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Analysis of Single Cell RNA-seq Data Revealed Interferon Gamma Signaling Alteration in Severe COVID patients

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Analysis of Single Cell RNA-seq Data Revealed Interferon Gamma Signaling Alteration in Severe COVID patients

A Thesis Submitted to

The Biotechnology Master's Program

In partial fulfillment of the requirements for

The degree of Master of Science

By: Ahmed Adel El-Baz

Under the supervision of:

Dr. Hassan Azzazy

American University in Cairo

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Dedication

I dedicate this research to my family members for their efforts and support along the process of my course of study.

Acknowledgments

First and foremost, I am incredibly grateful to my supervisor, Prof.Azzazy, for his invaluable support, patience, and advice during my MSc study. He has the substance of a genius: he convincingly encouraged me to be motivated, professional, and do the right thing even when I face challenging times. Without Dr.Azzazy's persistent help, the goal of this project would not have been realized. I would like to thank Ahmed Safwat Abouhashem for his unlimited support for me working on this project. He spent much effort and time providing a friendly and cooperative work environment. Ahmed was the one who advised me to join the program, and he was behind any single success during this journey. Special thanks to the American University in Cairo and CytoTalk LLC for providing me with the required learning environment during my course of study. My thanks are extended to my colleague Ahmed Yamany for his valuable encouragement. I would also like to acknowledge Manar El-Naggar, Fatma Z. Gharbia, Yomna Moqidem and Kanhaiya Singh for their contribution. Last but not least, I would like to thank my family for always being beside me. For you all, I dedicate this thesis which I wish to help in overcoming the pandemic very soon.

Abstract

Background: SARS-COV2 virus detected in December 2019, and was considered a pandemic in March 2020 by the WHO. Symptoms range from asymptomatic to life threatening ones. Studying cell-cell interactions in patients' blood samples may lead to novel diagnosis and treatment approaches.

Aim: This study aims to analyze single-cell RNA sequencing data to identify differences in cell-cell communications between healthy and COVID patients and differentially expressed T-cells genes that contributed to immune system antiviral activity.

Materials and methods: Single-Cell RNA sequencing data from seven COVID patients and five healthy individuals were collected from (GEO accession GSE155673). Cell types were identified and cell-cell interactions were inferred for each condition (healthy, moderate and severe COVID patients). Additionally, T cells differentially expressed genes between the three conditions were identified and pathways enrichment were performed.

Results: Eight cell types were identified. Percentage of T cells decreased from 32.76% in healthy individuals to 16% in severe COVID cases. Cell-Cell interactions analysis revealed significant alterations among healthy, moderate, and severe conditions such as reduction of overall incoming signaling in T cells of severe cases. Additionally, SN signaling pathway was identified only in COVID cases, which in turn was found to be in IFN-γ reduction in distinct cell types. Pathways enrichment analysis identified IFN-γ signaling to be upregulated in moderate cases, and to be downregulated in severe ones. Protein interacting with IFN-γ also shows downregulation such as IRF1. However, the negative regulator of IFN-γ -SOCS3- was upregulated in COVID patients T cells.

Conclusion: Cell-cell interactions alteration in COVID patients might have resulted in eliciting improper immune response. Not only, did T cells percentage decreased in severe COVID cases, but also T cells overall incoming

signaling was decreased. Additionally, cell-cell interaction alteration might have played a significant role in suppressing antiviral response through IFN-γ reduction which might contribute to the observed severity of COVID cases.

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List of abbreviations

ALCAM	Activated Leukocyte Cell Adhesion Molecule
BKV	Bk Virus
CCA	Canonical Correlational Analysis
cDNA	Complementary Di-Nucleic Acid
CHIKV	Chikungunya Virus
CITE-seq	Cellular Indexing of Transcriptomes and Epitopes by Sequencing
CMP	Common Myeloid Progenitor
DC	Dendritic Cell
EBOV	Ebola Virus
ECM	Extracellular Matrix
EM	Ensemble Mean
FDA	Food and Drug Administration
GAS	Gamma-Activated Sequence
HLA	Human Leukocyte Antigen
HLH	Haemophagocytic Lymphohistiocytosis
HPCA	Human Primary Cell Atlas
HSC	Hematopoietic Stem Cell
HSV	Herpes Simplex Virus Type
ICU	Intensive Care Unit
IFITM1	Interferon-Inducible Transmembrane Protein1
IFN	Interferon
IFNGR	Interferon Gamma Receptor
IPA	Ingenuity Pathways Analysis
IRF	Interferon Regulating Transcription Factor
ISG	Interferon-Stimulated Gene
ITGA4	Integrins Alpha-4

