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Intercellular adhesion molecules (ICAM)-1 ICAM-2 and ICAM-3 function as counter-receptors for lymphocyte function-associated molecule 1 in human immunodeficiency virus-mediated syncytia formation*

It has been previously demonstrated that lymphocyte function-associated molecule 1 (LFA-1) plays a major role in human immunodeficiency virus (HIV)-mediated syncytia formation. In the present study we investigated the involvement of intercellular adhesion molecule-1 (ICAM-1), ICAM-2 and ICAM-3 in the process. The ability of monoclonal antibodies (mAb) directed against ICAM-1, ICAM-2 and ICAM-3 to block syncytia was analyzed either in phytohemagglutinin (PHA)-activated lymphocytes infected *in vitro* with primary or laboratory strains of HIV or by coculturing a T cell line stably expressing HIV envelope with PHA-activated lymphocytes. Complete inhibition of syncytia formation was observed only by the simultaneous addition to the cell cultures of all (*i.e.* anti-ICAM-1, anti-ICAM-2 and anti-ICAM-3) mAb. These results indicate that the interaction between LFA-1 and ICAM is a critical step in HIV-mediated syncytia formation, and that ICAM-1, ICAM-2 and ICAM-3 are the receptor molecules for the LFA-1-dependent syncytia formation.

1 Introduction

Syncytia formation is a major mechanism responsible for the depletion of $CD4^+$ T lymphocytes in cultures of mononuclear cells acutely infected *in vitro* with human immunodeficiency virus type 1 (HIV-1) or HIV-2 [1–4]. Formation of syncytia has not been observed *in vivo* in humans. However, recent studies in primates have demonstrated extensive formation of syncytia in the lymphoid organs during acute infection [5]. Furthermore, in HIV-infected individuals the emergence of viral isolates that have a greater capacity to induce syncytia formation *in vitro* has been correlated with rapid progression of the disease [6, 7]. The specific interaction of the HIV envelope protein expressed on the surface of an infected cell with the CD4 molecule on the surface of another cell is a necessary step in the formation of HIV-mediated syncytia [8–12]. Moreover, it has been recently demonstrated that integrin lymphocyte function-associated molecule 1 (LFA-1) is a critical molecule involved in the formation of HIV-1 and HIV-2-mediated syncytia [13–15]. The importance of LFA-1 in this process was initially suggested by the observation that monoclonal antibodies (mAb) directed against the α or β chains of LFA-1 strongly inhibited syncytia formation [13, 14]. Direct proof of the regulatory role of LFA-1 in HIV-mediated cell fusion and syncytia formation was obtained by using leukocytes genetically deficient in LFA-1 expression as target cells for HIV infection [15]. In this latter study, LFA-1-negative $CD4^+$ lymphocytes were unable to fuse and to form syncytia when infected with HIV-1 or HIV-2 [15].

Three counter-receptors have been reported for LFA-1: intercellular adhesion molecule-1 (ICAM-1) [16, 17], ICAM-2 [18–20] and ICAM-3 [21–26]. All three molecules are members of the immunoglobulin gene superfamily. Their molecular, biochemical and functional characteristics have been described previously [27]. Although it has recently been shown that anti-ICAM-1 mAb partially inhibited HIV-induced syncytia formation [28], the role of interactions between LFA-1 and the ICAM in the process of LFA-1-dependent syncytia formation in HIV-infected $CD4^+$ lymphocytes has not been fully delineated.

In the present study we investigated the requirement of ICAM-1, ICAM-2 and ICAM-3 in HIV-1 or HIV-2-mediated syncytia formation, and we determined whether mAb directed against ICAM-1, ICAM-2 and ICAM-3, used alone or in combination, mimicked the inhibitory effect on syncytia formation obtained by blocking the interactions between LFA-1 and the ICAM with anti-LFA-1 mAb. We demonstrated that anti-ICAM-1, anti-ICAM-2 and anti-

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Abbreviations: **LFA-1:** Lymphocyte function-associated molecule 1 **HIV:** Human immunodeficiency virus **ICAM-1:** Intercellular adhesion molecule-1

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ICAM-3 mAb used in combination completely suppressed syncytia formation. These findings indicate that the process of HIV-mediated syncytia formation is dependent on the LFA-1/ICAM-1-2-3 pathway of cell adhesion and that ICAM-1, ICAM-2 and ICAM-3 function as counter-receptors for LFA-1 in this process.

