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Investigation of Neuropathogenesis in HIV-1 Clade B and C Infection Associated with IL-33 and ST2 Regulation

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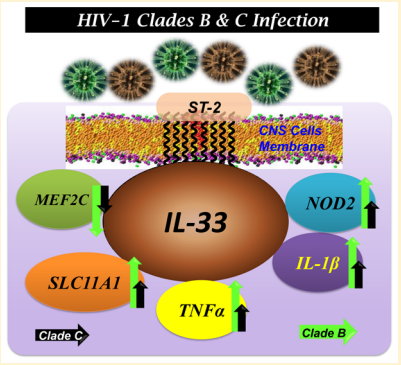
ABSTRACT: In present research work, for the first time, we demonstrate that neuropathogenesis in HIV-1 clade B and C infection is associated with IL-33 and ST2 dysregulation, that is, implication toward neuropathogenesis. It is known that neuropathogenesis of HIV infected individuals is clade dependent. Proinflammatory cytokines and related receptors play a significant role in the complex regulatory mechanisms of neuropathogenesis in HIV-1 infection. Among them, IL-33 is an inflammatory cytokine expressed in the central nervous system (CNS) and activates microglia cells and may affect neuroimmune inflammatory processes involved in HIV neuropathogenesis. Beside this, IL-33 receptor (ST2) plays a role in neuroinflammatory processes through the modulation of the biological action of IL-33. quantitative real time PCR (qRT-PCR), ELISA, Western blot (WB), and flow cytometry experiments were performed to elucidate the role of IL-33/ST2 in HIV neuropathogenesis in CNS cells. Apoptosis and mechanisms of IL-33 in neuronal cells were studied using caspase-3 assay and RT-PCR. Results of the studies suggest that the infection in CNS cells with HIV-1 clade B resulted in higher levels of IL-33/ST2L expression compared to HIV-1 clade C infection. Furthermore, higher concentrations of IL-33 were associated with a decrease in myocyte enhancer factor 2C (MEF2C) expression, a transcription factor that regulates synaptic function, and an increase in apoptosis, NOD2, and SLC11A1 in clade B infection. This led to neuroinflammation which dysregulates synaptic function and apoptosis. These parameters are common in neuroAIDS provoked by HIV infection.



KEYWORDS: *Neuroinflammation, synaptic plasticity, neuroAIDS, HIV-1, clade B, clade C, IL-33, ST2*

Manifestations of neuroAIDS between HIV-1 clades are differentially regulated. HIV viruses responsible for the pandemics belong to lineage M and are subdivided into 10 clades (from A to K). Clades B and C are part of that HIV subdivision, and clade classification is mainly based on nucleotide sequences derived from multiple subgenomic regions (gag, pol, and env). Interclades, env is the most variable gene encountered which is about 20−30%, while pol regions are less divergent. The env gene, which encodes the envelope surface glycoprotein 120 (gp120) and transmembrane glycoprotein 41 (gp41), can exhibit 35% amino acid diversity between subtypes with most of the genetic variation occurring in gp120. In addition, significant differences have been reported based on the nonstructural regulatory TAT protein among clades.[1,2]

In the Western world, clade B is the predominant HIV-1 subtype. An elevated percentage of HIV Associated Neurocognitive Disorders (HAND) has been found associated with clade B, while in regions where clade C is prevalent, a low or mild incidence of HAND is reported.[3−5] Many reports suggest lower neuropathogenesis in clade C infection than that of clade B infections.[7,8] Thus, studying different factors to understand clade-specific effects on neuropathogenicity is crucial and worth exploring.[6] A clear explanation is not proposed yet to elucidate mechanisms of the differential neuro-effects between clades. The regulation of proinflammatory cytokines and their receptors played a significant role in the complex regulatory mechanisms of HIV-1 neuropathogenesis.[1] Among cytokines, interleukin-33 (IL-33), a member of the IL-1 family,[7,8] plays a major role in a wide range of inflammatory responses.[8−11] IL-33 has been shown to induce T helper type 2 responses by activating NF-κB and MAP kinases via binding to its receptor ST2, which is a member of the toll-like receptor superfamily. Studies confirm that IL-33 and ST2 were found to be expressed in the central nervous system (CNS). Wherein, IL-33 activates microglia cells and increases M-CSF levels[8,11−13] to act as a potent mitogen facilitating increased phagocytosis.[13] The elevated IL-33 has been observed in various neurological disorders,[13,14] and no studies have been reported related to the role of IL-33/ST2 in HIV-I infection with clades B and C.

Recently, our group demonstrated that HIV-1 clade B is a major suppressor of synaptic plasticity genes compared to clade C.[15] Studies have also reported a relationship between inflammatory conditions and disparities of synaptic plasticity.[16−21]

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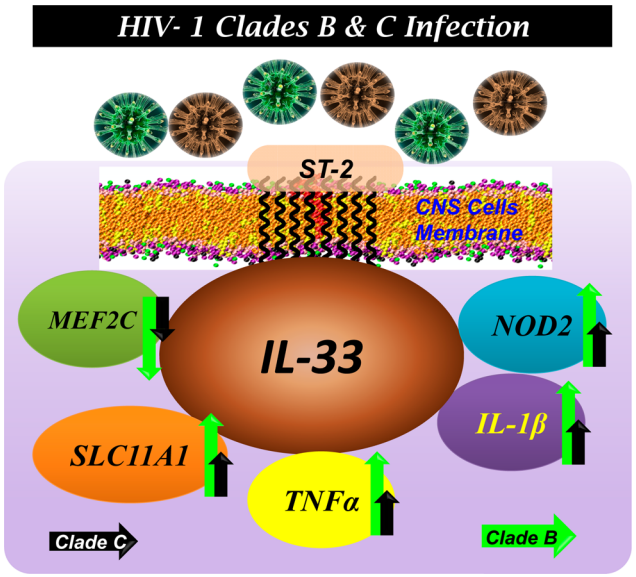
The transcription factor, myocyte enhancer factor 2 (MEF2), is known to regulate a variety of synaptic functions, including synapse weakening, maturation, and development.[21,22] In particular, one of the isoforms involved in hippocampal synaptic function is MEF2C, which has been correlated with synapse regulation, neuronal survival, and differentiation.[21,22] The rationale and hypothesis related to neuropathegensis HIV-1 clade B and C infection associated with IL-33 and ST2 dysregulation are illustrated in Scheme 1.

Scheme 1. Rationale and Hypothesis Related to the Neuropathogensis HIV-1 Clades B and C Infection Associated with IL-33 and ST2 Dysfunction



In this work, for the first time, we investigate the modulation of IL-33/ST2, with HIV-1 clade B and C infection in CNS cells. Outcomes suggest that increment of IL-33, mainly, by HIV-1 clade B is related with differential manifestations inneuroAIDS through the modulation of MEF2C expression, induction of neuronal apoptosis, and mechanisms of action of IL-33 leading to increased neuropathogenesis.[4]

RESULTS

Twofold HIV-1 Clade C Inoculum Yields Equivalent Clade B Infectivity of HA and SKNMC. Lower infectivity was achieved in HIV 1 clade C than in HIV-1 clade B infected cultures. To compensate, we standardized the inoculum to reach equivalent levels of infection. As measured using p24 ELISA, 200 ng of clade C and 100 ng of clade B produced an equal amount of p24 in human astrocytes and SKNMC. (Figure 1). All experiments were performed under the same conditions. Equal levels of infection were checked for each batch of experiment before proceeding with further gene and protein analyzes. Infections did not affect significantly the viability of the cells (data not shown).

