In primary human monocyte-derived macrophages exposed to Human immunodeficiency virus type 1, does the increased intracellular growth of Leishmania infantum rely on its enhanced uptake?

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Received 28 October 2005
Accepted 3 January 2006

INTRODUCTION
Leishmaniasis is a complex of diseases caused by protozoan parasites of the genus Leishmania. Humans are infected by Leishmania via the bite of sandflies, although person-to-person transmission through the sharing of needles also puts a significant population at risk of developing leishmaniasis. Over 20 species and subspecies of Leishmania can infect humans, each causing a different spectrum of symptoms ranging from a simple, self-healing skin ulcer (e.g. cutaneous leishmaniasis due to infection with Leishmania major) to a severe, life-threatening disease [e.g. visceral leishmaniasis (VL) caused by Leishmania donovani or Leishmania infantum]. Although people are often bitten by sandflies infected with Leishmania protozoans, most do not develop the disease. However, among people who are immunosuppressed as a result of immunosuppressive treatment for organ transplants, haematological malignancy, autoimmune diseases or, in particular, advanced human immunodeficiency virus type 1 (HIV-1) infections, the most severe form of leishmaniasis, VL, rapidly develops and disseminates. Due to the overlapping geographical distribution of leishmaniasis and HIV-1 infection, co-infection with both human pathogens is becoming a more common event and an extremely serious clinical health problem (Alvar et al., 1997).

Leishmania is an obligate intracellular protozoan parasite that infects an invertebrate vector, the phlebotomid sandfly, and diverse vertebrate hosts, including humans. There are two main developing forms of Leishmania parasite, the extracellular flagellated promastigote, which colonizes the alimentary tract of the sandfly vector, and the non-flagellated amastigote, which is the intracellular form in the vertebrate hosts. Macrophages are considered the main vertebrate host cells for Leishmania, implying a high degree of specificity for this cell type. Within the acidic environment of macrophage phagolysosomes, promastigotes are differentiated into amastigotes. Amastigotes are responsible for maintaining and spreading infection within the host (reviewed by Kane & Mosser, 2000). However, gamma interferon (IFN-γ)-activated macrophages can kill amastigotes (Murray et al., 1983), suggesting that any condition that causes macrophage dysfunction can lead to uncontrolled growth of the parasite.

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Macrophages play a pivotal role in the detection and elimination of pathogenic micro-organisms. Recognition of foreign micro-organisms by macrophages results ultimately in phagocytosis, a complex process leading to the engulfment and eventual destruction and elimination of the invasive pathogens by lysosomal enzymes, toxic reactive oxygen and nitrogen intermediates, and/or by nutrient derivational mechanisms (reviewed by Stafford et al., 2002). Generally, the opportunistic infections associated with advanced HIV-1 infection are controlled by host immune responses that involve both humoral and cellular immune responses. However, some opportunistic pathogens such as Toxoplasma gondii, Trypanosoma cruzi and various Leishmania species have developed unique adaptive mechanisms to ensure their survival in the harsh environment prevailing in macrophages. In AIDS patients, these protozoan parasites can reactivate and eventually develop to cause fatal opportunistic diseases, strongly suggesting an influence of underlying HIV-1-induced immune deficiencies. Indeed, it has been observed that, following HIV-1 infection, some important macrophage functions are impaired, including phagocytosis, intracellular killing, chemotaxis and cytokine production (reviewed by Kedzierska & Crowe, 2002).

Currently, there is minimal information on the ability of HIV-1-infected macrophages to control the growth of Leishmania. Given that phagocytosis of Leishmania by macrophages is a critical event in the parasite life-cycle and that HIV-1 can impair this effector function carried out by macrophages, it has been proposed that HIV-1 might affect the invasion of macrophages by the parasite to some extent. The purpose of this study was thus to investigate the ability of HIV-1 to affect the process of Leishmania infection in a cell type known to act as a natural reservoir for both pathogens, i.e. primary human macrophages. We report here that infection of macrophages with fully competent HIV-1 particles resulted in a higher growth of Leishmania infantum, as assessed using luciferase-expressing recombinant parasites. Despite the expected HIV-1-dependent impairment of phagocytosis, the observed enhancement of intracellular growth of Leishmania was found to be associated with a higher parasite uptake upon virus infection of macrophages. This finding could provide a partial explanation for the high prevalence of VL in persons with advanced HIV-1 infection.

