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HIV Nef enhances the expression of oncogenic c-MYC and activation-induced cytidine deaminase in Burkitt lymphoma cells, promoting genomic instability

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Abstract

Background: Non-Hodgkin lymphoma is of high prevalence among HIV-infected people. In particular, the incidence of HIV-associated Burkitt lymphoma (BL) remains high despite the advent of Highly Active Anti-Retroviral Therapy. Recent evidence shows that serum-soluble HIV proteins can enhance oncogenesis, particularly in lymphoid tissues. This study sought to define the role of HIV protein Negative regulatory factor (Nef) in BL development by assessing its effect on key lymphoma driver genes.

Methods: A recombinant Nef protein was used to assess changes in expressions of activation-induced cytidine deaminase (*AICDA*/AID) and c-MYC in B lymphocytes exposed extracellularly to the protein. Additionally, changes in the promoter activities of these genes were measured using a Nef-expressing cellular model and reporter assays. Confocal microscopy was used to observe c-MYC and AID expression and localization, and genomic integrity via the recruitment of phosphorylated γ-H2AX, in Nef-exposed cells.

Results: mRNA transcription of *c-MYC* and *AICDA* were significantly enhanced in lymphoma cells, up to 2-fold for *c-MYC* and up to 4-fold for *AICDA*, when exposed to varying concentrations of Nef (0–1000 ng/ml) and for different periods of time (3, 6 and 12 h). The protein expressions of AID and c-MYC followed a similar pattern and these effects were specific to BL but not lymphoblastoid cells. While the promoter activity of *c-MYC* was enhanced in the presence of Nef in a dose-dependent manner, the same was not observed for *AICDA*. Both AID and c-MYC accumulated within the cytoplasmic and nuclear spaces of Nef-exposed lymphoma cells, with a concomitant increase in DNA double strand breaks within the genome.

Conclusions: Exposure to HIV Nef leads to significant increases in AID and c-MYC, leading to genomic instability, potentially enhancing the oncogenic potential of Burkitt lymphoma. Our findings align with that of others to show that HIV proteins can directly contribute to the development and pathogenesis of HIV-associated lymphoma and accounts for the elevated incidence of BL observed in the HIV-infected population.

Keywords: HIV, Nef, Burkitt lymphoma, c-MYC, AID, Genome integrity

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Background

Burkitt Lymphoma (BL) is a highly aggressive Non-Hodgkin lymphoma (NHL) of B cell origin and the first haematological malignancy to be associated with HIV infection [1]. Its pathogenesis is strongly linked to the overexpression of c-MYC, an oncogenic transcription factor and an important regulator of apoptosis, cell growth and cellular metabolism [2]. The overexpression of c-MYC is described in a majority of cancer types and shown to contribute to the cause of at least 40% of tumours [3]. In BL, *c-MYC/IGH* translocation events are described as a hallmark of the disease, with t(8;14)(q24; q32) being the most frequent (70–80% of cases) [4]. Importantly, the *c-MYC* translocation event has been shown to be dependent on expression of the B-cell specific protein Activation-Induced cytidine Deaminase (AID), an essential enzyme in antibody diversification in B cell immune responses [5, 6]. Both somatic hypermutation and class switching require AID which converts deoxycytidines to deoxyuracils in single-stranded DNA. Therefore the expression of this enzyme warrants tight regulation to prevent aberrant expression. The deregulation of AID activity has been observed especially in cancers of the B cell lineage, and interestingly a study in Diffuse Large B Cell Lymphoma patients who underwent CHOP based chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) found that elevated AID expression can be used as a marker of unfavourable outcome [7, 8].

Compared to the HIV negative population, people living with HIV have a reported 70–200 times greater risk of developing an HIV-related lymphoma, and the risk remains high even after the start of antiretroviral therapy [9, 10]. A 2017 study of a national hospital-based cancer registry dataset in the United States found that HIV/AIDS continues to be independently associated with increased risk of death among patients with lymphoma [11]. Surprisingly, a few reports have found a significant proportion of HIV positive patients develop BL even when not severely immunosuppressed, having CD4 counts > 200 cells/mm^3 and suggest that the pathobiology of HIV-BL is unique despite similarities at the macroscopic and microscopic levels across the various BL subtypes namely sporadic, endemic and HIV-related [12, 13].

In recent years, new evidence has emerged supporting a direct role for HIV and its viral components in driving the oncogenic process. For instance, the HIV-1 matrix protein p17, which has long term persistence in the tissues of HIV positive patients on HAART, was reported to induce angiogenesis by binding to the chemokine receptors CXCR1 and CXCR2 [14]. Furthermore, recent reports show that HIV-1 transactivator of transcription (Tat) protein could contribute to the remodelling of the B-cell nucleus, inducing oxidative DNA damage and increasing the probability of c- *MYC* / *IGH* translocations [15]. While HIV does not infect B cells, studies suggest that serum-soluble HIV proteins could function as biologically active extracellular proteins released by infected cells and/or be transported inside uninfected B cells where they disrupt normal cellular function and act as contributors to oncogenesis [16, 17]. The HIV Negative regulatory factor (Nef), a multifunctional 27-kDa protein is one of the first HIV proteins to be produced in infected cells and the most immunogenic of the accessory proteins [18]. Nef interacts with the host cells' signal transduction proteins providing long term survival of the infected T cells. It can also promote apoptosis of uninfected T cells and the endocytosis and degradation of cell surface receptors such as CD4 and MHC proteins. This impairs cytotoxic T cell function which helps the virus evade the host's immune response. While the Nef genes of HIV are dispensable in vitro, they are essential for efficient viral spread and disease progression in vivo. Nef is detected in the serum of HIV infected patients, even those who are virally suppressed [19, 20]. In vitro, Nef has been shown to lead to overexpression of the B lymphocyte stimulator BLyS by monocyte-derived denditric cells, and has also been shown to travel from infected macrophages into B cells along actin-propelled conduits [21, 22].

