Article

Effects of Toll Like Receptor Agonists and SARS-Cov-2 Antigens on Interferon (IFN) Expression by Peripheral Blood CD3+ T Cells in COVID-19 Patients

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Abstract

Background: Signaling by toll like receptors (TLRs) initiates important immune responses against viral infection. The role of TLRs in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections is not well elucidated. Thus, we investigated the interaction of TLRs agonists and SARS-COV-2 antigens with immune cells *in vitro*. **Material & methods:** 30 coronavirus disease 2019 (COVID-19) patients (15 severe and 15 moderate) and 10 age and sex matched control (HC) were enrolled. Peripheral blood mononuclear cells (PBMCs) were isolated and activated with TLR3, 7, 8 and 9 agonists, the spike protein (SP) of SARS-CoV-2 and the Receptor Binding Domain (RBD) unit of SP. Frequencies of $CD3^+IFN\text{-}\beta^+$ T cells, and $CD3^+IFN\text{-}\gamma^+$ T cells was evaluated by flow cytometry. Interferon (IFN)-β gene expression was assessed by qRT-PCR. **Results:** The frequency of CD3+IFN-β+ T cells was higher in moderate and severe patients at baseline in comparison with HCs. Stimulation of PBMCs from moderate patients with SP and TLR8 agonist significantly upregulated the frequency of CD3+IFN-β+ T cells (P=0.0005 and 0.0024, respectively) when compared to non-stimulated (NS) samples. The greatest increase in $CD3^+IFN\text{-}\beta^+$ T cell frequency in PBMCs from severe patients was seen with TLR8 and TLR7 agonists when compared to NS (P= 0.003 and 0.0167, respectively). TLR

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stimulation did not significantly enhance the frequency of CD3+IFN-γ+ T cells generated from PBMCs from moderate and severe patients compared with unstimulated controls. However, the frequency of CD3+IFN-ɣ+ T cells in PBMCs from moderate patients was upregulated by agonists of TLR3, 8 and 9, SP and RBD when compared with NS samples from HCs. The expression of the IFN-β gene after stimulation of $CD3^{+}$T cells with the TLR8 agonist was also up-regulated in moderate than severe patients (moderate vs. severe: p=0.0006). In addition, stimulation of $CD3^{+}$ T cells with SP, up-regulated the expression of IFN-β gene expression in cells from patients with moderate disease (moderate vs. severe: p=0.01). **Conclusion:** Stimulation of PBMCs from COVID-19 patients with a TLR8 agonist and with SP enhanced IFN-β protein and gene levels. This may potentiate immune responses against SARS-CoV-2 infection and prevent viral replication and spread.

Keywords: TLR; IFN; COVID-19; SARS-CoV-2

Introduction

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel respiratory virus, triggers coronavirus disease 2019 (COVID-19) (1). SARS-CoV-2 is a positive-sense single stranded RNA virus (ssRNA) which encodes the spike (SP), envelope (EP), membrane (MP), and nucleocapsid (N) proteins as well as 16 non-structural proteins (NSP1–16) and nine accessory proteins (2, 3). Attachment of the receptor-binding domain (RBD) of the viral SP to angiotensin-converting enzyme 2 (ACE2), which is expressed on lung epithelial cells, facilitates virus entry (4). After internalization of virus by host cells, the subsequent clinical manifestations of infection include a spectrum of symptoms ranging from asymptomatic to acute respiratory distress syndrome (ARDS) (1, 5). As a first line of defense against the virus, viral particles are recognized by pattern recognition receptors (PRR) by the host innate immune system including toll-like receptors (TLRs), which are expressed on immune cells and non-immune cells, including airway and lung epithelial cells (6, 7).

Based on their cellular localization, TLRs are divided into cell surface and intracellular receptors. TLR1, 2, 4, 5, 6 and 10 are expressed on the surface of cells, whilst TLR3, 7,

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8 and 9 are intracellular endosomal receptors (8). A spectrum of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs) are recognized by TLRs (9). Intracellular TLR3, 7 and 8 recognize the nucleic acid-based structure of pathogens including single stranded RNA (ssRNA) and double stranded RNA (dsRNA) (10). In addition, unmethylated CpG motifs within pathogens are recognized by TLR9 within endosomes (11). Triggering of TLR downstream signaling requires recruitment of adaptor molecules containing Toll-IL-1R (TIR) domains and activation of interferon (IFN) regulatory factor (IRF)- and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-signaling pathways to generate the production of type I and III IFNs such as IFN-α and IFN-β and proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, and IL-12 (3, 12).

The ssRNA of SARS-CoV-2 virus possess viral structural proteins which, together, are recognized by PRRs within the respiratory immune system (13). Exposure to the virus activates immune cells to release IFN-γ and other mediators which limit viral replication (14). Following innate immune activation, an antigen specific response is required to develop acquired immunity. However, SARS-CoV-2 utilizes immune escape pathways including antagonism of the host IFN responses, resulting in detrimental effects on the body (15). Indeed, viral structural proteins and open reading frames (ORFs) of viral non-structural proteins (NSP), such as ORF6, hamper IFNs signaling and anti-viral responses (2, 16). The decreased IFN, pro-inflammatory cytokines and dysregulated immune responses is associated with disease severity (17).

