Experimental and Molecular Pathology 137 (2024) 104897

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Effects of toll-like receptor agonists and SARS-CoV-2 antigens on interferon (IFN) expression by peripheral blood CD3$^+$ T cells from COVID-19 patients

Samaneh Abdolmohammadi-Vahid [a], Behzad Baradaran [b], Armin Sadeghi [c], Gilina Bezemer [d], Fatemeh Kiaee [a], Ian M. Adcock [e,f], Gert Folkerts [d], Johan Garssen [d], Esmaeil Mortaz [a,*]

[a] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Tuberculosis and Lung Disease Research Center of Tabriz University of Medical Sciences, Tabriz, Iran
[d] Division of Pharmacology, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands
[e] Respiratory Section, Faculty of Medicine, National Heart and Lung Institute, Imperial College London, London, United Kingdom
[f] Immune Health Program at Hunter Medical Research Institute and the College of Health and Medicine at the University of Newcastle, NSW, Australia

ARTICLE INFO

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

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) infection 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 healthy 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) of SP. Frequencies of CD3$^+$IFN-β$^+$ T cells, and CD3$^+$IFN-γ$^+$ T cells were 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 PBMCs from moderate ($p < 0.0001$) and severe ($p = 0.009$) patients at baseline in comparison with HCs. The highest increase in the frequency of CD3$^+$IFN-β$^+$ T cells in cell from moderate patients was induced by TLR8 agonist and SP ($p < 0.0001$ for both) when compared to HC, while, the highest increase of the frequency of CD3$^+$IFN-β$^+$ T cells in sample of severe patients was seen with TLR8 and TLR7 agonists (both $p = 0.002$). The frequency of CD3$^+$IFN-γ$^+$ T cells was significantly increased upon stimulation with TLR agonists in cell from patients with moderate and severe COVID-19, compared with HC (all $p < 0.01$), except with TLR7 and TLR8 agonists. The TLR8 agonist did not significantly increase the frequency of CD3$^+$IFN-γ$^+$ T cells in PBMCs of severe patients, but did so in cells from patients with moderate disease ($p = 0.01$). Moreover, IFN-β gene expression was significantly upregulated in CD3$^+$T cells from moderate ($p < 0.0001$) and severe ($p = 0.002$) COVID-19 patients, compared to HC after stimulation with the TLR8 agonist, while, stimulation of T cells with SP, significantly up-regulated IFN-β mRNA expression in cells from patients with moderate ($p = 0.0003$), but not severe disease.

Conclusion: Stimulation of PBMCs from COVID-19 patients, especially patients with moderate disease, with TLR8 agonist and SP increased the frequency of IFN-β-producing T cells and IFN-β gene expression.

1. Introduction

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel respiratory virus, triggers coronavirus disease 2019 (COVID-19) (Huang et al., 2020). 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 (Li et al., 2020; Zhang et al., 2022b). 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 (Chen et al., 2020c; Gu et al., 2022). After the internalization of the virus

* Corresponding author.
E-mail address: e.mortaz@sbmu.ac.ir (E. Mortaz).

https://doi.org/10.1016/j.yexmp.2024.104897
Received 14 June 2023; Received in revised form 9 March 2024; Accepted 22 April 2024
Available online 30 April 2024

by host cells, the subsequent clinical manifestations of infection include a spectrum of symptoms ranging from asymptomatic to acute respiratory distress syndrome (ARDS) (Diamond and Kanneganti, 2022; Xu et al., 2020). As a first line of defense against the virus, viral particles are recognized by pattern recognition receptors (PRRs) 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 (Mortaz et al., 2020; Yi et al., 2020).

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, 8 and 9 are intracellular endosomal receptors (Kawasaki and Kawai, 2014). A spectrum of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs) are recognized by TLRs (O'neill et al., 2013). Intracellular TLR3, 7 and 8 recognize the nucleic acid-based structure of pathogens including single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) (Yokota et al., 2010). In addition, unmethylated CpG motifs within pathogens are recognized by TLR9 within endosomes (Huang et al., 2008). SsRNA recognition by TLR7/8 triggers recruitment of the MyD88 adaptor protein and signaling, which evokes interferon (IFN) regulatory factor (IRF)- and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-transcription factor activation and the production of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, IL-18, IL-17, tumor necrosis factor-alpha (TNF-α) and type I interferons (IFN-α and IFN-β), respectively (Farahani et al., 2022), as well as IFN- γ (Salerno et al., 2016). There are also alternative pathways for the induction of type I interferons. TLR4 recognizes NSP1 of SARS-CoV-2, as well as stress molecules originating from infection such as high-mobility group box 1 protein (HMGB1) and oxidized phospholipids (Imai et al., 2008). Endocytosis of activated TLR4 results in the recruitment of TIR-domain-containing adaptor-inducing interferon-β (TRIF), activation of IRF3 and production of IFNs (Akira and Takeda, 2004). Additionally, dsRNA, originating from the replication of SARS-CoV-2 ssRNA, is recognized by TLR3 resulting in the subsequent recruitment of the adapter molecules TRIF and TNF receptor-associated factor 3 (TRAF3) and activation of IRF3 (Farahani et al., 2022). In addition to TLRs, the other member of PRRs, such as retinoic acid-inducible gene I (RIG-1) also detects the viral RNA in infected cells. Activated RIG-1 interacts with the mitochondrial antiviral-signaling protein (MAVS), recruits associated adaptor molecules and eventually results in type I IFN production by activation of IRF3 (Chen et al., 2020b).

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 (Minkoff and tenOever, 2023). Indeed, viral structural proteins and open reading frames (ORFs) of viral NSPs, such as ORF6, hamper IFNs signaling and anti-viral responses (Kopecky-Bromberg et al., 2007; Li et al., 2020). The decreased IFNs, pro-inflammatory cytokines and dysregulated immune responses are associated with disease severity (Angelopoulou et al., 2020).

