A new model for post-integration latency in macroglial cells to study HIV-1 reservoirs of the brain

Martha Schneider, Bianca Tigges, Manja Meggendorfer, Markus Helfer, Christoph Ziegenhain and Ruth Brack-Werner

Objective: Macroglial cells like astrocytes are key targets for the formation of HIV-1 reservoirs in the brain. The 'shock-and-kill' HIV-1 cure strategy proposes eradication of reservoirs by clinical treatment with latency reversing agents (LRAs). However, virus activation may endanger the brain, due to limited cell turnover, viral neurotoxicity and poor penetration of antiretroviral drugs. Since the brain is not accessible to clinical sampling, we established an experimental model to investigate the LRA effects on HIV-1 latency in macroglial reservoirs.

Design: Human neural stem cells (HNSC.100) were used to generate a system that models HIV-1 transcriptional latency in proliferating progenitor, as well as differentiated macroglial cell populations and latency-modulating effects of LRAs and compounds targeting HIV-1 transcription were analysed.

Methods: HNSCs were infected with pseudotyped Env-defective HIV-1 viruses. HIV-1 DNA and RNA levels were quantified by qPCR. Expression of latent GFP-reporter viruses was analysed by confocal microscopy and flow cytometry. NF-κB signalling was investigated by confocal microscopy and chromatin immunoprecipitation.

Results: Two of the eight well known LRAs (tumour necrosis factor-alpha, suberoylanilide hydroxamic acid) reactivated HIV-1 in latently infected HNSCs. Tumour necrosis factor-alpha reactivated HIV-1 in progenitor and differentiated populations, whereas suberoylanilide hydroxamic acid was more potent in progenitors. Pre-treatment with inhibitors of key HIV-1 transcription factors (NF-κB, Cdk9) suppressed HIV-1 reactivation.

Conclusion: We conclude that latent HIV-1 in macroglial reservoirs can be activated by selected LRAs. Identification of small molecules that suppress HIV-1 reactivation supports functional cure strategies. We propose using the HNSC model to develop novel strategies to enforce provirus quiescence in the brain.

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Introduction

HIV-1 reservoirs prevent eradication of HIV-1 from infected individuals. They arise at a very early clinical phase and also occur in patients that initiated early antiretroviral therapy (ART) [1,2]. Virus reservoirs are long-lived cells with persisting HIV-1 genomes which support chronic viraemia even in optimally treated patients with undetectable plasma virus levels. Latently infected reservoirs contain silenced virus genomes that can be reactivated by extracellular stimuli or stochastic events. Resting memory $CD4^+$ T cells are currently the best characterized virus reservoirs, but other cells of the immune system like proliferating T cells [3], macrophages, monocytes or haematopoietic stem cells [4,5] can also form HIV-1 reservoirs. In addition to the immune

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system, the virus can also invade the brain within a few weeks after primary infection, presumably via the blood–brain barrier [6,7] and can persist in this organ lifelong [8,9]. HIV-1 infection can lead to HIV-associated neurocognitive disorders (HANDs), with a prevalence of up to 50% of HIV-1-infected individuals, irrespective of HAART [10]. Restricted immunological surveillance and longevity of brain cells favour establishment of virus reservoirs in the brain. Ex-vivo studies of brain tissues from HIV-1-infected individuals, as well as studies with cultured human brain cells, demonstrate HIV-1 infection of various brain cell types [8,11–14]. Productive infection was shown for brain macrophages, which include perivascular and non-resident macrophages, as well as resident microglia. In addition, macroglial cells like astrocytes and neural progenitor cells were shown to contain proviral DNA, although these cells rarely displayed HIV-1 production markers. Up to 19% of the astrocyte population in neurocognitively impaired individuals contained HIV-1 DNA [11], amounting to a substantial population of HIV-1-positive cells, considering the abundance of human astrocytes (total number estimated to be $>17 \times 10^9$ cells [15] and Pelvig *et al.* [16]). Early infection of astrocytes was demonstrated in macaques exposed to simian immunodeficiency virus [17], and numerous cell culture studies confirmed persistence of HIV-1 in astrocytes and neural progenitor cell populations [13,18,19]. Infected astrocytes exposed to pro-inflammatory cytokines were capable of releasing infectious virus into the culture supernatant [20] and of transmitting virus to T cells and monocytes [13]. Together, these findings suggest a key role for astrocytes and their precursors as reservoirs with latent HIV-1 in the brain.

Currently, the ‘shock-and-kill’ strategy is under intensive discussion for eliminating virus reservoirs. This strategy proposes killing of reservoir cells by compound-induced virus reactivation, combined with HAART intensification, to prevent de-novo infection [21,22]. Potential effects of ‘shock-and-kill’ strategies on HIV-1 reservoirs in the brain are still unknown. This issue is extremely critical for several reasons [23], including potential neurotoxicity of HIV-1 proteins [24,25]; poor penetration of antiretroviral drugs into the brain [26] and loss of irreplaceable cells in the brain [23].

Studies with $CD4^+$ T-cell models of HIV-1 latency [27–29] have identified several pharmacological agents for activation of latent HIV-1 [28,30], as well as complex mechanisms involved in suppressing HIV-1 expression during latency in $CD4^+$ T cells [31]. Whereas a model for latent HIV-1 infection in microglial cells has been established [32], a model for easy monitoring of HIV-1 latency in macroglial cells of neuro-ectodermal lineage is still lacking.

Here, we report the establishment of a model for transcriptional HIV-1 latency in neuro-ectodermal brain cells, using a human neural stem cell (HNSC) line that can be cultured as self-renewing progenitor populations, as well as differentiated astrocyte-enriched cell populations [19,33]. We show that selected latency reversing agents (LRAs) can increase virus expression in the HNSC model and provide proof of concept for inhibition of HIV-1 reactivation in macroglial brain reservoirs by treatment with small molecules. Thus, we propose incorporation of the HNSC model for the development of HIV-1 cure strategies.

Materials and methods

Cells

HNSC.100 is a clonal cell line established from human fetal neural stem cells [34,35]. HNSC cells were cultivated on poly-L-lysine-coated plastic ware and incubated at 37°C and 5% CO_2. Basal medium for proliferating HNSC.100 consisted of Dulbeccos Modified Eagle Medium (DMEM) F-12, 1% bovine serum albumin (BSA), 1× N2 supplement, 5 mmol/l HEPES, 0.5% fetal calf serum (FCS) (Gibco, Darmstadt, Germany). 1% PenStrep (Sigma–Aldrich, Taufkirchen, Germany), 20 ng/ml basic fibroblast growth factor 2 (FGF-2) and 20 ng/ml epidermal growth factor (EGF) (TebuBio, Offenbach, Germany). For differentiation, HNSC.100 cells were incubated for at least 2 weeks in the basal medium without growth factors. All other cells are described in the supplemental digital content (SDC) 1 (http://links.lww.com/QAD/A694).