The importance of TLRs agonists and signaling in the pathogenesis of SARS-CoV-2 infection is under investigation (18). Synthetic agonists of TLRs modify the immune response and trigger their specific downstream signaling pathways (19, 20). In patients with COVID-19, we hypothesize that application of TLR agonists may overcome the high pathogenicity of virus by potentiation of the immune system (21) and thereby, play an important role in the elimination of virus during the early stages of disease (22). In the present study we evaluated the effects of intracellular TLRs agonists, (TLR3, 7, 8 and 9) and SP protein and RBD of virus, on blood cells of the moderate and severe COVID-19 patients to explore interferons responses.

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Material & methods

Study design and subjects

Thirty COVID-19 patients, admitted to the Imam Reza Hospital of Tabriz University of Medical Sciences, Tabriz, Iran between February-May 2022 were enrolled in the study. All were confirmed by positive real time- polymerase chain reaction (RT-PCR) tests for SARS-CoV-2 (15 severe and 15 moderate) in nasal swabs. Ten age- and sex-matched healthy controls (HC) with negative RT-PCR test were recruited. The study protocol was approved by ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1400.715) and written informed consent was obtained from all the participants prior to the sampling.

COVID-19 patients were diagnosed according to the World Health Organization (WHO) interim guidance (23). The patients were classified into moderate and severe groups as described earlier (24). Patients younger than 18 and older than 75, pregnant or breastfeeding women, subjects with malignancies, autoimmune disease, hepatitis B (HBV), hepatitis C (HCV) and human immunodeficiency virus (HIV) infection and immunosuppressive drug administration were excluded.

Sample collection

15 ml of peripheral blood was obtained from participants in EDTA tubes for immunophenotyping studies and evaluation of IFN-β gene expression. All the demographic, clinical, laboratory and computed tomography (CT) scan findings for each subject were retrieved using electronic medical records which are summarized and presented in **Table 1**.

Isolation of peripheral blood mononuclear cells (PBMCs)

Density gradient centrifugation method was utilized for PBMC isolation of patients and control group by using Ficoll, as described before (25). Briefly, blood sample was diluted with sterile phosphate buffered saline (PBS) at a 1:1 ratio. The diluted blood was gently layered over Ficoll-Hypaque (lymphosep, biosera, UK) at a 2:1 ratio. Subsequently, the tube was centrifuged at 450 xg for 20 min at 18°C. After centrifugation, the interphase layer between Ficoll and plasma, which contained mononuclear cells, was carefully

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harvested and transferred to a fresh sterile tube. The cells were washed twice with PBS and re-suspended in 1 mL of RPMI 1640 medium (Sigma-Aldrich, NY, USA) containing 10% fetal calf serum (FCS) (Sigma-Aldrich) and 100 U/ml streptomycin. The isolated cells were counted and viability was evaluated using a hemocytometer and trypan blue dye exclusion.

Cell culture and stimulation

The isolated PBMCs were cultured in 96-well cell culture flat bottom plates in order to be stimulated with various agonists of TLRs (Invivogen, San Diego, CA, USA). For each experiment 1 $\times 10^6$/ml of PBMCs were cultured and stimulated with the agonists of TLR3 (Poly I:C), TLR7 (imiquimod), TLR8 (ssRNA40/LyoVec), TLR9 (ODN2006), SARS-CoV-2 SP (R&D systems, Abingdon, UK) and SARS-CoV-2 RBD (R&D systems, Abingdon, UK) were incubated for 24 hours at 37°C supplied with 5% CO_2. LPS (100 ng/ml) (Sigma-Aldrich, USA) and phytohaemagglutinin (PHA) (5 µg/ml) (Sigma-Aldrich, NY, USA) were served as positive controls. The concentrations used were selected based on the literature and the Manufacturer's recommendation (**Table 2**).

Brefeldin A (5µg/ml, Biolegend, San Diego, CA, USA), a protein transport inhibitor, was added to the PBMCs for the last 5 hours of incubation to improve intracellular staining (26). Thereafter, the cells were centrifuged at 1100xg for 5 minutes prior to surface and intracellular staining by flow cytometry.

Flow cytometry analysis

In order to evaluate the frequency of T cells containing intracellular IFN-γ and IFN-β, PBMCs were stained for surface and intracellular proteins as described earlier (25). The characterization of all the antibodies used is shown in **Table 3**.

For staining, the harvested PBMCs ($1x10^6$ /ml) were stained with anti-human CD3 antibody for 30 min in the dark at 4°C, subsequently, for intracellular staining, the cells were washed twice with cold PBS (1X) and fixed and permeabilized with fixation and permeabilization buffers (Biolegend, San Diego, CA, USA) as instructed by Manufacturer for 20 min and then FITC-IFN-β and PerCP-IFN-γ antibodies were added. Thereafter, the cells were incubated for 30 min in the dark at 4°C. The cells were then washed with cold

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PBS (1X) and ten thousand events were evaluated by flow cytometry (Milteny BiotecTM FACS Quant 10; Milteny Biotec, Bergisch Gladbach, Germany). Data were processed using Flow Jo software version 8.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Whole blood T cells were isolated from PBMC by magnetic cell separation using a $CD3^+$ T cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) to evaluate the gene expression of IFN-β. Isolated T cells were stimulated with the TLR8 agonist and SP for 24 hours, subsequently, RNA was extracted as described earlier (27). Extracted RNA was reverse transcribed using a cDNA synthesis kit (Sinaclon, Tehran, Iran). Total RNA was used as a template and was reverse transcribed using oligo d(T), dNTP and M-MuLV reverse transcriptase, according to the manufacturer's instructions.