The importance of TLRs agonists and signaling in the pathogenesis of SARS-CoV-2 infection is under investigation (Liu et al., 2022). Synthetic agonists of TLRs modify the immune responses and trigger their specific downstream signaling pathways (Domingues et al., 2015; Kwok et al., 2012). TLR ligands alone, in the absence of T cell receptors (TCRs) and costimulatory signaling, are capable of inducing cytokine and chemokine production by T lymphocytes, as well as their proliferation (Caron et al., 2005; Sharma et al., 2016). Therefore, TLRs ligation can induce activation and proliferation of T lymphocytes, alone, and in combination with costimulatory and TCR signaling. In patients with COVID-19, we hypothesize that the application of TLR agonists may overcome the high pathogenicity of the virus by potentiation of the immune system (Dahl, 2002) and, thereby, play an important role in the elimination of the virus during the early stages of the disease (Szeto et al., 2021). Since we focused on the effect of TLR ligation in the induction of innate responses from T cells, we used TLR agonists for the stimulation of PBMCs, which include T cells, as the most abundant cell type (60–70%). Furthermore, we evaluated the immune responses induced by intracellular TLRs ligation, as the downstream signaling of these receptors would result in the activation of IRF3, IRF5 and IRF7 transcription factors and the production of IFN responses, which are crucial in hampering viral replication and spread, whilst most of the signaling pathways stimulated by extracellular TLRs, lead to the production of pro-inflammatory cytokines and immunopathological consequences such as the cytokine storm (Nazerian et al., 2022).

In addition, the spike glycoprotein of SARS-CoV-2 is recognized by TLR1, TLR4, and TLR6 (Dai et al., 2022). The strongest attachment occurs between SP and TLR4 (Manik and Singh, 2022). Therefore, SP stimulates both the innate immune responses and acquired immune system through TLRs and TCR (with antigens that can be processed and presented to TCRs of specific T lymphocytes), respectively. However, there is no specific report of attachment of RBD to TLRs, therefore, we aimed to explore the effectiveness of SARS-CoV-2 components in stimulation of T lymphocytes, thus we tested viral proteins, SP and RBD, in comparison with TLRs agonist. Moreover, we investigated whether stimulation of immune cells with RBD induces activation of T cells.

In the present study, we evaluated the interaction of intracellular TLRs agonists and SARS-CoV-2 antigens with PBMCs of moderate and severe COVID-19 patients in order to explore interferons responses.

2. Material & methods

2.1. Study design and subjects

Thirty COVID-19 patients, admitted to the Imam Reza Hospital of Tabriz University of Medical Sciences, Tabriz, Iran, in 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 tests were recruited. The study protocol was approved by the 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 before the sampling.

COVID-19 patients were diagnosed according to the World Health Organization (WHO) interim guidance (World Health Organization, 2020). The patients were classified into moderate and severe groups as described earlier (Mortaz et al., 2022). 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.

2.2. 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.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs)

The density gradient centrifugation method was utilized for PBMC isolation of patients and the control group by using Ficoll, as described before (Dezfuli et al., 2021). Briefly, fresh blood samples obtained within 1 h of collection in EDTA tubes, were diluted with sterile phosphate-buffered saline (PBS) at a 1:1 ratio, in a 50 ml sterile conical tube (Ghosh et al., 2006; Ghosh et al., 2007). The diluted blood was

Table 1
Demographic and clinical data of the 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.

gently layered over Ficoll-Hypaque (lymphosep, biosera, UK) at a 2:1 ratio, in 3 different 15 ml sterile conical tubes, in order to enhance the mononuclear cells yield. Subsequently, the tube was centrifuged at 450 x*g* for 20 min at 18 °C. After centrifugation, the interphase layer between Ficoll and plasma, which contained mononuclear cells, was carefully 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), 100 U/ml streptomycin and 200 mM L-glutamine. The isolated cells were counted and viability was evaluated using a hemocytometer and trypan blue dye exclusion. The isolated PBMCs were cultured with stimulators immediately after isolation.

2.4. 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 1×10^6/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) and were incubated for 24 h at 37 °C, supplied with 5% CO_2. LPS (100 ng/ml) (from *Escherichia coli* O111:B4; Sigma-Aldrich, USA) and phytohaemagglutinin (PHA) (5 μg/ml) (Sigma-Aldrich, NY, USA) served as positive controls. The concentrations used were selected based on the literature and the manufacturer's recommendation (Table 2).

Brefeldin A (5 μg/ml, Biolegend, San Diego, CA, USA), a protein transport inhibitor, was added to the PBMCs for the last 5 h of incubation to improve intracellular staining (Moloudizargari et al., 2020). Thereafter, the cells were centrifuged at 1100 ×*g* for 5 min prior to the surface and intracellular staining by flow cytometry.

2.5. 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 (Dezfuli et al., 2021).

Table 2
Agonist concentrations used.

Receptor	Agonist	Agonist concentration	References
TLR3	Poly I:C	10 μg/ml	(Ghosh et al., 2007; Peng, 2016; Tang, 2016; Tang, 2015)
TLR4	LPS	100 ng/ml	(Meurs, 2011; Pu and Wang, 2014)
TLR7	Imiquimod	3 μg/ml	(Ghosh et al., 2007; Tang, 2016; Yang, 2005; Tang, 2015)
TLR8	ssRNA40/ LyoVec	3 μg/ml	(Ghosh et al., 2007; Yang, 2005)
TLR9	ODN2006	5 μM	(Yang, 2005)
Spike	Spike	5 μg/ml	(Liu, 2021)
RBD	RBD	100 ng/ml	(Hsu, 2020)
PHA	PHA	5 μg/ml	(De Groote, 1992; Galicia, 2003)

