CD4 (Fig. 3c). These studies indicated the role of CD4 expression on PDC in IFN induction.

In evaluating further the potential importance of the virus envelope on PDC IFN induction, polyclonal and monoclonal antibodies to gp120 and gp41 were added to the coculture of PDC with HIV-1_{SF33}-infected CD4+ cells. No effect on IFN induction was observed (data not shown). The anti-gp120 antibodies, however, reduced the ability of HIV-1_{SF33} to infect CD4+ cells by only 20%, reflecting a limited neutralizing activity. When neutralizing antibodies to HIV-1_{SF2} were used in coculture with HIV-1_{SF2}-infected CD4+ cells, a marked reduction in IFN production was observed (Fig. 3d). These antibodies block HIV-1_{SF2} infection of CD4+ cells by >90% (Levy et al., 1984). These findings suggest that gp120 on the surface of the virus in HIV-infected cells is directly involved in PDC IFN induction.

We also examined whether an HIV interaction with its chemokine coreceptor was needed for IFN release. Anti-CXCR4 antibodies reduced the infection of CD4+ cells by the X4-tropic virus HIV-1_{SF33} by 80% (data not shown). However, these antibodies had no effect on IFN induction by HIV-1_{SF33}-infected cells (Fig. 3e).

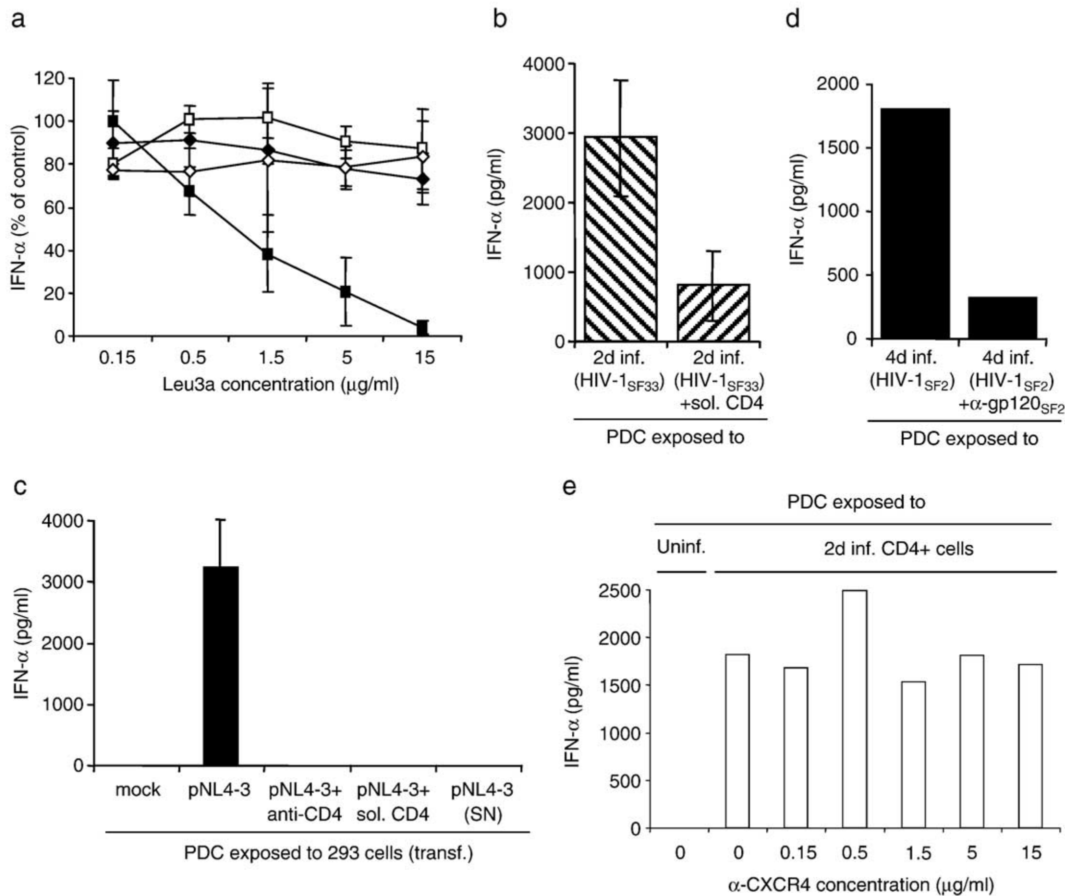

Fig. 3. Characterization of the PDC surface receptor interactions involved in the induction of IFN production. (a) Effect of sodium-azide free anti-CD4 (Leu3a) on the induction of IFN-α by PDC after exposure to four different stimuli: CD4+ cells 2 days after infection by HIV-1_{SF33} (■); irradiated herpes simplex virus type 1 (HSV-1) (□); CpG-A (♦); and the synthetic TLR7 agonist S-27609 (◊). Results are given as mean ± 1 SD, representing at least three separate experiments using PDC from different donors (control IFN-α levels for 2d HIV-1_{SF33}-infected cells (1802 pg/ml), HSV (4377 pg/ml), CpG-A (4542 pg/ml), and S-27609 (2899 pg/ml). (b) Effect of soluble (sol.) CD4 on the PDC IFN production during exposure to 2d HIV-1_{SF33}-infected (inf.) CD4+ T cells. Results are representative of three separate experiments. Bars are ±1 SD. Soluble CD4 did not block PDC IFN induction by HSV (data not shown). (c) PDC IFN production after exposure to 293 cells transfected (transf.) with medium (mock) or pNL4-3 in the absence and presence of anti-CD4 and soluble (sol.) CD4, as well as the supernatant (SN) from pNL4-3 transfected 293 cells. The mean RT activity in the supernatant of the transfected 293 cells was 264×10^3 cpm/ml. Results are representative of three separate studies. (d) Effect of HIV-1 $gp120_{SF2}$ antiserum (10 μl/well) on the PDC interferon production after coculture with CD4+ cells, 4 days after infection with HIV-1_{SF2} (4d inf.). (e) Effect of anti-CXCR4 antibodies on the PDC IFN production after exposure to uninfected (uninf.) CD4+ cells or CD4+ cells 2 days after infection (2d inf.) by HIV-1_{SF33}. The data are representative of two separate studies.

Role of endocytosis in PDC IFN induction

In attempts to determine if some aspect of endocytosis is involved in this induction of IFN, we examined the role of the PDC surface marker BDCA2. BDCA2 is a novel type II calcium-dependent lectin that has been reported to mediate antigen capture and uptake (Dzionek et al., 2000). Anti-BDCA2 antibodies blocked PDC IFN induction by HIV-infected cells, CpG-A, and HSV, whereas IFN induction by S-27609 remained unaffected (Fig. 4a). The effect of anti-BDCA2 was observed at 100-fold lower concentration compared to the isotype control antibody. PDC were also treated with chloroquine, which blocks TLR9-mediated signaling by inhibiting endosomal acidification and maturation (Lee et al., 2003). This treatment abolished IFN induction by HSV, CpG-A, and HIV-infected cells at a 10-fold lower concentration than the IFN induction by the synthetic TLR7 agonist (Fig. 4b). Importantly, these concentrations were 5- to 10-fold lower than those reported to inhibit HIV-1 replication in PBMC (Rayne et al., 2004).