IFN-β gene expression was evaluated using SYBR Green (Bio Rad, USA) using specific primers (Sinaclon, Tehran, Iran) and real-time PCR (Roche, Manheim, Germany). Primers sequences for IFN-β were (forward primer: CTTGGATTCCTACAAAGAAGCAGC) and (reverse primer: TCCTCCTTCTGGAACTGCTGCA) and for GAPDH (QIAGEN, MD, USA), as house-keeping gene, were (forward primer: CCAGGTGGTCTCCTCTGACTTC) and (Reverse primer: CACCCTGTTGCTGTAGCCAAA). The expression level of the IFN-β gene was normalized to the expression level of GAPDH as an internal control using the $2^{-\Delta\Delta Ct}$ method. The real-time PCR program is summarized in **Table 4**.

Statistical analysis

Statistical analysis was performed in GraphPad Prism (version 8; Graph Pad Software, Inc.) and in SPSS (version 16.0; SPSS, Inc. Chicago, USA). Normally distributed data was analyzed using one-way ANOVA and Tukey's multiple comparison tests (mean ± SD) and non-parametric data was analyzed using Kruskal Wallis and Dunn's multiple comparison test (Median, 95% confidence intervals (CI). P-values <0.05 were considered as statistically significant.

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Results

Immunophenotyping of IFNs bearing lymphocytes

The frequencies of IFN-γ and IFN-β containing T cells were evaluated among PBMCs isolated from COVID-19 patients and HC. Two subtypes of T cells were evaluated in this study, $CD3^+IFN\text{-}\beta^+$ T cells, and $CD3^+IFN\text{-}\gamma^+$ T cells. The gating strategy used to obtain these populations is depicted in **Fig. 1**.

Intracellular expression of IFNs by PBMC of COVID-19 patients

The frequency of $CD3^+IFN\text{-}\beta^+$ T cells was evaluated among PBMCs of severe and moderate COVID-19 patients in presence/absents of TLRs agonists, SP, and RBD. At baseline in the absence of any stimulus (NS), cells from patients with moderate (M, $p<0.0001$) and severe (S, all $p=0.009$) COVID-19 had a significantly greater frequency of $CD3^+IFN\text{-}\beta^+$ T cells than observed with healthy controls (HC, **Fig. 2A&B, Table 5**). The increase in $CD3^+IFN\text{-}\beta^+$ T cells was significantly greater in patients with moderate disease compared to that observed in patients with severe disease ($p<0.0001$, **Fig. 2A&B**, **Table 5**). Stimulation of PBMCs with all TLR agonists investigated resulted in significantly increased frequencies of $CD3^+IFN\text{-}\beta^+$ T cells in patients with moderate and severe COVID-19 (**Fig. 2A, Table 5**, all $p<0.003$).

The frequency of $CD3^+IFN\text{-}\beta^+$ T cells was significantly enhanced by the positive control PHA in the HC subjects ($p<0.05$, **Fig. 2B, Table 5**) but not by SP or RBD. The frequency of $CD3^+IFN\text{-}\beta^+$ T cells was significantly increased by SP and RBP in both patients with moderate and severe COVID-19 (all $p<0.001$, **Fig. 2B**).

The highest increase of the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in moderate patients, was seen with SP (45.45±4.21%, $p<0.0001$) and with the TLR8 agonist (43.87±4.73%, $p<0.0001$), when compared with healthy controls (**Fig. 2, Table 5**). The highest increase of the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in sample of severe patients was seen with TLR8, (25.25±3.74%, $p=0.001$) and TLR7 (23.94±4.39%, $p=0.003$) agonists, when compared with healthy controls (**Fig. 2A, Table 5**).

When comparing the effect of the NS controls versus that observed with the TLR agonists on $CD3^+IFN\text{-}\beta^+$ T cell frequency, there was no significant difference between the baseline induction of $CD3^+IFN\text{-}\beta^+$ T cells and that seen with any TLR agonist in the HC subjects,

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moderate and severe patients (data not shown). However, the stimulators significantly increased the $CD3^+IFN\text{-}\beta^+$ T cell frequency of moderate and severe patients than observed with the NS controls of healthy controls (NSHC) (**Table 6**).

The frequency of $CD3^+IFN\text{-}\gamma^+$ T cells was significantly increased upon stimulation with TLR agonists in patients with moderate and severe COVID-19, compared with HC (all $p<0.02$, **Fig. 3A, Table 5**), except with TLR7 agonist, which did not significantly increase the frequency of $CD3^+IFN\text{-}\gamma^+$ T cells of moderate patients, however, the frequency of $CD3^+IFN\text{-}\gamma^+$ T cells of sever patients increased upon stimulation with TLR7 agonist ($p=0.03$) (**Fig. 3A, Table 5**).

