Implication of p38 mitogen-activated protein kinase isoforms (α, β, γ and δ) in CD4+ T-cell infection with human immunodeficiency virus type 1

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The CD4+ T-cell reduction characteristic of human immunodeficiency virus type 1 (HIV-1) infection is thought to result, in addition to infected T-cell death, mainly from uninfected bystander T-cell apoptosis. Nevertheless, the immunological and virological mechanisms leading to T-cell death during HIV-1 infection are not yet fully understood. In the present study, we analysed the individual implication of the p38 mitogen-activated protein kinase (MAPK) isoforms (p38α, p38β, p38γ and p38δ) during apoptosis induced by HIV-1, taking into account that HIV-1 replication is known to be blocked by p38 inhibitors. For this purpose, we used the SupT1 cell line, where death induced by HIV-1 mainly occurs by uninfected bystander cell apoptosis. A variety of SupT1-based cell lines were constructed constitutively expressing, under the control of cytomegalovirus promoter (PCMV), each dominant-negative (dn) p38 isoform and each wild-type p38 isoform as a control. An enhanced green fluorescent protein marker gene, under the control of the HIV-1 promoter, was inserted in all of them. These cell lines were infected with HIV-1 and analysed by flow cytometry. We found that survival in SupT1-based cell lines infected by HIV-1 was increased by the p38αdn, p38γdn and p38δdn isoforms, but not by the p38βdn isoform. HIV-1 replication was delayed most by p38δdn and to a lesser extent by p38αdn and p38γdn. Moreover, these three isoforms, p38αdn, p38γdn and p38δdn, reduced apoptosis induced by HIV-1. These results suggest that, in SupT1-based cell lines, p38α, p38γ and p38δ, but not p38β, are implicated in both HIV-1 induced replication and apoptosis in infected and uninfected bystander cells.

A variety of HIV-1 proteins are implicated in T-cell death. Some proteins are involved in direct cytopathic effects, such as protease, others in bystander cell death, such as gp120/gp41 and HIV-1-negative factor (Nef), and others in both processes, such as Vpr, Vpu and Tat (Casella et al., 1999; Bartz & Emerman, 1999; Silvestris et al., 1999; Azad, 2000; Nie et al., 2002; Algeciras-Schimnich et al., 2002; Brenner & Kroemer, 2003; Yang et al., 2003; Castedo et al., 2003). Infected and bystander CD4+ T-cell death can be caused by several mechanisms; namely, the toxic effect of viral proteins, proapoptotic signalling as a result of interactions between the HIV-1 envelope glycoproteins and their receptors, and activation-induced cell death due to the increased expression of cytotoxic ligands and cytokines.

In recent years, several studies point to the participation of p38 mitogen-activated protein kinase (MAPK) in HIV-1...
infection. The p38 MAPK pathway regulates the production of cytokines and transcription factors implicated in HIV-1 replication (Kumar et al., 1996; Cohen et al., 1997). In fact, the presence of specific p38 MAPK inhibitors, such as CNI-1493, SB203580 or RWJ67657, decreases constitutive HIV-1 production (Cohen et al., 1997; Shapiro et al., 1998; Muthumani et al., 2004). However, it should be taken into account that these inhibitors are not absolutely specific to p38 and that they also affect, although to a lesser extent, other proteins like JNK. Another limitation to keep in mind is that all known inhibitors affect p38α and p38β, but not p38γ and p38δ isoforms.

Although the immunological and virological mechanisms leading to CD4+ T-cell death during HIV-1 infection are not yet understood, there is reason to believe that p38 is involved. It has been demonstrated that p53, a substrate of p38, plays a role in the apoptosis induced by HIV-1 (Gennini et al., 2001; Castedo & Kroemer, 2002; Castedo et al., 2001, 2002). This finding suggests p38 participation in this apoptotic pathway. In fact, two mechanisms that implicate p38 in uninfected bystander cell apoptosis have been described recently; one through the HIV-1 envelope (Perfetti et al., 2005) and the other, through Nef (Muthumani et al., 2005). None of these studies, however, confirm the individual implication of the various p38 isoforms during apoptosis induced by HIV-1. Therefore, in order to determine the specific role of each p38 isoform (both in HIV-1 replication and HIV-1-induced apoptosis), we pursued a genetic strategy to obtain CD4+ T cell lines expressing mutated versions of p38 isoforms, i.e. dominant-negative p38 (p38dn) isoforms, to be used in a model of HIV-1 multiple cycle infection.

