Nano-Immunotherapy Accelerates Recovery of Patient with Covid-19: Clinical Analysis and Metabolomics

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Abstract. In a severe COVID-19 patient case (78-year-old male) that was under active immunotherapy for high-grade non-muscle invasive bladder cancer was followed. He received a shorter hospitalization period, as hypothesis it was suggested that active immunotherapy played a crucial role together to anti-inflammatory and antiviral treatment in precluding the evolution of pulmonary symptoms to a poor prognosis.

1. Introduction

SARS-CoV-2 infected patients exhibit the so called COVID-19 pandemic which is a respiratory disease characterized by progressive respiratory failure, and may evolve to acute respiratory distress syndrome (ARDS), severe pneumonia, sepsis and septic shock with several kinds of dysfunctions (e.g. hepatic and vascular) [1,2].

Once cells are infected by a virus, viral pathogen-associated molecular patterns (PAMPs) can be recognized by several intracellular pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs). For example, TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 play important roles in the recognition and detection of nucleic acids (RNA and DNA) specific to the viral genome (40). Some viruses can avoid recognition by TLRs and may be recognized by other intracellular PRRs present in the cytosol [3]. Despite the different types of PRRs that can be activated, the antiviral immune response following the PAMP-PRR interaction involves common signaling pathways. In general, the recognition of viral pathogens is associated with the production of several inflammatory cytokines, in particular IFNs, that have important antiviral and anti-proliferative activity [3].
Strong adaptive immune response to viral removal through IFN-γ demands early interferon type-1 IFN response. Furthermore, previous knowledge demonstrated cytokine dysregulation as a crucial attribute on SARS-CoV premature infection; the functional of CoV-cell recognition and type-1 IFN response may define the pro- or anti-inflammatory immune response and damage severity [1,4]. Then, immunomodulators efficient of controlling cytokines initial response, such as effector cells enrolment and viral liberation, might be an excellent strategy against SARS-CoV-2. A synthetic nanostructured inorganic phosphate complex associated to a glycoside protein, called OncoTherad, with immunomodulatory and antitumoral properties synthesized at the University of Campinas/ Brazil for the cancer’s treatment [5,6]. Taking into account the immunoprotective action of OncoTherad, and the function of the interferon signaling on COVID-19 infection control, it was searched the potential effect of this nano-immunotherapy on the management of SARS-CoV-2 infection in an individual diagnosed with moderate ARDS associated to COVID-19 infection.

2. Materials and Methods

2.1. Study protocol: Case Report

A 78-year-old Brazilian man enrolled on the OncoTherad clinical trial (Brazilian Clinical Trials Registry – RBR-6swqd2) to treat BCG-refractory or relapsed HGNMIBC, with history of systemic arterial hypertension, previous revascularization of myocardium and former tabagism (34 years), arrived at Hospital Municipal de Paulinia in Brazil presenting COVID-19 symptoms. The patient reported dry cough, inappetence, coryza, and malaise, which appeared right after a cruise trip. The SARS-CoV-2 diagnosis was confirmed with local RT-PCR, IgM/IgG serological rapid screening and chest CT (Figures 1; 2-a, b, c). The reported patient was the only one with symptoms and a positive test to SARS-CoV-2 from the OncoTherad clinical trial protocol.

Before the COVID-19 symptoms, the patient was diagnosed with high grade papillary urothelial carcinoma. In January 2020, due to refractory tumor, the patient was enrolled in the OncoTherad clinical trial, developed at the University of Campinas. Consecutive weekly applications of OncoTherad (one intravesical and one intramuscularly applied per week, for four weeks) were used to treat the cancer before the patient interrupted the treatment for two weeks to travel.

On April 1st, 2020, after 24 hours of flu-like symptoms reporting, the patient’s clinical conditions evolved to fever (38.3°C), headache, dyspnea (pO2= 66.4 mmHg, SatO2 = 87%, respiratory frequency = 21 bpm) and basilar crackling and stertors in the lungs. A chest CT revealed areas of ground-glass opacities on lung parenchyma with bilateral distribution and predominance on right upper and middle lobes (Figure 2-a, b, c), pointing to an evolving acute lung inflammatory process.

