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Interferon gamma, TGF-β1 and RANTES expression in upper airway samples from SARS-CoV-2 infected patients

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ABSTRACT

Upper respiratory tract is the primary site of SARS-CoV-2 replication. Releasing of pro and anti-inflammatory mediators plays an important role in the immunopathogenesis of Coronavirus Disease 2019 (COVID-19). The aim of this study was to evaluate the early inflammatory response in upper airway by measuring of IFN-γ, TGF-β1 and RANTES at mRNA level. Forty five SARS-CoV-2 infected patients were enrolled, whose were divided in two groups: asymptomatic and symptomatic. Twenty healthy persons, SARS-CoV-2 negative were included as controls. Higher IFN-γ expression was detected in SARS-CoV-2 infected patients in comparison with controls ($p = 0.0393$). IFN-γ expression was increased in symptomatic patients ($p = 0.0405$). TGF-β1 and RANTES expressions were lower in SARS-CoV-2 infected patients than controls ($p < 0.0001$; $p = 0.0011$, respectively). A significant correlation between IFN-γ and TGF-β1 was observed in SARS-CoV-2 asymptomatic patients ($r = +0.61$, $p = 0.0014$). The findings suggest that imbalance between IFN-γ and TGF-β1 expression could be an impact in clinical expression of SARS-CoV-2 infection.

1. Introduction

Since March 2020, World Health Organization declared the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, causal agent of the Coronavirus Disease 2019 (COVID-19), as pandemic. Clinical spectrum of COVID-19 ranges from asymptomatic to symptomatic cases; and severe complications are more frequent in older age patients with comorbidities associated (diabetes mellitus, cardiovascular, obesity and renal diseases). Cytokine storm with an uncontrolled inflammatory response contribute to lung injury and multiple organ failure in the course of COVID-19 [1].

Cytokines play an important role in the immunopathogenesis of COVID-19, and high levels of pro-inflammatory mediators were found in sera and bronchial-alveolar washing of SARS-CoV-2 infected patients. Authors observed that up-regulation of interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α) causes damage of alveoli and microvasculature in lung, mediate by vascular leakage and edema [2].

Initial site of virus replication is the epithelium of superior respiratory tract, it means that innate immune response in this site is critical controlling the viral spreading and symptoms in early stage of COVID-19.

Recruitment and activation of inflammatory cells, secreting pro- and anti-inflammatory mediators, is indispensable to virus clearance. However, the pathogenesis appears with viral persistence and the imbalance of inflammatory response. Respiratory mucosa is compound by epithelial cells, globet cells, dendritic cells, invariant natural killer T (iNKT) cells and γδ T cells. These cells constitute the first line of defense against pathogens. Therefore, nasopharyngeal swabs, sample minimally invasive and necessary for SARS-CoV-2 diagnostic, could be used to evaluate immunological parameters at initial phase of infection.

Natural history of SARS-CoV-2 infection arises when the virus is binding to its receptor angiotensin converting enzyme 2 (ACE2) located in the surface of target cells. Cytosolic receptors such as toll-like receptors (TLR) with capacity to recognize molecular patterns of virus; triggers the production of pro-inflammatory cytokines such as interferons (IFN). Type I (α and β) and III (λ) IFNs releasing by virus infected cells, mediate the primary antiviral response. These IFNs are binding to their receptors and promote the transcription of IFN-

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stimulated genes (ISG), establishing the immunological synapse. Type II IFN gamma (IFN-γ) and regulated on activation normal T-cell expressed and secreted (RANTES) chemokine are part of ISG induced. IFN-γ mainly produced and secreted by iNKT cell and γδ T cell, has immunomodulatory activity and reinforces the antiviral estate [4]. High level of this cytokine has been associated with increased severity of human respiratory virus infection [3].

Studies in vitro suggested that transformer growth factor beta (TGF-β) act as a modulator of IFN production, in respiratory infections [4]. TGF-β is an inflammatory mediator necessary to keep the integrity and homeostasis of respiratory epithelium. TGF-β is a multifunctional and pleitropic cytokine, essential in the repairing process and suppression of immune response. TGF-β has three isoform (β1-β3), TGF-β1 is mostly implicated in the immune regulation [5]. Epithelial cells are the major source of TGF-β1, although others cellular subsets (innate lymphocyte, endothelial cells, infiltrating and effector cells) can produce it in respiratory mucosa. An incremented concentration of TGF- β1 leads an enhance Rinhovirus replication, due to suppression of IFN activity [6].

