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Amphotericin-B-mediated reactivation of latent HIV-1 infection

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Abstract

To date, attempts to eliminate HIV-1 infection from its latent reservoirs, a prerequisite for the development of a curative treatment strategy for HIV-1 infection, have been unsuccessful. We demonstrate that the FDA approved antifungal agent amphotericin B efficiently reactivates HIV-1 infection in THP89GFP cells, a model of HIV-1 latency in macrophages. Although amphotericin B does not directly reactivate latent HIV-1 infection in T cells (e.g., J89GFP), amphotericin-B-stimulated macrophages (THP89GFP cells or primary macrophages) when cocultured with J89GFP cells can induce HIV-1 reactivation in these cells in trans. Because of the close proximity of antigen presenting macrophages and T cells in the primary lymphoid organs, such interaction between antigen presenting macrophages and T cells are frequent, and it seems reasonable to assume that trans-reactivation strategies hold promise to also reactivate latent HIV-1 infection in vivo.

Keywords: HIV-1; Latency; Reactivation; Amphotericin B; Reporter cell lines; THP89GFP; J89GFP

Introduction

While efficiently suppressing HIV-1 replication, highly active antiretroviral therapy (HAART) cannot eradicate the virus from infected patients (Chun et al., 1999a, 2000; Davey et al., 1999; Wong et al., 1997). Present HIV-1 therapy would thus require a life-long treatment, which is hampered by drug side effects, development of drug resistant virus strains, and high treatment costs. In this setting, the development of a curative treatment strategy with the goal to eradicate HIV-1 from the infected patients is a major challenge. An essential step towards this goal is the depletion of the viral reservoirs in latently infected cells. According to the present understanding, HIV-1 latency develops primarily in memory T cells and macrophages (Chun et al., 1997a, 1997b, 1998; Crowe and Sonza, 2000; Lebargy et al., 1994; Mikovits et al., 1992; Sonza and Crowe, 2001) (reviewed in (Crowe et al., 2003). In vitro, reactivation of latent HIV-1 infection in both cell types can be achieved by a variety of physiological stimuli, such as TNF-α, IL-1β, IL-2 IL-6, IFN-γ, or CD154 (Biswas et al., 1992; Butera et al., 1991; Duh et al., 1989; Folks et al., 1987; Kutsch et al., 2003; Poli et al., 1994). Chemical compounds that reactivate latent HIV-1 infection, among others, are the phorbol esters PMA (Folks et al., 1988) and prostratin (Korin et al., 2002; Kulkosky et al., 2001), and the histone deacetylase inhibitor trichostatin A (TSA) (Quivy et al., 2002; Sheridan et al., 1997; Van Lint et al., 1996) and sodium butyrate (Laughlin et al., 1993, 1995). In addition, certain activating antibodies can mediate HIV-1 reactivation (anti-CD3) (Tong-Starkesen et al., 1989; Wong et al., 1997). Although these stimuli efficiently reactivate latent HIV-1 infection in vitro, severe side effects prevent their usage in vivo. To date, only IL-2 and anti-CD3 antibodies have been used in small clinical trials. However, initial studies in patients have shown no meaningful decay of the viral latent reservoir but significant adverse side effects (Chun et al., 1999b; Dybul et al., 2002; Kovacs et al., 2000, 2001; Kulkosky et al., 2002).

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To screen for and analyze the effect of pharmaceutical compounds with HIV-1 reactivating properties, we have recently established a cell-based reporter system that allows direct quantification of HIV-1 reactivation on a single cell basis, using EGFP as a read-out (Kutsch et al., 2002). The system consists of the monocytic THP89GFP cells and two T cell lines, J89GFP and JNLGFP, which contain an integrated but transcriptionally silent copy of molecular clones of HIV-1 89.6 and HIV-1 NL4-3, respectively, engineered to express EGFP.

In this study, we used these cell lines to study the effect of amphotericin B, an FDA-approved antifungal agent, on latent HIV-1 infection. Amphotericin B is the treatment of choice for a variety of mycoses and is used in the treatment of fungal infections in AIDS patients (Coker et al., 1993; Hsieh et al., 1998; Marty and Mylonakis, 2002; McKinsey et al., 1989). In vitro, amphotericin B and its derivate MS-8209 have been reported to induce TNF-α expression in macrophages and to increase HIV-1 expression in acutely infected macrophages (Chia and Pollack, 1989; Clayette et al., 2000; Tokuda et al., 1993). Other reports demonstrated that amphotericin B and its derivate MS-8209 have anti-HIV-1 properties, inhibiting viral replication in T cells in in vitro experiments (Cefai et al., 1991; Hansen et al., 1990; Konopka et al., 1999; Otake et al., 1989; Pleskoff et al., 1995, 1996; Pontani et al., 1989a, 1989b; Schaffner et al., 1986).

Here, we report the ability of amphotericin B to reactivate latent HIV-1 infection. Low doses of amphotericin B are sufficient to directly reactivate latent HIV-1 infection in the monocytic cell line THP89GFP. Although amphotericin B did not directly reactivate latent HIV-1 infection in T cells (J89GFP cells), cocultures of amphotericin-B-stimulated THP89GFP cells or primary macrophages with J89GFP cells were able to trigger HIV-1 reactivation in J89GFP in trans.

In vivo, such a "trans-reactivation" approach would exploit the frequent immunological interaction of T cells and macrophages during antigen presentation. Specific activation of macrophages could directly reactivate latent HIV-1 in macrophages, which upon cell-to-cell contact trigger reactivation of latent HIV-1 in T cells.

