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Mitochondrion-dependent caspase activation by the HIV-1 envelope

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Abstract

Cells expressing the envelope glycoprotein complex (Env) encoded by the human immunodeficiency virus can fuse with cells expressing Env receptors (CD4 and CXCR4). The resulting syncytia undergo apoptosis. We developed a cytofluorometric assay for the quantitation of syncytium formation and syncytial apoptosis. Using this methodology, we show that caspase activation in syncytia is inhibited by pharmacological or genetic intervention on cyclin-dependent kinase-1, p53, and mitochondrial membrane permeabilization (MMP). Thus, transfection of fusing cells with the viral mitochondrial inhibitor of apoptosis encoded by cytomegalovirus, a specific inhibitor of MMP, prevented the mitochondrial cytochrome *c* release and abolished simultaneously the activation of caspase-3. Conversely, inhibition of caspases did not prevent MMP. These results indicate that Env-elicited syncytial apoptosis involves the intrinsic (mitochondrial) pathway.

Keywords: AIDS; Cyclin-dependent kinase-1; Caspases; Human immunodeficiency virus; p53; Programmed cell death

1. Introduction

According to current understanding, at least two major pathways for activating the latent apoptotic machinery exist [1,2]. In the ''extrinsic'' pathway, the occupation of cell death receptors, such as CD95 or the TNF-α receptor, results into the receptor-proximal activation of caspases (mainly caspases-8 and -10) which can activate other caspases, and in particular caspase-3, one of the principal proteases participating in the apoptotic execution [3]. In the ''intrinsic'' pathway, MMP occurs as a primary event. Under normal circumstances, apoptosis is blocked due to the strict compartmentalization of catabolic hydrolases and their activators on both sides of the outer mitochondrial membrane [4]. Thus, for instance, cytochrome *c* is confined to the mitochondrial intermembrane space and therefore cannot bind to Apaf-1, a cytosolic protein. Upon permeabilization or rupture of the outer mitochondrial membrane, cytochrome *c* suddenly interacts with Apaf-1, which becomes an allosteric activator of pro-caspase-9, which in turn proteolytically activates caspase-3 [5].

Infection with the human immunodeficiency virus (HIV-1) is notoriously accompanied by an increase in the apoptotic turnover of $CD4^+$ T lymphocytes, resulting in their progressive depletion [6]. This phenomenon is mostly due to ''bystander killing'', meaning that the large majority of dying cells is not infected by HIV-1. Both the intrinsic and the extrinsic pathways have been accused to participate in the spontaneous or activation-induced apoptosis of $CD4^+$ T lymphocytes from HIV-1 carriers [7–10].

One of the principal apoptogenic products encoded by HIV-1 is the Env [11], which is found on the surface of infected cells, on the surface of viral particles, and as a soluble product in body fluids. Env kills uninfected cells expressing CD4 and/or the chemokine receptor CXCR4 (or CCR5) *via* at least three independent mechanisms. First, the soluble Env product gp120 can induce the

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Abbreviations: Casp-3a, activated caspase-3; Cdk1, cyclin-dependent kinase-1; $\Delta\Psi_m$, mitochondrial transmembrane potential; DAPI, diaminidophenylindol; Env, envelope glycoprotein complex; FSC, forward scatter channel; HIV-1, human immunodeficiency virus; MMP, mitochondrial membrane permeabilization; vMIA, viral mitochondrial inhibitor of apoptosis; Z-VAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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apoptotic cell death of lymphocytes, neurons, and myocardiocytes, *via* interaction with surface receptors, presumably through the elicitation of signals [12–14]. Second, Env present on the surface of HIV-1-infected cells can transiently interact with cells expressing CD4 and CXCR4/CCR5, thereby provoking a hemifusion event which results in the death of the uninfected cell [15–18]. Third, the interaction between Env on infected cells and its receptors on uninfected cells can result into syncytium formation, resulting into the progressive loss of $CD4^+$ T cells [19–25]. Such Env-elicited syncytia undergo apoptosis after a phase of latency [17,26–29].

For obvious reasons, syncytia are oversized and thus are difficult to be analyzed by cytofluorometry, meaning that most studies performed on the apoptotic death of syncytia are based on microscopic examination. We therefore decided to develop a cytofluorometric method for the assessment of syncytium formation and death. Using this method, we then reassessed the question as to whether Env-elicited syncytia die through the extrinsic or intrinsic pathway. As shown here, our data indicate that caspase activation occurs secondary to MMP, indicating that Env-elicited syncytia die through the intrinsic (mitochondrial) pathway of apoptosis.

