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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-RAG1-1/IL2rγ-/- 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 cell cultures and CD4+ T cell depletion was reduced in the spleen and peripheral blood of similarly infected NRG mice. Inflammatory cytokine release was also decreased in the sera of HIVNefsmr5a infected mice relative to HIVwt infected controls. These findings demonstrate the importance of Nef 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 to 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-/-IL2rγ-/- double mutant mice...

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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) mutant of pNL4-3 was generated by direct synthesis of the mutated sequence by Genscript (Piscataway, NJ).

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 μg of pNL4-3 and Arg-31 (Figure. 1B) similar to a previously described clone [49].

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). Viral supernatants were harvested 48h post transfection and stored at -80ºC.

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 100,000 × g to remove cells and cell debris. The supernatants were then subjected to sequential 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 Tat 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 μg/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.

Figure 1: The amino acid sequence of HIV-1 Nef and mutants. (A) HIVwt sequence (1-79aa) showing domains important for Nef-vesicle induced secretion (BAA-2, P25, 29G/931, T44, PACS motif, and SMR domain). (B) The HIVdsNef mutant contains two point mutations creating two premature termination codons, one 3’ of each initiator ATGs. (C) The HIVNefsmr5a mutant contains five alanine substitutions in the Secretion Modification Region (66-70aa).
centrifugation at 30,000 × g for 30 min, using a Type FX6100 rotor and
at 100,000 × g for 2 h, 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 100,000 × g pellets 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 2 h 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 2 h 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
days 2 post-infection, the supernatants were tested for
infection of U373-MAGI-CXCR4Δ531 cells plated in 12-well plates at 1.2
X 105 cells/well 24 h 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
5 min at room temperature. Following fixation, cells were washed with PBS
and stained for 60 min at 37°C with X-Gal solution (5-bromo-4-chloro-3-indolyl-β-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 analysis

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
5 min, blocked with 5% nonfat milk in TTB (TBS with 0.1% Tween
20) for 1 h 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
lumino 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 anti-
human 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-Carbocyanin, 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 Carbocyanin,
Succinimidyl Ester (Invitrogen). Flow cytometry analysis was performed on a BD® LSR II (BD Bioscience) cytometer. Flow cytometry data was
acquired with the Diva software and analyzed using FlowJo software
10.5.0 (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 (Miltenyi Biotec, Auburn, CA). A CD34+ cell purity of
>91% was routinely obtained. NOD-RAG1-/-IL2rg-/- 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 data was acquired 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 Bioscience). Flow cytometry data was
acquired 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 Bio-
systems, 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'-CTGGCTACTATTTCTTTTGCTA-3'
and 5'-TGGCATGGTGACCCAGACA-3' and probe 5'-TGGATCTATTT-
GTTCATTCCCTCCATTCTTT-3' (IDT DNA Technologies, Coralville,
IA). Quantitative-PCR was performed on an Applied Biosystems 7200
analyzer.
the N-terminal arm and the C-terminal loop, specifically amino acid within the protein. The motifs for CD4 downregulation are located at [13]. These functions of Nef have been mapped to structural motifs facilitate viral release from infected cells, and assist in immune evasion expression of these molecules is thought to prevent super-infection, cell surface expression of HIV co-receptors CD4, CXCR4, and major its numerous intrinsic functions, Nef is known to downregulate the viral particles were 100-fold less infectious (Figure 2B).

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 infectivity by MAGI assay. HIVNefsmr5a 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.

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 Nef-induced 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 [39-41]. 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,
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 HIVdsNef- cultures (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 × g for 30 min and 100,000 × g for 2 h, re-suspended in 1 X 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 nef-transfected 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 SD) which was essentially similar to that seen in Jurkat cells exposed to exosomes isolated from mock infected cultures (2.2%, p<0.768; Figure SD).

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 HIVwt-infected 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% pre-infection 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.

Figure 4: SMR is dispensable for viral replication and CD4+/CD3+ T cell depletion was reduced in HIVNefsmr5a infected PBMCs. (A) 4.0 X10^6 PBMCs from three HIV-1 seronegative donors were infected with HIVwt, HIVdsNef, or HIVsmr5A virus and assayed by ELISA for changes in p24 release in culture medium at 2-day intervals. p24 ELISA analysis revealed similar viral replication kinetics for HIVwt and HIVNefsmr5a viral clones for donors 1 and 2. (B) 4.0 X 10^6 PBMCs from nine HIV-1 seronegative donors were infected with HIVwt, HIVdsNef, or HIVsmr5A virus. Day 12 post infection, cells were harvested and analyzed for the percentage of CD3+ lymphocytes expressing CD4 by flow cytometry. The percentage of CD3+ lymphocytes expressing CD4 was significantly higher in HIVNefsmr5a infected cultures relative to HIVwt. Values reflect the means of three independent experiments with three different donors used per experiment. Error bars represent SEM of the mean. Differences between groups were determined using an unpaired student t-test *p<0.05, **p<0.01, ***p<0.001.
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 HIVwt-infected 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 the SMR motif resulted 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 was 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 compared to HIVNefsmr5a. Differences between groups were determined using an unpaired student t-test *p<0.05, **p<0.01, ***p<0.001.
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 known 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 10-fold less infectious than HIVwt. This observation may explain the delay in viral release from HIVNefsmr5a-infected A3.01 T cells suggesting this region may be 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 pQBF 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 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 three-dimensional structure of the Nef protein [45]. Thus, we reason that the change of residues 66-70 (VGFPV to AAAA) 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-1, 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, Nef-mediated 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 point 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 which 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 viruses.
[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 Nef-mediated 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 pro-inflammatory 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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