Chemokines secreted by respiratory virus-infected cells in upper airway, stimulate the recruitment of inflammatory cells, including neutrophils, eosinophils, NK cells, and macrophages from blood into infected tissues [7]. RANTES is a powerful chemotactic with activating properties for basophils, eosinophil and NK cells. RANTES has been used as surrogate marker for IFN activity, to demonstrate the integrity of downstream signal of IFN releasing by infected cells [8]. Several chemokine (MCP1, MIP1α, MIP1β, IP-10) are produced during SARS-CoV-2 infection; and different cells via chemokine/chemokine receptor interaction are recruited and activated during infection [9].

Symptomatology of SARS-CoV-2 infection could be determined by virus (mutations, viral load, and virus linage) and host (immune system, age, gender, nutritional status) factors [10]. Also, epigenetic modifications observed in autoimmune disease like systemic lupus erythematous increase viral entry to target cells, replication, viremia and loss of regulation of immune response in COVID-19. These events appear as result of deregulation in methylation of ACE2 gene and ISG [11].

Host-virus interaction and control capacity of immune system determinate the clinical presentation of COVID-19. The aim of this study was evaluate inflammatory response using the expression of immune mediators with antiviral, immunosuppression and chemotactic functions in the primary site of SARS-CoV-2 replication, at early stage of infection. Closing of June 112,020, Cuba reported 2219 SARS-CoV-2 confirmed cases, lethality rate reached 3,78% and around 52,95% individuals were asymptomatic at the moment of diagnostic.

2. Material and methods

2.1. Samples collection

A nasopharyngeal swabs specimens (NPS) were collected in universal transport medium (Puritan® Unitranz-RT®, USA), and sent to National Reference Laboratory of Influenza Virus and Other Respiratory Virus, at Institute of Tropical Medicine Pedro Kourí (IPK). Forty five patients diagnosed with SARS-CoV-2 (18 females, mean age = 39.14 years and 27 males, mean age = 40.22 years) since March to April 2020 were included (Table 1). Infected patients were divided in asymptomatic (24) and symptomatic (21); and referred between 3 and 10 days of onset clinical symptoms of COVID-19 or contact with confirmed cases. Patients declared symptoms such as fever, dry cough, anorexia, fatigue, anosmia, pharyngodynia and dyspnea. No chronic diseases were identified in enrolled subjects. Most of infected persons were recovered of SARS-CoV-2 infection, except one symptomatic patient who died. At the moment of collecting nasopharyngeal swabs, no prior antiviral and immunomodulator medications were used in the infected patients. As control group was included 20 individuals (13 females and 7 males, mean age = 45.31 years), during scheduled healthy check-ups for health workers. Patients and controls enrolled in this study were treated anonymously and identified by code. This study was approved by the Ethics Committee for Research of the IPK.

Table 1
Demographic characteristics of the SARS-CoV-2 infected patients.

Variables	Asymptomatic (n = 24)	Symptomatic (n = 21)	Total (n = 45)
Sex			
Female (n, %)	9 (37.50)	9 (42.85)	18 (40.00)
Male (n, %)	15 (62.50)	12 (57.14)	27 (60.00)
Age (m. of y. ± SD)	40.23 ± 20.21	39.15 ± 24.72	39.70 ± 22.23

Abbreviations: m, Mean; y, Years; SD, Standard Deviation.

2.2. SARS-CoV-2 RT rt-PCR

Real time reverse transcription-PCR (RT rt-PCR) was carried out following-up the protocol recommended by Pan-American Health Organization. Starting with 140 μl of sample, total RNA was isolated using a commercial specific kit, QIAamp Viral RNA Kit (QIAGEN, Germany). A 25 μl reaction was set up containing 5 μl of RNA, 2× reaction buffer with the One-Step RT rt-PCR system (Invitrogen, Life Technologies). Thermal cycling was performed at 55 °C for 10 min. For reverse transcription, followed by 95 °C for 3 min. and then 45 cycles of 95 °C for 15 seg., 58 °C for 30 seg., with primers and probe specific for envelop gene; with 5.2 RNA copies/reaction of limit of detection (L.O.D.). RT rt-PCR for polymerase region was used as confirmatory assay with L.O.D. = 3.8 RNA copies/reaction. As quality controls were included internal, positive and negative controls in each assay. Viral titer was estimate by threshold cycle (Ct) values detected in RT rt-PCR.