**METHODS**

**Cell cultures.** GP2-293 (catalogue no. 631530; Clontech) and 293T/17 (CRL-11268; ATCC) cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with l-glutamine and 4.5 g glucose (BioWhittaker) 1−1, supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% streptomycin/penicillin. MT2 (93121518; ECCAC) and SupT1 (95013123; ECCAC) cell lines were cultured in RPMI 1640 with l-glutamine and 25 mM HEPEs (BioWhittaker), supplemented with 10% (v/v) FBS and 0.4% (v/v) streptomycin/penicillin. Modified SupT1 cell lines expressing p38dn and p38 isoforms under the cytomegalovirus promoter (PCMV) were maintained in RPMI 1640 medium (BioWhittaker), supplemented with 10% (v/v) FBS, 0.4% (v/v) streptomycin/penicillin and 1 mg G418 (Amersham Biosciences) ml−1. All cell types were grown at 37 °C under a humidified atmosphere containing 5% CO2.

**Antibodies.** Anti-flag M2 murine IgG1 monoclonal antibody (mAb) was purchased from Sigma. Horseradish peroxidase (HRP)-labelled anti-mouse mAb was obtained from Amersham.

**Construction of retroviral vectors containing the p38/p38dn isoforms and the enhanced green fluorescent protein (EGFP) gene with the long terminal repeat (LTR5) HIV-1 promoter.** LTR5 promoter was amplified from the pNL4.3 vector (item no. 114; NIH AIDS Research and Reference Reagent Programme) and cloned as Aatt–Kpml fragment of 705 bp into pIRE2–EGFP vector (Clontech), using the following primers forward/reverse (Supplementary Table S1 available in JGV Online): F-LTR5′–R-LTR5′. Then, to obtain plasmid pQCXIN-LTR5′–EGFP, the LTR5′–EGFP fragment was amplified and cloned as an XbaI fragment into the retroviral expression vector pQCXIN (Clontech) using the following primers forward/reverse (Supplementary Table S1): F-LTR5′–EGFP, R-LTR5′–EGFP. Plasmids encoding p38α, p38β, p38γ, p38δn and p38δdn isoforms were obtained from Dr G. Bou (Juan Canalejo Hospital, A Coruña, Spain). All these isoforms were amplified with a flag-epitope tag and cloned as Agel–BamHI fragments (for p38α, p38δn and p38δ isoforms) and as Agel–EcoRI fragments (for p38γ, p38dn and p38δ isoforms) into the retroviral vector pQCXIN-LTR5′–EGFP, after PCMV. Therefore, we constructed plasmids pQCXIN-LTR5′–EGFPp38α, pQCXIN-LTR5′–EGFPp38δn, pQCXIN-LTR5′–EGFPp38δ and pQCXIN-LTR5′–EGFPp38δn (Fig. 1). In order to obtain these plasmids, restriction enzyme sites were included in primer sequences to facilitate subsequent cloning steps. The following set of primers of forward and reverse was used (Supplementary Table S1): F-p38α/2αx; R-p38α/2αx; F-p38β/2βx; R-p38β/2βx; F-p38γ/2γx; R-p38γ/2γx; F-p38δ/2δx; R-p38δ/2δx. The p38α dn and p38δdn isoforms were created from the corresponding wild-type isoforms with a flag-epitope tag, and cloned into pcDNA vector. For this purpose, direct mutagenesis was performed substituting Thr183 (in p38γ) and Thr180 (in p38δ) by Ala, and Tyr185 (in p38γ) and Tyr182 (in p38δ) by Phe. The mutagenesis consisted of combining two separate PCR products with overlapping sequences into one longer product. To obtain p38dn from p38γ, we used the ‘outside’ forward and reverse primers (Supplementary Table S1): F1-p38α/2αx; R1-p38α/2αx, and the overlapping ‘inside’ forward and reverse primers (Supplementary Table S1): F2-p38δ/2δx; R2-p38δ/2δx. And to obtain p38dn from p38δ, we used the ‘outside’ forward and reverse primers (Supplementary Table S1): F1-p38δ/2δx, R1-p38δ/2δx, and the overlapping ‘inside’ forward and reverse primers (Supplementary Table S1): F2-p38δ/2δx, R2-p38δ/2δx. Subsequently, p38α dn was cloned as an Agel–BamHI fragment and p38δdn as an Agel–EcoRI fragment into the retroviral vector pQCXIN-LTR5′–EGFP, after PCMV, to obtain plasmids pQCXIN-LTR5′–EGFPp38α and pQCXIN-LTR5′–EGFPp38δ. For this purpose, we