OncoTherad immunotherapy was resumed to treat the cancer condition with one intramuscular application. COVID-19 treatment regimen included oxygen therapy (nasal catheter flux 4 L/min), Ceftriaxone 2 g (for six days, intravenously), Azithromycin 500 mg (for six days, intravenously) and Tamiflu 75 mg (b.i.d., for five days, orally). After 72 hours of hospitalization, diminished coryza, cough and absence of fever were observed. However, the patient presented continued inappetence and fatigue on minimal exertions. Oxygen therapy was maintained, and, after the 6th day of hospitalization, a significant improvement of pulmonary inflammatory condition resulted in SatO2 = 94% without oxygen therapy. On the 8th day, oxygen therapy was removed, and the following parameters were evaluated: pO2 = 96.2 mmHg and SatO2 = 98%; decreased basilar crackling and stertors in the lungs; absence of fever, attenuated cough, and appetite enhancement. The patient was discharged on 10th day of hospitalization.

A new chest CT was performed on patient discharge and a comparative analysis with the chest CT acquired upon admission revealed areas of periphery pulmonary consolidations and fibrous stripes. Areas of ground-glass opacities were no longer observed (Figure 2-d, e, f). Moreover, treatment-related adverse events were Grade 1 or 2, such as pruritus and rash.

In this study, primary endpoints were:
a) Improvement in clinical status: evaluation through the ordinal scale of 7 categories: 1) Without activity limitation, hospital discharge; 2) Restriction of activities, hospital discharge; 3) Hospitalization without oxygen supplementation; 4) Use of a nasal O₂ catheter; 5) Non-invasive mechanical ventilation or high oxygen flow; 6) Intubation and mechanical ventilation; 7) Death;

b) Characterization of OncoTherad immunomodulatory efficacy on COVID-19 infection management and its relationship with lipid metabolism;

c) Adverse events resulting from treatment: the assessment of toxicity and adverse events was based on CTCAE (NCI Common Terminology Criteria for Adverse Events – http://ctep.info.nih.gov/reporting/ctc.html) version 4.0.

The secondary endpoints involved:

a) Time until normalization of oxygen saturation: defined as SpO₂ ≥ 94% sustained for at least 24 hours;

b) Time to fever normalization and hospitalization.

Figure 1. Rapid antibodies serological test (IgM/IgG) against SARS-CoV-2.

2.2. SARS-CoV-2 testing

The three parameters, such as, RT-PCR and rapid serological test positivity and chest CT findings confirmed the SARS-CoV-2 infection.

For RT-PCR testing, swab specimens (2 nasopharyngeal and 1 oropharyngeal) from patient were collected at day 4 and submitted to viral RNA extraction. A 25-μL reaction was set up containing 5 μL of extracted RNA, 12.5 μL of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each deoxyribonucleotide triphosphates (dNTP) and 3.2 mM magnesium sulfate), 1 μL of reverse transcriptase/Taq mixture from the kit, 0.4 μL of a 50 mM magnesium sulfate solution (Invitrogen), and 1 μg of nonacetylated bovine serum albumin (Roche). 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 s, 58°C for 30 s [1]. The SARS-CoV-2 was identified using the protocol developed by the US Centers for Disease Control and Prevention (2020), targeting the virus nucleocapsid (N) gene and the human RNase P gene as an internal control. The specimens were considered positive if both viral targets (ie, N1 and N2) showed cycle threshold lower than 40.00 [2].

2.3. Metabolic profiling using High Resolution Mass Spectrometry (HRMS) analysis

Metabolites from 20 μL of each serum were extracted by adding 200 μL of tetrahydrofuran (THF) and 780 μL methanol, followed by homogenization and centrifugation at 3400 rpm, 4°C for 5 minutes.
Ten microliters of clear supernatant were diluted in 990 µL of methanol and ionized using formic acid (0.1% final concentration). The final solution was directly injected in a HESI-Q-Orbitrap®-MS (Thermo, Bremen, Germany) and scanned with 140,000 FWHM of mass resolution on positive ion mode, using m/z range 150-1,700 and 20 mass spectral acquisitions per sample. MS parameters were set as follow: sheath gas flow rate 8 units, capillary temperature 320°C, aux gas heater temperature 33°C, spray voltage 3.70 kV, automatic gain control (AGC) at 1 × 106, S-lens RF level 50, and injection time < 2 ms [7].

