The NCOR2-Nurr1-CoREST Transrepression Axis Impairs HIV Reactivation in Latently Infected Microglial Cells

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ABSTRACT Whereas the incidence of HIV-associated dementia (HAD) has declined due to successful anti-retrovirals treatment, prevalence of milder forms of HAD, which include asymptomatic neurocognitive impairment (ANI) and minor neurocognitive disorder (MND) has increased. Both ANI and MND are part of what is known as HIV-associated neurocognitive disorders (HAND). However, the molecular mechanisms explaining regulation of HIV activation in the brain remain ill-defined. The Nurr1/CoREST transrepression pathway has been recently described as a regulator of glial cells response to brain inflammation by limiting over-reactivation of NF-κB-dependent pro-inflammatory genes. We report here that, unlike in latently-infected T-cells, in latently-infected microglial cells (CHME-5/HIV), HIV is induced by pharmacological inhibitors of the CoREST complex chromatin-modifying enzymes LSD1 and G9a/GLP; these inhibitors also sensitized CHME-5/HIV cells for LPS-mediated HIV reactivation. shRNA-mediated knockdown of Nurr1, LSD1, or CoREST yielded similar results. Chromatin immunoprecipitation analysis followed by high throughput next generation sequencing revealed that upon microglia treatment with TNFα, the nuclear receptor co-repressor 2 (NCOR2/SMRT), which strongly interacts with Nurr1 to facilitate transrepression, is present at the HIV promoter before activation, then recruited at the earliest time points, and then its presence fluctuates over time. Likewise, Nurr1, CoREST, LSD1, and G9a are recruited to the HIV promoter, changing the epigenetic signature (lower H3K4Me/higher H3K9Me2). The repressor role of these proteins in regulating HIV emergence from latency has been confirmed by unbiased shRNA screens for factors involved in maintaining HIV silenced in latently-infected microglial cells. Our data indicate that the NCOR2-Nurr1-CoREST axis plays a role in preventing HIV over-reactivation in microglial cells, and studying this mechanism in detail may provide therapeutic targets for the treatment of HAND.

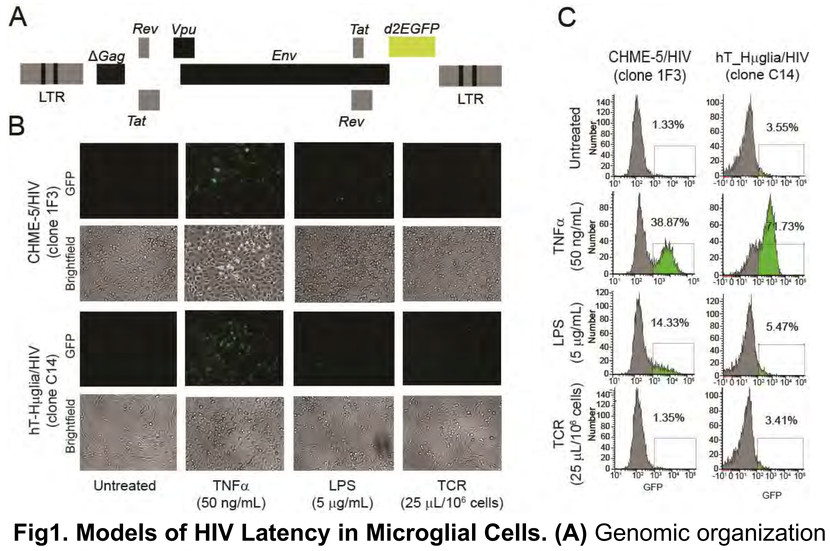


Fig1. Models of HIV Latency in Microglial Cells. (A) Genomic organization of the HIV lentiviral vector, showing a fragment of HIV-1_{pNL4-3} containing *Tat*, *Rev*, *Env*, and *Vpu* with the reporter gene d2EGFP cloned into the pHR' backbone. **(B)** TNF-α (50 ng/mL)- and LPS (5 μg/mL)-mediated reactivation of HIV measured by fluorescence microscopy. **(C)** FACS analysis 16 h post-treatment. LPS-mediated reactivation is restricted.