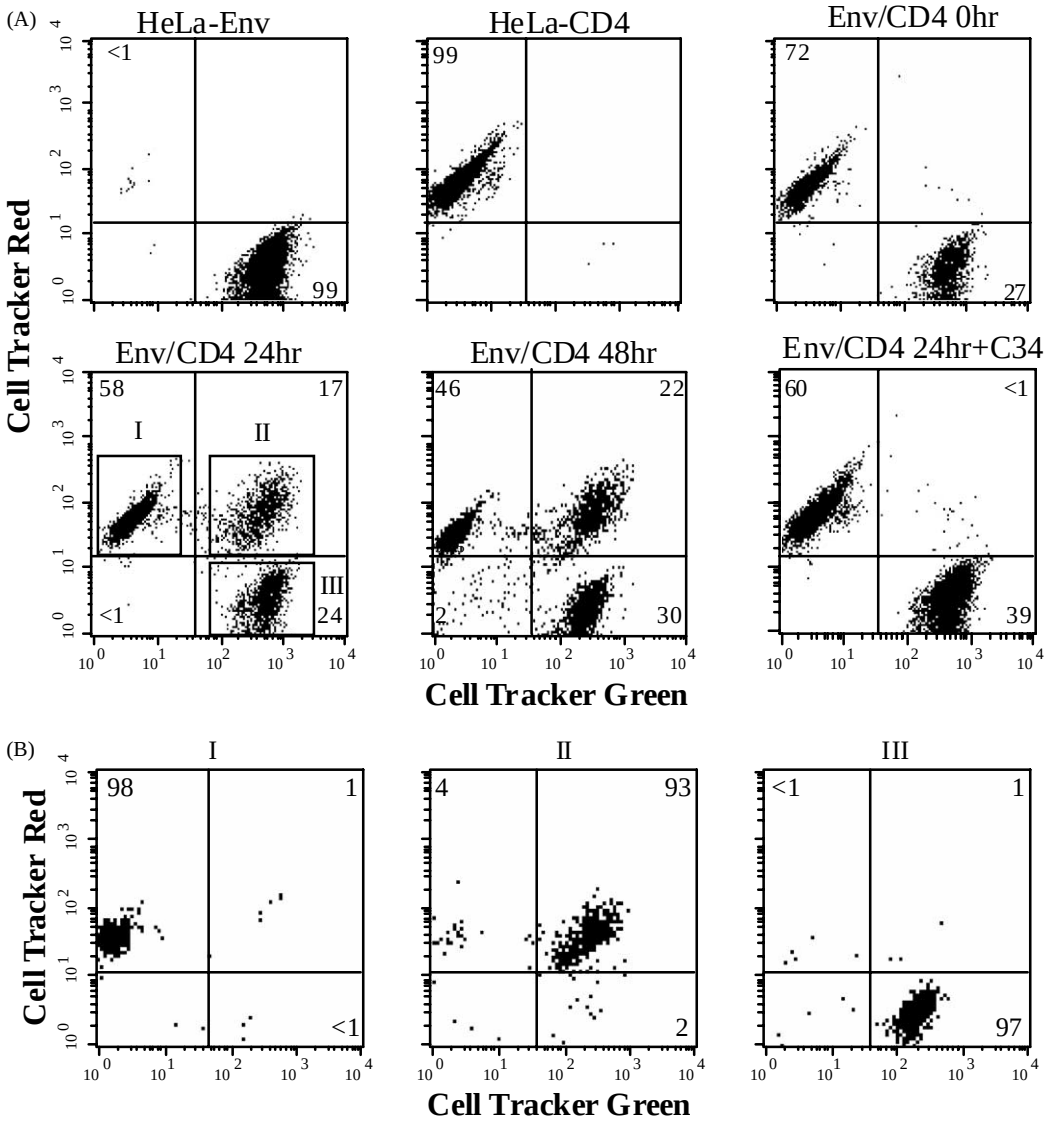

Fig. 1. FACS identification of Env-induced syncytia. (A) FACS-detectable formation of syncytia. HeLa-Env and HeLa-CD4 stained with CellTracker® green or red, respectively, were identified by cytofluorometry. A mixture of HeLa-Env and HeLa-CD4 cells (time 0) did not manifest major signs of cell aggregation, as indicated by the absence of double-positive events. However, upon co-culture for 24 or 48 hr, double-positive cells which are *bona fide* syncytia become detectable. The formation of syncytia was fully suppressed by addition of the fusion inhibitor C34 from the beginning of co-culture. Numbers indicate the percentage of cells in each quadrant. (B) FACS purification of syncytia. The three sub-populations (gates I, II, and III in panel A) corresponding to non-fused HeLa-Env cells (gate I, single positive for CellTracker® green), non-fused HeLa-CD4 cells (gate III, single positive for CellTracker® red) or syncytia (gate II, double positive for CellTracker® green and red) were sorted and then re-analyzed as a control of the reliability of this approach. Note that the overwhelming majority of cells gated on behave as to be expected upon re-analysis.

