

Research Article

Hallmarks of HIV-1 pathogenesis are modulated by Nef's Secretion Modification Region

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Abstract

CD4⁺ T cell depletion and immune activation are hallmarks of HIV infection. Despite extensive studies, the mechanisms underlying immune modulation remain elusive. HIV-1 Nef protein is secreted in exosomes from infected cells and is abundant in the plasma of HIV+ individuals. Exosomal Nef (exNef) was also shown to induce apoptosis in bystander CD4⁺ T cells. We hypothesized that exNef contributes to HIV pathogenesis. A HIV-1 NL4-3 virus containing alanine substitutions in the secretion modification region (SMR; amino acids 66 to 70; HIVNefsmr5a) was developed. Nef protein containing this modified SMR was shown to be deficient in exNef secretion in *nef*-transfected cells. Using both HIV-1 NL4-3 wild type (HIVwt) and HIVNefsmr5a, correlates of pathogenesis were evaluated in cell-lines, human peripheral blood mononuclear cells, and humanized NOD-RAG1 '/ IL2r '/ double mutant (NRG) mice. Disruption of the SMR did not affect viral replication or exNef secretion from infected cell cultures as compared with *nef*-transfected cells. However, T cell apoptosis was reduced in HIVNefsmr5a infected NRG mice. Inflammatory cytokine release was also decreased in the spleen and peripheral blood of similarly infected NRG mice. Inflammatory cytokine release was also decreased in the soft and the SMR motif in HIV pathogenesis and suggest a potential role for exNef in HIV-driven immune modulation.

Keywords: Immune activation; Apoptosis; CD4 T-cells; Exosomes; HIV; Nef; Cytokines

Introduction

Chronic immune activation and CD4⁺ T-cell depletion are hallmarks of HIV infection. Chronic immune activation has been well established as one of the strongest predictors of disease progression [1-9]. The source of such sustained immune activation has been ascribed to several factors that often use overlapping mechanisms including: (i) continuous plasmacytoid dendritic cell production of Type I IFN [10]; (ii) direct activation via the effect of viral proteins such as Tat, Nef and gp120 [11]; and (iii) bacterial translocation [5]. However, we submit that additional mechanisms underlie immune activation/immune dysregulation. One such candidate is the secretion of exosomes containing the HIV accessory protein Nef.

Nef is a 27-35 kDa protein that is expressed early in the replication cycle of HIV-1 [12]. Nef's myristoylated N-terminal anchor domain is critical for its association with membrane structures and essential for several of its intracellular functions including downregulation of cell surface expression of CD4, MHC-I, and CXCR4 molecules [13]. Several studies have revealed Nef to be a determinant of disease progression in both HIV and SIV infection: (i) Mice and rats transgenic for HIV-1 *nef* develop immunodeficiencies [14-22]; (ii) the clinical course and both acute and chronic viral loads were markedly attenuated or abolished in both humans and rhesus macaques infected with viral strains that harbor deletions in nef [23-27].

Most of the literature on Nef has centered on its intrinsic functions within infected cells. However, Nef is known to be secreted or released from cells, and has been found in the plasma of HIV positive individuals and SIV infected rhesus macaques [28-29]. Moreover, several investigators, including ourselves, have shown that Nef is excreted from cells and is thereby able to extend its immune regulation beyond infected

cells [30-33]. In particular, we have shown that Nef is secreted in small 50-100 nm diameter vesicular bodies called exosomes characterized by the presence of Alix, AChE, and CD45 [34]. Additionally, exposure of non-infected CD4⁺ T cells to exosomal Nef (exNef) resulted in activation induced apoptosis via engagement of the CXCR4 receptor [35]. Genetic analysis revealed several motifs critical for Nef-induced exNef secretion [34]. One previously uncharacterized motif spanning amino acid residues 66-70 (VGFPV), the secretion modification region (SMR), was required for secretion as alanine replacements of the amino acid residues in this motif resulted in abrogation of exNef secretion from *nef*-transfected cells. This region was also found to be 100% conserved across HIV-1 clades suggesting the importance of this sequence in a protein highly prone to mutation [34].

Given our findings on the role of exNef and the implication of specific Nef motifs (e.g., SMR) in Nef driven exNef secretion, we examined the role of the SMR motif in Nef-induced pathogenesis. Using HIV-1 NL4-3 virus containing alanine substitutions in the SMR developed in our lab (HIVNefsmr5a), we evaluated correlates of *in vitro* and *in vivo* pathogenesis in cell lines, human peripheral blood mononuclear cells (PBMCs), and humanized NOD-RAG-1^{-/-}IL2ry^{-/-} double mutant

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(NRG) mice. Disruption of the SMR motif in the context of a replication competent HIV isolate did not affect *in vitro* or *in vivo* viral replication or exNef secretion from infected cell cultures. This is in sharp contrast with our findings using transfection with plasmids expressing *nefsmr5a* where the SMR mutation abrogated exNef secretion [34]. However, T cell apoptosis was reduced in HIVNefsmr5a infected cell cultures and CD4⁺T cell depletion was reduced in the spleen and peripheral blood of similarly infected mice. Inflammatory cytokine release was also decreased in the sera of HIVNefsmr5a infected mice relative to HIV wild type (HIVwt) infected controls. These findings suggest that exNef, acting through the SMR motif, may play a significant role in Nef-induced HIV-1 pathogenesis.