Virus production

Vesicular stomatitis virus (VSV)-G protein (VSV-G)-pseudotyped lentiviral particles were produced in human embryonic kidney (HEK) 293T cells co-transfected with pSG3.1Δenv or pNL4-3-deltaE-EGFP and pMD2.G, using Extreme Gene HP (Roche, Mannheim, Germany) according to the manufacturer’s protocol (for plasmid details, see SDC 1). Virus-containing supernatants were harvested 72 h after transfection and concentrated with Amicon Ultra 100 K centrifugal filters (Millipore, Darmstadt, Germany). Virus preparations were diluted with HNSC basal medium, and viral titres were determined by HIV-1 p24 antigen capture assay according to the manufacturer’s protocol (ABL Inc., Rockville, Maryland, USA).

Infections

HNSC.100 cell lines were exposed to VSV-G-pseudotyped SG3.1Δenv virus particles at 2.2 pg p24/cell for 48 h, or to NL4-3-deltaE-EGFP virus particles at 0.4 pg p24/cell were treated as described for 6 h. Subsequently, the cells were washed twice with medium to remove inoculum, and incubation was continued in fresh medium. Cells were harvested and stored at −20°C for isolation of nucleic acids or fixed with 2% paraformaldehyde (PFA) overnight at 4°C for flow cytometry analysis.

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Trans-complementation assay

HNSCLatGFP1.2 cells were transfected with pMD2.G or a standard yellow fluorescent protein (YFP)-expressing plasmid, using Extreme Gene HP (Roche) as transfection reagent. Twenty-four-hour post-transfection, the medium was changed, and the cells were treated with compounds for 24 h before the medium was changed again. After additional 24 h, supernatants were collected and the cells were harvested for flow cytometry analysis. The p24 levels in the supernatants were determined by HIV-1 p24 antigen capture assay according to the manufacturer's protocol (ABL Inc.). To detect infectious virus, 20 μl of HNSC supernatants were used to inoculate 1×10^4 LC5-RIC cells. After 48 h, the red fluorescence signal was measured with a Tecan infinite M200 (Tecan, Männedorf, Switzerland) (for details see [36])

Flow cytometry analysis

Flow cytometry analysis was performed with a FACS CANTO II and the FACSDiva software (BD Bioscience, Heidelberg, Germany) using PFA-fixed cells.

Quantitative polymerase chain reaction (qPCR)

Levels of HIV-1 DNA or transcripts were quantified by quantitative polymerase chain reaction (qPCR) with the Roche LightCycler 1.5 System, using LightCycler FastStart DNA Master SYBR Green I-Kit and standard LightCycler software (Roche Diagnostics, Mannheim, Germany). RNA levels were quantified by relative qPCR with cDNA generated by reverse transcription with specific primers for HIV-1. HIV-1 DNA loads were determined by absolute qPCR quantification, using the TH4-7-5 cell line as external standard (for details and primer sequences, see SDC 1).

Immunofluorescent stainings and confocal microscopy

HNSCLatGFP1.2 cell lines were seeded on poly-L-lysine-coated glass cover slips. For detection of intracellular localization of p65, cells were left untreated or treated with nuclear factor-kappaB (NF-κB) inhibitors for 2 h before 10 ng/ml tumour necrosis factor (TNF)-alpha was added for 30 min. For GFP expression analysis, cells were left untreated, treated with 10 ng/ml TNF-alpha or 5 μmol/l suberoylanilide hydroxamic acid (SAHA) for 30 h. After treatments, cells were washed and fixed with 2% PFA for 4°C overnight before immunofluorescent stainings were performed as described in SDC 1. Localization of p65 or GFP expression was then analysed by fluorescence microscopy (Nikon TiE equipped with Perkin Elmer UltraView Vox System) using the Volocity 6.2.1-software (Perkin Elmer, Rodgau, Germany) (for details, see SDC 1).

Treatments with test compounds

TNF-alpha, Withaferin A (WTA), valproic acid (VPA), sodium butyrate (NaB), Prostratin A (Pro), 5-azacytidine (5-aza), Trichostatin A (TSA), phorbol 12-myristate 13-acetate (PMA), ammonium pyrrolidinedithiocarbamate (PDTC) and BMS-345541 were purchased from Sigma–Aldrich. SAHA/Vorinostat and Flavopiridol were obtained from the NIH AIDS Reagents Program (Cat. Nr.: 12130 and 9925). Bortezomib/Velcade (Biomol, Hamburg, Germany), R-Roscovitine (Adipogen AG, San Diego, California, USA), Fascaplysin (Santa Cruz Biotechnology, Heidelberg, Germany), PHA767491 hydrochloride (Tocris Bioscience, Wiesbaden, Germany) and all other substances were solved in DMSO and stored at −20°C. TNF-alpha was solved in PBS. LRAs were used at concentrations reported to reverse latency in HIV-1 latency immune cell models (compiled in Table S1, SDC2, http://links.lww.com/QAD/A694). Inhibitory compounds were used at concentrations reported to be effective in other studies (see Tables S2, SDC2, http://links.lww.com/QAD/A694 and S£, SDC2, http://links.lww.com/QAD/A694). Cell viabilities in treated proliferating and differentiated HNSCLatGFP1.2 populations were determined by MTT assay as described in Kremb *et al.* [36] and were generally at least 80% (see Fig. S1). Toxicities of compounds during J-Lat 8.4 treatment were determined by CellTox Green Cytotoxicity Assay (Promega, Mannheim, Germany) and were 20% or less (Fig. S2).

Chromatin immunoprecipitation assays

HNSCLatGFP1.2 and J-Lat 8.4 cells were treated with 10 ng/ml TNF-alpha or were left untreated for 30 min before chromatin was prepared from 1×10^7 cells. Chromatin immunoprecipitation (ChIP) assays were performed with the Imprint Chromatin Immunoprecipitation Kit (Sigma–Aldrich) according to the manufacturer's protocol (for details, see SDC 1).

Statistical analysis

Statistical analysis was performed with Prism software (Graph Pad Software, San Diego, California, USA) using the one-way analysis of variance (ANOVA) test for pairwise comparisons of selected columns referring to values of differently treated/differentiated cells to values of untreated/proliferating cells or values of untreated, reactivated samples to treated, reactivated cells, respectively. Statistical significance was also expressed (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; **** $P < 0.0001$; NS = not significant).

Results

Establishment of human neural stem cell progenitor populations with transcriptionally silenced HIV-1

To generate an experimental model for HIV-1 latency, proliferating HNSC.100 populations were infected with VSV-G-pseudotyped envelope (Env)-defective HIV-1.