Preparation of virus stocks. Fully infectious HIV-1 particles were produced by transient calcium phosphate transfection of human 293T cells with pNL4-3balexv. In this molecular construct, the env gene of the X4 (T)-tropic NL4-3 strain has been replaced with that of the R5 (macrophage)-tropic bal strain (Dornadula et al., 1999). Virus preparations underwent a single freeze-thaw cycle before infection. Virus stocks were normalized for virion content with an in-house sandwich ELISA assay specific for the major viral core p24 protein (Bounou et al., 2002). Values of p24 were calculated on the basis of regression analysis of p24 standards prepared from known-concentration samples.

Preparation of Leishmania infantum amastigotes. Experiments were performed with axenic amastigotes that were differentiated in vitro from stationary-phase promastigotes. The expression vectors pNEO-GFP and pGL2-neoLUC1.2 vectors have been described previously (Boucher et al., 2002a; Roy et al., 2000). The GFP-expressing and luciferase-encoding parasites were generated by transfection of purified pNEO-GFP and pGL2-neoLUC1.2, respectively, into Leishmania infantum (term Leishmania infantum–GFP and Leishmania infantum–LUC1.2). In the pGL2-neoLUC1.2 vector, a 3′ UTR element capable of inducing reporter-gene expression specifically in amastigotes has been cloned downstream of the luciferase gene (Boucher et al., 2002b). The culture and maintenance of axenic amastigotes have been described previously (El Fakhry et al., 2002). Briefly, promastigotes were cultured in RPMI 1640 supplemented with 10% FBS, buffered with 25 mM HEPES and 2 mM NaHCO3 at 25°C. Stationary Leishmania infantum–GFP and Leishmania infantum–LUC1.2 promastigotes were transferred into MAA/20 medium in order for them to differentiate into amastigotes. MAA/20 consists of modified medium 199 (Gibco-BRL) with Hanks' salts, supplemented with 0.5% soybean trypto-casein (Pasteur Diagnostics), 15 mM d-glucose, 5 mM L-glutamine, 4 mM NaHCO3, 0.023 mM bovine haemin, 25 mM HEPES (at a final pH of 6.5) and 20% FBS. Axenically grown amastigotes were maintained at 37°C with 5% CO2 by weekly subpassages in MAA/20 complete medium in 25 cm2 flasks. These amastigotes showed morphological, biochemical and biological characteristics similar to those of amastigotes isolated in vivo (El Fakhry et al., 2002).

Exposure of MDMs to HIV-1 and Leishmania parasites. The possible modulatory effect of HIV-1 on the biology of Leishmania parasites was measured by first infecting MDMs with NL4-3balexv (2 ng p24 per well seeded with monocytes) prior to Leishmania infection. Briefly, MDMs were initially exposed to HIV-1 for 2 h and uninternalized virions were eliminated by extensive washes with warm PBS. The cells were then incubated for another 5 days before exposure to either Leishmania infantum–GFP (30 min) or Leishmania infantum–LUC1.2 (2 h) at a 1:2 cell: parasite ratio. Free parasites were washed away with warm PBS. To measure the effect of HIV-1 on the intracellular growth of Leishmania infantum, MDMs that were inoculated with both HIV-1 and Leishmania infantum–LUC1.2 parasites were cultured for another 2 days before the cells were lysed to monitor luciferase activity. Luciferase units, which are directly proportional to Leishmania infection levels (Roy et al., 2000), were measured with a microplate luminometer (MLX; Dynex Technologies). For the Leishmania uptake assay, HIV-1-infected MDMs were exposed to Leishmania infantum–GFP parasites before treatment with trypsin and EDTA to eliminate parasites that had not entered the cells. The intracellular uptake of parasites was estimated by performing flow cytometry (Epics ELITE ESP; Coulter Electronics). In both experiments, MDMs that were not exposed to HIV-1 (mock-infected) were used as controls. To assess the percentage of MDMs carrying the parasite and/or infected with HIV-1, cells were infected with NL4-3balexv for 12 days before exposure for 30 min at 37°C to Leishmania infantum–GFP. Next, cells were fixed with 2% paraformaldehyde for 30 min on ice, washed with PBS and

