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Human immunodeficiency virus type 1-anchored CD40 ligand induces secretion of the chemokine interleukin-8 by human primary macrophages

Émilie Maurais, Réjean Cantin, Michel J. Tremblay *

Centre de Recherche en Infectiologie, Centre Hospitalier de l'Université Laval, Canada
Département de Biologie Médicale, Faculté de Médecine, Université Laval, Québec, Canada

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ABSTRACT

CD40 ligand (CD40L) is mainly expressed in activated $CD4^+$T cells and interacts with CD40 on antigen-presenting cells to regulate both humoral and cellular immune responses. We previously reported that CD40L is acquired by emerging HIV-1 particles. Here we demonstrate that both wild-type and a non-functional mutated form of CD40L are incorporated within HIV-1. Importantly, we show that wild-type CD40L remains functional since CD40L-bearing virions mediate NF-κB activation in a CD40-expressing reporter cell line and induce secretion of the chemokine IL-8 by monocyte-derived macrophages. These results suggest a possible means exploited by HIV-1 to attract susceptible target cells to the site of infection, a process that might promote viral dissemination.

Introduction

Interactions between CD40 and CD40 ligand (CD40L) play a pivotal, non-redundant role in the initiation and regulation of both humoral and cell-mediated immunity (reviewed in Mackey, Barth, and Noelle, 1998). The demonstration of the physiological importance of CD40/CD40L interactions came from the discovery that the hyper-IgM syndrome, an X-linked immunodeficiency, is due to a genetic alteration of the *CD40L* gene (Thusberg and Vihinen, 2007). This disease is characterized by a severe impairment of T cell-dependent antibody responses with no B cell memory, deficient induction of somatic mutations, and little or no circulating IgG, IgA, or IgE antibodies (Schneider, 2000). CD40L is a type II transmembrane trimeric protein that belongs to the tumor necrosis factor (TNF) family. It is now recognized that CD40L is primarily expressed on the surface of $CD4^+$T cells upon cellular activation but not on resting cells. As for CD40, a type I membrane protein in the TNF receptor family, it is constitutively expressed on antigen-presenting cells (APCs) (e.g. B cells, macrophages, and dendritic cells).

Macrophages, as APCs, are rapidly involved in the immune response directed against human immunodeficiency virus type 1 (HIV-1). These cells play a dominant role, linking innate to acquired immunity. Interactions between CD40, on macrophages, and CD40L, on $CD4^+$T lymphocytes, are crucial to the activation of these two cell types upon creation of the immunological synapse. This interplay results in the production of a vast array of soluble factors, such as interleukin (IL)-1, IL-8 and TNF-α, which all participate in the establishment of an inflammatory milieu (Bergamini et al., 2002; Bolacchi et al., 2001; Cotter et al., 2001; Kiener et al., 1995).

We reported previously that host-derived CD40L molecules are acquired by clinical HIV-1 isolates expanded in peripheral blood mononuclear cells (PBMCs) and lymphoid tissue (Martin and Tremblay, 2004). More recently, we demonstrated that the presence of CD40L at the surface of HIV-1 particles can induce homotypic cellular adhesion, immunoglobulin G production, as well as IL-6 secretion upon binding of virus to primary B lymphocytes (Martin et al., 2007). Given that macrophages do express CD40 and are considered as key targets for HIV-1 infection, we tested the capacity of virus-anchored CD40L to activate human primary monocyte-derived-macrophages (MDMs) by measuring the secretion of the CXC chemokine IL-8.

Results

Both wild-type and mutated CD40L are acquired by HIV-1

We first produced four different viral stocks by calcium-phosphate transfection of 293T cells with the R5-tropic infectious molecular clone pNL4-3Bal*env* that was co-transfected either with an empty control vector to produce virions lacking CD40L (parental 293T cells are negative for CD40L) or an expression vector coding for the human wild-type CD40L to generate viruses bearing host-derived CD40L. This task was achieved using a previously described transient transfection-

* Corresponding author. Laboratoire d'Immuno-Rétrovirologie Humaine, Centre de Recherche en Infectiologie, RC709, 2705 Boul. Laurier, Québec (QC), Canada G1V 4G2. Fax: +1 418 654 2212.
E-mail address: michel.j.tremblay@crchul.ulaval.ca (M.J. Tremblay).