In a recent study Nef was found to actively drive lung cancer in HIV infected patients in whom lung cancer develop on average a decade earlier than uninfected patients [23, 24]. In the presence of Nef, lung cells were found to have increased proliferative and invasive capabilities, and enhancement in expression of vascular endothelial growth factor A (VEGF-A), a key facilitator of angiogenesis. In Kaposi sarcoma Nef has been shown to synergize with the human herpesvirus 8 protein K1 to promote cellular proliferation and angiogenesis through the phosphatase and tensin homolog / protein kinase B / mammalian target of rapamycin (PTEN/AKT/mTOR) pathway mediated by miRNA-718 [25]. In B cells, the mechanisms by which Nef may contribute to lymphoma, either from external binding or via internalisation remains to be elucidated. One potential mechanism by which Nef could enhance B-cell lymphoma development is via enhancing the expression of proto-oncogenes. The aim of this study was to determine whether the HIV-1 protein Nef can affect the expression of AID and c-MYC, two important role players in Burkitt lymphoma development, and the impact that this may have on the B cell genome.

Methods

Cell lines, cell treatments and plasmid constructs

The Ramos cell line (ATCC® CRL-1596™) was obtained from the American Type Culture Collection (ATCC, USA) and cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal calf serum (FCS) (Biochrom, Germany) and 1% penicillin-streptomycin

(PS) (Sigma-Aldrich, USA). The EBV-immortalized B lymphoblastoid cell line (L1439A), was produced at the University of Cape Town (Division of Chemical Pathology) using an adapted protocol and cultured in Dulbecco's Modified Eagle Media (DMEM) medium containing 20% FCS with 1% PS [26]. The HT1080 cells were cultured in DMEM containing 10% FCS and 1% PS. All media were from Sigma-Aldrich (USA). All cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

The pcDNA-Nef construct was donated by Professor Mitra (National Centre for Cell Science, India) [27] and the pET-14b-NEF SF2 construct was kindly donated by Professor Smithgall (University of Pittsburgh, USA) [28]. The pGL3-cMyc plasmid was a kind gift from Professor Dai (University of California USA) [29]. pRL-TK was a kind gift from Professor Prince (University of Cape Town). The pGL3-hAID construct was produced by cloning of a ~ 2000 bp 5`-UTR sequence upstream of the Transcription start site (TSS) of the *AICDA* gene into the pGL3-Basic backbone (Promega, USA). For cell treatments, cells were plated and exposed to Nef (or control) by adding purified His-tagged protein directly to cell cultures at indicated concentrations and incubated for 3, 6 and 12 h.

Production of recombinant His-tagged HIV Nef

The *E.coli* strain BL21 pLysS was transformed with either pET-14b-NEF SF2 or p*ET21+* (control) and grown until mid-log phase when synthesis of the His-tagged recombinant HIV protein was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM final conc.) for 4 h at 25 °C. His-tagged protein was isolated using the His-Spin protein miniprep system (Zymo Research, USA). Lysate from cells transformed with the pET21+ empty vector was used as control. Bacterial endotoxins were removed using the High Capacity Endotoxin Removal Resin (Pierce, USA). Purified recombinant protein was quantified using a BCA Assay (Thermo Scientific™, USA) and specificity and purity were assessed using SDS-PAGE and western blotting.

SDS-PAGE and Western blot analyses

Total protein extracts were prepared using RIPA lysis buffer: 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl (pH 7.5), 1% deoxycholate, supplemented with protease inhibitors (Roche, Switzerland). Proteins were resolved on 8–12% SDS-polyacrylamide gels, transferred to Hybond ECL membranes (Amersham Biosciences, USA), probed with appropriate primary antibodies, detected using peroxidase-conjugated secondary antibodies and visualized by ECL (Pierce, USA). Antibodies: HIV-1 Nef antiserum (2949 - NIH AIDS Research and Reference Reagent Program; 1:1000); anti-c-MYC (SC-764, Santa Cruz Biotechnology, USA; 1:1000); anti-AID (392,500, Thermofisher Scientific, USA; 1:1000); anti-p38 (M0800, Sigma USA; 1:5000).