2.3. Relative quantification of cytokines and chemokine

Cells pellets were collected from NPS and total RNA was extracted using RNAeasy kits (QIAGEN, Germany), according to manufacture instruction. RNA samples were treated for removing genomic DNA with gDNA Wipeout Buffer from QIAGEN. Samples with A260/280 RNA absorption ratio > 2 in QIAxpert system were used for relative mRNA quantification. RT rt-PCR for IFN-γ quantification was carried in duplicates with TaqMan Universal One-Step qRT-PCR System (Invitrogen, Life Technologies) following the protocol described by Kim et al. [12]. Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as housekeeping gene. RANTES and TGF-β1 expression were measuring according to Ziklo et al. protocols [13]; first mRNA was reverse-transcribed using radom primers and SuperScript® Reverse Transcriptase Kit (Invitrogen, Life Technologies). cDNA synthesized was use as template in PCR reaction using QuantiFastTMSYBR® Green PCR Kit (QIAGEN, Germany). Beta actin (βactin) gene was used for normalization RANTES and TGF-β1 genes expression. All PCRs were running in Rotor Gene Q software 2.3.1.49. Relative cytokine mRNA concentration was controlled with constitutive genes cycle threshold (Ct) values; and transformed to quantify using $2^{-\Delta Ct}$ method (ΔCt = Ct target gene- Ct reference gene).

2.4. Statistical analysis

It was conducted a descriptive study; SARS-CoV-2 viral load and levels of expression of inflammatory mediators were expressed as median and interquartile range (m, IQR). Differences and similarity between the symptomatic and asymptomatic SARS-CoV-2 cases with control group was performed using nonparametrict tests, Mann-Whitney *U* test and Kruskal-Wallis with Dunn's multiple comparison test, to compare two or three variables respectively. Correlation between expression levels of IFN-γ and TGF-β1 was analyzed using