In contrast to the results seen with $CD3^+IFN\text{-}\beta^+$ T cells, the frequency of $CD3^+IFN\text{-}\gamma^+$ T cells was not affected by PHA in any subject group whereas RBD gave significant increases ($p=0.04$) in patients with severe disease compared with that seen in HC (**Fig. 3B, Table 5**). Overall, there was no significant increase in the frequencies with TLR agonists or with SP or RBD compared with the effects seen with unstimulated cells **(Table 6).**

Real-Time PCR (Q-PCR) analysis

Since we found high intracellular levels of IFN-β in PBMCs, we examined the expression of IFN-β at the gene level using RT-qPCR in isolated $CD3^+$ T cells stimulated with the TLR8 agonist or with SP for 24h. There was no difference in the expression of IFN-β mRNA expression in unstimulated CD3+ T cells between HC, M and S patients (**Fig. 4A**). IFN-β gene expression was significantly upregulated in moderate ($p<0.0001$) and severe ($p=0.002$) COVID-19 patients compared to HC after stimulation with the TLR8 agonist (**Fig. 4B)**. The expression of IFN-β mRNA was significantly greater in patients with moderate compared to severe disease ($p<0.0001$) upon stimulation of TLR8. In addition, stimulation of T cells with SP, significantly up-regulated IFN-β mRNA expression in patients with moderate ($p=0.0003$), but not severe, disease (**Fig. 4C**). There was a significantly lower expression of IFN-β mRNA in CD3+ cells from patients with severe compared to moderate COVID-19 (moderate vs. severe: $p=0.005$, **Fig. 4C**).

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Discussion

In the current study, we evaluated the intracellular expression of IFN-β and -γ in CD3+ T cells isolated from PBMCs of moderate and severe COVID-19 patients in unstimulated cells and in cells stimulated with TLR agonists or with SARS-CoV-2 SP and RBD. This is the first study which assessed the IFN responses of COVID-19 patients' immune cells upon in-vitro stimulation with these factors and also compared the results to those obtained with cells from healthy control subjects. The results show that unstimulated cells from patients with COVID-19 expressed significantly higher frequencies of $CD3^{+}IFN\text{-}\beta^{+}$ T cells with that in patients with moderate disease being greater than that patients with severe disease. Similar levels of $CD3^{+}IFN\text{-}\beta^{+}$ T cell frequencies were seen after stimulation with TLR agonists in HC subjects with all TLR agonists and SARS-CoV-2 proteins enhancing the frequency. TLR8 and SP gave the greatest increase in the frequency of $CD3^{+}IFN\text{-}\beta^{+}$ T cells especially in patients with moderate COVID-19. In contrast, the highest increase in the frequency of CD3+IFN-β+ T cells in severe patients was seen with TLR8 and TLR7 agonists.

Interferons, are a critical arm of the innate immune response against viral infections and are secreted upon recognition of the virus or viral proteins by PRRs, such as TLRs. Type I interferons, including IFN-α and IFN-β, block viral replication and spread in addition to promoting acquired immunity (28). There are accumulating data regarding the impaired IFN responses in SARS-CoV-2 infection (29, 30). IFN expression is enhanced by many factors including TLR agonists and the activity of these agonists may help combat SARS-CoV-2 infection in COVID-19 patients (31). IFN-β decreases the replication of SARS-CoV and MERS-CoV (32) and may be useful clinically in the early stages of COVID-19 (33, 34).

IFN-γ, is related to type II IFNs family, and is produced by T and NK cells. IFN-γ reduces viral replication and promotes the cytotoxic activity of T lymphocytes (35). IFN-γ expression is also suppressed by SARS-CoV-2 with IFN-γ expression by $CD4^{+}T$, $CD8^{+}T$, and NK cells being decreased in severe and moderate COVID-19 patients (36).

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Evaluation of immunologic responses across COVID-19 severity indicated that severe COVID-19 patients had a lower IFN-γ expression by $CD4^+$T cells than patients with moderate disease (36). However, the immune cells of these patients were not stimulated with an activator. In agreement with these data, we observed a lower expression of IFN-β by CD3+ T cells in severe patients following stimulation with TLR ligands as well as the SARS-CoV-2 SP and RBD. Immune cells from patients with moderate COVID-19 are more capable of expressing high levels of IFN-β than cells from patients with severe disease at baseline and in response to different cell activators. This may reflect the high rate of inborn errors in the type I IFN pathway (37) or the presence of neutralizing autoantibodies against type I IFNs in these subjects (38, 39).

We also evaluated the frequency of $CD3^+$IFN-γ^+ T cells in the PBMCs of moderate and severe COVID-19 patients at baseline and following the same stimuli. The frequency of $CD3^+$IFN-γ^+T cells at baseline was higher in COVID-19 patients compared with HC. Activation with TLR agonists, SP or RBD evoked a similar pattern of enhanced $CD3^+$IFN-γ^+ T cells in patients with moderate and severe COVID-19 which was not seen with PHA and SP. However, the impact of the various stimuli on the frequency of $CD3^+$IFN-γ^+ T cells was not significantly affected by any of these ligands compared to that seen in unstimulated cells. A reduced number of IFN-γ producing cells was previously observed in stimulated PBMCs from COVID-19 patients (40), which is most probably due to the suppression of IFN responses by SARS-CoV-2. In accordance with our findings, the TLR7/8 agonist (R-848) induced IFN-γ expression in $CD56^+$ cells but not in $CD4^+$ or $CD8^+$ T cells in peripheral blood cells from normal healthy subjects (41). Overall, *in vitro* stimulation of PBMCs with TLRs agonist enhanced IFN-β, but not IFN-γ, expression in cells from patients with COVID-19. In view of this, we infer that TLR agonists are not suitable candidates for increasing or restoring the defective IFN-γ expression seen in T cells of patients with COVID-19. However, TLR agonists did significantly enhance the expression of IFN-β in cells from patients with SARS-CoV-2 infection.