PDC maturation after exposure to different stimuli

In response to various stimuli, PDC can release interferon and/or mature into DC. In evaluating this process, PDC were exposed to HIV, uninfected and HIV-infected CD4+ cells, CD40L, HSV, CpG-A, and CpG-B. Expression of surface markers for activation (CD80 and CD86), maturation (CD83), and migration (CCR7) was determined after 24 and 48 h. Exposure to infectious HIV alone did not change surface marker expression, even after 48 h (Fig. 5). In contrast, all markers were upregulated within 24 h after exposure of the PDC to HSV, CpG-B, and to a lesser extent CpG-A. CpG-A readily induced IFN-α production by PDC, whereas CpG-B is primarily an activator of B cells and, as

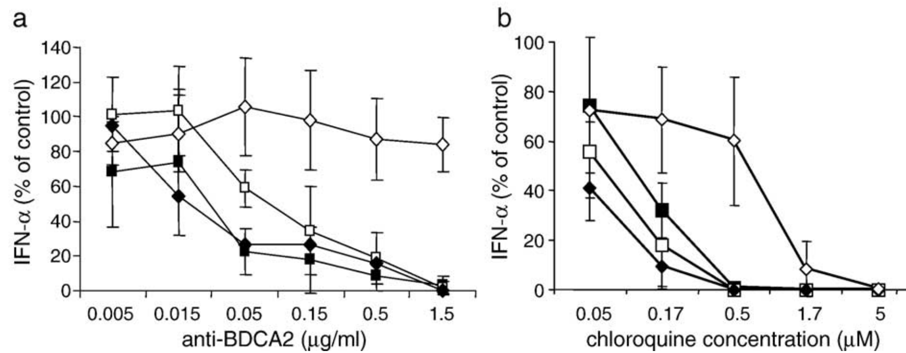

Fig. 4. Role of endocytosis in the induction of PDC IFN-α production. PDC were exposed to CD4+ cells 2 days after infection by HIV-1_{SF33} (■); UV-irradiated herpes simplex virus type 1 (HSV-1) (□); CpG-A (♦); and the synthetic TLR7 agonist S-27609 (◊). Effect of (a) anti-BDCA2 (control IFN-α levels for 2d HIV-1_{SF33}-infected cells (5037 pg/ml), HSV (2148 pg/ml), CpG-A (2625 pg/ml), and S-27609 (1648 pg/ml), and (b) chloroquine (control IFN-α levels for 2d HIV-1_{SF33}-infected cells (2301 pg/ml), HSV (4062 pg/ml), CpG-A (4498 pg/ml), and S-27609 (2900 pg/ml). All supernatants were removed 24 h after exposure of PDC to the four different stimuli. Results are given as mean ± 1 SD, representing at least three separate experiments using PDC from different donors.

was shown by others (Rothenfusser et al., 2004), induced maturation of PDC (Fig. 5).

The encounter of PDC with PHA-stimulated CD4+ cells enhanced activation and maturation of autologous and heterologous PDC after 24 h, whereas unstimulated CD4+ cells did not (Fig. 5). In contrast, upregulation of CCR7 expression was only observed with HIV-infected CD4+ cells, which similarly enhanced PDC maturation and activation. This observation was confirmed by studies with three other PDC donors (Table 2).

Discussion

Innate immune responses are the first line of defense against HIV infection and can be important in preventing transmission or limiting the infection through antiviral activity (Levy, 2001). One important member of the innate immune response, the plasmacytoid dendritic cell (PDC), is the major producer of type-1 IFN (Cella et al., 1999; Siegal et al., 1999). PDCs are found at higher numbers in HIV-infected individuals who are healthy, particularly long-term survivors of the infection (Soumelis et al., 2001). Previous studies have suggested that HIV itself can induce IFN production by cultured PDC (Fong et al., 2002; Fonteneau et al., 2004; Yonezawa et al., 2003). Our systematic approach exposing a low number of PDC to different stimuli for 24 h indicates that the virus-infected cells are better inducers of IFN-α production than HIV alone (Figs. 1a, b). This finding is supported by several observations: the absence of substantial IFN production by cell-free virus containing fluids (Figs. 1a, b, c), the abrogation of IFN induction by HIV-infected cells using transwell inserts (Fig. 1e), and the lack of correlation of IFN release with high particle-associated reverse transcriptase activity in cell culture fluids vs. the lower viral levels associated with virus-infected cells (Table 1).