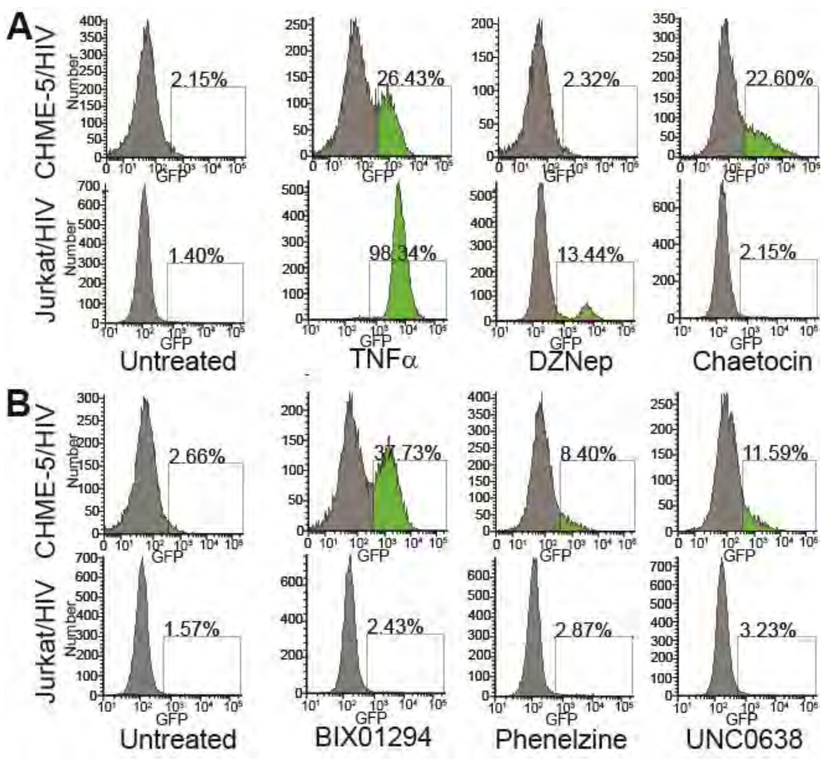


Fig 2. Orthogonal Role of Members of Different Repression Complexes in Maintaining HIV Latency in T cells vs. Microglia. (A) Whereas DZNep (50 μM), an inhibitor of the polycomb PCR2 repression complex protein EZH2, reactivated HIV expression in a significant portion of Jurkat/HIV cells (model for HIV latency in T cells), it failed to do so in CHME-5/HIV. **(B)** Inhibitors of the CoREST repression complex BIX01294 (5 μM; targets G9a/EHMT2), phenelzine (1 mM; targets LSD1/KDM1), or UNC0638 (10 μM; targets G9a/GLP heterodimer) reactivates HIV in CHME-5/HIV cells, but not in Jurkat/HIV cells.

