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THE STUDY OF THE MOUSE SCHLAFEN FAMILY MEMBERS AS MEDIATORS OF ANTIVIRAL ACTIVITY

JULIA F. HERNANDEZ

Master's Program in Biomedical Engineering

APPROVED:

Manuel Llano, Ph.D., M.D., Chair

Thomas Boland, Ph.D.

Binata Joddar, Ph.D.

Stephen L. Crites, Jr., Ph.D.
Dean of the Graduate School

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Julia F. Hernandez

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DEDICATION

I would like to dedicate this thesis to all of my family. Thank you mom and dad, for all the support, for helping me as much as you can, without you, this wouldn't be possible. To my brother and my sister, who have always being cheering me up. To my husband, for always being here for me, you bring up the best on me even on the hard times.

THE STUDY OF THE MOUSE SCHLAFEN FAMILY MEMBERS AS MEDIATORS OF ANTIVIRAL ACTIVITY

by

JULIA F. HERNANDEZ, B.S.

THESIS

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ABSTRACT

The Schlafen family of proteins was first characterized in 1988[1], and since then evidence of their relevance in different physiological and pathological situations have been accumulating although at a slow pace. Hence, the biological roles of these proteins remain ill-defined. The expression of these proteins is positively regulated by type 1 interferons, constituting a component of the innate immune response. Functions of these proteins vary from regulation of cell proliferation to restriction of viral infections. Recent studies have implicated SLFN11 and SLFN13 as inhibitors of HIV-1 infection[2-4]. The mechanism of action of SLFN11 resides in its capacity to regulate the size of the host tRNA pool, thus counteracting changes in the tRNA repertoire induced by viral infections[2,4]. However, no relevant cellular models of HIV-1 infection were used to asses this information. Moreover, only one additional report has confirmed the original observations made seven years ago. This situation has fueled doubts in the HIV-1 research community regarding the relevance of SLFN11 anti-HIV-1 mechanism. Importantly, in this work we have validated the tRNase-dependent anti-HIV-1 activity of SLFN11 in human CD4+ T cells and cells of myeloid origin, the main targets of HIV-1 *in vivo*. Furthermore, we revealed a novel tRNase-independent anti-HIV-1 activity in murine SLFN proteins. These are important contributions to the anti-viral innate immune response field, and they could be exploited in HIV-1 cure strategies.

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INTRODUCTION

HIV, Epidemic and Statistics. The first HIV case was reported in 1981, and since then no cure has been found. HIV-1 infection is still one of the most sexually transmitted diseases worldwide. Back in 2012, 35.3 million people were living with HIV in Southern Africa, representing 70.8% worldwide. Statistics show that globally, 37.9 million people were living with HIV in 2018. Out of the 37.09 million people infected with HIV, 18.8 million accounts for infection in women, and 17.4 million in men. Around 1.7 million are children of 15 years or less. 23.3 million were receiving anti-retroviral treatments by the end of 2018 [5]. However, 770 000 HIV- related deaths were reported in 2018, from where 670,000 occurred in adults, and 100,000 in children. These finding suggests a decrease in mortality compared to the number of HIV related deaths in 2000 (1.4 million people) and a 56% decrease in the number of people dying from HIV causes in 2004, when the highest peak of infection was registered [6,7]. The reduction of mortality is associated to the acquirement and access of antiretroviral therapy in the past years.

HIV Pathogenesis. HIV is a lentivirus that belongs to the family of retroviruses, it has a spherical structure of around 120 nm, and it is composed of two identical positive- sense single stranded RNA molecules of about 9200 nucleotides. These RNA molecules are enclosed within a capsid and a viral envelope. The viral envelope consists of two glycoproteins: gp120 and gp41. The interaction of the viral glycoprotein gp120 with the cell surface receptor CD4 and a co- receptor (CXCR4) or (CCR5) allows for a conformational change in which the N- terminal of the transmembrane protein gp41due to its hydrophobic nature inserts itself into the host cell membrane forming a channel, bringing the mature HIV particle in closer proximity to the host cell until fusion of the membranes is achieved. The viral capsid is translocated into the cytoplasm of the target cell, and viral RNA is released. Encoded in the viral RNA is the enzyme reverse transcriptase whose

function consists in the transcription of the single stranded RNA into complementary DNA (cDNA). A host tRNA (Lys3) used as a primer by RT[8]. Reverse transcriptase binds to the primer binding site, this is around 180 nucleotides from the 5′ of the genomic RNA[9]. As viral RNA is being transcribed as single-stranded cDNA, the RNA-DNA complex previously formed is degraded by the RNAse activity of RT. The DNA- dependent DNA polymerase activity of RT converts the single-stranded cDNA into double stranded DNA[10]. After DNA synthesis is completed, a pre-integration complex (PIC) is created, consisting of the protein integrase (IN), the viral protein R (Vpr), the matrix protein (MA), as well as the dsDNA and the protein reverse transcriptase[11]. Integrase carries the double stranded DNA, while Vpr plays an important role in nuclear transport. DNA is transported into the nucleus through the nucelar pores, however, due to the size of the PIC complex, a nuclear localization signal (NLS) is required. These NLS signals have been found in the proteins of the PIC (IN,MA and Vpr)[12]. Studies have demonstrated that the NLS signals present in MA introduce a positive charge crucial for nuclear targeting properties[11].Vpr is a multifunctional protein necessary for successful infection of CD4 cells, but it is also crucial for nuclear transport. Studies have shown that the interaction of Vpr with importin alpha targets the PIC to the nucleus, which promotes binding to nuclear pore proteins[13]. Other functions of the vpr protein include cell cycle arrest and and activation of HIV-1 LTR transcription[13]. Integrase (IN) has two necessary catalytic functions to successfully replicate HIV-1: Transport of dsDNA to the nucleus and transfer into the host DNA. Integrase binds to the double stranded DNA in a dimer manner to each end of the strand. Integrase then traverses a nuclear pore, thus accessing to the nucleus of the host cell. A transcriptional coactivator (LEDGF/p75) assists integrase serving as a tethering factor between integrase and chromatin thus promoting integration to the target sequence on the host genome[11,14]. Once transportation to the nucleus is achieved,

cleavage of the 3′ end of viral DNA is catalyzed. In this reaction known as 3′ processing, GT dinucleotides are removed, leaving a 5′ flanking at the end of the viral genome, called sticky ends. Another important reaction, strand transfer takes place when integrase attacks the 3′ hydroxyl groups at ends of the viral DNA on phosphodiester bonds in the host DNA[15]. After integration of the viral DNA into the host genome, repair of the gaps created in this process are repaired by cellular enzymes[16]. Once the viral DNA is integrated into the host DNA the infection is persistent, and this newly synthesized DNA is known as proviral DNA. Proviral DNA is flanked by long terminal repeats (LTR's) at both ends in which the promotor for transcription is encoded. Long terminal repeats are subsequently divided in 3 regions: U3, R and U5. Located 28 bp upstream of the transcription start site in the U3 region is the HIV-1 TATA box. The R region contains the TAR hairpin, which serves as the binding site of TAT, a trans-activator protein of HIV-1 that increases virus production by approximately 100-fold[17,18]. Adjacent to the 5′ LTR is the *gag* gene, encoding for the structural proteins p24, p17, p7 and p6. Then, the *pol* reading frame follows, encoding for the enzymes protease (p10), RNase H (p15) and reverse transcriptase (p51). Adjacent to the *pol* gene is the *env* reading frame, encoding for the envelope glycoproteins gp120 and gp41. The transactivator protein (p14) is enconded in the *tat* gene, followed by *nef* gene encoding for p19, an RNA splicing regulator. These proteins are said to be required for HIV-1 replication since they regulate gene transcription. The negative regulating factor (p27) encoded by *nef* is responsible for downregulation on CD4 cells, thus preventing the super infection of those. In addition, it has been demonstrated that *nef* enhances pathogenicity in vivo and is required for the progression of the infection[19]. Followed by the *nef* gene is the *vif* gene encoding for the viral infectivity protein (p23) that enhances the production of infectious virus. The regulatory proteins Vpr (p15) and Vpu (p16) have an influence in the rate of the production of viral particles[20-22]. Proteolytic cleavage of

precursor proteins (p55 and p160) takes place at the end of the budding process, in which the lipid envelop of the virion is acquired. Attachment of HIV to a target cell (CD4) varies between 30 minutes and 2 hours, and after 6 hours transcription is completed. Integration is also achieved in the following 6 hours and finally, 24 hours after infection viral particles are released[23].