Both the TLR8 agonist and SP stimulation upregulated IFN-β mRNA levels in isolated $CD3^+$ T cells in moderate and severe COVID-19 patients with the same pattern as observed for the $CD3^+$IFN-β^+ T cells. The elevated mRNA expression level of IFN-β at

 doi:10.20944/preprints202212.0577.v1

baseline in circulating leukocytes of COVID-19 patients was previously described evaluating the effect of SARS-CoV-2 infection on the expression of PRRs on peripheral blood cells and the release of associated cytokines (42). However, the frequency of immune cells expressing IFNs was not evaluated. Additionally, stimulation of PBMCs with a TLR9 agonists was previously reported to upregulate IFN-β mRNA expression (43) supporting earlier data showing IFN-β mRNA upregulation by TLR7, TLR7/8, TLR8, and TLR9 activation in cells from healthy volunteers (44). IFN-β protein expression was also induced by R848 in PBMCs from asthmatic and non-asthmatic subjects (45).

To our knowledge, there are no reports on IFN-β gene and/or protein expression following stimulation of PBMCs from COVID-19 patients with intracellular TLR agonists. The current study, is also the first study describing the effects of SP and RBD on up-regulating IFN-β expression in $CD3^{+}$T cells from COVID-19 patients. The data highlights the importance of innate immune responses in priming of acquired immunity against SARS-CoV-2. Further studies are required to evaluate the alterations of immunologic parameters including frequency of T cells subsets, gene expression of transcription factors and cytokines following stimulation of TLRs and the other PRRs, such as retinoic-acid inducible gene (RIG) in COVID-19 patients.

The limitations of the current study include the low number of participants and lack of parallel measurement of cytokines in the serum of the patients. The strength of our study is the novelty of the evaluation of the TLR agonist effects on IFN expression by immune cells from both moderate and severe COVID-19 patients. This opens a new window towards understanding the immunopathogenesis of SARS-CoV-2 and suggests the introduction of TLR8 agonists in the COVID-19 armamentarium. In conclusion, due to the impairment of IFN signaling by SARS-CoV-2, new approaches are required to limit the replication and spread of virus and disease progression. The current study demonstrated that the TLR8 agonist, ssRNA40/LyoVec, potentiates the attenuated IFN responses seen in patients with severe COVID-19 by upregulating IFN-β expression by T lymphocytes in-vitro. TLR8 agonists may improve IFN-β responses in COVID-19 patients.

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Footnotes:

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Authors contributions:

SAV did experiments, wrote the manuscript and carried out the statistical analysis

BB participated in the design of the study and revised the manuscript.

AS participated in patients' selection and data collection

GB comments and revised the manuscript.

FK participated in performing the experiments

IMA advised the protocol and revised the manuscript.

GF and JG revised the manuscripts.

EM conceived of the study, and participated in the design of the study and supervised the project. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends:

Figure 1: Gating strategy for identification of $CD3^+IFN\text{-}\beta^+$ and $CD3^+IFN\text{-}\gamma^+$ T cells.

Representative flow cytometric dot plots showing expression of IFN-β and IFN-γ in CD3+ T cells in a TLR8-stimulated PBMC sample from a severe COVID-19 patient.

Figure 2: Frequency of CD3+ interferon (IFN)-β+ peripheral blood T cells following stimulation with toll-like receptor (TLR) agonists, spike protein (SP) and receptor binding domain (RBD).

(A) The frequency of CD3+IFN-β+ T cells in the healthy control (HC, n=10) group and in patients with moderate (M, n=15) or severe (S, n=15) COVID-19 was assessed. Cells were unstimulated (NS) or stimulated with agonists against TLR3 (10µg/ml Poly I:C), TLR4 (100ng/ml LPS), TLR7 (3µg/ml Imiquimod), TLR8 (3µg/ml ssRNA40/LyoVec) and TLR9 (5µM ODN2006) for 24hrs and the frequency of CD3+IFN-β+ cells measured. (B) The peripheral blood mononuclear cells (PBMCs) stimulation by positive (5µg/ml phytohaemagglutinin, PHA) and negative controls (NS), SP (5µg/ml) and RBD (100ng/ml) on the frequency of CD3+IFN-β+ cells. Results are plotted as mean ± SD and differences determined using a one-way ANOVA and post-test analysis. A post-test p value <0.05 was considered statistically significant. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 3: Frequency of CD3+ interferon (IFN)-γ+ peripheral blood T cells following stimulation with toll-like receptor (TLR) agonists, spike protein (SP) and receptor binding domain (RBD).

(A) The frequency of CD3+IFN-γ+ T cells in the healthy control (HC, n=10) group and in patients with moderate (M, n=15) or severe (S, n=15) COVID-19 was assessed. Cells were unstimulated (NS) or stimulated with agonists against TLR3 (10µg/ml Poly I:C), TLR4 (100ng/ml LPS), TLR7 (3µg/ml Imiquimod), TLR8 (3µg/ml ssRNA40/LyoVec) and TLR9 (5µM ODN2006) for 24hrs and the frequency of CD3+IFN-γ+ cells measured. (B) The peripheral blood mononuclear cells (PBMCs) stimulation by positive (5µg/ml phytohaemagglutinin, PHA) and negative controls (NS), SP (5µg/ml) and RBD (100ng/ml) on the frequency of CD3+IFN-γ+ cells. Results are plotted as mean ± SD and

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differences determined using a one-way ANOVA and post-test analysis. A p value <0.05 was considered statistically significant. *p<0.05, **p<0.01.