Our findings with HIV alone contrast with previous reports (Del Corno et al., 2005; Fong et al., 2002; Fonteneau et al., 2004; Yonezawa et al., 2003) in which different approaches were used. For example, the induction of IFN-α production by HIV alone can be enhanced by exposing 40,000 PDC instead of 10,000 PDC to the virus and measuring the cytokine after 48 h instead of 24 h (Fig. 1d). These experimental conditions were used by Fong et al. (2002) and Yonezawa et al. (2003), respectively. In a recent study, a very large number of PDC (2.5×10^5 cells) were induced to release IFN after exposure to different stimuli (Del Corno et al., 2005). Moreover, substantial IFN induction by HIV alone was shown by Fonteneau et al. (2004) after purifying virions by sucrose gradient ultracentrifugation with a final 1000-fold concentration compared to the original cell culture supernatant. Our results with very high concentrations of AT-2 inactivated HIV-1 support these findings. However, our studies were designed to determine the relative importance of HIV vs. HIV-infected cells in the induction of IFN production by PDC. With our approach using low amounts of PDC and physiologically relevant HIV concentrations, optimal IFN production was found induced by HIV-infected cells.

Viability of the infected cells appeared to be necessary for this effect on PDC, because IFN was not induced by apoptotic uninfected cells nor by virus-infected cells killed by different methods. In the evaluation of other cellular and viral component(s) that could be important for this process, we did not observe IFN induction by the viral envelope and accessory proteins nor by DNA and RNA extracted from virus-infected cells. In this regard, some earlier studies noted IFN induction when PDC were exposed to the gp120 protein derived from HIV-1_{IIIB} (Ankel et al., 1994). This response could be blocked by antibodies to the principal neutralizing domains of HIV and the CD4 binding site, but not by antibodies to domains in the V3 loop that prevented HIV infection and cell fusion. This IFN induction was not observed with gp120s derived from other HIV isolates. Thus, our negative findings with recombinant gp120 proteins support these latter observations.

The induction of IFN production was considerably reduced by filtering viral stocks prior to PDC challenge (Fig. 1c). These findings, in addition to the substantial PDC release of IFN after exposure to HIV-infected cells, support a role of cell surface molecules in this process. The IFN induction by HIV-infected cells appears to be specially regulated since antibodies to CD4 (also Yonezawa et al., 2003), gp120, and soluble CD4 can

