Expression levels of the innate response gene RIG-I and its regulators RNF125 and TRIM25 in HIV-1 infected adult and pediatric individuals

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Objective: TLRs (toll-like receptors) and RLRs (RIG-I-like receptors) mediate innate immune responses by detecting microorganism invasion. RIG-I activation results in the production of interferon (IFN) type 1 and IFN responsive genes (ISGs). Since the ubiquitin ligases RNF125 and TRIM25 are involved in regulating RIG-I function, our aim was to assess whether the levels of these three genes vary between healthy and HIV-infected individuals and if these levels are related to disease progression.

Design: Gene expression analysis for RIG-I, RNF125 and TRIM25 were performed for HIV infected adults and children?s PBMCs.

Methods: RT-qPCRs were performed in order to quantify the expression levels of RIG-I, RNF125 and TRIM 25 from control or HIV infected individuals isolated PBMCs.

Results: Controls express higher levels of the three genes when compared to HIV-infected patients. These expressions are clearly distinct between healthy and progressors, and are reproduced in adults and children. In controls, RNF125 is the highest expressed gene while in progressors, RIG-I is either the highest expressed gene or is expressed similarly to RNF125 and TRIM25.

Conclusions: A pattern of expression of RIG-I, RNF125 and TRIM25 genes in HIV patients is evident. The high expression of RNF125 in healthy individuals reflects the importance of keeping RIG-I function off, inhibiting unnecessary IFN production. Consistent with this assumption, RNF125 levels are lower in HIV patients and importantly, the RNF125/RIG-I ratio is lower in patients that progress to AIDS. Our results might help to predict disease progression and unveil the role of poorly characterized host genes during HIV infection.

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Introduction

Over 34 million people are infected by HIV worldwide [1] and resistant strains have emerged since the development of the HAART therapy [2]. Recent focus on host proteins, that interfere with infection outcome, lead to the discovery of host restriction factors [3–7] and viral sensors (TLR and RLR) [8–11]. Recently, it has been shown that genomic HIV RNA can be detected by RIG-I [9], a RNA helicase. Upon RNA recognition, the CARD domains present in this protein, interact with similar ones in MAVS at the mitochondria [12] activating the transcription of IFN via NF-κB and IRF3. RIG-I CARD domains undergo post-translational modifications [13–16], including ubiquitination [17], that influence its capacity to regulate IFN production. TRIM25 and RNF125 are ubiquitin ligases that can mediate the latter process [18,19]. TRIM25 belongs to the tripartite motif family and its function activates RIG-I, hence increasing IFN production [19,20]. Several TRIM proteins participate in HIV infection [21], TRIM5α interferes with viral uncoating [22] and is differentially expressed in HIV^+ patients as is TRIM22 [23–25].

RNF125 targets RIG-I for destruction in the proteasome [18], is involved in T-cell activation and inhibits HIV transcription [26]. TRIM25 and RNF125 genes are regulated by IFN [18,23] providing regulatory mechanisms for the production of this cytokine, whose fine balance is essential to achieve a protective rather than an immunopathogenic effect in HIV^+ patients [27,28].

Data suggests that high levels of expression of ISGs are related to viral replication inhibition *in vitro*; however, *in vivo* data are scarce and controversial [25]. Higher levels of mRNA for RIG-I and RNF125 were found in hepatic tissues from HCV^+ patients [29]. However, the simultaneous analysis of RIG-I expression levels and those of its positive (TRIM25) and negative (RNF125) regulators have not been investigated in an HIV setting. This information could be useful to predict progression to AIDS. Here, we performed qPCR analysis of RIG-I, RNF125 and TRIM25 in HIV^+ adults and children. Our results suggest that the levels of these 3 genes vary according to disease progression.

Methods

Ethics statement

Subjects came from the University Hospitals at Federal Universities of Rio Grande (FURG) and Rio de Janeiro (UNIRIO)-Brazil. Children were from the Pediatrics Institute -Federal University of Rio de Janeiro (UFRJ). This study was approved by Institutional Review Boards of the 3 institutions.

Study subjects

Adults- 34; 14 HIV^- controls and 20 HIV^+ patients from which 10 were non-progressors (NP) and 10 progressors (P) as defined elsewhere [30]. Children?s group had 8 HIV^- controls and 12 HIV^+ children – (4 NP and 8 P as defined in the literature [32]) and samples were retrieved from a previous study [31].