2 Materials and methods

2.1 mAb

FITC-conjugated Leu3a+b mAb (anti-CD4) was purchased from Beckton Dickinson Immunocytometry Systems (San Jose, CA). The hybridoma cell lines for TS1/22 (anti-LFA-1α, IgG1) [29] and TS1/18 (anti-LFA-1β, IgG1) [29] mAb were purchased from ATCC (Rockville, MD). RR1/1 (anti-ICAM-1, IgG1) [16], CBR-IC2/1 (anti-ICAM-2, IgG2a) and CBR-IC2/2 (anti-ICAM-2, IgG2a) [19] mAb were previously described. CBR-IC3/1 (IgG1) [21] and CBR-IC3/2 (IgG2a) [22] mAb, directed against two different epitopes of ICAM-3, have been recently characterized and were used in combination in the experiments performed with anti-ICAM-3 mAb. In all the experiments TS1/18 and TS1/22 mAb were used at 1/4 dilutions of hybridoma cell culture supernatant; RR1/1, CBR-IC2/1, CBR-IC2/2, CBR-IC3/1 and CBR-IC3/2 (purified mAb) were used at 10 μg/ml.

2.2 Isolation of lymphoid cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient over Lymphocyte Separation Medium (Organon Teknika, Durham, NC) from leukapheresis preparations obtained from HIV-seronegative normal donors.

2.3 Cell cultures and cocultures

PBMC were stimulated with PHA (2 μg/ml) (Sigma Chemical Co., St. Louis, MO) for 72 h in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% FCS (Flow Laboratories, Mc Lean, VA). After stimulation the cells were washed twice, resuspended in tissue culture medium containing rIL-2 (50 U/ml) (Cetus, Emeryville, CA), plated (6×10^5 cells/ml) in 48-well plates (Costar, Cambridge, MA) and incubated with the appropriate mAb for 30 min at room temperature. After incubation the cell cultures were inoculated either with HIV-1_{MN} or HIV-2_{ROD} (10^{-3} final dilution of purified pelleted virus preparations purchased from Advanced Biotechnology Inc., Columbia, MD) and cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures were performed in triplicate and monitored daily for syncytia formation. Every 2 to 3 days 100 μl of culture supernatant was removed and stored at −70 °C until assayed for reverse transcriptase (RT) activity. In addition, 200 μl of cell suspension was collected and analyzed by cytofluorometry for the presence of $CD4^+$ cells. Fresh medium containing rIL-2 with or without the appropriate mAb was added to the cultures. In another set of experiments, PHA-activated PBMC from normal donors were resuspended in tissue culture medium containing rIL-2 (50 U/ml), plated in 48-well plates (6×10^5), inoculated with primary isolates (# 11911) of HIV-1 (10^{-1} final dilution of plasma) and cultured in the presence or absence of either anti-LFA-1 α+β or anti-ICAM-1+2+3 mAb. Cell cultures were monitored daily for syncytia formation and culture supernatants were collected every 2 to 3 days for RT activity assay. In co-culture experiments, a transfected T cell line (Jurkat) stably expressing gp120 and gp41 of HIV-1_{IIIB} (HIVenv$_{(2-8)}$, [30]) and PHA-activated T lymphocytes from HIV-negative donors were used. Equal numbers of each cell population (2×10^5) were resuspended in RPMI supplemented with 10% FCS and incubated separately for 30′ at 4 °C with the appropriate mAb. After incubation cells were mixed and cocultured in 48-well plates at 37 °C in humidified atmosphere containing 5% CO_2. Co-cultures were performed in duplicate and syncytia formation was assessed in 10 different microscopic fields per well after 20–24 h.

2.4 Assay for RT activity

Cell cultures were monitored for viral production by assay of culture supernatant for magnesium-dependent RT activity as previously described [31].

