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Regulation Of Schlafen 11 Expression

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DEDICATION

I would like to dedicate this work to my family, specially my mother that always gave me her support and love, I also want to dedicate this to my girlfriend that always believed in me, and last but not least to all my friends that always helped me during this processes.

REGULATION OF SCHLAFEN 11 EXPRESSION

by

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THESIS

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4. To PhD Carlos Valenzuela for sharing his knowledge especially about the Schlafen family field.

ABSTRACT

Schlafen 11 (SLFN11) is a member a family of proteins that can be found in mammals. Members of this family are involved in important functions. SLFN11 in particular has been identified as a restriction factor for several evolutionarily disparate viruses such as HIV-1 and Flaviviruses and to play an important role in DNA repair, sensitizing cells to chemotherapy. Despite its relevance SLFN11 remains largely uncharacterized. The aim of this research was to achieve a more comprehensive understanding the regulation mechanism of SLFN 11 expression. Before our research, it was only known that type I interferon (IFN) regulates SLFN11 levels. However, we have demonstrated that SLFN11 expression is controlled by type I interferon-dependent and -independent mechanisms. Among the latter protein kinase C- and calcium-regulated-signaling pathways are of relevance. More important, we have also established that during viral infection-induced SLFN11 upregulation the relevance of these mechanisms vary depending on the ability of the virus to block the type I IFN system. Therefore, our findings have revealed the complexity of the regulation of the expression of this innate immune protein and evidenced important gaps in our knowledge.

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INTRODUCTION

The Schlafen family

Schlafen (SLFN) is a family of proteins that can be found in mammals, these genes have been specially conserved in mice and humans[1,2], in mice these genes are confined to the chromosome 11 and in humans they are in the chromosome 17. SLFN proteins can be divided in three groups depending on their molecular weight, Group I goes from 37 to 42 kDa, Group II from 58 to 58 kDa and the last Group III from 100 to 104 kDa[1–3] **(Fig. 1)**. Group I and II of SLFN family can be found in the cytoplasm and group III is nuclear[4]. A unique domain can be found in all of the SLFN proteins called Slfn box which function is not known[3,4], there is another component of SLFN protein the poly AAA motif that help for GTP and ATP binding[5] also intervenes in protein degradation[6], more over there is an SWDAL domain composed by the sequence Ser-Trp-Ala-Asp-Leu[2]. When all motif work together they can lead to RNA remodeling and can metabolize RNA[2,3].

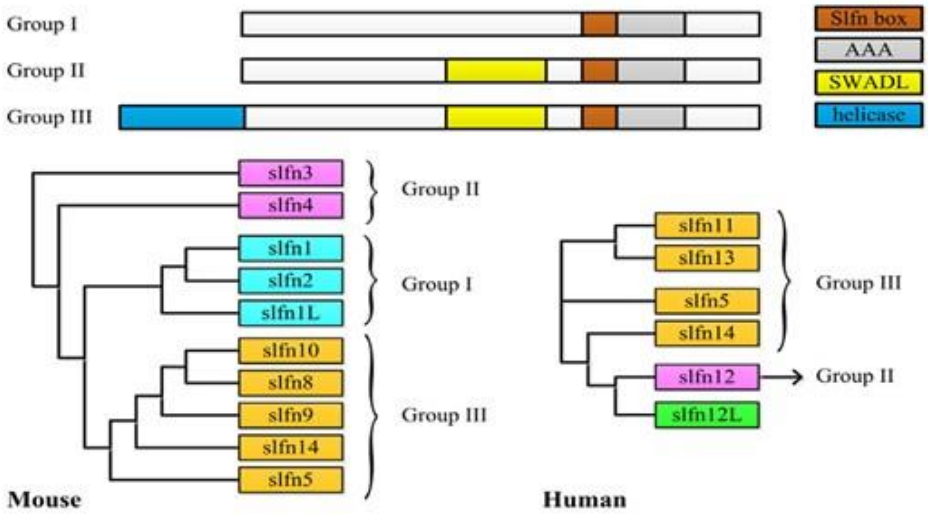

Fig. 1. Linear structural model of Schlafen family proteins. From: Liu, F., Zhou, P., Wang, Q., Zhang, M. & Li, D. The Schlafen family: complex roles in different cell types and virus replication. Cell Biol. Int. 42, 2–8 (2018).

In mice 10 different SLFNs have been identified (SLFN1, SLFN1L, SLFN2, SLFN3, SLFN4, SLFN5, SLFN8, SLFN9, SLFN10, and SLFN14), and five in human (SLFN5, SLFN11, SLFN12, SLFN13, SLFN14)[1,2,7,8].

Mouse SLFNs

Several functions of the SLFN proteins have described in mouse models and some of them have been studied in detail[5,9–12], SLFN2 has a role in innate immunity against viral and bacterial infections[13], and is also implicated in osteoclastogenesis. The latter case seems to be by the activation of osteoclast precursors via RAC1[10]. In addition mouse SLFN2 participates in suppression of cyclin D1 and inhibition of malignant cell growth[14]. SLFN1 has a similar activity on the suppression of cyclin D1 controlling cell proliferation[5]. Potentially through this mechanism SLFN1 also reduces the endothelial progenitor cell growth[15]. Moreover SLFN3 is downregulated upon activation of CD4+T cells [16], suggesting a regulatory role of this protein in maintenance of quiescence. Additionally SLFN3 has an important role in intestinal mucosa aging. The expression of this protein can be from 8 to 10 times lower in aged colon mucosa[12,17]. Importantly, overexpression of SLFN3 into colon cancer cells increases levels of cell differentiation, reduces the tumor sphere, and increases the effectiveness of chemotherapy by increasing the intracellular drug transport[6]. Similar to SLFN3, mouse SLFN4 mRNA is downregulated after macrophage activation and high levels of this protein are associated to reduce the myelopoiesis[8]. Therefore, group I and II mouse SLFNs seem to negatively regulate cell proliferation. In contrast, group III SLFNs are associated with control of immune responses and viral replication. For example, increased levels of SLFN8 are found in autoimmune encephalomyelitis[18] and SLFN14 impairs influenza virus and varicella zoster virus infections[19] .

Human SLFNs

Humans SLFNs belong to groups II (SLFN12 and 12L) and III (SLFN 5, 11, 13, 14) [2]. As the mouse counterparts, human SLFN12 is implicated in regulation of cellular processes linked to cell differentiation and apoptosis. For example this protein plays an important part during placenta development by impairing translation of specific ER proteins, leading to cellular apoptosis. Apoptosis is crucial for placenta implantation and growth[2021]. In addition SLFN12 influences enterocytic differentiation in intestinal cells[2223] and high levels of expression of SLFN12 in prostate cancer is associated to a less aggressive phenotype[24].