Figure 4: Interferon (IFN)-β gene expression following stimulation of peripheral blood CD3+ T cells with the toll-like receptor (TLR)8 agonist ssRNA40/LyoVec or spike protein (SP).

Gene expression of IFN-β was determined in CD3+ T cells from 5 patients with moderate (M) and severe (S) COVID-19 and from 5 healthy control (HC) subjects using RT-qPCR. The effect of 24hrs treatment with media (unstimulated, A), the TLR8 agonist (B) and the COVID-19 SP (C) are reported as the mean±SD of the fold-change compared to baseline. A p value <0.05 was considered statistically significant. *p<0.05, p<0.01 and p<0.001.

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Table 1: Demographic data of study population

		Moderate patients (n=15)	Severe patients (n=15)	Healthy control (n=10)	P value (severe vs. moderate)	P value (moderate vs. control)	P value (severe vs. control)
Age (mean ±SD) range	-	55.20±14.55 (27-70)	50.13±13.55 (30-74)	45.50±14.73 (30-69)	ns	ns	ns
Gender (n, %)	**Female**	7 (%46.6)	6 (%40)	5 (%50)	ns	ns	ns
	male	8 (%53.3)	9 (%60)	5 (%50)			
O2 saturation	**(So2)**	92.27±2.84	81.20±14.01	-	0.0001	-	-
Comorbidities (n)	**Hypertension**	4 (%26.6)	2 (%13.3)	-	-	-	-
	Diabetes	3 (%20)	3 (%20)	-	-	-	-
	Cardiovascular disease	2 (%13.3)	2 (%13.3)	-	-	-	-
	Chronic kidney disease	3 (%20)	1 (%6.66)	-	-	-	-
	COPD	2 (%13.3)	3 (%20)	-	-	-	-
	High BMI (≥30)	2 (%13.3)	3 (%20)	-	-	-	-
Lung involvement	**≥50%**	0	9 (%60)	-	-	-	-
	<50%	5 (%33.3)	0				
Symptoms (n, %)	**Fever**	4 (%26.6)	4 (%26.6)	-	-	-	-
	Cough	11 (%73.3)	9 (%60)				
	Dyspnea	8 (%53.3)	10 (%66.6)				
	Myalgia	5 (%33.3)	2 (%13.3)				
	Tiredness	3 (%20)	3 (%20)				
Laboratory Findings	**Lactate Dehydrogenase (LDH) (U/L)**	595.1±126.6	736.8±178.9	-	0.018	-	-
	C-Reactive protein (CRP) (mg/l)	82.71±12.82	102.3±85.61		0.037		
	Aspartate aminotransferase (AST)	28.44±13.43	39.22±16.46		ns		
	Alanine aminotransferase (ALT)	31.78±22.61	50.11±26.92		ns		
Death (n, %)		2 (%13.3)	5 (%33.3)	-	-	-	-

Abbreviation: COPD: Chronic obstructive pulmonary disease; BMI: Body mass index; ns: not significant.

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Table 2: Agonist concentrations used

Receptor	Agonist	Agonist concentration	References
TLR3	Poly I:C	10 μg/ml	(46-50)
TLR4	LPS	100 ng/ml	(51, 52)
TLR7	Imiquimod	3 μg/ml	(46, 48-50, 53)
TLR8	ssRNA40/LyoVec	3 μg/ml	(46, 49, 53)
TLR9	ODN2006	5 μM	(46, 53)
Spike	Spike	5 μg/ml	(54)
RBD	RBD	100 ng/ml	(55)
PHA	PHA	5 μg/ml	(56, 57)

Table 3. Information the antibodies used for cellular staining

Antigen	Fluorophore	Clone	Company	Isotype	Lot/Cat No.
CD3	APC	UCHT-1	Cytognos	IgG1	Lot 2010552 Cat: CYT-3AP10
IFN-γ	PerCp	4S.B3	Biolegend	Mouse IgG1, κ	Lot B340751 Cat: 502523
IFN-β	FITC	A1 (IFNb)	Invitrogen	Mouse IgG1, κ	Lot 270933-000 Cat: BMS1044FI

Table 4: The real-time PCR program

	Steps	Cycles	Temperature (°C)	Duration (seconds)
1	Pre-incubation	1	95	240
2	2 step amplifications	35	95	5
			60	30
3	Melting	1	95	10
			65	60
			97	1
4	Cooling	1	37	30

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Table 5: The frequency of CD3+ IFN-β+ and CD3 IFN-ɣ+ T cells among PBMCs of severe and moderate covid-19 patients and healthy control group after stimulation with different agonists