The harvested PBMCs (1×10^6/ml) were stained with Allophycocyanin (APC)-conjugated anti-human CD3 antibody (Cytognos, IgG1 isotype, UCHT-1 clone) for 30 min in the dark at 4 °C, subsequently, for intracellular staining, the cells were washed twice with cold PBS (1×), fixed and permeabilized with fixation and permeabilization buffers (Biolegend, San Diego, CA, USA), as instructed by the manufacturer for 20 min and then fluorescein isothiocyanate (FITC)-IFN-β (Invitrogen, San Diego, CA, USA; Mouse IgG1 κ isotype, A1 clone) and Peridinin Chlorophyll Protein Complex (PerCP)-IFN-γ (Biolegend, San Diego, CA, USA; Mouse IgG1 κ isotype, 4S·B3 clone) antibodies were added. Thereafter, the cells were incubated for 30 min in the dark at 4 °C. The cells were then washed with cold PBS (1×) and ten thousand events were evaluated by flow cytometry (Milteny BiotecTM FACS Quant 10; Milteny Biotec, Bergisch Gladbach, Germany). Doublet discrimination (FSC-H vs. FSC-A) was performed on all samples, as previously described (Cosma, 2020; Cossarizza et al., 2017) and identified negligible numbers. Data were processed using Flow Jo software version 8.

2.6. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Whole blood T cells were isolated from PBMCs 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 h, subsequently, RNA was extracted as previously described (Dezfuli et al., 2021). 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), specific primers (Sinaclon, Tehran, Iran) and real-time PCR (Roche, Manheim, Germany) and the following program: Pre-incubation (95 °C for 240 s), 2 step amplifications (35 cycles, 95 °C for 5 s and 60 °C for 30 s), melting (95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s) and cooling (37 °C for 30 s). Primer 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.

2.7. 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.

3. Results

3.1. Study participant characteristics

The demographic, clinical, laboratory and computed tomography (CT) findings for all subjects are presented in Table 1. Fifteen moderate COVID-19 patients, aged 27–70, 15 severe COVID-19 patients, aged 30–74 and 10 healthy controls, aged 30–69, with no significant difference in age and sex between 3 groups were enrolled. Laboratory biomarkers including C-reactive protein (CRP), which indicates general inflammatory status and lactate dehydrogenase (LDH), which is secreted upon necrosis of cell membrane after lung damage by SARS-CoV-2 infection, were significantly higher in severe patients in comparison with moderate group ($p = 0.03$ and 0.01, respectively), which reflects previously published data (Kermali et al., 2020). However, there were no significant differences in the levels of aspartate transaminase (AST) and alanine transaminase (ALT), indicators of liver function, between groups. Oxygen (O_2) saturation was significantly decreased in severe patients, while the moderate group showed a higher level of O_2 saturation ($p = 0.0001$). After 2 weeks from hospital admission, the survivors from COVID-19 were discharged, while, five out of 15 (33.3%) severe patients and two out of 15 (13.3%) moderate patients died.