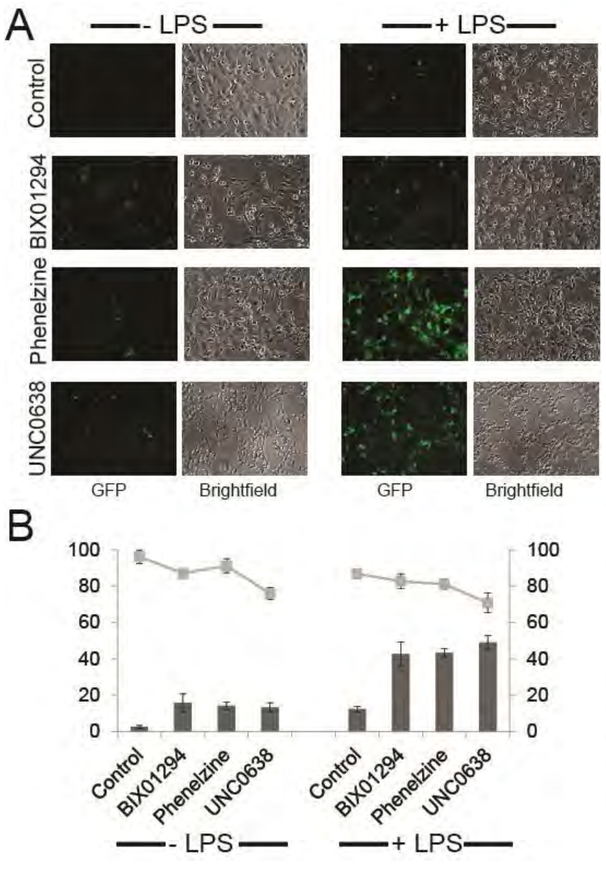
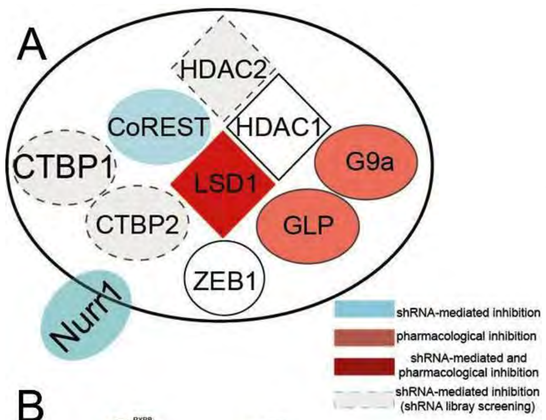


Fig 3. CoREST Complex Inhibition Sensitizes Microglial/HIV Cells for LPS-mediated HIV Reactivation. hT-Hμglia/HIV **(A)** or CHME-5/HIV **(B)** cells were pre-treated with either BIX01294 (2.5 μM), phenelzine (0.5 μM) or UNC0638 (5 μM) for 1 h prior to addition of LPS (5 μg/mL) and incubation for 16 h. HIV reactivation was verified by fluorescence microscopy **(A)** and FACS analysis **(B)**. These data suggest that disruption of repression complexes results in epigenetic changes leading to more accessibility of positive transcription factors and RNA Pol II.



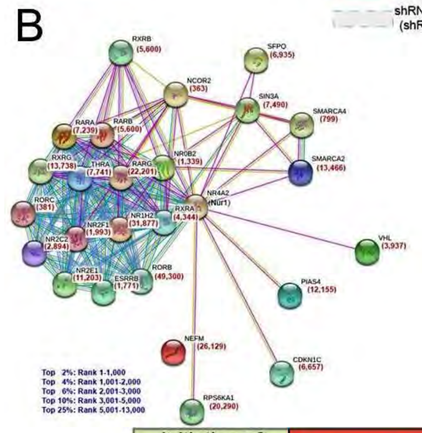


Fig 4. shRNA-mediated Knock-down of NCOR2/Nurr1/CoREST Leads to HIV Reactivation. (A) CHME-5/HIV cells were subjected to an unbiased shRNA library screen for factors involved in maintaining HIV silenced in latently-infected microglial cells as well as individual shRNA cell transduction. The results revealed and confirmed that Nurr1/CoREST is involved in HIV latency in microglial cells. (B) The screen top 10% of the factors were subjected to Ingenuity Pathway Analysis® for factors interacting with Nurr1. Among other factors, the repressor protein NCOR2 is shown as a strong interaction partner of Nurr1 involved in maintaining HIV in latent state.