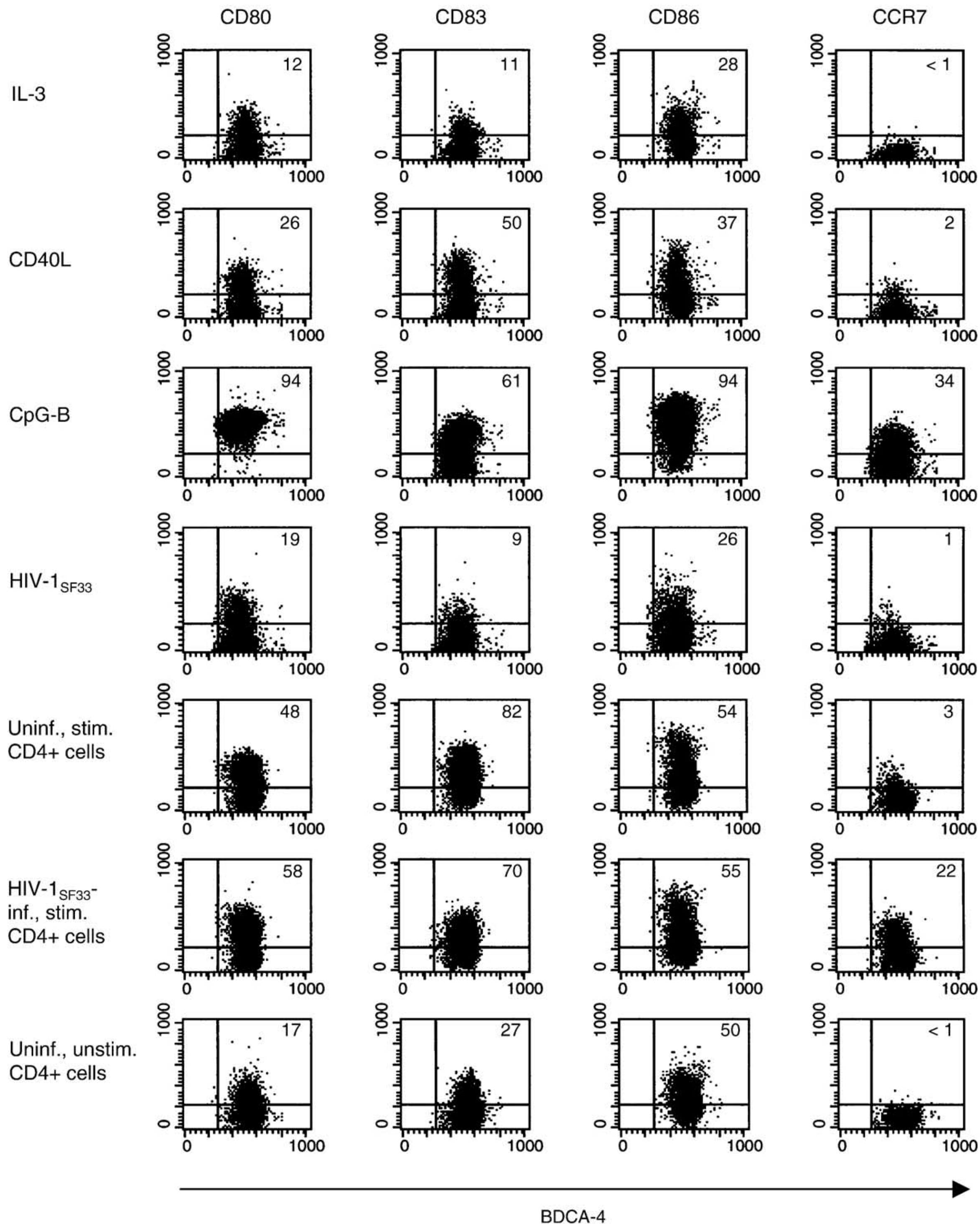

Fig. 5. Effect of different stimuli on the expression of PDC surface markers for cell activation (CD80, CD86), maturation (CD83), and migration (CCR7) after 24 h of exposure. Results are compiled from four different experiments using freshly isolated PDC from the same donor. Data are representative of studies with four different donors. Stimuli were IL-3 only, CD40 ligand (CD40L), CpG-B, HIV-1_{SF33} (10,000 $TCID_{50}$ per experiment), uninfected (uninf.) unstimulated (unstim.) or PHA-stimulated (stim.) CD4+ cells as well as HIV-1_{SF33}-infected (inf.) stimulated CD4+ cells. For exposure of PDC to CpG-A, the respective percentages were 65 (CD80), 60 (CD83), 71 (CD86), and 43 (CCR7).

block the effect on PDC (Figs. 3a, b, d). The results further suggested that the CD4 receptor on PDC rather than HIV-infected cells interacts with gp120 present on the surface of HIV-infected cells (Fig. 3c). In addition, coreceptor binding of viral particles to PDC and virus infection of PDC do not seem to be required since antibodies to CXCR4 did not affect IFN induction by HIV-1-infected cells (Fig. 3e). It cannot be excluded, however, that only the initial binding step, i.e. the attachment to the CD4 receptor, can be inhibited efficiently by antibody.

Table 2
Upregulation of CCR7 expression by PDC after exposure of these cells to uninfected and HIV-infected CD4+ cells

PDC donor	Percentage of CCR7-expressing PDC after exposure of these cells to		
	Mock	Uninfected CD4+ cells	HIV-infected CD4+ cells
01	<1	3	22
02	4	14	26
03	1	10	25
04	3	8	47

Results of donor 01 are identical to those shown in Fig. 5.
PHA-stimulated CD4+ cells were infected by HIV-1_{SF33} 2 days prior to coculture. 10^5 PDC were exposed to uninfected or HIV-infected CD4+ cells at a ratio of 1:2 (PDC/CD4) in 48-well flat-bottom plates. After 24 h, the percentage of CCR7-expressing BDCA4-positive cells was assessed using FACS analysis. Mock = IL-3 containing medium alone.