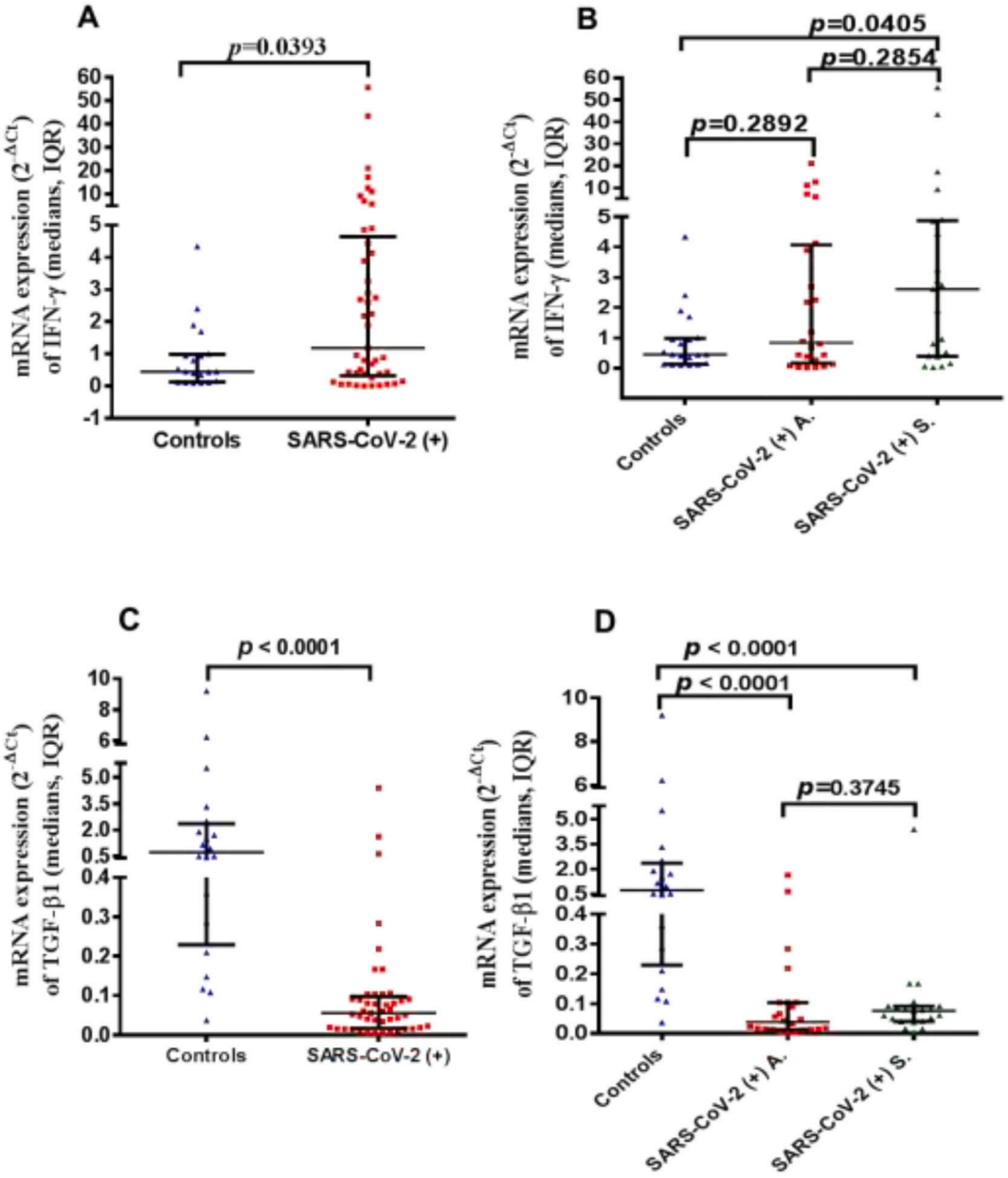

Fig. 1. Differential transcription of inflammatory mediators in cells recovered from NPS. Comparisons were performed between control group (n = 20) versus SARS-CoV-2 infected persons (n = 45) and between asymptomatic (SARS-CoV-2 (+) A, n = 24) and symptomatic (SARS-CoV-2 (+) S, n = 21) patients. A, B: mRNA expression of IFN-γ; C, D: mRNA expression of TGF-β1 and E, F: mRNA expression of RANTES. To compare two variables was used Mann-Whitney U test. Multiple comparisons were performed with Kruskal-Wallis test adjusting with Dunn's test. Differences p value < 0.05 were considered significant.

Spearman's rank correlation coefficient *rho* (r). In the graphics dots indicate individual data. Middle bars on scatters plot means medians and the ends show interquartile ranges. All data were analyzed with Prism 7 (GraphPad, La Jolla, CA) and p value < 0.05 was considered as statistically significant difference.

3. Results

3.1. SARS-CoV-2 viral load and expression of inflammatory mediators

Taking into account that Ct value has a correlation with the amount of RNA present in the samples, it was found that medians and IQR of SARS-CoV-2 viral titer was similar in asymptomatic (33.00, 29.00–37.00) and symptomatic (30, 27.00–37.00) cases; and comparison between groups did not show difference (p = 0.4373).

IFN-γ was significantly more expressed in SARS-CoV-2 infected individuals compared with control group (1.18, 0.32–4.64 vs. 0.44, 0.13–0.98; p = 0.0393) (Fig. 1A). Its expression was increased and significant in symptomatic SARS-CoV-2 patients; in contrast with healthy persons (2.61, 0.39–4.87; p = 0.0405). But, no different was found between symptomatic and asymptomatic (0.84, 0.16–4.07; p = 0.2892) cases in FN-γ expression (Fig. 1B).

Regarding to TGF-β1, the results showed that its expression was significantly lower in SARS-CoV-2 positive patients than control group (0.05, 0.01–0.09 vs. 0.73, 0.22–2.35; p < 0.0001) (Fig. 1C). According to symptoms, TGF-β1 mRNA concentration did not differ significantly between groups, although the expression decreased in asymptomatic persons in relation with symptomatic cases (0.03, 0.01–0.10 vs. 0.07, 0.04–0.09) (Fig. 1D).