2.4. Statistical analysis and pathway integration

Mass spectral data of samples upon hospitalization admission and prior to discharge were checked using the XCalibur software (v. 3.0, Thermo Scientific). A datasheet of peak intensities with m/z features versus sample replicates was generated and submitted to MetaboAnalyst 4.0 online software (https://www.metaboanalyst.ca/)[4].

Figure 2. Chest CT alterations found in case patient on hospital admission (a, b, c) and on discharge (d, e, f). Areas of ground-glass opacities on lung parenchyma with bilateral distribution and predominance on right upper and middle lobes agree with active COVID-19 lung affection (a, b, c). This characteristic is not observed after patient clinical improvement, with predominance of periphery pulmonary consolidations and fibrous stripes (d, e, f).
Mass peak intensities between both conditions (Admission/Discharge) were compared using Fold Change (FC) analysis with attributed p-values < 0.001. A threshold of 6.0 was applied to rank the most variable m/z features (log2(FC)) with the ratio Admission/Discharge and p-value < 0.001, FDR-adjusted. Metabolomic databases, METLIN (Scripps Center for Metabolomics, La Jolla, CA, www.metlin.scripps.edu) and LIPIDMAPS (Lipidomics Gateway, www.lipimaps.org), were used for compound identification with working mass accuracy lower than or equal to 5 ppm. Heat map analysis used Ward Clustering algorithm with Euclidean distance. Pathway analysis using MetaboAnalyst 4.0 was based on compound ID and homo sapiens metabolic database provided by KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/) [7].

3. Results and Discussion

It is known that when oxygen therapy is required, clinical manifestations of COVID-19 is considered severe. Upon admission, minimal exertions caused fatigue and respiratory distress to the patient due to low pO2 (66.2 mmHg) and low SatO2 (87%). Further, radiological alterations found support the characteristics of active COVID-19 in the lungs (Figure 2-a, b, c). In spite of clinical findings, patient’s evolution and recovery happen within a brief process (10 days) (Figure 2-d, e, f) than that described by former study [7]. In a Chinese study 86% of patients recruited were discharged after 16 days of hospitalization; the larger part of patients demonstrated radiological exacerbation on day 7 and amelioration on day 14, on which starts the absorption period [8].

On SARS-CoV-2 infection, the respiratory tract epithelium acts as a barrier actively modulating local immunity, but this virus provokes extensive host lipid recasting upon cell infection [9]. A 72 metabolites were classified based on m/z signals (features) ranked by -3.5<log2(FC)>3.5 and p-
value<0.001; molecules related to metabolic status on admission (33 molecules) and after clinical condition improvement and discharge (39 molecules) were identified. The metabolites were visually detached over hierarchical clustering analysis (non shown) (see similar to [7]). Additional investigation of metabolites’ relationship was grounded on the correlation network diagram created by MetaboAnalyst 4.0 software using KEGG compound ID (Figure 3-a, b). Entirely, our outcomes suggest extensive lipid dysregulation with pronounced glycerol-, glycerophospho-, sterol lipids and fatty acids metabolism disturbance. This lipid alterations have been previously discussed on HCoV-229E infected cells. Glycerophospholipids are abundant on cell membrane being transformed in lysophospholipids and fatty acids [9]. Mediators of arachidonic and linoleic acids metabolism, which play a role on inflammation were found (Figure 3-a). Beside this, patient clinical amelioration was characterized with upper levels of free fatty acids, acyl carnitines and phosphatidylglycerols, as previously reported on serum metabolome of recovered SARS-CoV patients [10].