BDCA-2, a type II calcium-dependent lectin expressed on PDC, mediates antigen capture and inhibits induction of type I IFN (Arce et al., 2001; Dzionek et al., 2001). Our findings also suggest a specific involvement of BDCA-2 in the process of IFN induction by HIV-infected cells (Fig. 4a). Importantly, the anti-BDCA-2 antibodies used did not affect IFN induction by the TLR7 agonist at these concentrations, thus indicating that IFN production was not generally blocked. The results could indicate that BDCA2 has a role similar to that of DC-SIGN on monocyte-derived dendritic cells (Kwon et al., 2002). It could mediate antigen transfer to endocytotic vesicles in PDC.

This observation on BDCA-2 could have relevance to Toll-like receptors (TLR), which are involved in the recognition of non-self antigens in innate immunity. PDC preferentially express TLR7 and TLR9 (Kadowaki et al., 2000); which are located intracellularly (Krieg, 2002). TLR9 is activated via unmethylated CpG DNA (Hemmi et al., 2000). The CpG oligonucleotides are internalized into early endosomes and subsequently transported to a tubular lysosomal compartment where they encounter TLR9 redistributed from the endoplasmic reticulum (Latz et al., 2004); thus endocytosis is involved in the function of TLR9.

The activity of TLR9 in comparison to TLR7 is blocked at lower concentrations of compounds such as chloroquine that prevent endosomal acidification and maturation (Lee et al., 2003). Thus, our observations on the inhibitory effect of chloroquine on the induction of IFN by HIV-infected cells (Fig. 4b) are relevant. They resemble findings by others showing a chloroquine sensitivity of HSV and HSV-infected cells in their induction of interferon production (Feldman et al., 1994; Lebon, 1985). In this regard, recognition of HSV-2 by PDC has been reported to be mediated by TLR9 in the mouse model (Lund et al., 2003). Therefore, our results with chloroquine (Fig. 4b) provide indirect evidence for a role of TLR9 on human PDC in the IFN induction by HIV-infected cells. However, further studies are required to establish direct evidence of a role for specific TLRs on PDC in the IFN induction by HIV-infected cells.

The IFN-α induction by PDC resulted in reduced HIV-1 replication in the infected CD4+ cells (Figs. 2a, b, c), which was similarly reproduced using exogenous recombinant IFN-α (Fig. 2d) and could be reversed by neutralizing antibodies to IFN-α (Fig. 2e). These data indicate that PDC can have anti-HIV activity mediated by IFN-α production. Notably, this virus suppression was observed using CD4+ cells which had been infected with HIV 2 days prior to coculture with PDC. It is likely that the PDC secreted IFN-α will have an even greater antiviral effect on uninfected or recently infected CD4+ cells.

Another important finding from our studies was that the expression of CCR7 associated with cell migration was more readily enhanced by contact of PDC with HIV-infected CD4+ cells than CD4+ cells alone (Fig. 5 and Table 2). These results suggest that PDC mature upon contact with CD40L expressed by stimulated CD4+ cells, whereas trafficking of PDC to secondary lymphatic tissue (via CCR7) is greatly enhanced by contact with HIV-infected CD4+ cells. In HIV-infected individuals, about 1 in 1000 to 10,000 circulating CD4+ cells is HIV-infected. This number increases with progression to disease, which enhances the chance of PDC encountering these cells. The contact with HIV-infected cells will also occur within secondary lymphatic tissue, which may contribute to retention of PDC in these tissues. Thus, a reason for the depletion of circulating PDC in late stages of HIV infection (Soumelis et al., 2001) may be the transit to and maintenance of PDC in the lymph nodes after contact with HIV-infected cells. Understanding further the mechanism by which virus-infected cells induce IFN production by PDC could thus be helpful for the development of therapeutic approaches directed at maintaining and increasing the number of PDC in HIV-infected individuals.