![Fig. 1. Vector map of the retroviral vector pQCXIN with inserts corresponding to the EGFP gene with LTR5 HIV-1 promoter as well as the various p38 isomorph genes (X=p38α/2αx; Y=p38γ/2γx; Z=p38dn/2δx).](https://www.microbiologyresearch.org/.../Fig1.pdf)
used forward and reverse primers (Supplementary Table S1): F-p38/γdn, R-p38/γdn; F-p38β/δdn, R-p38β/δdn.

In the present study, all PCRs were performed using a high-fidelity DNA polymerase (PfuUltra; Stratagene) with the following parameters: 95 °C for 2 min, then 35 cycles with a denaturation step at 95 °C for 30 s, hybridization at 55 °C for 30 s and elongation at 72 °C for 2 min, with a final elongation at 72 °C for 7 min. A ‘touch down’ PCR was needed when F1-p38/δdn and B2-p38/δdn primers were used in p38β mutagenesis. For this purpose, we used the following parameters: 94 °C for 2 min; then 5 cycles with a denaturation step at 94 °C for 1 min, hybridization at 65 °C for 30 s and elongation at 72 °C for 2 min; 10 cycles with a denaturation step at 94 °C for 1 min, hybridization at 60 °C for 30 s and elongation at 72 °C for 2 min; 20 cycles with a denaturation step at 94 °C for 1 min, hybridization at 55 °C for 30 s and elongation at 72 °C for 2 min; and finally, an elongation at 72 °C for 7 min. The constructions were transformed into Escherichia coli (DH5α competent cells; Clontech) at 30 °C, and purified by an alkaline extraction procedure (High Plasmid Plasmid Isolation Kit; Roche Applied Science). DNA cloning and manipulation were carried out using standard procedures (Sambrook et al., 1989; Seidman et al., 1994). All p38 isoforms cloned into the retrovector pQXCIN-LTR5–EGFP were verified by automated sequencing on the CEQ 2000 DNA Analysis System (Beckman Coulter).

**Generation of stable modified SupT1 cell lines.** The retroviral vectors pQXCIN LTR5–EGFP-p38/δdn, pQXCIN LTR5–EGFP-p38β/δdn were co-transfected with an expression plasmid for vesicular stomatitis virus protein (pSVS-G) in the packaging cell line GP2-293 (Clontech), by calcium phosphate co-precipitation. The pseudotyped virions were collected 48 h after transfection. Culture supernatants supported PVDF membranes and probed with anti-flag M2 mAb (1:15,000), followed by incubation with a secondary HRP-labelled antibody (1:5000). Bound antibodies were detected by chemiluminescence with the ECL Plus Western Blotting Detection Reagent (Amersham).

**Western blot analysis.** Cell aliquots (10⁶ cells) were solubilized in ice-cold lysis buffer containing 0.1% (v/v) Triton X-100, 0.5 mM DTT, 25 mM HEPES pH 7.5, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM Na2EDTA and 1% (v/v) of protease inhibitor cocktail. After 10 min on ice, the lysates were clarified by centrifugation. Protein concentrations of the lysates were determined with the Pierce Bicinchoninic acid protein assay reagent. In each sample, 20 µg protein was resolved by SDS-PAGE (12% acrylamide gel), transferred to supported PVDF membranes and probed with anti-flag M2 mAb (1:15,000), followed by incubation with a secondary HRP-labelled antibody (1:5000). Bound antibodies were detected by chemiluminescence with the ECL Plus Western Blotting Detection Reagent (Amersham).