Materials and Methods

HIV-1 plasmid clones (pNL4-3 HIVwt, HIVdsNef, HIVNefsmr5a)

The HIV-1 pNL4-3 clone was obtained through the NIH AIDS Reagent Program (ARRRP, Figure 1A; 67). To construct the HIVNefsmr5a clone, alanine replacement mutations were generated in pNL4-3 by primer-directed mutagenesis (Figure. 1C) [18]. The HIVdsNef (double stop Nef) mutant of pNL4-3 was generated by direct synthesis of the mutated sequence by Genscript (Piscataway, NJ). The synthetic DNA fragment, containing stop codon mutations (nt 37 and nt 61) and the flanking restriction sites BamHI and XhoI was then ligated into BamHI /XhoI - digested pNL4-3. The resulting dsNef plasmid encodes a Nef reading frame with TGA codons replacing Trp-13 and Arg-31 (Figure. 1B) similar to a previously described clone [49].

Production of HIV-1 viral stocks from plasmid clones

HIV-1 viral stocks were generated by transfection of HEK 293 cells with plasmid DNA. Briefly, HEK 293 cells, grown in RPMI 1640 medium containing 10% fetal bovine serum, were transfected with 6 μ g of HIVwt, HIVdsNef, or HIVNefsmr5a NL4-3 viral constructs in T75 flasks by the Effectene transfection reagent (Qiagen, Inc., Valencia, CA). Viral supernatants were harvested 48h post transfection and stored at -80°C. The concentration of HIV-1 p24 was measured using a p24 capture enzyme-linked immunosorbent assay (ImmunoDiagnostics).

Cell culture. A3.01 CD4⁺ T cell lines were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated exosome depleted fetal bovine serum, streptomycin (100 U/ml), penicillin (100 U/ml), L-glutamine (2 mM), and HEPES-buffered saline solution (10 μ M). Exosomes were depleted from fetal bovine serum by centrifugation at

100,000 × g for 16h at 4°C. HEK 293 cells derived from a human primary embryonic kidney transformed with adenovirus type 5, obtained from ARRRP, were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (100 U/ml), penicillin (100 U/ml), L-glutamine (2 mM), and HEPES-buffered saline solution (10 µM). Peripheral blood mononuclear cells (PBMCs) were isolated from HIV seronegative blood donors, obtained from Emory Center for AIDS Research, with Ficoll-Paque. Isolated PBMCs were maintained at 37°C in RPMI 1640 medium supplemented with 20% heat-inactivated exosome depleted fetal bovine serum, streptomycin (100 U/ml), L-glutamine (2 mM), HEPES-buffered saline solution (10 μM), and IL-2 (20 U/ml). U373-MAGI-CXCR4 $_{_{CEM}}$ cells, obtained from the NIH AIDS ARRRP, were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, G418 (0.2 mg/ml), hygromycin B (0.1 mg/ml), puromycin (1 $\mu g/ml),$ penicillin (100 U/ml), and streptomycin (100 U/ ml). All participants gave written informed consent, and the study was approved by the institutional review boards of Emory University and Morehouse School of Medicine.

In vitro HIV-1 replication assay

A3.01 cells maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated exosome depleted fetal bovine serum, streptomycin (100 U/ml), penicillin (100 U/ml), L-glutamine (2 mM), and HEPES-buffered saline solution (10 µM), were exposed to HEK-derived viral supernatants (25 ng/p24) for 24h. The cells were than pelleted at 1,250 rpm for 10min at 25°C and re-suspended in fresh media. Viral replication was monitored at 3-day intervals by measuring the amount of p24 present in the culture supernatant. PBMCs maintained at 37°C in RPMI 1640 medium supplemented with 20% heat-inactivated exosome depleted fetal bovine serum, streptomycin (100 U/ml), L-glutamine (2 mM), HEPES-buffered saline solution (10 μ M), and IL-2 (20 U/ml), were exposed to A3.01-derived viral supernatants (50 ng/p24) for 24h. The cells were than pelleted at 1,250 rpm for 10min at 25°C and re-suspended in fresh media. Viral replication was monitored at 2-day intervals by measuring the amount of p24 present in the culture supernatant.

Isolation and purification of exosomes from virus

For the preparation of exosomes on flotation gradients, 40 ml of supernatants (from uninfected cells or NL4-3 infected cells supernatants) were first centrifuged for 5 min at $600 \times g$ to remove cells and cellular debris. The supernatants were then subjected to sequential



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centrifugation at 30,000 × **g** for 30min, using a Type FX6100 rotor and at 100,000 × g for 2h, using a Type 50.2 ultracentrifuge rotor (Beckman Instruments, Inc., Fullerton, CA). The 100,000 × **g** pellets were resuspended in 1 ml of 1X PBS. OptiprepTM velocity gradients in PBS were prepared by layering as 11 steps in 1.2% increments ranging from 6 to 18%. The resuspended 100,000 × **g** pellets were layered onto the top of the gradients and centrifuged for 2h at 250,000 × **g** in a SW40 Ti rotor (Beckman Instruments, Inc.). Gradient fractions containing exosomes (fractions 2-4 = 7.2-9.4%) were collected, diluted 1:3 with phosphate-buffered saline (PBS), and centrifuged for 2h at 400,000 × **g** using a TLA 100.4 rotor (Beckman Instruments, Inc.). The resultant exosome pellets were used in the TUNEL assay.