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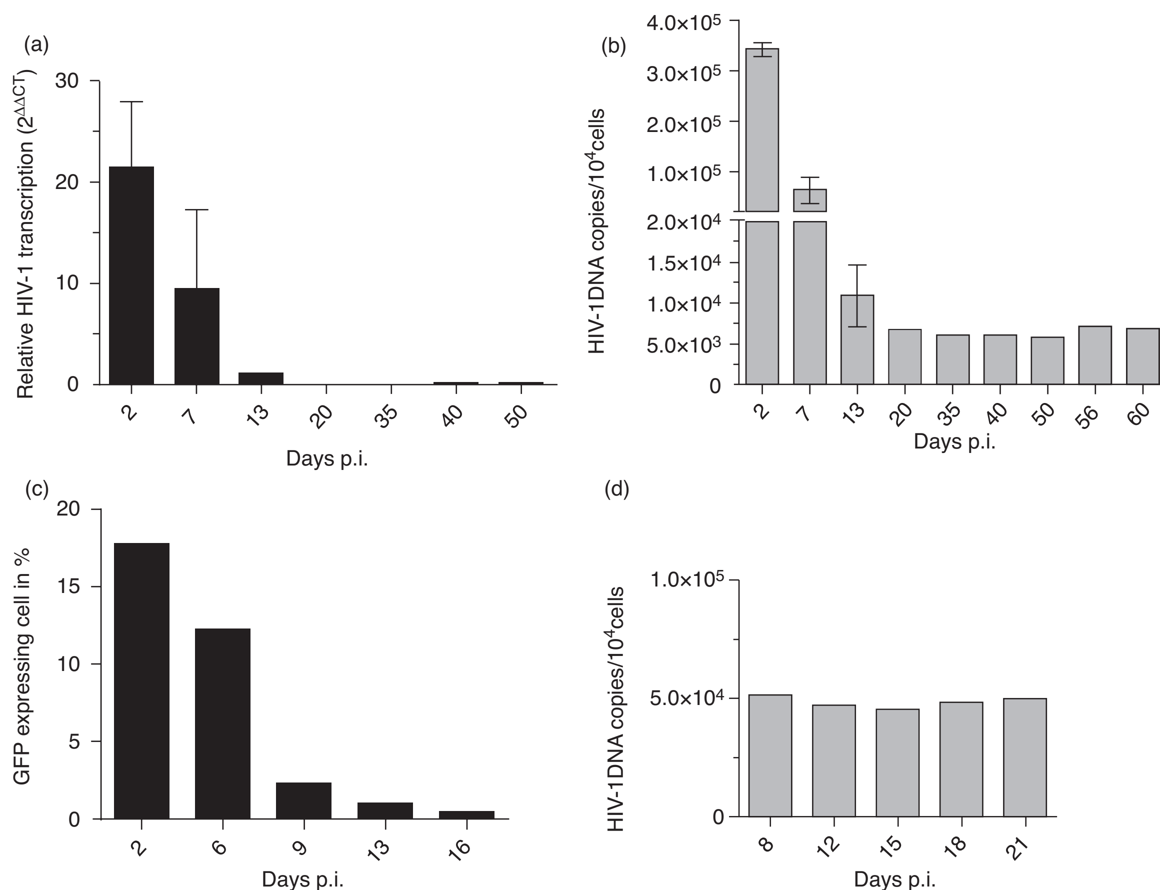

Fig. 1. Establishment of stable human neural stem cell progenitor populations with transcriptionally silenced HIV-1 genomes. Human neural stem cell populations with stable HIV-1 genomes were established by exposing proliferating HNSC.100 cells to VSV-G-pseudotyped virus particles containing SG3.1ΔEnv (a, b) or the HIV-1 reporter virus NL4-3-deltaE-EGFP genomes (c, d), resulting in HNSCLat and HNSCLatGFP1.2 populations, respectively. HIV-1 infection of HNSCLat populations was monitored by qPCR analysis of HIV-1 RNA (a) and DNA levels (b) at different time points post-infection (p.i.). HNSCLatGFP1.2 populations were analysed for GFP-expressing cells by flow cytometry (c) and for levels of HIV-1 DNA by qPCR (d). Data are from one infection experiment for each latent cell population, with columns indicating mean values for each time point. Standard errors are shown for the mean of triplicate samples. HNSC, human neural stem cell.

Monitoring quantities of HIV-1 nucleic acids for over 50 days in HNSC populations infected with SG3.1ΔEnv (HNSCLat) showed that HIV-1 RNA levels decreased to undetectable levels within 13 days, whereas HIV-1 DNA levels remained stable from day 20 onward (Fig. 1a, b). Similarly, HNSC.100 populations infected with the green fluorescent protein (GFP)-reporter virus NL4-3-deltaE-EGFP (HNSCLatGFP1.2) showed declining proportions of GFP-positive cells, whereas the copy numbers of HIV-1 DNA again remained stable (Fig. 1c, d). These results confirm generation of HNSC.100 populations with persisting, quiescent HIV-1 proviruses.

Effects of latency reversing agents on provirus quiescence in human neural stem cell progenitor populations

To investigate reactivation of HIV-1, HNSCLat and HNSCLatGFP1.2 were exposed to a panel of potential LRAs at concentration reported to reactivate HIV-1 in latently infected T cells (Table S1, SDC2). Initial experiments showed that exposure to TNF-alpha or SAHA clearly increased GFP expression in HNSCLatGFP1.2 cultures (Fig. 2a, b). In contrast, treatment with other compounds, including several histone deacetylase inhibitors (NaB, VPA and TSA), protein kinase C agonists (Pro, PMA) and the DNA methyltransferase inhibitor 5-aza led to no or only very low HIV-1 activation in both HNSCLatGFP1.2 and HNSCLat populations (Fig. 2b, c).

Next, we investigated possible synergistic effects of the compounds. Co-treatment of HNSCLatGFP1.2 with TNF-alpha and SAHA resulted in about 50% GFP-positive cells, compared to approximately 10% obtained by treatment with the single compounds (Fig. 2d). This indicated that at least 50% of the HNSCLatGFP1.2 population contained inducible HIV-1 proviral DNA. Synergistic effects were also observed for co-treatment

