Mutational analysis of ORF-8 of SARS-CoV-2 - a window to immunotherapy

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ABSTRACT

Background: TGF-Beta plays an important role in immune evasion in oncology. Similarly, SARS-Cov-2, the causal agent of the COVID-19 pandemic, also has an immune evasion function. This is mediated by ORF-8 through its interaction with multiple immune regulatory elements, including TGF-beta. This is a mutational analysis of ORF-8.

Methods: We took advantage of the database of millions of SARS-CoV-2 genomes are archived and organized in phylogenetic relationships to show the evolution of ORF-8. Site numbering and genome structure use Wuhan-Hu-1/2019 as reference. The phylogeny is rooted relative to early samples from Wuhan. Temporal resolution assumes a nucleotide substitution rate of 8 × 10^-4 subs per site per year. (https://nextstrain.org/). The epidemiological data provided at https://ourworldindata.org/coronavirus was used to determine the property of the variants using mortality and infectivity data at the site.

Results: Scan of ORF-8 revealed a high rate of mutation at aa119 and aa120. More importantly, the mutation at 120 or 119 that resulted in null ORF8 clearly delineates the pre-Delta and Delta SARS-Cov-2. In fact, all the delta lineages exhibited the null mutation at 119/120. This region is important for the dimerization of ORF-8 and possibly its interaction with host TGF-beta. All other variants, including the alpha variants, are wild type (aa120 = F). Monitoring the mutations over the last several months indicated that the delta variants have now picked up the wild type F at aa120 (Faa120) in Egypt or the L at aa 120 (Laa120) in India. The epidemiology of Egypt and India indicates that the Faa120 is more immune evasive and suggestive that more infectious but not more lethal.

Conclusions: This is an opportunity to monitor in real-time the evolution of ORF-8 and how it is interacting with the host immune system. Additionally, since our current clinical trial on TGF-beta inhibitors is in India and Latin America, it is an opportunity to correlate clinical findings to molecular and epidemiological data for these variants. If we are correct, the Faa120 will emerge as the dominant variant in the next wave of COVID-19.

INTRODUCTION

In late 2019, a novel coronavirus causing respiratory infection and pneumonia lead to a global pandemic. This virus, now termed Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) is responsible for millions of deaths world-wide. Despite efforts in vaccine development and distribution, the virus remains pervasive with continual identification of new strains recalcitrant against current vaccines. The genomic information for SARS-CoV-2 is known and has been shared. The SARS-CoV-2 genome encodes 28 confirmed proteins. Open reading frame 1ab (ORF1ab) encodes polyproteins PP1ab and PP1a which are cleaved into 16 nonstructural proteins (Nsp1 to Nsp16). Additionally, there are four structural proteins (spike [S], envelope [E], membrane [M], and nucleocapsid [N]) and eight accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, and ORF9b). Mutational conservation is highest for the Nsp polyproteins, while the genome sequence encoding the accessory factors (ORFs) diverges greatly. It is hope that study the evolution of the virus would shed light on it pathology and treatment options.

We previously proposed two central hypotheses: 1) TGF-β shuts down cellular replication and promotes viral replication and 2) TGF-β plays a pivotal role in development of life-threatening complications of COVID-19, including cytokine storm, ARDS, thromboembolic complications, and Kawasaki disease. Coronavirus entry into cells is followed by suppression of cellular replication and redirection of cellular machineries to the replication of the virus. Cell cycle arrest is centrally mediated by up-regulation of TGF-β. Suppression of TGF-β expression by OT-101 or Artemisinin effectively suppressed SARS-CoV-1 and SARS-CoV-2 replication in the viral replication assays at nanomolar concentrations. Therefore, it is most likely that induction of TGF-β following infection results in cell cycle arrest to allow for diversion of the cellular machinery to promote virus production. As the viral load increases, there would be a proportional increase in TGF-β levels which in turn would accelerate the progression of the COVID-19 disease and development of potentially lethal complications. By targeting TGF-β, Artemisinin/OT-101 would shut off the "driving engine" behind COVID-19 allowing patients to recover without developing respiratory failure.