MAGI infectivity assay

Differences in infectivity due to the SMR motif mutation were tested in single round infection of the MAGI cell assay [50]. HIVwt NL4.3, HIVdsNef and HIVNefsmr5a mutant NL4.3 were used to infect A3.01 CD4⁺ T cells. At 12 days post-infection, the supernatants were tested for infection of U373-MAGI-CXCR4 $_{\rm \scriptscriptstyle CFM}$ cells plated in 12-well plates at 1.2 X 10⁵ cells/well 24h prior to infection. HIV-1 virions (1 ng/p24) were added to each well in duplicate, along with DEAE-dextran at a final concentration of 20 µg/ml. Forty-eight hours post-infection, cells were fixed by incubation with 0.2% glutaraldehyde and 1% formaldehyde for 5min at room temperature. Following fixation, cells were washed with PBS and stained for 60min at 37°C with X-Gal solution (5-bromo-4chloro-3-indolyl-\beta-D-galactosidase [Invitrogen] dissolved in dimethyl sulfoxide, 4 mM potassium ferrocyanide, 4 mM ferricyanide, and 2 mM MgCl₂ in PBS). The cells were washed twice with PBS, and positive syncytia (blue-stained cells) were counted with an inverted microscope (model TMS-F; Nikon, Melville, NY). Results are reported as the total number of blue cells per ng of p24.

TUNEL assay

Jurkat CD4⁺ T cells were assayed for apoptosis using a TUNEL assay as described previously [35]. The cells were visualized by epifluorescence on a computer-controlled microscope system (Zeiss microscope; Carl Zeiss, Thornwood, NY). Microscopic images were processed using a charge-couple device camera, MC 100 SPOT (Photonic Science, East Sussex, United Kingdom). Further image processing was conducted with the Image-Pro Plus 2.0 software (Media Cybernetics, Silver Spring, MD).

Immunoblot analysis

Cells and exosome proteins were analyzed using Western blot analysis. The protein samples were separated by SDS-PAGE on 4-20% Tris-HCL Criterion precast gel (Bio-Rad Laboratories, Hercules, CA) and electrophoretically transferred to a nitrocellulose membrane. The membrane was washed in Tris-buffered saline (TBS; Bio-Rad) for 5min, blocked with 5% nonfat milk in TTBS (TBS with 0.1% Tween 20) for 1h by shaking at room temperature, and processed for immunoblotting using a specific primary antibody with shaking at 4°C overnight, followed by a secondary HRP-conjugated IgG (H+L) antibody. Protein bands were detected by using the Western blotting luminol reagent (Santa Cruz Biotechnology) and imaged with the Image Quant LAS 4000 (GE Healthcare Biosciences, Pittsburgh, PA). Images were arranged for publication in Adobe illustrator 10 (Adobe Systems, San Jose, CA). The following antibodies were used: (i) murine monoclonal anti-Nef antibody (Immuno Diagnostics, Inc., Bedford, MA); (ii) murine monoclonal anti-p24 antibody (Immuno Diagnostics, Inc.); (iii) goat anti-mouse IgG (H+L) labeled with horseradish peroxidase (HRP) antibody (Thermo Fisher Scientific, Inc., Rockford, IL).

Flow cytometry analysis

To evaluate the effect that mutation of the SMR motif had on CD4 and CXCR4 downregulation, A3.01 CD4⁺ T cells were stained with antihuman antibodies and analyzed by flow cytometry. Cells were stained with allophycocyanin (APC)/cyanine 7 (CY7)-labeled anti-CD4 (OKT4; Biolegend, San Diego, CA) and phycoerythrin (PE)/CY7-labeled anti-CXCR4 (12G5; Biolegend). Viability was assessed with Alexa Fluor 430-Carboxylic Acid, Succinimidyl Ester (Life Technologies, Grand Island NY). To evaluate the percentage of CD3⁺/CD4⁺ T cells in infected PBMC cultures, T cells were stained with Alexa Fluor 700-labeled anti-CD3 (UCHT1; BD Bioscience), APC/CY7-labeled anti-CD4 (OKT4; Biolegend). Viability was assessed with Alexa Fluor 430 Carboxylic Acid, Succinimidyl Ester (Invitrogen). Flow cytometry analysis was performed on a BDTM LSR II (BD Bioscience) cytometer. Flow cytometry data was acquired with the Diva software and analyzed using FlowJo software 10.0.5 (Tree Star, Inc, Ashland, OR).

Humanized mouse studies

The humanized mouse model used is described in detail in Walker et al., 2012 [51]. Briefly, CD34+ hematopoietic stem cells (HSCs) were obtained from umbilical cord blood obtained after normal delivery (NDRI, Philadelphia, PA) using Ficoll-Paque (GE Healthcare, Piscataway, NJ). HSCs were purified by immunomagnetic bead separation (Miltenvi Biotec, Auburn, CA). A CD34⁺ cell purity of >91% was routinely obtained. NOD-RAG1-/-IL2ry-/- double mutant (NRG) mice (stock number 007799) were obtained from The Jackson Laboratory (Sacramento, CA). Mice were used in compliance with institutional and IACUC guidelines. Newborn NRG pups (2-5 days old) were sublethally irradiated with 150 cGy of gamma irradiation and subsequently intrahepatically injected with 3.5 x 10⁵ cells/mouse. Three months post-transplant, mice were bled and the peripheral blood was analyzed by flow cytometry for human leukocytes with PE-CY7-labeled anti-CD45 (HI30; BD Bioscience), APC-labeled anti-CD3 (HIT3A; BD Bioscience), and PE-labeled anti-CD4 (RPA-T4; BD Bioscience). Flow cytometry was performed on a Beckman Coulter FC-500.