**Virus stock preparation and virus titre.** Virus stock of HIV-1 was prepared by transfecting 293T/17 cells with the pCHUS plasmid (Abad et al., 2004) by using calcium phosphate precipitation (CalPhosTM Mammalian Transfection kit; Clontech), and following the manufacturer’s guidelines. Culture supernatants containing HIV-1 were collected 48 h later and were filtered through a 0.45 µm pore-size filter. Virus was concentrated 10-fold by ultracentrifugation and kept frozen at −80 °C. Titration of each virus stock was performed by MT2-cell infectivity. Six serial 10-fold dilutions of each virus stock were titrated in quadruplicate in 96-well flat-bottomed tissue-culture plates (Nunclon; Nunc). MT2 cells were adjusted with growth medium to a concentration of 10⁶ cells ml⁻¹, and 5 x 10⁴ cells in 50 µl were added to each well. Dilutions of virus were made in RPMI 1640 with l-glutamine and 25 mM HEPES, supplemented with 10% (v/v) FBS and 0.4% (v/v) streptomycin/penicillin, and 50 µl each dilution was added to cells in each well, with the exception of four negative control wells to which 50 µl medium without virus was added. Plates were incubated at 37 °C under a humidified atmosphere containing 5% CO₂. On days 3, 6 and 9 syncytium formation was monitored by microscopy. TCID₅₀ was calculated by the Spearman–Karber method, based on cumulative positives and negatives for each dilution set.

**HIV-1 infection.** Modified SupT1 cell lines were infected with HIV-1 by incubation for 4 h at 37 °C in the presence of polybrene (4 µg ml⁻¹). Infections were performed at a ratio of 10².₅ TCID₅₀ ml⁻¹ (10⁶ cells ml⁻¹). Infected cells were washed once to remove polybrene and were then plated in a 24-well tissue culture plate in 0.5 ml medium. Twice a week, cells were passaged to keep the total cell density at 10⁶ cells ml⁻¹. On different days post-infection (p.i.), aliquots of cells were harvested from the culture and analysed by flow cytometry.

**Flow cytometry analysis.** Differentiation from dead cells was performed with 7-aminoactinomycin D (7AAD) as specified by the manufacturer’s instructions. Infected cells were detected by EGFP fluorescence. Phosphatidylserine on the outer membrane of the apoptotic cells was detected with phycoerythrin (PE)-conjugated annexin V (BD Via-Probe; BD Biosciences Pharmingen). This method allows quantitative determination of the percentage of cells within a population that are actively undergoing apoptosis. Flow cytometry analysis was carried out on an EPICS ALTRA Flow Cytometer (Beckman Coulter) by using the following filters: 448, 525, 575 and 610 nm. Data are displayed in a log dot-plot enabling the enumeration of viable, apoptotic and dead cells. To define the corresponding squares in the plot, we treated cells with camptothecin solution (Sigma). All the results obtained in the different analysis were processed with the Expo32 analysis package (Beckman Coulter).

**Statistical analysis.** Data are expressed as the mean of several values. Differences in measured variables between clones expressing p38δdn isoforms and control clones were assessed using Student’s t-test. Statistical difference was accepted at P<0.05.

**RESULTS**

**Survival in the SupT1-based cell lines infected with HIV-1 was increased by the p38α/δdn, p38β/δdn and p38β/δdn isoforms, but not the p38β/δdn isoform**

To examine the effect of p38 isoforms in CD4⁺ T-cell infection with HIV-1, we began by analysing the implication of individual p38δdn isoforms in SupT1 cell line survival. For this purpose, a number of constructs (Fig. 1) were used to generate various modified SupT1 cell lines expressing each of the p38δdn isoforms under PCMV, as well as various modified control SupT1 cell lines expressing each of the wild-type p38 isoforms under PCMV. The generated cell lines were also made to express the EGFP protein under the LTR5’ HIV-1 promoter. This was used as a sensitive marker for HIV-1 cell infection because EGFP expression is triggered by the early viral protein Tat.
A retroviral method was used to insert constructs into the SupT1 cell line. The modified cell lines were cloned and infected with HIV-1 virions obtained from the pCHUS plasmid. At several days p.i., 7AAD and annexin V-PE assays were performed and cells were analysed by flow cytometry. It was found that death occurred mainly by apoptosis in the SupT1 cell line infected by HIV-1 (Fig. 2).