Group III human SLFNs are implicated in control of viral replication. In particular SLFN11 and 13 has been demonstrated to restrict infection by HIV-1 and flaviviruses. Central in the anti-HIV-1 activity these proteins degrades tRNAs[25,26]. Interestingly, SLFN5 that lacks this activity is not active against HIV-1 and instead seems to have a role in negatively controlling cancer cells [14,27,28]. SLFN14 seems to have a role in platelet generation since mutations in this protein are clinically associated to low count of platelets and their function[29,30].

Role of SLFN11 in human diseases

The most well characterized protein in the SLFN family is SLFN11[31,32]. This protein has been demonstrated to act as a viral restriction factor against evolutionarily divergent viruses such as HIV-1 and flaviviruses and to impair DNA damage repair in cancer cells. The role of SLFN11 in both diseases depend on the ability of this protein to prevent changes in the tRNA repertoire. In the case of HIV-1 and DNA damage repair SLFN11-induced tRNA degradation prevents translation of viral or host DNA damage repair proteins that are encoded in codon biased messengers[32–34]. In the case of flavivirus infection SLFN11 opposes to viral-induced tRNA downregulation, although the mechanism is unknown[35]. The ability of SLFN11 to negative

regulate the levels of ataxia-telangiectasia-mutated and Rad3-related, proteins required for DNA damage repair, determine that this protein is a main predictor (biomarker) of cancer response to chemotherapy agents[36–42].

Regulation of expression of SLFN proteins

The relevance of the SLFN proteins in controlling cell cycle, cell differentiation, apoptosis, DNA damage repair and viral infections indicate the importance of understanding the mechanisms that regulate the cellular levels of these proteins. This is the main goal of my research. When I started this work, the only mechanism described to regulate SLFN protein levels was the type I interferon (IFN) system[2]. This group of interferons is composed by IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, and all of them are encoded in the chromosome 9 in humans[43]. Almost all nucleated human cells can produce IFN-α and IFN-β[43]. Upon binding to a common receptor, type I IFNs activate three of the Janous kinases (JAKs): JAK1,JAK2 and tryrosin kinase 2 (TYK2), that in turn activate signal transducers and activators of transcription (STATs) 1 and 2. Phosphorylated STAT1 and STAT2 binds to interferon regulatory factor 9 (IRF9) forming a complex that bind to promoter of a group of genes called interferon-stimulated genes (ISG) **(Fig. 2)**. SLFN proteins are part of the ISG [43]. Furthermore, MAPK signaling has been implicated in the type I IFN signaling pathway that regulates expression of some of the SLFN proteins[2,14]

Significance.

SLFN11 is a type I interferon (IFN)-induced protein that has anti-viral activity against phylogenetically distant viruses such as HIV-1[42] and flaviviruses[35]. Additionally new studies have extensibility showed that SLFN11 have an important role during cancer chemioresistance development in response to treatment.

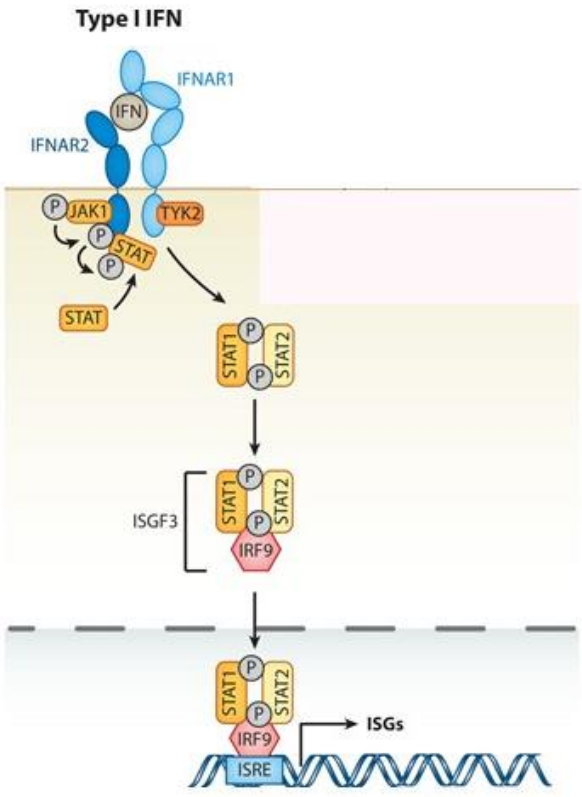

Fig. 2. Type I interferon signaling cascade. Modified from Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-stimulated genes: a complex web of host defenses. *Annu. Rev. Immunol.* **32**, 513–545 (2014).

Therefore, SLFN11 is a biomarker or predictor for relevant diseases. Understanding the molecular mechanism that regulates SLFN11 expression will allow us to modulate the levels of this protein affecting these diseases. However, the mechanisms that regulate SLFN11 expression are ill-defined, and the goal of this research was to understand better these mechanisms at the molecular level. In order to fulfill this goal I completed the following aims:

Specific Aim 1: **To determine mechanisms of regulation of SLFN11 expression during viral infection**

We focused on three phylogenetically distant viruses that are differentially affected by SLFN11. West Nile Virus (WNV) is a positive-sense single-stranded virus of the flaviviridae

family, vesicular stomatitis virus (VSV) is a negative-sense single-stranded virus of the rhabdoviridae family, and human immunodeficiency virus-1 (HIV-1) a retrovirus. In contrast to VSV, which escapes SLFN11 restriction, the other two viruses are impaired by this host protein. Furthermore, infection by VSV and WNV, but not by HIV-1 robustly triggers type I IFN signaling, a major regulator of the expression of SLFN11.

Specific Aim 2: **To establish the effect of type I IFN-dependent and -independent stimuli on the activity of the SLFN11 promoter.**

A 1.3 kb SLFN11 promoter (chr17 33,700,532 - 33,700,847) was reported. This DNA region was cloned upstream eGFP to generate a simple reporter system that allows us study promoter regulation by Fluorescence-activated cell sorting (FACS) analysis. Different stimuli discovered in aim 1 to regulate SLFN11 protein levels in cells will be evaluated in this system.

MATERIALS AND METHODS

Cell culture.