HIV-1 infection of engrafted NRG mice

Successfully engrafted NRG mice were infected intra-peritoneally with 200,000 TCID₅₀ of HIV-1 (either NL4.3 HIVwt or NL4.3 HIVNefsmr5a). The infected mice were monitored for HIV replication *in vivo* by measuring human CD4⁺ cells in blood and plasma viremia every two weeks. Peripheral blood was analyzed for total human CD4⁺ cell percent by flow cytometry with PE-CY7-labeled anti-CD45 (HI30; BD Bioscience), APC-labeled anti-CD3 (HIT3A; BD Bioscience), and PE-labeled anti-CD4 (RPA-T4; BD Biosciences). Flow cytometry was performed on a Beckman Coulter FC-500.

To determine levels of HIV plasma viremia, viral RNA was extracted from the blood of infected mice using a Qiagen Viral RNA extraction kit (Qiagen, Valencia, CA). Reverse transcription was performed with oligo dT primers using a Taqman RT Reagents kit (Applied Biosystems, Carlsbad, CA). Quantitative-PCR was performed using SYBR Green (Applied Biosystems, Carlsbad, CA) and a probe and primer pair specific for the HIV *pol* gene: 5'-CTGGCTACTATTTCTTTGCTA-3' and 5'-TGGCATGGGTACCAGCACA-3' and probe 5'-TTTATCTACTT-GTTCATTTCCTCCATTCCTT-3' (IDT DNA Technologies, Coralville, IA). Quantitative-PCR was performed on an Applied Biosystems 7200 analyzer.

Luminex multiplex assay

Twenty cytokines/chemokines were measured in plasma from HIV-1 infected and uninfected NRG mice using a high-sensitivity human cytokine/chemokine 20-plex magnetic bead kit (Affymetrix, Inc, Santa Clara, CA) comprising CD40 ligand, GM-CSF, IFN- α 2, IFN- β , IFN- γ , IL-1 β , IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IP-10, MCP-1, MIP-1 α , MIP-1 β , TNF- α , Fas ligand and ICAM-1. Data was acquired using a luminex-200 system and analyzed using Bio-plex Manager software, 6.0 (Bio-Rad).

Data analysis

The numerical and graphical analyses of all *in vitro* data were based on at least three repetitions of each experiment. Statistical analysis was performed and graphs were generated using either SigmaPlot 10 (Systat, San Jose, CA) or GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Statistical significance was evaluated with an unpaired two-factor *t* test set at a *p* level of <0.05. On account of the relative small sample size, NRG mice data was log-transformed prior to analysis.

Results

Mutation of the SMR motif results in delayed viral release and decreased infectivity

We have previously identified motifs in Nef that are required for Nef-induced exNef secretion [34]. One of those motifs, the secretion modification region, is conserved across all HIV-1 clades. This motif was found to be important for secretion as alanine replacements of its five amino acids completely abolished exNef secretion in nef-transfected cells [34]. Based on the reports suggesting a role for exNef in Nefinduced pathogenesis, we sought to examine the effect of the mutation of the SMR motif on correlates of pathogenesis. To evaluate the effect of the SMR motif on HIV replication and infectivity, we constructed a replication competent HIV-1 NL4-3 clone expressing a nef gene with five alanine replacements in the SMR motif (HIVNefsmr5a; Figure. 1C). As controls, we used the wild type HIV-1 NL4-3 clone (HIVwt; Figure 1A), and a clone expressing a nef gene with point mutations resulting in two premature stop codons (HIVdsNef; Figure 1B). A3.01 T cells were infected with viral supernatants corresponding to the same amount of p24 prepared from HEK 293 cells transfected with each of these viral clones. The cells were cultured for 12 days and monitored at 3-day intervals for viral release by p24 ELISA and for infectivity by MAGI assay. While the levels of p24 released from HIVwt infected A3.01 cells started to increase by day 3, peaking by day 7, there was a slight delay in p24 release from cells infected with HIVNefsmr5a as well as HIVdsNef, with peak p24 release observed at day 9 (Figure 2A). To examine the effect of the SMR mutation on infectivity, culture supernatants harvested from infected A3.01 cells were assessed for infectivity by MAGI assay. HIVNefsmr5a viral particles were found to be 10-fold less infectious than HIVwt viral particles, while HIVdsNef viral particles were 100-fold less infectious (Figure 2B).

SMR motif is necessary for CXCR4 downregulation: Among its numerous intrinsic functions, Nef is known to downregulate the cell surface expression of HIV co-receptors CD4, CXCR4, and major histocompatibility complex (MHC) class I [13]. The reduction in the expression of these molecules is thought to prevent super-infection, facilitate viral release from infected cells, and assist in immune evasion [13]. These functions of Nef have been mapped to structural motifs within the protein. The motifs for CD4 downregulation are located at the N-terminal arm and the C-terminal loop, specifically amino acid residues W57 and L58 [36-38], and a dileucine motif L164-L165 [39-



Figure 2: Mutation of the SMR motif results in delayed viral release and decreased infectivity. (A) 5.0 X10⁵ A3.01 T cells were infected with HIVwt, HIVdsNef, or HIVNefsmr5a virus and assayed by ELISA for changes in p24 release in culture medium at 3-day intervals. Differences in viral replication kinetics were observed between the various viral clones. (B) A3.01-derived viral preparations were assessed for infectivity by MAGI assay. HIVNefsmr5a and HIVdsNef viral particles were found to be less infectious than HIVwt viral particles by 10-fold and 100-fold respectively. Plots shown are a representation of three independent experiments.