2. Materials and methods

2.1. Cell lines, culture conditions, and transient transfections

HeLa 243 Env cells stably transfected with a vector containing the *Env* gene of HIV-1 LAI as well as Tat were cultured in complete culture medium (DMEM supplemented with 2 mM glutamine, 10% FCS, 1 mM pyruvate, 10 mM HEPES, and 100 units/mL penicillin/streptomycin) containing 2 mM methotrexate. HeLa cells stably transfected with CD4 as well as the LacZ gene under the control of the HIV-1 long terminal repeat (LTR) (HeLa-CD4) were selected in medium containing 500 mg/mL G418. HeLa-Env and HeLa-CD4 cells were cultured alone or together (1:1 ratio) for 24, 48, or 72 hr to allow for syncytium formation. In some experiments, cells were cultured in the presence of cyclin-dependent kinase-1 (Cdk1) inhibitor roscovitin (1 μM; Calbiochem), the p53 inhibitor cyclic pifithrin α (10 μM, Calbiochem) or the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) (100 μM; Enzyme Systems). Tranfections were performed with lipofectamine, with pcDNA3.1 vector only, or vectors containing a dominant-negative p53 mutant (H175) (kind gift of T. Soussi), p35 (kind gift of Guy Salvesen), or viral mitochondrial inhibitor of apoptosis (vMIA) (kind gift of Victor Goldmacher).

2.2. Generation and evaluation of Env-dependent syncytia

Prior to co-culture, HeLa-Env cells were pre-stained with 5 μM 5-chloromethylfluorescein diacetate (CellTracker® green) and HeLa-CD4 cells pre-stained with 5 μM 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CellTracker® red), both from Molecular Probes. Preliminary experiments showed that neither of these dyes had toxic effects on HeLa cells during a 72-hr culture period. After 24, 48, or 72 hr of co-culture, cells were harvested and stained with Hoechst 33342 for cell cycle analysis (10 μg/mL, 20 min), diaminidophenylindol (DAPI) for viability analysis (5 μg/mL, 15 min), and subjected to cytofluorometric analysis on an FACS Vantage (Becton Dickinson) equipped with a 100 μm nozzle, allowing for the analysis of relatively large cells. Alternatively, syncytia were fixed with paraformaldehyde (4% w/v) and picric acid (0.19% v/v in PBS), then stained with a polyclonal rabbit antiserum specific for cleaved caspase-3 antibody (pAb 9661 from Cell Signaling, detected by a goat anti-rabbit IgG conjugated with Alexa® fluor) or a monoclonal antibody specific for cytochrome *c* (mAb 6H2.B4 from Pharmingen, detected by a goat anti-mouse IgG conjugated with Alexa® fluor) and subjected to FACS analysis and/or fluorescence microscopic examination [30,31].

2.3. Determination of β-galactosidase activity

For the determination of β-galactosidase activity *in situ*, cells were fixed with a mixture of formaldehyde (0.37%) and glutaraldehyde (0.2%) in PBS solution for 5 min, then treated overnight with buffer containing 200 mM potassium ferrocyanide, 1 M $MgCl_2$, and 50 mg/mL X-Gal (ProMega), and examined by contrast phase microscopy. Alternatively, FACS-sorted cells were lysed and subjected to the determination of β-galactosidase activity using the Enhanced β-Galactosidase assay kit (CPRG), from Gene Therapy Systems, as well an MRX II microplate reader from Dynex Technologies.

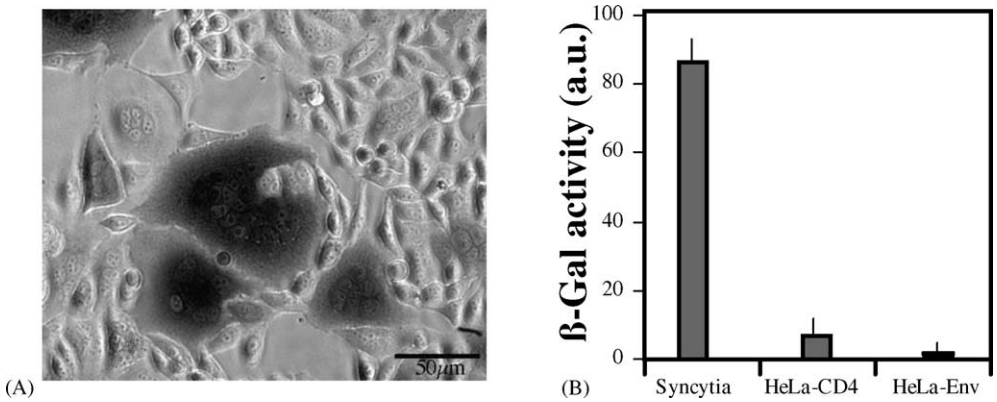

Fig. 2. Transcomplementation between a transactivator (Tat) and a promoter (LTR upstream of the β-Gal gene) in syncytia defined with CellTrackers. (A) β-Galactosidase transactivation by LTR as a consequence of Env-induced syncytia formation. HeLa-Env cells expressing Tat and HeLa-CD4 cells transfected with a β-Gal gene under the LTR promoter were co-cultured, followed by an *in situ* histochemical detection of β-Gal using X-Gal as a substrate. Note that only multinucleated cells express β-Gal. (B) Transcomplementation is restricted to *bona fide* syncytia. Sorted sub-populations of co-cultured (24 hr) cells, identified by prior staining of HeLa-Env and HeLa-CD4 with CellTracker® green or red, respectively, followed by FACS purification of single cells or syncytia (as in Fig. 1) were subjected to the determination of β-galactosidase activity. Results ($X \pm$ SD of triplicates) are representative of three independent experiments.