Viability decreased progressively over a period of 10 days p.i. in the controls, with respect to p38α, p38γ and p38δ isoforms. On the other hand, cell clones expressing the p38αdn, p38γdn and p38δdn isoforms maintained a much higher viability (Fig. 3). This indicates that the p38α, p38γ and p38δ isoforms are implicated in T-cell death. In the case of p38β, viability decreased both in the infected control and in the infected clones expressing the p38βdn isoform (Fig. 3). This leads to the conclusion that p38β is not implicated in T-cell death.

It is known that p38 MAPK has a participation in HIV-1 replication (Cohen et al., 1997; Shapiro et al., 1998; Muthumani et al., 2004). The implication of p38 has also been reported in apoptosis induced by HIV-1 envelope protein (Perfettini & Kroemer, 2005; Perfettini et al., 2005). Thus, protection from cell death by the p38αdn, p38γdn and p38δdn isoforms may be due to inhibition of HIV-1 replication (by competition with wild-type endogenous isoforms) and/or to inhibition of HIV-1-induced apoptosis. We proceeded to analyse both possibilities.

**Fig. 2.** Flow cytometric analysis on day 10 of apoptosis and cell death in modified SupT1 cell clones infected by HIV-1 viruses. Cells were infected as described in Methods and stained with annexin V-PE and 7AAD on different days p.i.
p38\textsubscript{adn}, p38\textsubscript{ydn} and p38\textsubscript{d\textsubscript{2}dn} isoforms, but not p38\textsubscript{y/dn} isoform, inhibited HIV-1 replication and decreased induced apoptosis

In order to study the implication of the various p38 MAPK isoforms in both HIV-1 replication and apoptosis, one clone corresponding to each p38dn isoform and one control clone corresponding to each p38 isoform expressed under PCMV were used for infection. Cytometric analysis was carried out with EGFP as a marker of infected cells and annexin V-PE fluorescence as a marker of apoptotic cells. Regarding the p38\textsubscript{x} isoform (Fig. 4), the clone expressing p38\textsubscript{adn} isoform significantly decreased EGFP expression with respect to the control throughout infection. These results showed that the p38\textsubscript{x} isoform contributes to HIV-1 replication. For its part, annexin V fluorescence was significantly lower in the clone expressing the p38\textsubscript{adn} isoform than in the control. This reflects the implication of p38\textsubscript{x} in the apoptotic pathway induced by HIV-1 in CD4\textsuperscript{+} T lymphocytes.

In order to quantify the protective effect of p38\textsubscript{adn} in apoptosis and HIV-1 replication over time, an HIV-1 infection was followed up for 23 days (Fig. 5). The apoptosis protection of the clone expressing the p38\textsubscript{adn} isoform dropped off between days 19 and 23 p.i., probably due to the involvement of other apoptotic pathways. Nevertheless, EGFP expression stayed at around 10%. These results indicate that the effect of p38\textsubscript{adn} is greater in HIV-1 replication than in apoptosis. There is likely to be an

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Fig. 3. Flow cytometric analysis of viability in the following modified SupT1 cell clones infected by HIV-1 viruses: control \( a_{1.8} \) expressing the p38\textsubscript{a} isoform, \( x_{d2.6} \) and \( x_{d2.1} \) expressing the p38\textsubscript{adn} isoform, control \( j_{3.10} \) expressing the p38\textsubscript{b} isoform, \( j_{d2.4} \) and \( j_{d4.10} \) expressing the p38\textsubscript{bdn} isoform, control \( \gamma_{4.4} \) expressing the p38\textsubscript{y} isoform, \( \gamma_{d3.1} \) and \( \gamma_{d2.6} \) expressing the p38\textsubscript{ydn} isoform, control \( \beta_{3.6} \) expressing the p38\textsubscript{b} isoform and \( \delta_{d4.10} \) and \( \delta_{d2.10} \) expressing the p38\textsubscript{bdn} isoform. Cells were infected by HIV-1 as described in Methods and stained with annexin V-PE and 7AAD on different days p.i. As negative control, we used the corresponding clones without infection. Results are representative of two independent infections and the experiment was repeated three times.
Implication of p38α in the pathways of other p38 isoforms, and for this reason, the p38αdn isoform may decrease the entire p38 activity in HIV-1 replication.

