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High multiplicity HIV-1 cell-to-cell transmission from macrophages to CD4⁺ T cells limits antiretroviral efficacy

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Introduction

Macrophages constitute up to 7% of the HIV-1-infected cell population *in vivo* [1], and their contribution to HIV-1 pathogenesis is increasingly recognized [2] [3]. Multiple lines of evidence support a key role for macrophages in HIV-1 persistence: infected macrophages resist viral cytopathic effects [4], harbour replication competent virus for weeks to months [5], reside in tissues where ART penetration is reduced [6] and are capable of efficient cell-to-cell viral transmission to uninfected CD4⁺ T cells [7] [8].

Cell-to-cell infection increases the probability of escape from antiretroviral inhibition, and has been proposed to account for ongoing replication in the face of ART [9], a potential explanation for viral persistence [10]. However this proposal is controversial since others have suggested that ART has equivalent efficacy against both cell-free and cell-to-cell spread between T cells [11]. A related unanswered question that is central to this debate is whether resistance to antiretroviral inhibition is quantitative, resulting from a greater number of viruses transferred per cell [9], or whether qualitative differences inherent to the mechanism of cell-to-cell infection between different target cell types are involved [12]. The ability of ART to control HIV-1 replication and spread is of central importance to design of ART regimens [13], and has direct relevance to establishment and maintenance of viral reservoirs and potential HIV-1 cure [14]. For this reason, and given the potential importance of the macrophage reservoir [10] together with evidence that infected tissue macrophages persist in patients during ART [15], we sought to establish whether cell-to-cell transmission from MDM to autologous primary CD4⁺ T cells is amenable to antiretroviral inhibition. Our approach contrasts with previous studies that have used highly permissive reporter cell-lines and/or readout of viral Gag transfer between cells that may not accurately represent productive infection in the *in vivo* situation [9, 11, 16].

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Conflicts of interest

No conflicts of interest are declared.

Methods

MDM isolation and infection

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples from healthy HIV-1-uninfected donors by density gradient centrifugation (Histopaque, Sigma) and monocytes were enriched to high purity (> 95% CD14⁺) by untouched magnetic selection (MACS Monocyte Isolation Kit II, Miltenyi Biotech). Monocytes were seeded in 24 or 96 well plates at 5×10^5 cells/mL and differentiated to macrophages for 7 days in X-VIVO 10 (Lonza) medium supplemented with 1% human serum as previously described [7]. MDM were subsequently infected for 7 days [7] with replication-competent infectious molecular clones which stably express *Renilla reniformis* luciferase and macrophage-tropic Env from the BaL or YU-2 isolates [17] in *cis* with all HIV-1 viral proteins in an isogenic NL4.3 backbone (known as NL-LucR.T2A [17], kindly provided by J. Kappes and C. Ochsenbauer). Viral stocks were prepared by 293T transfection with polyethyleneimine and titered on TZM-bl (JC53) cells as described [18].

CD4⁺ T cell isolation and infection and ART treatment

Autologous CD4⁺ T cells were enriched from PBMCs by untouched magnetic selection (MACS CD4⁺ T cell Isolation Kit, Miltenyi Biotech) to high purity (> 95% CD3⁺CD4⁺) and stimulated for 3 days with 1 µg/mL PHA and 10 IU/mL IL-2 (Centre for AIDS Reagents, NIBSC, UK) in RPMI with 10% fetal bovine serum and 1% penicillin/streptomycin (RPMI-10) [7]. Activated CD4⁺ T cells were then pre-incubated for 1 h with non-toxic concentrations of the nucleoside reverse transcriptase (RT) inhibitor azidothymidine (AZT), the non-nucleoside RT inhibitor nevirapine (NVP), the integrase inhibitor raltegravir (RAL, all from Centre for AIDS Reagents, NIBSC) prior to coculture with infected MDM at a donor to target cell ratio of 1:2, or cell-free infection as described [7]. Cocultures were left static or gently shaken at 75 rpm at 37°C at 5% CO₂ [18, 19]. MDM viability under these conditions was assessed with the MTS viability assay (Promega) as previously described [13]. Infections were allowed to proceed for 48 h (approximating a single cycle of replication) during which time antiretroviral drugs were maintained in the medium. At 48 h CD4⁺ T cells were gently removed (<0.1% macrophage contamination determined by flow cytometry staining for CD3 and CD14), washed in PBS and lysed with Glo-Lysis buffer (Promega). For luciferase quantification cell lysates were mixed 1:1 with Ren-Glo assay solution (Promega) at room temperature and luminescence measured at 1000 ms⁻¹ integration on a SpectroMax M5 plate reader. After subtracting background values, data were normalised to untreated controls to derive the transmission index [9] (T_x): $\text{infection}_{\text{drug}}/\text{infection}_{\text{control}}$. Antiretroviral titration data were fitted to a sigmoidal-dose response curve in GraphPad Prism 5.0 to obtain the 50% inhibitory concentration (IC₅₀). Repeat experiments in multiple independent donors were compared using *t*-test, one-way ANOVA with Dunnett's post-test or two-way ANOVA with Tukey's post-test, and two-tailed $\alpha < 0.05$ was considered significant. Statistical analysis was performed in Prism 5.0.