HEK293, SupT1, and A172 cells were obtained from the American Type Culture Collection (Manassas, VA). Human peripheral blood mononuclear cells (PBMCs) where obtained from two donors using peripherical blood samples and purified using Ficoll Paque™. HEK293 and A172 cells were maintained in Dulbecco's modified Eagle's medium (DMEM); PBMCs and SupT1 cells were maintained in RPMI 1640. These culture media were supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% nonessential amino acids (NEAA), and 1% sodium pyruvate. VSV engineered to express eGFP. A replication-defective HIV-1 reporter virus (Hluc) was used, which expresses long terminal repeat (LTR)-driven luciferase from the negative factor (NEF) slot and contains a large deletion in envelope (ENV)[44]. Hluc was generated by calcium phosphate transfection of the corresponding HIV-1 expression plasmid (pHluc; 15μg) and the VSV glycoprotein G (VSV-G)-encoding plasmid pMD.G (5μg) into HEK293T cells, as described previously[44]. All work involving VSV and 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.

Immunoblotting.

Full procedures for protein detection by immunoblotting have been described previously[45]. Briefly, cellular lysates were obtained by lysing cells with 2X Laemmli buffer(prepared with : 4% SDS, 20% Glycerol, 0.004% bromophenol blue 1.25M Tris-Cl, pH6.8 and 10% 2-mercaptoethanol) and boiling for 10 min. Cell lysates were resolved by SDS-PAGE for 2:30 hrs. at 100 volts using 1x running buffer (10 x running buffer was prepared with: 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O adjust pH to 8.3 and transferred using 1x

transfer buffer (10x transfer buffer was prepared with: Tris 15.2g Glycine 72.1g SDS 5.0g and 500ml H2O) overnight(18 hrs) onto polyvinylidene difluoride (PVDF) membranes(bought from Biorad) 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 SLFN11 was detected with anti-Slfn11 antibody E-4 (Santa Cruz Biotechnology) (1/500). The Slfn11 was detected with anti-Slfn11 antibody D-2 (Santa Cruz Biotechnology) (1/500). Tetherin (BST-2) was detected with anti-BST-2 antibody (Santa Cruz Biotechnology) (1/500). α -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 α -tubulin monoclonal antibody (MAb) was incubated for 30min 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 chemiluminescence detection. Densitometry of selected bands was quantified based on their relative intensities using Image Studio software (Li-Cor, Lincoln, NE).

IFN-α ELISA.

IFN- α levels were quantified in the cell supernatants of infected cells by an ELISA (catalog numbers 41115-1 for IFN-α; PBL Assay Science). ELISAs were performed according to the manufacturer's instructions, 1:7- point standard curve where prepared in a sample matrix, sample buffer, standard and samples where added into the well and the incubation were performed at room temperature shaking at 550 rpm 30 sec then incubated at 4°C for 18hrs, samples were washed one time, antibody was added and incubated at room temperature for 1hr then washed 3 times, HRP was added and incubated 1hr at room temperature, TMB substrate is added for 30min and incubated in the dark at room temp, stop solution is added and samples were read using 450nm.

Transient Transfection

Transient transfection where performed seeding HEK293 cells at $3x10^6$ cells in a T75, 24hrs after seeding were calcium-phosphate transfected with the corresponding transfer plasmid derived from pEZX-LvPFO2 (slfn11 promoter) using 12µg, and incubated at 37°C for 48hrs, culture media must be replaced with fresh media 24hrs after transfection.

Cell treatments

HEK293 transient transfected with pEZX-LvPFO2 plasmid where seeded in a 24 well plate at 1x10^5 cell per well in a total volume of 400µl, cell where incubated for 24hrs at 37°C, 50µl of media was removed for each treatment used, treatment where prepared in a total volume of 50µl and then added into the well, treatments where used at this concentrations: PMA at 100µM using 8µl, Ionomycin 1/2000 dilution using 4µl, IFN-α at 5,000 units, IFN-β at 5,000 units, N-Fat at 1.187mM using 33.9µl, SR302 at 100µM using 4ul, CSA at 1/1000 using 4.8µl and Jak at 250µM using 1.6µl, after treatment cells where incubated for 24hrs and 48hrs.

FACS assay.

HEK293 cells were seeded onto 24-well plates ($1x10^5$ cells/well) and allowed to grow overnight. The next day, the cells were treated with different compounds and incubated at 37°C for 24hrs and 48hrs. Cells were harvested and resuspended in a total volume of 500µl. The cells were then analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter) using fluorescent detector channel FL1-A and a total of 10,000 cells were counted for each sample.

Virus infectivity assay.

All cell lines infected with WNV, VSV were seeded in T25 cell culture flasks (2.5x10^5 cells in a 2-ml total volume) and allowed to grow overnight. The following day, the cells were infected with the respective viruses and incubated at 37°C for 1h. Cells were subsequently washed

three times with serum-free medium to remove input virus, replenished with maintenance medium and incubated at 37°C, after 48hrs cell lysates were collected.

Single-round infectivity assay.

A172-derived cells were seeded onto 24-well plates (2x10^4 cells/well) and allowed to grow overnight. The next day, cells were infected with HΔEeGFP and HΔELuc and incubated at 37°C, after 48hrs cell lysates were collected.

RESULTS

Virus infection upregulates SLFN11 expression.

It has been reported that SLFN proteins are induced by type I-interferon **(**I IFN) signaling[2]. Our laboratory recently discovered that West Nile virus (WNV) infection induces SLFN11 an effect that coincide in time with a virus-induced surge of type I IFN secretion[35]. However, the induction mechanism is unknown. In order to investigate the role of type I IFN in the ability of WNV to induce SLFN11, cells were infected at a multiplicity of infection of 10 (MOI:10) in the presence of different inhibitors including Cyclosporin A (0.1uM), ethylene glycol tetra acetic acid (EGTA) (0.5mM), NFAT inhibitor (Cayman 13855, 100uM) and JAK (tofacitinib 100nM). Forty-eight hours later expression of SLFN11 was evaluated in these cells by immunoblotting. α-tubulin was detected as loading control in these experiments. Cell lysates obtained from untreated cells were serially diluted to find SLFN11 protein concentrations that were non-saturating. For example we selected amounts of protein that give a clear signal in the X-ray films in about 5 mins of exposure. Typically they were in the linear part of the detection system, since two-fold dilutions of these lysate amounts correspondingly reduced the amount of SLFN11 detected by half. Using these conditions we obtained the results in **Figure 3**. This experimental strategy was applied to all the immunoblots included in this work.