HIV-1 Latency. Latent infection is the main obstacle for curing HIV-1 infection. Upon integration HIV-1 infection can be eliminated only by eliminating the infected cells. However, latently infected cells cannot be eradicated by the cytopathic effects of the virus, the host immune system or anti-retroviral drugs because latently infected cells do not express viral proteins. Latency is achieved when the proviral genome is replicated with and as part of the host cell genome without viral protein expression[23]. However, once latency is established in a CD4 memory cell it will have the ability to escape from the host immune response and even from highly active antiretroviral therapy (HAART) [24]. The primary reservoir for HIV-1 latent proviruses are naïve CD4 T cells, which exists in a resting state, until they are activated after encountering antigens. Subsequently, some of these cells persist as memory T cells, and will respond to the same antigen in future infections[25]. Induction of transcription factors such as NFAT and NF- K by cytokines or antigens promote the activation of HIV-1 proviruses [25]. Molecular mechanisms of HIV-1 latency include transcriptional interference, insufficient levels of transcriptional activators, presence of transcriptional repressors, insufficient TAT activity, nucleosome positioning among others[26]. Moreover, research has shown that stability of HIV-1 latency is attributed to cpG methylation of the HIV-1 5′ LTR, a mechanism that promotes resistance to reactivation signals[24]

HIV Proteins are codon biased. The interaction between host and pathogen has been remarkably studied to understand how adaptive genetic changes affect fitness of species. Molecular resources on the host are exploited by pathogens to assure survival and growth, meanwhile the host fight to

eliminate such pathogen. Virulence, ability to hide within the host and infectivity are the main factors that pathogens maximize to maintain replicative fitness[27]. Humans have acquired complex mechanisms of defense to attack and eliminate pathogens. In contrast, pathogens developed the ability to mutate, thus evading the host immune response. It is known that the human code is degenerated, due to the fact that 64 codons encode for 20 amino acids, meaning that multiple codons code for a specific amino acid. Frequency of usage of synonymous codons, also called codon bias is exhibited in most organisms, and they contribute to mutation- selection balance[27]. Synonymous mutations do not change the primary sequence of the protein; however it changes the base composition of genes. In fact, it has been demonstrated that enriched genes for the preferred codons have higher translational efficiency. Precisely, some bacteriophage genes have been enriched in the codons preferred by their bacterial host. However, not all pathogen-host associations and codon usage are defined in the same way. A number of RNA viruses display low association with their host in codon usage and base composition[27]. The study of the codon usage pattern between HIV-1 and its host has been used as a suitable model to understand the pathogen genome evolution in such interaction. It is known that in coding regions, the human genome is GC- rich. In contrast, HIV-1 has an A- rich genome. Codon usage pattern of 9 HIV-1 genes were compared in respect to its host, results demonstrate that the *rev*, *tat* and *nef* genes are closest to humans, and that *env*, *gag* and *pol* as well as *vif* and *vpu* cluster away from humans[27]. Furthermore, relative neutrality plots for HIV-1 and general human genes show that all HIV-1 genes have more than 50% of their preferentially used codons as A/T ending, indicating extreme bias[27]. This means, that the codons rarely used by the host are used by the HIV-1 late expressing genes, this is *env, gag, pol, vif* and *vpu.* In contrast, *rev, tat* and *nef,* the early expressing genes of HIV-1 showed less preference for A/T ending codons[27]. Increased usage of human preferred codons in these three

HIV-1 genes suggest a role in translation selection, which explains how HIV-1 has developed an strategy to evade restrictions based in codon usage, such as Slfn11. I will describe this innate immune antiviral strategy below.

The Schlafen Family of Proteins, Classification and Characterization. The Schlafen family of proteins was first characterized by David A. Schwarz and its research group in 1988 at the University of California at San Diego when they identified 4 mouse SLFNs: mSLFN1, mSLFN2, mSLFN3 and mSLFN4. Further studies lead to the classification of other mouse and human schlafen genes based on their size and function (**Fig. 1**).10 SLFN proteins have been identified in mice: mSLFN1, mSLFN1L, mSLFN2, mSLFN3, mSLFN4, mSLFN5, mSLFN8, mSLFN9, mSLFN10 and mSLFN 14[28,29]; whereas that only 5 in humans: SLFN5, SLFN11, SLFN12, SLFN13, and SLFN14[30]. These proteins have been classified in groups based on their structural characteristics.

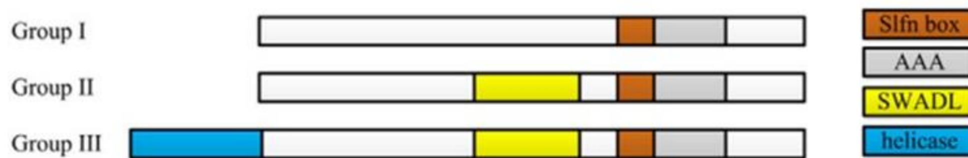

Fig. 1. SLFN proteins architecture. Liu, F., Zhou, P., Wang, Q., Zhang, M. & Li, D. The Schlafen family: complex roles in different cell types and virus replication (2018).

Group I SLFNs include proteins with molecular masses between 37 to 42 kDa. mSLFN1, mSLFN1L, and mSLFN2 correspond to this group, which like all SLFN genes, they contain a slfn box that lies under an AAA domain[30,31]. Group II consists of mSLFN3, mSLFN4 and SLFN12, whose molecular masses range between 58 to 68 kDa [30]. One of the most important features of this group is the presence of a specific domain called SWADL, which is defined by the amino acid sequence (Ser-Trp-Ala-Asp-Leu)[29,31]. The largest SLFNs belong to group III, whose molecular masses are between 100 to 104 kDa. In addition to the slfn box, the SWADL domain and the AAA

domain, this group preserves a C- terminal domain homologous to the DNA/ RNA helicases[32], which suggests that these proteins have a function in the nucleus [33]. mSLFN5, mSLFN8, mSLFN9, mSLFN10, mSLFN14, SLFN5, SLFN11, SLFN13 and SLFN14 belongs to this group.