JAK	Janus Protein Tyrosine Kinase
KNNs	K-Nearest Neighbors
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NIH	National Institution of Health
NK	Natural Killer
NSP	Non-Structural Protein
ORF	Open Reading Frame
PCA	Principle Component Analysis
PRR	Pattern Recognition Receptor
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RELMs	RESISTIN-Like Molecules
RRV	Ross River Virus
RSV	Respiratory Syncytial Virus
SARS-COV2	Severe Acute Respiratory Syndrome Coronavirus 2
scRNA-seq	Single Cell Ribonucleic Acid Sequencing
SIGLEC1	Sialic Acid-Binding Immunoglobulin Ig Lectin-1
SNNs	Shared Nearest Neighbor
SOCS	The Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
TCR	T-Cell Receptor
TLR4	Toll-Like Receptor 4
TNF	Tumor Necrosis Factor
TSNE	T-Distributed Stochastic Neighbor Embedding
VTE	Venous Thromboembolism
WHO	World Health Organization

CHAPTER 1: Introduction

1.1 COVID

1.1.1 Current Status

More than 150 million confirmed cases of COVID-19 disease (with more than 79 thousand daily new cases are reported as of 1st May 2021), and more than three million deaths were reported as of April 2021 [https://covid19.who.int]. According to Johns Hopkins Coronavirus Resource Center, India and Brazil reported the highest cases since March 2021 [https://coronavirus.jhu.edu/]. Due to the pandemic, the world economies are struggling even after more than one billion individuals are vaccinated (from which more than 200 million are fully vaccinated) with Food and Drug Administration (FDA) emergency authorized vaccines [https://www.fda.gov].

COVID-19 disease common symptoms include fever, fatigue, dry cough, headache, sore throat, hemoptysis, and diarrhea (Huang et al. 2021). Most patients are asymptomatic or show mild symptoms, while others show moderate to severe symptoms (Tang et al. 2021). Individuals with risk factors (old age, obesity, or diabetes) develop a cascade of acute biological events that may lead to hospitalization and the need for ventilation (Laforge et al. 2020). Covid-19 has led to several complications, including lung injury, cardiac injury, myocardial damage, and ischemia (Bénard et al. 2021, Garcia-Beltran et al. 2021),

1.1.2 SARS-CoV2

SARS-CoV2 (similar to SARS-COV, MERS-COV) is a single-stranded positive RNA virus (Chen et al. 2020b). It belongs to the Orthocoronavirinae subfamily(Pal et al.), which causes coronavirus disease that became a global pandemic and health emergency in 2019 (Dong et al. 2020, Huang et al. 2021). Coronavirus-2 has four structural proteins: envelope (E) protein, membrane (M) protein, nucleocapsid (N) protein, and (S) trimeric protein, which consists of two main subunits (S1) and (S2) (Ou et al. 2020).

1.1.3 Classification of COVID-19 disease severity

The WHO proposed a classification of Covid-19 patients that includes three main severity groups. The first group comprises Critical COVID-19 patients who mainly require life-sustaining therapies provisions such as vasopressor therapy or mechanical ventilation, with criteria of ARDS (Acute respiratory distress syndrome) and lung injuries. The second group is severe COVID-19 patients related to low oxygen saturation and respiratory rate levels and severe respiratory distress symptoms. On the other hand, non-severe COVID-19 patients as the third group have none of the critical and severe defined criteria. [https://www.who.int/]