This approach, here exemplified by the use of amphotericin B, adds to the strategies that should be further investigated as an alternative approach to eradicate the pool of latent HIV-1 from infected patients.

Results

Amphotericin-B-mediated reactivation of latent HIV-1 infection

It is an accepted concept that specific activation of cells harboring latent HIV-1 can result in reactivation of HIV-1 infection. Unfortunately, presently no FDA approved drugs are described that could be used for this purpose. As amphotericin B, a potent approved antifungal drug, has previously been described to activate macrophages, we thought to determine the effect of amphotericin B on latent HIV-1 infection in THP89GFP cells, a model for latently infected macrophages, and the latently infected T cell lines J89GFP and JNLGFP.

Treatment of the latently infected T cell lines J89GFP and JNLGFP with amphotericin B did not result in reactivation of latent HIV-1 infection. However, HIV-1 reactivation in THP89GFP cells was seen at amphotericin B concentrations as low as 0.3 μg/ml. Near-complete reactivation of HIV-1 infection in THP89GFP cells on a population level was achieved at an amphotericin B concentration of 3 μg/ml. At a concentration of 10 μg/ml of amphotericin B, levels of HIV-1 expression measured as EGFP mean channel fluorescence were comparable to those seen following stimulation with TNF-α (10 ng/ml) (Fig. 1). As seen following stimulation with TNF-α, amphotericin-B-mediated HIV-1 reactivation was accompanied by a pronounced increase in the cell surface expression of ICAM-1, but in contrast to stimulation with IFN-γ, no induction of MHC class II expression was seen, suggesting that amphotericin B exerts its function mostly by activating the NF-κB pathway (Fig. 2).

Augmentation of amphotericin B triggered HIV-1 reactivation in THP89GFP cells by autocrine secreted TNF-α

It has been previously reported that stimulation of macrophages with amphotericin B results in TNF-α secretion, which in turn could contribute to HIV-1 reactivation (Chia and Pollack, 1989; Tokuda et al., 1993). TNF-α secretion from macrophages following stimulation has been reported to be controlled by the ERK MAPK pathway (Andersson and Sundler, 2000; Pearson et al., 2001; Suttles et al., 1999). To investigate the possible contribution of TNF-α secretion to amphotericin-B-mediated HIV-1 reactivation in THP89GFP cells, we thus pretreated the cells for 1 h with various concentrations of the specific ERK inhibitor U0126 (0.01–1 μM), or the inactive control compound UO124 (1 μM), and then stimulated the cells with amphotericin B (10 μg/ml). Levels of TNF-α in the culture supernatants were determined by ELISA and compared to levels of HIV-1 expression in THP89GFP cells, as measured by flow cytometry. 50 pg/ml TNF-α were detected in the supernatants of unstimulated THP89GFP cells. Forty-eight hours after stimulation, TNF-α levels in cultures treated with amphotericin B alone were increased to 300 pg/ml. Addition of increasing amounts of U0126 (0.1–1.0 μM) gradually inhibited TNF-α secretion to baseline levels but had no effect on EGFP fluorescence as a marker of HIV-1 expression, indicating that the presence of TNF-α in the supernatants is mostly augmenting HIV-1 expression but is not necessary to trigger HIV-1 reactivation. Higher concentrations of U0126 also affected the levels of HIV-1 reactivation. As concentrations of U0126 up to 10 μM did not alter the viability of the cells, this suggests that the