Sample preparation and qPCR

RNA was extracted by TRizol (Invitrogen) from Ficoll-paque isolated PBMCs and cDNA was synthesized using SuperScript II (Promega) and random primers (Invitrogen) following manufacturer?s instructions. Taqman primers, probes and equipment (ABI 7500) were from Applied Biosystems. Results are shown as the ratio: target gene/GAPDH expressions ($2^{-\Delta Ct}$). Mann-Whitney *U*-tests and Spearman's *rho* test were used for statistical data and correlations analysis, respectively.

"in vitro" IFN treatment, HIV infection and western blots

Jurkat cells were treated for different times with IFNα (100ng/ml) or IFNγ (50ng/ml)-(data shown as $2^{-\Delta\Delta Ct}$). For HIV infections, 10^6 PBMCs were spinoculated at 1200 g for 2 hours with 50ng of NL4.3-Luc (normalized by p24 ELISA-Zeptometrix Kit-Retrotek). Frozen PBMCs were used to perform the western blots. RIG-I and α-tubulin antibodies were from Abcam.

Results

Subjects' clinical data

Adult patients were followed up for at least 3 years with quarterly quantification of HIV VL and $CD4^+$ T-cells. Ages, $CD4^+$ T cell counts and HIV VL averaged 40.4 years ± 10.7; 582.1/mm^3 ± 343.53 and 4.33 log ± 1.31, respectively. For the children cohort, ages, $CD4^+$ T-cell counts and VL averaged 7.16 yrs ± 4.59; 19.75% ± 9.8 and 4.09 log ± 1.22, respectively (Supplementary Table 1).

Expression of RIG-I, RNF125 and TRIM25

The genes studied here are involved in antiviral signaling, they respond to IFN and their proteins products directly interact, so we assessed their expression levels in HIV^- and

HIV^+ subjects to verify if these reflected HIV infection status and could be linked to disease progression.

HIV^- controls expressed higher levels of the 3 genes and in these subjects, RNF125 was as the highest expressed gene (78% of the adults and 75% of the children), reaching twice the levels of TRIM25 in more than 62% of these (Fig. 1a–e). This expression was 4 and 2.5 times higher in HIV^- compared to HIV^+ adults and children, respectively. In both cohorts, P and NP expressed similar levels of RNF125, indicating that RNF125 expression does not reflect disease progression (Supplementary Figs 1 and 2). In contrast, RIG-I was the highest expressed gene in progressor adults (70%) and children (50%) (Supplementary Figs 1 and 2). In adult progressors the ratio RNF125/RIG-I is significantly lower than this ratio in HIV^- controls (Fig. 1f). This pattern of higher expression of RIG-I in HIV^- controls compared to HIV^+ subjects was confirmed at protein levels for some of the samples which were still available (Fig. 2a and b).

In order to elucidate if the expression patterns observed could be seen at the beginning of the infection, *in vitro* HIV infections using PBMCs isolated from 2 HIV^- blood donors were performed, followed by qPCRs. As seen in Fig. 2c and d, RNF125 and RIG-I doubled in the first 48hrs, however no major increases were detected for TRIM25 and the two other ISGs APOBE3G and 3F after HIV exposure.

The results suggest that the genes for RIG-I?s regulators and RIG-I itself are expressed differently in progressors compared to HIV^- subjects, following a specific pattern which is independent of age, mode of transmission or ART (samples were collected at a time when only 2 of the 19 adults (F7 and F12) were subjected to therapy). However, this pattern is only observed in an established chronic disease, suggesting the participation of other players of the immune system. To summarize, in controls, expression of RNF125 > RIG-I = TRIM25; in progressors RIG-I > RNF125 = TRIM25.

Although a negative correlation exists between the levels of RIG-I and $CD4^+$ T-cell counts, no significant correlation was found for any of the gene?s expressions alone and HIV VL. These results show that RIG-I is not the sole determinant of viremia (Supplementary Table 2).

Levels of RIG-I and TRIM25 were correlated, but this was not found for RIG-I and RNF125. Since the 3 genes are regulated by IFN, their expressions should all be correlated, unless they respond differently to this cytokine. To clarify this issue, Jurkat cells were treated with IFNα or IFNγ and q-PCR were performed. RIG-I