Regulation of SLFN proteins Expression. Previous research has shown that human IFN type 1 receptors are involved in the induction of SLFN genes, some of these genes include SLFN5, SLFN11, SLFN12 and SLFN13[34]. mSLFN protein expression is also induced by interferons. Specifically, when mouse cells are treated with IFNα, mRNA expression of SLFN1, SLFN2, SLFN5 and SLFN8 is highly induced [34]. Stat 1, and Stat 3 proteins were required for the induction of mSLFN genes, suggesting that mSLFN gene induction occurs in the same manner as classic interferon-stimulated genes[34,35]. However, neither Stat 3 nor p38 MAPK were required for mSLFN 5, suggesting that another mechanism of regulation might be followed[34]. In the other hand, activation of T cells significantly downregulate SLFN genes. Activation of T lymphocytes with anti-CD3 or a combination of CD28 or CD3 antibodies exhibit downregulation of most SLFN genes. SLFN 5 was downregulated gradually, while SLFN12 was downregulated by 50% 6 hours after T cell activation. Expression of SLFN12 and SLFN11 was not altered[36]. . The expression of SLFN2 is upregulated when mouse macrophages are treated with LPS and unmethylated CpG dinucleotides (CpG-DNA) by activating the NF-kB and AP-1 pathways [37].

SLFN Proteins Biological Functions. SLFN and mSLFN genes are highly present in immune cells and their importance has been remarkable because of their role in immune responses, control of cell cycle proliferation as well as regulation of viral replication[38]. Particularly, the expression of SLFN1 inhibits cyclin D1 function in NIH3T2T murine fibroblasts, resulting in cell cycle arrest prior to the G1/S transition[39]. Cyclin D1 protein is crucial for the G1/S stage, and the overstimulation of this protein in SLFN1 expressing cells showed an increase in cell growth.

Therefore, cyclin D1 is one of the main targets of SLFN1 protein.mSLFN2 contributes to cell growth, differentiation and immune response. mSLFN2 mutations in mice increases susceptibility to bacterial and viral infections[40]. Silencing of SLFN2 decreases activation of c-Jun ad expression of NFATc1 results in decreased osteoclastogenesis. mSLFN proteins are also capable to modulate differentiation of T cells and macrophages.[33] Expression of mSLFN3 has been found in CD 4 and CD25 circulating T cells, however activation and proliferation of them results in mRNA downregulation. Moreover, it been demonstrated that mSLFN3 exerts a function in intestinal development and maturation. [41]. Ectopic expression of mSLFN3 results in anti-proliferative properties, by decreasing cell proliferation as well as decreased levels of proliferating cell nuclear antigen.[32] Sensitization of cells to chemotherapy resistance is another characteristic of mSLFN3. Ectopic expression of mSLFN3 inhibits characteristics of cancer stem cells. In regards to functions of human SLFNs, research demonstrates that SLFN5 possess negative regulatory effects on anchorage- independent melanoma cells in collagen[35], whereas SLFN11 appears to have anti-viral effects, by impairing the production of retroviruses. Moreover, SLFN11 also sensitizes malignant cells to topoisomerase inhibitors and DNA- damaging agents [42] [43].

SLFN proteins are involved with virus replication, acting on open reading frames to suppress viral replication[44].One of the typical targets of SLFN proteins are HIV and camel pox virus[45,46]. In addition, research demonstrates that mSLFN14 impairs Influenza A and Varicella Zoster virus by affecting nuclear trafficking of nucleoproteins[47].

In December 2019, a large number of cases reported as "pneumonia of unknow etiology" were reported in Wuhan, China. This led to an intensive investigation program, which attributed those cases to a novel virus belonging to the Coronavirus family. Viruses on this family are known for their potential to develop respiratory disease outbreaks, causing illness ranging from common

colds to MERS and SARS[48]. Literature postulates the probability of origin of this virus from bats, until they were able to infect other mammalian hosts before infecting humans. Transmission of this pathogen occurs from respiratory droplets and exposure to elevated aerosol concentrations in closed spaces. In addition, the virus can be transmitted human- to- human by asymptomatic individuals, and therefore isolation has been recommended to counteract this epidemic.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive sense single-stranded [(+)ss] RNA virus of around 60-140 nm. Its viral structure presents a round or elliptic from with a crown-like appearance due to the presence of glycoproteins on its envelope which guides the virus to the host receptors[49]. Enclosed by nucleocapsid proteins is its genome, consisting of 29,891 nucleotides. At least 14 open reading frames (ORFs) have been identified, coding for viral proteins[50]. A single ORF at the end of its genome codes for a polyprotein that is subsequently cleaved into 16 non-structural proteins, thus allowing for the formation of the replicase-transcriptase complex[49]. Until now, there is no specific treatment or vaccine for SARS-CoV-2. However, previous work and publications from our laboratory demonstrates that SLFN11 is able to impair (+)ssRNA viruses, such as West Nile, dengue and zika viruses[4]. In addition, SLFN11 impairs HIV-1 as I have demonstrated in this work, exploiting the fact that HIV-1 genome is codon usage biased. Importantly, SARS-CoV-2 is both (+)ssRNA and the genome is codon usage biased, therefore its is possible that SLFN11 could impair SARS-CoV-2 infection, a current active research in my laboratory.

Effects of SLFN proteins on HIV-1 infection. Viral infections induce type I interferons, a group of signaling proteins known for their potent anti-viral activity. Schlafen genes are a subset of early response genes, induced also by pathogens via the interferon regulatory factor 3 pathway[37]. A member of the Schlafen family, SLNFN11 impairs HIV-1 protein expression[2]. Research

demonstrates that SLFN11 does not affect integration, transcription or reverse transcription of HIV, but it rather acts in the late stage of the infection cycle by inhibiting the expression of viral proteins of the codon-biased HIV-1 genome. [2]. SLFN11 binds to tRNA, thus counteracting upregulation of the tRNA pool elicited by HIV to subsequently inhibit viral protein synthesis[2]. Recently, another member of the schlafen family, SLFN13 has been proposed as a tRNA/rRNA targeting endoribonuclease, which possesses a unique U-pillow- shaped structure in its N- domain, which enables base-paired cleavage of RNAs[3]. Cleavage takes place at the acceptor steam of the substrate tRNA, by removing 11 nucleotides at the 3′ terminus [3]. Nine residues are implicated in the nucleolytic activity of SLFN13, and 8 of them are conserved in SLFN11, which suggest that the antiviral mechanism of SLFN11 involves tRNA nucleolytic activity. In addition, it was demonstrated that this enzymatic activity is essential for SLFN13 to restrict HIV-1 protein expression.

This thesis focuses on the study of mSLFN proteins and the study of the mechanisms implicated in their anti-viral activity.

Significance.

Although effective antiretroviral drugs have helped to slow the progression of new HIV-1 infections and control the multiplication of HIV-1 in infected patients preventing AIDS development, still HIV-1 infection is incurable and patients require treatment for life [51]. The main obstacle to cure HIV-1 infection is that the current treatment does not eliminate a reservoir of latently infected cells. These are long-lived cells in which no viral proteins are expressed and therefore these cells can neither be eliminated by the immune system nor viral cytopathic effects. Two different strategies are envisioned for a cure. One strategy is based in the so called "Berlin Patient", the only HIV-1 patient cured. This patient received a successful allogenic bone marrow

transplant of cells carrying a mutation in one of the HIV-1 co-receptors and therefore naturally resistant to HIV-1. This case sparkled interest in the development of *ex vivo* modified patient cells resistant to HIV-1 for autologous transplant by engineering the stable overexpression of HIV-1 restriction factors. The second cure strategy intended to trigger latency reactivation leading to death of the latently infected cells by the toxicity of viral replication or immune clearance.

The anti-HIV-1 mechanism of SLFN11 has potential use in both cure strategies. Patient cells constitutively overexpressing SLFN11 could restrict HIV-1 infection. This is particularly important considering that type 1 IFN is the main induction mechanism of SLFN11 during viral infections and HIV-1 efficiently impair secretion and activity of this cytokine. In addition, limitation of the SLFN11 anti-viral activity could eliminate post-transcriptional barriers during latency reactivation.