2.5 Flow cytofluorometric analysis and calculation of $CD4^+$ cell viability

Analysis of $CD4^+$ cells in uninfected versus HIV-1-infected cell cultures was performed on an EPICS Profile (Coulter, Hialeah, FL) as previously described [32]. Control samples were stained with FITC-conjugated goat anti-mouse IgG1 or IgG2. Ten thousand events were recorded. The $CD4^+$ T lymphocyte viability in the cultures was calculated for each time point and for each experimental condition by the formula:

$$\frac{\%\ \text{of CD4}^+\ \text{cells in the infected culture}}{\%\ \text{of CD4}^+\ \text{cells in the uninfected culture}} \times 100$$

3 Results and discussion

The role of the interaction between LFA-1 and ICAM-1-2-3 in HIV-1 or HIV-2-mediated syncytia formation was determined in two different experimental systems. The effect of mAb directed against LFA-1, ICAM-1, ICAM-2 and ICAM-3 on HIV-1-mediated syncytia formation was examined by co-culturing a transfected T cell line stably expressing HIV env [HIVenv$_{(2-8)}$] with PHA-activated PBMC obtained from normal HIV-negative donors in the absence or presence of the individual mAb either alone or in various combinations. Transfected HIVenv$_{(2-8)}$ cells have complete down-regulation of surface CD4 expression and do not fuse spontaneously in culture [30]. However, HIVenv$_{(2-8)}$ cells can be induced to fuse when co-cultured with $CD4^+$ cells [30]. Extensive syncytia formation was observed by co-culturing HIVenv$_{(2-8)}$ cells with PHA-activated PBMC (Fig. 1). Syncytia formation was strongly inhibited in the presence of mAb against the α or the β chains of LFA-1. Rare and isolated syncytia were observed in the co-cultures treated with either anti-LFA-1α or anti-LFA-1β used alone, whereas inhibition of syncytia formation was complete

when the two mAb were used in combination (Fig. 1). These results confirm previous studies demonstrating the primary role of the LFA-1 molecule in the process of HIV-mediated cell fusion [15]. In the same co-culture experiments, syncytia formation was partially prevented by anti-ICAM-1 mAb alone, but was totally unaffected by either anti-ICAM-2 or anti-ICAM-3 mAb alone. A significant increase in the inhibition of syncytia was obtained by combining either anti-ICAM-2 or anti-ICAM-3 with anti-ICAM-1, whereas the combination of anti-ICAM-2 and anti-ICAM-3 showedd no inhibitory effect (Fig. 1). Complete inhibition of syncytia formation was observed only when cells were simultaneously treated with anti-ICAM-1, anti-ICAM-2 and anti-ICAM-3 mAb (Fig. 1). These results were confirmed in five separate experiments (Fig. 2).

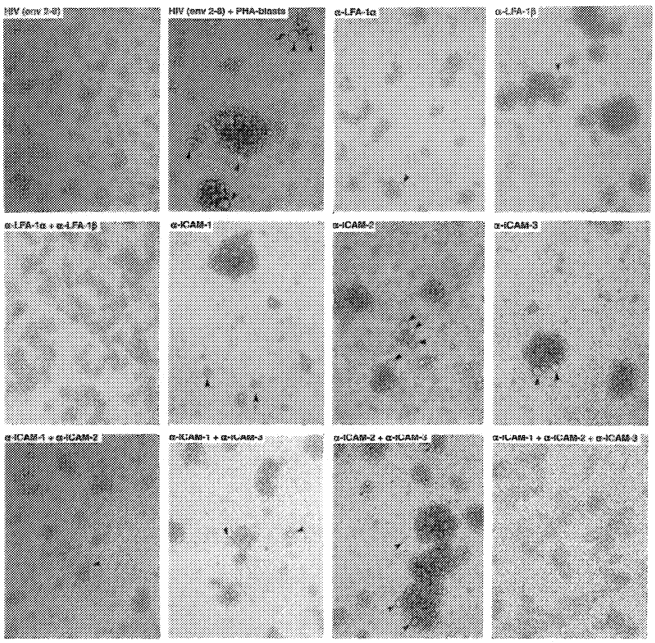

Figure 1. Effect of anti-LFA-1, anti-ICAM-1, anti-ICAM-2 and anti-ICAM-3 mAb on HIV-1-mediated syncytia formation. HIVenv$_{(2-8)}$ cell line was co-cultured with PHA-activated PBMC in the presence or absence of the individual mAb used alone or in various combinations. Co-cultures were performed in duplicate as described in Sect. 2.3. Photomicrographs (× 100) were taken 20–24 h after co-culture.