METHODS

Isolation and culture of monocyte-derived macrophages (MDMs). Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors by density-gradient centrifugation on Ficoll-Hypaque. Monocytes were purified by adherence to plastic in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Briefly, PBMCs (3 × 106 cells ml−1) were first seeded into 48-well plates (0.5 ml per well) and, after 2 h, non-adherent cells were removed by several washes with warm PBS. Freshly isolated monocytes were allowed to differentiate into MDMs in complete RPMI 1640 supplemented with human recombinant macrophage colony-stimulating factor (100 ng ml−1) for 6 days before HIV-1 infection.
permeabilized with PBS containing 0·1% saponin for 5 min at 37 °C. Cells were labelled with a commercial monoclonal anti-p24 antibody (R-phycocerythrin-labelled KC57; Coulter Clone) or a control isotype antibody for 30 min on ice. After several washes with PBS supplemented with 1% BSA and 0·01% saponin, cells were resuspended in PBS and analysed by flow cytometry to estimate GFP (an indication of the engulfed parasites) and p24 (an indication of HIV-1 infection) staining.

**Phagocytosis assay.** To assess the impact of HIV-1 on phagocytic function of macrophages, fluorescein-labelled *Escherichia coli* K-12 BioParticles (Vybrant Phagocytosis Assay kit, V-6694; Molecular Probes) were incubated with MDMs that had been infected with HIV-1 for 5 days. Phagocytosis was terminated after 30 min incubation at 37 °C according to the manufacturer’s instructions. Cells not infected with HIV-1 were used as a control.

**Statistical analyses.** The results presented are expressed as the mean value ± SD of experiments performed with triplicate samples. Statistically significant differences between groups were determined by analysis of variance. Calculations were made using Microsoft Excel software. P values of less than 0·01 were considered statistically significant.

**RESULTS**

**Leishmania growth is increased in HIV-1-infected MDMs**

To approximate more closely the complex molecular events taking place in a cell type known to act as a natural cellular reservoir for both HIV-1 and *Leishmania*, we performed infection studies in primary human MDMs. The susceptibility of such cells to infection with the HIV-1 strain NL4-3balenv was first assessed, as previous work has revealed that there is a wide spectrum of variability to virus infection from donor to donor (Crowe *et al.*, 1994). We found that infection of MDMs with NL4-3balenv resulted in a productive virus infection, as defined by p24 measurements (Fig. 1). The kinetics of virus production were comparable for two other healthy donors (data not shown). For experiments aimed at defining the putative modulatory effect of HIV-1 on the intracellular growth of *Leishmania*, MDMs were infected with NL4-3balenv for 5 days before addition of the parasites being tested.

Next, we assessed the ability of *Leishmania* parasites to propagate in MDMs, as primary human cells usually ingest this parasite less readily than established cell lines (Wolday *et al.*, 1998). It should be noted that the present study was conducted with *Leishmania infantum* as it is the most common *Leishmania* species found in individuals co-infected with both HIV-1 and *Leishmania*. Standard procedures to measure *Leishmania* infection in MDMs *in vitro* are labour-intensive, cumbersome and not quantitative (e.g. Giemsa staining). However, stable expression of the reporter firefly luciferase gene in *Leishmania* has provided a new tool to quantify *Leishmania* rapidly and sensitively once inside primary human MDMs. Indeed, a previous study revealed that there is a linear correlation between the number of *Leishmania* parasites and luciferase activity (Roy *et al.*, 2000). In addition, a previous quantification of infection levels with this approach indicated that as few as 100 parasites can be detected, and luciferase activity measured in extracts prepared from 10²–10⁶ parasites was found to be within a linear range when using the murine macrophage cell line RAW 264.7 (St-Denis *et al.*, 1999). Fig. 2 shows the kinetics of the
intracellular growth of *Leishmania infantum* in primary human MDMs. A peak level of reporter gene activity was reached after 2–3 days following infection with *Leishmania infantum*–LUC1.2 parasites.

We next monitored the possible impact of HIV-1 on the intracellular survival of *Leishmania infantum*. A representative experiment shown in Fig. 3 demonstrated that the parasite growth was enhanced in MDMs infected first with the HIV-1 R5-tropic variant NL4-3balenv. It should be noted that the HIV-1-mediated upregulatory effect on *Leishmania* growth was seen when using cells from three different healthy donors.