Quantitative real time PCR

Total RNA was extracted using the Highpure RNA isolation kit (Roche, Switzerland). Reverse transcription (1 μg) was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). Real-time PCR was performed on a LightCycler® 480 (Roche, Germany) using KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, South Africa). Relative mRNA expression levels were normalized to GAPDH for each reaction with PCR efficiency correction calculated using the formula Ratio = $(\text{Etarget})^{\text{CPtarget(control − sample)}}$/ $(\text{Eref})^{\text{CPref(control − sample)}}$; E: real-time PCR efficiency, CP: crossing-point. Primers used to amplify the human *AICDA*, *c-MYC* and *GAPDH* genes were manufactured by IDT (Qiagen, Germany). Primers: AID-forward 5`-CCAA ACCATCTCTCCAAAGC-3`, AID-reverse 5`- CATCCC CACCCATAACAATC-3`; c-MYC-forward 5`- CTGAGA CAGATCAGCAACAACC-3`, c-MYC-reverse 5`- TTGT GTGTTCGCCTCTTGAC-3`; GAPDH-forward 5′- CAG-GAGGCATTGCTGATGAT-3′; GAPDH-reverse 5′-GAAGGCTGGGGCTCATTT-3′.

Transfections and luciferase assays

Transient transfections were performed using Xtreme-Gene HP (Roche, Switzerland). Cells were plated to achieve 70–80% confluency at the time of transfection. HT1080 cells were transfected with 500 ng per well of luciferase reporter constructs for human AID (pGL3-hAID) or c-MYC (pGL3-cMYC) and 0–500 ng of pcDNA-Nef or empty vector per well. The vector pRL-TK was used as an internal control for transfection efficiency. Cells were cultured for 30 h and extracts were assayed for firefly and renilla luciferase activity using the dual luciferase assay system (Promega, USA). Luciferase activities were measured using the Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA, USA). Firefly luciferase values were normalised to the renilla luciferase activity and expressed relative to empty vector control.

Immunocytochemistry and confocal microscopy

Cells were plated and treated as indicated and thereafter transferred to microfuge tubes, pelleted, washed 2x in PBS and fixed using 4% PFA. Cells were washed again in PBS, permeabilized with 0.3% Triton x-100 in PBS, and then washed in PBS containing 0.1% Tween-20. Cells were blocked for 1 h in PBS + 5% swine serum and incubated overnight at room temperature in primary antibody (anti-c-MYC/anti-AID/anti-phospho-Histone H2A; Cell signalling technology, USA; 1:100 dilution) diluted in blocking buffer. After 2x washes in PBS + 0.1% Tween-20, cells were incubated in fluorescently labelled (Cy3) secondary antibody for 1 h at room temperature.

Cells were thereafter spun and the pellet washed 2x in PBS and then stained with 1 μg/ml DAPI in PBS for 10 min, mounted on a slide with Mowial mounting medium containing anti-fade (Sigma, USA) and examined the next day using confocal microscopy.

Statistical analysis

All cell treatments, qPCR and luciferase reporter assays were performed at least in triplicate. Statistical analysis was done using the GraphPad Prism software and significance between groups was determined using the Mann-Whitney U test (two-tailed) and p values < 0.05 were considered significant.

Results

Extracellular exposure to recombinant HIV Nef leads to upregulation of c-MYC and AID in lymphoma cells

To mimic an in vivo scenario where B cells are exposed to soluble HIV proteins found in the serum of HIV infected individuals, B lymphocytes were extracellularly exposed to varying concentrations of recombinant Nef protein for various lengths of time. The amount of Nef present in the serum of a patient is dependent on several factors including viral load and studies have reported up to 1000 ng/ml of Nef being detected in the serum of HIV positive individuals, and in vitro studies have used up to 1000 ng/ml recombinant Nef protein in exogenous assays [30, 31]. In our study, cells were exposed to 0, 200, 500 and 1000 ng/ml of recombinant Nef, over a period of 3, 6 and 12 h. As can be seen in Fig. 1a, exposure of lymphoblastoid cells (LCLs) to Nef led to a marginal increase in the expression of *c-MYC* mRNA at the earliest time point of 3 h, and thereafter, at 6 h and 12 h, no major increases were recorded, and interestingly, it appeared that *c-MYC* mRNA levels decreased slightly below control levels. In the Burkitt lymphoma cell line Ramos, Nef exposure led to a higher increase in *c-MYC* transcription compared to LCLs at the 3 h time point, and was sustained at 6 h and 12 h, especially at 200 and 500 ng/ml of recombinant Nef exposure. This difference in response between the LCLs and the Ramos cells is even more apparent for *AICDA* expression, with the shortest exposure of 3 h showing the largest differential response. Up to a 4.6-fold increase in *AICDA* mRNA was recorded for Ramos cells when exposed to 500 ng/ml Nef for 3 h, compared to a marginal increase of 1.2 in the LCLs. At the 6 and 12 h time points, the response was less robust in the Ramos cells, albeit still slightly higher than in LCLs. Once again, lower concentrations of Nef (200 and 500 ng/ml) lead to larger increases in *AICDA* in the Ramos cells, compared to the higher concentration of 1000 ng/ml.

To verify that changes in transcription led to changes in protein expression, western blotting was performed. We chose to only assess changes in protein expression in the Ramos cells, and at the 3 h and 6 h time points only, since these were significantly more responsive to Nef exposure compared to the LCLs. We found that although the expression of both the c-MYC and AID protein were increased in Ramos cells exposed to Nef, these increases did not correlate exactly to the mRNA levels (Fig. 1b). At the 3 h time point, the c-MYC protein expressions were generally higher than the control (0 ng Nef) although a similar trend as *c-MYC* mRNA increase was not observed. At 6 h post-exposure, c-MYC protein expression was only enhanced when cells were exposed to the lower Nef concentration of 200 ng.