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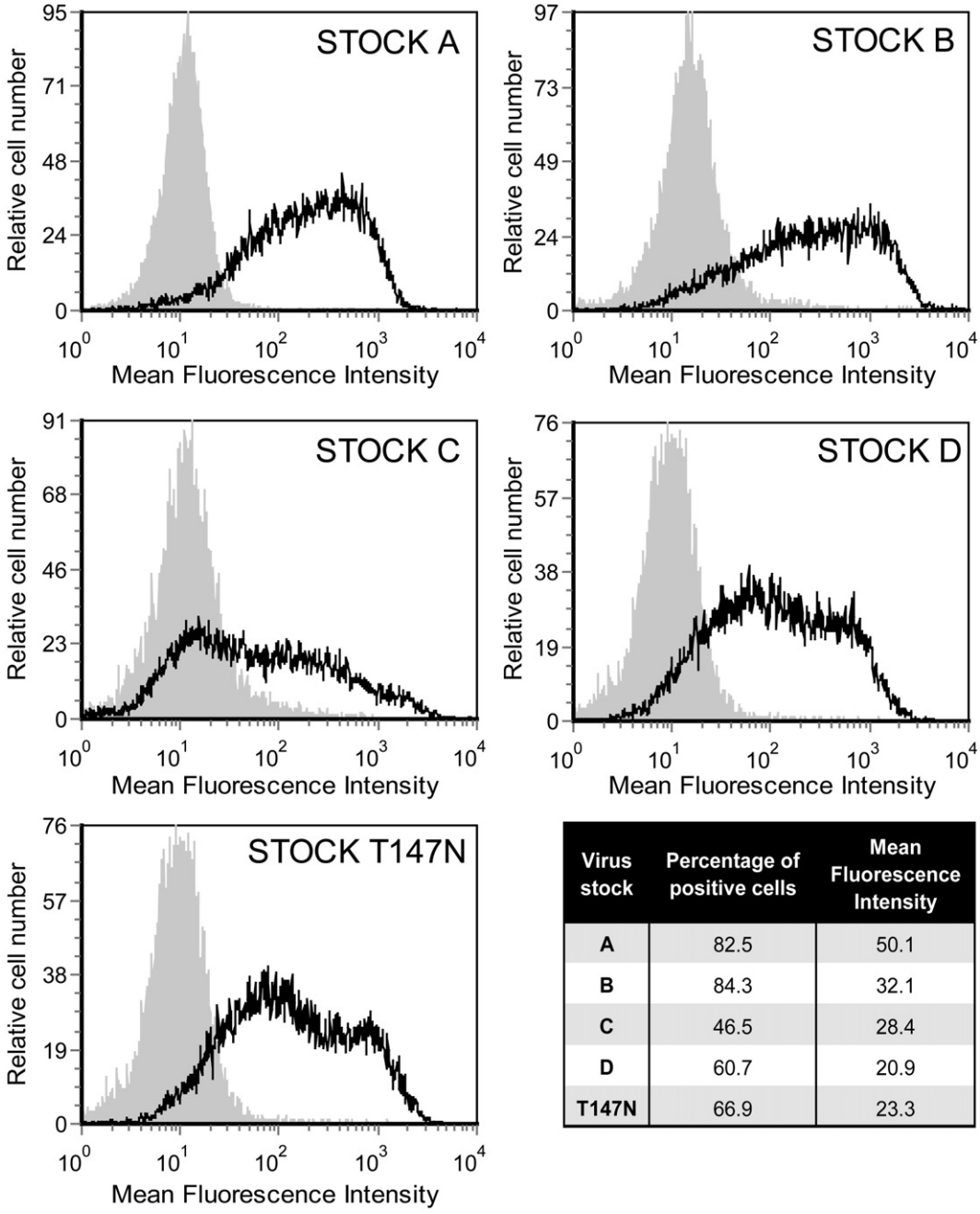
and-expression system (Fortin et al., 1997). We also produced a virus stock carrying a mutated version of CD40L, which is characterized by a point mutation consisting in the substitution of an asparagine for a threonine at position 147 (T147N). This single-point mutation is found in patients afflicted with the X-linked hyper-IgM syndrome and results in a non-functional CD40L that can no longer interacts with CD40. As depicted in Fig. 1, surface expression levels of wild-type CD40L and the T147N mutant vary amongst the virus producer 293T cells.