### HIV-1 infection mediates a higher uptake of *Leishmania* parasites through an indirect process

The subsequent experiments were aimed at shedding light on the putative process by which HIV-1 infection can increase the intracellular growth of *Leishmania infantum* in MDMs. Phagocytosis is an important weapon in the arsenal of the innate immune system that targets microbes into a cellular compartment where they can be eliminated. It is known that *Leishmania* is taken up by phagocytosis into phagosomes following initial attachment to the macrophage (Handman & Bullen, 2002). Therefore, we measured the uptake of *Leishmania* parasites by MDMs. To this end, uninfected and HIV-1-infected primary human MDMs were incubated with genetically modified *Leishmania infantum* amastigotes that constitutively expressed GFP in their cytoplasm. Using flow cytometry, we examined the percentage of GFP-positive MDMs after a brief exposure (30 min) to GFP-loaded parasites. Internalization of *Leishmania infantum* inside primary human MDMs was augmented in HIV-1-infected target cells compared with MDMs that were not exposed to the virus (Fig. 4a). As a control, the capacity of MDMs to phagocytize fluorescently tagged *E. coli* was also measured, as HIV-1 infection of this cell type is known to result in a reduced ability to engulf numerous pathogens. Thus, MDMs were infected with HIV-1 for 5 days and subjected to a phagocytosis test exploiting fluorescently

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**Fig. 3.** Effect of HIV-1 on the intracellular growth of luciferase-encoding *Leishmania infantum* amastigotes in MDMs. Primary human MDMs were infected with NL4-3balenv for 5 days before being exposed to *Leishmania infantum*–LUC1.2 amastigotes for 2 h. Cells were washed to remove any free parasites and further cultured for an additional 2 days. Finally, cells were lysed to estimate luciferase activity. Data are shown as means±SD of triplicate samples for each healthy donor (P values of 0.006, 0.008 and 0.006 in cells infected with HIV-1 compared with mock-infected cells for donors 1, 2 and 3, respectively). RLU, Relative luciferase units.

**Fig. 4.** Effect of HIV-1 on uptake of GFP-labelled parasites and fluorescently tagged *E. coli* by MDMs. (a) MDMs were either left uninfected (Mock) or infected with NL4-3balenv for 5 days prior to incubation with *Leishmania infantum*–GFP amastigotes for 30 min. Cells were washed gently and treated with trypsin/EDTA to eliminate cell-surface-bound parasites. Cells were next subjected to flow cytometry to monitor GFP expression. Data are shown as means±SD of triplicate samples for each healthy donor (P values of 0.006, 0.008 and 0.006 in cells infected with HIV-1 compared with mock-infected cells for donors 1, 2 and 3, respectively). (b) MDMs were either left uninfected (Mock) or infected with NL4-3balenv for 5 days before being subjected to a phagocytosis assay as described in Methods. Data are shown as means±SD of triplicate samples for each healthy donor (P values of 0.001, 0.006 and 0.006 in cells infected with HIV-1 compared with mock-infected cells for donors 1, 2 and 3, respectively).
labelled *E. coli* bacteria. Our results suggested that the capacity of MDMs to engulf the tested agent was impaired following infection with the macrophage-tropic strain of HIV-1, NL4-3balenv (Fig. 4b).

In an attempt to provide additional information on the mechanism through which HIV-1 might affect the biology of *Leishmania infantum*, the levels of MDMs harbouring the studied pathogens were assessed by infecting target cells with HIV-1 for 12 days prior to exposure to GFP-labelled *Leishmania infantum*. The percentage of cells productively infected with HIV-1 was defined through the use of an anti-p24 antibody specific for the major viral core protein. The data shown in Fig. 5 indicated that 19% of the studied MDMs were carrying *Leishmania infantum* (as indicated by GFP staining) compared with 24.4% that were productively infected with HIV-1 (as monitored by p24 staining). More importantly, we found that 6.2% of the studied MDMs were positive for both *Leishmania infantum* and HIV-1, thus suggesting that the virus is probably exerting its effect via an indirect phenomenon. A lower percentage of MDMs harbouring GFP-loaded parasites was detected when HIV-1 was omitted, in agreement with our previous observations (data not shown).

**DISCUSSION**

Previous studies have shown that both *Leishmania* and HIV-1 are able to infect and multiply within macrophages. A possible infection of the same cell target by these two different pathogens might have an important effect on susceptibility to infection with the intracellular parasite *Leishmania*. In the present study, we demonstrated that the intracellular growth of *Leishmania* was augmented in MDMs that were inoculated with HIV-1 before exposure to *Leishmania infantum*. This observation is in line with results from clinical studies demonstrating a high incidence of disseminated leishmaniasis in AIDS patients (Lopez-Velez et al., 1998). It was found that >50% of patients dually infected with *Leishmania* and HIV-1 displayed peripheral parasitaemia (Martinez et al., 1993), which is indicative of uncontrolled parasite growth. A similar enhancement of the intracellular multiplication of another pathogen, *Mycobacterium avium*, has also been observed following infection of macrophages with HIV-1 (Kallenius et al., 1992).