However, exploiting the SLFN11 anti-HIV-1 activity requires a deeper understanding of the importance of this innate immune restriction pathway in cells relevant to HIV-1 infection *in vivo* and of the SLFN11 mechanism of action. My work in specific aims 1 and 2, respectively, will contribute to eliminate these gaps in knowledge.

Specific Aims. This thesis focuses on the evaluation of the anti-viral activity of the group III mouse SLFNs. Specifically, mSLFN5, mSLFN8, mSLFN10 and mSLFN14 will be tested against HIV-1. Our laboratory has provided evidence that SLFN11 impairs replication of West Nile, Dengue and Zika Virus and confirmed the anti-HIV-1 effect previously reported. However, the structural determinants of the anti-HIV-1 activity are ill-defined. To uncover this, we have determined the anti-HIV-1 activity of a panel of mSLFN proteins that have some degree of homology at the protein level with SLFN11. To this end, we have completed the following specific aims:

Specific Aim 1. *To generate A172 KD cell lines stably expressing different mSLFN proteins*

A172 cells are permissive to single-round HIV-1 infection. Our laboratory previously demonstrated that knockdown of SLFN11 in these cells increases their susceptibility to HIV-1 infection that is reduced upon re-expression of this protein. Therefore, A172 KD cells are a suitable reporter system to evaluate the anti-HIV-1 activity of mSLFN proteins.

Specific Aim 2. *To determine the anti-HIV-1 activity of mSLFN proteins*

For this specific aim we evaluated HIV-1 infection susceptibility of the mSLFN generated cell lines. We took advantage of an HIV-1-derived virus that express from the viral promoter Gag and firefly luciferase. In contrast to firefly luciferase, Gag exhibits a marked codon bias therefore it was expected that Gag but not luciferase expression will be affected by SLFN11 like mSLFN. Gag was determined by measuring HIV-1 p24 by enzyme-linked immunosorbent assay and luciferase by a luminescence-based enzymatic assay.

MATERIALS AND METHODS

Cell lines and viruses. HEK293T, SupT1, THP-1 U87 and A172 cells were obtained from the American Type Culture Collection (Manassas, VA). A172 and HEK293T cell lines were maintained in modified Eagle's medium (DMEM); THP-1 and SupT1 cells were maintained in RPMI 1640. Culture media were supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% nonessential amino acids (NEAA), and 1% sodium pyruvate. U87 Cells were maintained in DMEM and supplemented with FBS 15% and 300 µg/ml G418.

(Hluc), a replication- defective HIV-1 reporter virus was used. This virus expresses long terminal repeat (LTR)-driven luciferase from the negative factor (NEF) slot and contains a large deletion in envelope (ENV)[52]. Generation of Hluc virus was performed by calcium phosphate transfection of the corresponding HIV-1 expression plasmid d (pHluc; 15 µg) and the VSV glycoprotein G (VSV-G)-encoding plasmid pMD.G (5 µg) into HEK293T cells, as described previously[52].

Concentration of viruses was performed by using Lenti- X concentrator (Takara Bio). In brief, Supernatant from lentiviral packing cells was briefly centrifuged at 500 x g for 10 minutes, and filtered through a 0.45 µm filter (ThermoScientific). Clarified supernatant was transferred to a sterile container and combined with 1 volume of lenti-X concentrator with 3 volumes of clarified supernatant and mixture was incubated 4°C for 30 minutes. Then, sample was centrifuged at 1,500 x g for 45 minutes at 4°C. Finally, supernatant was removed and the pellet was re-suspended 1/100th of the original volume using complete DMEM. Samples were stored at –80°C for further use.

All work involving HIV-1, was performed in a BSL-2 laboratory in accordance with biosafety practices described in *The University of Texas at El Paso Biological Safety Manual.*

Single-round infectivity assay. A172-derived cells were seeded onto 24-well plates (1 x 10 ^5 cells/well) and allowed to grow overnight. The next day, cells were infected with Hluc, and 24 h later, the cells were extensively washed with basal DMEM to remove the input virus. Four days later, the cell culture supernatant was collected for HIV-1 p24 quantification, and cell lysates were prepared in a buffer containing 1% Triton X-100 for luciferase activity quantification (Bright-Glow luciferase assay system; Promega), according to the manufacturer's instructions. Luciferase activity was determined in samples using a microplate luminometer reader (Luminoskan Ascent; Thermo Scientific). Luciferase and HIV-1 p24 samples were derived from at least three independent infections.

HIV-1 replication. 10^5 SUPT1- or U87-derived cells lines were incubated overnight with HIV-1 strain NL4-3 (25ng of p24) in 0.5 mls of culture medium. Subsequently, the input virus was removed by extensively washing and cells were placed in 4 mls of culture medium. After day 4 post-infection supernatant was collected every other day until cells die due to cytopathic effects. Then, HIV-1 p24 was measured by ELISA in the cell supernatants collected.

Immunoblotting. Full procedures for protein detection by immunoblotting have been described previously[53].Briefly, Cellular lysates were obtained by lysing cells with 2 X Laemmli buffer and boiling for 10 min. Cell lysates were resolved by SDS-PAGE and transferred overnight onto polyvinylidene difluoride (PDVF) membranes at 100 mA at 4°C. Membranes were blocked in Tris-buffered saline (TBS) containing 10% milk for 1 h and then incubated with the corresponding primary antibody diluted in TBS–5% milk– 0.05% Tween 20 (antibody dilution buffer). Full-length SLFN 5, 8,9,10 and 14 were detected with anti-Flag antibody (Sigma- aldrich) (1/500). In addition, anti-SLFN11 antibody D-2 (Santa Cruz Biotechnology) (1/500) was used to detect SLFN11. Alpha-Tubulin was detected as a loading control with antibody from clone B-5-1-2

(Sigma) (1/4,000). Membranes were incubated overnight at 4°C with primary antibodies, whereas anti- -tubulin monoclonal antibody (MAb) was incubated for 30 min at 25°C. Primary antibody-bound membranes were washed in TBS– 0.1% Tween 20, and bound antibodies were detected with goat anti-mouse IgG-horseradish peroxidase (HRP) (Sigma) (1/2,000, followed by chemiluinescence detection. Densitometry of selected bands was quantified based on their relative intensities using Image Studio software (Li-Cor, Lincoln, NE).

Plasmids. Plasmids needed for the generation of HIV-1 derived viral vectors were obtained from the Eric Poeschla laboratory (Mayo Clinic, Rochester, MN)[53]. These lentiviral vectors were used to express mSLFN5, mSLFN8, mSLFN10 and mSLFN14 proteins. They were generated with packaging plasmid pCMVΔR8.91, a transfer plasmid derived from pTRIP (described below), and the envelope plasmid pMD.G encoding VSVG.

mSLFN expression plasmids.

mSLFN5, mSLFN8, mSLFN10 and mSLFN14 pCMV6-Entry expression plasmids were purchase from OriGene. These mSLFN cDNAs were subcloned into an HIV-1-derived vector (TRIP-IRES-P) by PCR using the Phusion- Site directed mutagenesis kit (Thermo Fisher Scientific).