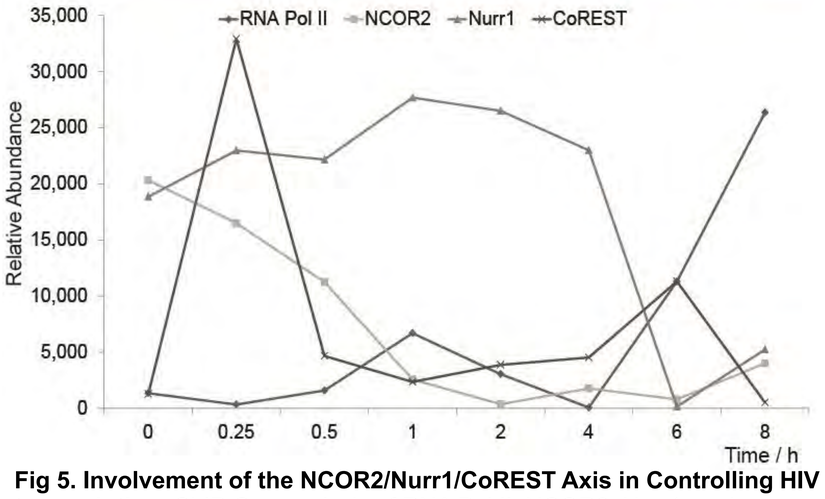


Fig 5. Involvement of the NCOR2/Nurr1/CoREST Axis in Controlling HIV transcription. ChIP-Seq analysis of RNA Pol II, NCOR2, Nurr1, and CoREST abundance at the HIV LTR Nuc 1 region. Significant increase of RNA pol II coincides with clearance of NCOR2/Nurr1/CoREST at Nuc 1.



CONCLUSIONS

- LPS-mediated reactivation of HIV is restricted in microglial/HIV cells
- Whereas the PRC2 is predominately involved in maintaining HIV silenced in latently-infected T cells, other repressor complexes with distinct epigenetics enzymes (G9a/GLP, LSD1) are predominant in microglial/HIV cells
- Pharmacological and shRNA-mediated inhibition of G9a/GLP and LSD1, and other members of the CoREST complex, result in HIV reactivation
- NCOR2 down-regulation results in HIV reactivation
- NCOR2/Nurr1/CoREST axis controls HIV transcription in microglial/HIV cells

Distinct Patterns of TLR-Mediated HIV Reactivation in Latently-Infected Microglial Cells and Monocytes

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ABSTRACT Toll-like receptors (TLRs) recognize molecules derived from microbes and play a key role in mediating innate immune responses. Multiple TLRs are typically expressed in cells of the monocytic lineage, including microglia. Microglial cells constitute the major reservoir for HIV infections in the brain, where inflammatory conditions in the central nervous system (CNS) are believed to induce HIV-associated neurocognitive disorders (HAND). Using HIV-latently infected microglial cell lines, we investigated whether TLR stimulation can induce HIV transcription. Treatment with a panel of TLR ligands, including *Mycobacterium tuberculosis (Mtb)*-derived molecules, we found that, unlike in monocytic cells, reactivation of HIV by TLR ligands was significantly restricted in microglia. Flagellin (TLR5 agonist) and, to a lesser extent, lipopolysaccharide (LPS; TLR4 agonist) were able to reactivate HIV in hTERT-immortalized glial (hT_Hμglia/HIV) and in SV40-immortalized (CHME-5/HIV) human fetal microglial cells. By contrast, agonists for TLR1, 2, 4, 5, 6, or 8 (but not for TLR3, 7, or 9), potently reactivated HIV in THP-1/HIV cells and, to a lesser extent, in U937/HIV and SC/HIV monocytic cells in an NF-κB-dependent manner. Mtb-derived molecules PIM_6 and LprG, which are potent TLR2 agonists, reactivated HIV in THP-1/HIV, but not in U937/HIV or SC/HIV cells which, unlike THP-1/HIV, showed no significant expression of TLR2. We conclude that TLR signaling probably plays only a minor role in activating HIV replication in the CNS, but can potentially drive replication in peripheral monocytic cells. Also, pro-inflammatory conditions in the brain may sensitize microglial cells for exaggerated immune responses.