3. Results and discussion

3.1. A cytofluorometric assay for the assessment of syncytium formation

HeLa cells stably transfected with a lymphotropic HIV-1 *Env* gene (HeLa-Env) were fused by co-culture with CD4/CXCR4-expressing HeLa cells (HeLa-CD4). We chose this model of fusion-induced cell death because it furnished valuable information on the pathway of Env-elicited syncytia apoptosis [17,26–29]. At difference with other models of cell death induced by fusogenic viral proteins [32], Env-elicited syncyta undergo apoptosis with caspase activation [17,26–29]. Fusion events, which depend on the Env/CD4 interaction and can be blocked with C34 [18], were monitored by means of two stable, non-toxic

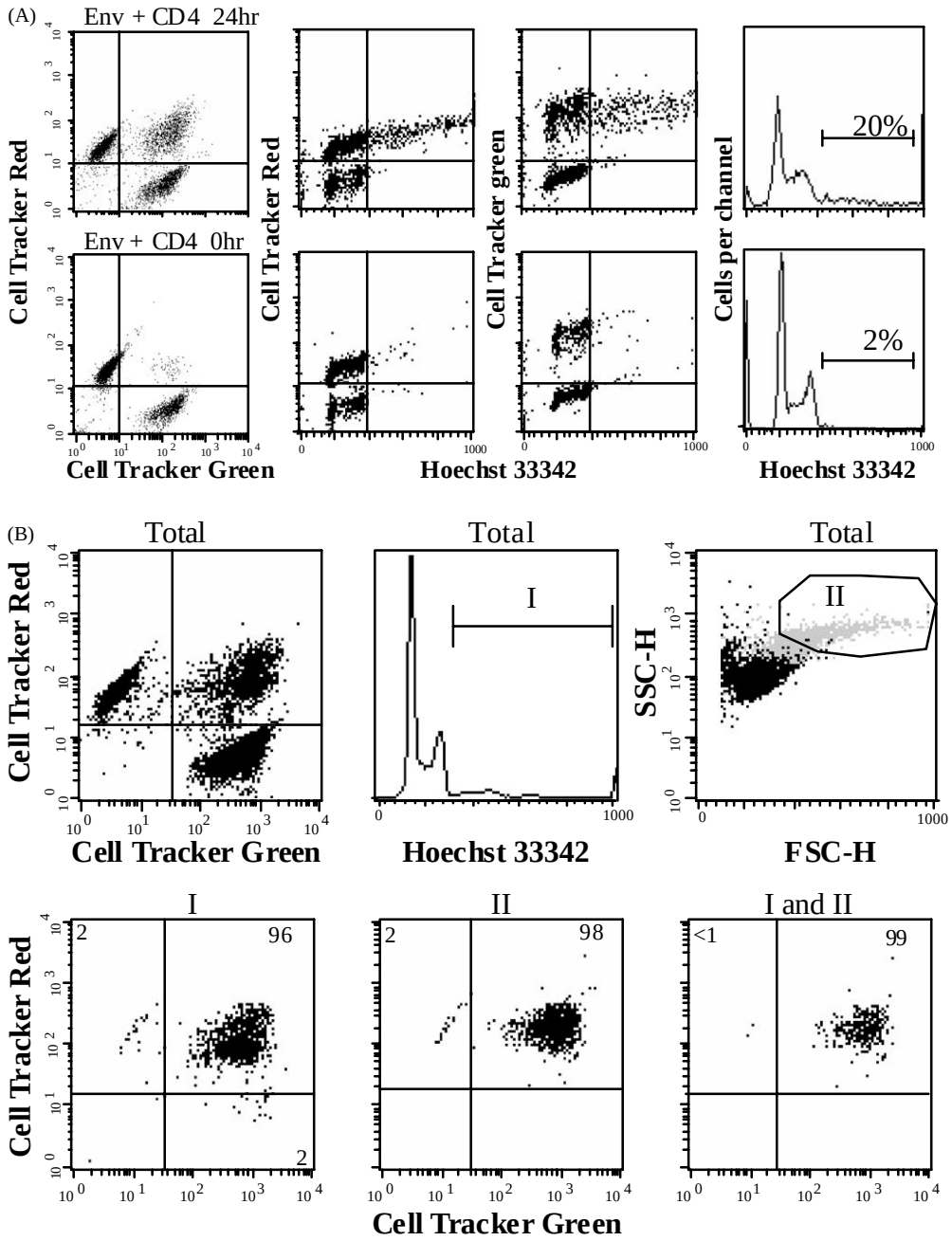

Fig. 3. Hyperploidy and cell size as surrogate markers of syncytia. (A) After co-culture of CellTracker® pre-stained cells (HeLa-Env in green and HeLa-CD4 in red), the entire population of co-cultured cells (0, 24 hr) was stained with Hoechst 33342 prior to FACS analysis. Note that only after 24 hr of co-culture, cells exhibiting a ploidy >4N become detectable. Such cells are positive for both CellTrackers. (B) Both hyperploidy (gate I) and forward scatter characteristics (gate II) allow for the definition of syncytia. When the entire population was subjected to monoparametric analyses for the determination of the chromatin/DNA content (after Hoechst 33342 staining) or cell size (FCS), polyploid (gate I) or oversized cells (gate II) turn out to be mostly double positive for the two CellTrackers and thus are *bona fide* syncytia. Simultaneous gating on both polyploid and oversized cells (I and II) does not provide a substantial advantage for the definition of syncytia.

CellTracker® fluorescent dyes with which HeLa-Env (CellTracker® green), or HeLa-CD4 (CellTracker® red) cells were pre-incubated. The co-culture of HeLa-Env and HeLa-CD4 cells for 24 or 48 hr led to the formation of double-positive (red/green) cells, which was blocked by C34 (Fig. 1A). Such double-positive cells could be sorted in the FACS and turned out to be stable among re-analysis (Fig. 1B). To further substantiate that the double-positive population is actually constituted by syncytia (rather than by cellular aggregates), we assessed the functional