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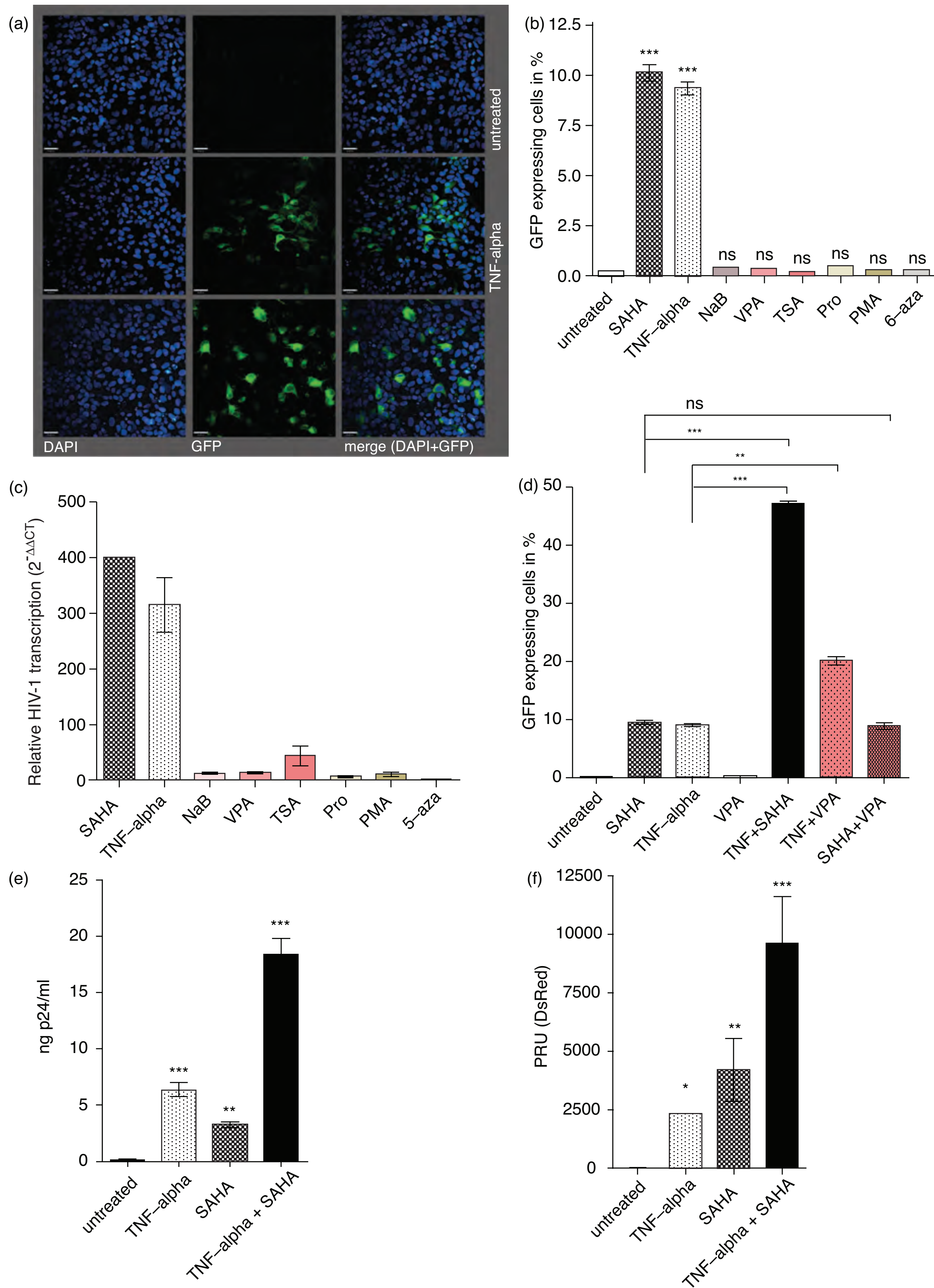

Fig. 2. Effects of latency reversing agents on provirus quiescence in progenitor HNSCLatGFP1.2 populations. Proliferating HNSCLatGFP1.2 (a, b, d, e, f) or HNSCLat (c) were treated with SAHA (5 μmol/l), TNF-alpha (10 ng/ml), NaB (500 μmol/l), VPA (1 mmol/l), TSA (100 nmol/l), Pro (1 μmol/l), PMA (50 ng/ml) or 5-aza (1 μmol/l) alone or in the combinations indicated and analysed for HIV-1 expression in comparison to untreated controls. (a) Detection of GFP-expressing cells. HNSCLatGFP1.2

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with TNF-alpha and VPA, although VPA treatment alone did not activate HIV-1 expression. Co-treatment with TNF-alpha and SAHA also markedly increased levels of Gag-p24 in the culture supernatant of HNSCLatGFP1.2 cells, confirming production of virus-like particles (Fig. 2e). Release of infectious virus particles by HNSCLatGFP1.2 cells transfected with plasmids directing expression of VSV-G envelope proteins was also measurable upon co-treatment (Fig. 2f).

These results demonstrate differential effects of known LRAs on latent HIV-1 in HNSC populations.

HIV-1 latency in differentiated human neural stem cells

To investigate HIV-1 latency in differentiated HNSC populations, HNSCLatGFP1.2 were cultured without growth factors for at least 2 weeks. Differentiation was verified by up-regulated expression of the astrocyte marker GFAP (Fig. S3, SDC3) and by termination of cell proliferation beginning at day 13 after growth factor withdrawal (Fig. 3a). qPCR analysis confirmed that, after growth factor withdrawal (Fig. 3b) HIV-1 DNA levels remained constant over time (Fig. 3b). Treatment of differentiated HNSCLatGFP1.2 with the same panel of LRAs and under the same conditions used to test activation of HIV-1 latency in proliferating HNSCLatGFP1.2 populations revealed elevated GFP expression only after TNF-alpha treatment (Fig. 3c, d). In agreement, HIV-1 transcript levels in differentiated HNSCLat were strongly increased by TNF-alpha treatment, whereas SAHA had only moderate effects on transcript levels, and VPA was ineffective to induce HIV-1 transcription (Fig. 3e). Combined treatment of HNSCLatGFP1.2 with TNF-alpha and SAHA yielded similar GFP induction levels as single treatment with TNF-alpha (Fig. 3f), indicating that TNF-alpha and SAHA do not synergistically activate HIV-1 in differentiated HNSC populations. These results show that HNSC populations maintain latent HIV-1 proviruses after differentiation, and are less responsive to HIV-1 reactivation by SAHA than progenitor populations.

Involvement of the NF-κB pathway in tumor necrosis factor-alpha-mediated HIV-1 induction in latently infected human neural stem cell populations

Activation of the NF-κB pathway with binding of p65/RelA to NF-κB-binding sites in the HIV-1 ong terminal repeat (LTR) is a key feature of HIV-1 transcription initiation in latently infected T cells [31,37,38]. Since TNF-alpha is an inducer of the NF-κB pathway, we elucidated a possible involvement of NF-κB in HIV-1 activation in HNSCLatGFP1.2 by investigating nuclear translocation and binding of p65 to the HIV-1 in TNF-alpha-treated cells. Immunofluorescence analysis revealed that TNF-alpha treatment strongly increased nuclear localization of p65, compared to untreated cells (Fig. 4a). Next, we compared levels of p65 associated with HIV-1 LTR sequences in TNF-alpha-treated and untreated HNSCLat and HNSCLatGFP1.2 populations by ChIP assays. TNF-alpha treatment clearly increased the association of p65 to the HIV-1 LTR compared to untreated cells (Fig. 4b, c). These results confirmed that TNF-alpha treatment of latently infected HNSC populations activated the NF-κB signalling pathway and increased association of p65 with the HIV-1 LTR.

Small molecule inhibitors of NF-κB signalling and Cdk9 diminish activation of latent HIV-1