As seen for c-MYC, AID protein expression was enhanced at both the 3 h and 6 h time points. While a correlation with mRNA expression was not seen, it can be noted that at the earlier time point of 3 h, AID protein expression was most pronounced when cells were exposed to the lower concentrations of Nef, while at the later time point of 6 h, it would appear that AID protein levels at 200 ng Nef had gone back down to control levels, but were enhanced for the 500 ng and 1000 ng Nef exposures.

Nef enhances the promoter activity of *c-MYC* but not that of *AICDA*

We sought to investigate whether the changes in *c-MYC* and *AICDA* expressions observed upon exposure to Nef were as a result of changes in the activity of the promoter of these genes in the presence of Nef. While B lymphocytes are not hosts for HIV, the viral proteins have been reported to become internalised within these cells [32]. Moreover, Nef has been shown to modulate the transcription of cellular genes and to cooperate with cellular proteins such as transcription factors to regulate genes [33, 34]. We used a luciferase reporter assay system to investigate whether Nef affects the promoter activities of both *c-MYC* and *AICDA*. The HT1080 cell line was transiently transfected with a constant amount of a luciferase reporter containing either the human *c-MYC* or *AICDA* promoter, as well as varying doses of a mammalian expression vector expressing Nef. We found that the activity of the *c-MYC* promoter significantly increased in a dose-dependent manner in the presence of increasing amounts of Nef protein, with a maximum increase of 2.5 fold observed when 500 ng of the Nef expressing plasmid was used (Fig. 2a – top panel). On the other hand, the activity of the *AICDA* promotor was not significantly altered, even at high doses (Fig. 2a – bottom panel). Western blot analysis was used to demonstrate that increasing amounts of Nef-expressing plasmid correlated with increasing expression of the Nef protein within the cells (Fig. 2b).