Regarding p38β participation in HIV-1 replication, no significant differences were observed in EGFP expression between the control and the clone expressing the p38βdn isoform (Fig. 4). This behaviour showed that the p38β isoform is not implicated in HIV-1 replication. Similarly, viability simultaneously increased in both clones, shown with annexin V (Fig. 3), indicating that the p38β isoform is not implicated in apoptosis induced by HIV-1. In the case of the p38γ isoform (Fig. 4), increases in EGFP expression were delayed in the clone expressing the p38γdn isoform in comparison with the control. However, EGFP expression finally increased after day 10, indicating that HIV-1 replication was reactivated after an initial inhibition, probably through endogenous p38 isoforms. With respect to apoptosis, the clone expressing the p38γdn isoform maintained a value for annexin V below 20% for 14 days, even when EGFP expression was higher than 40% (day 14 p.i.). On the other hand, the control reached 70% of apoptosis even when EGFP expression was still at 20% (day 7 p.i.). Hence, we conclude that the p38γdn isoform competes with the endogenous isoforms in delaying HIV-1 replication and differentially inhibits apoptosis in spite of reactivating HIV-1 replication.

Fig. 4. Flow cytometric analysis of EGFP+ cells and total apoptosis in the following modified SupT1 cell clones infected by HIV-1 viruses: control α1.8 expressing the p38α isoform, αdn2.6 expressing the p38αdn isoform, β3.10 expressing the p38β isoform, βdn2.4 expressing the p38βdn isoform, γ4.4 expressing the p38γ isoform, γdn3.1 expressing the p38γdn isoform, control δ3.6 expressing the p38δ isoform and δdn4.10 expressing the p38δdn isoform. Cells were infected by HIV-1 as described in Methods and stained with annexin V-PE on different days p.i. As negative control, we used the corresponding clones without infection. Data for the negative control were subtracted from the corresponding data of the apoptotic cells. Results are representative of two independent infections and the experiment was repeated three times. *, P<0.05; **, P<0.01; ***, P<0.001. Regarding the p38α and p38γ isoforms, there are no P-values on day 10 p.i. because the high percentage of dead cells in the control clones made it difficult to obtain enough observations.
Regarding the p38δ isoform (Fig. 4), the clone expressing p38δdn significantly decreased, from the day 7, EGFP expression and annexin V percentage with respect to the control. It is worth noting that, in the clone expressing the p38δdn isoform, EGFP expression and annexin V percentage remained lower than 7 and 15%, respectively, for the 38 day follow-up. This experiment showed that the p38δdn isoform is able to inhibit HIV-1 replication on its own despite the endogenous p38 isoforms.

The greater participation of p38δdn in the inhibition of HIV-1 replication (compared with p38xdn and p38γdn) could be due to the fact that the p38δdn protein expression in the clone is higher than p38xdn and p38γdn protein expression in their corresponding clones (Fig. 6). It is worth noting that all clones tested expressing both p38δ (controls) and p38δdn isoforms under PCMV showed higher protein expression than all clones tested expressing the three other wild-type and dn isoforms under PCMV (data not shown).

Anti-apoptotic effect of the p38αdn, p38γdn and p38δdn isoforms was found in infected and uninfected bystander cells

Due to the fact that apoptosis in HIV-1 infection of CD4+ T cells can occur in uninfected bystander cells in addition to infected cells, we analysed the anti-apoptotic effect of the p38xdn, p38γdn and p38δdn isoforms in infected and bystander cells. Throughout the experiments described in the previous section, EGFP expression, i.e., infection, was different in the control clones expressing the p38α, p38γ and p38δ isoforms under PCMV than in the clones expressing their corresponding dn isoforms (Fig. 4). Therefore, cytometric analysis was carried out to compare the percentage of apoptosis (annexin V-positive cells) in infected (EGFP+ cells) and uninfected bystander cells (EGFP− cells) of the clones expressing the p38dn isoforms compared to their controls (Table 1).