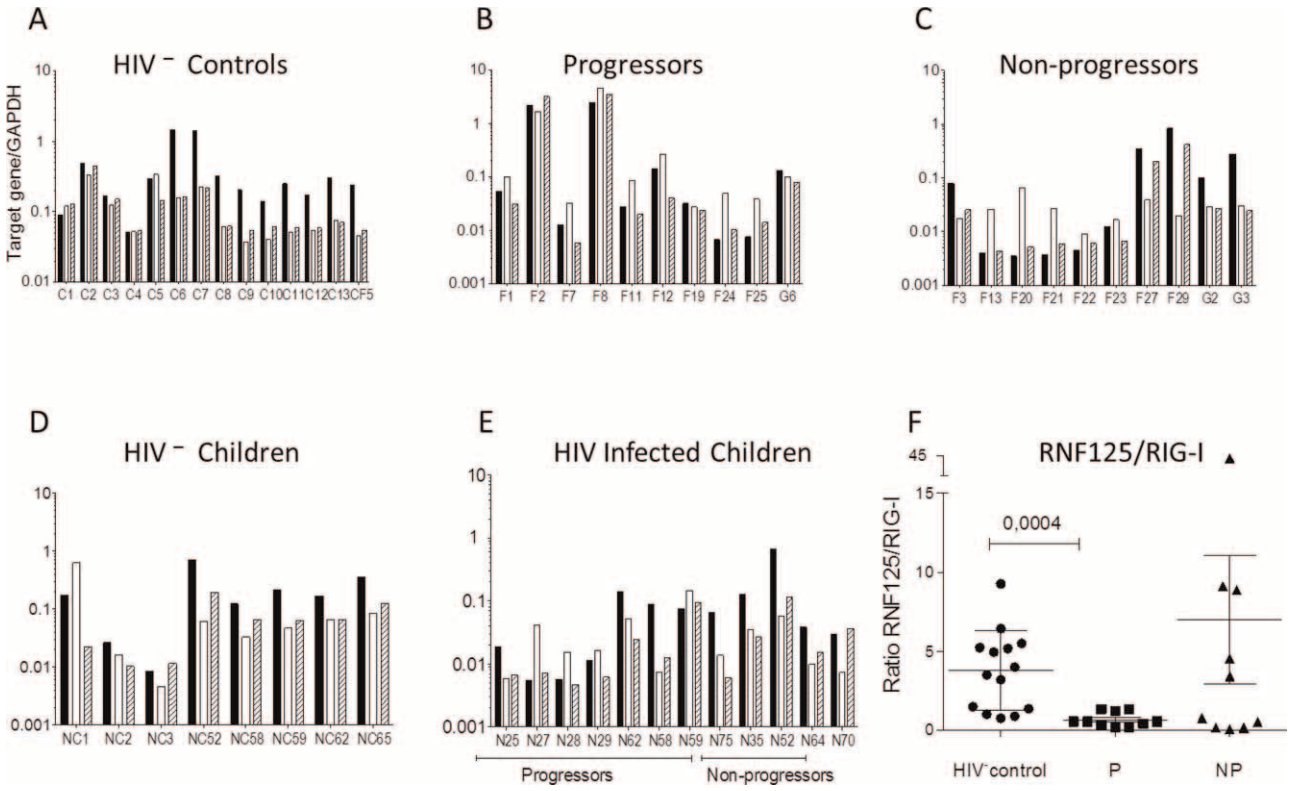

Fig. 1. Pattern of expression for RNF125, RIG-I and TRIM25 for each subject analyzed in the study. Black bars- RNF125 mRNA levels; White bars- RIG-I mRNA levels and Hatched bars- TRIM25 mRNA levels. (a, d) HIV^- controls; (b, c and e) HIV^+ subjects. P-progressors; NP, Non-progressors. All gene?s expression levels relative to GAPDH. Patient ID indicated in the X axis; (f) Ratio RNF125/RIG-I from the individuals shown in A to E. Mann Whitney *U* test was performed, $p = 0.004$.