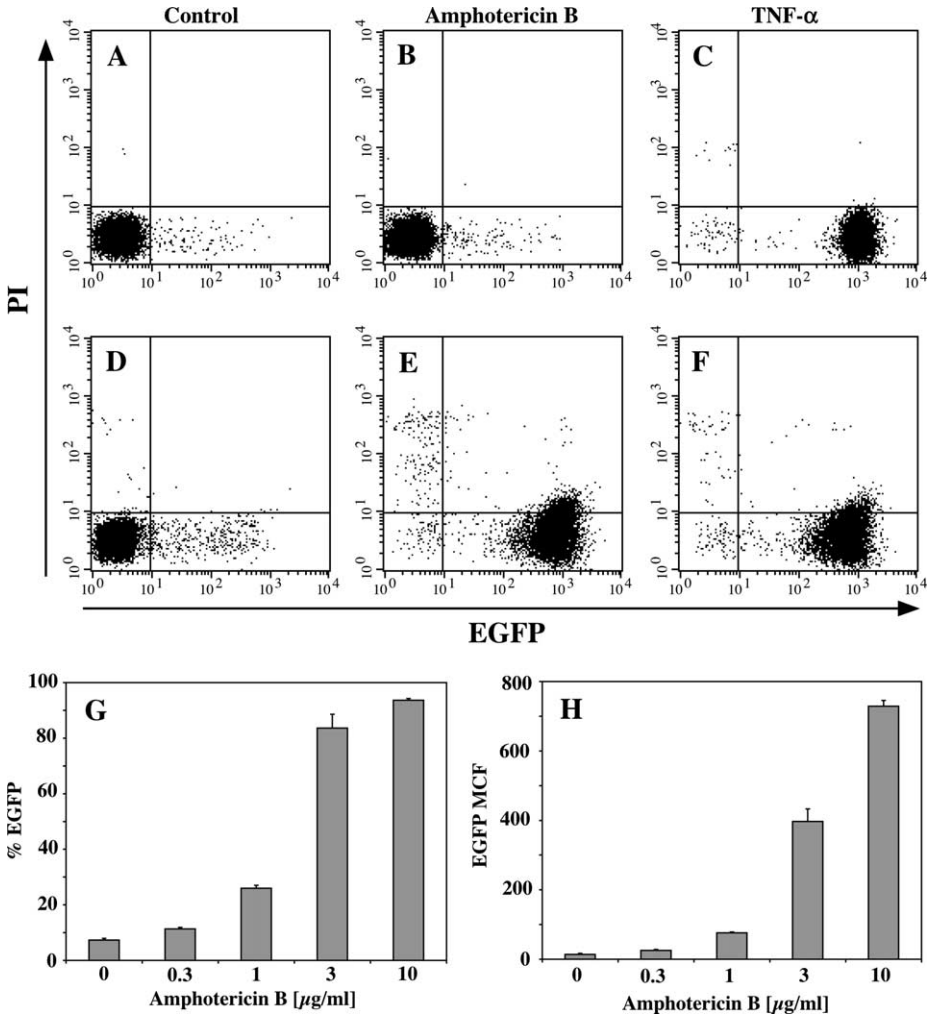

Fig. 1. Amphotericin-B-mediated reactivation of latent HIV-1 infection in THP89GFP cells. J89GFP and THP89GFP cells, respectively, were left untreated (A and D), stimulated with amphotericin B (10 μg/ml) (B and E), or as a positive control stimulated with TNF-α (10 ng/ml) (C and F) and EGFP fluorescence as a marker of HIV-1 expression was determined by flow cytometry after 48 h. Quantification of HIV-1 reactivation as indicated by EGFP expression determined as (G) the percentage of EGFP-positive cells or (H) the EGFP mean channel fluorescence intensity of the treated cell population. Results represent the mean ± SD of three independent experiments.

ERK MAPK pathway may also be part of the amphotericin B triggered signaling cascade in THP89GFP cells (Fig. 3). With respect to inhibition of HIV-1 reactivation, similar results were obtained when THP89GFP cells were stimulated with amphotericin B in the presence of neutralizing anti-TNF-α antibodies (Fig. 3B). These results suggest that amphotericin B triggers HIV-1 reactivation in a TNF-α independent manner, but the secreted TNF-α then augments virus expression.

Trans-reactivation of latent HIV-1 in J89GFP T cells

Although amphotericin B did not directly induce HIV-1 reactivation in latently infected T cells (J89GFP), we investigated the possibility that factors secreted by amphotericin-B-stimulated monocyte/macrophages (THP89GFP cells, primary macrophages) could reactivate latent HIV-1 in bystander T cells (J89GFP). Forty-eight hours following amphotericin B stimulation of THP89GFP cells (1.0–10 μg/ml), we conducted supernatant transfer experiments using the latently HIV-1-infected reporter cell line J89GFP. Despite the demonstrated presence of TNF-α, supernatants from THP89GFP cultures stimulated with amphotericin B concentrations as high as 10 μg/ml did not, or only slightly triggered, HIV-1 reactivation in J89GFP cells (12% EGFP-positive cells at 48 h; data not shown). A possible explanation for this finding is that the secreted TNF-α is mostly bound and functionally neutralized by secreted soluble TNF-receptor (sTNFR). To test this hypothesis, we used supernatants from the cultures of THP89GFP cells stimulated with 10μg/ml of amphotericin B. We then treated samples of these culture supernatants with anti-TNFR I- or TNFR II-specific neutralizing antibodies that recognize the respective TNF-α binding side. As these antibodies have a much higher affinity to the respective TNFRs than TNF-α, bound TNF-α is released from the TNFR/TNF-α complexes