Production of lentiviral vectors. Assembly of the pTRIP derived transfer plasmids was performed by using Nebuilder HiFi DNA Assembly Master Mix kit (New England Biolabs). Entire procedures for transfection and production of lentiviral vectors have been described previously[52-54]. In brief, HEK293T cells were calcium-phosphate transfected with the corresponding transfer plasmid derived from pTRIP (15µg), packaging plasmid pCMVΔR8.91 (15µg), and VSV-G envelope expression plasmid pMD.G (5 µg).72 h post transfection viral supernatants were harvested and concentrated with Lenti-X concentrator (Takara Bio) following manufacturer protocol.

Expression of full-length mSLFN proteins in SLFN11-deficient cell lines.A172 cells were manipulated by to express mSLFN5 protein by plating 1.3 x 10 ^6 cells in a T 75 flask. Followed by transfection with PEI 1 X. 48 hours post transfection cells were selected in the presence of geneticin. In addition, A172 KD cells were engineered to express mSLFN8, mSLFN10 and mSLFN14 proteins by transduction with lentiviral vectors expressing each of the previously mentioned proteins and the puromycin resistance gene. Briefly, viral vectors were produced in n HEK293T cells by transfection with the pTRIP-IRES-P- Slfn transfer plasmid expressing full-length SLFN proteins (15 μg) and the packaging and envelope expression plasmids described above Viral supernatants were concentrated with Lenti-X (Takara Bio).A172 KD cells were seeded in a 24-well plate (1 x 10 ^5 cells/well in 400 uL- total volume) and transduced the next day with different M.O.I's. After three days transduced cells were selected in the presence of puromycin (3 μg/ml) for A172 KD cells. Surviving cells were expanded as necessary.

HIV-1 p24 ELISA. HIV-1 p24 in the supernatants of the infected cells was measured by ELISA. (ZeptoMetrix Corporation). ELISAs were performed according to the manufacturer's instructions.

Statistical analysis. Statistical significance of data represented in bar graphs was determined using one-way ANOVA with Tukey HSD test, and in data represented as replication curves with multiple experimental points was determined by two-way ANOVA with Bonferroni post-hoc test.

RESULTS

SLFN11 impairs HIV-1 replication. SLFN11 knockdown (KD) was reported to enhance HIV-1 replication in the human CD4+ T cell line CEM[55] . However, since this original Nature report in 2012[55], no other publication has indicated an effect of SLFN11 on HIV-1 replication sparkling general doubts on the relevance of the anti-HIV-1 activity of this protein in the HIV-1 research community. In order to clarify this important issue we evaluated the effect of SLFN11 on HIV-1 replication in the human CD4+ T cell line SUP-T1 and in the human astrocytoma-derived U87 CD4+CXCR4+ reporter cell line. The latter although is not a target for HIV-1 *in vivo* has been widely used in HIV-1 research. These cells were stably transfected with either a shRNA against a scrambled sequence or SLFN11. Expression of SLFN11 was evaluated in these cells by immunoblotting with a specific antibody and as a loading control alpha tubulin was detected in these experiments. Levels of SLFN11 in U87 CD4+CXCR4+ shRNA cell line decreased dramatically (**Fig. 2B**), whereas in SUP-T1 some expression is still appreciated (**Fig. 2A**).

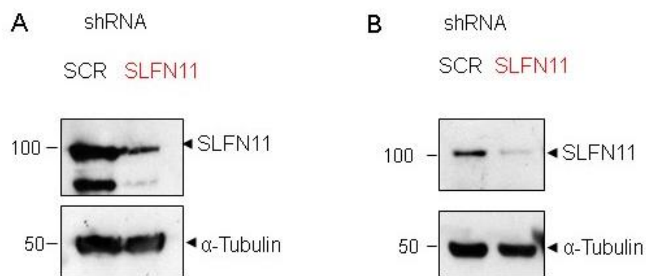

Fig. 2. Expression of SLFN11 in SUP-T1- and U87 CD4+ CDCR4-derived cell lines. (A) SUP-T1 and **(B)** U87 CD4+ CXCR4+ cells were stably transfected with an scrambled sequence (SCR) or an SLFN11-specific shRNA. SLFN11 expression was evaluated by immunoblot with an specific antibody. α-tubulin was detected as a loading control.

Subsequently, these cells were infected with HIV-1 wild type, the input virus removed, and after day 4 post-infection supernatant was collected every other day until cells die due to cytopathic effects. Then, HIV-1 p24 was measured by ELISA in the cell supernatants collected. U87 CD4+CXCR4 and SUP-T1 SCR cell lines demonstrated lower p24 levels in comparison to the

corresponding SLFN11 KD cell lines (**Fig. 3**). Importantly the differences between each pair of control and KD cell lines in SLFN11 directly correlated with their differences in p24 (**Fig. 3**). These results indicated that SLFN11 impairs HIV-1 replication in relevant cell lines.

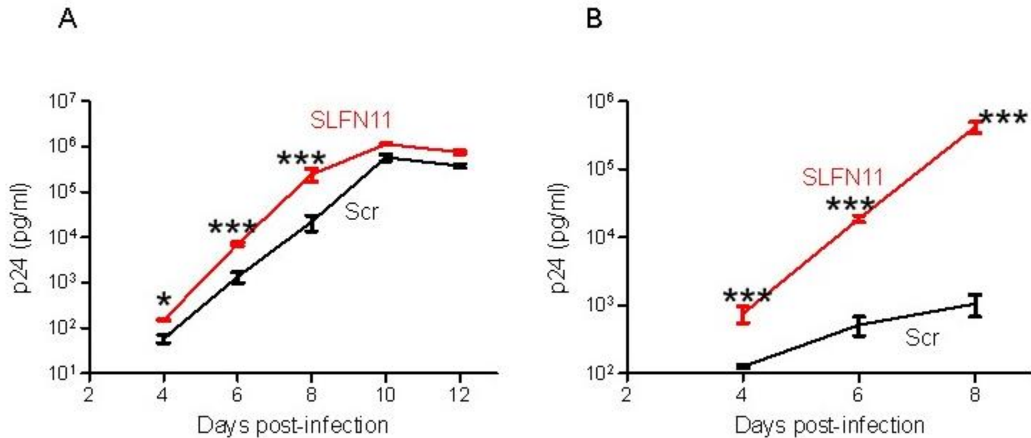

Fig. 3. Effect of SLFN11 on HIV-1 replication. **(A)** SUP-T1- and **(B)** U87 CD4+ CXCR4+ -derived cells analyzed in figure 1 were infected with HIV-1 NL4-3. Viral replication was followed by measuring HIV-1 p24 in the cell supernatant by ELISA. Error bars represent the variability observed in three biological replicates. Statistical significance was determined by two-way ANOVA with Bonferroni post-hoc test. All values are mean +/- S.D. (error bars). *, P<0.05; **,P<0.01; ***, P<0.001.

SLFN11 impairs HIV-1 infection in human myeloid cells. In further support of a role of SLFN11 in HIV-1 infection our laboratory have demonstrated that SLFN11 KD enhances HIV-1 single-round infection, in the human A172 (glioblastoma) and HEK293 cells[4]. For this thesis I repeated the experiments with A172-derived cell lines. Then, A172-derived cells expressing an shRNA with an shRNA against SLFN11 (KD) or KD cells re-expressing SLFN11 (BC) were infected with an HIV-1-derived reporter virus that lacks Env and has replaced Nef with the luciferase cDNA (HΔEluc). This virus can undergo only one round of infection. The reporter gene luciferase was determined in cell lysates and p24 was measured in the supernatants of infected cells by ELISA at day 4 post-infection. It has been reported that SLFN11 affect selectively the translation of codon biased messengers[3,55], then we normalized HIV-1 p24 to HIV-1 luciferase

levels to exclude any effect of the genetic manipulation introduced in the target cell lines used in these experiments that could affect the steps of the viral life cycle prior to viral-encoded translation. This strategy allows focusing only on the effect of the genetic perturbations on HIV-1 protein translation. Using this model we observed that SLFN11 KD cells produced close to six fold more p24 than SCR cells and that these differences were substantially removed when SLFN11 was re-expressed in the KD cells (BC cells) (**Fig. 4**).