3.2. Flowcytometric evaluation of IFNs bearing lymphocytes

The frequencies of IFN-γ- and IFN-β-producing T cells were evaluated

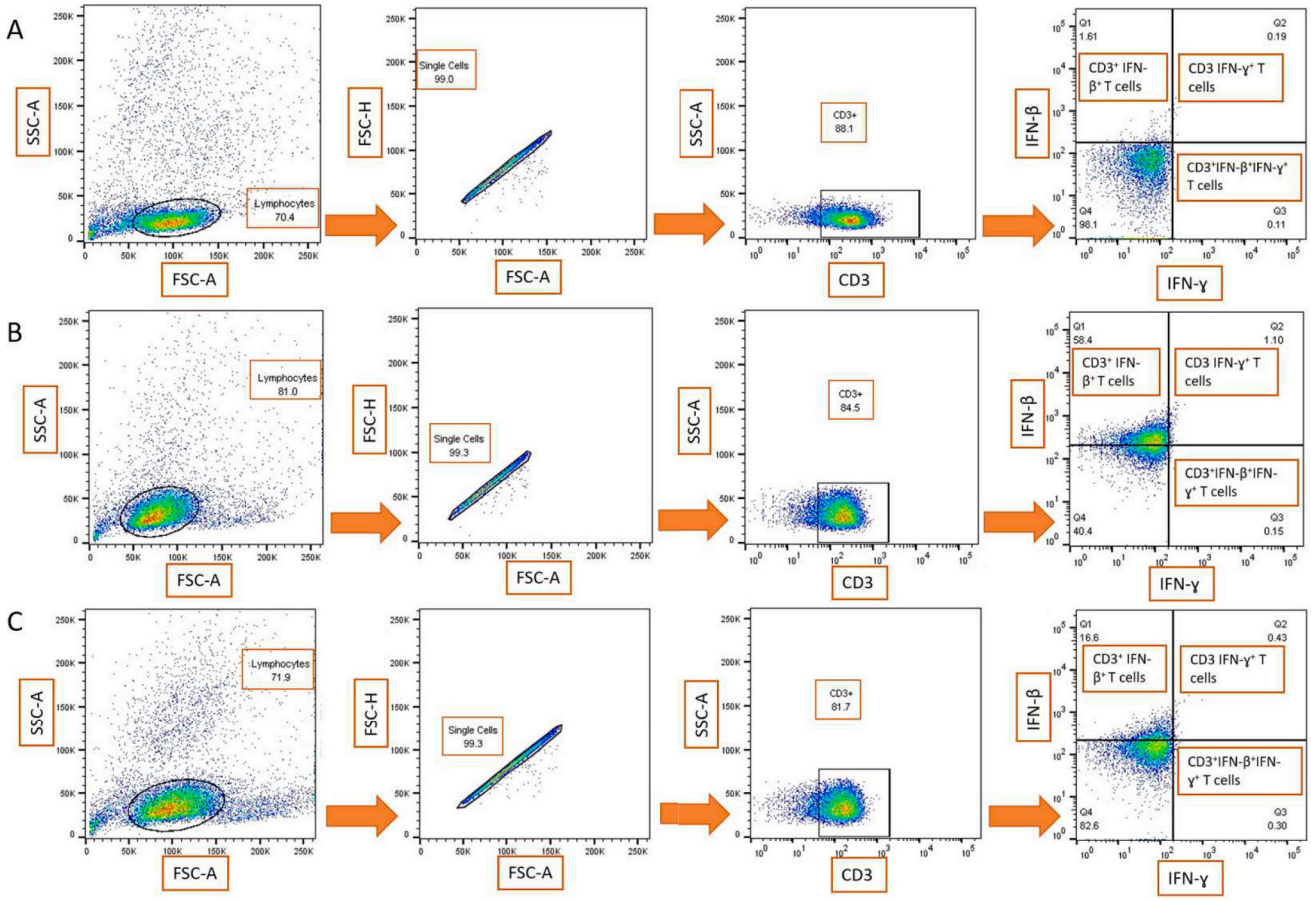

Fig. 1. Gating strategy for identification of $CD3^+IFN\text{-}\beta^+$, $CD3^+IFN\text{-}\gamma^+$ T cells and $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells. Representative flow cytometric dot plots showing expression of IFN-β and IFN-γ in $CD3^+$T cells in a TLR8-stimulated PBMC sample from a A) healthy control, B) moderate and C) severe COVID-19 patient.

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

3.3. Population of IFN-producing T cells in stimulated PBMCs of COVID-19 patients

3.3.1. $CD3^+IFN\text{-}\beta^+$ T cells

The frequency of $CD3^+IFN\text{-}\beta^+$ T cells was evaluated among PBMCs of severe and moderate COVID-19 patients in the presence/absence 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, $p = 0.009$) COVID-19 had a significantly greater frequency of $CD3^+IFN\text{-}\beta^+$ T cells than observed with HC, also the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in the PBMCs from moderate group was significantly higher than in severe patients in NS ($p = 0.02$) (Fig. 2A & B).

The increase in $CD3^+IFN\text{-}\beta^+$ T cells was significantly greater in cell from patients with moderate disease compared to that observed in patients with severe disease, upon stimulation with TLR8 and 9 agonists and RBD ($p = 0.001$, $p = 0.04$, and $p = 0.001$, respectively, Fig. 2A and B). Although the other stimuli increased the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in moderate patients' cells to a higher degree than in the severe group, this increase was not significant. Stimulation of PBMCs with all TLR agonists investigated, resulted in significantly increased frequencies of $CD3^+IFN\text{-}\beta^+$ T cells in samples from patients with moderate and severe COVID-19 (Fig. 2A, all $p < 0.007$).

The frequency of $CD3^+IFN\text{-}\beta^+$ T cells was significantly enhanced by the positive control PHA, in cells from both patients with moderate and severe COVID-19, when compared to HC (all $p < 0.0001$, Fig. 2B). The frequency of $CD3^+IFN\text{-}\beta^+$ T cells was significantly increased by SP and RBD in cells from both patients with moderate and severe COVID-19, when compared to HC (all $p < 0.005$, Fig. 2B). The increase in $CD3^+IFN\text{-}\beta^+$ T cells was significantly greater in samples from patients with moderate disease compared to that observed in patients with severe disease, upon stimulation with RBD ($p = 0.001$, Fig. 2B), however, although stimulation with SP increased the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in cells from moderate patients to a higher degree than in the severe group, this increase was not significant (Fig. 2B).

The highest increase in the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in PBMCs from moderate patients, was seen with the TLR8 agonist (44.42 ± 4.68%, $p < 0.0001$) and with SP (42.43 ± 5.06%, $p < 0.0001$), when compared with HC (Fig. 2). The highest increase in the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in sample of severe patients was seen with TLR8 (23.46 ± 3.96%, $p = 0.002$) and TLR7 (22.16 ± 4.57%, $p = 0.002$) agonists when compared with HC (Fig. 2A).

When comparing $CD3^+IFN\text{-}\beta^+$ T cell frequency in NS controls versus TLR agonist-treated cells, there was no significant difference between the baseline induction of $CD3^+IFN\text{-}\beta^+$ T cells and that seen with any TLR agonist in samples of the HC subjects (**Supplementary Fig. S2**), moderate (**Supplementary Fig. S3**) and severe patients (**Supplementary Fig. S4**). However, the stimuli significantly increased the $CD3^+IFN\text{-}\beta^+$ T cell frequency in cells from moderate (**Supplementary Fig. S3**) and severe (**Supplementary Fig. S4**) patients compared to that seen with the NS controls of healthy controls (NSHC). A comparison of the frequency of $CD3^+IFN\text{-}\beta^+$ T cells between unstimulated and stimulated cells indicated that TLR8, TLR9 agonist, SP and RBD significantly increased the frequency of $CD3^+IFN\text{-}\beta^+$ T cells, when compared to NS samples, in the moderate group (all<0.02, **Supplementary Fig. S10**). In