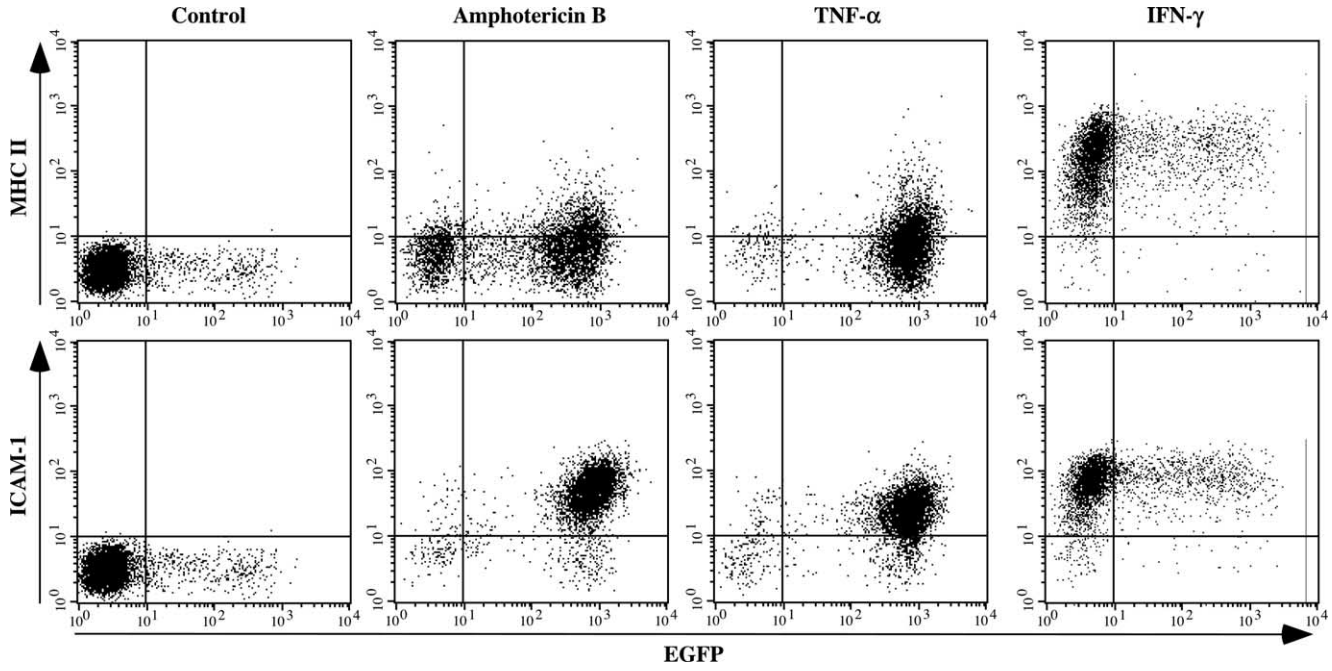

Fig. 2. Correlation of cellular and viral activation in THP89GFP. The effects of amphotericin B (10 μg/ml), TNF-α (10 ng/ml; NF-κB-dependent activation), and IFN-γ (500 U/ml; NF-κB independent activation) on the macrophage activation marker MHC class II (MHC II) and ICAM-1 and on HIV-1 infection (EGFP) were assessed 48 h following stimulation with optimal concentrations of the respective agent. The results are representative for two independent experiments.

following addition of these antibodies. Following removal of the antibody-TNFR complexes by protein G sepharose precipitation, we used these supernatants and the corresponding original untreated supernatants to determine the amount of free TNF-α. We found that the majority of the secreted TNF-α is indeed bound to either sTNFRI or sTNFRII. At the 12-h time point, which would be relevant for TNF-α-mediated reactivation at a later 24- or 48-h time point, only 200 pg/ml of free TNF-α could be detected in the cultures, which is insufficient to fully reactivate latent HIV-1 in J89GFP cells, whereas 400 pg/ml were functionally neutralized by sTNFR (Fig. 4) (Kutsch et al., 2002). At the 48-h time point, still more than 60% of TNF-α was TNFR-bound and thus functionally neutralized, suggesting that TNF-α activity would be very local and mostly restricted to cell-to-cell contact.

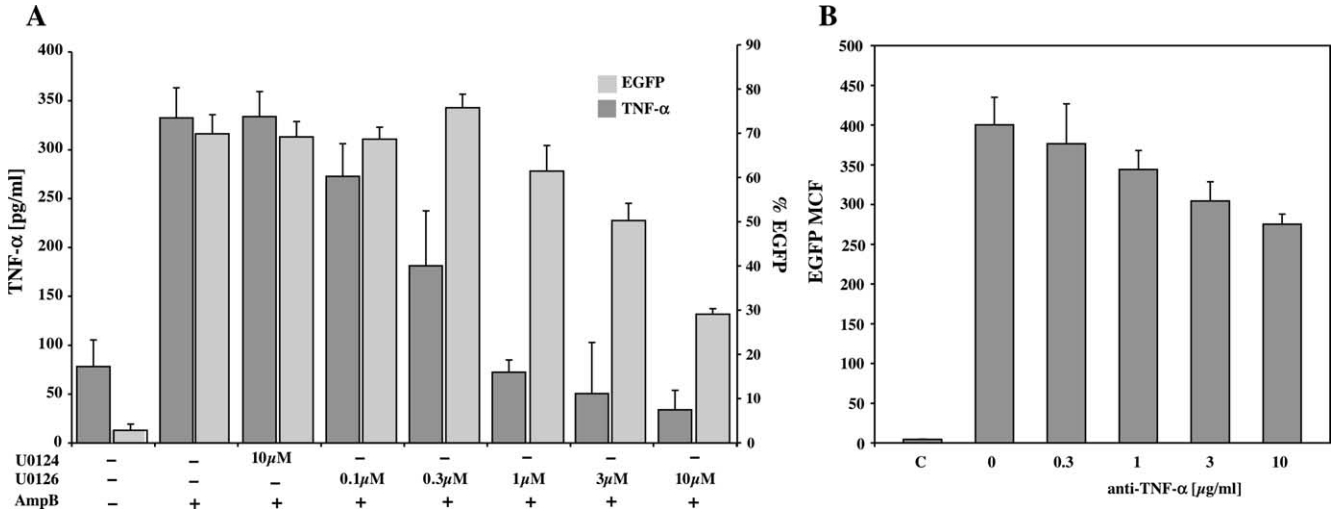

Fig. 3. Amphotericin B triggers HIV-1 reactivation independent of autocrine TNF-α secretion. (A) THP89GFP cells were left untreated or for 2 h pretreated with various concentrations of the ERK-inhibitor U0126 or the inactive control compound U0124, and then stimulated with amphotericin B (10 μg/ml). Forty-eight hours after amphotericin B stimulation, levels of HIV-1 reactivation were assessed by flow cytometric determination of EGFP expression (light gray bars) and compared to the levels of TNF-α expression as determined by ELISA (dark gray bars). The results represent the mean ± SD of three independent experiments. (B) Neutralizing anti-TNF-α antibody was added to THP89GFP cells at the various concentrations indicated. The cells were then stimulated with amphotericin B (10 μg/ml), and 48 h poststimulation levels of HIV-1 reactivation were determined by flow cytometric analysis and depicted as EGFP mean channel fluorescence. The results represent the mean ± SD of three independent experiments.