41]. Nef-mediated downregulation of CXCR4 and MHC-I are located in the phosphofurin acidic cluster sequence (PACS) and the Src homology domain 3 (SH3) motifs [42-43]. The SMR motif is located between the PACS and SH3 motifs, which prompted us to investigate the impact of mutation of the SMR motif on Nef-mediated down regulation of CD4 and CXCR4. Aliquots of the A3.01 T cell line were infected with either HIVNefsmr5a, HIVwt, or HIVdsNef and subsequently analyzed for surface expression of CD4 and CXCR4 by flow cytometry. While mutation of the SMR motif only modestly affected CD4 downregulation relative to HIVwt, it abolished the ability of Nef to downregulate CXCR4 (Figure 3B and 3C). To rule out the possibility that decreased expression of Nef within HIVNefsmr5a infected cells contributed to this observation, similar amounts of cell lysates, measured by total protein, from infected cultures were assessed for expression levels of Nef by western blot analysis. Nef was found to be expressed in equal amounts in HIVNefsmr5a infected cultures as compared to HIVwt infected cultures (Figure 3A).

SMR motif is dispensable for viral replication in human PBMCs: To extend the analyses of the role of the SMR in primary cell cultures,



representative image from three independent experiments. (B-C) A3.01 T cells infected with HIVNefsmr5a, HIVwt, and HIVdsNef were analyzed for surface expression of CD4 and CXCR4 by flow cytometry. Mutation of the SMR motif abolished the ability of Nef to downregulate CXCR4. Nef-mediated downregulation of CD4 was slightly affected. Values reflect the means of three independent experiments, and error bars represent SEM of the mean. Differences between groups were determined using an unpaired student *t*-lest *p<0.05, **p<0.01, ***p<0.001.

viral replication was compared in primary cultures of human PBMCs. Isolated PBMCs were infected with equal amounts, based on p24 content, of HIVNefsmr5a, HIVdsNef, or HIVwt viral supernatants. The cells were cultured for 12 days and monitored at 3-day intervals for viral release by p24 ELISA. Unlike the infection of the A3.01 cell line, p24 release from HIVNefsmr5a- and HIVwt-infected PBMCs displayed similar viral replication kinetics for donors 1 and 2, while viral release was delayed in HIVdsNef-infected cultures (Figure 4A). Conversely, donor 3 displayed similar kinetics as observed using the A3.01 infected cell line (Figure 4A).

CD4⁺ T cell depletion was reduced in HIVNefsmr5a infected PBMC cultures

To determine whether infection with HIVNefsmr5a virus resulted in depletion of CD4+ T cells in culture, isolated PBMCs were infected with equal amounts of HIVNefsmr5a, HIVdsNef, or HIVwt viral supernatants. The cells were cultured for 12 days, harvested, and examined for viable levels of CD4⁺/CD3⁺ T cells by flow cytometry. Compared to PBMC cultures infected with HIVwt in which only 27.8% of CD4⁺/CD3⁺ T cells remained at 12 days post infection, HIVNefsmr5a-infected cultures exhibited 51.2% of CD4⁺/CD3⁺ live T cells and HIVdsNef cultures 63.6% (Fig. 4B). The difference between HIVNefsmr5a and HIVwt cultures was statistically significant (p<0.027), while the difference observed between HIVNefsmr5a and HIVdsNef was not (p<0.231). However, compared to mock infected cultures (79.9%), a significant difference was observed in HIVwt- (p<0.001) and HIVNefsmr5a- (p<0.021) cultures, while no significant difference was observed in HIVdsNefcultures (p<0.116; Fig. 4B).

ExNef is released from HIVwt and HIVNefsmr5a infected cells

Nef is known to be released in exosomes from HIV-infected and nef-transfected cells [30-33]. In previous studies from our group, an important SMR motif for nef-induced exNef secretion was identified using a nef-transfection assay [34]. To examine the effect of mutation of the SMR motif on exNef release during the course of a viral infection, PBMC were infected with HIVNefsmr5a virus and culture supernatants were assayed for exNef. To separate exosomes from HIV-1 particles, conditioned cell-free media, 12 days post-infection, was harvested and concentrated by ultracentrifugation at $30,000 \times g$ for 30 min and 100,000 \times g for 2h, re-suspended in 1X PBS then separated by centrifugation through a 6-18% Optiprep[™] velocity separation gradient. Fractions from each gradient were collected and assayed for AChE, an exosomal marker, by enzymatic assay, and p24 to confirm purification of exosomes from viral particles. Fractions were also assayed by Western blot analysis, probing for Nef and p24. Using AChE and p24 to identify exosomes and virus respectively, exosomes sediment in fractions 2-4 (7.2-9.4%) (Fig. 5B), while HIV-1 viral particles sediment in fractions 8-10 (14.4-16.8%; Fig. 5A). Contrary to our previous finding in neftransfected cells, Nef was detected in both the exosomes and the viral particles collected from cells infected with HIVwt and HIVNefsmr5a (Fig. 5C).