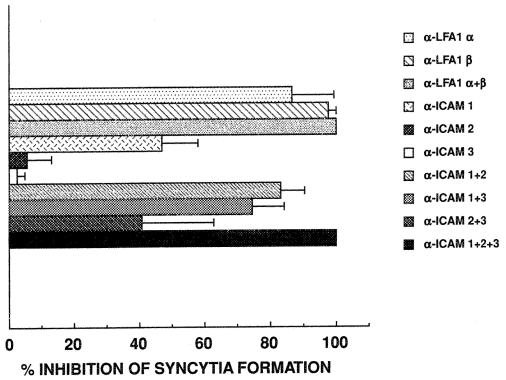

Figure 2. Effect of anti-LFA-1, anti-ICAM-1, anti-ICAM-2 and anti-ICAM-3 mAb on HIV-1-mediated syncytia formation in co-cultures of HIVenv$_{(2-8)}$ cells with PHA-activated PBMC. Co-cultures were performed in duplicate and ten different microscopic fields per well were scored for syncytia formation after 20–24 h. Results (mean ± SD) of five separate experiments are shown.

The effect of mAb directed against LFA-1, ICAM-1, ICAM-2 and ICAM-3 on HIV-2-mediated syncytia formation was then determined. PHA-activated PBMC obtained from normal donors were inoculated *in vitro* with HIV-2$_{ROD}$, an HIV strain that induces syncytia very efficiently in cell cultures. Extensive syncytia formation was observed by day 4 in untreated cultures (Fig. 3). Syncytia were rarely observed in the cultures treated with anti-LFA-1 α or anti-LFA-1 β mAb and were completely inhibited when the combination of the two anti-LFA-1 mAb was used (Fig. 3). Anti-ICAM-1, anti-ICAM-2 or anti-ICAM-3 mAb alone had no detectable effect on syncytia formation. Partial inhibition was obtained by combining anti-ICAM-1 with either anti-ICAM-2 or anti-ICAM-3, and a similar degree of inhibition was observed with the simultaneous addition of anti-ICAM-2 and anti-ICAM-3 mAb (Fig. 3). In contrast, syncytia formation was never detected in cultures treated with mAb directed against ICAM-1, -2 and -3 molecules added together in culture (Fig. 3). The degree of inhibition observed with the different combinations of anti-LFA-1 and anti-ICAM mAb was confirmed in two independent experiments, one of which is shown in Fig. 4.

The degree of anti-HIV activity mediated by different agents against laboratory strains of HIV may be greatly reduced, and sometimes completely lost, against primary isolates of HIV-1 [33]. For this reason, the inhibitory

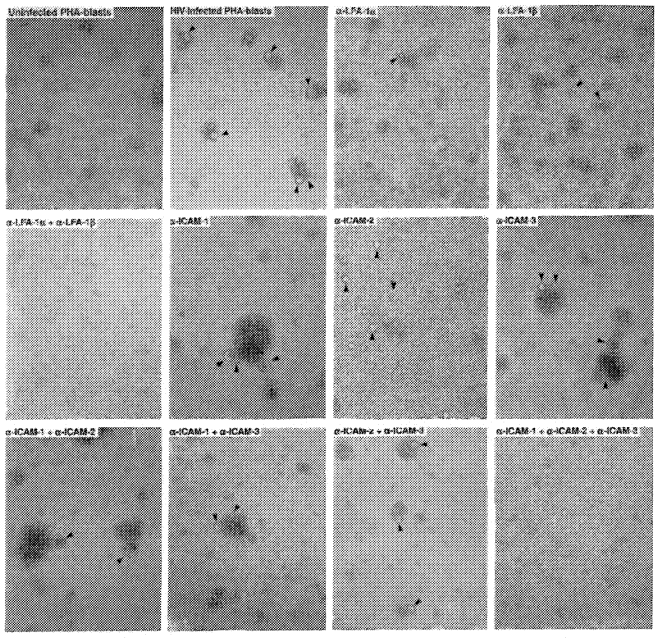

Figure 3. Effect of anti-LFA-1, anti-ICAM-1, anti-ICAM-2 and anti-ICAM-3 mAb on HIV-2-mediated syncytia formation. PHA-activated normal PBMC were inoculated with HIV-2$_{ROD}$ and cultured in the presence or absence of the individual mAb used alone or in various combinations. Cultures were performed in triplicate as described in Sect. 2.3. Photomicrographs (× 100) were taken at day 4 after infection.