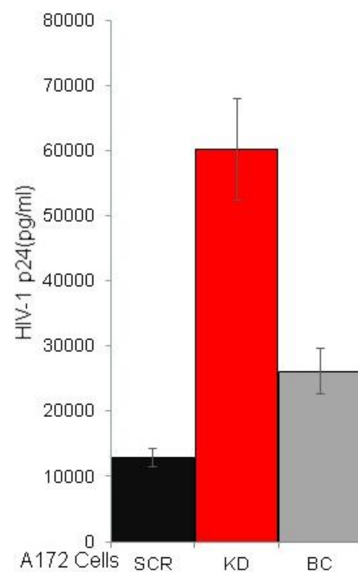

Fig. 4. Role of SLFN11 in HIV-1-driven p24 expression. Single-round infection on A172 SCR, KD and BC cell lines with an HIV-1 derived vector expressing luciferase. HIV-1 p24 was determined in supernatants of infected cells at day 4 post-infection. SLFN11 levels in these cells can be observed in figures 9 and 11.

These findings are important because the work previously reported in Nature[55] was conducted in an experimental model that do not include viral infection. Instead this group cotransfected in HEK293T cells both a SLFN11 and an HIV-1 expression plasmids in the absence of infection. We will refer here to this model as co-transfection model. However, an important caveat of the published[4,55] and figure 3 data is the use of cells that are not representative of cell types infected by

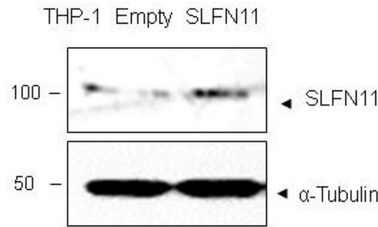

Fig. 5. Expression of SLFN11 in THP-1-derived cell lines. THP-1 cells transduced with retroviral vectors expressing or not SLFN11 were analyzed by immunoblotting for SLFN11 and α-tubulin.

HIV-1 *in vivo*, i.e. A172, HEK 293, and HEK 293T cells. Therefore to verify further the relevance of the anti-HIV-1 activity of SLFN11 in a relevant cell type we chose THP-1 cells. This model is relevant since HIV-1 *in vivo* infect CD4+ T cells and macrophages, and THP-1 are myeloid cells that resemble macrophages/monocytes. In addition we (**Fig. 2A**) and others[55] have demonstrated a role of SLFN11 in CD4+ T cells but myeloid cells have not been evaluated yet. Then, THP-1 cells were transduced by spin-inoculation with two different retroviral vectors containing a puromycin resistant cassette. One of this vectors expressed SLFN11, while the control or empty vector did not express SLFN11. After transduction, cells were selected in the presence of puromycin and expression of SLFN11 was analyzed by immunoblotting (**Fig. 5**). Tubulin was also detected in the same immunoblot membranes to verify equal loading. These cells were infected with HΔEluc and analyzed as described above by normalizing HIV-1 p24 levels to luciferase activity. Using this model we observed that overexpression of SLFN11 impairs HΔEluc p24 production (**Fig. 6**).

To further verify the role of SLFN11 in these cells, we also transduced THP-1 cells with a retroviral vector expressing an SLFN11-specific shRNA. As expected, these cells expressed lower levels of SLFN11 as detected by immunoblot analysis (**Fig. 7**).

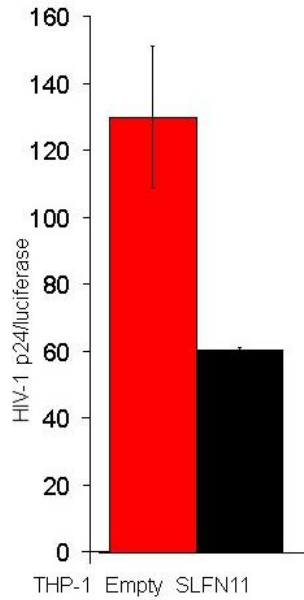

Fig. 6. Effect of SLFN11 on HIV-1 infection. THP-1 Empty and THP-1 SLFN11 cell lines analyzed in figure 5 were infected with a saturating dose of HΔEluc. Luciferase activity and p24 were measured in cell lysates and supernatants 4 days after infection. Error bars represent the variability of three biological replicates.

Infection of these and control THP-1 cells with HΔEluc was tracked as described above. Importantly, HIV-1 p24 production normalized to luciferase values was higher in the SLFN11 KD cells (**Fig. 8**), further demonstrating the anti-HIV-1 activity of this host protein.

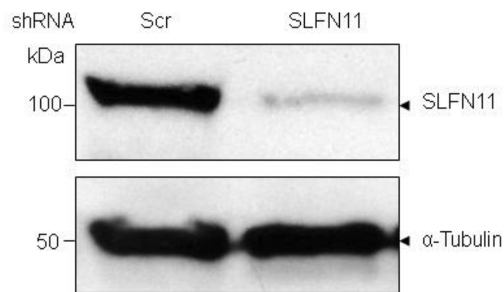

Fig. 7. Expression of SLFN11 in THP-1-derived cells. THP-1 cells were transduced with a retroviral vector expressing scrambled sequence or SLFN11-specific shRNAs. Expression of SLFN11 and α-tubulin was detected by immunoblot analysis.

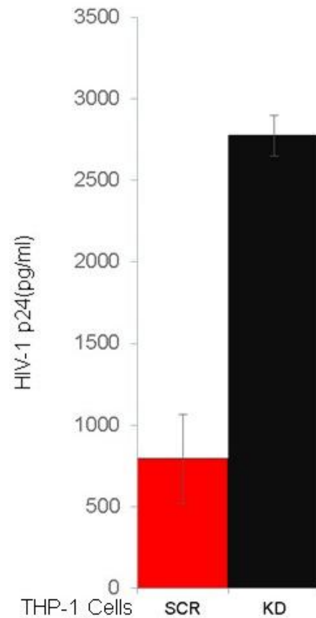

Fig. 8. Single-round infection of THP-1-derived cells. Cells analyzed in figure 7 were infected with HΔEluc and 4 days post-infection HIV-1 p24 levels were measured in cell supernatants by ELISA.

tRNase activity of SLFN11 is essential to impair HIV-1 infection. It has been proposed that the catalytic activity of SLFN11 is required for the anti-HIV-1 activity[55]. However, as mentioned above, these data were obtained in the HEK293T cotransfection model in the absence of infection. Therefore, in order to clarify the role of tRNase in the anti-HIV-1 activity of SLFN11 in a more relevant experimental model, we determined the effect of SLFN11 tRNase-dead mutant on HΔEluc infection in A172 KD. This is a good for this experimental question as we demonstrated above (**Fig. 4**). Then, this mutant was generated by substituting the catalytic residues E209 and E214 [3] with A and A172 KD cells were transduced with a retrovirus expressing this mutant genetically linked to the puromycin resistance cassette. The SLFN11 catalytic mutant was robustly expressed in A172KD cells as shown by immunoblot in **figure 9**. A172 KD cells and these cells expressing SLFN11 wild type or catalytic mutant were infected with HΔEluc and infection was monitored as described above. HIV-1 p24-luciferase normalized data in **figure 10** demonstrated

that SLFN11 catalytic mutant failed to impair HIV-1 p24 production. Therefore, our results also confirmed the relevance of tRNase in the anti-HIV-1 activity of SLFN11.