The next step was to verify whether both wild-type and mutated CD40L were effectively acquired by HIV-1 through the use of a plate immunocapture assay coated with an antibody specific for CD40L (clone M90.1, BD Pharmingen) as previously described (Beauséjour and Tremblay, 2004). Results from the virus capture test indicate that the levels of incorporation of wild-type CD40L are variable amongst the viral stocks tested (Fig. 2A). Interestingly, the T147N mutant is incorporated as efficiently in emerging virions as wild-type CD40L (Fig. 2B). Altogether data from flow cytometry analyses and the virus capture assay indicate that the variability in the incorporation process of host-derived CD40L does not seem to be linked with the amounts of CD40L expressed on the surface of 293T cells (compare Figs. 1 and 2).

CD40L remains functional once incorporated within viruses

Next, we addressed the biological activity of host-derived CD40L molecules once inserted within virions. This goal was reached by using a 293-derived reporter cell line engineered to express CD40 (i.e. CD40-293-SEAP), the natural counter-receptor of CD40L.



Virus stock	Percentage of positive cells	Mean Fluorescence Intensity
A	82.5	50.1
B	84.3	32.1
C	46.5	28.4
D	60.7	20.9
T147N	66.9	23.3

Fig. 1. Surface expression of CD40L on transiently transfected 293T cells. Cell surface expression of CD40L on virus producer 293T cells was monitored by flow cytometry. Cells were first labeled with a primary anti-CD40L monoclonal antibody (M90.1) followed by a secondary fluorochrome-labeled antibody (black lines). An isotype-matched irrelevant antibody was used as a control (gray histograms). The percentages of CD40L-expressing cells and the mean fluorescence intensity values are presented in the table.

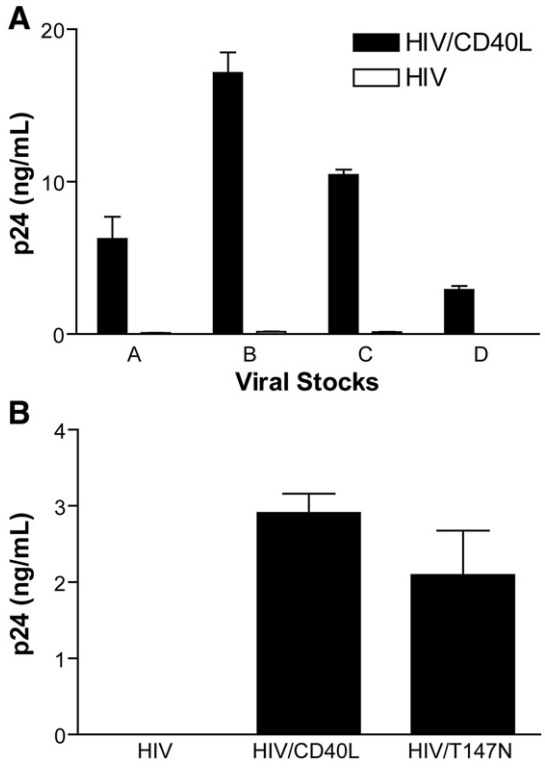


Fig. 2. Wild-type and mutated CD40L are acquired by HIV-1. (A) 293T cells were either cotransfected with pNL4-3Bal*env* and an empty control vector (HIV) or cotransfected with pNL4-3Bal*env* and the pcDNA3.1-CD40L expression vector (HIV/CD40L). Virus preparations originated from 4 independent transfections. (B) In some experiments, virus preparations were made by cotransfection of 293T cells with an expression vector coding either for wild-type (HIV/CD40L) or mutated CD40 (HIV/T147N). Similar amounts of the indicated virus stocks, standardized in terms of p24, were next incubated in a plate immunocapture assay. The levels of captured viruses were estimated with the use of a p24 test. The data shown represent the means±SEM of triplicate samples. The baseline values obtained from an isotype-matched control antibody have been subtracted from each value.