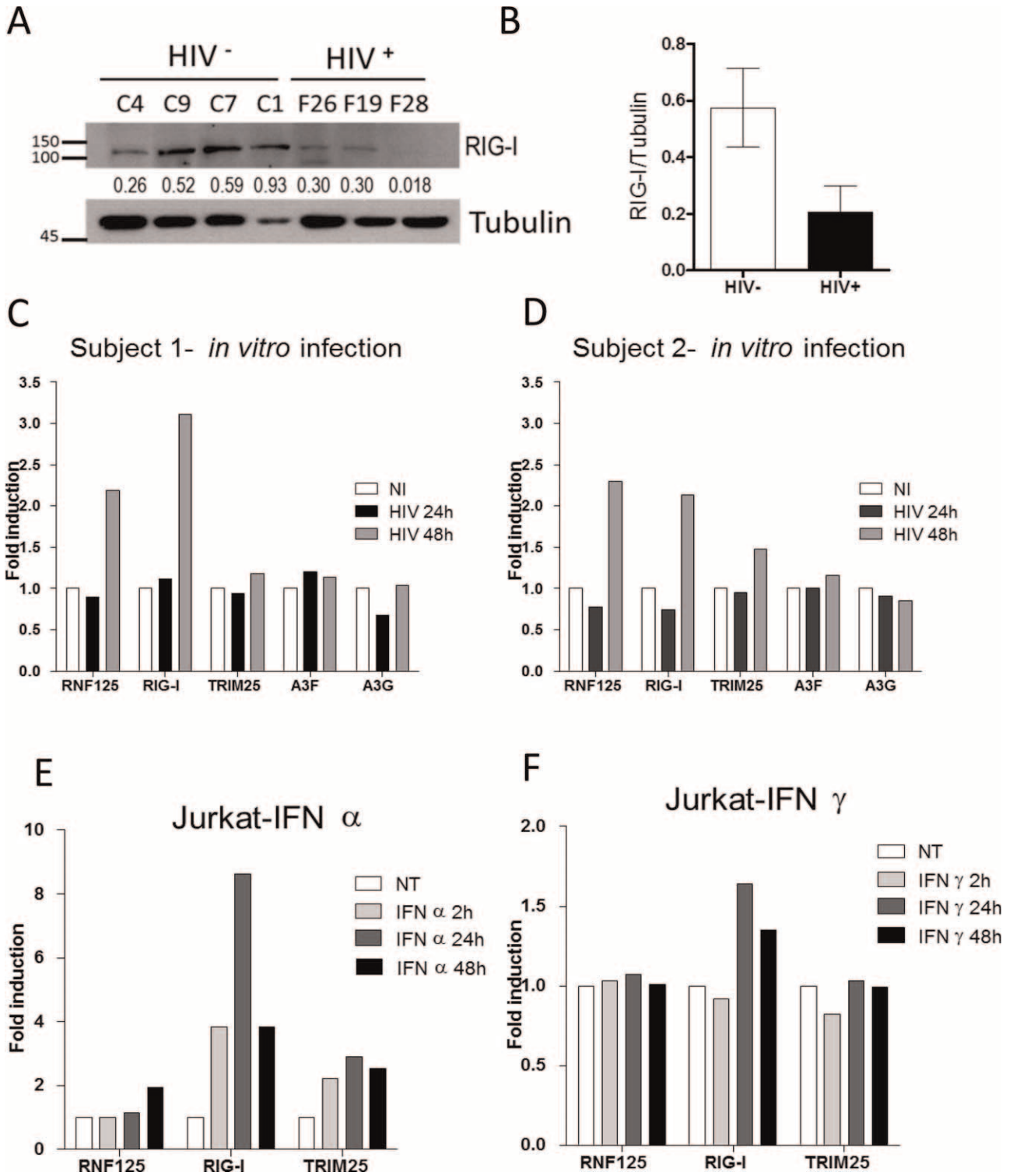

Fig. 2. RIG-I protein levels and Effect of early infection and IFN treatment on gene expression. (a)-Top panel- Western blot for RIG-I; bottom pannel- Western blot for Tubulin. (b) Densitometry results obtained from A. Numbers under each blot correspond to the ratio of RIG-I over tubulin, measured by band densitometry (n = 1). Patients IDs on top of each lane; samples from patients F26 and F28 were only used to perform the Western blots and do not appear in other figures or tables. Densitometry analyses were done using Image J. (c and d)-PBMCs from 2 donors were *in vitro* infected with NL4.3-Luc. Samples were processed 24 and 48 hrs post infection and qPCRs were performed (n = 1). (e and f)- Expressions of RNF125, RIG-I and TRIM25 from Jurkat cells treated for the indicated times with 100ng/μl of IFNα or 50ng/μl of IFNγ (n = 2).

and TRIM25 expressions were detected 2hrs after IFNα treatment, while that of RNF125 doubled only at 48hrs (Fig. 2e). RIG-I?s response to IFNα was more intense (8 fold increase after 24 hrs), while TRIM25 mRNA levels triplicated at this time point. Hence, RIG-I and TRIM25 genes are more sensitive to IFNα *in vitro* than RNF125 gene. Of these 3 ISGs, only RIG-I responded to IFNγ (Fig. 2f).

Discussion

Our hypothesis was that innate immunity genes levels would reflect HIV infection and disease progression. Hence levels of RIG-I (a viral sensor) and two of its regulators, RNF125 and TRIM25 (ubiquitin ligases with opposing functions) were quantified by qPCR. Our study is the first to demonstrate the potential relevance of RIG-I and the ubiquitin ligases RNF125 and TRIM25 together in an HIV scenario using HIV^+ adults and children.