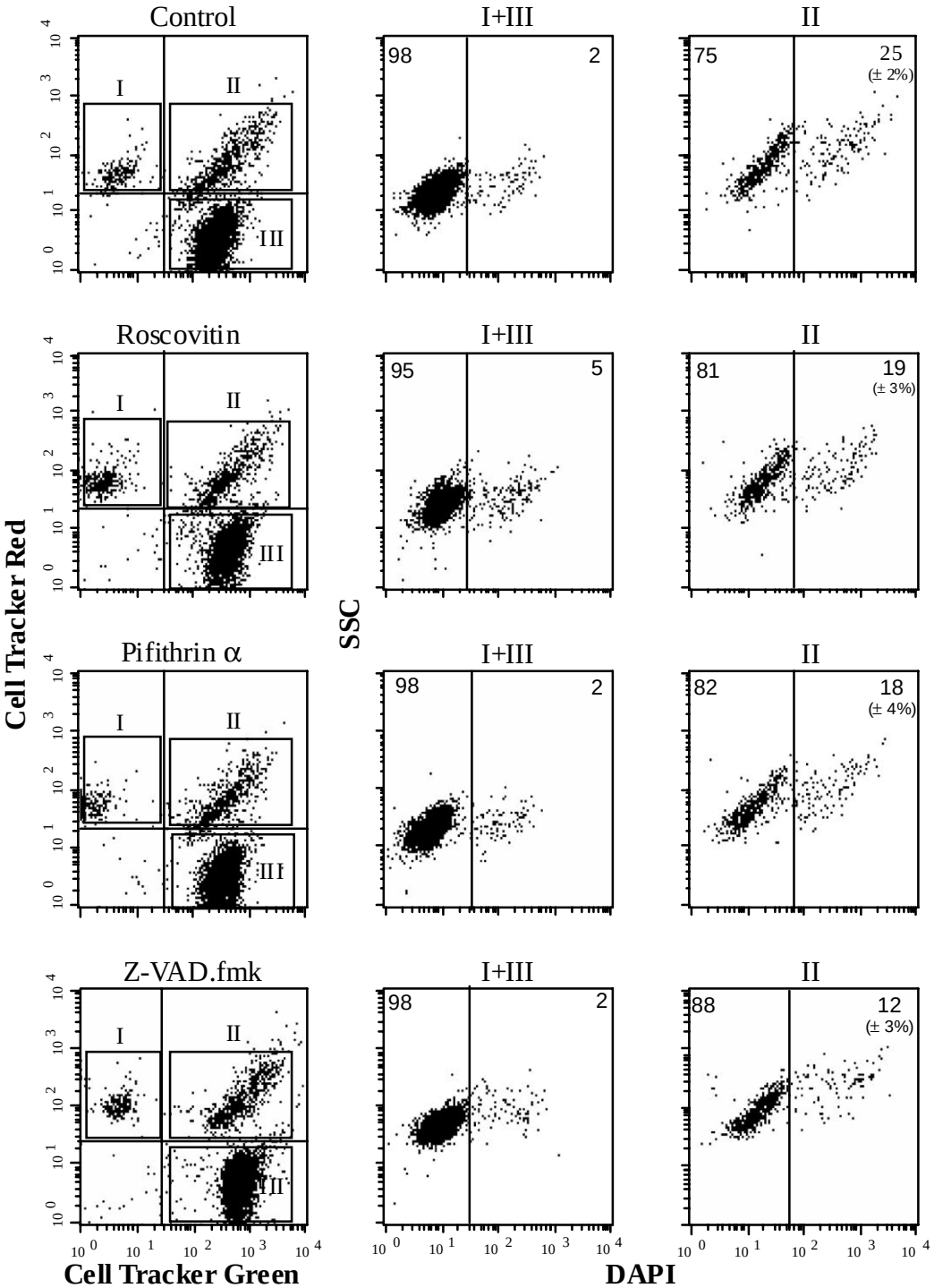

Fig. 4. DAPI staining for the determination of syncytial viability. After co-culture of CellTracker® pre-stained cells (HeLa-Env in green and HeLa-CD4 in red) for 48 hr, cells were harvested and DAPI (blue fluorescence) was added to the medium. This molecule only enters permeabilized dead cells. Gates were set on single cells (I + III) and syncytia (II). Note that single cell viability was near-to-complete, while a fraction of syncytia were dying. Numbers are mean percentage values ± SEM (N = 3) in each gate. Roscovitin, cyclic pifithrin α, and the pan-caspase inhibitor Z-VAD.fmk, significantly (paired Student's *t* test, $P < 0.01$) improved syncytia survival when added from the beginning of co-cultures.

transcomplementation between nuclei from the two cell types [33]. The HeLa-CD4 cell line used was stably transfected with the β-galactosidase gene (β-Gal), under the control of the promoter of the HIV-1 LTR, while the HeLa-Env cell line was transfected with the HIV-1 Tat gene. In such a system, only syncytia (but not single cells) generated by co-incubation of the two cell lines expressed the β-Gal gene as a result of the Tat-mediated transactivation of LTR [33] (Fig. 2A). Upon FACS-mediated purification of single-positive or double-positive cells (as in Fig. 1B), only *bona fide* syncytia (but not single cells) were found to express β-Gal (Fig. 2B), indicating functional transcomplementation between the nuclei from both cell lines. Accordingly, syncytia were found to be polyploid upon staining with the chromatin dye Hoechst 33342, with a DNA content >4N (Fig. 3A). In subsequent experiments (Fig. 3B), we independently determined the formation of syncytia (using the CellTracker® green/red method), the production of polyploid cells (with Hoechst 33342) and the size of cells (by means of the forward scatter channel, FSC). Gating on the polyploid and oversized population, independently (I, II) or simultaneously (I and II in Fig. 3B) revealed that these parameters could be employed as surrogate markers with a >95% confidence interval for the cytofluorometric identification of syncytia.