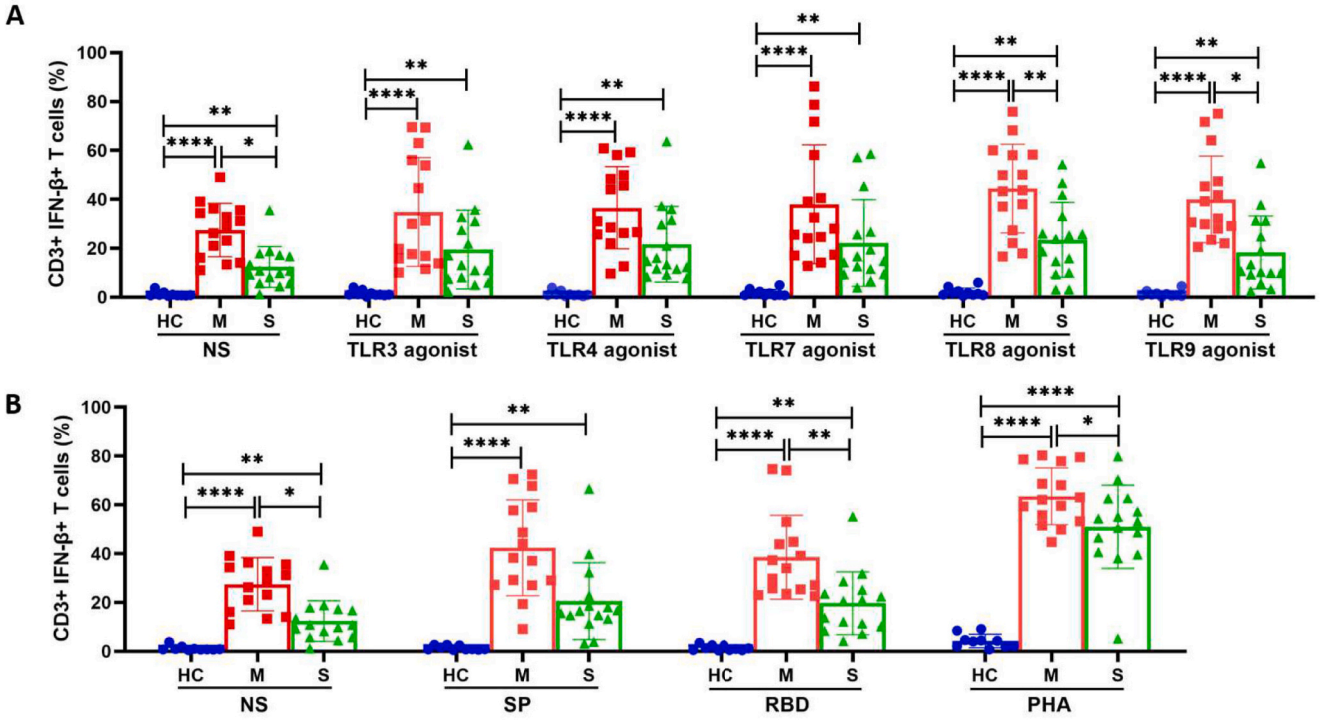

Fig. 2. Evaluation of the frequency of $CD3^+$ interferon (IFN)-β^+ peripheral blood T cells following stimulation with toll-like receptor (TLR) agonists, spike protein (SP) and receptor binding domain (RBD) by flowcytometry. (A) The frequency of $CD3^+IFN\text{-}\beta^+$ T cells in cells from healthy control (HC, $n = 10$) group and 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 (10 μg/ml Poly I:C), TLR4 (100 ng/ml LPS), TLR7 (3 μg/ml Imiquimod), TLR8 (3 μg/ml ssRNA40/LyoVec) and TLR9 (5 μM ODN2006) for 24 h and the frequency of $CD3^+IFN\text{-}\beta^+$ cells measured. (B) The effects of peripheral blood mononuclear cells (PBMCs) stimulation by positive (5 μg/ml phytohaemagglutinin, PHA) and negative controls (NS), SP (5 μg/ml) and RBD (100 ng/ml) on the frequency of $CD3^+IFN\text{-}\beta^+$ cells. Results are plotted as mean ± SEM and differences are determined using a one-way ANOVA test and multiple comparison tests. *P* value <0.05 was considered statistically significant. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

the samples of severe group, TLR8 agonist and SP also significantly increased the frequency of $CD3^+IFN\text{-}\beta^+$ T cells in stimulated samples compared to unstimulated ones (both<0.04, **Supplementary Fig. S11**). In the samples of HC group, only the TLR8 agonist significantly increased the frequency of $CD3^+IFN\text{-}\beta^+$ T cells ($p = 0.009$, **Supplementary Fig. S9**).

3.3.2. $CD3^+IFN\text{-}\gamma^+$ T cells

The frequency of $CD3^+IFN\text{-}\gamma^+$ T cells was significantly increased upon stimulation with TLR agonists in cells from patients with moderate and severe COVID-19, compared with HC (all $p < 0.01$, Fig. 3A), except the TLR7 and TLR8 agonist. The TLR8 agonist did not significantly increase the frequency of $CD3^+IFN\text{-}\gamma^+$ T cells in PBMCs of severe patients, but did so in PBMCs of patients with moderate disease ($p = 0.01$) (Fig. 3A).

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 the samples of any subject group, whereas RBD gave significant increases ($p = 0.04$) in cells from patients with moderate disease compared with that seen in HC (Fig. 3B). Overall, there was no significant difference between the baseline induction of $CD3^+IFN\text{-}\gamma^+$ T cells and that seen with any TLR agonist in cells from HC, moderate and severe groups (data not shown). However, TLR3 and TLR4 agonists significantly increased the $CD3^+IFN\text{-}\gamma^+$T cell frequency in cells from moderate patients (**Supplementary Fig. S5**) compared with NSHC. Overall, there was no significant difference in the frequency of $CD3^+IFN\text{-}\gamma^+$ T cells between unstimulated and stimulated cells in PBMCs of HC, moderate and severe group (data not shown).

3.3.3. $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells

The frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells was significantly increased upon stimulation with TLR agonists in cells from patients with moderate and severe COVID-19, compared with HC (all $p < 0.007$, **Supplementary Fig. S1A**). The frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells was also significantly increased upon stimulation with the positive control PHA, in PBMCs of both patients with moderate and severe