1.1.4 Treatment and management

There is an urgent need for novel treatments of COVID-19 disease in its early and late stages (Bose et al. 2020). Treatment and management should rely on the onset of infection and disease severity, which correlates with clinical symptoms and complications. For outpatient management of acute COVID-19 infected cases, the U.S. National Institution of Health (NIH) guidelines recommended treatment plans according to patient's vital signs, physical examinations, and risk factors for disease progression. Anti-pyretic, analgesics, and anti-tussive drugs are used to relieve symptoms like fever, headache, and cough for outpatient cases' management. Rest is required in the early stages of infections, and then other activity forms should be increased during recovery. In mild to moderate COVID-19 outpatient cases with a high risk of developing severe progression, NIH guidelines recommended using a combination of anti-SARS-CoV2 monoclonal antibodies. The two FDA approved combinations of anti-SARS-CoV2 monoclonal antibodies are bamlanivimab plus etesevimab or casirivimab plus imdevimab. These combinations are highly recommended for outpatient COVID-19 cases with higher risk of clinical progression. However, with patients who received the antibody combination therapy,

vaccination should be deferred for at least 90 days after treatment; to avoid the disease progression resulted from antibody and vaccine interference [https://www.nih.gov/].

For the hospitalized COVID-19 patients, the only drug approved by the FDA is the antiviral Remdesivir in addition to supplemental oxygen (Eastman et al. 2020). Moreover, anticoagulant drugs are recommended for COVID-19 hospitalized cases with clinical indications of developing venous thromboembolism (VTE) or arterial thrombosis in COVID-19 cases. Despite the recommendations of WHO to use low doses of anticoagulants to prevent thromboses, there is no sufficient evidence for the requirement for prophylactic doses with hospitalized COVID-19 cases (Tiwari et al. 2020). Due to lack of sufficient data about safety and efficacy, the NIH guidelines recommended not to use Antibacterial therapies like Azithromycin with or without chloroquine against COVID-19 disease in outpatient or hospitalized cases [https://www.nih.gov/]. Thus, current interventions and guidelines for COVID management are related to isolation, and supportive medicine for infected patients, besides symptomatic and respiratory failure management in severe cases (Marini and Gattinoni 2020).

1.1.5 Vaccination against COVID-19 disease

Safe and effective vaccines for COVID-19 infection are crucial to building antiviral protection and very useful weapons to stop the pandemic (Menni et al. 2021). Different vaccine types show numerous immune-system stimulated responses. An effective vaccine should trigger the immune system to produce memory T-cells and B-cells (Teijaro and Farber 2021). Memory T-cells and memory B-cells are essential components of protective immunity against viral infection. So, the immune system will gain the ability to fight against future viral infections more precisely and effectively (Cox and Brokstad 2020). Different COVID-19 developed vaccines could be categorized according to the mechanism of triggering a future viral recognition by the immune cells, such as mRNA vaccines that contain specific genetic particles from the SARS-CoV2 virus. The viral genomic particles trigger the immune system to produce harmless viral proteins, stimulating the formation of memory T-cells and B-cells that will activate adaptive immune responses against the virus

(Teijaro and Farber 2021). Also, a formation of memory T-cells and B-cells could be gained via protein subunit-based vaccines, including harmless viral proteins or protein fragments (Pollet et al. 2021). A viral vector is a modified version of different virus that contains SARS-CoV2 genetic materials. The viral vector-based vaccines also activate specific antiviral immune responses (Lundstrom 2021).

No FDA-approved vaccine prevents COVID-19 as of 10th May 2021 [https://www.fda.gov/]. Despite that, the FDA had authorized the emergency usage of different vaccines such as Pfizer-BioNTech, Moderna and Johnson, Johnson / Janssen, Novavax, and Vaxzevria (formerly COVID-19 Vaccine AstraZeneca) (Singh and Upshur 2021). The types of currently FDA authorized vaccines are represented in Table 1. According to the Centers for disease control and prevention (CDC), everyone 12 years old or older is recommended to be vaccinated the first available vaccine from the currently authorized and available vaccines [https://www.cdc.gov].

Table1: FDA authorized vaccines against COVID-19 for emergency use.