Interestingly, this cell line can serve to measure the bioactivity of CD40L since it secretes embryonic alkaline phosphatase (SEAP) upon NF-κB activation through the stimulation of CD40. The validity of CD40-293-SEAP cells was assessed by measuring the functional response of this reporter cell line to increasing proportions of paraformaldehyde-fixed CD40L-expressing 293T cells. In this set of experiments, similarly treated parental CD40L-negative 293T cells were used as a negative control. In contrast to what is seen with parental CD40L-negative 293T cells, reporter gene activity was increased in a dose-dependent manner upon co-culture of CD40-293-SEAP cells with CD40L-positive 293T cells (Fig. 3A). Thereafter, we monitored the ability of CD40L-bearing viruses to drive reporter gene activity in CD40-293-SEAP cells. Briefly, the reporter cells were first plated in each well of a 96-well plate the day before performing the experiment. Next, cells were exposed for 24 h to isogenic viruses either lacking or bearing host-derived wild-type CD40L. In such studies, controls consisted of the same volume of supernatant from 293T cells transiently transfected with the CD40L expression vector (mock). We tested with this reporter cell line the various viral stocks depicted in Fig. 2A. Interestingly, a statistically significant increase in SEAP production was seen with the four CD40L-bearing virus preparations tested but not with the corresponding isogenic virus stocks lacking host-derived CD40L (Fig. 3B). Moreover, the T147N mutant of CD40L was unable to induce the production of SEAP (Fig. 3C), which confirms its inability to interact with CD40. Therefore, the incorporation of CD40L within HIV-1 does not alter its natural capacity to interact with CD40 and to drive signal transduction leading to NF-κB activation.

CD40L-bearing viruses induce secretion of IL-8 by MDMs

It has been well established that stimulation of MDMs by soluble CD40L induces the secretion of IL-8 (Kiener et al., 1995), a well-known chemoattractant for neutrophils, and to a lesser extent, for T lymphocytes (Larsen et al., 1989; Leonard et al., 1990). It can be