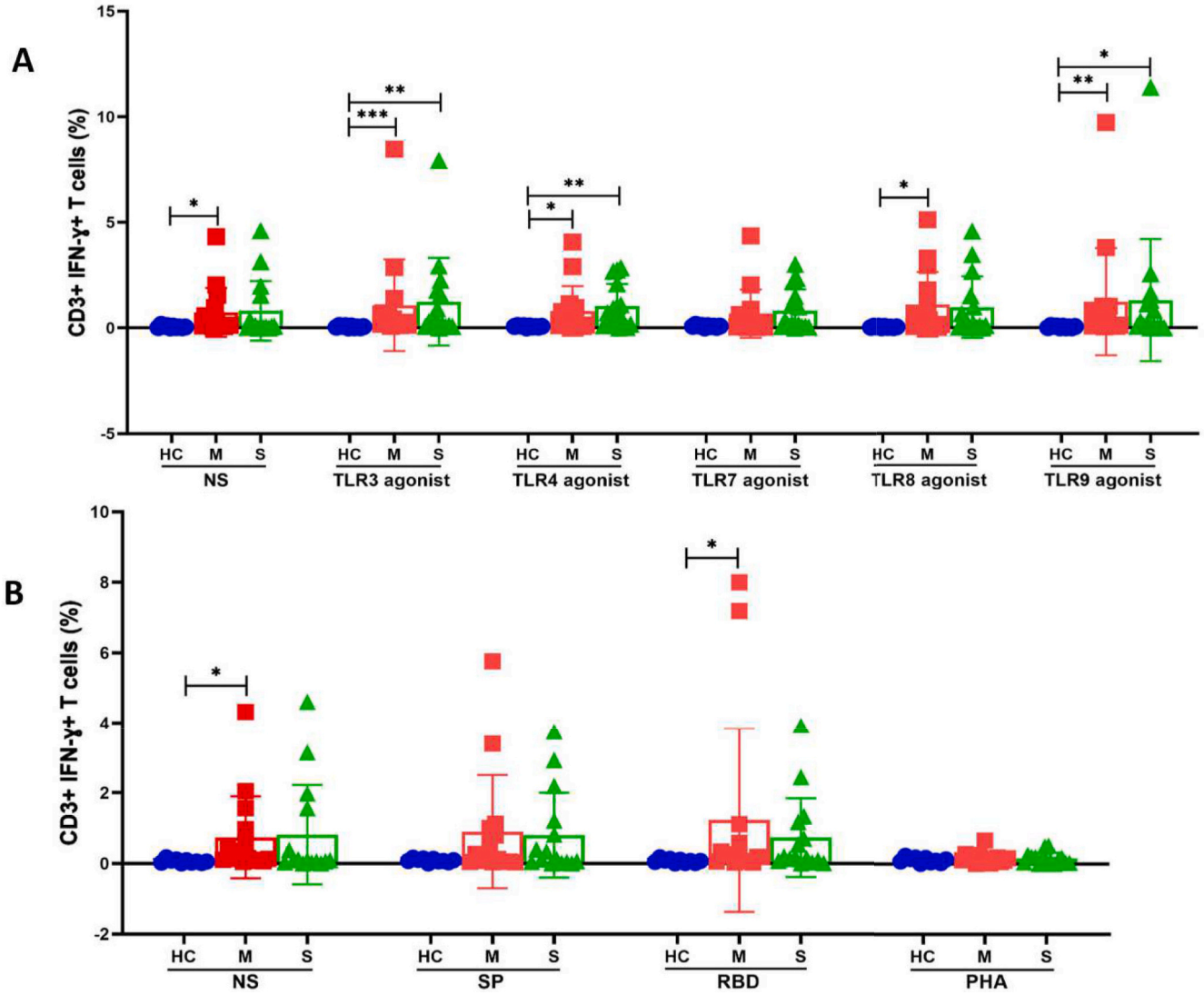

Fig. 3. Evaluation of the frequency of $CD3^+$ interferon (IFN)-γ^+ peripheral blood T cells following stimulation with toll-like receptor (TLR) agonists, spike protein (SP) and receptor binding domain (RBD) by flowcytometry. (A) The frequency of $CD3^+IFN\text{-}\gamma^+$ T cells in cells from healthy control (HC, n = 10) group and 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 (10 μg/ml Poly I:C), TLR4 (100 ng/ml LPS), TLR7 (3 μg/ml Imiquimod), TLR8 (3 μg/ml ssRNA40/LyoVec) and TLR9 (5 μM ODN2006) for 24 h and the frequency of $CD3^+IFN\text{-}\gamma^+$ cells measured. (B) The effects of peripheral blood mononuclear cells (PBMCs) stimulation by positive (5 μg/ml phytohaemagglutinin, PHA) and negative controls (NS), SP (5 μg/ml) and RBD (100 ng/ml) on the frequency of $CD3^+IFN\text{-}\gamma^+$ cells. Results are plotted as mean ± SEM and differences are determined using a one-way ANOVA and multiple comparison tests. P value <0.05 was considered statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

COVID-19, when compared to HC (both p < 0.01, **Supplementary Fig. S1B**). Moreover, RBD gave significant increases in the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in cells from patients with moderate and severe disease compared with that seen in HC (both $p < 0.001$, **Supplementary Fig. S1B**). However, stimulation with SP significantly increased the frequency of these cells, only in samples of moderate patients compared with HC group ($p < 0.0001$, **Supplementary Fig. S1B**). The highest increase of the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in samples from moderate patients, was observed with TLR9 agonist (3.82 ± 1.12%, p < 0.0001) and with TLR8 agonist (3.62 ± 1.01%, $p < 0.0001$), when compared with HC (**Supplementary Fig. S1A**). The highest increase of the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in cells from severe patients was seen with TLR8 (2.4 ± 0.61%, $p = 0.002$) and TLR7 (1.93 ± 0.62%, $p = 0.0007$) agonists, when compared with HC (**Supplementary Fig. S1A**).

When comparing the effect of the NS controls with the TLR agonists on frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells, there was no significant difference between the baseline induction of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells and that seen with any TLR agonist in cells from HC subjects (**Supplementary Fig. S6**), moderate (**Supplementary Fig. S7**) and severe patients (**Supplementary Fig. S8**). Nevertheless, the stimuli were capable of increasing the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in PBMC from moderate (**Supplementary Fig. S7**) and severe (**Supplementary Fig. S8**) patients when compared with the NSHC. Furthermore, the TLR7, TLR9 agonist, SP and RBD significantly decreased the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in cells from HC group (all p < 0.007, **Supplementary Fig. S12**), whilst TLR3, 4 and 8 agonists significantly induced the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in PBMC from patients with severe disease when compared to NS samples (all $p < 0.04$, **Supplementary Fig. S14**). However, none of the stimuli significantly increased the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in PBMC from the moderate group (**Supplementary Fig. S13**).