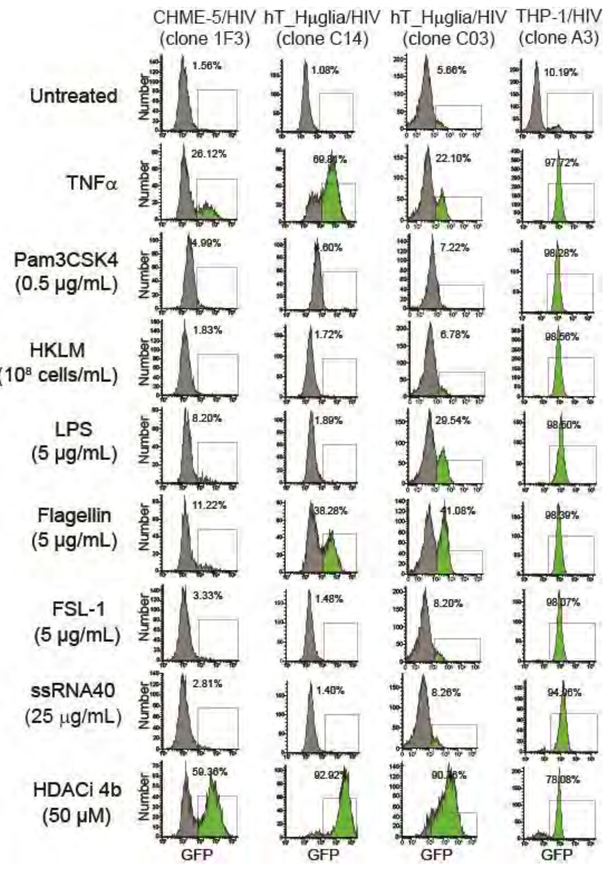

Fig 2. TLR-mediated HIV Reactivation in Latently-infected Microglial Cells is Mainly Restricted to TLR4 and TLR5. Microglial cells are known to express all TLRs at low but detectable levels in the human CNS. However, significant TLR activation in hT-Hμglia/HIV cells was only achieved with the TLR5 ligand, flagellin (~ 40% positive cells). In clone C03, LPS reactivated HIV to ~ 30% of the cells. In the CHME-5/HIV cells, we found that responses to the TLR agonists were generally low and fairly uniform against a broad range of receptors. THP-1/HIV cells (monocytes) used as positive control.

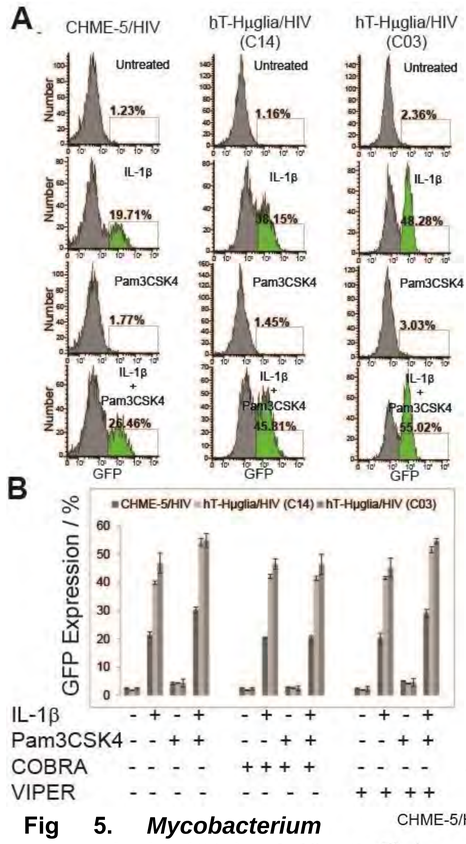

Fig 4. IL-1β Synergizes with Pam3CSK4 for Induction HIV Reactivation in Microglial/HIV Cells. Apart from TNF-α, IL-1β is also highly expressed in the CNS of patients with HAD, a fact that correlates with neuronal injury defining IL-1β as an important pro-inflammatory factor involved in HAD pathogenesis. **(A)** CHME-5/HIV and hT-Hμglia/HIV cells were untreated or treated with IL-1β (100 pg/mL), Pam3CSK4 (0.1 μg/mL), or a combination of IL-1β and Pam3CSK4 for 16 h prior to measuring GFP-expressing cells by flow cytometry. **(B)** Similar experiments were performed in the absence of presence of 20 μM of either COBRA (TLR2 or TLR4 inhibitor; IMGENEX, Inc.) or VIPER (TLR4 inhibitor; IMGENEX, Inc.). COBRA, but not of VIPER, abolished the effect of Pam3CSK4, but not of IL-1β, indicating that Pam3CSK4 is indeed responsible for the potentiation of HIV reactivation.