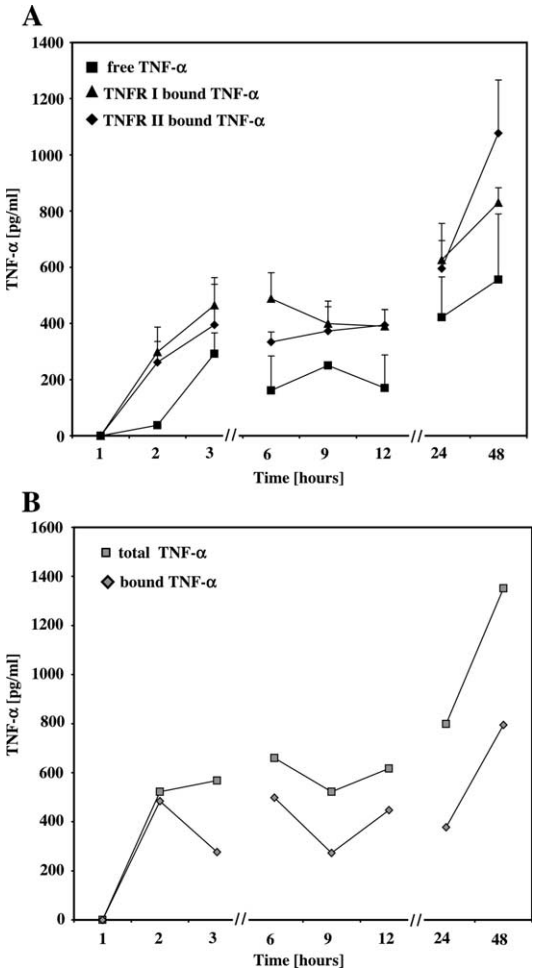

Fig. 4. Contribution of sTNFR1 and sTNFRII to functional neutralization of secreted TNF-α. Supernatants from THP89GFP cells treated with amphotericin B (10 μg/ml) were harvested at the indicated time points. Supernatants were either directly applied to a TNF-α-specific ELISA or following pretreatment with neutralizing anti-TNFRI or anti-TNFRII antibody. Due to their extremely high affinity, these antibodies release TNF-α bound to the respective TNFR and thus allow for the determination of the level of receptor bound TNF-α. (A) Levels of free TNF-α compared to the levels of TNF-α bound to TNFRI or TNFRII. (B) Levels of total receptor-bound TNF-α in comparison to total TNF-α. The results represent the mean ± SD of three independent experiments.

Indeed, while supernatant transfer experiments had little effect on HIV-1 infection in J89GFP cells, latent HIV-1 infection in J89GFP cells was reactivated if the cells were cocultured with amphotericin-B-stimulated THP89GFP cells or noninfected primary human macrophages. For these experiments, we labeled THP89GFP cells with the red-fluorescent live-dye PKH26 and cocultured the stained cells with J89GFP cells. Due to the uniform red fluorescence signal of the PKH26 stained THP89GFP cells, the monocytes can be distinguished from the unstained J89GFP cells in the second fluorescence using flow cytometric analysis. Latent HIV-1 infection in J89GFP cells cocultured treated with THP89GFP cells was efficiently reactivated in the presence 10 μg/ml of amphotericin B. Again, addition of neutralizing anti-TNF-α antibody did only slightly alter the percentage of THP89GFP cells that exhibited reactivated HIV-1 expression, but decreased the level of EGFP MCF in these cells by 50%. At the same time, reactivation of latent HIV-1 in the J89GFP cells was abrogated in the presence of anti-TNF-α antibodies in these cultures, demonstrating that the trans-reactivating effect here was entirely dependent on the presence of TNF-α (Fig. 5).

Similar results were obtained using primary macrophages. Coculture of primary macrophages with J89GFP cells already revealed slightly elevated levels of reactivation in J89GFP cells in the absence of amphotericin B stimulation (15% EGFP-positive cells). Stimulation of the cocultures resulted in efficient reactivation of latent HIV-1 infection in the entire J89GFP population. Addition of neutralizing anti-TNF-α antibody to these cocultures greatly decreased the EGFP MCF in J89GFP cells, demonstrating the contribution of TNF-α to the observed HIV-1 reactivation, but did not alter the level of EGFP-positive cells, indicating that in this case also other factors may be involved in HIV-1 reactivation.

Influence of amiloride on amphotericin-B-mediated HIV-1 reactivation in THP89GFP cells

At this time, very little is known about the mechanism and signal transduction events associated with amphotericin B treatment. However, several reports indicate that amiloride, which at low concentrations acts as a potent and specific inhibitor of transmembrane Na^+ entry and Na^+, K^+-ATPase at higher concentrations, blocks the Na^+/H^+ exchange pathway and can counteract the effect of amphotericin B in vitro and in vivo. We thus pretreated THP89GFP cells for 2 h with various concentrations of amiloride (3–300 μM) and then stimulated the cells with a suboptimal concentration of amphotericin B (3 μg/ml). EGFP fluorescence as a marker of HIV-1 expression was assessed 48 h poststimulation using flow cytometric analysis. Whereas low concentrations of amiloride (3–10 μM) exerted no effect on amphotericin-B-mediated HIV-1 reactivation, we found that higher concentrations (100–300 μM) decreased the level of HIV-1 reactivation on a population basis by 40% (Fig. 6). At the same time, amiloride had no influence on the level of EGFP fluorescence intensity in the cells with active HIV-1 expression. This finding suggests that alteration of the intracellular ion concentrations, in particular the intracellular pH, may play a role in the observed amphotericin-B-mediated HIV-1 reactivation (Makutonina et al., 1996).