activity of anti-LFA-1 and anti-ICAM-1, -2 and -3 mAb on syncytia formation was analyzed in PHA-activated normal PBMC that were inoculated *in vitro* with a syncytia-inducing primary isolate of HIV-1. Syncytia formation peaked at day 5 in untreated cell cultures (Fig. 5). In contrast, syncytia formation was completely abolished in cultures treated either with the combination of anti-LFA-1 α and β mAb or with mAb directed to ICAM-1, -2 and -3 molecules (Fig. 5). Comparable results were obtained in two experiments performed with different isolates (data not shown).

Finally, the effects of anti-LFA-1 α and β, anti-ICAM-1, ICAM-2 and ICAM-3 mAb were analyzed on HIV replication and HIV-mediated depletion of $CD4^+$ T lymphocytes in culture. PHA-activated PBMC were inoculated with $HIV\text{-}1_{MN}$. Viral replication, as measured by RT activity, reached a peak at day 10 in untreated cultures (Fig. 6 A). Anti-ICAM-1, -2 and -3 mAb exerted minimal effects on viral replication, whereas anti-LFA-1 α and β delayed and partially (40%) inhibited the peak of RT activity similar to previous studies [34] (Fig. 6 A). HIV-mediated depletion of $CD4^+$ lymphocytes was assessed in untreated cultures versus those treated with the different mAb. In HIV-infected untreated cultures a decrease in $CD4^+$ T cell viability was evident at day 10 and by day 16 the depletion of $CD4^+$ cells was ~80% (Fig. 6 B). In the cell cultures treated with anti-LFA-1 α+β mAb the depletion of $CD4^+$ T cells was significantly reduced. In fact, in these latter experiments, $CD4^+$ T cells were completely viable at day 10 after infection and a minor depletion (~20%) was observed at day 19 (Fig. 6 B). Similar results were obtained in cultures treated with anti-ICAM-1, -2 and -3 mAb. However, in these cultures, with the progression of HIV infection the protective effect mediated by anti-ICAM mAb on $CD4^+$ T cell depletion was lower compared to that observed with anti-LFA-1 mAb (~50% depletion at day 19; Fig. 6 B). Three different experiments were performed with comparable results.

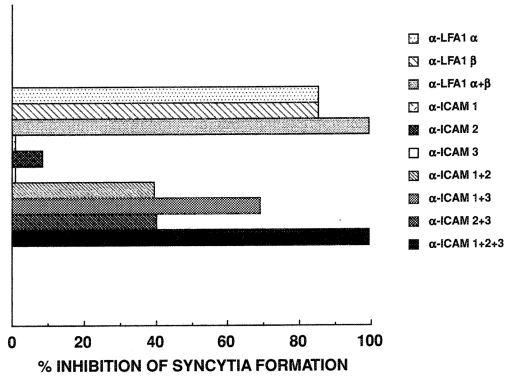

Figure 4. Effect of anti-LFA-1, anti-ICAM-1, anti-ICAM-2 and anti-ICAM-3 mAb on HIV-2-mediated syncytia formation in one of two representative experiments. PHA-activated PBMC were inoculated *in vitro* with $HIV\text{-}2_{ROD}$ and observed daily for syncytia formation. In this experiment the peak of syncytia formation was at day 4. Ten different microscopic fields per well were scored for syncytia.

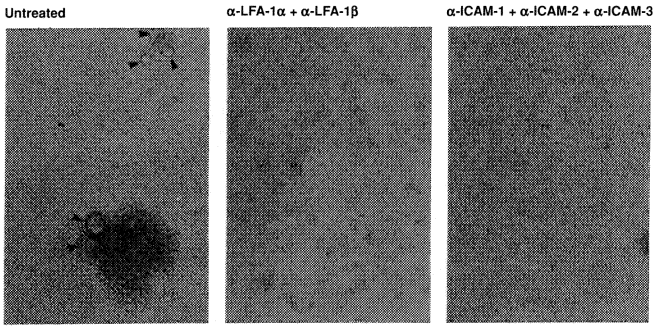

Figure 5. Effect of anti-LFA-1α+β and anti-ICAM-1+2+3 mAb on syncytia formation in activated PBMC inoculated *in vitro* with a syncytium-inducing primary isolate of HIV-1. Infection was performed as described in Sect. 2.3. Photomicrographs (× 100) were taken at day 5 after infection. Comparable results were obtained in two experiments performed with two different isolates.