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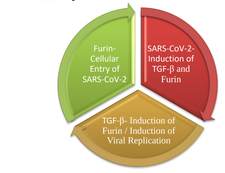
TGF-β In Covid and Cancer

TGF-beta surge previously described for influenzae virus is probably driving the pathology of COVID in particular the IgA conversion and the resulting Kawasaki syndrome noted in children infected with COVID. The multitude of downstream effects from the TGF-beta surge explain why targeting a single branch within this tree of TGF-beta cascade was not effective ie. the use of IL-6 inhibitors. Our hypothesized TGF-beta surge was recently confirmed with COVID pts. And it was reported that the use of dexamethasone suppressed this TGF-beta surge somewhat opening the possibility that dexamethasone is working through suppression of TGF-beta and there could be synergy of TGF-beta inhibitor / dexamethasone combination [Wang EY, Chen H, Sun BQ, Wang H, Qu HQ, Liu Y, Sun XZ, Qu J, Fang ZF, Tian L, Zeng YF, Huang SK, Hakonarson H, Liu ZG. Serum Levels of the IgA Isotype Switch Factor TGF-β1 are Elevated in Patients with COVID-19. FEBS Lett. 2021 May 7. doi: 10.1002/1873-3468.14104. Epub ahead of print. PMID: 33961290.].

- **SAR-CoV-2 infection upregulates TGF-β and Furin. Stukalov A et al. bioRxiv.2020.06.17.156455**
- **TGF-β locks cell cycle allowing the virus to replicate.**
- **TGF-β drives the expression of Furin- a protease required for the cellular entry of SARS-CoV-2. In well-differentiated primary human 1 bronchial epithelial cells, TGF-β1 and TGF-β2 induce expression of furin. Michael J. O'Sullivan, Jennifer A. Mitchel, Chimwemwe Mwase, Maureen McGill, Phyllis Kanki, Jin-Ah Park**
- **Furin /SARS-CoV-2/ TGF-β constitutes the positive loop that keeps spinning off TGF-β resulting in TGF-β surge**
- **TGF-β surge drives the pathology of COVID-19**
- **Nothing is more dangerous than an out of control positive feedback loop**



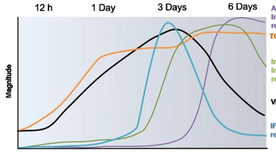


Table 1. Similarities between COVID-19 and Cancer from the perspective of TGF-β.

Cancer and COVID-19 patients are similar as inflammatory driven processes that resulted in clinical sequalae including death. The pivotal element in both COVID-19 and Cancer is TGF-beta. The similarities between the two indications are summarized in Table 1.

Direct Impact on TGF	TGF upregulation in COVID-19	TGF upregulation in CANCER
Neutrophil recruitment	TGF-β recruits neutrophils into the site of inflammation – lung of COVID pts	TGF-β recruit neutrophils into the tumors with high intra-tumor neutrophil density
Tissue Fluid Accumulation	TGF-β inhibits ENaC and causes fluid accumulation in the lung and ARDS/pneumonia	A pathologically elevated interstitial fluid pressure (IFP) is a characteristic of both clinical and experimental carcinoma.
Fibrosis	TGF-β induces late stage fibrosis compromising lung capacity	TGF-β induces fibrotic response and activation of the cancer stroma.
IL-6	TGF-β induces IL-6 leading to systemic inflammation and "cytokine storm".	IL-6 level is associated with tumor invasion level, disease severity, and metastasis
TGFBIp	TGF-β induces TGFBIp leading to vascular inflammation	TGFBIp is overexpressed in human tumors
IgA class switch	TGF-β induces IgA class switching leading to elevated IgA in COVID and IgA vasculitis in children.	The prevalence of malignancy among patients with adult onset IgA vasculitis is as high as 29-43%.

RESULTS

Method

- **Global SARS-CoV-2 genomic sequencing efforts have contributed large amounts of sequencing data from several variants into various public databases such as, GISAID and Nextstrain and NCBI SARS-CoV-2 Resources. These databases have allowed the interrogation of viral diversity with associated disease transmission in different countries.**
- **In Nextstrain, the data is organized into a phylogeny tree showing evolutionary relationships of SARS-CoV-2 viruses. The site subsamples available genome data for these analysis views with ~600 genomes per continental region (~200 prior to the last 4 months, and ~400 from the most recent 4 months) in order to display a balanced global sequence distribution. Site numbering and genome structure uses Wuhan-Hu-1/2019 as a reference and the phylogeny rooted relative to early samples from Wuhan. Temporal resolution assumes a nucleotide substitution rate of 8 × 10-4 substitutions per site per year.**
- **For each position, Nextstrain calculated the Shannon entropy of the distribution of amino acids where a score of 0 corresponds to no variation and higher scores correspond to sites with increasing amino acid diversity.**
- **Epidemiological data was obtained through https://ourworldindata.org/coronavirus**

Mutational Wave in SARS-CoV-2

Figure 1, there are high entropy residues across the SARS-CoV-2 genome.