Discussion

In the course of an HIV-1 infection, macrophages are among the first cells to be exposed to the virus (Meltzer and Gendelman, 1992; Meltzer et al., 1990) and later on provide a reservoir for HIV-1 in the lower body as well as in the brain (Bagasra et al., 1996; Clements et al., 2002; Demuth et

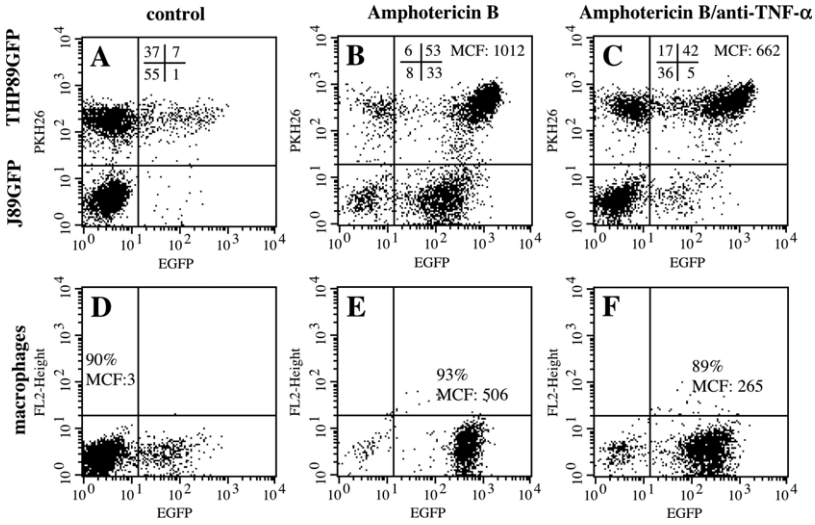

Fig. 5. Trans-reactivation of latent HIV-1 infection in T cells. To allow for analytical separation using flow cytometry, THP89GFP were stained with PKH26 and then cocultured with J89GFP cells for 48 h. (A) Cultures were left unstimulated, (B) treated with amphotericin B (10 μg/ml), or (C) stimulated with amphotericin B in the presence of neutralizing anti-TNF-α antibody. Levels of HIV-1 reactivation under the respective culture conditions were determined by flow cytometric analysis of EGFP expression. Numbers indicate the percentage of cells in each quadrant and the EGFP mean channel fluorescence (MCF). J89GFP cells were cocultured for 48 h with primary macrophages that were differentiated for 5 days in tissue culture. Cultures were (D) left unstimulated, (E) stimulated with amphotericin B, or (F) amphotericin B treated in the presence of neutralizing anti-TNF-α antibody. Levels of HIV-1 reactivation observed in J89GFP cells were determined by flow cytometry (EGFP). Numbers indicate the percentage of EGFP-positive cells and the EGFP MCF. The results are representative for three independent experiments.

al., 2000). The significant contribution of macrophages to ongoing HIV-1 infection has recently been demonstrated in a rhesus monkey/SHIV model (Igarashi et al., 2001), where following systemic depletion of the $CD4^+$ T cell pool that usually sustains virus replication, viremia persisted at levels of 10^6–10^7 RNA copies/ml. This persistence was apparently maintained by infected tissue macrophages in the lymph nodes, spleen, gastrointestinal tract, liver, and kidney. Due to the reported long half-life of macrophages (Spector and Ryan, 1969), the noncytopathic nature of HIV-1 infection in macrophages (Meltzer and Gendelman, 1992; Meltzer et al., 1990; Orenstein et al., 1997), and the relative insensitivity of