Stimulation with	Parameters	Healthy Control (N=10)	Moderate (N=15)	Severe (N=15)	P value (ANOVA /Kruskal Wallis)	P value (Tu
					P value (Healthy control vs. moderate)	
TLR3 agonist	CD3+ IFN-β+ T cells (%)	1.639±0.39	38.38±4.99	21.33±3.76	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.129±0.02	0.871±0.38	1.03±0.38	0.007	0.01
TLR4 agonist	CD3+ IFN-β+ T cells (%)	1.441±0.37	37.19±4.03	22.93±3.70	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.137±0.03	0.643±0.2	0.971±0.2	0.0015	0.02
TLR7 agonist	CD3+ IFN-β+ T cells (%)	1.727±0.25	40.91±5.47	23.94±4.39	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.134±0.03	0.568±0.22	0.814±0.21	0.03	ns (0.2)
TLR8 agonist	CD3+ IFN-β+ T cells (%)	2.179±0.4	43.87±4.73	25.25±3.74	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.098±0.02	0.732±0.3	0.960±0.34	0.007	0.009
TLR9 agonist	CD3+ IFN-β+ T cells (%)	1.515±0.33	41.35±3.81	20.68±3.77	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.092±0.02	0.827±0.35	0.980±0.39	0.002	0.004
SARS-CoV-2 Spike protein	CD3+ IFN-β+ T cells (%)	1.667±0.29	45.45±4.21	22.28±3.41	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.13±0.02	0.832±0.36	0.760±0.24	ns (0.055)	ns (0.09)
SARS-CoV-2 RBD subunit	CD3+ IFN-β+ T cells (%)	1.884±0.32	39.32±3.91	19.93±2.95	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.151±0.03	0.896±0.39	0.816±0.27	0.03	ns (0.06)
PHA	CD3+ IFN-β+ T cells (%)	5.369±0.89	60.34±3.10	50.19±3.97	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.204±0.04	0.238±0.06	0.228±0.06	ns (0.9)	ns (>0.99)
Negative sample (un-stimulated)	CD3+ IFN-β+ T cells (%)	1.177±0.25	25.59±2.77	11.63±1.53	<0.0001	<0.0001
	CD3+ IFN-ɣ+ T cells (%)	0.141±0.03	0.608±0.21	0.773±0.3	ns (0.1)	ns (0.1)

Abbreviation: TLR3: toll-like receptor 3; TLR4: toll-like receptor 4; TLR7: toll-like receptor 7; TLR8: toll-like receptor 8; TLR9: toll-like receptor 9; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; RBD: receptor binding domain; PHA: Phytohaemagglutinin; ns: not significant. Data are reported as mean± SEM. p value < 0.05 was considered statistically significant.

Table 6: **The frequency of CD3+ IFN-β+ and CD3 IFN-ɣ+ T cells among PBMCs of severe and moderate covid-19 patients and healthy control group at the baseline in the absence of any stimulus (NS) and after stimulation with different agonists**

			Stimulators								P value (ANOVA/Kruskal Wallis)
		NS	TLR3 A.	TLR4 A.	TLR7 A.	TLR8 A.	TLR9 A.	SP	RBD	PHA	
cD3+ IFN-β+	HC (N=10)	1.177±0.25	1.639±0.39	1.441±0.37	1.727±0.25	2.179±0.4	1.515±0.33	1.667±0.29	1.884±0.32	5.369±0.89	0.004
	MP (N=15)	25.59±2.77	38.38±4.99	37.19±4.03	40.91±5.47	43.87±4.73	41.35±3.81	45.45±4.21	39.32±3.91	60.34±3.10	<0.0001

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	SP (N=15)	11.63±1.53	21.33±3.76	22.93±3.70	23.94±4.39	25.25±3.74	20.68±3.77	22.28±3.41	19.93±2.95	50.19±3.97	<0.0001
CD3+ IFN-γ + T cells	**HC** (N=10)	0.141±0.03	0.129±0.02	0.137±0.03	0.134±0.03	0.098±0.02	0.092±0.02	0.13±0.02	0.151±0.03	0.204±0.04	ns
	MP (N=15)	0.608±0.21	0.871±0.38	0.643±0.2	0.568±0.22	0.732±0.3	0.827±0.35	0.832±0.36	0.896±0.39	0.238±0.06	ns
	SP (N=15)	0.773±0.3	1.03±0.38	0.971±0.2	0.814±0.21	0.960±0.34	0.980±0.39	0.760±0.24	0.816±0.27	0.228±0.06	0.04

Abbreviation: HC: healthy control; MP: moderate patients; SP: severe patients; NS: not stimulated; NSHS: not stimulated sample of healthy controls; TLR3 A.: toll-like receptor 3 agonist; TLR4 A.: toll-like receptor 4 agonist; TLR7 A.: toll-like receptor 7 agonist; TLR8 A.: toll-like receptor 8 agonist; TLR9 A.: toll-like receptor 9 agonist; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; RBD: receptor binding domain; PHA: Phytohaemagglutinin; ns: not significant. Data are reported as mean± SEM. p value < 0.05 was considered statistically significant.