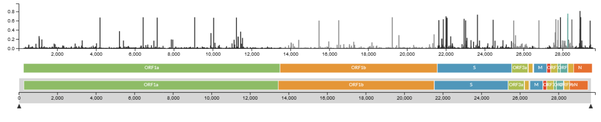
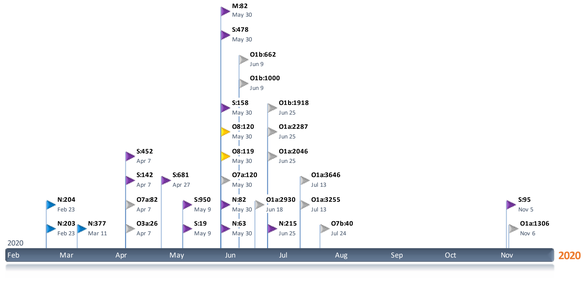


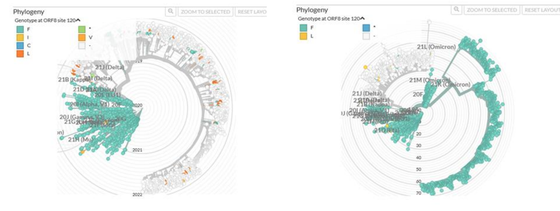
Figure 2. Residues with entropy greater than 0.6 were examined for their ability to generate offshoots from the Delta backbone. Each of the 30 high entropy residues examined was able to separate out Delta variants away from pre-Delta variants such as Alpha, Beta, and Omicron. these mutational changes occur in waves. The first wave consists of the structural proteins with the N protein leading follow by the S protein. The second wave consists of ORFs proteins with ORF8 happening early.



Mutational waves observed with SARS-CoV-2. Structural proteins are either purple (Spike protein) or blue (N and M proteins). Nonstructural ORF proteins are either in yellow (ORF8) or grey (all other ORFs). The amino acid residue and the date of divergence are indicated in text.

ORF8

- **Through interactome analysis, ORF8 has been shown to modulate various pathways including the complement and coagulation cascades and cardiovascular pathology (FDR <0.3%. ORF8, in conjunction with ORF3a, also binds to TGF-β-associated factors that suggests a functional role in inducing a pro-inflammatory state. Furthermore, multi-omics analysis highlighted the ability of ORF8 to perturb Integrin-TGF-β-EGFR-RTK signaling. Together, the dysregulation of TGF- β signaling, likely cause by ORF8 interactions, leads to common symptoms of SARS-CoV-2 such as lung fibrosis and oedema.**
- **In addition to modulating signaling cascades, ORF8 also aids in the immune evasion of SARS-CoV-2. This is achieved through downregulation of MHC-1 molecules and suppression of interferon-mediated responses within the host.**
- **We scanned each residue of ORF8 for its ability to form new clades. Only residues 27, 52, 73, 92, 119 and 120 were able to branch off into a new clade. Additionally, 52, 119 and 120 being part of the covalent dimer interface; 73 and 92 are part of the alternate dimeric interface.**
- **On submission of the abstract last year, we predicted that the Faa120 will emerge as the dominant variant in the next wave of COVID-19.**
- **Figure 3 confirmed our prediction fast forward to today (right panel).**



CONCLUSIONS

1. **ORF8 protein is abundantly secreted as a glycoprotein in vitro and in patients with newly diagnosed SARS-CoV-2. The levels of ORF8 protein in the blood correlate with disease mortality in patients with acute infection, and fatality in hospital patients is associated with higher serum levels of ORF8. Glycosylated ORF8 stimulates PBMCs to produce SARS-CoV-2 specific (IL1b, IL6, IL8) cytokines but not IL2. ORF8 induces proinflammatory cytokines through activation of NLRP3-mediated inflammasome pathways.**
2. **SARS-CoV-2 interaction with TGF-β caused dysregulation of NK functions. Although TGF-β is thought to be an suppressed excessive immune response to the virus and its suppression would be detrimental to COVID-19 patients, we have proven this to be incorrect and that suppression of TGF-beta is a therapeutic option against the virus and it has been demonstrated that an untimely early production of TGF-β and associated NK cell dysfunction is a hallmark of severe SARS-CoV-2.**
3. **Our TGF-β antisense molecule, OT-101, has completed a phase 2 clinical trial against hospitalized SARS-CoV-2 patients with promising efficacy data. At the same time, Artemisinin, a known inhibitor of TGF-β was tested in our ARTI-19 clinical trial in India with similarly good clinical efficacy. When added to SOC, Artemisinin accelerated the recovery of patients. [35]. The data from these clinical studies indicate the targeting of TGF-β as a feasible strategy in aiding the recovery of patients with mild-moderate SARS-CoV-2.**
4. **The availability of large number of sequences spanning years into this pandemic present us with a unique opportunity to gain insight into how COVID evade immune system through TGF-beta signaling.**
5. **This knowledge should be useful in cancer immunotherapy given the similarity between the COVID and cancer**