RANTES is a well-established, monocytes, NK and T cell

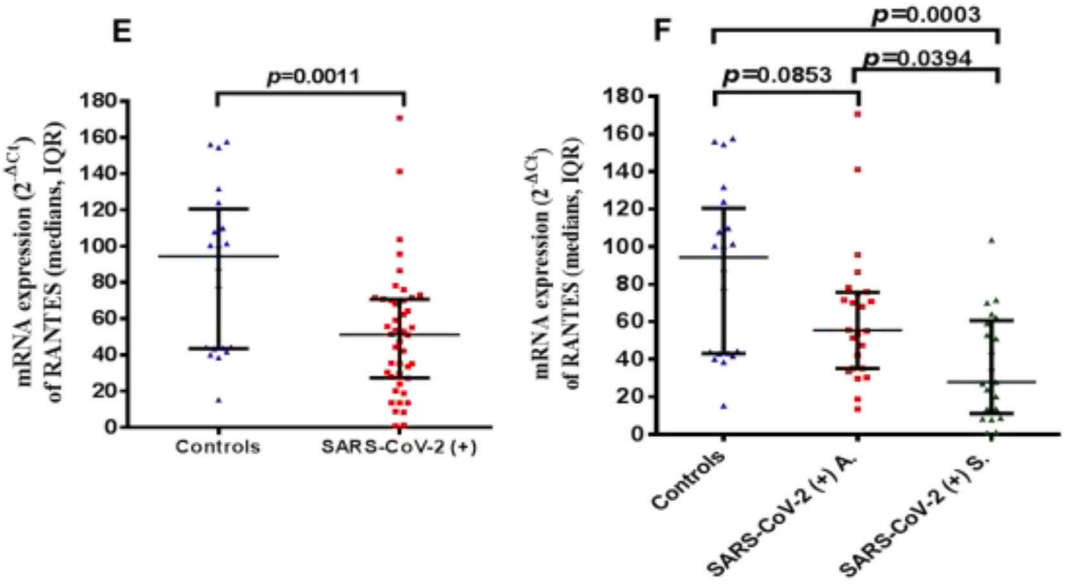

Fig. 1. (*continued*)

chemoattractant and important link between innate and adaptive immune response. In comparison with control group, RANTES decreased significantly in SARS-CoV-2 infected persons (100.50, 43.38–124.00 vs. 50.91, 27.11–70.11; $p = 0.0011$) (Fig. 1E). Similarly, the difference between healthy persons with symptomatic cases was relevant (31.73, 13.50–61.42; $p = 0.0003$). However, median and IQR (55.15, 35.16–75.94; $p = 0.0853$) did not change considerably between asymptomatic patients with control individuals (Fig. 1F). The lowest RANTES expression was detected in symptomatic SARS-CoV-2 patients.

3.2. Correlation between IFN-γ and TGF-β1 in asymptomatic and symptomatic SARS-CoV-2 infected individuals

Taking into account, the balance between inflammatory and anti-inflammatory cytokines could be related with clinical expression and pathogenesis of SARS-CoV-2 infection, we analyzed the relationship between IFN-γ and TGF-β1. It was found a positive correlation between both markers in healthy persons ($r = +0.2723$) and asymptomatic SARS-CoV-2 infected persons ($r = +0.6141$) (Fig. 2A-B). Interestingly, the correlation between IFN-γ and TGF-β1 in asymptomatic cases was statistically significant ($p = 0.0014$) (Fig. 2B). While, a negative correlation was observed in symptomatic patients ($r = -0.0390$, $p = 0.8667$) (Fig. 2C). Also, it was evaluated the balance between inflammatory mediator (IFN-γ) and anti-inflammatory (TGF-β1) mediator using the median ratio of IFN-γ:TGF-β1 by groups. These values increased notably from control group (0.44/0.73 = 0.60), passing through asymptomatic (0.84/0.03 = 28.00), up to symptomatic (2.61/0.07 = 37.28) SARS-CoV-2 individuals.