Regarding the p38α isoform, Table 1(a), shows that from day 4, p38xdn significantly decreased apoptosis in EGFP− cells ($P<0.01$–0.001), and to a lesser extent, in EGFP+ cells ($P<0.05–0.01$). With respect to the p38γ isoform, it was observed, that on day 7, the clone expressing the p38δdn isoform presented a significant decrease in apoptosis with respect to the control ($P<0.01$) in both EGFP+ and EGFP− cells (Table 1b).

Finally, we analysed apoptosis related to the p38δ isoform. Table 1(c) shows that the p38δdn isoform significantly decreased apoptosis from day 5 in EGFP+ cells ($P<0.05–0.001$) and from day 7 in EGFP− cells ($P<0.05–0.001$). These results indicate protection from apoptosis in both infected (EGFP+) and uninfected bystander (EGFP−) cells by the p38xdn, p38γdn and p38δdn isoforms.

All results were confirmed by independent statistical analysis of the raw data made with the Prada Bioconductor package.

**DISCUSSION**

The CD4+ T-cell reduction characteristic of HIV-1 infection is thought to result, in addition from infected T-cell death, mainly from uninfected bystander T-cell apoptosis. Nevertheless, the immunological and virological mechanisms leading to T-cell death during HIV-1 infection are not yet fully understood. When cell lines are used, there are discrepancies about the relative importance of direct cytopathicity and apoptosis of uninfected bystander cells (Cao et al., 1996; Carbonari et al., 1997; Herbein et al., 1998a, b; Grivel et al., 2000; Esser et al., 2001; LaBonte et
Table 1. Apoptosis percentage in infected (EGFP+) and uninfected bystander (EGFP−) cells of the following modified SupT1 cell clones infected by HIV-1 viruses

Data for the negative control were subtracted from the corresponding data for the apoptotic cells. Results are mean values obtained from 4 to 6 infections. NEO, Not enough observations.

<table>
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<th>Day</th>
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<th>Apoptosis in EGFP+ (%)</th>
<th>Apoptosis in EGFP− (%)</th>
<th>Significance</th>
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</table>

al., 2003; Holm et al., 2004). These apparent discrepancies may, in fact, reflect differences in the experimental systems used regarding such aspects as T-cell activation level, microenvironment of the cell culture, the affinity between cell receptor and viral envelope, the exposition of the union site to co-receptor and the presence of membrane proteins in the viral surface.

The tumoral line employed in our experiments, SupT1 cell line, is prone to strong activation due to the expression of a wide variety of T antigens: CD1a, CD3, CD4, CD5, CD7, CD8 and CD38. This can promote apoptosis in bystander cells during X4 HIV-1 infection. Moreover, the SupT1 cell line expresses β2-microglobulin, which may be incorporated into the viral envelope and improve cell adhesion.

Our results showed that, in SupT1 cell line infection by HIV-1, death occurred mainly by apoptosis (Fig. 2). In spite of this observation, which is in line with numerous studies reflecting apoptotic changes in cultures infected by HIV-1 (Gandhi et al., 1998; Ferri et al., 2000), opposing opinions exist that defend the predominance of necrotic death over apoptotic death in CD4+ T cell line infection (Cao et al., 1996; Lenardo et al., 2002). Nevertheless, in studies presenting this latter opinion, cytopathicity occurred almost exclusively in infected cells and there was hardly any cytopathic effect in uninfected bystander cells. These apparent contradictions may, once again, be related to specific activation of each cell line, to the affinity of incorporated molecules into the viral envelope with regard to the target cell, or to the apoptotic system of detection used. For example, TUNEL assay would not be the most suitable system to use if independent caspase apoptosis is present because there is no DNA nicking.