IL-33 and ST2 Are Upregulated in HIV-1 Clade B Infection. Epidemiological[22−24] and in vitro studies[1] have reported differences in neuroAIDS and cytokines during HIV-1 clade B and C infection. In particular, recent reports have shown a role for IL-33 in the immune response of cells in the CNS.[8,13,25] Therefore, we decided to compare the effects of both clades on IL-33, and its receptor, ST2, in HA and SKNMC (Figures 2 and 3). CNS cells were infected with HIV and harvested 7 days post infection. RNA was extracted, and the expression of IL-33 and ST2 was examined. Clade B infection upregulated both genes, while the induction of IL-33 (Figure 2A, E) and ST2 (Figure Figure 3A, E) in clade C infected cells was less significant.

In order to see whether increased gene expression truly correlated with elevated protein levels, the effects of HIV-1 infection on the secretion of IL-33 and ST2 were checked by ELISA, and intracellular expression was investigated using Western blot (WB) and flow cytometry. Outcomes showed the secreted IL-33 production was significantly higher in HIV infected cultures compared to noninfected controls (Figure 2B, F). Clade B significantly increased the secretion of IL-33 (Figure 2B, F) compared to clade C; nevertheless, no significant differences were found for the soluble receptor ST2, in both CNS cell lines (Figure 3B, F). The intracellular IL-33 (Figure 2C, D, G, H) and ST2 (Figure 3C, D, G, H) proteins were differentially upregulated in HA and SKNMC with respect to both clades as compared with uninfected controls. Statistical differences were found between clades when they were analyzed by WB (Figure 2C, G, and Figure 3C, G) and flow cytometry (Figure 2D, H, and Figure 3D, H). Further, the flow cytometry results show significant increases in IL-33 and ST2 in clade B infected cells; however, in clade C infected cells, nonsignificant levels of IL-33 and ST2 upregulation were observed compared to controls. Results reveal that no doublets appeared during flow cytometry experiments.

IL-33 Induction Correlates with a Decrease in Synaptic Plasticity As Measured by MEF2C Expression. Neuronal plasticity has been shown to be modulated by inflammation, and MEF2C has been correlated with synapse regulation, neuronal survival, and differentiation.[26,27] Given the role of MEF2C, we assessed the impact of IL-33 on MEF2C expression using RT-PCR (Figure 4A) and flow cytometry (Figure 4B). To determine whether IL-33 directly modulated MEF2C expression, SKNMC cells were treated with various concentrations of IL-33 (120, 240, and 480 pg/mL) selected from the ELISA results after HIV-1 infection (Figure 2F). Our data indicates that IL-33 and MEF2C expression were inversely proportional (Figure 4A, B). Further, a significant downregulation of MEF2C gene and protein were obtained after clade B infection (Figure 4C, D). During experiments no doublets were visible.

IL-33 Contributes to HIV-Induced Apoptosis in CNS Cells. It is well-known that HIV infection promotes inflammation and apoptosis; therefore, we wanted to determine the potential role of inflammatory conditions induced by IL-33 in this process. To determine whether IL-33 promotes apoptosis in HIV-infected neuronal cells, we tested the caspase-3 levels in SKNMC cells infected with HIV clade B or C. The level of apoptosis as measured by caspase-3 activity was significantly higher after HIV-1 infection (Figure 5). Cells infected with HIV-1 clade B showed significantly higher apoptosis than cells infected with clade C. Similar results were observed for IL-33 treatment of SKNMC where an increased in IL-33 levels resulted in elevated SKNMC apoptosis suggesting a role for IL-33 in the induction of neuropathogenic mechanisms. Taken together, these results suggest that the clade B infection is more neuropathogenic to neuronal cells than clade C.

IL-33 Induces Immune Response Activation in CNS Cells. Response to the treatment of 2 ng of IL-33 by SKNMC was analyzed using human innate and adaptive immune response RT PCR array. Six genes (CSF2, MX1, NOD2, RAG1,

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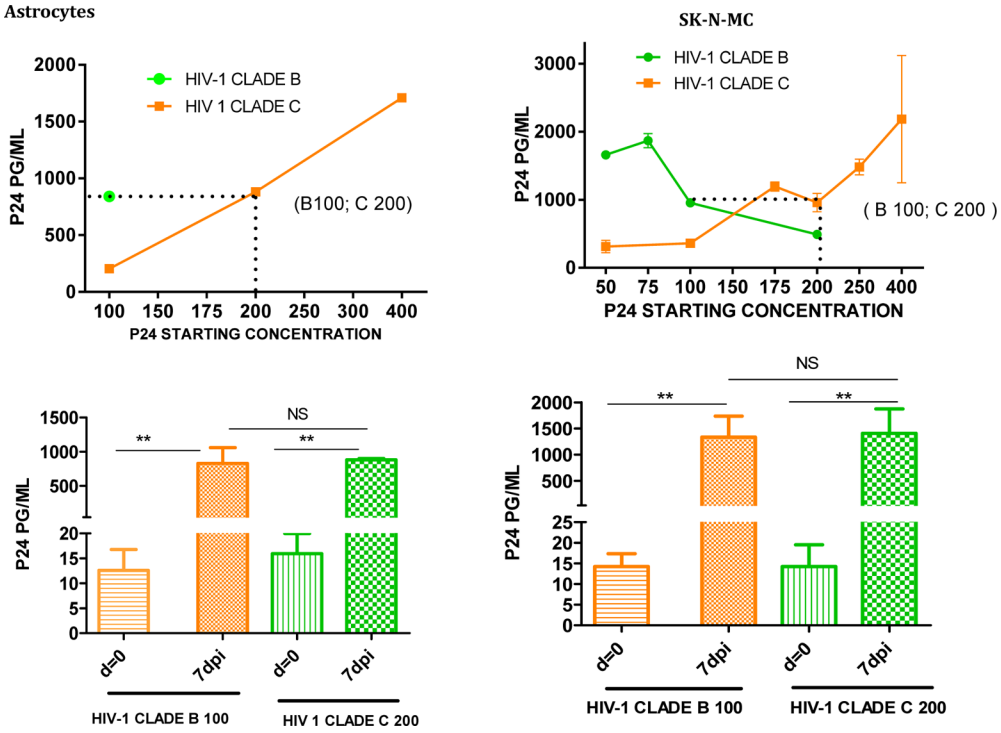

Figure 1. HIV clade B and C infection of HA and SKNMC yield equal p24 levels after 7 days of infection. HA and SKNMC cells were infected separately at different p24 starting concentration for HIV-1 B and C clades overnight with previous Polybrene activation. On day 7 postinfection, the supernatants were collected and tested with p24 ELISA Zeptometrix (Cat#: NC9130878). Data represent the means ± standard error of three independent experiments. All the data were analyzed using GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA and Tukey's multiple comparison post test. Differences were considered significant at $p \leq 0.05$.

SLC11A1, and TLR7) were found to be upregulated with significant increment of NOD2 (nucleotide-binding oligomerization domain containing 2) and SLC11A1 (solute carrier family 11 proton-coupled divalent metal ion transporters, member 1), while three genes were downregulated (CXCR3, TLR2, and TLR9). Data are shown in Table 2. The major impacts were found in NOD2 (fold change = 6.63) and SLC11A1 (fold change = 3.97) compared to control. Downregulated genes had no more than 3.0-fold change. TLR2 had the highest downregulation with a fold change of 2.69 compared to control.