Results

Reduced ART efficacy against cell-to-cell HIV-1 infection of CD4⁺ T cells

We measured luciferase activity to quantify expression of integrated virus in CD4⁺ T cells because this is a direct measure of productive infection not confounded by measuring non-infectious transfer of Gag by flow cytometry, or post-integration latency and/or the presence of nonintegrated viral DNA forms by PCR. To assess the relative efficiency of cell-to-cell and cell-free infection we first cocultured infected MDM and CD4⁺ T cells under gentle shaking conditions (75 rpm) that permit cell-free diffusion but dramatically reduce cell-to-cell transmission by preventing sustained cell-cell contacts [19], and compared the resulting infection levels to parallel co-cultures without gentle shaking. Infections were maintained for 48 h as this approximates a single cycle of infection by both cell-to-cell and cell-free routes [9, 11]. Luciferase production when cell-to-cell transmission was inhibited was approximately 20-fold lower ($P < 0.001$ *t*-test, Fig. 1a), extending our previous observation that cell-to-cell viral transfer from macrophages is considerably more efficient [7]. Shaking had no effect on cell viability as measured by MTS assay (Fig. 1b) as has been reported previously [18, 19]. As expected, antiretroviral agents effectively inhibited conventional low-multiplicity cell-free infection at the approximate maximum plasma concentrations in patients (C_{max}) [20-22]. By contrast, inhibition of luciferase production after cell-to-cell transmission was significantly impaired for all antiretrovirals tested (Fig. 1c), which was reflected in a significantly higher transmission index (T_x , Fig. 1d).

ART efficacy is dependent upon multiplicity of infection of the target cell

We reasoned that the impaired efficacy of ART against cell-to-cell transmission could result from qualitative differences in the mechanism of viral transfer at the macrophage-T cell interface as a result of virological synapse formation [12], or might simply reflect a higher HIV-1 MOI by direct cell-to-cell spread compared to cell-free spread [9]. To address this, we first infected CD4⁺ T cells with increasing MOI cell-free inocula in the presence or absence of AZT, which resulted in a stepwise increase in T_x , reflecting the reduced probability of inhibition with increased virus challenge (Fig. 2a). To directly address the effect of MOI under cell-to-cell transmission conditions, we titrated initial macrophage inocula to yield equivalent T cell infection by the cell-free and cell-to-cell routes (Fig. 2b and POS control in Fig. 2c). When MOI was controlled in this way, we observed that inhibition by antiretroviral drugs was equally effective by either route (Fig. 2c-d). Under these conditions, titration of ART in both cell-to-cell and cell-free infections demonstrated that the effectiveness of inhibition is not influenced by the mode of viral transmission across a range of antiretroviral concentrations (Fig. 2e-h).

Discussion

These findings have important implications for HIV-1 treatment and eradication strategies. Available evidence suggests that the enhanced efficiency of cell-to-cell transmission derives from polarized budding of HIV-1 from the donor cell towards the target cell [23], viral receptor clustering on the target cell [7, 24] and the close proximity of donor and target cells at the virological synapse [7, 12, 18]. If cell-to-cell infection were qualitatively drug-

insensitive, the implication is that efforts to drive transcription from latently infected cells under ART would fail due to uninhibited expansion of the infected cell population [9]. However, our data demonstrate that cell-to-cell infection is equally susceptible to antiretroviral inhibition when the target cell viral challenge ‘dose’ is equivalent, suggesting that the increased efficiency is not qualitative, but quantitative. This agrees with recent data on productive T cell-to-T cell spread [9, 11, 25] and supports the concept that aspects of HIV-1 cell-to-cell transmission relevant to antiretroviral activity are qualitatively similar to cell-free infection [12]. However the precise mechanisms governing high multiplicity macrophage HIV-1 transmission, particularly the synaptic molecular architecture and the influence of target cell/viral phenotype, require further definition.