Since TNF-alpha-mediated reactivation of HIV-1 in HNSC populations depends on NF-κB signalling, we investigated whether NF-κB inhibitors can prevent TNF-induced HIV-1 activation. To this end, HNSCLatGFP1.2 cells were treated with NF-κB inhibitors (BMS-345541; Bortezomib, PDTC and Withaferin A) for 2−4 h (for

cultures were treated with the LRAs for 30 h, and GFP-expressing cells were visualized by confocal microscopy. Shown are representative images of DAPI stainings of nuclei (blue, panels in left column), GFP expression (green, middle column) and merge (right column) of untreated (panels in upper row), TNF-alpha-treated (middle row) and SAHA-treated (lower row) cells. Exposure times: GFP 390 ms, DAPI 175 ms. Scale bar: 36 μm. (b, d) Quantification of proportions of GFP-expressing cells. HNSCLatGFP1.2 populations were treated with different LRAs for 30 h, and GFP expression was analysed by flow cytometry. Columns represent the means ± standard errors of at least three independent experiments (8 replicates). (c) Quantification of HIV-1 transcript levels. RNA was isolated from HNSCLat treated with LRAs for 24 h, cDNA prepared and HIV-1 transcripts quantified by relative qPCR. The data are expressed as fold enrichment of HIV-1 RNA levels in treated versus untreated cells. Columns represent the means ± standard errors of three independent experiments (in triplicate). (e) Quantification of Gag-p24 production. Levels of Gag-p24 were determined in supernatants of HNSCLatGFP1.2 cells cultured for 24 h after treatment with LRAs. Columns represent the means ± standard errors of five independent experiments performed in triplicate. (f) Levels of infectious virus in supernatants of HNSCLatGFP1.2 complemented with VSV-G envelope proteins. HNSCLatGFP1.2 populations were transfected with the VSV-G expression plasmid pMGD2. After removing the transfection reagent, cells were treated with the indicated LRAs for 24 h, the LRA-containing medium was removed and the culture continued for an additional 24 h. Levels of infectious virus particles in 20 μl of HNSC culture supernatants were determined with the HIV-1 indicator cell line LC5-RIC as described [36]. Columns represent the means ± standard errors of four independent experiments performed in triplicate showing relative fluorescence units above background fluorescence. 5-aza, 5-azacytidine; HNSC, human neural stem cell; LRAs, latency reversing agents; NaB, sodium butyrate; PMA, phorbol 12-myristate 13-acetate; Pro, Prostratin A; SAHA, suberoylanilide hydroxamic acid; TNF, tumour necrosis factor; TSA, Trichostatin A; VPA, valproic acid.

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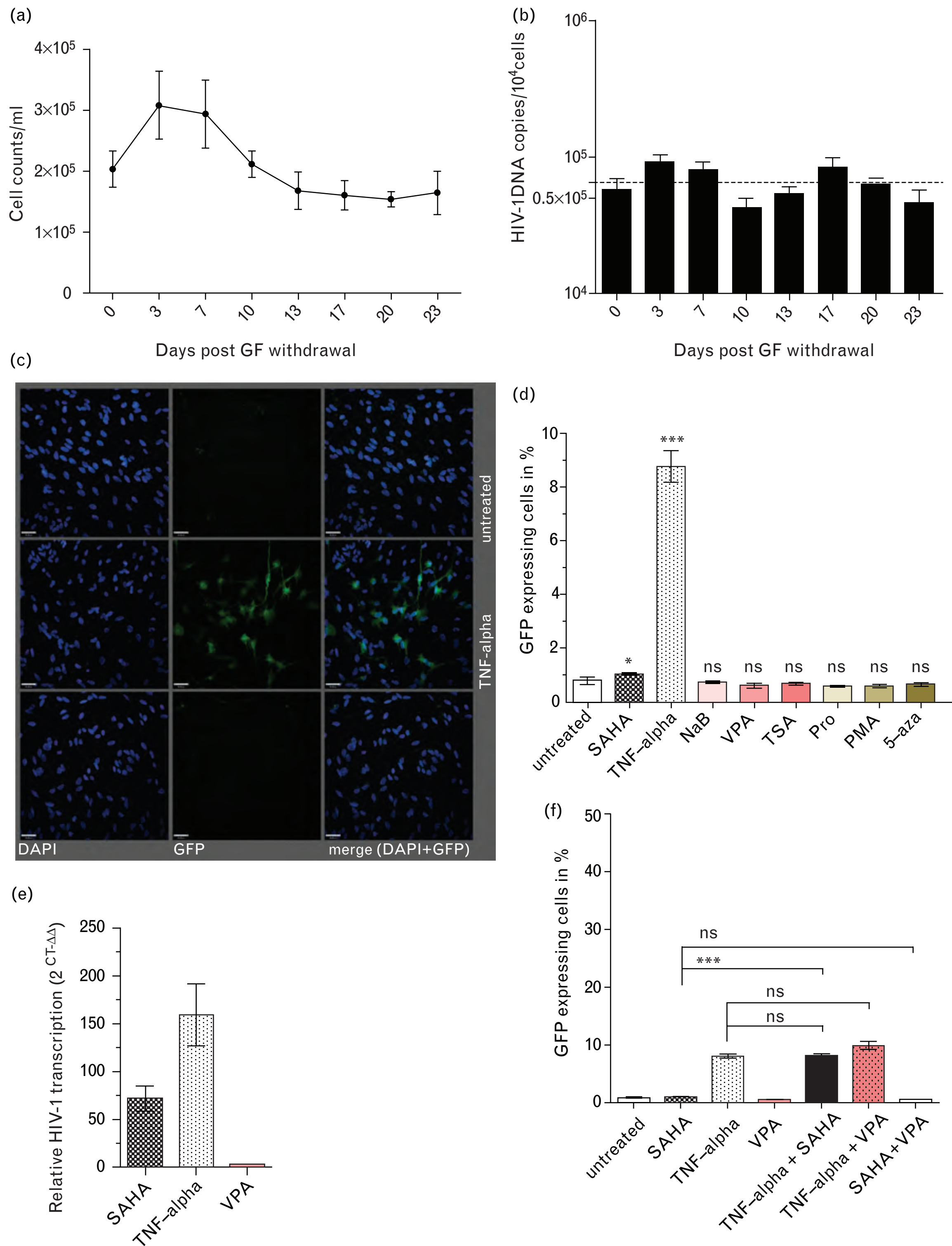

Fig. 3. Maintenance of HIV-1 latency in differentiated human neural stem cells and effects of treatment with latency reversing agents on HIV-1 expression. HNSCLatGFP1.2 populations were differentiated by culture without growth factors (GFs) for 23 days. Cell numbers (a) and HIV-1 DNA copy numbers (b) were monitored during the differentiation process. Terminally differentiated HNSCLatGFP1.2 (day 23 of culture without GFs) were exposed to various LRAs, and effects on HIV-1 expression were examined (c–f). (a) Cells were harvested and counted (cell counts/ml) at the indicated time points after growth factor withdrawal. Dots represent the means ± standard errors of three different cell samples. (b) Cells were harvested at different time points, genomic DNA isolated and proviral copies/cell determined by absolute qPCR. Columns represent the means ± standard errors of three samples. (c) Differentiated HNSCLatGFP1.2 populations were treated with SAHA (5 μmol/l) or TNF-alpha (10 ng/ml) for 30 h, fixed

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concentrations, see Table S2, SDC2) before NF-κB was activated by TNF-alpha. Immunofluorescent stainings showed that TNF-alpha-exposed cells treated with NF-κB inhibitors had lower levels of nuclear p65 than cells exposed to TNF-alpha without inhibitor treatment (Fig. 5a). NF-κB inhibitors did not affect p65 localization in cells which were not exposed to TNF-alpha. Next, we examined the effect of NF-κB inhibition on TNF-alpha-mediated activation of latent HIV-1 in HNSCLatGFP1.2 populations. All inhibitors reduced the proportions of GFP-positive cells in both progenitor and differentiated HNSCLatGFP1.2 populations compared to populations exposed to TNF-alpha alone (Fig. 5b). In progenitors, bortezomib, WTA, PDTC or BMS-345541 reduced HIV-1 activation levels to less than 40%. In differentiated HNSCLatGFP1.2 cells, treatment with BMS-345541 most effectively suppressed HIV-1 activation. For further proof of concept, we investigated whether these inhibitors could also prevent reactivation of HIV-1 in a Jurkat T-cell model for post-integration latency (J-Lat 8.4). Indeed, all NF-κB inhibitors potently reduced TNF-alpha mediated reactivation in J-Lat 8.4 cells (Fig. 5c).