DISCUSSION

The present study established a relation between IL-33 and ST2 dysfunction and HIV clade B and C infection. An upregulation of IL-33 produced by HIV-1 clade B infected cells was observed in comparison to clade C, under identical in vitro infection levels. These clade-specific responses can be associated with neuropathogenesis increment,[1,15,20] and other inflammatory cytokines were upregulated with respect to clade B than that of clade C.[1,20] On the contrary, higher levels of ST2 and low levels of IL-33 were found in plasma samples of HIV infected patients.[28] However, other details of clade differences and associated dependent factors, coinfections besides HIV, and the use of ARV were not considered in presented work.

We focused only on in vitro neuropathogenesis in HIV clade B and C infection, and clade-specific effects on IL-33 and ST2. Obtained findings manifested an increment in the production of IL-33 (Figure 2) and ST2 (Figure 3) in HIV infection on comparing with controls. The upregulation of IL-33 was consistently higher in HIV clade B infected astrocytes and SKNMC, whereas it was lower in the case of HIV-1 clade C infection (Figure 2). Clade B significantly increased IL-33 compared to clade C (Figure 2B, F). Nevertheless, no significant differences were found for the soluble receptor ST2, in both CNS cell lines (Figure 3B, F). This can be associated with an over production of secreted IL-33 that may block soluble ST2 receptors.

Though, studies reported that the soluble form of the receptor ST2 is generated and associated with IL-33. Also IL-33 blocks ST2-dependent signaling including the immunological effects of IL-33.[8,13,25] However, no significant levels of soluble receptors were observed in our study. Beside this, the significant impact in IL-33 production and function were observed. For example, MEF2C modulation and apoptosis, leading us to conclude that secreted ST2 levels have no effects on the inflammation induced by HIV clade B and C infection (Figure 3B, F). Inflammatory conditions aggravate the effects induced by HIV infection and our results have demonstrated that clade B and C are differentially inducing IL-33 and ST2 (Figure 2 and 3). In our work, SKNMC cells were treated with different IL-33 concentrations to explore the functional role of IL-33 in synaptic plasticity. Since MEF2C has been correlated with synaptic regulation, neuronal survival, and differentiation,[26,27] MEF2C expression analysis was made after HIV infection in the presence of increased levels of IL-33.

Findings of our research demonstrated a differential regulation on MEF2C when cells were infected using clades B and C. Higher expression of MEF2C was observed in HIV-1 clade C and lower in HIV-1 clade B infected cells. This can be related with different HIV associated neurocognitive disorders as reported earlier.[1] Further, we reported clades B and C as having differential effects on synaptic plasticity gene and spine density on CNS cells.[15]

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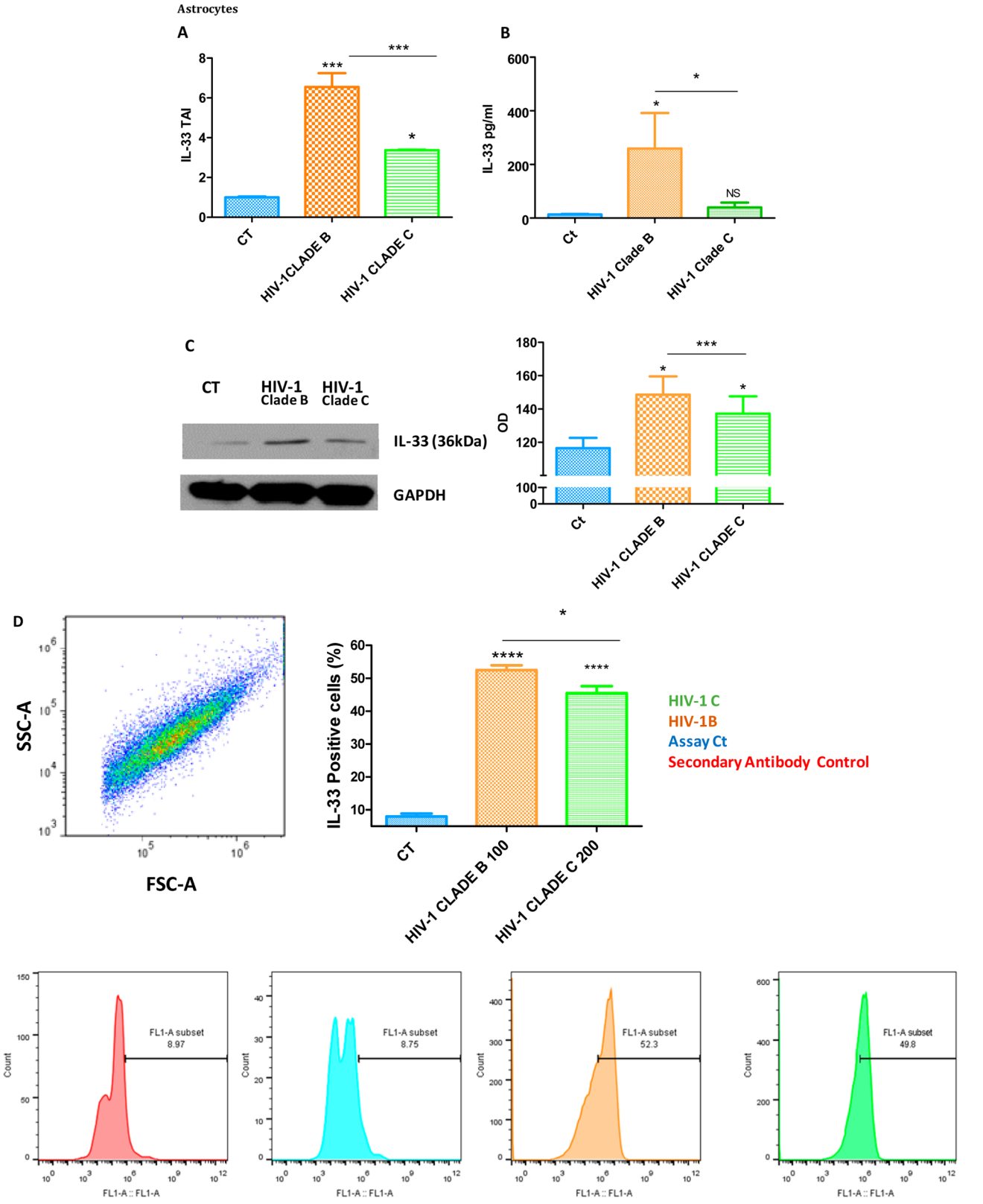
Cytokines have been associated with the impairment of synaptic plasticity as in the case of IL-1, TNFα, IL-18, and IL-6. Unfortunately, there are no reports elucidating the role of IL-33 and its receptor ST2 on synaptic plasticity. Our study showed a role of IL-33 on synaptic plasticity modulation as indicated by an inverse correlation of IL-33 and MEF2C (Figure 4). Doses were selected according to the different concentrations obtained in IL-33 ELISA in the cases of HIV 1B infection in SK-N-MC cells. Surprisingly, dose response data demonstrated that IL-33 and MEF2C are inversely correlated since at a lower