ExNef from HIVNefsmr5a infected cells results in reduced apoptosis

We and others have shown that exNef induces apoptosis in HIV naïve and uninfected CD4 $^+$ T cells [31-35]. Given the finding that



exNef was released in the culture supernatant of HIVNefsmr5a infected cells, we next investigated whether exNef from HIVNefsmr5a infected cells were able to induce apoptosis in HIV naïve cells similar to exNef released from HIVwt infected cells. To remain in the physiological range in which Nef exosomes are found in the plasma of HIV positive individuals (10 ng/ml) [28], Jurkat cells were exposed to 30 ng/ml of exosomes isolated from HIVNefsmr5a or HIVwt infected PBMC cultures for 48h. The cultures were subsequently screened for apoptosis by TUNEL assay. Compared to exNef from HIVwt (7.5%) infected cells, there was significant reduction in the number of cells undergoing apoptosis exposed to exNef from HIVNefsmr5a (2.6%) infected cells (p<0.036; Figure 5D) which was essentially similar to that seen in Jurkat cells exposed to exosomes isolated from mock infected cultures (2.2%, p<0.768; Figure 5D).

CD4⁺ T cell depletion was reduced in HIVNefsmr5a-infected NRG mice

Although effects of SMR mutations were observed *in vitro*, it was unclear whether mutation of the SMR motif would have any consequences *in vivo*. This issue prompted us to use the humanized NRG mouse model. We examined the effect of infection with HIVNefsmr5a or HIVwt on viral loads, human CD4⁺ T cell dynamics and cytokine/ chemokine responses using this model. Surprisingly, the difference in

viral dynamics between HIVwt and HIVNefsmr5a-infected NRG mice were minimal and similar to the kinetics observed in *in vitro* infected PBMC cultures from donors 1 and 2 (Fig. 6A and 4A). In fact, the levels of HIV-1 RNA copies per milliliter were between 7 and 8 log units in HIVNefsmr5a-infected mice at 9 weeks post-infection, which was equivalent to the levels of HIV-1 RNA copies per milliliter in HIVwtinfected controls.

In contrast to plasma viremia, significant differences were observed for human CD4+ T cell dynamics in mice infected with HIVwt compared to those inoculated with HIVNefsmr5a (Figure. 6B, C, and D). The peripheral blood at various weeks post infection was monitored for levels of CD4⁺ T cells using standard flow cytometry. While mice infected with HIVwt displayed a significant, gradual decline (Figure. 6B), in the frequencies of human CD4⁺ T cells (as determined on the gated population of human CD45⁺ leukocytes) from an average of 79.45% pre-infection to an average of 26.2% at 9 weeks post-infection (*p*<0.05; Figure. 6C), mice infected with HIVNefsmr5a maintained relatively stable or slightly increasing frequencies of human CD4⁺ T cell levels throughout the 9 weeks of follow-up (Figure. 6B), from 54.26% preinfection to 68.7% at 9 weeks post-infection (Figure. 6C). To verify that the CD4⁺ T cell levels measured in blood were not a reflection of altered trafficking patterns, the levels of human CD4+ T cells were evaluated in the spleen of infected mice at the end of the challenge experiments.

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Figure 5: ExNef is released from HIVNefsmr5a infected cells. (A) 4.0×10^6 HIV seronegative PBMCs were infected with HIVwt, HIVdsNef, or HIVNefsmr5a virus. 12 days post-infection, conditioned cell-free media was harvested and subjected to differential centrifugation at $30,000 \times g$ for 30min and $100,000 \times g$ for 2h. The $100,000 \times g$ pollet was re-suspended in 1 ml of 1X PBS, loaded onto OptiprepTM velocity separation gradient, and subjected to flotation centrifugation at $250,000 \times g$ for 2h. (A-C) Fractions were assayed by p24 and AChE enzymatic assay, a marker for exosomes, and western analysis for Nef and p24 to confirm purification of exosomes from viral particles. While Nef was not detected in the exosome, or the viral fractions of supernatants collected from cells infected with HIVdsNef, Nef was detected in both the exosome, and the viral fractions of supernatants collected from donor #3 was screened for apoptosis by TUNEL assay. Exosomes were added to target cell cultures at 30 ng/ml as measured by total protein. Compared to exNef from HIVwt infected cells, there was a significant reduction in the number of cells undergoing apoptosis after 48h of exposure to exNef from HIVNefsmr5a infected cells. Error bars represent SEM from the mean. Differences between groups were determined using an unpaired student *t*-test **p*<0.05, ***p*<0.01.

There was a significant decrease (p<0.05) in the percentage of human CD4⁺ T cells in the spleen of HIVwt infected mice (31.5%) compared to HIVNefsmr5a infected mice (70.8%; Figure 6D), indicating that the decrease in CD4⁺ T cells observed in blood was generalized.

Pro-inflammatory cytokine levels were elevated in HIVwtinfected NRG mice but not in HIVNefsmr5a-infected NRG mice.