Company	Type	Doses	Storage
Oxford Uni-AstraZeneca	Viral vector	Two doses	2°-8° C
Pfizer - BioNTech	mRNA based	Two doses	-70° C
Moderna	mRNA based	Two doses	-20° C
Novavax	Protein based	Two doses	2°-8° C
Janssen	Viral vector	One dose	2°-8° C

1.2 Immune response

1.2.1 Immune response against viral infection

The innate and adaptive antiviral immune responses play critical roles against viral invasions through cellular components, immune molecules, and intrinsic blood components (Huang et al. 2021). The immune system antiviral response alteration significantly affects disease severity (Ren et al. 2021). Innate immunity is the host's first line defense mechanism against viral infections (Dzananovic et al. 2018). The innate immune response could be activated within minutes to several hours after pathogen recognition. Depending on the type of activated cells after the pathogen infection, the innate immune response could be further classified into an immediate innate immune response or early induced innate immune response. In most RNA viruses like coronaviruses, the innate immune response is activated by viral antigens recognition with pattern recognition receptors (PRRs). PRRs are expressed by the innate immune system cells like monocytes, neutrophils, and epithelial cells (Iwasaki and Medzhitov 2015). During innate immunity response, infected cells produce cytokines like interferons (IFNs) molecules, especially The IFN-α (alpha) and IFN-β (beta), which act as signaling molecules to activate other immune cells surrounding those who were infected (Rao et al. 2020).

1.2.2 Immune response in COVID-19 patients

Immune responses against SARS-CoV2 include hyper-inflammatory responses involving CD4+ T-cells and CD8+ T-cells differentiation and reactivity with viral antigens (Azkur et al. 2020, Catanzaro et al. 2020). CD4+ T-cells responses were directed against S, M, and N proteins and partially against non-structural protein 3 (nsp3), nsp4, and open reading frame 8 (ORF8) (Grifoni et al. 2020). Also, CD8+ T-cells responses were significantly reactive with antigens like nsp6, ORF3a, and the N protein (Grifoni et al. 2020).

Within a specific period of unsuccessful elimination of the pathogen by the innate immune system, the host activates adaptive immune responses against the invasion (Rao

et al. 2020). During the adaptive immune response, the antigens are translocated to the lymphoid organs where B-Cells and T-Cells recognize them. B- and T-Cells become activated through released cytokines and differentiate into effector cells (Chaplin 2010, Marshall et al. 2018, Ghaffari et al. 2020).