Cyclosporin A and EGTA +were included in the study for their role in counteracting WNV-induce calcium-signaling and NFAT inhibitor because this is the main transcription factor activated by calcium signaling, whereas JAK is central in the initiation of type I IFN signaling.
In correspondence with a previous report[35] data in **Figure 3** indicated that WNV induces SLFN11. Importantly inhibition of JAK signaling completely abrogated this effect. Upon type I IFN receptor binding JAK is activated initiating a signaling cascade that end in transcriptional activation of

interferon-stimulated genes, such as SLFN11. Therefore, these results indicate that WNV induces SLFN11 in a type I IFN-dependent manner. Interestingly, Nuclear factor of activated T-cells(NFAT) inhibitor only partially reduced WNV-induced SLFN11 upregulation, but in contrast EGTA and Cyclosporin A(CsA) which prevent Ca-signaling required for NFAT activation, failed to mimic the effect of NFAT inhibitor. These data suggest that NFAT could be activated in a Ca-independent manner via I IFN signaling during WNV infection. In further support that JAK and NFAT inhibition were acting via type I IFN signaling, we observed that these inhibitors favored WNV replication 8- and 2-fold, respectively, as compared to untreated cells of cells exposed to EGTA or CsA, at 48h post-infection (Data not shown).

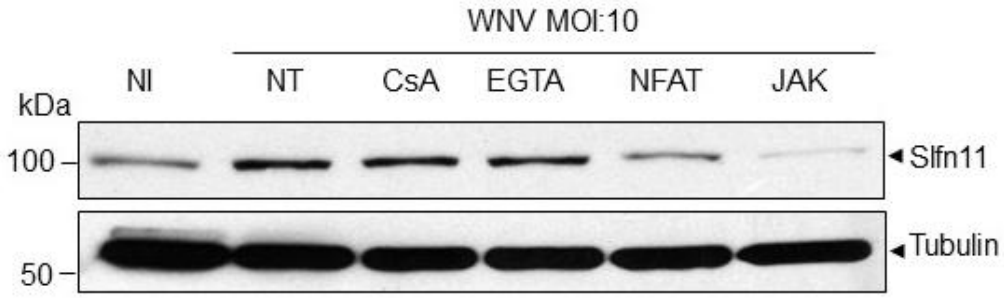

Fig. 3 Effect of WNV infection on Slfn11 expression. (**A**) A172 cells were infected with WNV in the absence of any treatment or the presence of cyclosporin A (CsA) EGTA, or inhibitors of NFAT or JAK. Infected cells were analyzed 48 hrs post-infection. The treatments were kept during these 48hrs.

To better understand the regulation of SLFN11 expression during viral infections we determined the effect of vesicular stomatitis virus (VSV) infection and HIV-1 on the levels of this host protein. VSV infection triggers an important type I IFN response, but HIV-1 notoriously block type I IFN at multiple steps[46].

Furthermore, SLFN11 impairs HIV-1 but not VSV infection[35]. Therefore, these two model viruses are expected to be informative about the viral regulation of SLFN11 expression. A172 cells were infected with VSV (MOI 1) or HIV-1 (MOIs 1 and 10) and 48 hrs later the levels of SLFN11 in these cells were determined by immunoblot analysis. Data in **Figure 4** indicated that VSV infection potently upregulated SLFN11 expression.

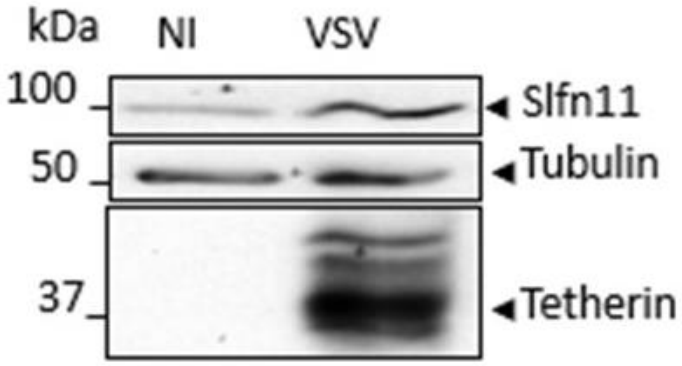

Fig. 4 Effect of VSV infection on Slfn11 expression. A172 cells were infected with VSV and analyzed 48 hrs later. Proteins were detected in total cell lysate by immunoblotting analysis.

Densitometry analysis (**Fig. 5**) showed a 2.6-fold increase in the α-tubulin-normalized SLFN11 levels. Similarly two single-round infection HIV-1 also induced SLFN11 (**Fig. 6**) and this effect was MOI-dependent. HΔEeGFP at MOI 10 induced 1.4 fold the expression of SLFN11 whereas at MOI 1 SLFN11 levels were not modified (**Fig. 7**).

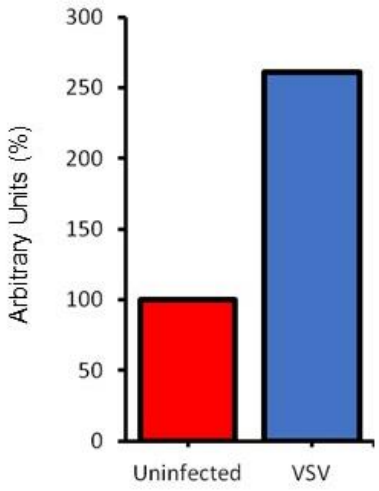

Fig. 5 Densitometry analysis of immunoblot in figure 2. SLFN11 signals were normalized to those of α-tubulin and values are expressed in %. Values in control cells were considered 100%.

HΔELuc that was used a saturating concentration (MOI cannot be calculated for this virus) upregulated SLFN11 around 10-fold. Therefore, these data demonstrate that viral infection induces SLFN11 expression.

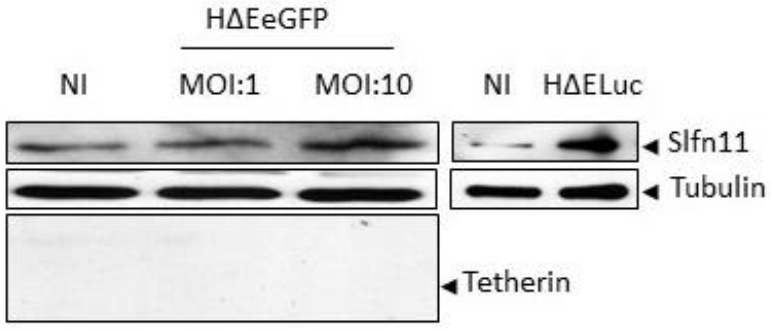

Fig. 6 Effect of HIV-1 infection on Slfn11 expression. A172 cells were infected with HΔEeGFP, and HEK293 cells with HΔELuc and analyzed 48 hrs later. Proteins were detected in total cell lysate by immunoblotting analysis.