4. Discussion

It is known that most of the initial symptoms of COVID-19 are focusing in upper airway. Therefore, an equilibrium between the dose of viral exposure and efficiency of the local innate immune response; might be crucial blocking the spreading of SARS-CoV-2 from upper to lower respiratory tract, at early stage of infection [14]. Our finding showed that viral titers did not differ between asymptomatic and symptomatic SARS-CoV-2 infected persons, in concordance with the results obtained by Zou et al. [15]. On other hand, some authors detected that viral titer increase in the early stage and decrease during recovery phase of COVID-19 in NPS [16]. On critically ill COVID-19 patients viral load in serum was correlated with IL-6 levels, inflammatory mediator in respiratory failure [17]. Additionally, therapies with antivirals have demonstrated its efficacy reducing the recovery time of SARS-CoV-2 infected patients. In spite of viral titer has been related with early viral clearance; its association with symptoms and severity of COVID-19 must still to be elucidated.

Role of host cytokines determining the differences between symptomatic and asymptomatic individuals was demonstrated in an experimental influenza virus challenge [18]. As show our results, SARS-CoV-2 infection induced high IFN-γ expression in swabbed cells from upper airway; its expression was higher in symptomatic patients in comparison with asymptomatic individuals. IFN-γ is a pivotal cytokine in the cell mediated immunity against virus diseases and amplifies the antigen recognition. Also, the role of IFN-γ in Coronavirus infection was already documented. In the fact, it can induce a set of antiviral proteins that restricting viral uncoating, entry into the host cell and interfere with the access to cytoplasm of phagocyted virions in endosomes [19]. This cytokine has strong antiviral and immunomodulatory activity, but at the same time its action requires to be regulated. An over-production of IFN-γ conduces to excessive inflammation, contributing to COVID-19 pathogenesis [20].

It was observed that tightly balance between pro- and anti-inflammatory cytokines could determinate symptoms expression, at early stage of SARS-CoV-2 infection [21]. High expression level of TGF-β1 was found at baseline in control group, which probably contributing to keep the homeostasis and tolerance for commensal agents in respiratory mucosa. A significant decreasing in TGF-β1 expression detected in SARS-CoV-2 infected patients, could lead a reduction of its immunosuppressant effect. TGF-β1 inhibit IFN-γ expression, inducing the phosphorylation of SMAD2 and/or SMAD3 signaling proteins, which with SMAD4 are translocated to the nucleus and bind to the promoters region of IFN-γ gene, blocking its transcription [22].

TGF-β1 had the lowest expression in asymptomatic cases; contrarily we would expect that inflammatory response expressed as presence of symptomatology was higher in this group. However, expression of IFN-γ and ratio IFN-γ:TGF-β1 were lower in comparison with symptomatic patients. Also, positive correlation between IFN-γ and TGF-β1 provided evidence of immune response control could determinate the asymptomatic presentation of SARS-CoV-2 infection. Denney et al. suggested that relationship between IFN and TGF-β has an important role in the evolution of respiratory viral infection [23].

In COVID-19, dynamic expression of TGF-β cytokine should be evaluated, since the respiratory distress and lung fibrosis have been