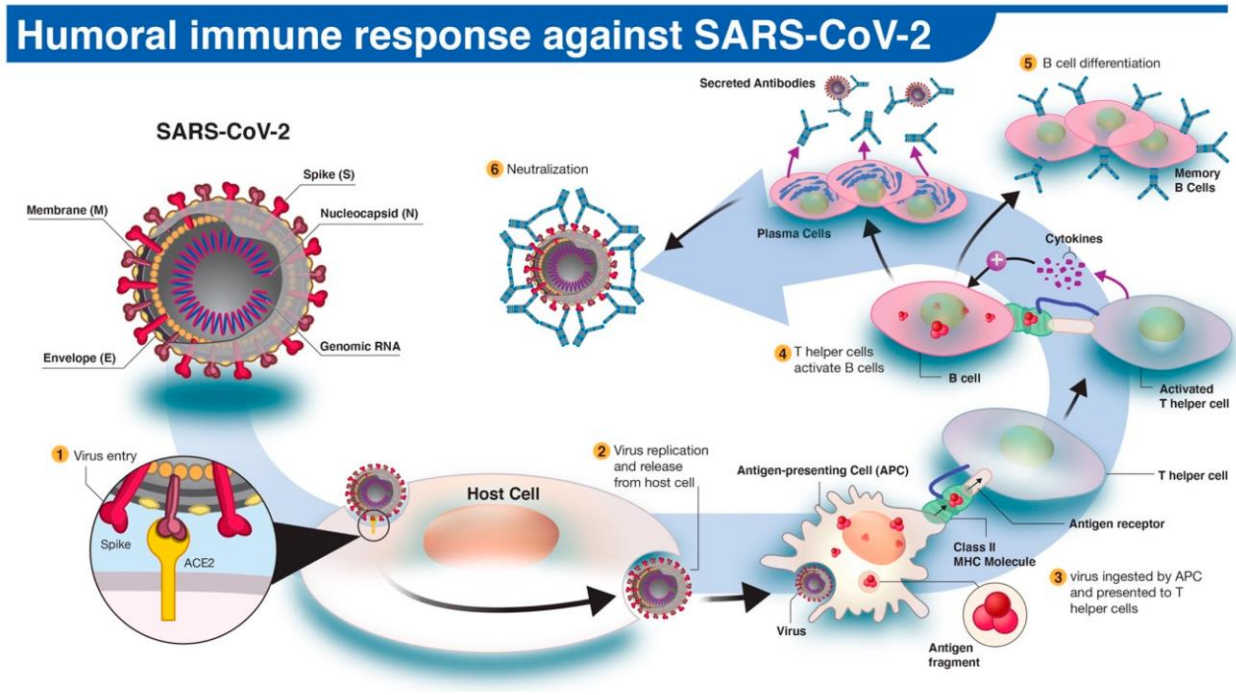


Figure 1. Schematic diagram of SARS-CoV2 activation of host adaptive immune response. SARS-CoV2 molecular structure, (M) membrane proteins, (S) spike proteins, (N) nucleocapsid proteins, (E) envelope proteins, and viral genomic RNA. (1) SARS-CoV2 viral entry to the host cell through interaction between viral (S) proteins and the angiotensin-converting enzyme inhibitor 2 (ACE2) receptors of the host cell. (2) Viral replication and release from the host cells. (3) Antigen fragments presentation to T helper cells by the antigen-presenting cells like macrophages and dendritic cells (DCs) after viral engulfment and digestion. (4) Activated T helper cells release cytokines that activate B cells. (5) Activated B cells differentiation into plasma or memory B cells with high-affinity binding receptors to the SARS-CoV2. (6) Plasma B cells secrete IgM, IgG, and IgA antibodies that mediate neutralization when bind to SARS-CoV2 antigens and prevent viral entry into host cells (Ghaffari et al. 2020).

1.2.3 Effect of Disease Severity on eliciting immune response

Crevia et al., 2021 suggested four antibody response grades depending on COVID-19 disease severity include (1) mucosal antibody response (IgA) during mild cases that

show few symptoms of COVID-19 disease; (2) delayed systemic IgA and IgG production in mild to moderate cases; (3) elevated serum levels of IgA and IgG in severe cases; and (4) critical COVID-19 cases shown very high serum titers of IgA and IgG (Cervia et al. 2021). The high titers of SARS-CoV2 specific antibodies are consistent with higher B-cells expression of the genes encoding for the constant regions of immunoglobulins IgA1, IgA2, IgG1, or IgG2 (Ren et al. 2021). Furthermore, serological analysis of innate and adaptive immune responses reported that the highest antibodies' levels were observed in severe COVID-19 patients (Carsetti et al. 2020). Innate and adaptive immune responses were reported to be related to disease severity. Other factors like age, obesity, and diabetes may also alter immune responses against COVID-19 disease. Elderly patients showed decreased production of IFNs, which led to altered immune responses (Sridharan et al. 2011, Rao et al. 2020). Also, significantly higher antibodies' responses were detected in severe COVID-19 cases than in mild ones (Rao et al. 2020, Zhang et al. 2020b, Cervia et al. 2021, Garcia-Beltran et al. 2021). Previous studies described differences between COVID-19 disease stages in peripheral immune cells (Su et al. 2020, Ren et al. 2021). Such differences revealed novel biological signatures of disease severity, such as dysregulation of JAK/STAT, MAPK/mTOR, and NF-.B immune signaling networks (Feyaerts et al. 2021).