Methods

Isolation of PDC

Using Ficoll–Hypaque gradients (Sigma Diagnostics Inc., St. Louis, MO), peripheral blood mononuclear cells (PBMC) were obtained from EDTA-containing blood of uninfected volunteers or from buffy coats provided by the Blood Centers of the Pacific (San Francisco, CA). This project received the approval of the Committee for Human Research, University of California, San Francisco, CA.

The PDC were purified from the PBMC using a BDCA-4 Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) (Dzionek et al., 2000). After two steps with LS and MS columns, these cells were >95% pure as determined by flow cytometry. From a buffy coat of 500×10^6 cells, about 5×10^5 PDC could be recovered. The isolated PDC were cultivated in RPMI 1640 medium containing 10% heat-inactivated (56 °C, 30 min) fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, supplemented with 20 ng/ml IL-3 (R&D Systems, Minneapolis, MN). For these studies, the PDC were generally plated at a density of 10^4 cells/well in 96-well flat bottom plates. For the FACS experiments, the PDC were plated at a density of 10^5 cells/well in 48-well flat bottom plates, and for the transwell experiments at a density of 2.5×10^4 cells/well in 24-well flat bottom plates.

CD4+ T cell isolation and infection

CD4+ T cells were isolated from the separated PBMC using CD4 MicroBeads (Miltenyi Biotec, Auburn, CA) by standard procedures (Mackewicz et al., 1991). The CD4+ cells were then cultured in the RPMI 1640 medium described above, supplemented with 10 ng/ml human IL-2 (Roche Diagnostics, Indianapolis, IN). They were stimulated with 3 μg/ml of phytohemagglutinin (PHA) for 3 days, washed and pretreated for 30 min with 2 μg/ml of polybrene (Sigma Chemicals, St. Louis, MO) prior to virus inoculation. The cells were then infected with the SF33 isolate of HIV-1 at a multiplicity of infection (MOI) of 0.0001 unless stated otherwise. This virus is an X4 syncytium-inducing (SI) chemokine-insensitive isolate (Mackewicz et al., 1996). Other HIV-1 isolates used for these studies were SF_{162}, SF_{128A}, and ME1, all NSI (R5) viruses, and ME46 and SF2, SI (X4) viruses (Levy et al., 1985). ME1 and ME46 were obtained from the AIDS Research and Reference Reagent Program, NIH. The HIV-2_{UC2} isolate was also used (Castro et al., 1990). After 1 h of infection, the cells were washed and cultured at a concentration of 3×10^6 cells/ml for 6 h to 5 days prior to PDC coculture. Virus replication was assessed by reverse transcriptase (RT) activity in the culture fluids (Hoffman et al., 1985), prior to and after adding the HIV-infected CD4+ cells to the PDC.

Virus stocks were generated by acutely infecting PHA-stimulated CD4+ cells, then adding PBMC when high viral production occurred. The supernatants were harvested at peak viral replication, which usually occurred within 2–3 days of passage. The $TCID_{50}$ was quantified in PBMC stimulated by PHA and IL-2 as described (McDougal et al., 1985).

Coculture with PDC

PDC were cocultured with HIV-infected or uninfected CD4+ cells at a ratio of 1:6. For some experiments, PDC were inoculated with 100 μl of supernatant from the HIV-infected CD4+ cells (Fig. 1b) or pNL4-3-transfected 293 cells (Fig. 3c) or with virus pelleted from these supernatants by centrifugation at 12,000 rpm for 1 h. For other experiments, viral particles adherent to the CD4+ cell surface were removed by treating the cells with 0.15% trypsin (Tang and Levy, 1991) 1 h after virus inoculation. For the cell maturation experiments, PDC were mixed with stimulated or unstimulated CD4+ cells at a ratio of 1:2. Transwell inserts for 24-well flat bottom plates were purchased from Corning Costar Corporation (Cambridge, MA).