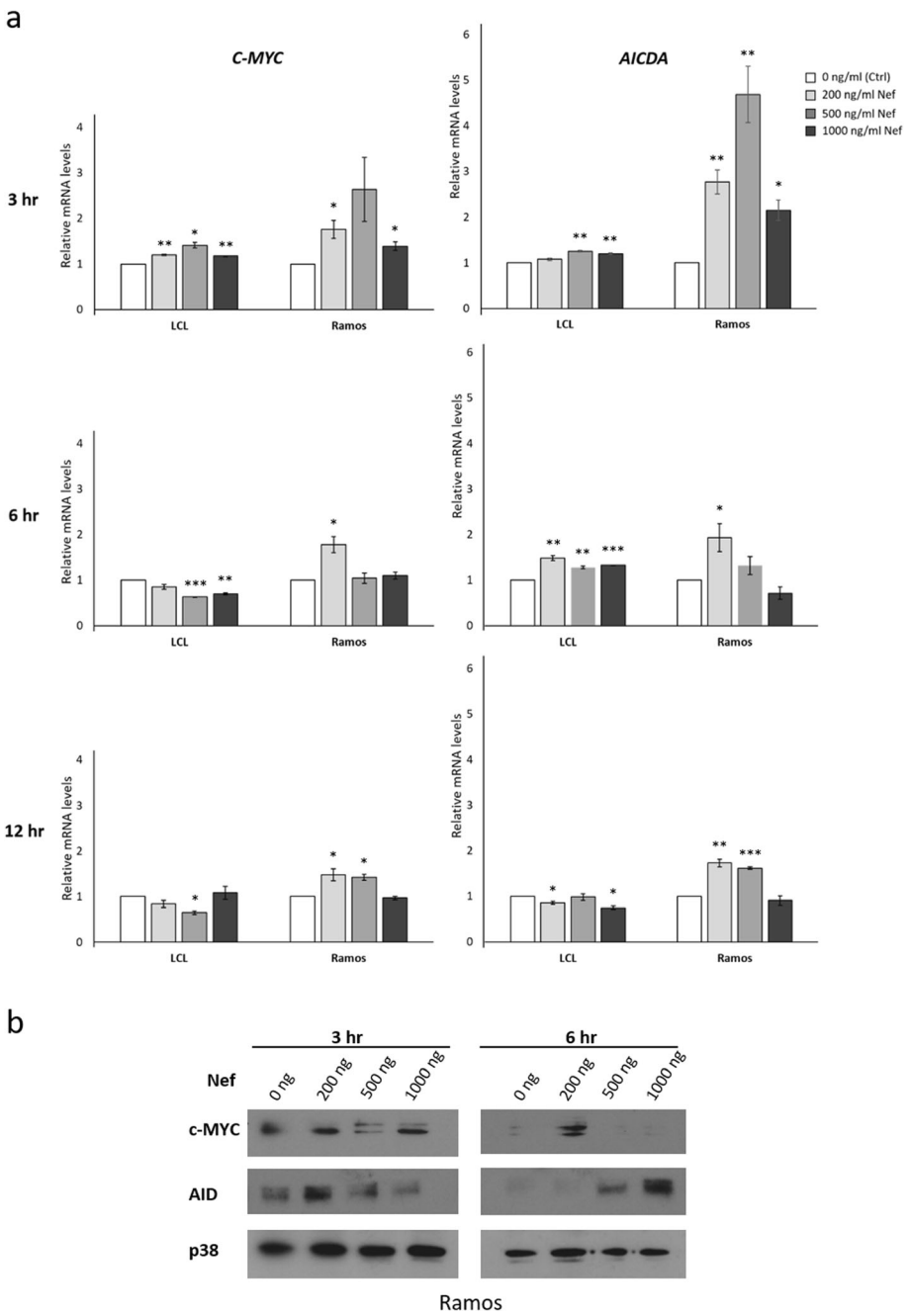

Fig. 1 AID and c-MYC expressions are altered in B cells upon exposure to HIV Nef. a. Bar graphs showing relative mRNA expression of *c-MYC* (left panel) and *AICDA* (right panel) at 3 h, 6 h and 12 h exposure to HIV Nef, in lymphoblastoid cells (LCLs) and Burkitt lymphoma cells (Ramos). Cells were treated at the indicated concentrations of recombinant Nef, total RNA was isolated and qPCR was performed on reverse transcribed mRNA using primers specific to *c-MYC* or *AICDA*. The levels were normalised to the internal control *GAPDH* and plotted relative to mock (0 ng/ml) treated cells. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$. All experiments were performed in triplicate. Bars indicate standard deviation. b. Ramos cells were treated at the indicated concentrations of recombinant Nef, total protein was isolated, separated on 12% SDS-PAGE and analysed by western blotting with antibodies against c-MYC and AID. p38 was detected as a loading control. All experiments were performed in triplicate

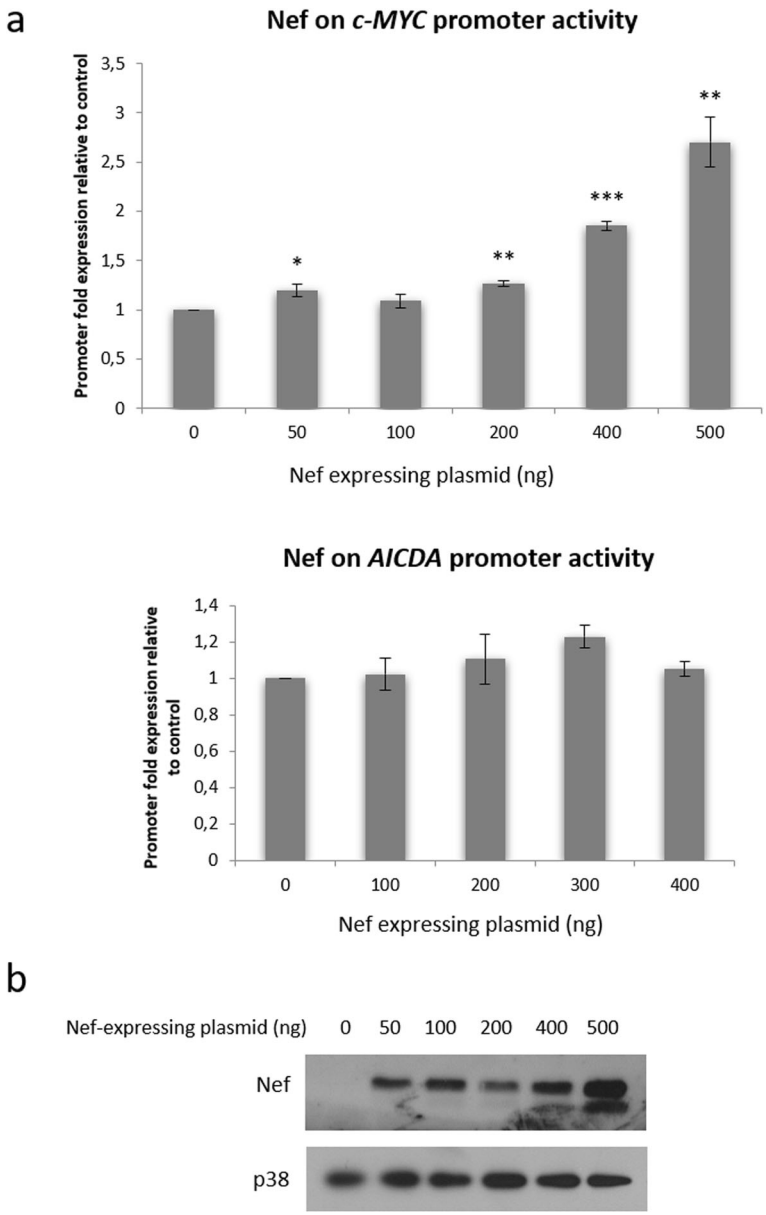

Fig. 2 HIV Nef activates the human *c-MYC* promoter, but not the *AICDA* promoter. **a**. Bar graphs showing promoter activities of *c-MYC* (top panel) and *AICDA* (bottom panel) when exposed to Nef, relative to control (empty vector). In both cases, the promoter-luciferase reporter construct (500 ng per well in 35 mm dish) was co-transfected into HT1080 cells with the internal control pRL-TK (50 ng) in the presence of increasing concentrations of the Nef expressing vector pcDNA-Nef (0–500 ng) or empty vector (pcDNA3.1). Luciferase activity was measured 30 h post-transfection and normalized to renilla luciferase activity. Activation fold values were calculated by setting promoter activity exposed to pcDNA3.1 empty (500 ng) to 1. Mean values (±) were calculated from three independent experiments. Bars indicate standard deviation. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$. All experiments were performed in triplicate. **b**. To demonstrate that increasing amounts of Nef expressing plasmid leads to increasing expression of Nef protein, western blot analysis of whole proteins lysates from luciferase assays performed in HT1080 cells above was performed. Proteins were separated on 15% SDS-PAGE, transferred onto nitrocellulose membranes and analysed using an antibody specific to Nef. p38 was detected as a loading control to demonstrate equal loading of protein lysates