Macrophages play a dual role during the process of *Leishmania* infection, acting as both the principal cellular reservoirs and the major effector cells to combat this obligate intracellular protozoan parasite (Pantaleo & Fauci, 1995). Therefore, we investigated whether the observed enhancement of intracellular growth of *Leishmania* could be linked with the reported HIV-1-mediated impairment of some effector functions carried out by macrophages, such as phagocytosis, intracellular killing, chemotaxis and cytokine production (reviewed by Kedzierska & Crowe, 2002). More emphasis was put on phagocytosis, as it is considered a fundamental host defence mechanism for the uptake and degradation of infectious agents and senescent cells (Swanson & Baer, 1995). Defective phagocytic capture of opportunistic pathogens by cells of the macrophage lineage following HIV-1 infection *in vivo* and *in vitro* has been well documented (Biggs et al., 1995; Kedzierska et al., 2000, 2002, 2003a). For example, HIV-1-infected MDMs display an
impaired capacity to phagocytize numerous pathogens such as *M. avium* (Kedzierska et al., 2003b), *Candida albicans* (Crowe et al., 1994), *Toxoplasma gondii* (Biggs et al., 1995), *Aspergillus fumigatus* (Koillides et al., 1993), *Histoplasma capsulatum* (Chaturvedi et al., 1995) and *Staphylococcus aureus* (Musher et al., 1990). The virus seems to impair FcγR-mediated phagocytosis via a CAMP-dependent signalling pathway (Kedzierska et al., 2001; Thomas et al., 1997). Moreover, HIV-1-mediated impairment of macrophage phagocytic ability has been suggested to be linked with the ability of some specific virus proteins (e.g. Nef) to target signalling molecules involved in phagocytosis such as the Src kinases Hck and Lyn (Greenway et al., 1995). Our observations showing that phagocytosis of fluorescently tagged *E. coli* is reduced following infection of MDMs with HIV-1 is thus in line with the reported literature. Surprisingly, in spite of the reduced capacity of HIV-1-infected MDMs to internalize fluorescently labelled *E. coli*, we found that *Leishmania* uptake was increased upon virus infection. Although such findings might appear contradictory at first sight, it should be noted that studies of the cell biology of microbial uptake by macrophages has revealed that several micro-organisms can enter mammalian cells by diverse phagocytic mechanisms (reviewed by Aderem & Underhill, 1999; Underhill & Ozinsky, 2002). For example, *Salmonella typhimurium* is internalized by macrophagocytosis through an actin-independent mechanism, whilst *Legionella pneumophila* gains entry into macrophages by a ‘coiling phagosome’ (Aderem & Underhill, 1999; Horwitz, 1984). Moreover, there is evidence that the two developmental forms of *Leishmania*, i.e. the motile promastigote and the amotile amastigote, are internalized via distinct endocytic pathways in macrophages. The uptake of *Leishmania* under the flagellated promastigote form by macrophages takes place through a classical receptor-mediated endocytic event (reviewed by Kane & Mosser, 2000). The limited amount of published information regarding amastigote uptake by mammalian cells suggests that there are aspects of this interaction that may not be consistent with classical receptor-mediated phagocytosis, as little or no respiratory burst is generated in macrophages following amastigote engulfment and the receptors that participate in this process are largely unknown (Love et al., 1998). It has been shown that amastigote internalization by macrophages is dependent on the expenditure of host-cell energy and on the localized polymerization of host-cell actin (Love et al., 1998). Taken together, our data indicate that, although HIV-1 can impair FcγR-mediated phagocytosis by human MDMs, phagocytosis of *Leishmania infantum* amastigotes is instead upregulated in virus-infected macrophages, which might be due to the fact that this process exhibits differences from classical receptor-mediated phagocytosis. Further study is warranted to shed light on this phenomenon.