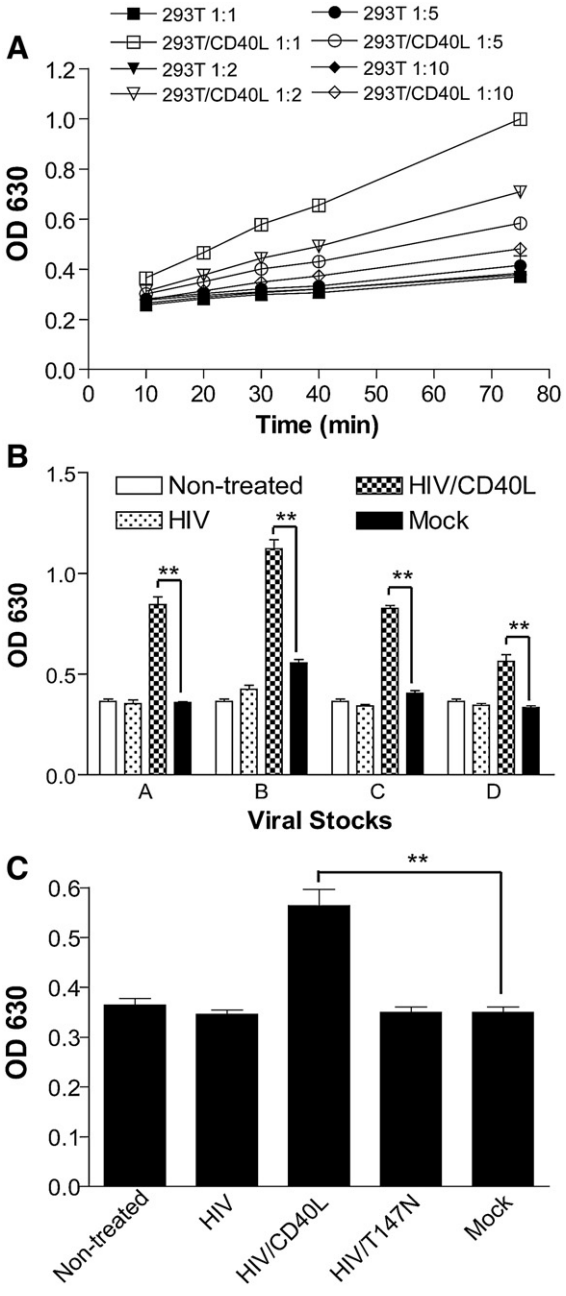


Fig. 3. Virus-associated CD40L remains functional. (A) Paraformaldehyde-fixed 293T cells either negative (i.e. parental) or positive for CD40L (i.e. transiently transfected with the vector coding for wild-type CD40L) were co-cultured with CD40-expressing 293 reporter cells (i.e. CD40-293-SEAP) at the listed ratio (i.e. 1:1, 1:2, 1:5 and 1:10). (B) CD40-293-SEAP reporter cells were either left unexposed or exposed to virions lacking CD40L, CD40L-bearing virions, or cell-free supernatants from 293T cells transfected with the CD40L expression vector only (mock). Virus stocks from the 4 independent transfections were tested. (C) In some experiments, comparative studies were made between viruses bearing either host-derived wild-type or mutated CD40L (viral stock D). The data shown represent the means±SEM of triplicate samples. The statistical significance of the obtained values were analyzed with a one-way ANOVA test followed by a Dunnett's multiple comparison post-test with the mock as a control and performed using GraphPad Prism 4.01 (**, $P<0.01$).