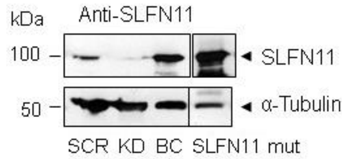

Fig. 9. SLFN11 levels in A172-derived cell lines. SLFN11 was detected by immunoblot analysis in cell lysates obtained from A172 SCR, KD, BC and from KD cells stably expressing SLFN 11 catalytic mutant.

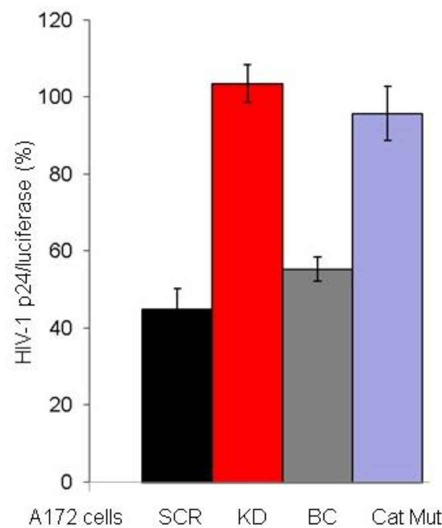

Fig. 10. Effect of SLFN11 Catalytic Mutant on HIV-1 infection. Cells analyzed in figure 9 were infected with HΔEluc and 4 days post-infection luciferase activity and HIV-1 p24 were measured.

tRNase-dependent and -independent mechanisms mediate the anti-HIV-1 activity of murine SLFN proteins. To further determine the role of tRNase activity on the anti-HIV-1 activity of SLFN proteins we also evaluated the anti-HIV-1 activity of murine (m) SLFNs belonging to the group III of this family. Importantly, SLFN11 and 13 that has been reported to impair HIV-1 infection [3,55] and both belong to this same group of the SLFN family. The anti-HIV-1 activity of SLFN11 resides in the N-terminal portion of this molecule[55]. Comparison of the amino acid

sequence of these humans and mSLFN proteins indicate between 53-69% of homology (**Table 1**). Importantly, analysis of the conservation of the catalytic triad amount these SLFNs (**Table 2**) predict that mSLFN 5 and 10 will lack tRNase activity. This prediction is supported by the fact that human SLFN5 that only lacks one of the three catalytic residues does not have tRNase activity and does not impair HIV-1 p24 expression[55].

Table 1. Percent of homology of the N-terminus of SLFN11 with other SLFN proteins.

Protein	SLFN13	mSLFN5	mSLFN8	mSLFN9	mSLFN10	mSLFN14
Homology(%)	65%	53%	69%	68%	69%	56%

However, mSLFN8, 9, and 14 are expected (**Table 2**) or have been demonstrated (SLFN13)[3] to have tRNase activity. mSLFN9 and 8 arrived by gene duplication and has 85% homology in their N-terminus. Therefore, they are expected to be functionally equivalent, based on this fact we decided to explore the function of mSLFN8 variant 2 that represent only the n-terminus of mSLFN8 rather than of the full-length variant 1. This strategy will also allow us to define if for the mSLFN proteins the N-terminus was sufficient to mediate their anti-HIV-1 function.

Table 2. Analysis of the conservation of the catalytic sites among SLFN proteins. Letter "h" refers to human, "m" to mouse and "r" as rat. Circles indicate SLFN proteins whose tRNase activity has been evaluated experimentally.

Protein	hSLFN11	rSLFN13	hSLFN11	mSLFN5	mSLFN8	mSLFN9	mSLFN10	mSLFN 14	hSLFN5
Residue	209	E	E	K	E	E	E	E	E
	214	E	E	E	E	E	Q	E	E
	251	D	D	H	D	D	D	D	H
tRNase	+ ●	+ ●	+	-	+ ●	+	-	+	- ●

Then, we generated A172 KD-derived cell lines expressing mSLFN5, mSLFN8, mSLFN10 and mSLFN14 by transducing these cells with lentiviral vectors expressing these flag-tagged mSLFN proteins and a puromycin resistance cassette. Transduced cells were expanded as necessary and a cell lysates were obtained from each of the established cell lines. Then, mSLFN protein expression was determined by immunoblotting using an anti-flag antibody, and alpha tubulin was detected as a loading control (**Fig. 11**).

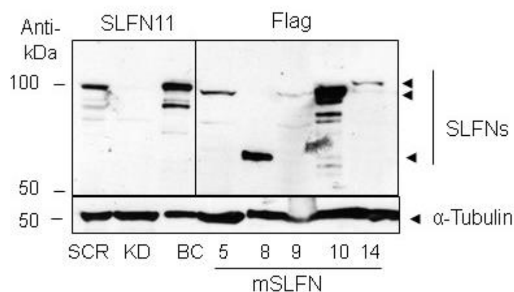

Fig. 11. Expression of SLFN proteins in A172-derived cells. A172 SCR, KD, BC and KD cells stably expressing different mSLFNs were evaluated for SLFN11and Flag-tagged mSLFNs by immunoblotting. α-tubulin was detected as a loading control.

Lower expression of mSLFN5, mSLFN9 and mSLFN14 than of mSLFN10 was achieved. These cell lines were infected with HΔEluc and 4 days post-infection luciferase (**Fig. 12**) and p24 values were measured and further normalized to luciferase levels (**Fig. 13**).

As expected, mSLFN8, 9, and 14 that conserve the tRNase catalytic residues impaired HIV-1 p24 production but did not modify luciferase levels (**Fig. 12 and 13**) indirectly supporting further the role of tRNase in the anti-HIV-1 activity of these proteins Moreover, mSLFN8 variant 2 that represents the N-terminus of this protein, were the catalytic triad is located, was sufficient to impair HIV-1.

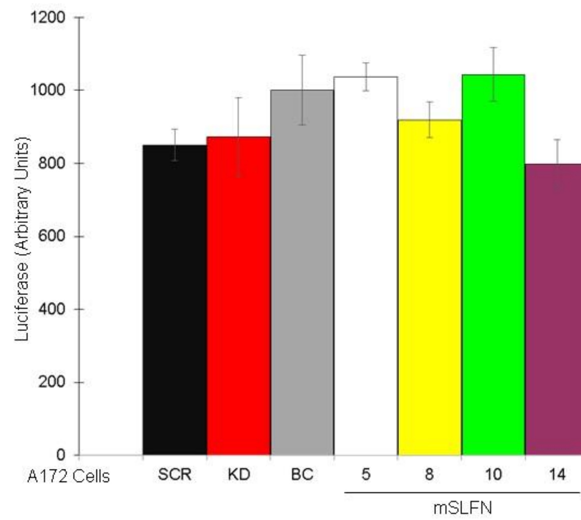

Fig. 12. Effect of SLFN proteins on HIV-driven luciferase activity. A172-derived cells evaluated in figure 10 were infected with HΔEluc and 4 days post-infection luciferase levels were measured.