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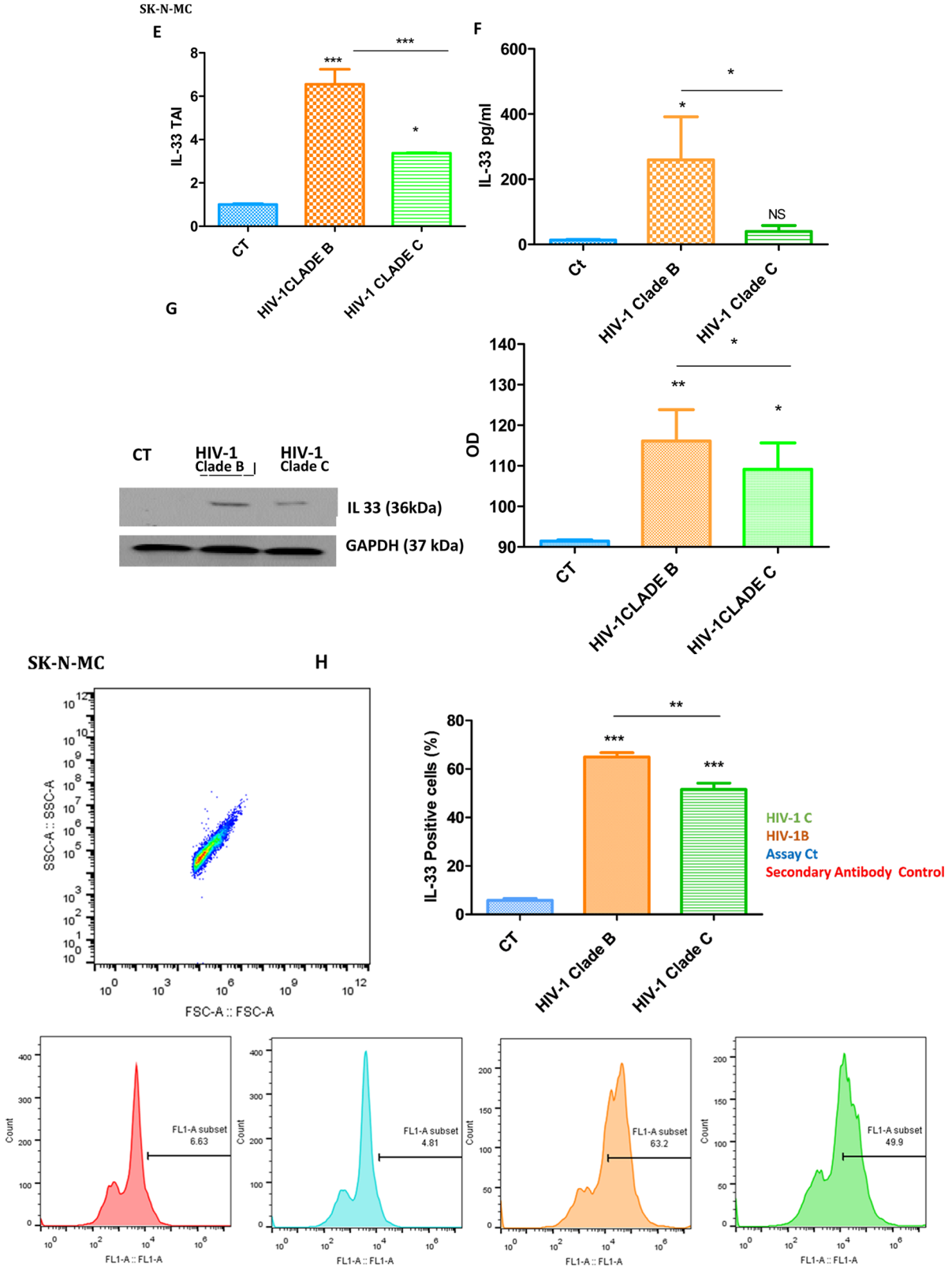

Figure 2. IL-33 expression is upregulated in HIV-1 clade B infection. HA and SKNMC cells were infected separately with HIV-1 B and C clades as described in Methods. On day 7 postinfection, RNA was extracted and reverse transcribed followed by quantitative real time PCR for IL-33 gene (A, E). Supernatants were used for ELISA (B, F), while cell lysates were used for Western blot (C, G). Cells were stained to determine IL-33 protein expression by flow cytometry (H). Data represents the means ± standard error of three independent experiments. All the data were analyzed using GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA and Tukey's multiple comparison post test. Differences were considered significant at $p \leq 0.05$.

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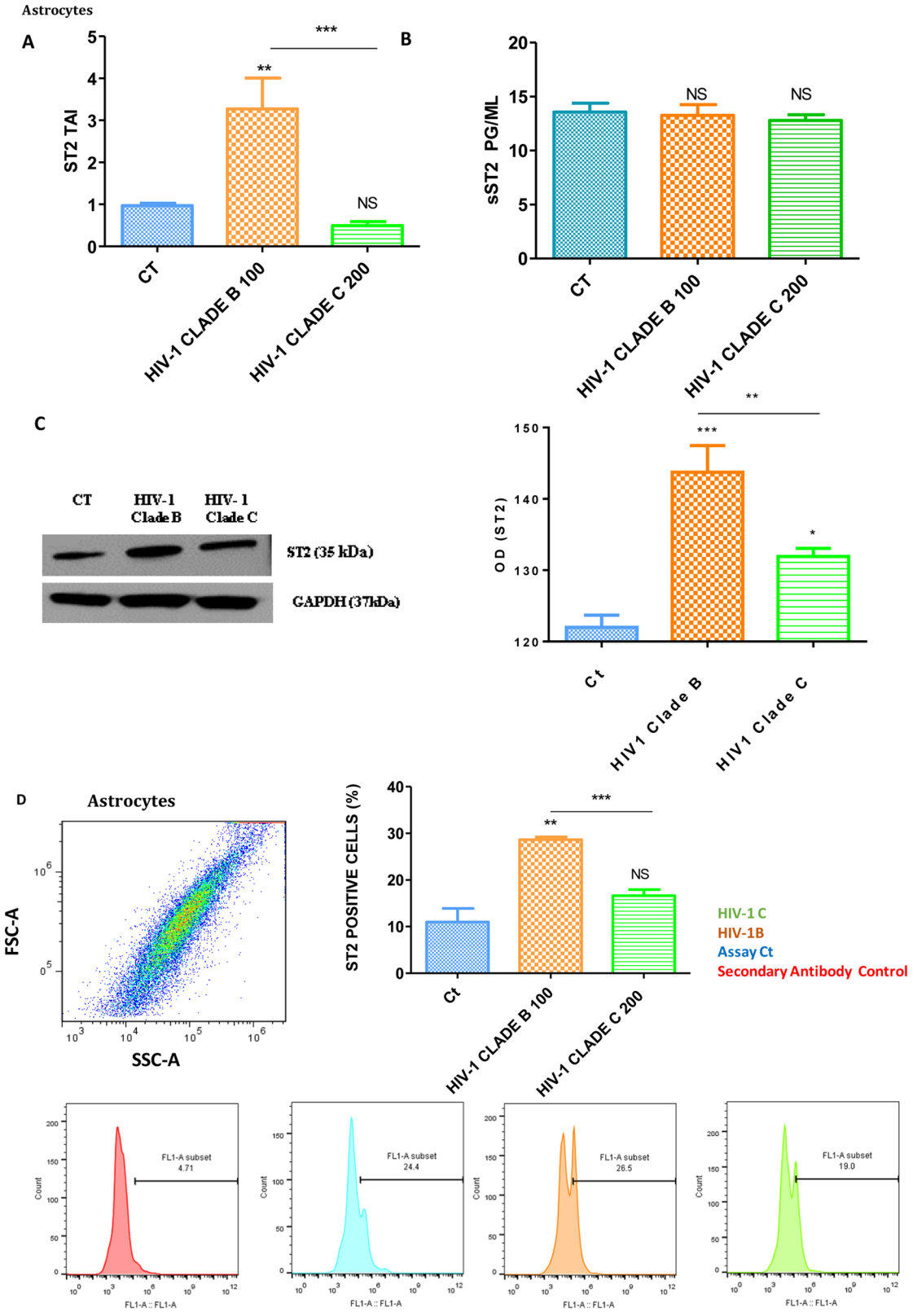
concentration of IL-33 (120 pg/mL) there was an increase in MEF2C gene expression, while the higher dose of IL-33 (480 pg/mL) produced a significant downregulation of MEF2C.[20,29−31]