Suberoylanilide hydroxamic acid reactivated HIV-1 in HNSC progenitor populations much more potently than other HDAC inhibitors. Recent studies in proliferating T cells link HIV-1 activation by SAHA with the activation of the positive transcription elongation factor b (p-TEFb), which is a complex of the cyclin-dependent kinase Cdk9 and cyclin T1 or T2 [39,40]. Thus, we investigated whether exemplary Cdk inhibitors can reduce HIV-1 reactivation by SAHA in proliferating HNSCs. Therefore, proliferating HNSCLatGFP1.2 were either pre-treated with Cdk9 inhibitors (i.e. Flavopiridol, Roscovitine and PHA-767491) [41–43] or with the highly selective Cdk4 inhibitor Fascaplysin for 2–3 h (for concentrations see Table S3, SDC2) before HIV-1 expression was activated with SAHA. Pre-treatment with Roscovitine, Flavopiridol and PHA-767491 substantially diminished HIV-1 reactivation by SAHA, with Flavopiridol showing the strongest inhibition (Fig. 5d). In contrast, treatment with Fascaplysin did not reduce HIV-1 reactivation by SAHA. Cdk9 inhibitor treatments also reduced HIV-1 reactivation by SAHA in the J-Lat model, whereas Fascaplysin was also ineffective (Fig. 5e). These results demonstrate that small molecules that interfere with NF-κB signalling or Cdk9 activity can potently inhibit TNF-alpha or SAHA-induced activation of latent HIV-1 in proliferating and terminally differentiated HNSCLatGFP1.2, as well as in latently infected T cells.

Discussion

Cells of the neuro-ectodermal lineage, particularly astrocytes and their precursors, are key candidates of HIV-1 reservoirs in the brain. Here, we report a novel model for HIV-1 proviral latency that consists of human neural progenitor cell populations (HNSC.100) carrying stable envelope-defective HIV-1 proviral genomes. The HNSC model has several properties that support its use in investigating HIV-1 latency in the brain. It mirrors the reactivation potential of an entire cell population, bypassing heterogeneity of latency responses in clones of individually selected cells [29,44]. Furthermore, the HNSC model allows investigation of HIV-1 latency in dividing progenitor as well as in non-dividing, differentiated brain cell populations. This is important since the astrocyte population in the brain consists of cells at different developmental stages and with different mitotic potentials [45–47].

Macroglial HIV-1 reservoirs show both differences and similarities to T-cell reservoirs in their sensitivities towards individual LRAs. Whereas several LRAs with high reactivation potential in T-cell reservoirs (Table S1, SDC2) did not reactivate HIV-1 in HNSCs, the pro-inflammatory cytokine TNF-alpha potently induced latent HIV-1 also in HNSCs. TNF-alpha is increased in different body compartments of HIV-1-infected individuals, including the brain [48,49], and is a hallmark of the chronic neuroinflammation accompanying HIV-1 infection of the brain [7,50]. Activation of latent HIV-1 by TNF-alpha in progenitor and terminally differentiated HNSC, as well as in T cells, strengthens its role as a 'pan' inducer of transcriptionally latent HIV-1 in different virus reservoirs.

Suberoylanilide hydroxamic acid is a primary LRA candidate for clinical flush-out of HIV-1 reservoirs and

and GFP-expressing cells visualized by confocal microscopy. Shown are representative images of DAPI staining of nuclei (blue, left column), GFP expression (green, middle column) and the merge (right column) of untreated (upper row), TNF-alpha-treated (middle row) and SAHA-treated (lower row) cells. Exposure times: GFP 611 ms, DAPI 485 ms. Scale bar: 36 μm. (d, f) Differentiated HNSCLatGFP1.2 populations were treated with SAHA (5 μmol/l), TNF-alpha (10 ng/ml), NaB (500 μmol/l), VPA (1 mmol/l), TSA (100 nmol/l), Pro (1 μmol/l), PMA (50 ng/ml) or 5-aza (1 μmol/l) alone or with indicated combinations for 30 h, harvested, fixed and GFP expression was analysed by flow cytometry. Columns represent the means ± standard errors of at least three independent experiments (eight replicates). (e) Differentiated HNSCLat populations were treated as described above with SAHA, TNF-alpha and VPA for 24 h. Cells were harvested, RNA was isolated, cDNA prepared and HIV-1 transcripts were quantified by relative qPCR which is expressed as fold enrichment of HIV-1 RNA levels in treated versus untreated cells. Columns represent the means ± standard errors of at least three independent experiments (eight replicates). 5-aza, 5-azacytidine; HNSC, human neural stem cell; LRAs, latency reversing agents; NaB, sodium butyrate; PMA, phorbol 12-myristate 13-acetate; Pro, Prostratin A; SAHA, suberoylanilide hydroxamic acid; TNF, tumour necrosis factor; TSA, Trichostatin A; VPA, valproic acid.