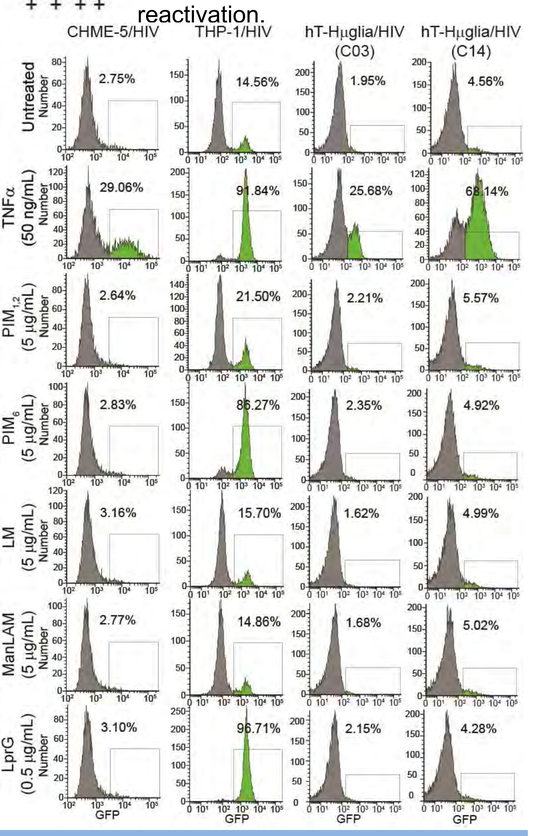

Fig 5. *Mycobacterium tuberculosis* (Mtb) TLR2 Ligands do not Reactivate HIV in Latently-infected Microglial Cells. Mtb-derived lipoproteins such as LprG and the glycolipid lipomannan (LM), but not mannosylated lipoarabinomannan (ManLAM), are potent TLR2 agonists that trigger inflammatory and microbicidal innate immune responses. In addition, Mtb-derived polar glycolipid phospatidylinositol mannoside 6 (PIM_6), but not $PIM_{1,2}$, triggers TLR2 signaling in a reporter HEK cell system. In contrast to microglial/HIV cells, HIV was reactivated by PIM_6 or LprG in THP-1/HIV cells. $PIM_{1,2}$ was a weak activator of HIV in THP-1/HIV cells whereas LM and ManLAM were not activators.