We speculate that lower doses of IL-33 may exert a protective effect on synaptic plasticity and the accumulation of this cytokine impacts negatively the synaptic plasticity transcriptional factor probably as a feedback mechanisms in response to chronic inflammation.[20,29−31] For instance, in the case of other inflammatory cytokines such as TNFα, an upregulation of AMPA receptors is produced at physiological levels of TNFα,



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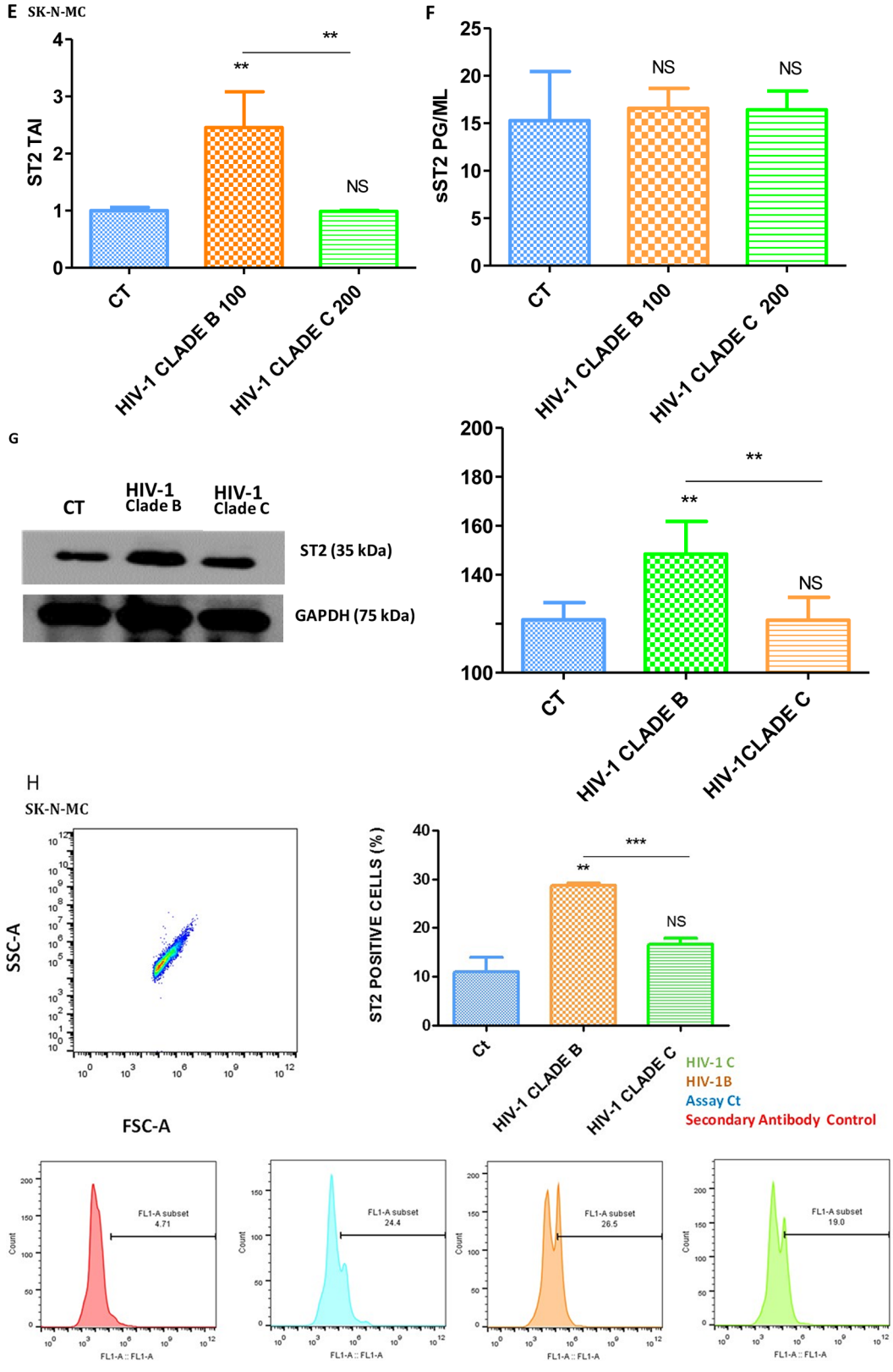

Figure 3. ST2L expression is upregulated in HIV-1 clade B infection. No differences were found in soluble ST2. HA and SKNMC cells were infected separately with HIV-1 B and C clades as described in Methods. On day 7 postinfection, RNA was extracted and reverse transcribed followed by quantitative real time PCR for ST2 gene (A, E). Supernatants were used for ELISA (B, F), while cell lysates were used for Western blot (C,G). Cells were also stained to determine ST2 expression by flow cytometry. Data represents the means ± standard error of three independent experiments. All the data were analyzed using GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA and Tukey's multiple comparison post test. Differences were considered significant at $p \leq 0.05$.

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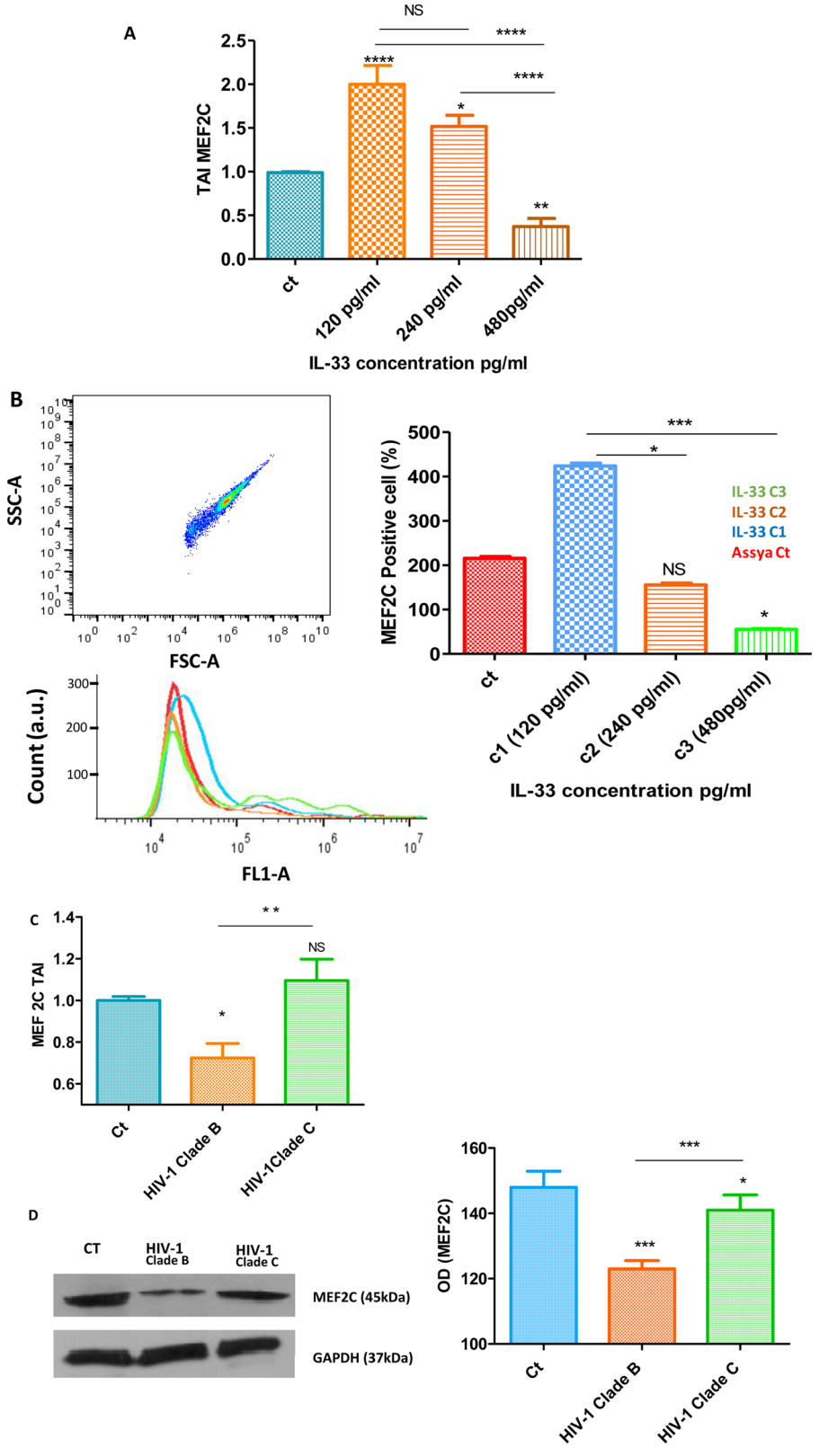