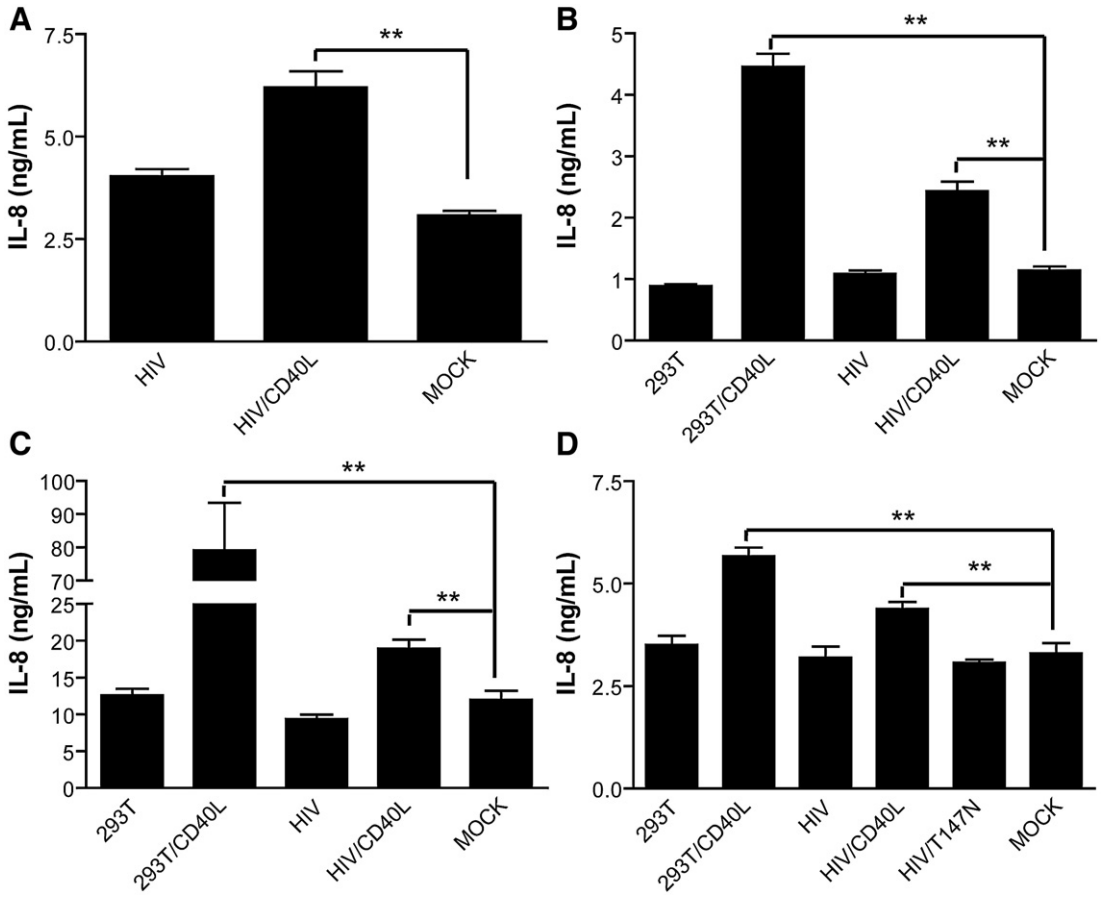


Fig. 4. HIV-1-associated CD40L induces secretion of IL-8 by MDMs. Similar amounts of virus preparations originating from the 4 independent transfections were incubated for 24 h at 37 °C with MDMs (panels A to D). Levels of IL-8 were then quantified through the use of a commercial ELISA test. In some experiments, MDMs were incubated with paraformaldehyde-fixed parental 293T cells (CD40L-negative) or 293T cells transfected with the CD40L expression vector (293T/CD40L) (panels B to D), whereas some studies were performed with isogenic viruses bearing either host-derived wild-type or mutated CD40L (panel D). The data shown represent the means ± SEM of triplicate samples. The statistical significance of the obtained values were analyzed with a one-way ANOVA test followed by a Dunnett's multiple comparison post-test with the mock as a control and performed using GraphPad Prism 4.01 (**, $P<0.01$).

proposed that a putative IL-8-mediated attraction of $CD4^+$T lymphocytes by macrophages interacting with CD40L-bearing viruses could facilitate the propagation of HIV-1. Our next objective was thus to determine if the presence of host-derived CD40L on the exterior of HIV-1 particles can promote the secretion of this chemokine by MDMs. The cells were derived from a 7-day long differentiation of monocytes purified by plastic adherence in a culture medium made of RPMI-1640 supplemented with 5% heat-inactivated autologous serum and 25 ng/mL of macrophage-colony stimulating factor. Fully differentiated MDMs (2×10^5) were then exposed to the different virus preparations listed in Fig. 2 (a final concentration of 40 ng of p24 was used). Again, cell-free supernatants from 293T cells transiently transfected with the vector coding for wild-type CD40L were used as controls in each instance (mock). In some experiments, additional controls were included that consisted of paraformaldehyde-fixed 293T cells (1×10^5) either transiently transfected with an empty control vector or the molecular construct coding for wild-type CD40L. Finally, production of IL-8 was monitored with a commercial sandwich ELISA test (human CXCL8/IL-8 DuoSet, R and D Systems, Minneapolis, MN). A statistically significant production of IL-8 by MDMs was seen when using CD40L-bearing virus preparations (Fig. 4). Secretion of IL-8 was more important in such samples as compared to what is seen in mock-infected MDMs and cells exposed to virions lacking CD40L. In addition, viruses carrying the T147N mutant of CD40L were unable to induce any significant production of IL-8 by MDMs. However, we did observe some variability in the induction of IL-8 secretion between the different viral preparations and donors tested (data not shown). For example, a single virus preparation bearing wild-type CD40L could trigger IL-8 production in MDMs from only one of the two different donors studied (data not shown), therefore implying that secretion of IL-8 is influenced primarily by the responding MDMs and less by the CD40L-bearing virus preparation. Additional studies are necessary to address this issue in a more detailed manner and to define for example if the surface expression levels of CD40 in MDMs could account for the different responsiveness to CD40L-bearing viruses.