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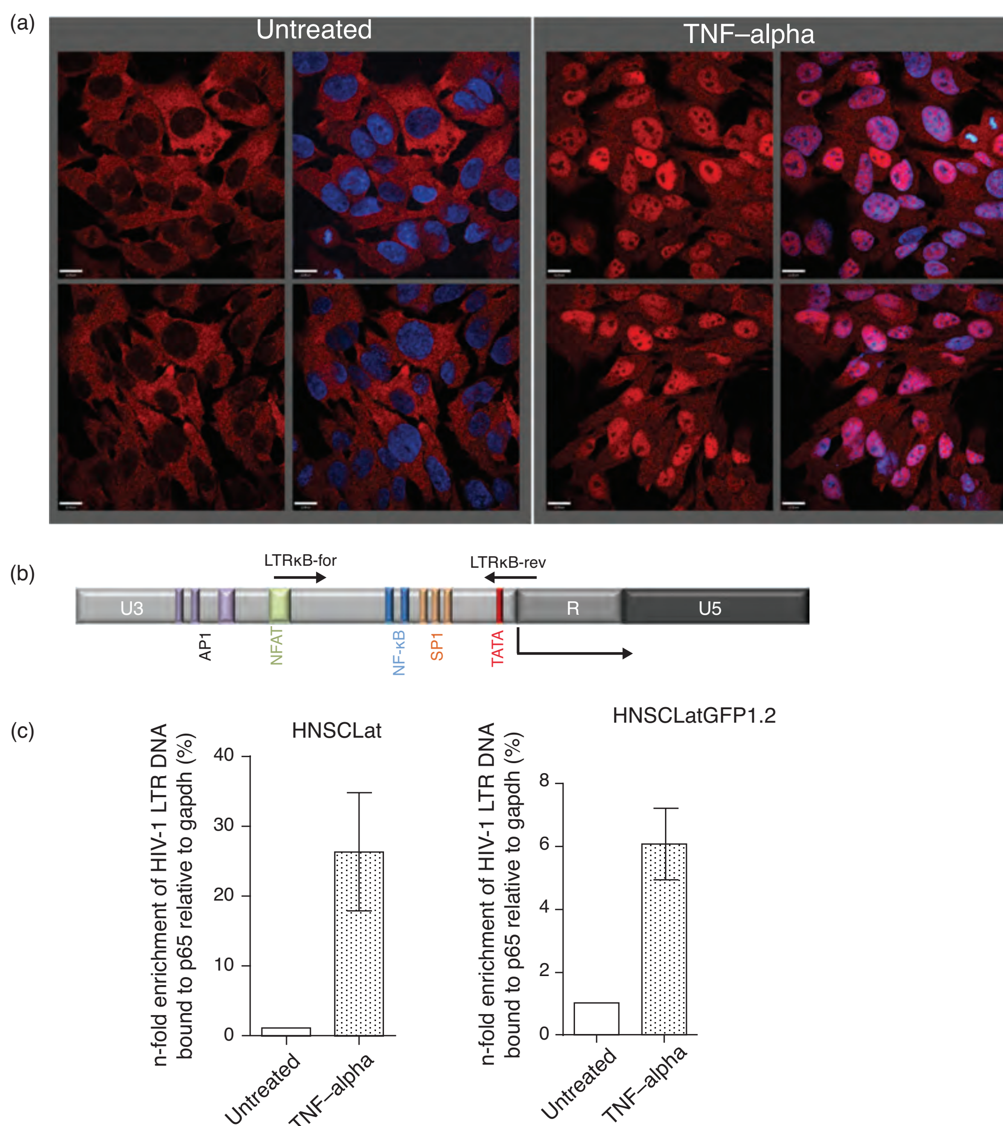

Fig. 4. Induction of HIV-1 expression in HNSCLatGFP1.2 by tumor necrosis factor-alpha is mediated by activation of the NF-κB pathway. (a) Nuclear translocation of p65 in HNSCLatGFP1.2 cells treated with TNF-alpha (10 ng/ml; 30 min). Cells were analysed by immunofluorescent staining with antibodies recognizing p65 and confocal microscopy. Shown are representative images of p65 (red) and DAPI (blue) staining of untreated (left panel) or treated (right panel) cells. Exposure times: Cy3 106 ms, DAPI 127 ms. Scale bar: 12 μm. (b, c) Increased association of p65 with the HIV-1 LTR in TNF-alpha treated cells. Chromatin was isolated from untreated and TNF-alpha treated (2 h, 10 ng/ml) HNSCLat and HNSCLatGFP1.2 cells and ChIP performed with antibodies against p65 and with IgG antibodies as control. The precipitate was analysed by qPCR to quantify DNA levels of a segment of the HIV-1 LTR spanning the NF-κB binding sites [primer binding sites shown in (b)] and the cellular *gapdh* locus as reference. The data show *n*-fold enrichment of HIV-1 LTR DNA associated with p65 relative to the reference locus *gapdh*. Mean values (bars) and SD are indicated for four (HNSCLat) and five (HNSCLatGFP1.2) independent experiments. ChIP, chromatin immunoprecipitation; HNSC, human neural stem cell; TNF, tumour necrosis factor.

has been shown to activate virus expression in HIV-1-infected individuals [51] and in animal models [52]. Studies in rodents also support access of systemically administered SAHA to the brain [53]. SAHA potently reactivated HIV-1 in proliferating HNSCs, but showed reduced capacity in terminally differentiated HNSCs. This suggests differential responses of subsets of latently infected brain reservoir populations to SAHA treatment. Selective latency reversing activity of SAHA may also occur in T-cell populations. Thus, SAHA-reversed HIV-1 latency in only a minor fraction of resting $CD4^+$ T cells derived from patients [54], but showed efficient latency reversing activity in various T-cell lines [55]. SAHA's weak reactivation potential in non-proliferating cells may

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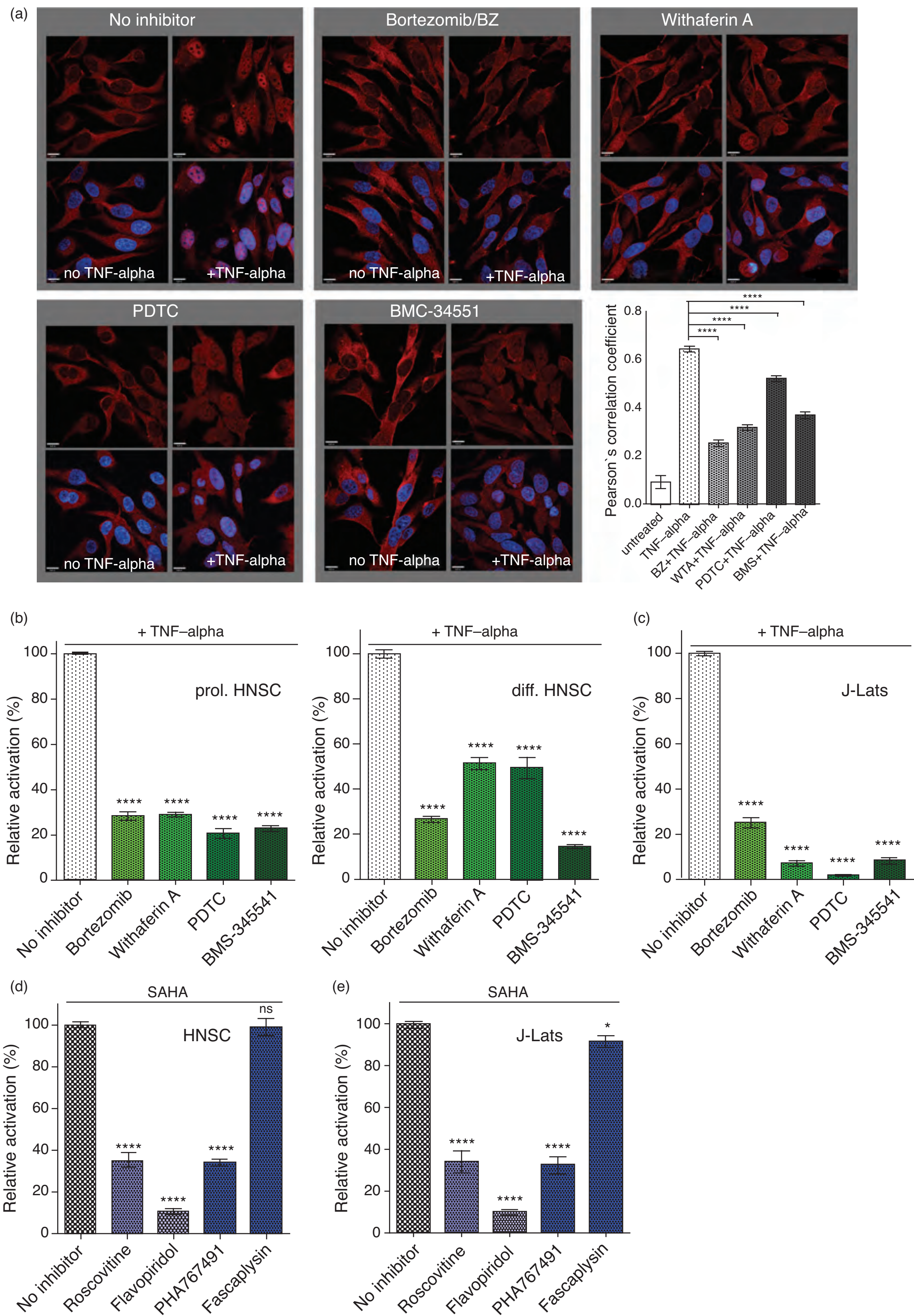