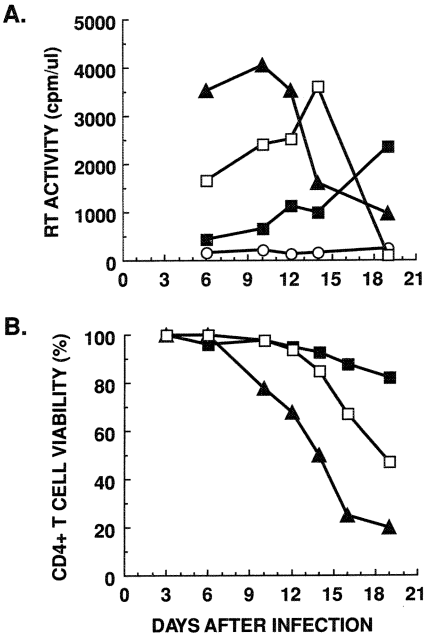

Figure 6. Effect of anti-LFA-1α+β and anti-ICAM-1+2+3 mAb on HIV replication and HIV-mediated cytopathicity. Panel A. Levels of RT activity in uninfected (○) and HIV-1-infected untreated cultures (▲). Cultures treated with anti-LFA-1α+β (■) or anti-ICAM-1+2+3 (□) mAb. Panel B. $CD4^+$ T cell viability in HIV-1-infected untreated cultures (▲) and in infected cell cultures treated with anti-LFA-1 α+β (■) or anti-ICAM-1+2+3 (□) mAb. The $CD4^+$ T cell viability was calculated as described in Sect. 2.5. One of three representative experiments is shown.

4 Concluding remarks

It has been shown in non-HIV systems that the contribution of ICAM-1, ICAM-2 andd ICAM-3 to LFA-1 binding may vary in different types of cells, in resting versus activated lymphocytes and in immune responses [19, 21, 26, 27, 35]. Our results demonstrate that ICAM-1, ICAM-2 and ICAM-3 all function as counter-receptors for LFA-1 in the process of HIV-driven syncytium formation. In this regard, the interaction between LFA-1 and the three ICAM in syncytium formation appears identical with other processes

of lymphocyte adhesion. The HIV-mediated process is, nevertheless, more complex insofar as it requires an initial interaction between gp120 and CD4, followed by an interaction between gp41 and a second cell [36–39]. Our results do not distinguish between the two possibilities that these HIV envelope-specific events either activate the cells in preparation for LFA-1/ICAM adhesion, or that they contribute directly to the interaction. Our results rule out the possibility that the HIV envelope proteins can substitute for the ICAM in interacting with LFA-1.

Since little information is available concerning syncytia formation in human lymphoid organs during acute infection and extensive syncytia formation has been difficult to document in sequential lymph node biopsies, the precise role of syncytia formation in the pathogenesis of HIV disease currently remains unclear. This difficulty may reflect the rapidity by which the reticuloendothelia system would be expected to clear such dying cells, because, even *in vitro,* syncytia rarely persist for more than several days. Histopathologic and virologic examination of lymphoid tissue at various stages of HIV disease clearly demonstrate that free virions trapped on the villus processes of follicular dendritic cells of lymph node germinal centers are in close contact with uninfected cells within the microenvironment of the lymphoid tissue [40, 41]. This proximity between infected and uninfected cells could potentially create an optimal milieu for the formation of syncytia.

Recent studies hve indicated that viral isolate showing a "syncytia-inducing" phenotype *in vitro* are associated with rapid disease progression [6, 7] which suggests that syncytia-induction at least measure a parameter for *in vivo* pathogenicity. Our current results extend previous observations demonstrating that syncytia formation correlated well with the rate and extent of $CD4^+$ T cell depletion in cultures, but not with virus replication and spreading. Thus, delineation of the mechanisms of syncytia formation and the role of cellular adhesion molecules in this process may improve our understanding of the pathogenic mechanisms of HIV disease and provide further targets for potential therapeutic interventions.

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