Multi-omics of 139 COVID-19 patients significantly correlated a loss of specific metabolite classes and metabolic processes to the shift from mild to moderate disease state. The same study reported emerging and amplifying multiple unusual immune cell phenotypes due to disease severity (Su et al. 2020). Moreover, immune system responses to COVID-19 disease severity are accompanied by changes in B-cells subsets frequencies, subpopulation, and differentiation (Sosa-Hernández et al. 2020, Su et al. 2020). Another extensive analysis of 32 COVID-19 patients' immune responses reported a significant increase in mature natural killer (NK) cells, low T-cell numbers, and exhausted T-cell overexpressed mucin domain-3 (TIM-3) (Varchetta et al. 2021). Henry et al. (2020) described the prediction of in-hospital mortality via neutrophil to lymphocytes ratio, which is significantly increased in critical COVID-19 patients. Thus, altered immune responses due to COVID-19 severity with the contribution of the cytokine storm can be detrimental

and cause immune-mediated tissue injury (Song et al. 2020, Ren et al. 2021). Much work has been aimed to clarify the clinical characteristics (Chen et al. 2020a) and virological features (Lu et al. 2020, Wu et al. 2020). However, little is known about COVID-19 severity association with patients' immunological and inflammatory profiles.

1.2.4 T-cells and cytokines production

Two types of T lymphocytes are involved in the adaptive immune response: CD8+' killer' or 'cytotoxic' (CD8+T_c) cells and CD4+' helper' (CD4+T_h) cells (Kos and Engleman 1995). CD8+T_c cells can directly destroy the pathogen-infected cells and recruit different types of immune cells through cytokine signaling. CD8+T_c cells recognize specific viral antigens with their expressed T-cell receptors (TCRs). An infected cell displays the antigen on its surface with class I major histocompatibility complex (MHC), which recruits the binding with a specific type of CD8+T_c cell TCR. This binding activates the cytotoxic role of CD8+T_c cells (Varela-Rohena et al. 2008). CD4+ T_h cells bind with class II MHC on the antigen-presenting cell. An important function of CD4+ T_h cells is to recruit B-cells (lymphocytes that produce antibodies). Also, CD4+ T_h cells release cytokines that activate CD8+ T_C cells and their proliferation (Oxenius et al. 1998).

The excessive level of released cytokines (cytokine storm) in response to SARS-CoV2 infection is related to COVID-19 severity complications, hospitalization, and even mortality (Skinner et al. 2019, Sanli et al. 2021). Patients with the severe progression of COVID-19 showed signs of hyperinflammatory secondary hemophagocytic lymphohistiocytosis (HLH) syndrome. This syndrome is accompanied by a fatal cytokine storm and multiorgan failure (Skinner et al. 2019, Ruan et al. 2020). Significant increases in circulating cytokine levels such as TNF-α, IL-6, IL-2, IL-7, IL-10, TNF-a, G-CSF, IP-10, MCP-1, and MIP-1A were reported among the more severely COVID-19 patients compared to mild and moderate cases (Huang et al. 2020a).

Effector T-cells are vital for an effective immune response against viral infections. Despite that, recent studies showed relatively low circulating T-cell count in COVID-19

patients (Diao et al. 2020a, Zhang et al. 2020a). Cytokine storm and the marked reduction in T-cells counts, growth, and viability might be highly correlated to COVID disease fatality (Kang et al. 2021). However, T cells' number and functional state in COVID-19 patients need to be more investigated (Diao et al. 2020b).

1.2.5 Interferons

Interferons (IFNs) are autocrine and paracrine secreted proteins. IFNs regulate several intercellular and intracellular molecular mechanisms, like tumor cell fate, innate and adaptive antiviral immune responses (Ahmed and Xiang 2011). The main three types of IFNs are type I, II, III, which are structurally and functionally different in humans (Hoffmann et al. 2015). Type I interferons consist of IFN-α, β, ε, κ, and ω and are produced by infected cells and the immune system (Ivashkiv and Donlin 2014). Type II is also produced by immune cells; however, it includes only one member, IFN-γ. Type III interferons consist of 3 subtypes of IFN-λ, produced by some immune cells and cells in the developmental pathway of epithelial cells or epithelial cells themselves (Lazear et al. 2019). IFNs were initially described as molecules that interfere with viral replication (Seo and Hahm 2010). The three types activate distinct STAT (signal transducer and activator of transcription) complexes in addition to several signaling cascades, such as immune response signaling pathways (Figure 2) (Takeuchi and Akira 2010, Schoggins et al. 2011, Garcia-Diaz et al. 2017). IFNs activate cellular interactions among other immune cells such as macrophages and natural killer cells (Huang et al. 2019).