We concluded that none of the gene levels *per se* can be used to predict disease progression. However, when the expressions of the 3 are analyzed together, a disease progression-dependent pattern was detected. RNF125 was the highest expressed gene in HIV^- controls, while RIG-I was the highest expressed gene in progressors and the ratio RNF125/RIG-I was higher in the former group (Fig. 1f). We speculate that in a chronically infected individual, with detectable viral load, the presence of the virus maintains the innate response activated via RIG-I, resulting in IFN production, which on its turn, maintains RIG-I and TRIM25 transcription ongoing, explaining the positive correlation found for RIG-I and TRIM25 mRNAs. High levels of RIG-I would keep IFN production on, resulting in $CD4^+$ T-cells apoptosis [27]. In fact, a negative correlation was found between $CD4^+$ counts and RIG-I levels, but none was found between RIG-I?s expression and VL, suggesting the participation of other host restriction factors in the control of viremia. This is also corroborated by the suggested role of RNF125 in inhibition of HIV transcription [26].

Although RNF125 has been described to respond to type I IFNs [18], in our hands this induction was approximately 4 times smaller than that observed for RIG-I, leading us to conclude that RNF125 expression is regulated by factors other than IFN. This conclusion is also corroborated by the higher levels of RNF125 found in controls, whose levels of circulating IFN should not be high in the absence of infection. We believe that RNF125 protein has a major role in maintaining the innate immunity signalling off (by targeting RIG-I for destruction). In fact, when RNF125 is exogenously expressed, endogenous levels of RIG-I decrease [18]. RNF125 also has a short half-life [33] so cells would have to continually transcribe and translate its gene to maintain its functional cellular levels. The identification and characterization of the RNF125's promoter would help to elucidate its regulation.

Although it would have been interesting to sort the different cell types from the patients PBMCs to evaluate how each one contributes to the expression of genes studied here, the fact that Jurkat cells (immortalized $CD4^+$T-cell) analysis lead to similar expression pattern found for PBMCs from controls suggests that T-lymphocytes are the main contributors to the results found here (data not shown). This is also supported by the expression data published in BioGPS site, where the expression of the RNF125 and RIG-I genes were higher in $CD8^+$ and $CD4^+$ cells. Two features detected during HIV infection are the depletion of the $CD4^+$ T cell population and the expansion of the $CD8^+$ T cell one. Since both $CD8^+$ and $CD4^+$ cells express the highest levels of both RIG-I and RNF125, the sorting of these cell types from patients might not have been clarifying here [34,35].

In a HCV^+ scenario, sustained non-virological responders (NVR), express more RIG-I and less RNF125 (lower RNF125/RIG-I ratio) compared to sustained virological responders (SVR) [29,36]. Similarly, we found that in an HIV scenario, progressors have a lower RNF125/RIG-I ratio when compared to non-progressors and controls (Fig. 1f). Hence these ratios can be useful both to predict both NVR and progression in a HCV and HIV scenarios, respectively.

Although at present we are unable to explain the higher expression of the 3 genes in PBMCs from controls, this has been also found for IRF1 in a HCV scenario [36]. Besides TLR3, TLR7, RIG-I and IFN-α mRNA levels are significantly downregulated in patients with chronic HCV infection when compared with healthy controls [37]. Also, there has been controversy concerning ISGs expression during HIV infection [38–40]. The different virus biology and escape routes might account for these differences [41–45]. Nevertheless, our results support the findings of others and start to unveil the important role of yet uncharacterized players in HIV infections.

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Authors contributions: Authors Britto AMA, Amoedo N and Afonso AO contributed to the purifications of PBMCs, RNA, cDNA and the performance and analysis of the qRT-PCR reactions. Pezzuto P performed the IFN

treatment of Jurkat cells and prepared their cDNAs. Martinez AMB and Silveira J organized the clinical data from adults enrolled in the Dr. Miguel Riet Corrêa Junior University Hospital-Federal University of Rio Grande (FURG). Sion FS and Machado ES were responsible for the clinical data collection from the adult patients from the Gaffrée and Guinle University Hospital-Federal University of the Rio de Janeiro State (UNIRIO) and the pediatric individuals from the Martagão Gesteira Institute of Pediatrics-Federal University of Rio de Janeiro (UFRJ). Giannini ALM and Soares MA and were responsible for the design of the experiments and preparing the manuscript.

Conflicts of interest

None declared.

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