The discrepancy between viral loads and CD4 loss suggested a difference in pathogenic mechanisms. We therefore investigated whether mutation of the SMR motif resulted in difference in the elicitation of pro-inflammatory cytokines in HIVNefsmr5a- versus HIVwt-infected mice during acute and chronic infection. Plasma specimens were collected from mice at 2 and 9 weeks post-infection, and analyzed by multiplex Luminex array for a panel of 20 human cytokines/chemokines (Supplementary Table 1). Among these 20 analytes at week 2, IL-13 (p<0.001) and IL-15 (p<0.01) were significantly elevated in HIVwt-infected mice but not in HIVNefsmr5a-infected mice relative to uninfected controls (Figure. 7A). At week 9 (Figure. 7B), IL-7 (p<0.001), IL-10 (p<0.01), IL-15 (p<0.001), and IP-10 (p<0.001) were significantly elevated in HIVwt-infected mice but not in HIVNefsmr5a-infected mice. In fact, HIVNefsmr5a- infected mice did not show significant elevation for any of the tested analytes at the specified time points relative to uninfected controls.

Discussion

Nef is known to be secreted from HIV-infected and *nef*-transfected cells, though the exact role of extracellular Nef in HIV pathogenesis remains to be fully elucidated [14-17]. One report in the nonhuman primate model of HIV showed that secreted SIV Nef was able to



Figure 6: CD4*/CD3* T cell depletion was reduced in HIVNefsmr5a infected NRG mice. NRG mice successfully transplanted with CD34* HSCs were infected with either HIVwt (n=2) or HIVNefsmr5a (n=3) NL4-3 virus. (A-C) At various weeks post-infection, mice were bled and the plasma was analyzed by Q-PCR using a primer/ probe pair specific for HIV *pol* gene and PBMCs were analyzed by flow cytometry for total CD45* human CD4*/CD3* T cell percentage. (A) Disruption of the SMR motif did not impair *in vivo* viral replication and (B-C) disruption of CD4*/CD3* T cells in a significant reduction in depletion of CD4*/CD3* T cells relative to HIVwt. (D) Disruption of the SMR motif also resulted in preservation of CD4*/CD3* T cells in the spleen of HIVNefsmr5a infected mice relative to HIVwt. Error bars represent SEM from the mean. Differences between groups were determined using an unpaired student *t*-test **p*<0.05.



Figure 7: Pro-inflammatory cytokine levels were reduced in HIVNefsmr5a infected NRG mice. NRG mice successfully transplanted with CD34⁺ HSCs were infected with either HIVwt (*n*=2) or HIVNefsmr5a (*n*=3) NL4-3 virus. At various weeks post-infection, mice were bled and the plasma was analyzed for pro-inflammatory cytokine and chemokine expression by 20-plex luminex array. Of the 20 pro-inflammatory cytokines and chemokines measured, (A) Week 2 post-infection: IL-13, and IL-15 were significantly increased in HIVwt infected mice relative to HIVNefsmr5a and (B) Week 9 post-infection: IL-10 and IP-10 was significantly elevated in HIVwt infected mice groups were determined using an unpaired student *t*-test **p*<0.05, ***p*<0.01, ****p*<0.001.

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affect hematopoiesis [29], although the authors did not test whether Nef was or was not released in exosomes. Importantly, the secretory function was mapped to specific motifs near the N-terminus of the Nef protein. One of these motifs, the previously uncharacterized secretion modification region, was found to be conserved across all HIV-1 clades [34]. However, despite this observation, little is know about this region and its involvement in exNef secretion and Nef-induced pathogenesis.

In this study, we investigated the role of the SMR motif in correlates of pathogenesis during the course of an HIV infection using both *in vitro* and *in vivo* model systems. Alanine replacements of the five amino acid residues of the SMR did not attenuate viral replication in A3.01 T cells, similar results were seen in Jurkat T cells (data not shown), but instead resulted in delayed viral release compared to HIVwt control with virus eventually reaching levels similar to those in the control. Conversely, mutation of this region did affect Nef-mediated enhancement of infectivity. In fact, infectivity of HIVNefsmr5a viral particles was 10fold less infectious than HIVwt. This observation may explain the delay in viral release from HIVNefsmr5a-infected A3.01 T cells suggesting this region maybe important for post-entry steps in HIV-1 target cells.

One surprising finding in our study was the release of Nef in exosomes from HIVNefsmr5a infected cells. In our previous analysis [34], mutation of the SMR in NL4-3 *nef* cloned into expression vector pQBI resulted in abolition of exNef secretion in *nef*-transfected cell. But as shown here, mutation of this region in the context of the replicative did not result in abrogation of exNef secretion. This finding suggests that in the presence of the full-length virus, other compensatory mechanisms may rescue exNef release, further underscoring the importance of this pathway for the virus.

While Nef was produced and released in exosomes from HIVNefsmr5a-infected cells, exNef from these cells induced far less apoptosis in bystander cells, than exNef from HIVwt infections. Interestingly, this functional phenotype was mapped to two apoptotic motifs, one of which (Nef amino acids 50 to 60) is located five amino acids upstream of the SMR motif [44]. The The second motif was mapped to amino acids 170 to 180. Recently, we showed by molecular modeling that mutation of the SMR region of Nef affected the threedimensional structure of the Nef protein [45]. Thus, we reason that the change of residues 66-70 (VGFPV to AAAAA) in the HIVNefsmr5a mutant would most likely alter the function of the adjacent apoptotic motif (Nef amino acid residues 50-60). In fact, intracellular and extracellular Nef within and released from HIVNefsmr5a appeared to have a higher molecular weight than HIVwt upon separation by SDS-PAGE. This observation was true for both human T cell lines and human primary cells (data not shown). Taken together, our findings suggest that mutation of the SMR region could have altered the presentation of the apoptotic motif in exNef to target cells thereby resulting in a reduction of Nef-induced apoptosis.