Transfection of 293 cells

293 cells were plated in 60 × 15 mm tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ). The cells were transfected at 50% confluency using 3 μl of the FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN) with 1 μg of DNA (pNL4–3). Virus production was assessed by RT activity (Hoffman et al., 1985) in the cell culture supernatants 48 h after transfection, immediately before the 293 cells were cocultured with PDC.

Cytokine assays

Culture supernatants were generally harvested 24 h after exposure of PDC to different stimuli unless indicated otherwise. The supernatants were assayed for IFN-α activity using a sandwich enzyme linked immunosorbent assay (ELISA) (Biosource International, Camarillo, CA) according to the manufacturer's instructions. All samples were evaluated in duplicate and diluted if appropriate. Each experiment was performed at least twice with different PDC donors.

Biologic reagents

The following reagents were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD): HIV-1_{PB1MN} antiserum, goat; HIV-1 $gp120_{SF2}$ antiserum, goat; MAb (human) to HIV-1 gp120 (F105), MAb to HIV-1 gp120 (C2G12); MAb (human) to HIV-1 gp41 (98-6); MAb to HIV-1 gp41 (F240); HIV-1_{IIIB} gp120; HIV-1_{SF2} gp120; HIV-1_{BaL} gp120; HIV-1_{SF162} gp120; HIV-1 Tat; HIV-1_{SF2} p55 Gag, and recombinant soluble CD4. Sodium-azide free antibodies to CD4 (Leu3a) were kindly provided by Becton Dickinson (San Jose, CA). Antibodies were generally used at 15 μg/ml unless stated otherwise, by preincubating PDC or HIV-infected CD4+ cells at 37 °C for 60 min. Proteins were added to PDC at amounts of 0.2–1 μg per well. CD40L (received from Immunex Corp., Seattle, WA) was added at a concentration of 0.5 ng/ml. The IFN-α antibody (cat. no. G026-501-568) and the isotype control (cat. no. G027-501-568) were obtained from the NIAID Research Resources; both antibodies were resuspended in 0.5 ml of PBS and used at a final dilution of 1:200. Monoclonal antibodies for FACS analysis were obtained from BD Biosciences, San Jose, CA (HLA-DR and CD4) and BD PharMingen, San Diego, CA (CD80, CD86, and CCR7). Flow cytometry was performed on a FACSort with CellQuest Software (BD Biosciences). CpG-A (ODN 2336; provided by Coley Pharmaceutical Group, Wellesley, MA) and CpG-B (5′-tcgtcgttttgtcgttttgtcgtT-3′, lower cases indicate phosphorothioate linkage, synthesized by Invitrogen Corporation, Carlsbad, CA) were used at final concentrations of 1 and 5 μM, respectively. UV-irradiated HSV-1 was used at a final concentration of 10^6 PFU/ml. Aldrithiol-2 inactivated HIV-1 (Arthur et al., 1998) (isolate 97ZA012 from the NIH AIDS Research and Reference Reagent Program) was used at a non-toxic concentration of 10^9 RNA copies/ml. Chloroquine (purchased from Sigma-Aldrich CO, St. Louis, MO) was used at 0.05–5 μM. The synthetic TLR7 agonist S-27609, provided by Richard Miller (3M Pharmaceuticals, St. Paul, UK), was used at a concentration of 5 μM. Recombinant IFN-α was obtained from Biosource.

Extraction of viral and cellular DNA and RNA

PHA-stimulated CD4+ cells were infected with HIV-1_{SF33} as described above and cultured for 3 days. Control uninfected CD4+ cells were also cultured. The DNA or RNA was extracted from 3×10^6 cells using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) or the TRIzol Reagent (Invitrogen, Carlsbad, CA), respectively. For the studies described, concentrations of 0.5–5 μg/ml of the DNA obtained from infected or uninfected cells were used.

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