In this and other model systems MOI appears to be the principal *in vitro* determinant of antiretroviral activity in the absence of resistance mutations [9, 11]. The extremely high titer extracellular concentrations of virus needed to saturate antiretroviral activity are unlikely to be reached *in vivo* by cell-free spread, where virion infectivity is limited by fluid phase dilution and diffusion coupled with rapid Env degeneration [12]. By contrast, sufficiently high multiplicities might be achieved in tissues in which target cells are densely packed, cell-to-cell transmission occurs [26, 27], and antiretroviral penetrance is reduced [6].

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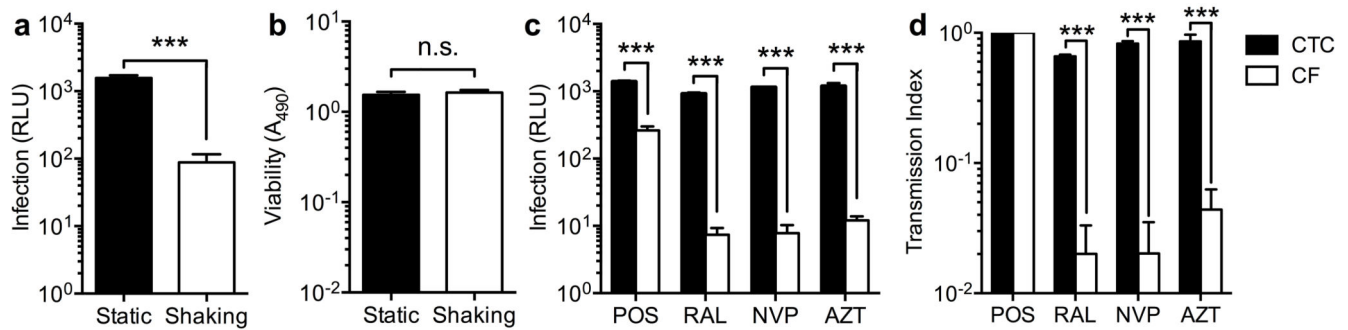


Figure 1. Reduced ART inhibition of CD4⁺ T cell infection by cell-to-cell spread from MDM compared to cell-free infection

(a) CD4⁺ T cells were infected by coculture with MDM infected with HIV-1-LucR in either static (cell-to-cell spread) or gently shaking cultures (cell-free spread), T cells gently washed off and infection quantified by assay of *Renilla* luciferase activity (relative light units, RLU) in T cell lysates. Shaking culture resulted in productive CD4⁺ T cell infection above background in all donors ($P=0.036$ *t* test, not shown). Bars represent mean \pm S.E.M. of experiments in $n=4$ independent donors, *** $P<0.001$ *t* test. (b) Gentle shaking for 48h did not affect MDM viability by MTS assay; bars represent mean \pm S.E.M. of experiments in $n=5$ independent donors, n.s. = non-significant by *t* test. (c) The reverse transcriptase (RT) inhibitors azidothymidine (AZT, 10 μ M) and nevirapine (NVP, 10 μ M) and the integrase inhibitor raltegravir (RAL, 2 μ M) reduced cell-free HIV-1 infection of CD4⁺ T cells robustly, but showed minimal impact on luciferase expression during cell-to-cell transmission. Bars represent mean \pm S.E.M. of experiments in $n=3$ independent donors, *** $P<0.001$ two-way ANOVA plus Tukey's post-test. (d) Antiretroviral inhibition efficiency was defined by the transmission index (T_x): $\text{infection}_{(\text{drug})}/\text{infection}_{(\text{no drug})}$. Cell-to-cell transmission reduces ART efficacy and T_x approaches 1, whereas conventional cell-free infection results in a significantly lower T_x . Bars represent mean \pm S.E.M. of data from (b), *** $P<0.001$ two-way ANOVA plus Tukey's post-test.