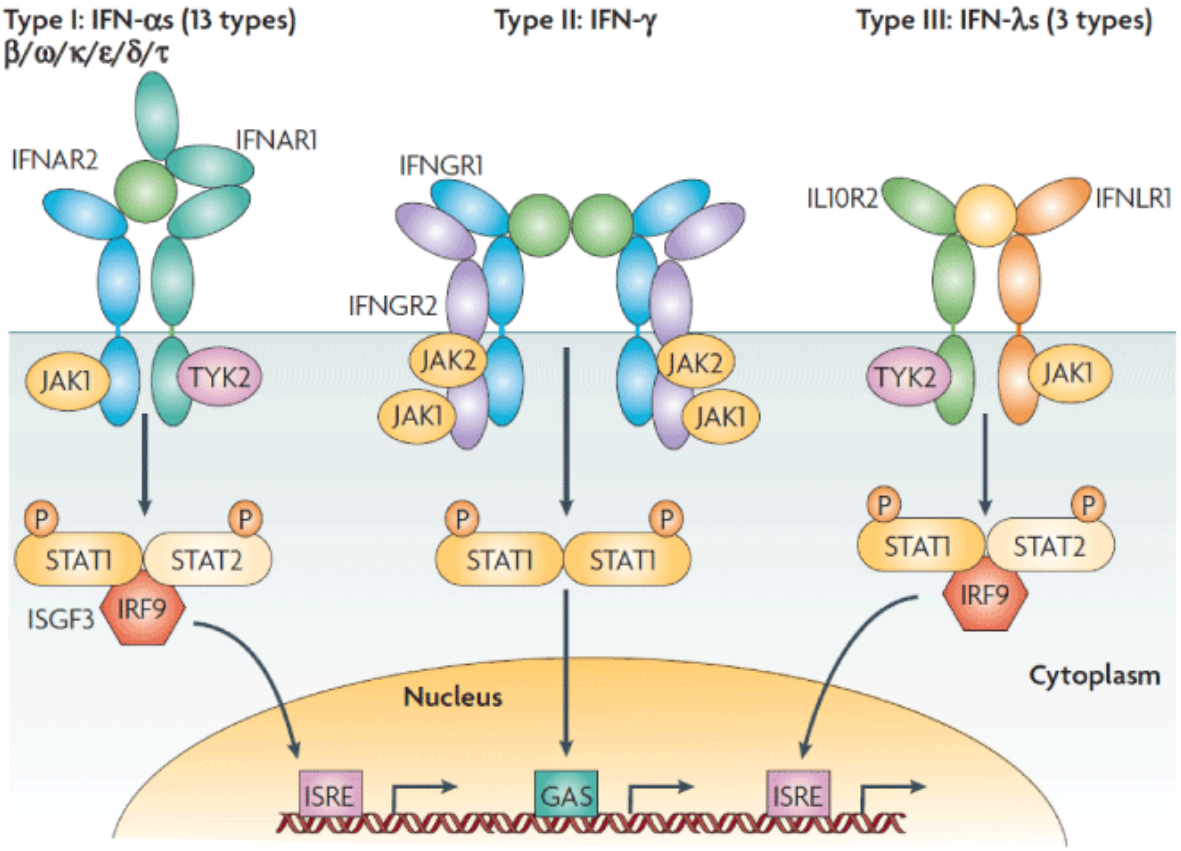


Figure 2. Schematic diagram of the three types of interferons (I, II and III) receptor activation signaling pathways. Type I interferons (IFNs) include 13 types in mammalian cells such as (α, β ω, κ, ε, δ, and, τ) that interact with IFN receptor 1 (IFNAR1) and IFNAR2. Type II has only IFN-γ that interacts with IFN-γ receptor 1 (IFNGR1) and IFNGR2. Type III IFN-λs include three types that interact with IFN-λ receptor 1 (IFNLR1) and interleukin 10 receptor 2 (IL10R2). Type II IFN-γ binding to IFNGR1 form a complex which is stabilized by two IFNGR2 chains. The IFNs binding to IFNs receptors causes confirmational changes associated with two kinases from JAK family: JAK1 and TYK2 for IFNs type I and III; JAK1 and JAK2 for IFN-γ. Phosphorylated STAT1/2 bind to (GAS) interferon-gamma activated site. (IRF9) interferon regulatory factor 9; (ISGF3) interferon-stimulated gene factor 3; (ISRE) IFN-stimulated response element ;(P) phosphate; (STAT1/2) signal transducer and activator of transcription 1/2 (Zhang 2017).

1.3 Single-Cell RNA Sequencing

The first single-cell transcriptome analysis based on next-generation sequencing was published in 2009 (Tang et al. 2009). In recent years, single-cell RNA sequencing (scRNA-seq) had advanced our knowledge of biological systems as it opened the gate for

studying cellular heterogeneity (Plass et al. 2018) and discovering previous hidden cellular populations (Montoro et al. 2018). scRNA-seq inspired computational biologists to evolve a wide range of analysis tools (Rostom et al. 2017). Accurate gene expression differences' assessment between individual cells helped identify more cellular populations and cell-cell communications that cannot be detected from pooled cells' analysis (Shaffer et al. 2017).

Several protocols are generating transcriptomic information from an isolated sample. The general scheme incorporates the following steps, single-cell isolation, complementary DNA (cDNA) library construction, and sequencing (Rostom et al. 2017, Vieth et al. 2017). 10x Genomics is a Droplet-based method that involves capturing each single cell from the sample in a microfluidic droplet. Computational methods are being applied to overcome detecting and excluding stressed cells or droplets containing more than one cell. Each droplet contains the necessary enzymes and chemicals to reversely transcribe the mRNAs for each cell while labeling them with a unique barcode of 16 nucleotides. Next, all cDNAs are pooled together for sequencing (Olsen and Baryawno 2018).