In order to investigate the role of type I IFN in the effect of these viruses on SLFN11 expression, we measured levels of this cytokine in the cell supernatant by ELISA (**Fig. 8**). This increase in SLFN11 expression correlated with a marked production of IFNα in the supernatant of the infected cells (3,978.6 pg/ml) as measured by ELISA. In addition, VSV infection triggers expression of tetherin, another type I IFN-stimulated gene[43]. However, the levels of type I IFN in the HIV-1 infected cells were similar to those in non-infected cells (**Fig 8**). These results further supported the concept that SLFN11 is induced by viral infections in type I IFN-dependent and -independent manners. (All of these results were done in triplicates during the same experiment).

Type I IFN-independent mechanisms of SLFN11 upregulation.

To investigate type I IFN-independent pathways in the regulation of SLFN11 expression primary cells were evaluated. Due to their role in HIV-1 infection *in vivo* we determined the effect of several stimuli on the expression of this protein in primary CD4+ T cells isolated from two donors. These cells were treated with Phorbol myristate acetate (PMA) (20nM), ionomycin (5nM), Phytohemagglutinin (PHA) (5ug/ml), PMA plus ionomycin (20nM/5nM), TNFα (10ng/ml) and IL-4 (50 ng/ml). At 48hrs post stimulation, levels of SLFN11 in these cells were determined by immunoblot analysis using non-saturating amounts of cell lysates as described above.

In these experiments α-tubulin was detected as loading control. Data in **Figure 9-10** showed that treatments with PMA, ionomycin, PHA, PMA pus ionomycin and IL4 upregulates SLFN11 expression. In donor I, densitometry analysis indicated that PMA increased in 2-fold SLFN11 expression compared with non-treated whereas ionomycin only activated 1.7 folds.

A synergistic effect was found between these two drugs since 2.2-fold increase were observed with their combination. PHA also potently (2.5 fold) activated SLFN11 expression whereas the effect of IL4 was more modest (1.5 folds). Similar results were observed in donor II.

Although PMA or ionomycin alone failed to upregulate SLFN11 expression, their combination activated 1.7. PHA was also potent causing a 2-fold increase but IL4 failed. Notoriously, TNFα failed to enhance SLFN11 expression in both donors.

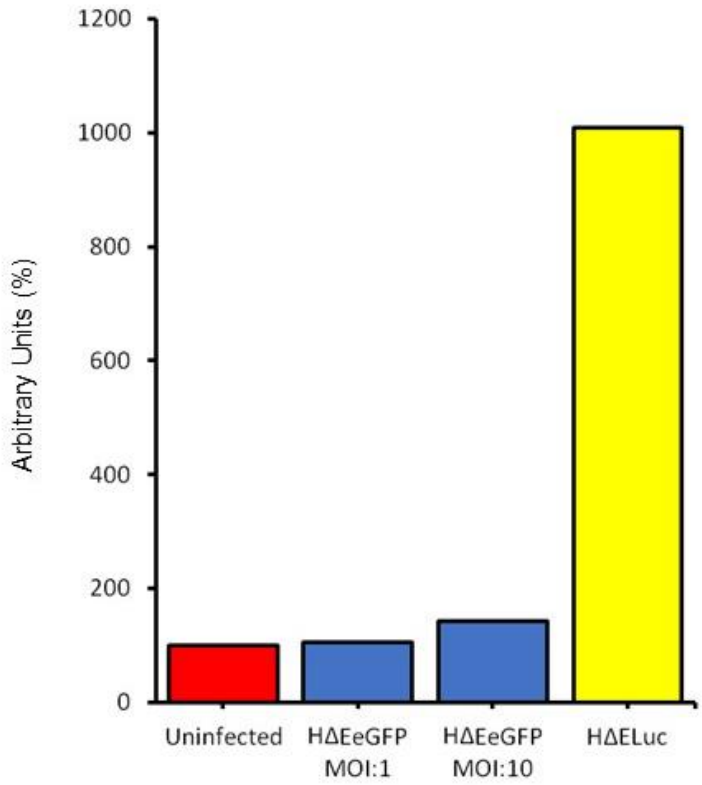

Fig. 7 Densitometry analysis of immunoblot in figure 4. SLFN11 signals were normalized to those of α-tubulin and values are expressed in %. Values in control cells were considered 100%.

These results indicated that PMA in combination with ionomycin, and PHA potently upregulate SLFN11 in two different donors. These stimuli are known to act independently of the type I IFN signaling. PHA and ionomycin trigger Ca-dependent signaling and PMA is a protein kinase activator.

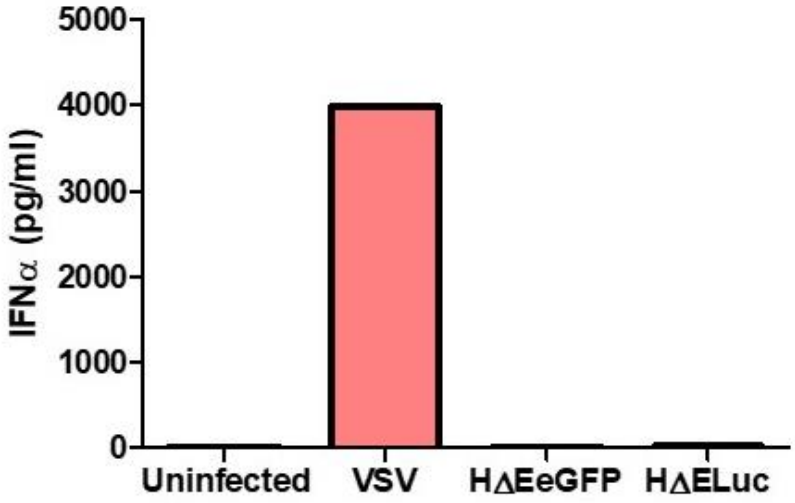

Fig. 8 Levels of IFNα in virus cells infected. Levels of IFNα were measured by ELISA in the Supernatant of cells characterized in figures 2 and 3.

To determine whether type I IFN-independent of SLFN11 was relevant in different cell types we determine the effect of these compounds in the human cell lines HEK293 (epithelial cells) and SUPT1 (CD4+ T cells). Cells were stimulated with a panel of compounds and SLFN11 and α-tubulin were measured by immunoblotting as described above. PMA (20nM), ionomycin (5nM) and PMA + ionomycin (20nM/5nM) stimulated SLFN11 production at both 24 and 48 hrs post-treatment in both cell lines, although the response in HEK293 was more evident (**Fig. 11**). However, TNFα (10ng/ml), IL-4 (50 ng/ml), and PHA (5ug/ml) failed to do so (data not shown).

Quantification of these immunoblots (**Fig. 12**) indicated that at 48 hrs HEK293 SLFN11 levels increased in 2.3 fold for PMA (2 at 24 hrs), 2 for ionomycin (1.3 at 24 hrs), and 2.2 for PMA plus ionomycin (1.2 at 24 hrs).