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Fig. 1

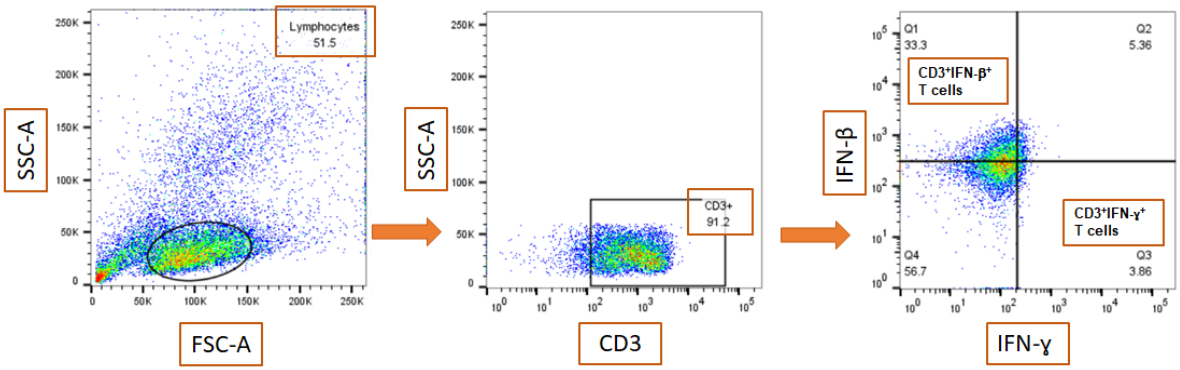

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Fig. 2

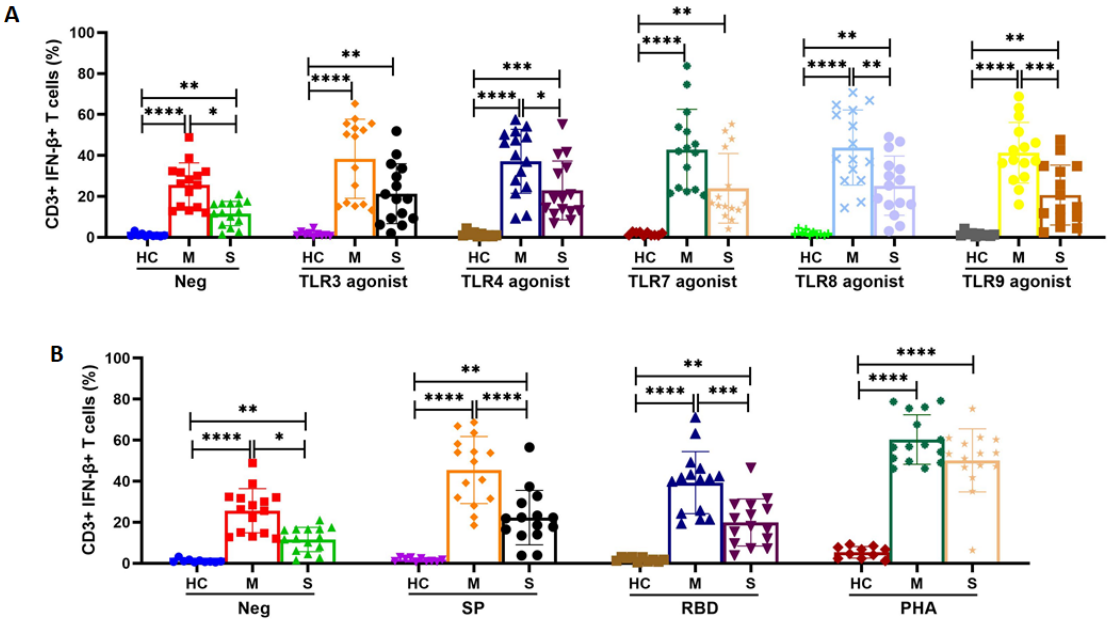

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Fig. 3

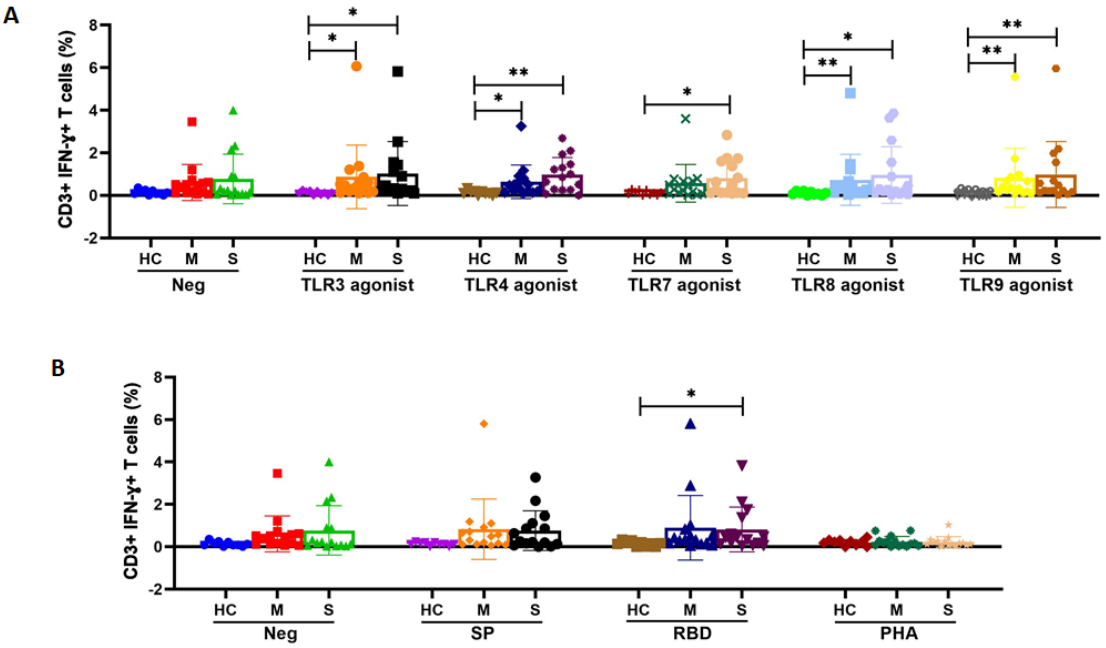

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Fig. 4