Figure 4. IL-33 induction correlates with a decrease in synaptic plasticity as measured by MEF2C expression. SKNMC cells were treated with different IL33 concentrations (120, 240, and 480 pg/mL) for 24 h. (A) RNA was extracted and reverse transcribed followed by quantitative real time PCR for MEF2C gene. (B) Intracellular MEF2C was measured by flow cytometry. The histogram shows an overlay of total cells. The bar graph represents the mean ± standard error of percent of mean fluorescence intensity. (C) SKNMC cells were infected with HIV-1 clades B and C. (D) After 7 days of infection, cell lysates were used for RT-PCR and Western blot. The figure represents the gene expression. Data represents the means ± standard error of three independent experiments. All the data were analyzed using GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA and Tukey's multiple comparison post test. Differences were considered significant at $p \leq 0.05$.

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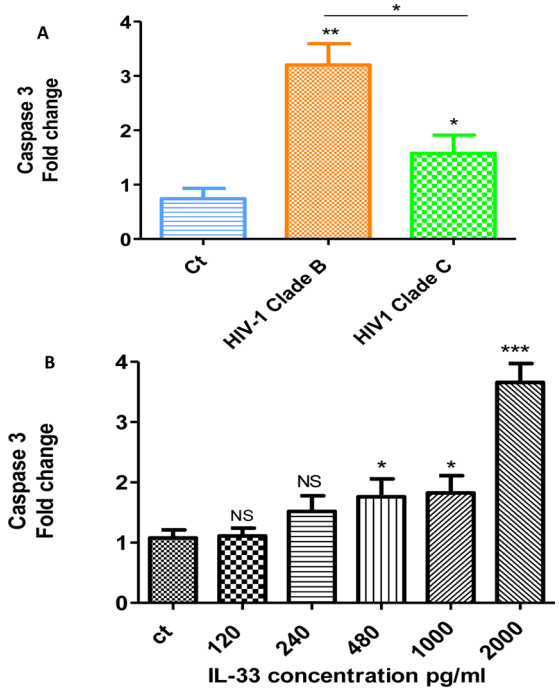

Figure 5. HIV infection induces apoptosis of CNS cells through the contribution of IL-33. (A) SKNMC cells were infected with HIV-1 clades B and C. Cytosol extracts were quantified. Caspase-3 activity was measured using a colorimetric kit from Invitrogen, following manufacturer instructions (Cat# KHZ0021). Untreated population was used to define the basal level of apoptotic and dead cells. (B) SKNMC cells were treated with different IL-33 concentrations for 7 days, following the procedure described above. Untreated population was used to define the basal level of apoptotic and dead cells. Bar graph represents the mean ± standard error of percent of mean fluorescence intensity. Data represents the means ± standard error of three independent experiments. All the data were analyzed using GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA and Tukey's multiple comparison post test. Differences were considered significant at $p \leq 0.05$.

and as a consequence of this, the synaptic strength is favored. While higher levels can negatively impact long-term potentiation (LTP).[20] In addition, low levels of IL-1 help in the maintenance of short-term plasticity and long-term potentiation and higher levels damage LTP and depressive-like behavior can be observed in animal models.[20]

The results of our study showed a correlation between IL-33 and synaptic plasticity as indicated by obtained inverse effect of IL-33 and MEF2C after HIV-1 clade B infection (Figure 4). This suggests a major role of IL-33 in the reduction of MEF2C and possibly disruption of synaptic plasticity.

Proinflammatory cytokine, such as TNFα and IL-1β, production is higher in cases of HIV-1 clade B as compared to clade C.[1,32] TNFα and IL-1β induce apoptosis in neurons[33] and CNS cells, and their production increases on infection with HIV-1 clade. In this research, the contribution of IL-33 to induce apoptosis was assessed (Figure 5B). IL-33 was found to induce apoptosis as measured by caspase-3 assay. This can be explained by the ability of IL-33 to activate the production of other proinflammatory cytokines such as TNFα, IL-1β, IL-6, and MCP-1 in glial cultures.[12,13,34] Therefore, production of IL-33 may be implicated in the induction of apoptosis observed in our results and may be correlated with the clade-specific neuroAIDS manifestations.

SKNMC cells were treated with human recombinant IL-33 for 7 days to investigate the role of other activated genes involving in innate and adaptive immune response on the effects observed after IL-33 treatment, and to elucidate the mechanisms of IL-33 in neuronal cells (Tables 1 and 2). The results showed an increment in the regulation of nucleotide-binding oligomerization domain (NOD2). NOD-like receptor (NLR) signaling represents a major class of cytosolic pattern recognition receptors (PRR) that, like their cell-surface toll-like receptor counterparts, recognize a wide variety of pathogens and immunogenic biological products. Activation of one of four PRR family members initiates the formation of an inflammasome. These protein complexes in turn activate caspase-1, leading to upregulation of the proinflammatory cytokines IL1B and IL18 and pyroptosis, or caspase-1-dependent programmed cell death.[35]

The activation of NOD2 was produced by treatment with the higher concentrations of IL-33, which was observed during HIV 1 clade B infection, only. IL-33 may be considered as NOD2 agonist, inducing the activation of the inflammatory markers c-Jun N terminal kinase, ERK1/2, and p38 MAPK, degradation of inhibitor of kBα,[35] and induction of NF-κB signaling pathway and inflammatory cytokines.[36] According to previous work, HIV infection can upregulate the production and activity of NF-κB, leading to an increment of proinflammatory cytokines, including IL-33, which is considered a beneficial environment to increase the HIV infection.[37] This mechanism may be related with the induction of clade specific neuroAIDS manifestation.[38]

The solute carrier family, with 11 proton-coupled divalent metal ion transporters (SLC11A1), was activated with higher IL-33 concentration treatment in SKNMC with a fold change of 3.97. An upregulation of SLC11A1 was reported on treatment with toxins leading to the activation of mechanisms that support host defense against pathogens including activation of inflammatory responses.[39] Transcriptional activation of SLC11A1 leads to apoptosis, in accordance with the notion that genes that deplete the iron content of the cell cytosol antagonize cell growth.[40] The activation of SLC11A1 using IL-33 is related to the neurological disorders manifested in clade.