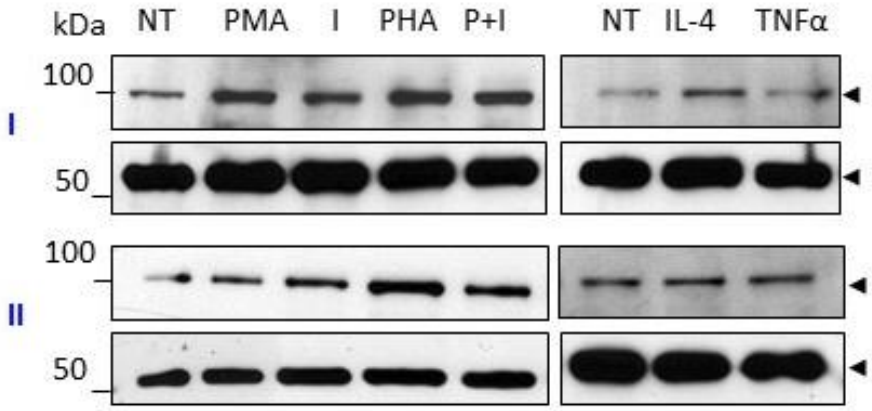

Fig. 9 Pharmacological stimulation of Slfn11 expression in human primary cells. CD4+ T lymphocytes isolated from two different donors (I and II) were stimulated for 48 hrs with different compounds and SLFN11 measured by immunoblotting. α-tubulin was measured as loading control. NT is non-treated control cells, I is ionomycin.

At this time point in SUPT1 cells levels increased by 2.2 folds for PMA (1.4 at 24 hrs), 4.6 folds for ionomycin (1.9 at 24 hrs), and 4 for PMA pus ionomycin (1.8 at 24 hrs). Importantly, PMA and ionomycin upregulated SLFN11 expression in SUPT1 and HEK293 cells without increasing Tetherin (**Fig. 11**), suggesting that these stimuli acted in a type I IFN-independent way. In sum, these results indicated that PMA and ionomycin upregulate SLFN11 expression.

Signaling pathway analysis (**Fig. 13**) indicate that PMA/PKC could promote SLFN11 upregulation via AP-1- or Nf-Kβ-mediated transcription and inomycin via NFAT and AP-1. However, the lack of an effect of TNFα on SLFN11 upregulation argues against an important role for Nf-Kβ. AP-1 activation could proceed via PKC/SEK1/JNK or PKC/ERK1/2, and Ca/Calcineurin/SEK1/JNK while NFAT is activated by Calcineurin (**Fig. 13**).

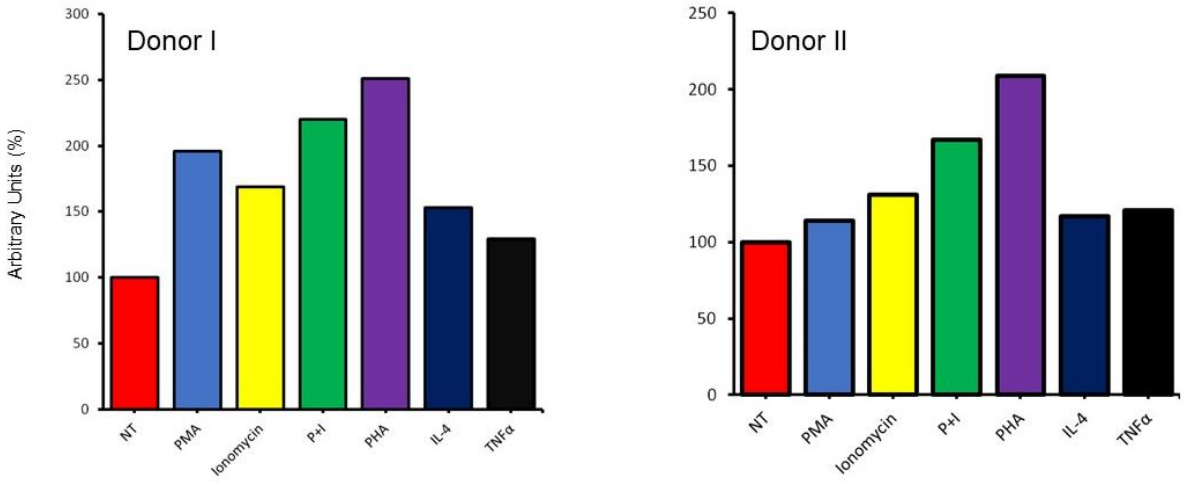

Fig. 10 Densitometry analysis of immunoblot in figure 7. SLFN11 signals were normalized to those of α-tubulin and values are expressed in %. Values in control cells were considered 100%.

This plasmid was transfected into HEK293 cells and 24 hrs later the cells were distributed to different treatments, and 24 and 48 hrs post-stimulation eGFP mean intensity of fluorescence (MFI), an indication of the amount of eGFP molecules in the whole culture, were calculated by FACS. Treatments included PMA (20nM), ionomycin (5nM), PMA + ionomycin (20nM/5nM), IFNα (5,000 U/ml) and IFNβ (5,000 U/ml).

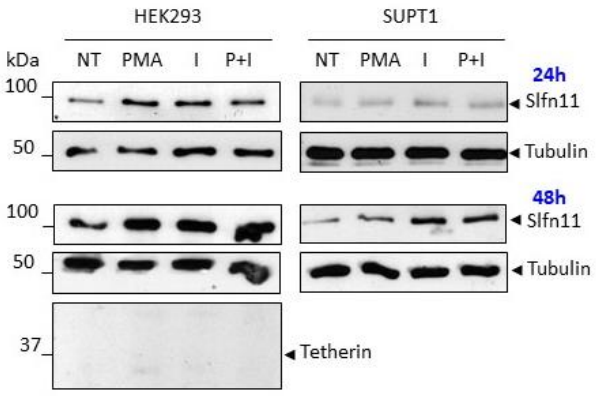

Fig. 11 Pharmacological stimulation of Slfn11 expression in human cell lines. HEK293 and SUPT1 cells were stimulated for 24 and 48 hrs with different compounds and SLFN11 measured by immunoblotting. α-tubulin was measured as loading control and tethering as type I IFN activity control. NT is non-treated control cells, I is ionomycin. P+I is PMA plus ionomycin.