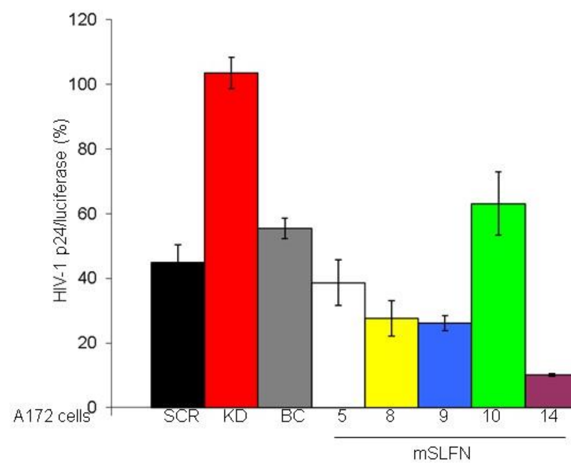

Fig. 13. Effect of SLFN proteins on HIV-1-driven p24 expression. A172-derived cells evaluated in figure 11 were infected with HΔEluc and 4 days post-infection HIV-1 p24 and luciferase levels were measured. HIV-1 p24/luciferase values normalized to the values found in KD cells are shown for each cell line. Error bars represent the variability of three biological replicates. The differences observed between mSLFN proteins 5, 8, 9, or 10 and BC cells are not statistically significant ($p>0.22$) . However, statistically significant differences were found between BC cells and mSLF14 ($p=0.03$), or KD cells ($p=0.001$). Data were analyzed using one-way ANOVA with Tukey HSD test.

However, mSLFN5, which naturally lacks two of the catalytic residues, and mSLFN10 that lack one on the critical residues **(table 2)** potently inhibited HIV-1 p24 production in a similar fashion than SLFN11. A potential explanation for the findings with mSLFN5 or mSLFN10 is that endogenous SLFN11 inadvertently was re-express in the A172KD cells during the generation of these cell lines. We were not able to detect endogenous SLFN11 in any of the cell lines expressing

the murine proteins but SLFN11 levels below the detection level of our immunoblots could be functional, for example notice that low levels of mSLFN9 and 14 (**Fig. 11**) potently impair HIV-1 p24 production (**Fig. 13**). Therefore, to exclude this possibility we expressed mSLFN5 in A172 SCR cells that express wild type levels of SLFN11 (**Fig. 11**) and determined the effect ofmSLFN5 overexpression on HIV-1 p24 production. These cells were infected with HΔEluc and evaluated as described above. Importantly, mSLFN5 potently inhibit HIV-1 p24 production in the presence of wild type SLFN11 (**Fig. 14**).

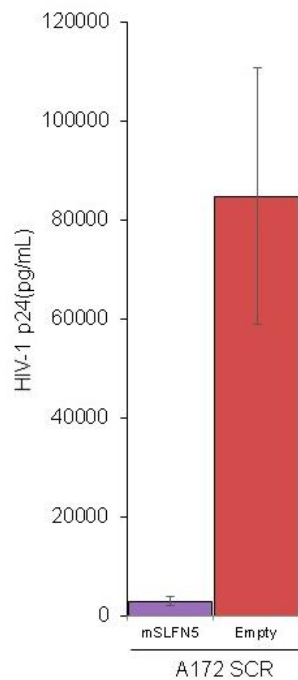

Fig. 14. Effect of mSLFN5 overexpression on HIV-1-driven p24 expression. A172 SCR cells stably expressing or not mSLFN5 were infected with HΔEluc and p24 production was measured 4 days later.

DISCUSSION

Our data confirmed the relevance of SLFN11 in HIV infection in cells that are relevant to the HIV-1pathogenesis. The role of SLFN11 in HIV-1 infection is a topic in discussion in the HIV-1 research community. The first report of this relevant mechanism appeared in Nature in 2012[2] and not until 2019[4] was any original report on this activity. Last year report was published in Journal of Virology by our lab and confirmed the original observations in a different *in vitro* HIV-1 infection model. However, these observations were made in cell types that were not relevant to HIV-1 infection *in vivo* keeping the lack of interest in the field. Therefore, one of my specific aims was to determine the role of SLFN11 in HIV-1 infection CD4+ and myeloid cells, the main targets of HIV-1 in vivo. My data confirmed that both over expression or downregulation of SLFN11 impair HIV-1 infection of wild type and single-round infection HIV-1. These findings are expected to eliminate doubts in the HIV- research community on the role of SLFN11 in HIV-1 infection.

We also have advanced our understanding of the anti-HIV-1 mechanism of action of SLFN proteins. We demonstrated that mSLFN proteins conserve the ability of human SLFN11 to impair HIV-1 p24 production acting at a post-transcriptional step of the virus life cycle. We also confirmed in a relevant *in vitro* HIV-1 infection model that in SLFN11 the tRNase activity is necessary. These findings also provide significance to the role of SLFN11 in HIV-1 infection since the Nature 2012 mechanistic studies were made in non-infected cells. However, more relevantly we have demonstrated that mSLFN proteins lacking the tRNase catalytic residues exhibit a potent anti-HIV-1 activity indicating the existence of tRNase-dependent and -independent mechanisms. Importantly both mechanisms affect HIV-1 at a post-transcriptional level. We speculate that HIV-1 has evolved an strategy to evade the tRNAse-independent mechanism present in SLFN11, but fails to counteract the murine SLFNs. HIV-1 has also evolved a mechanism to escape the tRNase-

dependent anti-viral activity of SLFNs. The mechanism consists in maintaining the early viral proteins encoded in open reading frames with low codon bias. This is the case of Tat, Rev, and Nef. Therefore production of these proteins is less restricted by SLFN11; once Tat is produced viral transcription is significantly amplified overcoming the effects of SLFN11 on translation of the codon biased viral open reading frames. Similarly Rev contribute to a more efficiently export of these viral mRNAs guaranteeing that the translational machinery is flooded with viral messengers. The role of Nef in this viral strategy that we postulate is not known yet. However, Nef is a multifunctional protein that drastically affect multiple signaling pathways in the cell and that is essential in vivo. Perhaps these potentially SLFN11-indpendent early functions have fueled the codon adaption of Nef. We postulate that drugs inhibiting these viral proteins (not available yet) will act at lower doses in cells expressing higher levels of SLFN11. Similarly HIV-1 carrying mutations that diminish the activity of these proteins will be more sensitive to the inhibitory effect of SLFN11. Therefore, we envision that, despite of HIV-1 escapes SLFN11 restriction, its anti-HIV-1 activity could be enhanced by conditions that decrease the levels of functional HIV-1 early proteins.

Conclusions.

Our data evidenced that SLFN11 impairs HIV-1 infection in CD4+ and myeloid cells, the main targets of HIV-1 in vivo, by reducing HIV-1 p24 production in a tRNase-dependent manner. These results highlight the relevance of this innate immune anti-HIV-1 mechanism.

In addition, we found that mSLFN proteins lacking tRNase catalytic activity are capable of impairing HIV-1 infection at a pot-transcriptional step of the viral life cycle, illustrating the complexity of the anti-HIV-1 activity of the SLFN proteins at a mechanistic level.

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VITA

Julia Hernandez was born in Saltillo Coahuila, Mexico. Daughter of Brenda Galvan and Mario Hernandez. Moved to Cd. Juarez in 1995 and did most of her education in Cd. Juarez, Chihuahua. Graduated from CBTIS 128 in 2012, and then enrolled at the University of Texas at El Paso. While pursuing her bachelor's degree, she was working at LACIT, for the liberal arts department. During her studies, she appeared multiple times in the dean's and deans plus list. She graduated in 2017 with a degree in microbiology and cum laude honors. In the same year, she enrolled in the master's program in biomedical engineering. Julia obtained her master's degree in May 2020. Now, her future goal is to work in the industry for biotech companies.