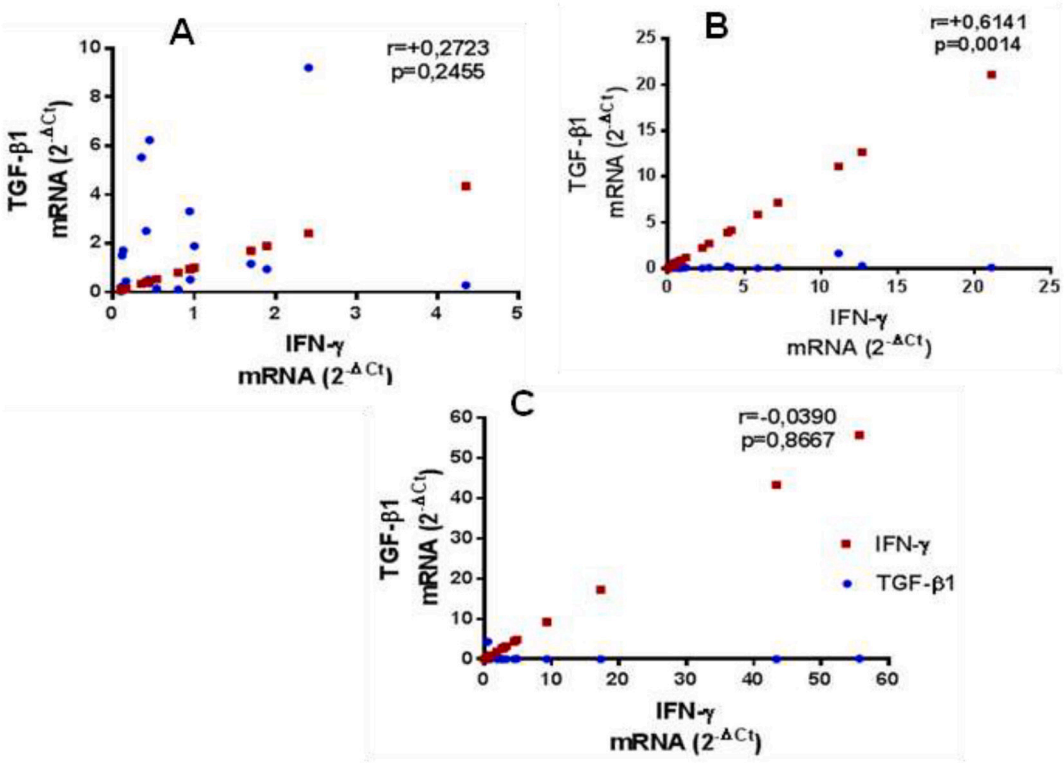

Fig. 2. Correlation between TGF-β1 and INF-γ: A, in healthy group (n = 20); B, asymptomatic SARS-CoV-2 cases (n = 24) and C, symptomatic SARS-CoV-2 patients (n = 21). Results were analyzed using nonparametric Spearman correlation; a 2-tailed p value < 0.05 was considered statistically significant.

mainly attributed to cytokine storm and exacerbated production of TGF-β [24]. It is known that TNF-α up-regulates TGF-β1, using mechanisms involving both increased its transcription and stabilizing mRNA [25]. Probably, the kinetic of TGF-β is changing with synergistic activity of cytokines and the migration of inflammatory cells into the lung. These cells could be responsible of local increasing of TGF-β, in late and severe phases of SARS-CoV-2 infection. On the other hand, the inhibitory effects of TGF-β could trigger regulatory T cells, suppressing innate and adaptive immune response.

Type I and II IFNs promote immune cell migration trough expression of several chemokines like RANTES; and different types of inflammatory cells are moving to respiratory epithelium by this immune mediator [19]. In our study, it was found a reduced expression of RANTES in SARS-CoV-2 positive cases compared to healthy persons; and symptomatic patients showed the lowest quantification. RANTES act as a chemotactic factor to induce the migration of monocytes, neutrophils, dendritic cells, NK cells and T cells from the bloodstream to the sites of infection [7].

An inhibition of the traffic and recruitment of activated immune cells and memory lymphocytes to upper airway mucosa, even with normal expression of its receptors, could be related with decreasing of RANTES concentration. In this context, some authors reported that arresting the differentiation, recruitment and activation of inflammatory cells may reduce the COVID-19 pathogenesis. Several therapeutic strategies have been used with hihg efficacy like blocking granulocyte macrophage-colony stimulating factors and IL-6 [26]. Also, the treatments with complement C3 inhibitors, point where all complement activation pathway converge, decreasing the anaphylatoxins production and improving the clinical status of COVID-19 patients [27]. Asymptomatic SARS-CoV-2 infection and atypical infection were observed in patients with long-term glucocorticoids medication; potent immunosuppressive and antiinflammatory drugs [28].

RANTES expression indicates the integrity of downstream signals in response to IFN production by infected cells. However, Kawka et al. showed that IFN-γ alone did not induce RANTES mRNA; this was reverted rapidly with a concurrent increasing of TNF-α [29]. In addition, in alveolar epithelial cells the RANTES expression depended of synergic TNF-α and IFN–γ stimulations. TNF-α induces nuclear translocation of nuclear factor κB and interferon regulatory factor 3, which bind to the RANTES promoter region and triggers its expression [30].