Discussion

Overall our data confirm the previous observations demonstrating that host-derived CD40L molecules are still functional when found embedded within HIV-1 particles (Martin et al., 2007). In the present work, the capacity of CD40L-bearing virions to elicit IL-8 production by human primary MDMs was illustrated through the use of viral stocks produced following transient transfection of 293T cells. The artificial nature of the experimental cell system used to generate the tested virus preparations might weaken the physiological significance of our observations. However, this strategy carries a significant advantage since it allows performing side-by-side comparative studies with isogenic viruses that are either lacking or bearing host-derived CD40L. Importantly it permits also to test virions carrying a mutated CD40L molecule that can no longer interact with its normal

counter-receptor CD40 (i.e. T147N mutant). This unique tool has allowed us to validate the biological activity of virus-associated host CD40L. It is important to state that it is difficult to perform similar experiments with PBMCs-derived clinical and/or laboratory isolates of HIV-1 because the functionality of virus-associated host CD40L would have to be addressed with the use of antibodies blocking the natural interaction between CD40 and CD40L. In such studies, an anti-CD40 antibody would have to be used to avoid any steric hindrance phenomenon mediated by an antibody specific for virus-anchored host CD40L. Indeed, an anti-CD40L antibody might jeopardize the natural interactions between virus-associated gp120 and CD4. On the other hand, the use of an anti-CD40 to demonstrate the ability of virus-anchored host CD40L to mediate secretion of IL-8 in human primary MDMs is also subjected to a severe limitation. Indeed, it has been shown that antibody-mediated homotypic aggregation of Fc receptors on monocytic cells induces the expression of several soluble factors including IL-8 (Fernandez et al., 2002). Therefore, although viruses expanded in human primary cells are likely to better reflect the natural conditions, there are several factors that impede their use to demonstrate the functionality of virus-anchored cell surface molecules of host origin.

Since IL-8 acts as a chemoattractant for $CD4^+$T cells, the results of the present study suggest a possible means exploited by HIV-1 to attract such preferential target cells to the infection site, a phenomenon that might promote virus replication. Moreover, a previous work demonstrated that IL-8 stimulates viral production in both macrophages and $CD4^+$T lymphocytes (Lane et al., 2001). Importantly, it has been reported that the basal expression of CD40L is increased in T cells from HIV-1-infected individuals with high blood CD4 counts (Muller et al., 1998; Sousa et al., 1999; Zhang et al., 2004), which provides credence to our findings. This might increase the probability for the virus to incorporate host-derived CD40L during the budding process. Because of its known physiological functions, IL-8 participates in the establishment of a pro-inflammatory microenvironment that causes a systemic immune activation and an overexpression of matrix metalloproteinases (reviewed in Mauviel, 1993). These proteolytic enzymes are playing a key role in a wide range of processes including tissue remodeling and release of biological factors. Therefore, the induction of IL-8 secretion by HIV-1-associated host CD40L might represent another factor possibly involved in hyperactivation of the immune system and structural destruction of lymphoid organs, which are two features commonly seen in the course of AIDS (Frost and McLean, 1994; Pantaleo et al., 1994). Overall our findings confirm that host-derived cell surface constituents are still functional when found embedded within the viral envelope and suggest that such foreign molecules can modulate HIV-1 pathogenesis.

Materials and methods

Cell culture

Human embryonic kidney 293T cells were cultured in complete DMEM medium (i.e. DMEM supplemented with 10% fetal bovine serum/FBS, 2 mM L-glutamine, 100 U/mL penicillin G and 100 µg/mL streptomycin). The indicator CD40-293-SEAP cells have been engineered to overexpress wild-type human CD40 and to produce the secreted embryonic alkaline phosphatase (SEAP) upon NF-κB activation through the stimulation of cell surface CD40 (kindly supplied by R.S. Kornbluth). These cells were cultured in DMEM medium supplemented with 10% FBS and appropriate selective agents (i.e. 500 µg/mL of G418 and 100 µg/mL of Zeocin). Human primary monocytes were purified by plastic adherence in complete RPMI-1640 medium supplemented with 5% heat-inactivated autologous serum. Briefly, peripheral blood mononuclear cells (1.5×10^7 cells/mL) were plated in 75 cm^2 flasks. After 1 h, non-adherent cells were removed by several washes with PBS and freshly isolated monocytes were further cultured in the same medium for 7 days in the presence of 25 ng/mL of macrophage-colony stimulating factor (GenScript Corporation, Piscataway, NJ) to derive MDMs. Thereafter, MDMs were recovered by scraping cells with a soft cell scraper following a 15 min incubation period with a solution made of PBS and 5 mM EDTA and cells were plated in 24-well plates at a final concentration of 2×10^5 cells per well in complete RPMI-1640 medium supplemented with 5% autologous human serum.