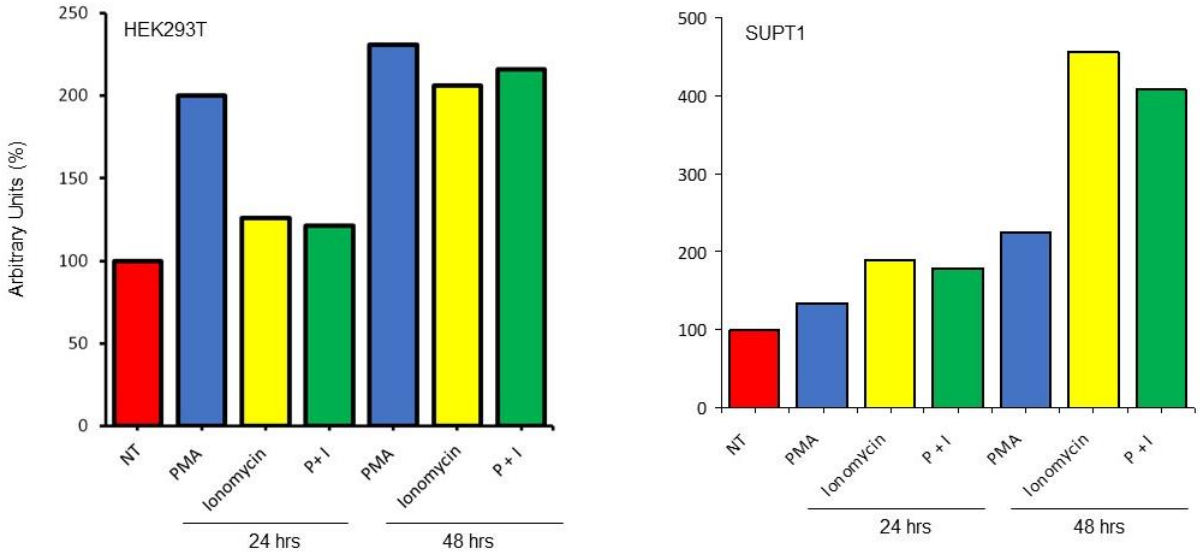

Fig. 12 Densitometry analysis of immunoblot in figure 9. SLFN11 signals were normalized to those of α-tubulin and values are expressed in %. Values in control cells were considered 100%.

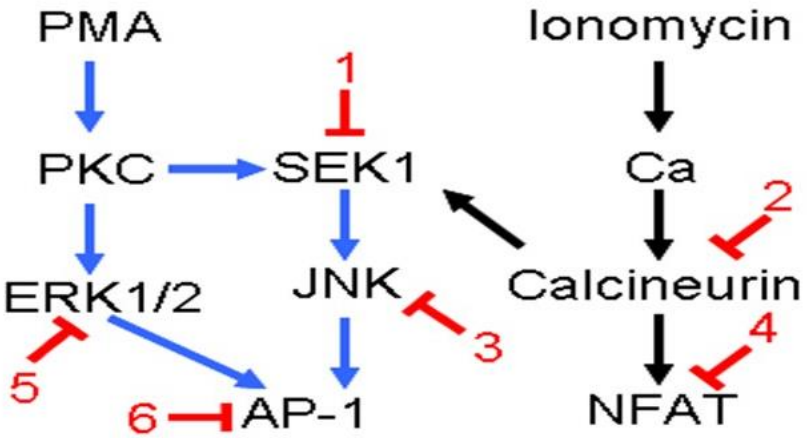

Fig. 13 PMA- and Ionomycin-triggered signaling pathways. Inhibitors are indicated: (1) PD98059, (2)CsA, (3) SP600125, (4) Cayman 13855, (5)PD98059, and (6) SR11302.

As MFI values in **Figure 14**. indicate, the SLFN11 promoter expression was stronger at 48 hrs post-stimulation although the pattern of response was similar to that of 24 hrs. Surprisingly

PMA was a stronger stimulus for this promoter than type IFNs. PMA augmented by 1.4 fold the promoter activity and this effect did not increase in combination with ionomycin that alone did not have effect on the promoter activity. In contrast, IFN β but not α stimulated the promoter activity but in a lower magnitude (1.2 folds). At 48 hrs PMA and IFN β also increased the activity of the promoter by 1.6 and 1.2 folds respectively (**Fig. 14**). These findings demonstrated that the SLFN11 promoter analyzed here responds to PMA stimulation, as the endogenous promoter, but not to ionomycin or type I IFN indicating that important regulatory elements of the endogenous promoter are missing in the 1.3 kbs sequence.

Next we characterized the transcription factors implicated in the PMA stimulation of this promoter. PMA activates PKC that could promote SLFN11 upregulation via AP-1(**Fig. 13**) since a role of Nf-Kβ is excluded, as discussed above.

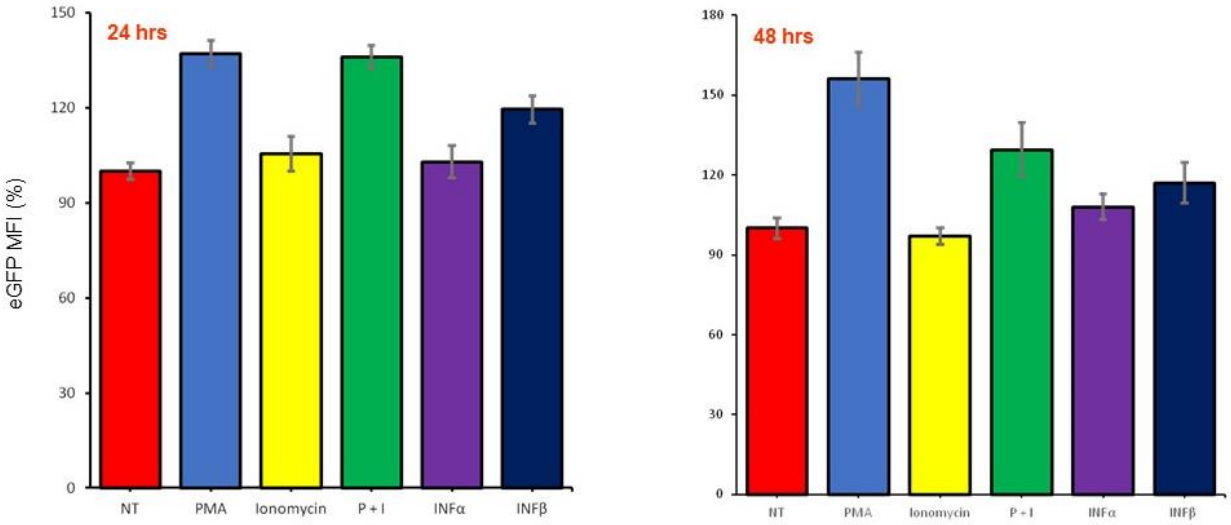

Fig. 14 Pharmacological regulation of the SLFN11 promoter. % of eGFP mean fluorescence intensity (MFI) of HEK293 cells transiently expressing a reporter containing the SLFN11 promoter driving the expression of eGFP and treated with different compounds is represented. Non-treated (NT) control cells values were considered 100%. P+I is PMA plus ionomycin.