Pro-inflammatory cytokines increase the inflammation to control the viral replication in upper respiratory airway. RANTES mediates the influx of mononuclear cells, including T cells, which are the main source of IFN–γ. However, we could not discard that SARS-CoV-2 disrupt RANTES mRNA as evasion strategy at early stage of infection, trying to block the migration of immune cells to the sites of infection; delaying the immediate immune response. Menachery et al., described that MERS-CoV bypass immune response since gene expressions of antigen presentation were down-regulated [31]. However, RANTES kinetic is quite different in late phase of COVID-19, Paterson et al. detected an elevation of RANTES in sera collected from critical patients, and observed that inflammatory molecules (IL-6 and IFN-related genes) declined with the blocking of CCR5 using monoclonal antibody [32]. This finding indicates that RANTES amplify the inflammation disorder in severe cases of SARS-CoV-2 infection.

Innate and adaptive immune response determinates the clinical expression and prognosis of SARS-CoV-2 infection. T lymphocyte CD8+ has a pivotal role eliminating the infected cells and low count of these cells increase the fatal outcome of COVID-19. However, this effect is attenuated increasing the efficacy of its cytotoxic activity [33].

Regarding to specific humoral response, early and high antibodies titers against SARS-CoV-2 has been associated with unfavorable progression of COVID-19. While, undetectable and low anti-SARS-CoV-2 levels were observed in asymptomatic cases. Authors reported that effective control of virus replication in infected cells related to ACE2 expression could reduce the stimulation of Th2 subset, with pathogenic role in SARS CoV-2 infection. Pathogenicity of anti-SARS-CoV-2 immunoglobulin has been attributed to accumulation and deposit of

immune complex or type 3 hypersensitive. As result, complement cascade is triggered by classical pathway, amplifying and perpetuating the inflammatory disease [34].

Concerning to type of cells taken from NPS to evaluate mRNA expression, Chua et al. identified dendritic cells, macrophages, cytotoxic T cells, NKT cells, and neutrophils in NPS specimens from SARS-CoV-2 infected subjects. These authors found a high percentage of epithelial cells in comparison with inflammatory cells in healthy individuals [35]. This means that epithelial cells could be the majority in homeostasis conditions, and expression of antivirals genes in upper airway could be detected for immune cell infiltration in presence of viral infection.

In congruency with our results, differential gene expression of cytokines and chemokine genes were found in monocyte infected with SARS-CoV; TGF-β2 and RANTES showed a down-regulation 24 h post infection [36]. We thinking that TNF-α is crucial cooperating and stimulating RANTES and TGF-β1 transcription. Equally, apoptosis of infected cells constitute an additional stimulus to induce expression of TGF-β1.

One of the limitations of this study were the small numbers of studied patients and we did not know if asymptomatic cases remained in this status during the whole time of SARS-CoV-2 infection. Further studies will be necessary to correlate our finding with circulating levels of inflammatory mediators, associated with systemic symptoms of COVID-19.

This work explored the host-pathogen interaction in at initial phase of SARS-CoV-2 infection. It was demonstrated the influence of unbalance immune response in the presence or absence of COVID-19 symptoms, in patients with viral titer similar. Asymptomatic patients were not immunologically quiescent; however they had an early control of inflammatory response in the primary site of infection.

5. Conclusions

Finally, we concluded at onset SARS CoV-2 infection resident cells in the upper respiratory epithelium increased de novo expression of IFN–γ, trying to control the virus replication and spreading at initial site of infection. These cells reduced their capacity of TGF-β1 and RANTES production de novo, in response to stimulation with IFN-γ. Contrary to symptomatic patients, in asymptomatic cases TGF-β1 and RANTES expressions were sufficient to attenuate the inflammatory effect of IFN–γ, preserve and renew an optimum influx of mononuclear cells; with virucidal activity. Mononuclear cells were the main sources of cytokines that can act synergistically, to reduce the inflammatory injury of respiratory mucosa.

Also, it was demonstrated the value added of NPS for both viral diagnostic and evaluation of host immunological genes expression. We suggest that evaluation of IFN-γ:TGF-β1 axis in the primary site of viral replication could be used to predict the course and outcome of COVID-19, at early phase of SARS-CoV-2 infection.

Declarations of Competing Interest

The authors have no conflict of interest.

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