Flow cytometry analysis

To monitor cell surface expression of CD40L, transiently transfected 293T cells were incubated with an antibody specific for human CD40L (clone M90.1, BD Pharmingen) or an appropriate isotype-matched irrelevant control antibody for 30 min at 4 °C. Cells were next washed with phosphate-buffered saline (PBS) and then incubated with an R-PE-conjugated goat anti-mouse IgG for 30 min at 4 °C. After two washes with PBS, cells were fixed in 2% paraformaldehyde and analyzed by FACS (Epics ELITE ESP, Coulter Electronics, Burlington, ON).

Molecular constructs

pNL4.3Bal*env* construct was kindly provided by R.J. Pomerantz (Thomas Jefferson University). The cDNA3.1-CD40L molecular construct codes for wild-type human CD40L, whereas the cDNA3.1-CD40L/T147N plasmid codes for a mutated version of CD40L in which an asparagine is substituted for a threonine at position 147 (T147N). Both cDNA3.1-CD40L and cDNA3.1-CD40L/T147N vectors were generous gifts from R.S. Kornbluth (University of California, San Diego).

Viral production

Virus particles differing only by the absence or presence of host-derived CD40L proteins on their outer membranes were produced by calcium-phosphate transfection of 293T cells as previously described (Fortin et al., 1997). It should be noted that 293T cells do not express CD40L constitutively (data not shown). Briefly, 293T cells were cotransfected with a plasmid coding for the human wild-type CD40L molecule (i.e. pcDNA3.1-CD40L) and a plasmid coding for a R5-tropic strain of HIV-1 (pNL4.3Bal*env*) at a 1:4 ratio. We also produced viruses bearing a mutated version of CD40L where pcDNA3.1-CD40L was replaced by pcDNA3.1-CD40L/T147N. Viruses lacking host-derived CD40L were made by substituting an empty control vector for pcDNA3.1-CD40L. Virus-containing supernatants were filtered through a 0.22 µm filter (Millipore) and the amount of viruses produced was measured by an in-house enzyme-linked immunosorbent assay (ELISA) specific for the major core p24 protein (Bounou, Leclerc, and Tremblay, 2002).

Immunocapture assay

The immunocapture assay was performed as described previously (Beauséjour and Tremblay, 2004). Briefly, flat-bottom 96-well plates were coated either with an anti-CD40L antibody or isotype-matched control antibody (IgG1) (5 µg/mL) for 2 h at room temperature and blocked for 1 h with a solution made of phosphate-buffered saline (PBS) and BSA 1%. Then, a viral input corresponding to 25 ng of p24 was added per well. The plates were incubated overnight at 4 °C and washed twice with a solution of PBS. Next, 200 µL of PBS and 50 µL of a 5% Triton-X solution were added per well and the plates were incubated for 30 min at room temperature. Finally, 125 µL were transferred to a plate coated for our homemade p24 test. This assay quantifies the viral protein p24 recovered from the captured viral particles, that is, the p24 associated with CD40L-bearing viruses.

Detection of SEAP and IL-8

CD40-293-SEAP cells were seeded in 96-well plate (2×10^4 per well) in DMEM medium supplemented with 10% FBS only. The next day, isogenic virus preparations either lacking or bearing CD40L (wild-type or mutated) were added to each well (16 ng of p24), and left for a 24 h period. In parallel, the same volume of mock-infected supernatant was used as a control (i.e. supernatant from 293T cells transfected with the expression vector coding for CD40L only). The production of SEAP was monitored according to the manufacturer's protocol with 20 µL of cell-free culture supernatant and the medium for detection and quantification of SEAP (i.e. Quanti-Blue™, Invivogen, San Diego, CA), after a 3 h incubation period. In some studies, fully differentiated MDMs (2×10^5) were exposed either to the tested virus stocks (40 ng of p24), an equivalent volume of mock, or, as controls, 1×10^5 paraformaldehyde-fixed 293T cells, transfected or not with wild-type CD40L, and incubated for a 24 h time period. The presence of IL-8 in the supernatant was determined using the IL-8 Duo-Set ELISA from R and D Systems (Minneapolis, MN).

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