With regard to the results presented in this paper, we conclude that in SupT1 cell line, the p38α, p38γ and p38δ isoforms, but not the p38β isoform, are implicated in both HIV-1 replication and apoptosis induced by the virus in infected and uninfected bystander cells. We will go on to propose several mechanisms of p38-isoform participation in SupT1 cell line infection by HIV-1.

In infected and uninfected bystander cells, a possible explanation for the different behaviour of the p38δdn isoform with respect to the other three p38dn isoforms is that signalling may be mediated by MAPK kinase 3. This MAPK kinase only activates p38α, p38γ and p38δ, but not p38β, which we found neither to be implicated in HIV-1 replication nor in HIV-1 apoptosis.

Taking into account previous studies about apoptosis induced by HIV-1 envelope and its relationship with transcription factor p53 and p38 activation (reviewed by...
Castedo et al., 2005; Genini et al., 2001; Perfettini et al., 2004, 2005), our findings suggest that p33 is activated, at least, during apoptosis by the p38z isofrom in uninfected bystander cells. In turn, p33 mediates transcription of proapoptotic proteins like Puma and Bax, and leads to mitochondrial liberation of cytochrome c, caspases activation and apoptosis.

We cannot rule out the participation of p38 in caspase-independent apoptosis of uninfected bystander cells because our experiments were carried out with X4 HIV-1 strains, which leads to caspase-dependent death by interaction with the CD4 receptor, or caspase-independent death by interaction with the CXCR4 co-receptor (Vlahakis et al., 2001). This latter process may involve oncoprotein 18 (Op18), also called stathmin, which was first identified as a p38δ substrate (Parker et al., 1998). In assays with SB203580, it has been suggested that p38z may contribute to Op18 phosphorylation (Mizumura et al., 2006). In terms of p38γ results, no relationship has yet been established between this protein and Op18.

The most notable observation concerning p38 signalling in infected cells is that p38z, p38γ and p38δ may participate in HIV-1 replication through the transcription factor NF-κB, which contributes to cis activation of the HIV-1 LTRs’ promoter (Alcamí et al., 1995; Wu et al., 1997; Asin et al., 2001). Once viral proteins give up protection of infected cells, these p38 isoforms may contribute to cell apoptosis, probably through p53 or Op18. A viral protein that could be implicated in apoptosis of infected cells is Vpr. This protein acts directly in the mitochondria on a molecular complex formed by Bax, and on the adenine nucleotide translocator leading to caspase-independent or caspase-dependent apoptosis (Jacotot et al., 2001; Roumier et al., 2002; Lum et al., 2003; Brenner & Kroemer, 2003). Vpr can activate transcriptional factors dependent on p53 (Chowdhury et al., 2003). In this way, Vpr may cooperate in caspase-dependent apoptosis through p38 and p53 and caspase-independent apoptosis, through p38 and Op18. These possibilities may explain differences in the p38zd, p38yd and p38dn results. In addition, differences between these pathways involving p53 and Op18 and the pathway involving NF-κB may explain why the p38γ isoform exhibits differential activity with respect to HIV-1 replication and apoptosis (Fig. 4).

Finally, recent research has shown that Nef stimulates FasL expression in infected cells through the p38 pathway with participation of the transcription factor AP-1 (Muthumani et al., 2005). At least in macrophages, p38δ and p38δ inhibit transcription that is dependent on AP-1 (Pramanik et al., 2003). The fact that p38β is not implicated in SupT1 cell line apoptosis induced by HIV-1 suggests that the p38z isoform in infected cells contributes to uninfected bystander cell apoptosis by inducing FasL transcription through AP-1.

Understanding the cellular pathways implicated in HIV-1 infection is of great importance for new therapies. It is known that the capacity of HIV-1 to mutate limits the effectiveness of anti-retroviral therapies. A MAPK inhibition strategy has the advantage of limiting the ability of HIV-1 to develop resistance to inhibitor agents, because the target is a cellular molecule and is not virally coded. Our findings suggest that this therapeutic alternative could involve the inhibition of endogenous p38z, p38δ and p38δ isoforms in combination with conventional anti-retroviral HIV-1 agents.

Until it becomes possible to eradicate HIV-1, the search for ways to block its invasion and the destruction it causes of the immunological system is of great importance for prolonging infected patients’ lives.

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