3.4. Real-time PCR (Q-PCR) analysis of IFN-β mRNA expression in stimulated T cells of COVID-19 patients

Since we found increased number of IFN-β-producing T cells 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 24 h. 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 cells from 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 cells from 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 cells from patients with moderate ($p = 0.0003$), but not severe disease (Fig. 4C). There was a significantly lower expression of IFN-β mRNA in $CD3^+$T cells from patients with severe compared to moderate COVID-19 (moderate vs. severe: $p = 0.005$, Fig. 4C).

The TLR8 agonist significantly increased IFN-β gene expression in cells from patients with moderate disease compared to unstimulated samples (p = 0.005, **Supplementary Fig. S15B**). However, neither the TLR8 agonist nor SP significantly increased IFN-β gene expression in PBMCs from HC subjects (**Supplementary Fig. S15A**) or patients with severe disease (**Supplementary Fig. S15C**) compared with unstimulated cells.

4. Discussion

The current study, to our knowledge, is the first study that assessed the IFN responses of COVID-19 patients' immune cells, upon in-vitro stimulation with TLR agonists and SARS-CoV-2 antigens. The results show that unstimulated cells from moderate COVID-19 patients expressed significantly higher frequencies of $CD3^+IFN\text{-}\beta^+$ T cells than either the HC and severe patient groups. After stimulation with TLR agonists, SP and RBD, the same pattern of higher frequencies of $CD3^+IFN\text{-}\beta^+$T cells was observed in the PBMCs from moderate group than in the HC and severe patient groups. TLR8 and SP induced the greatest increase in the frequency of $CD3^+IFN\text{-}\beta^+$ T cells from moderate patients, while the highest increase in the frequency of $CD3^+IFN\text{-}\beta^+$ T cells from severe patients was seen with TLR8 and TLR7 agonists. Higher levels of IFN-β from moderate group than patients with severe disease at baseline and in response to different stimuli could reflect the high rate of inborn errors in the type I IFN pathway (Tangye et al., 2023) or the presence of neutralizing autoantibodies against type I IFNs in subjects with more severe disease (Zhang et al., 2022a).

Type I interferons, IFN-α and IFN-β, are secreted upon recognition of the virus or viral proteins by PRRs, such as TLRs, and block viral replication and spread, in addition to promoting acquired immunity (Chu

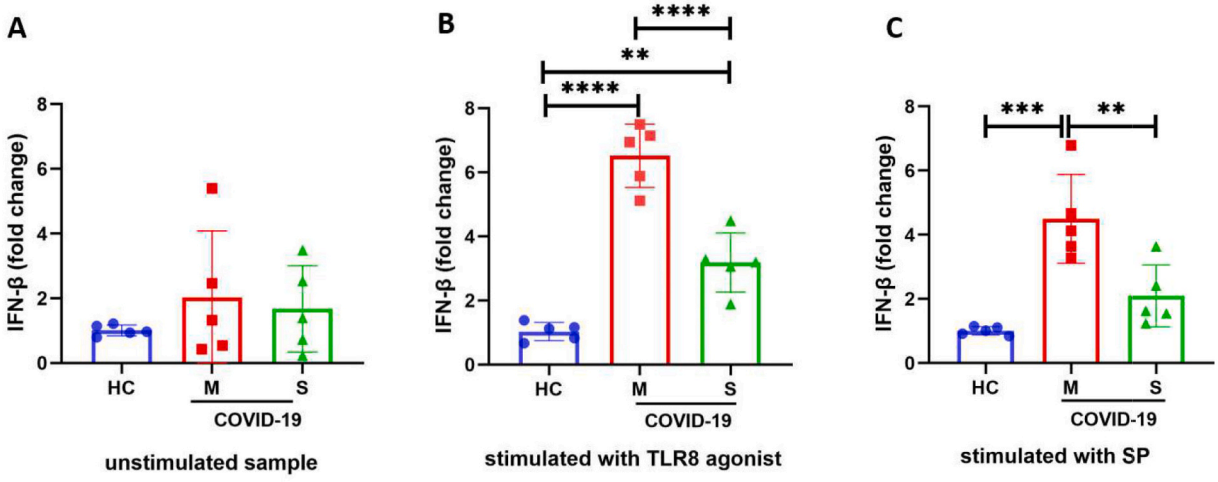

Fig. 4. Evaluation of interferon (IFN)-β gene expression following stimulation of peripheral blood $CD3^+$ T cells with the toll-like receptor (TLR8 agonist ssRNA40/LyoVec or spike protein (SP), by RT-PCR. Gene expression of IFN-β was determined in $CD3^+$T cells from 5 patients with moderate (M) and 5 patients with severe (S) COVID-19 and 5 healthy control (HC) subjects using RT-qPCR. The effect of 24 h treatment with media (unstimulated, A), the TLR8 agonist (B) and SP of SARS-CoV-2 (C) are reported as the mean ± SEM of the fold-change compared to baseline. A p value <0.05 was considered statistically significant. **p < 0.01, ***p < 0.001 and ****p < 0.0001.

et al., 2020). There are accumulating data regarding the impaired IFN responses in SARS-CoV-2 infection (Blanco-Melo et al., 2020; Park and Iwasaki, 2020). IFN expression is enhanced by TLR agonists, which would help combat SARS-CoV-2 infection (Girkin et al., 2022), as IFN-β has been reported to decrease the replication of SARS-CoV and MERS-CoV (Lu et al., 2020) and could be useful clinically in the early stages of COVID-19 (Bosi et al., 2020; Sosa et al., 2021).