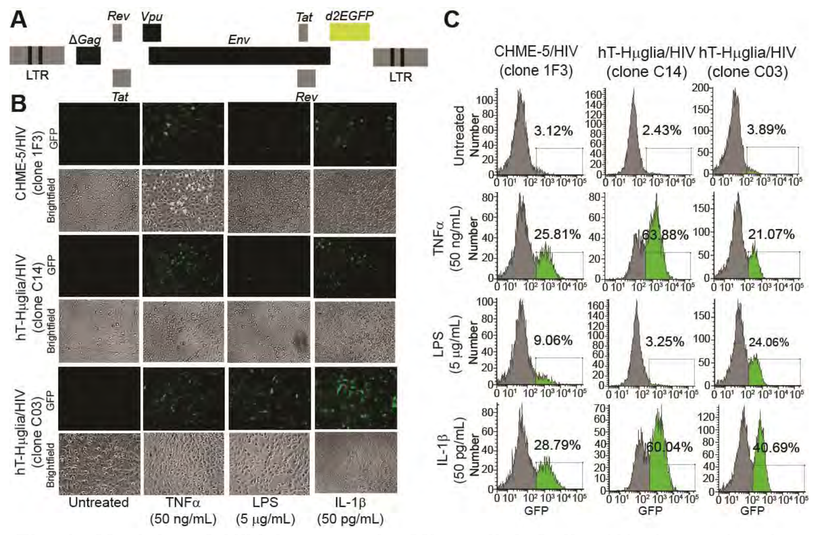

Fig 1. Models of HIV Latency in Microglial Cells. We have developed models of HIV latency out of glial cell lines previously immortalized with hTERT, since this enzyme can immortalize without causing cancer-associated changes or altering phenotypic properties. **(A)** Genomic organization of the HIV lentiviral vector, showing a fragment of HIV-1_{pNL4-3} containing *Tat*, *Rev*, *Env*, and *Vpu* with the reporter gene d2EGFP cloned into the pHR' backbone. **(B)** TNFα (50 ng/mL)-, LPS (5 μg/mL)-, and IL-1β (50 pg/mL)-mediated reactivation of HIV measured by fluorescence microscopy. **(C)** FACS analysis 16 h post-treatment. Individual clones were selected and tested for the presence of latent HIV proviruses. Results from representative clones (hT-Hμglia/HIV C14 and C03), using CHME-5/HIV (clone 1F3) as control, are shown. Differential responses among different clonal populations to the same stimuli were observed.

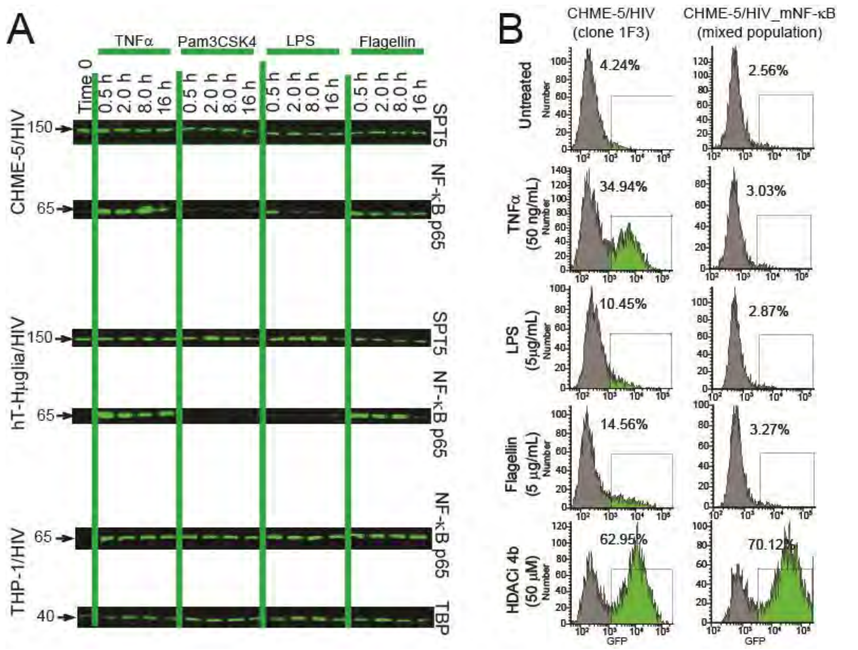

Fig 3. TLR-mediated HIV Reactivation is NF-κB-dependent. (A) Western blot analysis of nuclear extracts isolated from CHME-5/HIV or hT-Hμglia/HIV cells, using THP-1/A3 cells as control, either untreated or treated with TNFα, Pam3CSK4 (TLR1/2), LPS (TLR4), or flagellin (TLR5) for 30 min, 2, 8, or 16 h. NF-κB p65 nuclear translocation coincides with HIV reactivation. **(B)** CHME-5 cells were infected with a PHR' derivative carrying inactivating mutations in the NF-kB binding site of the HIV LTR (CHME-5/HIV_mNF-κB). CHME5/HIV_mNF-κB cells were treated with TNFα, LPS, flagellin or HDACi 4b for 16 h prior to flow cytometry analysis. TNFα, LPS or flagellin were unable to activate HIV in CHME-5/HIV_mNF-κB cells, as compared to the activation achieved in CHME-5/HIV cells. By contrast, HDACi 4b was a potent inducer of the proviruses in these cells.

CONCLUSIONS

- Differential response to TLR agonists by different clonal populations of microglial/HIV cells
- TLR agonist-mediated HIV reactivation in restricted in microglial/HIV, but not in monocytic/HIV cells; flagellin (TLR5) is only exception
- In the presence of IL-1β, Pam3CSK4 potentiates HIV reactivation