Nef-driven AID over-expression leads to accumulation of c-MYC and increased genomic instability in lymphoma cells

The translocation and overexpression of oncogenic c-MYC in B lymphocytes is intimately linked to the overexpression of AID, a protein which is known to induce double strand breaks in DNA [5, 35]. To further explore the downstream physiological consequences of Nef exposure, the Ramos BL cells were exposed to Nef and immunocytochemistry was performed followed by

confocal fluorescence microscopy to study c-MYC and AID expression and localization, as well as genomic integrity using a specific marker for double strand breaks. As can be seen in Fig. 3, a clear increase in both c-MYC and AID can be seen in the BL cells exposed to the purified recombinant Nef protein, compared to the control cells. Within the Nef exposed cells, AID and c-MYC accumulated both within the cytoplasm and the nucleus. We investigated the integrity of the genome by assessing the presence of DSBs within the cells. H2AX phosphorylation is an early event in the DNA damage response (DDR) to different genotoxic stresses that induce DSBs and gets recruited to sites of DSBs within the genome [36]. Lymphoma cells exposed to HIV Nef harboured a significantly higher number of DSB sites within the DNA as observed by the increased detection of γ-H2AX-associated foci within these cells.

Discussion

HIV as an oncogenic virus and a driver of lymphoma is an emerging concept which is rapidly gaining credibility as more reports emerge supporting this. Numerous studies, including ours, do not only implicate Nef but also other soluble HIV proteins including Tat and p17 [14, 15, 24, 25]. In the latter cases, research has advanced to show that these viral proteins have the ability to exploit cellular components and pathways in complex ways to advance oncogenic events.

The ability of Nef to alter the expression of two important genes in the development of Burkitt lymphoma, namely *AICDA* and *c-MYC*, was the focus of this study. We demonstrated an increase in the expression of these two markers at both the mRNA and protein levels in BL cells when exposed to HIV Nef. Previous studies have