Interesting that the metabolism of sterol- and glycerolipids is disarranged after clinical condition enhancement, particularly on respect to triacylglycerols and cholesterol esters (Figure 3-b). Macrophages activate pathogen recognition through Toll-like receptors, which stimulate early response through cytokine signaling to virus liberation and prolonged storage of fatty acids as triacylglycerols. Furthermore, macrophages exhibit CE and TG storages as lipid droplets with a decisive role in oxidized low-density lipoprotein (LDL) uptake [11]. A decreases level of LDL was associated to poor prognosis of COVID-19 patients. It was reported [12] that a decrease in serum LDL and cholesterol occurred during SARS-CoV-2 infection and progression, but, a restoration to basal levels during recovery was also observed. Still, progressive decrease on LDL and cholesterol serum levels was found in patients with poor prognosis [12]. On infection, cholesterol metabolism is accurately controlled by interferon signaling, as the host’s response to the pathogen. It was demonstrated a shift from decreasing cholesterol synthesis to increasing cholesterol cell import through a mechanistic study with influenza virus infection in macrophages [13], which in turn spontaneously engaged type-I IFN signaling and protection against the virus in mice. Remarkably, macrophages activation also causes LDL oxidation favoring its uptake [11, 13].

4. Conclusions

All data presented in this research indicate that the OncoTherad appears as important effect as immunomodulator in SARS-CoV-2 infection [14]. The ethically approval pre-clinical and Phase I/II clinical trials using OncoTherad disclosed the efficiency of this immunomodulator in the activation of innate immune system mediated by Toll-like receptors, giving onto an enhanced interferon signaling. (e.g. TLR4, TRIF, IRF-3, IFN-γ and iNOS immunoreactivities) [5, 6].

Totura et al. [15] showed that stimulation of TLR4 results in protective immune response and TRIF-related adaptor molecule -/ mice were more responsive to coronavirus infection, and manifested molecular signs like to those of SARS-CoV and MERS-CoV with poor prognosis. Moreover, SARS-CoV may obstruct on IRF-3 signaling, retarding IFN activity [4].

OncoTherad leads to the distinct stimulation of the innate immune system mediated by TLR2 and TLR4, resulting in an increased activation of the IFN signaling pathway [TLR4, TIR-domain-containing adapter-inducing IFN-β (TRIF), interferon regulatory factor (IRF)-3, IFN-α and -γ], which is associated to the superior efficacy of this nano-compound in the management of NMIBC compared to the standard treatment with BCG [3, 5, 6, 14]. More specifically, the OncoTherad-induced stimulation of the immune system via TLR2 and TLR4 occurs through the phosphorylation of hydroxylated amino acids, such as tyrosine, threonine and serine by compounds that contain phosphate salts [3, 5, 6, 14], resulting in the activation of stimulator of interferon genes (STING), with a consequent increase in the production of IFN-α and IFN-γ. The increase in the production of IFNs mediated by TLRs-2 and 4 promotes the activation of CD8+ cells, dendritic cells and M1 macrophages, culminating in the superior effectiveness of OncoTherad in the therapy of bladder cancer when compared to BCG [3, 5, 6, 14]. In 5 patients who developed severe COVID-19 while undergoing treatment for NMIBC, the administration of OncoTherad with corticosteroids and
antibiotics mitigated the exacerbated inflammatory response in the lungs, decreased the average hospital stay from 18 to 10 days, and prevented the need for intubation [8, 14]. Then, OncoTherad as an immunomodulator may represent a hopeful therapy to COVID-19 infection control and reduction of lung impairment severity.

Now an investigation with a cohort is in progress in order to understand OncoTherad immunomodulatory efficacy on SARS-CoV-2 infection management and its correlations with lipid metabolism and other found metabolites. Our data suggested that OncoTherad immunotherapy played a protective role against COVID-19 disease, preventing the infection evolution to a life-threatening condition. Then, due to the reliability shape and self-assurance of OncoTherad [5], the research of its prophylactic use or under the early stages of COVID-19 infection would be a relevant step to define the effectivity of OncoTherad for patient care in Brazilian hospitals during this pandemic.

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