3.2. Determination of cell death and apoptosis of Env-elicited syncytia

In contrast to Hoechst 33342 (which readily penetrates the plasma membrane), DAPI can be used as a vital stain, which only incorporates into dead cells. As shown in Fig. 4, syncytia (but not single cells), as identified with the CellTracker® green/red method, die upon prolonged co-culture (48 hr). Thus, in contrast to other cell types (e.g. U937 cells) [18], HeLa-CD4 cells do not die as single cells upon transient exposure to Env-expressing cells. Incubation of these cells with inhibitors of Cdk1 (roscovitin), the transcriptional activity of p53 (cyclic pifithrin α), or caspases (Z-VAD.fmk), significantly reduced the percentage of dying syncytia, thus confirming our previous finding that these inhibitors can enhance the survival of syncytia, as assessed by microscopic (rather than cytofluorometric) observation [28,29]. In a further series of experiments, we co-cultured unlabeled HeLa-Env and HeLa-CD4 cells, then stained them for the detection of activated caspase-3 (Casp-3a) and identified syncytia upon Hoechst 33342 counterstaining (as in Fig. 3), based on the criterion of hyperploidy (Fig. 5A). Upon prolonged incubation (48 hr), syncytia (but not single cells) and an increasing percentage of syncytia progressively exhibited caspase-3 activation. This capase-3 activation was partially inhibited by roscovitin, cyclic pifithrin α, and Z-VAD.fmk (Fig. 5B). Similar results were obtained, when cells were transfected with a dominant-negative p53 mutant or the baculovirus-encoded caspase inhibitor p35 (Fig. 5B), meaning that the caspase activation is, at least in part, secondary to the activation of Cdk1 and p53.