Fig. 5. Small molecule inhibitors of NF-κB signalling and Cdk9 diminish reactivation of latent HIV-1. (a) Diminished TNF-alpha-induced nuclear translocation of p65 in HNSCLatGFP1.2 cells by treatment with inhibitor compounds. HNSCLatGFP1.2 cells were treated with Bortezomib (BZ, 1 nmol/l), Withaferin A (WTA, 2 μmol/l), PDTC (50 μmol/l) or BMS (7.5 μmol/l), or left

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be caused by low expression or activity of HIV-1 key transcription factors in these cells. Thus, resting $CD4^+$ T cells express only low levels of cyclin T1, which is a component of active p-TEFb complexes [56] and is essential for HIV-1 transcription. Alternatively, non-dividing cells may increase expression of HIV-1 transcription inhibitors like the bromodomain-containing protein 4 (BRD-4) or the negative elongation factor NELF, which are also linked to p-TEFb activity [57,58]. In proliferating HNSCs, co-treatment with SAHA and TNF-alpha led to substantially higher HIV-1 reactivation levels than treatment with single compounds. This synergism indicates that multiple cellular pathways contribute to and cooperate in reactivation of HIV-1 in proliferating macroglial reservoirs.

Small molecules that target the NF-κB pathway or Cdk9 inhibited HIV-1 reactivation in latently infected HNSCs and in the J-Lat 8.4 model of latently infected T cells. Thus, our study supports the existence of common cellular effectors/pathways for the control of HIV-1 expression and latency in the lymphoid system and the brain. Drugs targeting these pathways could prevent HIV-1 reactivation from multiple virus reservoirs and thus could be part of functional cure strategies. These primarily aim at maximum suppression of viraemia rather than elimination of cellular reservoirs, and may therefore be safer for organs with limited cell replacement like the brain. Several of the drugs used here are already clinically relevant: The Cdk inhibitors Roscovitine and Flavopiridol are in clinical development for treatment of haematologic malignancies [41]. The proteasomal inhibitor Bortezomib (Velcade/PS-341) is approved for the treatment of multiple myelomas and mantle cell lymphoma [59]. Furthermore, Roscovitine, Flavopiridol and Bortezomib inhibit HIV-1 replication in various experimental models [60,61]. However, the suitability of these drugs for clinical applications in HIV-1-infected individuals needs to be established in future studies.

We conclude that reactivation of HIV-1 latency in macroglial reservoirs displays both similarities and differences to T-cell reservoirs. Similarities include common cellular pathways for regulation of HIV-1 transcription. These provide targets for simultaneous manipulation of HIV-1 latency in multiple reservoirs. Our study supports blocking activation of HIV-1 proviruses as a strategy for functional HIV-1 cure and provides a powerful model for comparison of latency responses in the brain and the immune system.

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untreated for 3 h, followed by exposure to TNF-alpha (10 ng/ml) for 30 min. Cells were fixed and stained with rabbit anti-p65 antibody and anti-rabbit Cy3 for confocal microscopy analysis. Shown are representative images of p65 (red) and DAPI (blue) staining of cells treated with inhibitors, either without (no TNF-alpha) or with (+TNF-alpha) subsequent exposure to TNF-alpha. Imaging exposure times: Cy3 ranging from 264 to 295 ms, DAPI ranging from 348 to 390 ms for different treatments, but identical for samples without and with TNF-alpha. Scale bar: 12 μm. Pearson's correlation of red and blue fluorescence was determined for at least 200 cells, with factor 1 or 0 representing total or no colocalization, respectively. (b, c) Diminished TNF-alpha-induced reactivation of latent HIV-1 by treatment with inhibitors of NF-κB signalling. (b) Proliferating (left) and differentiated (right) HNSCLatGFP1.2 cells were treated with BZ (1 nmol/l), WTA (2 μmol/l), PDTC (50 μmol/l) or BMS (7.5 μmol/l) for 3 h before HIV-1 reactivation was induced by exposure to TNF-alpha (10 ng/ml) for 30 h. Cells were fixed and GFP expression was analysed by flow cytometry, and related to GFP expression values of cultures exposed to TNF-alpha without inhibitor treatment (100% reactivation). Columns represent the means ± standard errors of at least three independent experiments (eight replicates). Statistical significances were determined by referring values for TNF-alpha-exposed cells pre-treated with inhibitors to untreated cells (no inhibitor). (c) J-Lat 8.4 cells (latently infected Jurkat T cells) were treated with the same inhibitors as in (b) and exposed to TNF-alpha for 30 h. Cells were fixed and GFP expression was analysed by flow cytometry and referred to those treated with TNF-alpha alone (100% reactivation). Columns represent the means ± standard errors of at least three independent experiments (eight replicates). Statistical significances were determined as described for (b). (d, e) Reduced SAHA-induced reactivation of latent HIV-1 by treatment with inhibitor compounds. (d) Effects of Cdk inhibitors on HIV-1 reactivation in HNSCLatGFP1.2 cells. Proliferating HNSCLatGFP1.2 were treated with Cdk inhibitors that target Cdk9 [R-Roscovitine (10 μmol/l), Flavopiridol (50 nmol/l), PHA-767491 (PHA, 200 nmol/l)] or with a highly selective Cdk4 inhibitor [Fascaplysin (350 nmol/l)] for 3 h before HIV-1 reactivation was induced by exposure to SAHA (5 μmol/l) for 30 h. Cells were fixed and GFP expression was analysed by flow cytometry. Activation levels were referred to those of cultures treated with SAHA alone (100% reactivation). (e) Inhibition of HIV-1 activation in J-Lat 8.4 cells. The same experimental conditions were used as in (d). Columns represent the means ± standard errors of at least three independent experiments (eight replicates). Statistical significances were determined by referring untreated/reactivated cells to inhibitor treated/reactivated cells. HNSC, human neural stem cell; PDTC, pyrrolidinedithiocarbamate; SAHA, suberoylanilide hydroxamic acid; TNF, tumour necrosis factor.

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Author contributions: Conceived and designed the experiments: M.S., B.T., M.M., M.H., R.B.W.; performed experiments: M.S., B.T., M.M., C.Z.; analysed and interpreted the data: M.S., B.T., M.M., M.H.; conceived, drafted and finalized the manuscript: M.S., R.B.W.

Conflicts of interest

The authors have no conflict of interest.

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