IFN-γ, is related to the type II IFNs family and produced by T and NK cells. IFN-γ reduces viral replication and promotes the cytotoxic activity of T lymphocytes (Robinson et al., 2010). 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 (Gozzi-Silva et al., 2022). In contrast with type I IFNs, type II IFN genes were not detected in the pelleted cells derived from oropharyngeal and/or nasopharyngeal swabs of COVID-19 patients (Antonelli et al., 2020). 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 moderate patients (Chen et al., 2020a). In light of these findings, SARS-CoV-2 could impair the type II IFN responses in COVID-19 patients, to a higher extent in severe patients, as observed in the current study.

Evaluation of the frequency of $CD3^+IFN\text{-}\gamma^+$ T cells in the PBMCs of moderate and severe COVID-19 patients at baseline and following the same stimuli indicated that at baseline, the frequency of these cells was higher in the samples from COVID-19 patients compared with HC. Activation with TLR agonists, SP or RBD evoked a similar pattern of enhanced $CD3^+IFN\text{-}\gamma^+$ T cells in samples from 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 $CD3^+IFN\text{-}\gamma^+$ 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 (Remy et al., 2020).

The third investigated population, $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells, were significantly increased upon stimulation with TLRs agonists in cells from both moderate and severe COVID-19 patients, when compared to the HC group. Furthermore, activation of PBMCs with RBD and PHA evoked a similar pattern of enhanced $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in samples from moderate and severe patients, while SP significantly increased the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells in cells from moderate, but not severe group. As with the other two T cell populations examined here, the frequency of $CD3^+IFN\text{-}\beta^+IFN\text{-}\gamma^+$ T cells was not significantly affected by any of the stimuli used.

Evaluation of IFN-β mRNA levels among isolated $CD3^+$ T cells indicated that both the TLR8 agonist and SP stimulation upregulated IFN-β mRNA levels in samples from moderate and severe COVID-19 patients with the same pattern as observed for the $CD3^+IFN\text{-}\beta^+$ T cells. However, despite the increase in mRNA level of IFN-β in samples from moderate patients at baseline, to a higher extent than the severe group and the levels in both patients groups being higher than HC, there was no significant difference between the mRNA level across these groups. This may be due to the low number of evaluated samples. The elevated mRNA expression level of IFN-β at baseline in circulating leukocytes of COVID-19 patients was previously described (Menezes et al., 2021). Additionally, stimulation of PBMCs with TLR9 agonist was previously reported to upregulate IFN-β mRNA expression (Peng and Zhang, 2015) supporting earlier data showing IFN-β mRNA upregulation by TLR7, TLR7/8, TLR8, and TLR9 activation in cells from healthy volunteers (Ghosh et al., 2006).

Overall, in vitro stimulation of PBMCs with TLRs agonist enhanced IFN-β and to some extent IFN-γ (not as much as IFN-β) expression in cells from patients with COVID-19. Given 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.

Evaluation of laboratory markers in COVID-19 patients indicated a negative correlation between the level of CRP, LDH and frequency of $CD3^+IFN\text{-}\beta^+$ T cells in COVID-19 patients; as severe patients with significantly higher level of CRP and LDH than moderate group, showed a lower frequency of $CD3^+IFN\text{-}\beta^+$ T cells. In contrast, moderate patients had significantly higher $CD3^+IFN\text{-}\beta^+$T cells frequency upon stimulation in comparison with the severe group and demonstrated a lower level of CRP and LDH. 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 of IFN-β expression in $CD3^+$T cells from COVID-19 patients. Our 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 cells from COVID-19 patients. Moreover, it would be interesting to evaluate IFNs production by other immune cells such as dendritic cells and macrophages upon stimulation with TLRs agonists in cells from COVID-19 patients, in future investigations.

The methodology limitations of the current study include the low number of participants, especially in real-time PCR analysis, which was conducted on 15 samples (5 HC, 5 moderate and 5 severe patients), due to financial limitations. The other possible limitations of the current study are the lack of parallel measurement of cytokines in the serum of the patients and the lack of evaluation of IFN-γ mRNA expression. 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.

In conclusion, due to the impairment of IFN signaling by SARS-CoV-2, new approaches are required to limit the replication and spread of the virus and disease progression. The current study demonstrated that T cells of moderate patients are more potentiated in combat against SARS-CoV-2 in baseline and are more responsive to TLR stimulators, than the ones from severe patients. Moreover, our results provide evidence for the TLR8 agonist, ssRNA40/LyoVec, to be an inducer for more robust IFN-β responses in COVID-19, especially in cells from moderate patients, by upregulating IFN-β expression by T lymphocytes in vitro.

CRediT authorship contribution statement

Samaneh Abdolmohammadi-Vahid: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Behzad Baradaran:** Writing – review & editing, Validation, Resources, Data curation. **Armin Sadeghi:** Investigation, Methodology. **Gilina Bezemer:** Validation, Writing – review & editing. **Fatemeh Kiaee:** Data curation, Formal analysis, Methodology. **Ian M. Adcock:** Investigation, Methodology, Project administration. **Gert Folkerts:** Funding acquisition, Investigation, Resources, Writing – review & editing. **Johan Garssen:** Data curation, Resources, Writing – review & editing. **Esmaeil Mortaz:** Writing – review & editing, Writing – original draft, Formal analysis, Investigation, Methodology, Project administration, Validation.

Declaration of competing 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.

Data availability

Data will be made available on request.

Acknowledgments

This article has been extracted from Ph.D. thesis (number 31611), written by Samaneh Abdolmohammadi-Vahid in School of Medicine, Shahid Beheshti University of Medical Sciences and was supported by a grant of Shahid Beheshti University of Medical sciences, Tehran-Iran (Ethics committee approval ID is: IR.SBMU.MSP.REC.1400.715 and registration number: 14001115). We are grateful to (Clinical Research Development Unit, Imam Reza General Hospital, Tabriz University of Medical Sciences, Tabriz, Iran) for their help in accessing patients' demographic and clinical data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2024.104897.

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