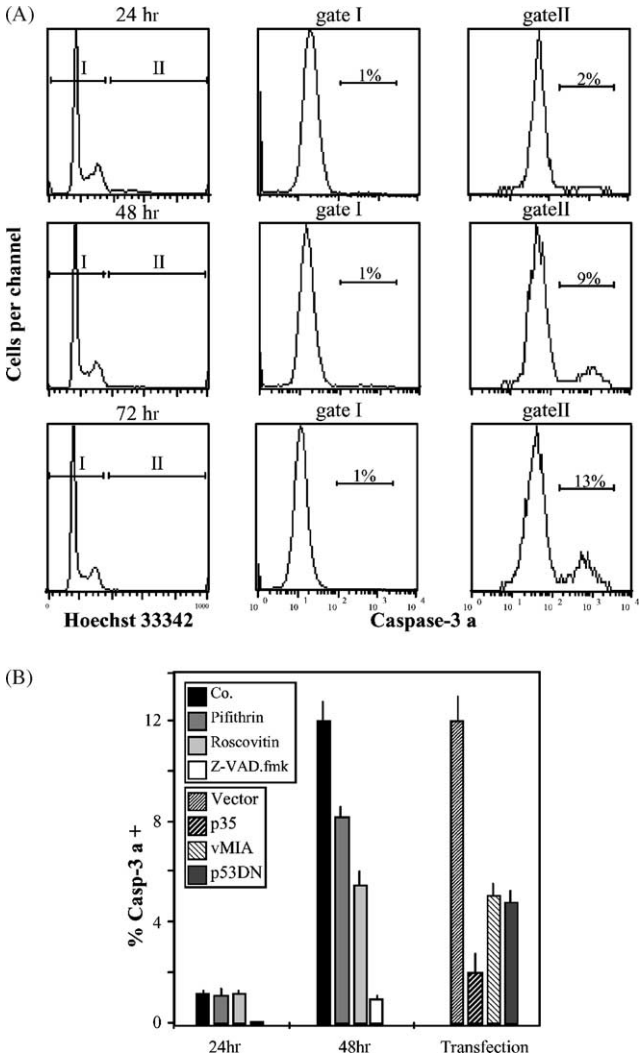

Fig. 5. FACS analysis of caspase-3 activation among syncytia. (A) Detection of Casp-3a in syncytia. HeLa-Env and HeLa-CD4 co-cultures were harvested, fixed, and permeabilized after 24, 48, or 72 hr of co-culture. Then, cells were stained for the detection of Casp-3a using an immunofluorescence technique and counterstained with Hoechst 33342 staining. This letter staining allowed for the discrimination between unfused cells (ploidy 4N) and Env-induced syncytia ploidy >4N, as shown in Fig. 3. Gates were set on single cells (gate I) and syncytia (gate II), and the green fluorescence corresponding to Casp-3a was plotted. Note that Casp-3a-positive cells only are found among syncytia and that they accumulate in a progressive fashion. (B) Pharmacological or genetic inhibition of caspase-3 activation in syncytia. Syncytia were left untreated (control) or were treated with cyclic pifithrin α, roscovitin, or Z-VAD.fmk, throughout the period of co-culture (24 or 48 hr), followed by determination of the frequency of Casp-3a-positive cells as in panel A. Alternatively, both fusion partners were transfected 24 hr prior to the initiation of co-culture (48 hr) with vector only, p35, vMIA, or the dominant-negative (DN) p53 mutant H175, followed by determination of the frequency of caspase-3-positive cells after co-culture (72 hr). The transfection efficiency was 80% and data are expressed as mean percentage values ± SEM (N = 3). The effects of cyclic pifithrin α, roscovitin, or Z-VAD.fmk, p53, vMIA, and p53 H175 were significant (paired Student's *t* test, $P < 0.01$) as compared to untreated or vector-only transfected controls.