Results from experiments aimed at assessing whether HIV-1 is exerting a direct or an indirect effect on parasite entry suggest that it probably occurs through an indirect process. Indeed, only a very low percentage of MDMs was found to be productively infected with HIV-1 and also to harbour GFP-tagged *Leishmania infantum*. Therefore, it can be proposed that HIV-1 infection of MDMs favours the production of an as yet unidentified soluble factor that can modulate the uptake of *Leishmania infantum*. In turn, this factor might affect expression of cell-surface receptor(s) known to be involved in attachment of *Leishmania* parasites to macrophages. For example, macrophage complement receptor type 3 (CR3, Mac-1), CR1, mannose–fucose receptor, fibronectin receptor and the macrophage receptor for advanced glycosylation end-products have been shown to be of paramount importance in attachment, invasion and intracellular survival of both *Toxoplasma gondii* (Joiner et al., 1990) and *Leishmania infantum* in macrophages (Guy & Belosevic, 1993; Kedzierski et al., 2004). On the other hand, the ability of macrophages to mount a pro-inflammatory response is critical to achieve control and eventual resolution of *Leishmania* infection (Engwerda et al., 1998; Kane & Mosser, 2001; Murphy et al., 2001; Sacks & Noben-Trauth, 2002). IFN-γ, interleukin 10 and transforming growth factor (TGF)-β are known to influence replication of the parasite in macrophages (reviewed by Barral-Netto & Barral, 1994; Jones et al., 1998). Moreover, HIV-1 is able to increase the production of anti-inflammatory cytokines such as TGF-β (Marshall et al., 1999), which may exert an inhibitory influence on *Leishmania*-specific immune responses. Therefore, it might be of interest to investigate whether HIV-1 can affect the surface expression level of some of these receptors. A possible relationship between HIV-1-mediated changes in the cytokine profile and the virus-induced increase in the intracellular growth of *Leishmania* also deserves to be studied. These two hypotheses are currently under investigation.

The HIV-1-mediated augmentation of intracellular growth of *Leishmania infantum* in MDMs might have a certain impact on the overall parasitaemia, as this phenomenon will take place over numerous rounds of replication during the course of the disease. Moreover, the demonstrated HIV-1-directed increase in *Leishmania* uptake in primary human macrophages may have physiological significance for the pathogenesis of both infections. For example, it is known that, once inside the macrophage, *Leishmania* has developed unique adaptive mechanisms to disrupt macrophage activation in order to be protected from phagolysosome degradation (reviewed by Cunningham, 2002). More specifically, *Leishmania* has been shown to inhibit phagosome–endosome fusion, hydrolytic enzymes, cell-signalling pathways, nitric oxide production and cytokine production. *Leishmania* can thus manipulate the host immune response to avoid destruction by the host immune system. A more important intracellular growth and dissemination of *Leishmania* may in turn amplify virus production, based on the reported *Leishmania*-mediated enhancement of HIV-1 production via an upregulating effect on virus regulatory sequences (i.e. the long terminal repeat domain) (Bernier et al., 1995, 1998; Zhao et al., 2004a, b). Therefore, it can be postulated that the course of both diseases is affected in patients dually...
infected with Leishmania and HIV-1 due to uncontrolled multiplication of the pathogens. Our findings help to explain the high prevalence and frequency of relapses of leishmaniasis in persons also infected with HIV-1, and also provide further support for the generally held concept that abnormal functions of macrophages play a key role in the pathogenesis of HIV-1 infection.

ACKNOWLEDGEMENTS

We are grateful to Ms Sylvie Méthot for editorial assistance. We thank Dr M. Dufour for his technical assistance in flow cytometry studies. This study was performed by C. Z. and S. T. in partial fulfilment of their PhD degree in the Microbiology-Immunology Programme, Faculty of Medicine, Laval University. C. Z. holds a Doctoral Award from the Fonds de la Recherche en Santé du Québec (FRSQ), whilst S. T. is the recipient of a Doctoral Award from the Canadian Institutes of Health Research (CIHR) HIV/AIDS Research Programme. This study was supported by a grant to M. J. T. and B. P. from the CIHR HIV/AIDS Research Programme (#HOP-37781) and the CIHR Group Grant to M. J. T., M. O. and B. P. (#MG-C-14500). M. J. T. holds the Senior Canada Research Chair in Human Immuno-Retrovirology, M. O. is a Burroughs Wellcome Fund Scholar in Molecular Parasitology and holds a Senior Canada Research Chair in antimicrobial resistance. B. P. is a Burroughs Wellcome Fund New Investigator in Molecular Parasitology and holds an FRSQ Scholar (Senior level).

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