One well established intrinsic function of Nef is to downregulate the cell surface expression of the HIV co-receptors CD4 and CXCR4, and the major histocompatibility complex MHC-I. Since SMR is located between the motifs responsible for Nef-mediated down regulation of CXCR4 and MHC-I, PACS and SH3, we investigated the effect of mutation of this region on cell surface expression of these molecules in infected cells. Similar to Meuwissen et al., the HIVNefsmr5a mutant failed to completely down regulate CXCR4 [46]. Conversely, Nefmediated down regulation of CD4 was not affected. Our results, in line with Meuwissen and coworkers, indicate the SMR is essential for CXCR4 down regulation, but is not required for Nef-mediated CD4 down regulation [46]. As a final set of *in vitro* studies, we investigated whether the SMR motif was necessary for viral replication in human PBMC. In our assay, the SMR motif was dispensable for viral replication as HIVNefsmr5a virus replicated as efficiently as the HIVwt, while there was a slight delay in viral release from HIVdsNef-infected cells. In contrast, Meuwissen *et al.* reported a significant attenuation in replicative ability of virus containing a mutation in the SMR region (VGF to AAA) and premature stop codons in Nef [46]. Since the same proviral backbone was used in our study, we speculate that the difference stems from the culture methods for infecting primary cells. Our PBMC cultures were not activated prior to infection and cultures were infected with a larger concentration of virus. This may explain the discrepancy between our results and their findings. In fact, infecting cell cultures with virus stocks greater than 300ng of p24 resulted in matched replication kinetics for HIVwt, HIVNefsmr5a, and HIVdsNef (data not shown).

One salient finding in the infection of primary human PBMCs in vitro and in vivo with HIVNefsmr5a was while its replication kinetics matched HIVwt, HIVNefsmr5a appeared to spare CD4+ T cells (Figure. 4B and 6). While the reasons for this dichotomy are unclear, one may posit this is a reflection of a direct effect exerted in infected cells. This indeed may be true, but would indicate markedly altered intrinsic Nef functions since HIVNefsmr5a appeared to produce intracellular levels of Nef equivalent to HIVwt (Figure. 3A and 5C). However, our data also clearly points to differences in apoptosis induction capacity of exNef released from HIVNefsmr5a- versus HIVwt-infected cell cultures (Figure. 5D). It is possible that mutation of the SMR altered the structure of Nef both intracellularly and in exosomes leading to functional alterations both intracellularly and extracellularly. Indeed, mutation of the SMR resulted in the loss of the virus ability to downregulate CXCR4 (Figure. 4C). In addition, mutation of the SMR could have also affected the three-dimensional structure of Nef leading to alteration in the function of the adjacent apoptotic motif shown to be responsible for the exNef apoptotic effect [44]. Taken together, this may help in explaining in vitro and in vivo preservation of CD4+ T cells during infection with HIVNefsmr5a despite matched viral replication kinetics with HIVwt.

The alteration of Nef function was also reflected in the absence of detectable immune activation in mice infected with HIVNefsmr5a, relative to HIVwt- and mock-infected mice. Serum levels of proinflammatory cytokines IL-7, IL-10, IL-13, IL-15, and IP-10, which were significantly elevated in HIVwt-infected mice, were found to be at levels similar to uninfected controls in mice Infected with HIVNefsmr5a. Interestingly, IL-7 and IL-15 were shown to be elevated in plasma from women with acute HIV-1 infection [47]. Further, IL-7 was found to be associated with rapid CD4 loss [47], likely reflecting compensatory secretion. In the same study, elevated levels of IP-10 was significantly associated with higher HIV viral loads, and levels of IL-10 correlated directly with acute infection viral loads and a greater risk of CD4⁺ T cell loss. Collectively, our findings suggest the SMR motif may influence the pro-inflammatory cytokine storm observed during acute infection, which is thought to contribute to the rate of disease progression in HIV infection.

In a study conducted by Stoddart et al. mutations of the regions involved in CD4 down regulation, amino acid residues 57/58 and 164/165, resulted in attenuation of pathogenesis in severe combined immunodeficiency mice implanted with human fetal thymus and liver (SCID-hu Thy/Liv mice) [48]. Alternatively, SCID-hu Thy/liv mice infected with virus containing a mutation in the PACS region displayed pathogenic potential comparable to that of wild type virus

[48]. These findings demonstrated that a loss in the ability of the virus to down regulate cell surface expression of MHC-I is dispensable for pathogenesis, while down regulation of CD4 is required for Nefmediated pathogenesis. In our study, mutation of the SMR did not affect the ability of the virus to down regulate CD4. It is therefore interesting to speculate whether this virus would exhibit an attenuated phenotype in a model of human HIV pathogenesis. The reduced levels of proinflammatory cytokines and preservation of CD4⁺ T cells certainly argues in favor of attenuation, though further confirmation is required given the limited sample size in our *in vivo* study. Overall, our *in vitro* and *in vivo* findings suggest that the SMR motif may play an important role in HIV-1 pathogenesis.

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