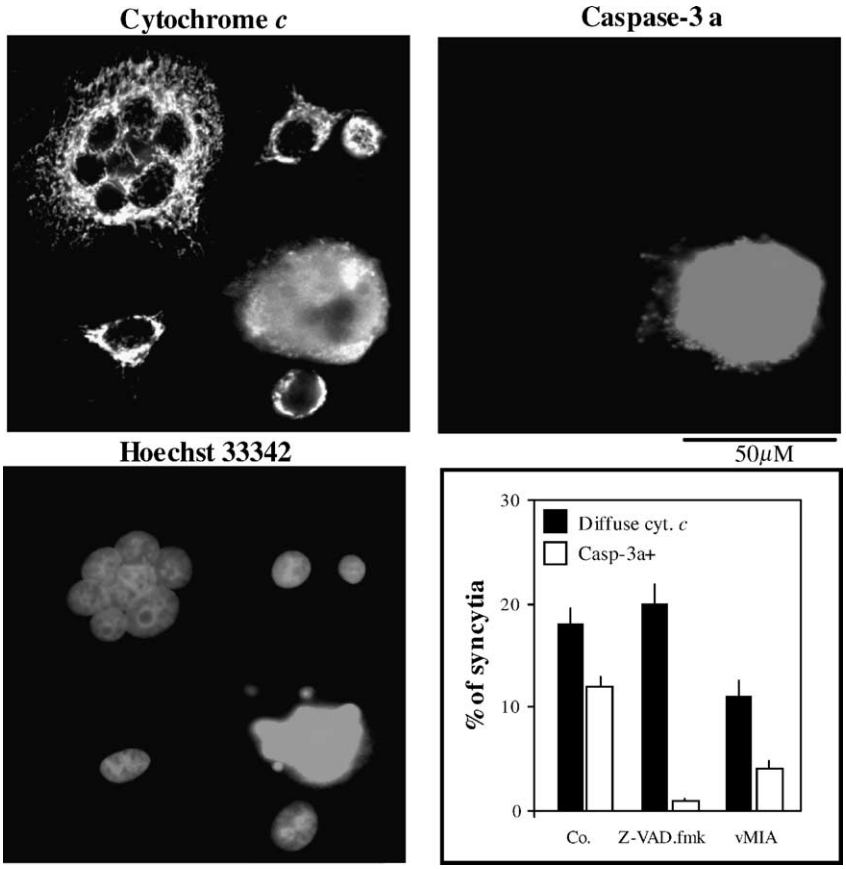

Fig. 6. Relationship between mitochondrial cytochrome *c* release and caspase-3 activation in syncytia. HeLa-Env and HeLa-CD4 were cultured together (48 hr) on a cover slip, fixed, permeabilized, stained for the immunofluorescence detection of cytochrome *c* and Casp-3a, and counterstained with Hoechst 33342. Quantitation of the positive Casp-3a staining among cells exhibiting a mitochondrial (punctate cytoplasmic) or diffuse cytochrome *c* staining pattern was performed by fluorescence microscopy. A representative microphotograph of untreated syncytia is shown. All cells that are positive for Casp-3a manifest a diffuse cytochrome *c* staining. In addition, the percentage of cells exhibiting diffuse cytochrome *c* staining or positive Casp-3a staining was determined among untreated cells or cells which had been transfected with vMIA 24 hr before co-culture or had been incubated with Z-VAD.fmk during the period of co-culture. Note that Z-VAD.fmk fully abolishes caspase-3 activation while having no effect on the relocalization of cytochrome *c*. In contrast, vMIA inhibits both the release of cytochrome *c* and the activation of caspase-3.

3.3. Caspase activation downstream of mitochondrial cytochrome c release

To further assess the mechanism of caspase activation in syncytia, we performed double staining experiments in which the cells were stained for both cytochrome *c* and Casp-3a. As shown in Fig. 6, caspase-3 activation was only found in syncytia in which cytochrome *c* had been released from mitochondria and thus exhibited a diffuse staining pattern throughout the cell. Inhibition of caspase-3 by Z-VAD.fmk or p35 had no effect on the release of cytochrome *c*, although Z-VAD.fmk completely inhibited caspase-3 activation as an internal control of its efficacy. This result, suggested that the event causing cytochrome *c* release, MMP, would occur in a caspase-independent fashion. To further substantiate the molecular order between MMP and caspase activation, we took advantage of a specific inhibitor of MMP, namely the vMIA encoded by the cytomegalovirus UL37 gene [34]. At difference with Bcl-2, vMIA is strictly confined to mitochondria [35,36], where it locally inhibits MMP [34,37]. While reducing the release of cytochrome *c*, vMIA also inhibited the activation of caspase-3 (Fig. 6). This result places the activation of caspase-3 downstream of mitochondrial cytochrome *c* release.

3.4. Concluding remarks

Based on microscopic observations, we reported in the past that, in Env-elicited syncytia, signs of MMP (such as the loss of the mitochondrial transmembrane potential, $\Delta\Psi_m$) occurred upstream of late (presumably caspase-dependent) signs of apoptosis, such as chromatin condensation [17,26–29]. Here, we employed a cytofluorometric method for the unequivocal quantitation of syncytial cell death, namely by defining syncytia using a variety of criteria (double positivity for stable markers of the two individual cells used for fusion, hyperploidy determined with the chromatin dye Hoechst 33342, oversize detectable as a strong FSC signal) as well as by defining apoptosis (expression of the neo-epitope created by proteolytic activation of caspase-3, loss of viability). Using this methodology,

as well as specific interventions on caspase activation and MMP, we tentatively determined the molecular order between caspase activation and MMP. Whereas, caspase activation is not required for MMP (as measured by the assessment of cytochrome *c* release from mitochondria), the vMIA-inhibitable MMP appears to be required for caspase activation. Accordingly, the activation of caspase-3 could only be detected in cells that exhibited a diffuse cytochrome *c* staining pattern indicative of MMP. These experiments thus underscore that, at least in this particular *in vitro* model, Env-induced apoptosis follows the intrinsic (mitochondrial) pathway. Activation of MMP (and caspase activation) appears to be, at least partially, under the control of Cdk1 and p53, as suggested by inhibitory experiments. Importantly, it appears that caspase inhibition also fails to prevent MMP of HIV-1-infected lymphocytes *in vitro* [38], suggesting that the robust *in vitro* model of Env-induced HeLa cell apoptosis may reflect important facets of HIV-1-induced T cell death. Using a semi-automatic read-out for the assessment of apoptotic parameters in such a model may provide invaluable insights into the pathophysiology of AIDS.

Acknowledgments

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