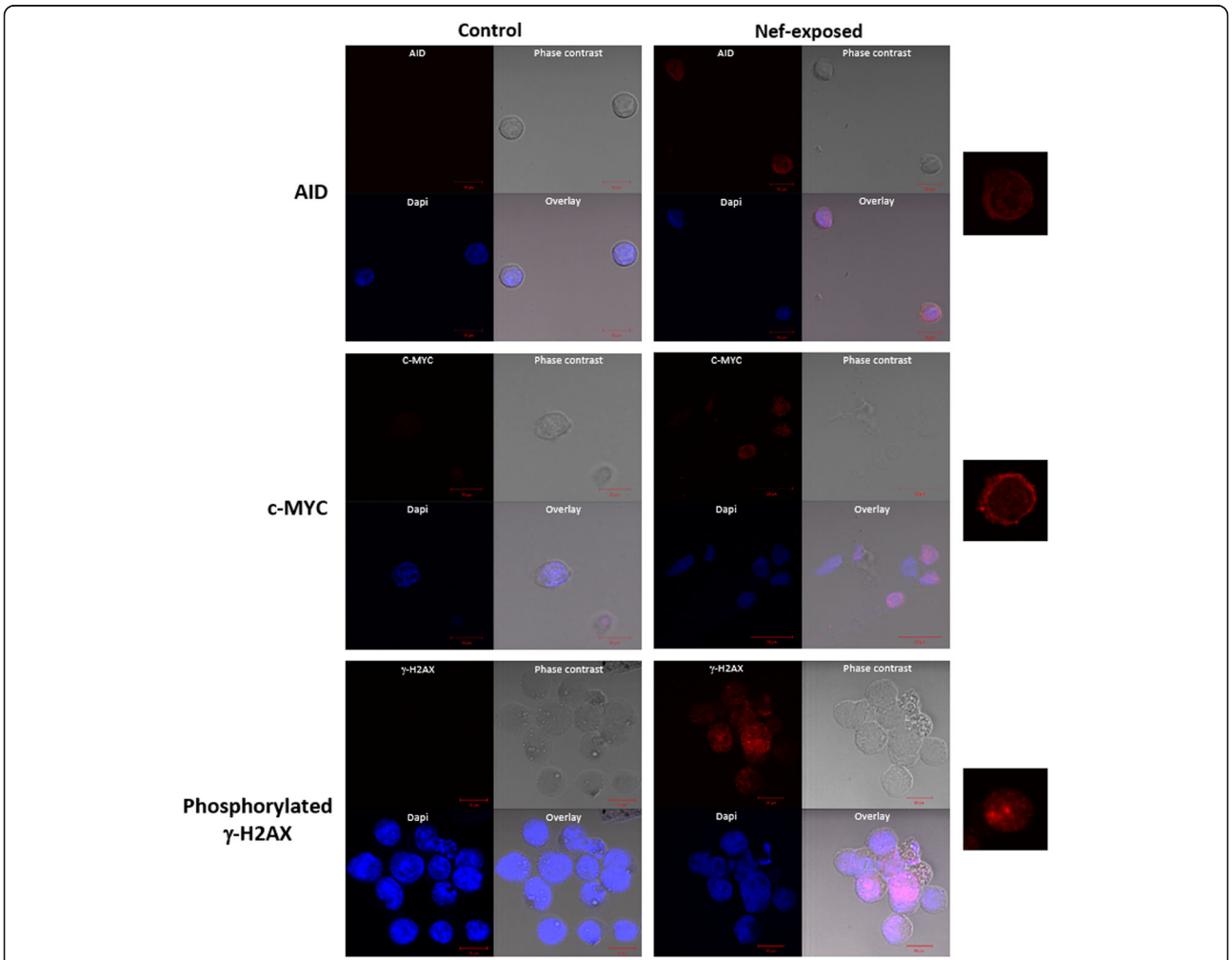

Fig. 3 Exposure to Nef leads to accumulation of c-MYC and AID in the cytoplasm and nuclei of lymphoma cells, enhancing genomic instability. Ramos cells were exposed to 500 ng/ml of HIV Nef for 3 h. Thereafter cells were fixed and permeabilized. The presence of c-MYC, AID and γ-H2AX were detected with specific primary antibodies and fluorescently tagged (Cy3 - red) secondary antibodies. DNA was stained with Dapi (blue). Cells were mounted on slides and images were captured using confocal microscopy. Left hand panels represent cells which were mock-treated (protein prepared using empty vector), and right hand panels represent cells exposed to recombinant HIV Nef. On the far right hand side of each panel is a zoomed image of one representative Ramos cell from that panel

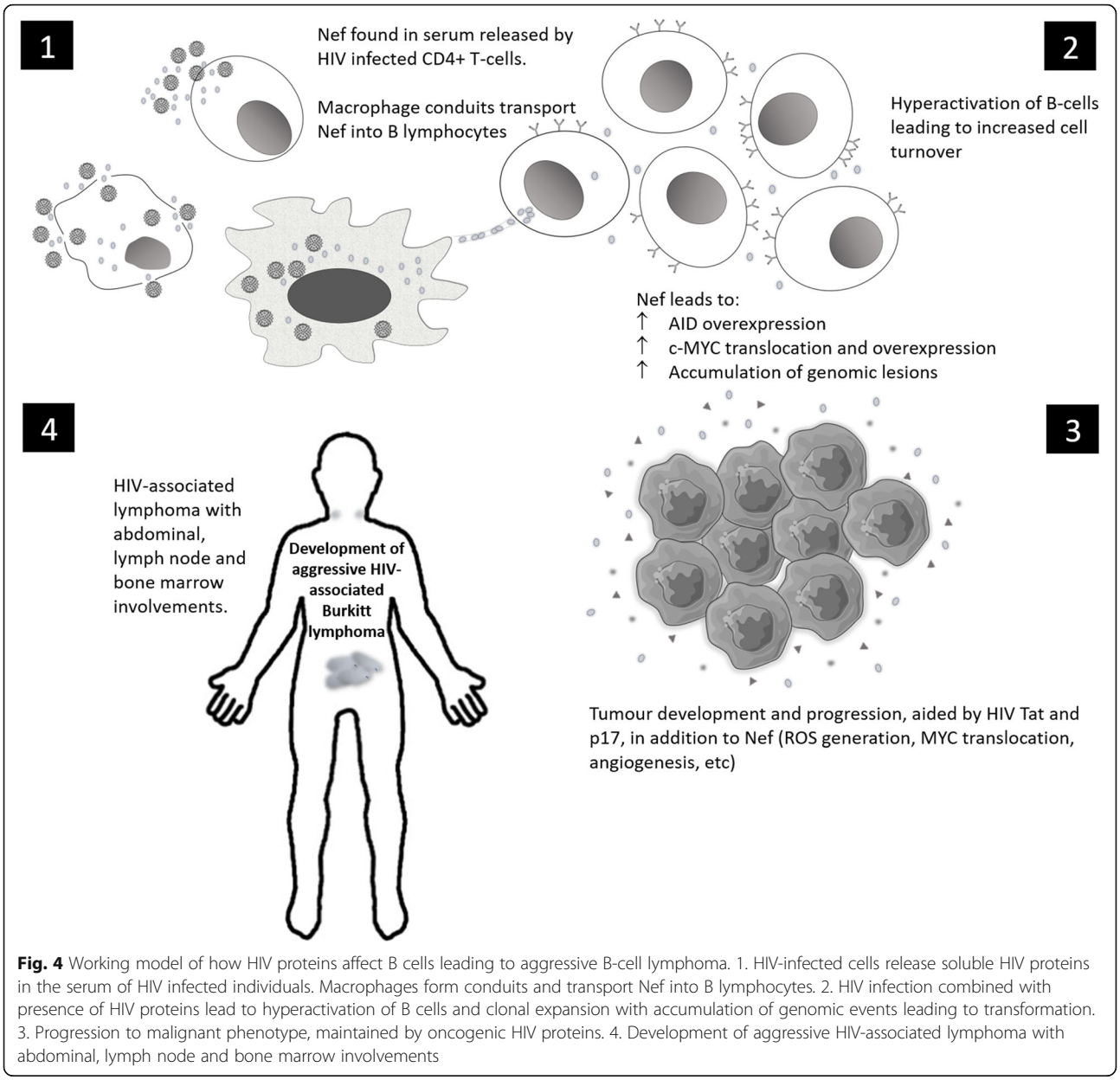

Fig. 4 Working model of how HIV proteins affect B cells leading to aggressive B-cell lymphoma. 1. HIV-infected cells release soluble HIV proteins in the serum of HIV infected individuals. Macrophages form conduits and transport Nef into B lymphocytes. 2. HIV infection combined with presence of HIV proteins lead to hyperactivation of B cells and clonal expansion with accumulation of genomic events leading to transformation. 3. Progression to malignant phenotype, maintained by oncogenic HIV proteins. 4. Development of aggressive HIV-associated lymphoma with abdominal, lymph node and bone marrow involvements