In turn, AP-1 activation could proceed via PKC/SEK1/JNK or PKC/ERK1/2. Using pharmacological interference, we evaluated the role of AP-1 in the ability of PMA to activate the SLFN11 promoter in HEK293 cells. As described above, these cells were transfected with the

SLFN11 reporter promoter plasmid and the next day were equally distributed among different treatments and eGFP MFI were determined 48 htrs post-stimulation. The treatments evaluated included PMA, ionomicin, PMA plus ionomicin and IFNα and β at the same doses indicated above alone or in combination with different inhibitors such as PMA (20nM) plus the AP-1 inhibitor SR11302(100 μM), ionomycin (5nM) plus NFAT inhibitor (1.187mM), ionomycin plus Cyclosporin A (.1μM), PMA (20nM) plus ionomycin (5nM) plus SR11303 (100 μM), IFNα (5,000 U/ml) plus JAK (tofacitinib 100nM), INFβ (5,000 U/ml) plus JAK (tofacitinib 100nM). Results from this experiment corroborated our findings in **Figure 15**, although here IFNβ failed to activate the promoter. Combination of ionomicin with PMA reached the same activation levels than PMA alone as previously we found. Importantly PMA consistently enhanced the promoter activity, increasing eGFP MFI levels by two-fold. Because in this experiment only PMA was able to significantly activate the reporter promoter, we show in **Figure 15** only the data of the PMA with and without the AP-1 inhibitor SR11302. Data in **Figure 15** indicate that PMA increased by two-fold the promoter activity and that effect that was partially reduced by SR11302 by 20%, suggesting that PMA activates the SLFN11 promoter at least partially via AP-1[47,48].

In further support that the studied SFN11 promoter likely respond to PMA via AP-1, sequence scanning of the reporter promoter with the PROMO software[49,50] identified one AP-1 site (CAACAGTCA). The predicted site has a dissimilarity margin of only 14.2 % with the canonical AP-1 binding sequence (TGAGTCA).(All of these results where done in triplicate during the experiment).

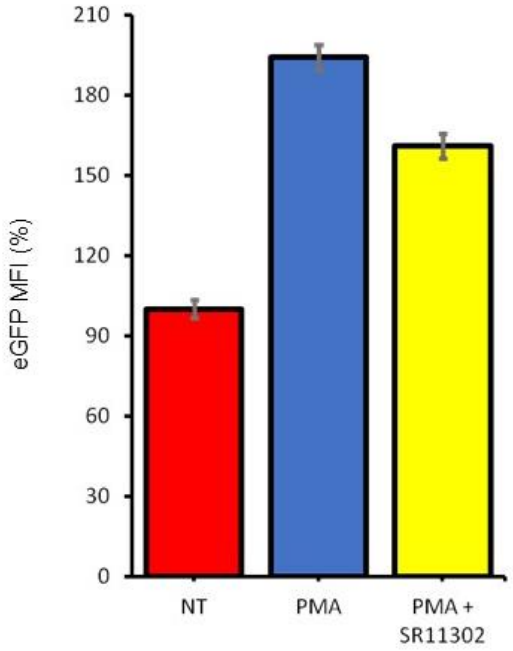

Fig. 15 Role of the PMA/AP-1 signaling pathway in the regulation of the activity of the SLF11 promoter. % of eGFP mean fluorescence intensity (MFI) of cells transiently expressing a reporter containing the SLFN11 promoter driving the expression of eGFP treated with different compounds is represented. Values found in non-treated (NT) control cells were considered 100%.

DISCUSSION

Our results illustrate the complexity of the regulation of the expression of SLFN11. As previously reported[2] SLFN11 is regulated by type I IFN signaling in virus infected cells. However, HIV-1 that potently blocks the interferon system[51] still induces SLFN11 expression. The mechanism employed by HIV-1 is unknown. Nevertheless, exploration of type I IFN-independent mechanisms indicate that stimuli that activate PKC or calcium-signaling stimulate SLFN11 expression in human CD4+ T lymphocytes (primary cells) and in human cell lines of different histological origin.

Our data also highlight that regulatory regions in the SLFN11 promoter remain unidentified. The reported 1.3 kb promoter (chr17 33,700,532 - 33,700,847) lacks important type I IFN-dependent and -independent regulatory regions. Yet, characterization of this promoter indicates that the transcription factor AP-1, at least partially, regulates promoter activity in response to PMA/PKC signaling.

It is important to identify the full-length promoter to be able to characterize in details the regulatory elements implicated in the type I IFN-dependent and -independent mechanisms. In collaboration with Dr. Jarrod Johnson, University of Utah, he has searched for additional SLFN11 transcriptional regulatory regions using assay for transposase-accessible chromatin (ATAC-seq) of HIV-1 infected primary human monocyte-derived dendritic cells. In this analysis he identified a 10.6 kb region (chr17 35,377,555 - 35,366,945) flanking the SLFN11 transcription start site that contains four regions of HIV-1-induced open chromatin. These changes in chromatin accessibility correspond with an innate immune response and induction of ISGs such as SLFN11[46]. The 10.6 kb genomic DNA is predicted to harbor binding sites of transcription factors regulated by PKC-, Ca- and IFN-I-stimulated pathways[51]. Although two of these regions partially overlap with the 1.3

kb promoter, we hypothesize that other sites are essential for full regulatory control of SLFN11 expression in cells.

It has been reported that SLFN11 tRNase and anti-HIV-1 activity is regulated by phosphorylation[51] and SLFN11 although necessary is not sufficient for the anti-viral activity[35]. Therefore, the diversity of signaling pathways that we have found in this work to regulate SLFN11 expression could impact the repertoire of post-translational modifications and SLFN11 interactors, modulating the functions of this protein. Investigation of these aspects of SLFN11 regulation and function deserve attention.

In summary, our data demonstrated the complexity of the SLFN11 regulation and identify relevant gaps in our knowledge of this relevant innate immune protein.

CONCLUSION

In sum, this research indicates that viruses can upregulate SLFN11 via type I IFN-dependent and –independent mechanisms. Characterization of the later highlights the relevance of PMA/AP-1 and calcium-driven signaling pathways in the regulation of SLFN11 expression. Our results also revealed that the SLFN11 promoter remains partially identified since relevant type I IFN-dependent as –independent regulatory regions are not present in the reported 1.3 kbs promoter.

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VITA

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