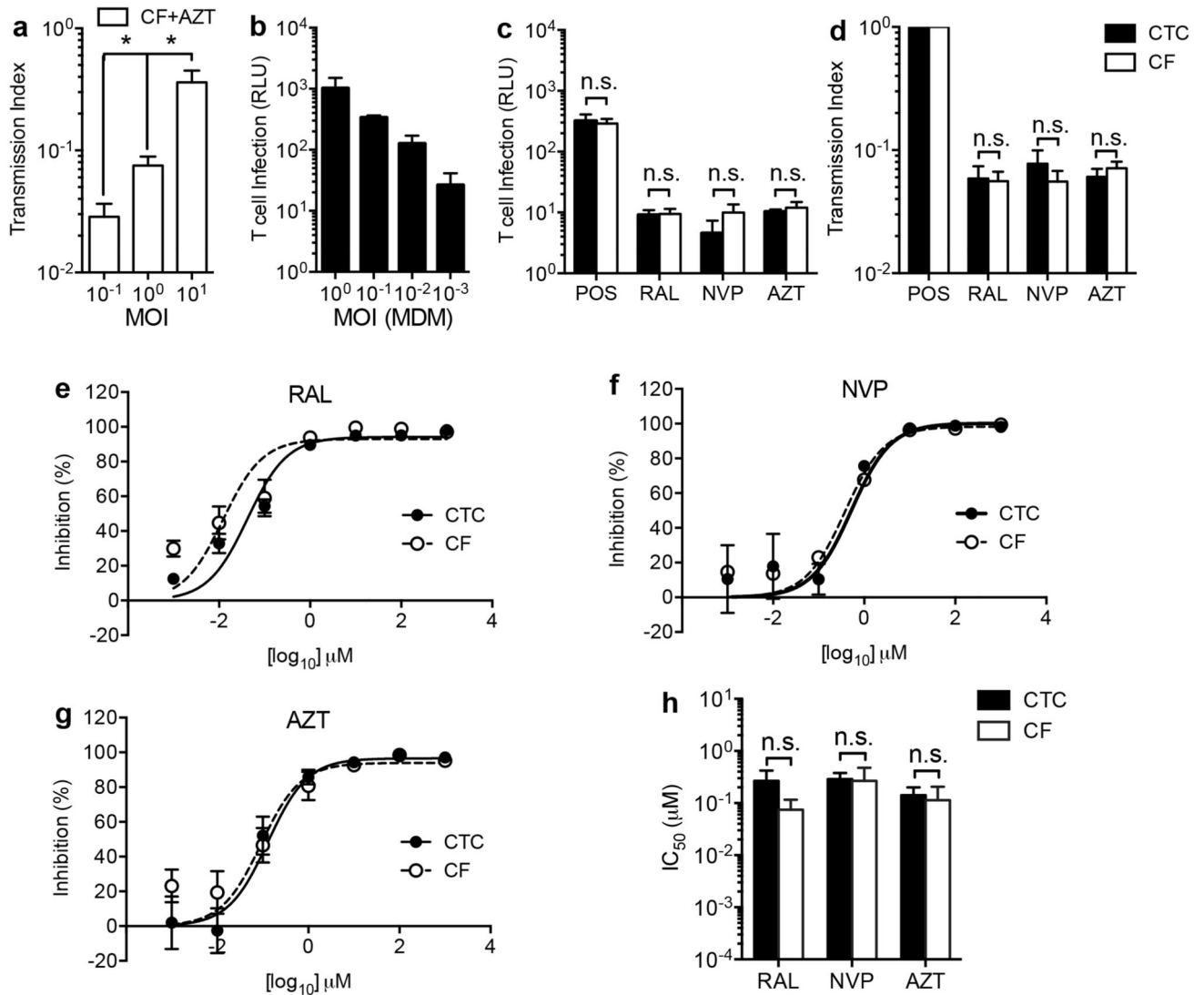


Figure 2. ART efficacy is dependent upon multiplicity of infection of the target cell

(a) Increasing MOI of CD4⁺ T cell infection with cell-free HIV-1 ± 10 μM AZT results in a stepwise increase in *Tx*. Bars represent mean ± S.D. of experimental replicates from a representative experiment in n=1 donor, *P<0.05 one-way ANOVA plus Bonferonni post-test. (b) Titration of MDM HIV-1 inocula permitted control of CD4⁺ T cell infection levels in the cell-to-cell (CTC) conditions to achieve equal multiplicity to cell-free (CF) conditions (see untreated positive control, POS in (c)). Bars represent mean ± SEM of experiments in n=3 donors. (c) Titration of CTC infections in turn yielded equivalent levels of antiretroviral inhibition of luciferase expression at approximate *in vivo* C_{max} concentrations (RAL 2 μM, AZT and NVP 10 μM,); bars represent mean ± SEM of experiments in n=5 independent donors; n.s. = non-significant by two-way ANOVA plus Tukey's post-test. (d) Equalising MOI of cell-free and cell-to-cell infection resulted in equivalent *Tx*; Bars represent mean ± S.E.M. of data from (c); n.s. = non-significant by two-way ANOVA plus Tukey's post-test.

Titration of antiretroviral agents RAL (**e**), NVP (**f**) and AZT (**g**) resulted in equivalent IC_{50} values (**h**, inhibition % expressed as $100 \times (1 - [\text{luciferase}_{\text{drug}}/\text{luciferase}_{\text{control}}])$). Lines are best-fit sigmoidal dose-response curves, bars represent mean \pm SEM of experiments in n=3 (CF) and n=5 (CTC) independent donors. No significant difference in IC_{50} was observed by two-way ANOVA plus Tukey's post-test (n.s. = non-significant).