shown that AID is elevated in the PBMCs of HIV-infected individuals who later developed lymphoma and more recently, B-cells treated with recombinant Tat had an increase in the *AICDA* gene [17, 37]. Our study shows yet another HIV protein, with demonstrated oncogenic potential, having a similar effect.

The oncogenicity of AID, via its mutagenic activity, is well described, not only in lymphoma but also in cancers of organs including the colon, lung and skin [38–41]. An enhancement in AID levels leads to cancer promoting features such as uncontrolled cellular proliferation and development of tumours in mice. Our findings confirm the well reported close relationship between AID and MYC expressions and we further demonstrate enhancement of MYC activity via its translocation into the nucleus where it can perform its primary function as a transcription factor. Future studies should focus on Nef's ability to affect cellular features including proliferation, apoptosis and invasiveness in lymphoma cells, which has recently been reported in lung cancer cells [24]. Additionally, HIV Nef and Tat have both been demonstrated to contribute to oncogenesis through cooperation with cellular miRNAs as well as other oncogenic viruses, and this too is another important area of investigation in the pathogenesis of HIV-associated lymphoma.

The recruitment of phosphorylated γ-H2AX at numerous DSB foci within the nucleus of Nef exposed cells show a more unstable genome in the presence of the viral protein. We attribute this phenomenon to aberrant

AID accumulation since the latter triggers class switch recombination (CSR) in B cells by initiating DNA double strand breaks. Genomic instability facilitates the accumulation of genetic lesions required to maintain the cancer phenotype and our study is supported by that of Germini et al. who showed that HIV Tat can induce DNA damage in B-cells in vitro [42].

Compared to lymphoma cells, the non-malignant lymphoblastoid cells showed very mild increases in *AICDA* and *c-MYC* upon exposure to Nef. This could indicate that the response is dependent on cell membrane components and that malignant cells become more responsive to oncogenic stimuli through the transformation process. Indeed, studies have shown that there are variations in the cell membrane carbohydrates between cancer and normal cell membranes and these differences translate into differential pathways and cellular uptake abilities [43].

We show that Nef could enhance the promoter activity of *c-MYC* but not that of *AICDA*. It is likely that extracellular exposure to Nef could affect a greater repertoire of genes, perhaps through ligand binding and activation of signalling pathways. However, upon internalisation the activity of Nef could be more specific and targeted. This is consistent with what has been observed for HIV p17 which has been shown to interact with the chemokine receptors CXCR1 and CXCR2 of B-cells, but the protein can also become internalized by a process not yet characterized [14]. We are invested in further defining the association between Nef and *c-MYC* transcription. While Nef may be acting as a transcription factor by directly binding to and enhancing the promoter activity, several reports support a role for Nef in cooperating with cellular transcription factors to alter transcription, including AP-1 and NF-kB [30].

While more investigations are needed to further define the mechanism of Nef-driven lymphoma, it is clear that HIV infection provides an additional layer of complexity in cancer development and progression. The illustration in Fig. 4 summarises our findings as well as that of other studies involving HIV Tat and p17, and provides a working model of how HIV proteins could be contributing to the development of aggressive B-cell derived malignancies in HIV infected individuals.

Abbreviations

AICDA / AID: Activation-Induced cytidine Deaminase; ATCC: American Type Culture Collection; BCA: Bicinchoninic acid; BL: Burkitt Lymphoma; CHOP: Cyclophosphamide, doxorubicin, vincristine, and prednisone; CSR: Class switch recombination; CXCR1: C-X-C motif chemokine receptor 1; CXCR2: C-X-C motif chemokine receptor 2; DAPI: 4′,6-diamidino-2-phenylindole; DMEM: Dulbecco's Modified Eagle Media; DSB: Double Strand Break; EBV: Epstein Barr Virus; ECL: Enhanced Chemiluminescence; FCS: Fetal Calf Serum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HAART: Highly active antiretroviral therapy; HIV: Human Immunodeficiency Virus; IDT: Integrated DNA technologies; IGH: Immunoglobulin heavy chain; LCL: Lymphoblastoid cells; Nef: Negative regulatory Factor; NHL: Non-Hodgkin lymphoma; PBS: Phosphate Buffered Saline; PCR: Polymerase Chain Reaction; PS: Penicillin-streptomycin; RPMI: Roswell Park Memorial Institute; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; VEGF-A: Vascular endothelial growth factor A

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Authors' contributions

SM - Conceptualized the research, developed the methodology, performed experiments and data analysis, sourced the funding, curates the data, supervised the research, and wrote and edited the manuscript. AN – Assisted with the development of some methodology and assisted with the execution of some experiments. NM – Performed experiments and some data analysis. KS – Wrote an early draft of the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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