In summary, higher levels of IL-33 and ST2 were observed in HIV-1 clade B infected cells than in clade C infected cells. The augmentation in IL-33 affects the expression of important genes and proteins such as MEF2C. The induction of IL-33 and ST2 also affects neuronal cell functions by inducing apoptosis, as estimated using caspase-3 assay and inflammatory conditions and seen as a regulation of innate adaptive immune genes (NOD2, SLC11A1). Neuropathogenesis in clade B infection is associated with IL-33/ST2 levels leading to neuroinflammation and resulting in dysregulation of synaptic function and apoptosis which are found neuroAIDS.

METHODS

Cell Culture. Primary human astrocytes (HA) were obtained from Sciencell Laboratories (Cat# 1800; Carlsbad, CA), and the neuronal cell line, SKNMC cells, was purchased from ATCC (Cat# HTB-10; Manassas, VA). HA were characterized by immunofluorescent method using antibody against glial fibrillary acid protein (GFAP). Primary HA were obtained at passage 2 and used for all experiments between passages 2 and 8. Cells were cultured as per instructions provided by the company.

HIV Infection. Cells were infected with HIV-1Ba-L (National Institutes of Health AIDS Research and Reference Reagent Program;

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Table 1. Functional Gene Grouping Included in Human Innate and Adaptive Immune Responses Gene Array[a]

functional gene grouping	
innate immunity	
pattern recognition receptors	DDX58 (RIG-I), NLRP3, NOD1 (CARD4), NOD2, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9
cytokines	(MCP-1), CCL5 (RANTES), CSF2 (GM-CSF), CXCL10, IFNA1, IFNB1, IL18, IL1A, IL1B, IL2, IL8, TNF
other genes	APCS, C3, CASP1 (ICE), CD14, CD4, CD40 (TNFRSF5), CD40LG (TNFSF5), CD8A, CRP, HLA-A, HLA-E, IL1R1, IRAK1, IRF3, IRF7, ITGAM, LY96 (MD-2), LYZ, MAPK1 (ERK2), MAPK8 (JNK1), MBL2, MPO, MX1, MYD88, NFKB1, NFKBIA (I?Ba/Mad3), STAT1, TICAM1 (TRIF), TRAF6
adaptive immunity	
Th1 markers/immune response	CCR5, CD80, CXCR3, IFNG, IL18, IL23A, SLC11A1, STAT4, TBX21, TLR4, TLR6
Th2 markers/immune response	CCR4, CCR8, CD86, GATA3, IFNB1, IL10, IL13, IL18, IL4, IL5, IL6, NOD2, STAT6
Th17 markers	CCR6, IL17A, RORC, STAT3
Treg markers	CCR4, CCR8, FOXP3, IL10
T cell activation	CD80, CD86, ICAM1, IFNG, IL23A, IL6, SLC11A1
cytokines	CCL2 (MCP-1), CCL5 (RANTES), CSF2 (GM-CSF), CXCL10 (INP10), IFNA1, IFNG, IL10, IL13, IL17A, IL18, IL2, IL23A, IL4, IL5, IL6, IL8, TNF
other genes	CD4, CD40 (TNFRSF5), CD40LG (TNFSF5), CD8A, CRP, FASLG (TNFSF6), HLA-A, IFNAR1, IFNGR1, IL1B, IL1R1, IRF3, IRF7, ITGAM, JAK2, MAPK8 (JNK1), MBL2, MX1, NFKB1, RAG1, STAT1
humoral immunity	C3, CCL2 (MCP-1), CCR6, CRP, IFNB1, IFNG, IL6, MBL2, NOD2, TNF.
inflammatory response	APCS, C3, CCL5 (RANTES), CRP, FOXP3, IL1A, IL1B, IL4, IL6, MBL2, STAT3, TNF
defense response to bacteria	IFNB1, IFNG, IL23A, IL6, LYZ, MBL2, MYD88, NOD1 (CARD4), NOD2, SLC11A1, TLR1, TLR3, TLR4, TLR6, TLR9, TNF
defense response to viruses	CD4, CD40 (TNFRSF5), CD86, CD8A, CXCL10 (INP10), DDX58 (RIG-I), HLA-A, IFNAR1, IFNB1, IL23A, IL6, IRF3, NLRP3, TICAM1 (TRIF), TLR3, TLR7, TLR8, TYK2

[a]This table shows the list of functional genes. Highlighted are genes regulated by treatment with 2 ng of human recombinant IL-33.

Table 2. IL-33 Induces Immune Response Activation in CNS Cells: Fold-up and Fold-down Changes[a]

abbreviation	name	fold-up change	function
CSF2	colony stimulating factor 2 (granulocyte macrophage)	+2.31	cytokine
MX1	myxovirus (influenza virus) resistance 1, infection-inducible protein p78 (mouse)	+2.03	innate other genes
NOD2*	nucleotide-binding oligomerization domain containing 2	+6.63	protein recognition receptor humoral immunity
RAG	recombination activation gene 1	+2.85	adaptive response
SLC11A1*	Solute carrier family 11 (protein coupled divalent metal ion transporters) member 1	+3.97	T cell activation
TLR7	toll-like receptor 7	+2.38	pattern recognition receptor defense to virus
abbreviation	**name**	**fold-down change**	**function**
CXCR3	chemokine (C-X-C motif) receptor 3	−2.71	Th marker
TLR2	Toll-like receptor 2	−2.69	pattern recognition receptor
TLR9	toll-like receptor 9	−2.06	pattern recognition receptor

[a]SKNMC cells were treated with 2 ng of human recombinant IL-33 for 7 days. Cells were collected, RNAs were extracted, and human innate and adaptive immune responses gene array was done using 96-well format. This array interrogates 84 genes related to the innate and adaptive immune response.

Cat# 510) and HIV-1 98CN006 (National Institutes of Health AIDS Research and Reference Reagent Program; Cat# 4164). First, the viruses were propagated using standard protocol. The supernatants were further collected and assayed with p24 ELISA Zeptometrix (Cat# NC9130878) to determine the concentration of p24 protein secreted in the supernatant during 15 days. The collected supernatants were used as a source and stoke of HIV viruses.

HA and SKNMC cells were infected separately at different p24 starting concentrations for HIV-1 clades B and C overnight with previous Polybrene activation for 8 h. Media was removed, and cells were washed extensively to eliminate the unbound virus before addition of fresh medium. After adding fresh media, aliquots were collected. On alternate days, the cultures were supplemented with fresh media. On day 7 postinfection, the supernatants were collected and tested with p24 ELISA Zeptometrix (Cat# NC9130878). The p24 concentration obtained from day zero were compared with aliquots collected at 7 day postinfection to know the increment of p24. The initial amount of p24 selected for future infections was the one that produced a similar level of infection between clades, considered as concentration of p24. The standardization experiments were performed in triplicate with the same batch of viruses and a similar number of passages of cells (Figure 1). The selected concentrations of p24 were 100 and 200 ng for further experiments for clades B and C, respectively. The infection was confirmed and significant when obtained differences were more than double as the initial p24 at day zero.

ELISA. Concentration of HIV-1 p24 antigen in HIV-infected cell culture supernatants was determined using the Retro-tek HIV-1 p24 antigen ELISA kit (Cat# 0801111; Zeptometrix, Buffalo, NY). IL-33 and ST2 secreted levels were also detected using commercially available human IL-33 DuoSet (Cat#DY3625) and human ST2/IL-1 R4 DuoSet (Cat#DY523, R&D systems, Minneapolis, MN) ELISA kits. Protocols for each ELISA were followed according to the manufacturer's instructions.

Quantitative Real Time PCR (qRT-PCR). Gene expression was quantitated using real time qRT-PCR method. Total RNA from HA and SKNMC obtained after 7 days of HIV 1 clade infection (100 ng of clade B and 200 ng of clade C) was used to study IL-33, ST2, and MEF2C gene expression. Taqman assays for IL-33 (Hs01125942_m1; Applied Biosystems, CA), ST2 (Hs00545033_m1; Applied Biosystems, CA), MEF2C (assay ID Hs00231149_m1; Applied Biosystems, CA), and GAPDH (Hs99999905_m1) were used.