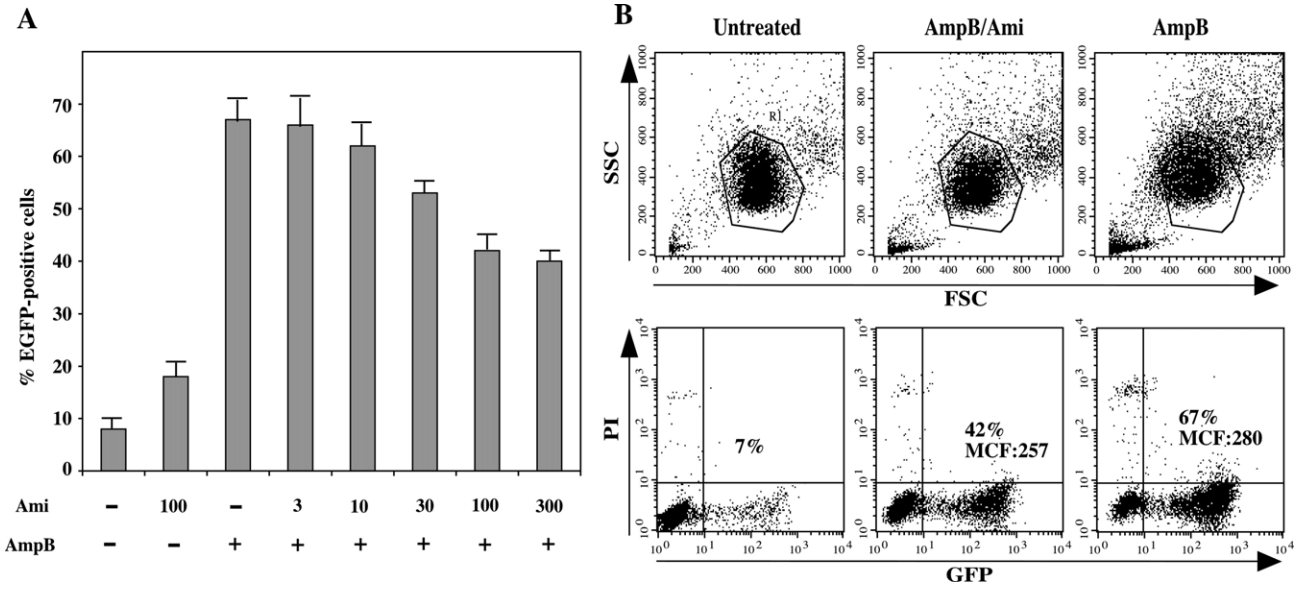

Fig. 6. Influence of amiloride on amphotericin-B-mediated HIV-1 reactivation in THP89GFP cells. (A) THP89GFP cells were left untreated or pretreated for 2 h with various concentrations of amiloride (0–300 μM) and then, where indicated (+), stimulated with a suboptimal concentration of amphotericin B (3 μg/ml). Forty-eight hours poststimulation, levels of EGFP fluorescence were assessed by flow cytometric analysis and are depicted as the percentage of EGFP-positive cells in the culture. The results represent the mean ± SD of three independent experiments. (B) Flow cytometric analysis of THP89GFP cells that remained untreated as negative controls were treated with a combination of amiloride (Ami; 100 μM) and amphotericin B (AmpB; 3 μg/ml), or with amphotericin B alone (3 μg/ml). Forward scatter/side scatter (FSC/SSC) dot plots allow to assess the level of cell viability (cells in the live gate R1), whereas propidium iodide (PI)/EGFP dot plots allow to directly determine the level of active HIV-1 expression in the viable THP89GFP cell population.

macrophages to HIV-1 protease inhibitors (Perno et al., 1998), these cells can propel infection for an extended period of time, eventually even in the presence of antiretroviral therapy. In addition, alveolar macrophages have early on been demonstrated to harbor integrated but transcriptionally silent viral genomes that can be reactivated to produce infectious virus (Lebargy et al., 1994). The possibility that HIV-1 latency can occur in macrophages and may not be limited to memory T cells is further supported by the genotypic discordance between rebounding virus that can be isolated if HAART is halted and HIV-1 isolated from the memory T cell pool (Zhang et al., 2000). Also, the kinetics of virus proliferation following therapy cessation cannot be explained under the assumption that latently infected memory T cells are the sole source of virus (Chun et al., 2000; Davey et al., 1999).

Unfortunately, the precise contribution of infected macrophages to the pool of latently infected cells is difficult to assess. Macrophages, which are readily accessible in the peripheral blood or the lymph nodes, per definition must be activated and should not harbor latent virus, whereas resting macrophages that reside in the body tissues or the brain are usually not accessible for experimental purposes. Irrespective of whether HIV-1 infection in macrophages persists in a latent state or simply in a state of very low level transcriptional activity, novel means to activate infection are required to efficiently target and deplete these cells.

To this end, we here demonstrate that the FDA approved antifungal agent amphotericin B efficiently reactivates latent HIV-1 infection in THP89GFP cells, a model for HIV-1 latency in macrophages (Kutsch et al., 2002, 2003). In contrast, no activating effect of amphotericin B on latent HIV-1 was seen in a T cell model of HIV-1 latency (J89GFP and JNLGFP cells) (Kutsch et al., 2002).

Nevertheless, amphotericin-B-mediated HIV-1 reactivation in T cells could be achieved in trans in cocultures of J89GFP cells with either THP89GFP cells or primary macrophages. In the case of J89GFP/THP89GFP cocultures, the trans-reactivating effect was apparently mostly dependent on membrane bound TNF-α, as addition of neutralizing anti-TNF-α antibodies abrogated HIV-1 reactivation in the T cell line, while supernatant transfer from amphotericin-B-stimulated THP89GFP cells to J89GFP cells did not result in HIV-1 reactivation. In cocultures of J89GFP cells with primary macrophages, HIV-1 reactivation was only partially mediated by amphotericin-B-stimulated TNF-α secretion, as even addition of high concentrations of neutralizing anti-TNF-α antibody to the cocultures could only decrease the level of HIV-1 expression in J89GFP cells, but did not inhibit reactivation on a population level. As we were not able to specify a second soluble factor contributing to HIV-1 reactivation in J89GFP cells in cocultures with primary macrophages, we speculate that interaction of costimulatory receptors such as B7 on the macrophage site and CD28 on J89GFP cells may play a role.

The approach to target latent HIV-1 infection in T cells in trans by activating antigen-presenting cells that confer the reactivation trigger upon cell-to-cell contact adds to the strategies that may be employed to deplete HIV-1 from its latent reservoirs in patients. In addition, activation of potentially existing low-level viral replication in macrophages may also allow for eradication of these cells following immune recognition or would improve the ability of HIV-1-specific immunotoxins to target these cells (Chaudhary et al., 1988; Hamer et al., 2003; McHugh et al., 2002; Root and Hamer, 2003).

Agents such as amphotericin B and its derivate MS-8209 would be particularly suited for this task, as they activate latent or low-level HIV-1 expression, but at the same time have been described to inhibit HIV-1 replication in T cells (Cefai et al., 1991; Otake et al., 1991; Pontani et al., 1989a; Schaffner et al., 1986). However, very little is known how amphotericin B actually exerts its activity in macrophages. It has been reported that amphotericin B complexes membrane cholesterol and increases potassium efflux. However, we demonstrate that amiloride, which at the experimental concentrations used is known to block the Na^+/H^+ exchange pathway, specifically counteracts the HIV-1 reactivating effect of amphotericin B. Although these data suggest that intracellular K^+, Na^+, or H^+ concentrations may actually play a significant role in the observed amphotericin B effect on latent HIV-1, and indeed it has previously been shown that HIV-1 infection induces changes in intracellular K^+, Na^+, or H^+ concentrations (Makutonina et al., 1996; Voss et al., 1996a,b), the data do not allow to draw any conclusion whether changes in the intracellular ion milieu are triggering virus replication or are simply required to maintain viral expression.

Clearly, high-dose amphotericin B treatment as administered, for example, for systemic candidosis, is associated with a variety of side effects. These are usually transient in nature, and McKinsey et al. (1989) have demonstrated that even long-term amphotericin B treatment is rather well tolerated in HIV-1 patients. As for cancer chemotherapy, potential adverse side effects would have to be balanced with the benefits of such a treatment. There are, however, alternatives to the commonly used high-dose treatment. Liposomal formulations of amphotericin B have demonstrated improved tolerability in patients while maintaining full efficacy against the respective fungal agents and the HIV-1 inhibitory capacity of amphotericin B (Pontani et al., 1989a; Reyes et al., 2000). Also, amphotericin B derivates such as MS-8209 seem to be less cytotoxic and could provide treatment alternatives (Clayette et al., 2000). In fact, MS-8209 has been successfully used to treat various prion-related diseases in a variety of animal models (Demaimay et al., 1997, 1999). The ability of MS-8209 to slow down the progress of the brain resident effects of various forms of prion disease in animal models also points towards another major consideration for successful HIV-1 reactivating drugs. As HIV-1-infected macrophages have also been detected in

the brain, the compound's ability to permeate through the blood-brain barrier is potentially important (Clements et al., 2002). Amphotericin B would also fulfill this requirement, as it is commonly used to treat fungus induced forms of meningitis (Coker et al., 1993; Guiloff and Tan, 1992; Hsieh et al., 1998).

In summary, the presented data establish a novel strategy to simultaneously reactivate latent HIV-1 or low-level HIV-1 infection in macrophages and T cells. Generalized activation of antigen presenting cells, here macrophages, achieved in a chemotherapy-like manner, is used to convey cell activation of T cells by cell-to-cell contact, exploiting the basic principle of continuous immune surveillance. Elimination of the cells harboring the previously latent HIV-1 infection would then be assisted by the application of suitable HIV-1-specific immunotoxins. Whether amphotericin B would indeed be a suitable drug candidate for such an endeavor and could be used to develop a treatment strategy for HIV-1 with curative intent remains to be seen, but it clearly possesses the required drug characteristics: (i) capacity of inducing HIV-1 expression; (ii) capacity to induce systemic activation of macrophages; (iii) permeates the blood brain barrier and can thus target brain resident reservoirs of latent HIV-1.

In a monotherapeutic approach, amphotericin B or drugs with similar characteristics will probably not be capable of depleting the entire pool of latently infected cells, but in combination with, for example, low-level IL-2 treatment, the described approach of trans-reactivation should be seriously entertained.

Materials and methods

Cell culture and reagents

THP89GFP cells and J89GFP cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, as previously described (Kutsch et al., 2002).

Macrophages were generated by plating 10×10^6 PBMCs/well in a 6-well plate and culturing the cells in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, 10% human serum, and 10% giant cell medium. On day 5, the nonadherent cells were removed by several stringent washing steps using ice-cold medium. The purity of the obtained macrophage cultures is generally 95% or higher, as determined by fluorescence microscope analysis following staining for CD14.

Amphotericin B was purchased from GibcoBRL (Langley, OK). Neutralizing anti-TNF-α antibody was obtained from Biosource (Camarillo, CA), anti-TNFR I and anti-TNFR II from R&D Biosciences (Minneapolis, MN), and the specific ERK inhibitor U0126 and the noninhibitory control compound U0124 from Calbiochem (San Diego, CA). The live dye PKH26 was purchased from Sigma (St. Louis, MO).

Flow cytometric analysis

Flow cytometric analysis was performed using a FACScan or a FACStarPlus and CellQuest software (Becton Dickinson). If the cells were not stained for the expression of cell surface markers, all samples were treated with propidium iodide to allow for the exclusion of dead cells during data acquisition and analysis.

ELISA

Detection of human TNF-α in the culture supernatants was performed by ELISA exactly to the manufacturer's instructions (Biosource). The sensitivity of the ELISA is >6 pg/ml TNF-α. To determine a potential neutralizing effect of soluble TNF-receptor I (TNFRI) or soluble TNF-receptor II (TNFRII), we performed antibody competition experiments. Ten micrograms of either neutralizing anti-TNFRI or TNFRII antibody was added to 500 μl of supernatants from amphotericin-B-stimulated THP89GFP cells and incubated for 1 h. As both TNF-α and the respective anti-TNFR antibodies compete for the same binding site (epitope), the high affinity antibody releases the receptor-bound TNF-α. Following precipitation of the antibody-TNFR complexes with protein G sepharose beads, the TNF-α concentration of the supernatants was determined by ELISA. The difference in TNF-α concentration between untreated and antibody treated supernatants then allowed for the calculation of the level of TNF-α bound to either soluble TNFR.

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