Western Blot (WB). IL-33 and ST2 protein levels were tested using WB analysis. The cells were infected with 100 ng of clade B and 200 ng of clade C. After 7 days, the cells were harvested and the cell

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lysates were prepared in protein extraction reagent (Pierce Biotechnology, Rockford, IL) containing protease inhibitor (Pierce Biotechnology) following the manufacturer's recommendations.[41] The protein levels were quantified using the protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal quantities of protein (50 μg) were denatured, subjected to SDS-PAGE, transferred into a nitrocellulose membrane (Bio-Rad Laboratories), blocked with 10% nonfat dry milk, washed with Tris-buffered saline/Tween 20, and incubated overnight with primary antibodies against IL-33 or ST2 (Cat# SAB3500439 and PRS3363, respectively; Sigma-Aldrich). After overnight incubation, the membranes were washed and incubated for 1 h with secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Millipore). The WB was developed using the Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology). Primary goat anti-MEF2C and secondary donkey anti-goat HRP (Cat# sc-13266 and sc-2020; Santa Cruz) were used for the detection of MEF2C protein expression after HIV infection.

Flow Cytometry. HA and SKNMC cells were infected with HIV-1 clades B and C as described above. Golgi inhibitor was used for the cases of determination of IL-33 and ST2. Cells were harvested according to manufacturer's recommendations (Sciencell) using trypsin-EDTA to detach cells and pipeting accordingly to break cell clumps. After cells were counted using a TC20 automated cell counter (Biorad), cells were distributed accordingly and blocked for 10 min on ice using inactivated serum from the species in which the secondary antibody was made. In addition, human inactivated serum was used as a blocker, too. Cell pellets were fixed and permeabilized with Cytofix/Cytoperm solution (BD Bioscience, San Jose, CA), and then they were incubated with primary antibody for 30 min, followed by washing three times and then staining with secondary antibody.

Primary rabbit anti-IL-33 (Cat# SAB3500439; Sigma-Aldrich) or rabbit anti-ST2 (Cat# PRS3363; Sigma-Aldrich) followed by secondary goat anti-rabbit IgG-FITC (Cat# AP132F; Millipore) were used for the staining of IL-33 and its receptor. For flow cytometry experiments, SKNMC cells were treated with different IL-33 concentrations for 24 h, and intracellular levels of MEF2C were detected using goat anti-human MEF2C as a primary antibody and donkey anti-goat FITC as secondary antibody (Cat# sc-13266 and sc2024, respectively; Santa Cruz). Cells were gated based on unlabeled and secondary controls. Cells were acquired using an Accuri Cytometer instrument (Ann Arbor, MI) and analyzed using FlowJo software (FlowJo v9; Ashland, OR). In order to assess cell doublets (clumping) and discriminate them, the provider recommended the plotting of FSC-A against FSC-H. Signals from single cells in the flow cell pass through the laser beam, and FSC-A vs FSC-H signals correlate linearly ^{2}. Data shows that cell clumps were not visible during the experiments.

Caspase-3 Activity Assay. Caspase-3 activity was measured in lysates from HIV-infected SK-N-MC cells using a colorimetric assay (Cat# KHZ0021; Invitrogen). Camptothecin was used as a positive control (Sigma-Aldrich; Cat# C9911). SKNMC cells were infected with HIV-1 clade B (100 ug) and clade C (200 ug) following protocols described above. Separately, SKNMC cells were treated with different IL-33 concentrations for 7 days (120, 240, and 480 pg/mL; 1 and 2 ng/mL). All the cells were harvested and lysed, and caspase-3 activity was quantitated in the cell lysates. Caspase-3 activity in samples was read at 400 or 405 nm in a microplate reader (Synergy HT, Biotek).

RT^2 Profiler PCR Array Human Innate and Adaptive Immune Responses. SKNMC cells were treated with 2 ng of human recombinant IL-33 (Cat# 3625-IL-010; R&D systems, Minneapolis, MN) during 7 days and harvested. The pellets were used for the mRNA isolation using the Illustra triplePrep kit (GE Healthcare Life Sciences, U.K.; Cat# 28-9425-44), and on-column DNase treatment step was also performed in the procedure. RNA was measured via microspot RNA reader (Synergy HT Multi-Mode Microplate Reader from BioTek). A total of 1 μg of RNA was used for the first strand cDNA synthesis using SA Biosciences's RT2 First Strand kit (Cat# 330401) as per the supplier's protocol. Genomic DNA elimination step was performed before proceeding with reverse transcription.

Human innate and adaptive immune responses was done using 96-well format (Qiagen; Cat# PAHS-052Z) using the Stratagene Mx3000p qRT-PCR instrument. This array interrogates 84 genes related to the innate and adaptive response. Innate immunity includes pattern recognition, cytokines, as well as other genes involved in the innate response, while adaptive immunity includes Th1, Th2, Th17, Treg markers/immune response, T cell activation, humoral immunity, inflammatory response, and defense response to bacteria and viruses. Functional grouping genes are shown in Table 1. Relative abundance of each mRNA species was assessed using RT2 SYBR Green/ROX PCR Master mix (SABiosciences, Cat# 330520), used for the real-time PCR arrays. The real time PCR cycling program (as indicated by the manufacturer) was run on a Stratagene Mx3000p qRT-PCR instrument. Ct data were analyzed in the data analysis template on the manufacturer's Web site (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Controls are also included in each array for genomic DNA contamination, RNA quality, and general PCR performance.

Statistics. Experiments were performed at least three times in duplicate unless otherwise indicated in the figure legend. The values obtained were averaged, and data are represented as the mean + standard error. All the data were analyzed using GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA and Tukey's multiple comparison post test. Differences were considered significant at $p \leq 0.05$.

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Author Contributions

Conceived and designed the experiments: A.Y., M.A., and M.N. Performed the experiments: A.Y. and A.K.. Analyzed the data: A.Y., M.A., A.R., S.K.S., and V.S.A. Contributed reagents/materials/analysis tools: A.K., M.A., and M.N. Wrote the paper: A.Y.

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Notes

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ABBREVIATIONS

Cat#, catalog number; CNS, central nervous system; CSF2, colony stimulating factor 2; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular-signal-regulated kinase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acid protein; HA, human astrocytes; HAND, HIV associated neurocognitive disorders; HIV-1, human immunodeficiency virus; HRP, horseradish peroxidase; IgG-FITC, fluorescein isothiocyanate; IL-1β, interleukin-1 beta; IL-33, interleukin 33; IL-6, interleukin 6; MAP, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein 1; M-CSF, macrophage colony-stimulating factor; MEF2C, myocyte enhancer factor 2C; MX1, myxovirus (influenza virus) resistance 1; NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; NOD2, nucleotide-binding oligomerization domain containing 2; P24, capsid protein; p38 MAPK, P38 mitogen-activated protein kinases; pg/mL, pico gram per milliliters; qRT-PCR, real-time quantitative reverse transcription PCR; RAG1, recombination

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activation gene 1; RNA, ribonucleic acid; SDS-PAGE, polyacrylamide gel electrophoresis; SKNMC, brain cell line; SLC11A1, solute carrier family 11; ST2, suppression of tumorigenicity 2/Interleukin 1 receptor-like 1; TAI, transcript accumulation index; TLR7, toll-like receptor 7; TNFα, tumor necrosis factor alpha; WB, Western blot

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