1.4 Cell-cell Interactions

Cell to cell communications via soluble and membrane-bound factors is fundamental for many cellular decisions, such as activating the cell cycle, programmed cell death, and immune response pathways against pathogens (Hermanowicz et al. 2020). Moreover, ligand-receptor interactions mediate other physiological and pathological signaling like cell adhesion, cellular recognition, and communication (Zhang et al. 2021). These regulated communications significantly impact many disease severity like cancer, autoimmune, and viral infection-related diseases (Oviedo-orta et al. 2000, Fruman and Walsh 2007, Rouse and Sehrawat 2010, Oktay et al. 2015). Thus, the coordination between cellular activities during cell infection by microorganisms directly affects the multicellular response, starting from the initial sensing of infection, mediated by innate PRRs, including Toll-like, RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors (Takeuchi

and Akira 2010). Continuously, the intracellular signaling cascade following this recognition triggers the expression of inflammatory mediators that act as signaling molecules in the process of pathogen elimination (Takeuchi and Akira 2010).

Moreover, cell-cell communications between immune cells such as macrophages, DCs, T-cells, and others are essential for the induction of antiviral responses through innate and adaptive immune responses, as well as the prevention of undesired immune system effects like hyperinflammatory lung disease in the case of COVID infected patients (Melenotte et al. 2020). So far, available treatment options for severely diagnosed COVID-19 patients are limited (Bénard et al. 2021). Thus, understanding such communications may lead to a better understanding of the disease progression, leading to better diagnosis, treatment, and disease prevention. Fortunately, single-cell RNA-seq data analysis approaches open the gate for closer insights into cell types and cellular differentiation trajectories. Several approaches have been developed recently to infer cell-cell communication from scRNA-seq data (Kumar et al. 2018, Raredon et al. 2019, Wang et al. 2019, Browaeys et al. 2020, Ren et al. 2020).

CHAPTER 2: Hypothesis and Objectives

2.1 Hypothesis:

The clinical picture of COVID-19 patients is ranging from developing mild and moderate symptoms to severe ones. The immune response in severe cases is believed to be altered, and patients suffer from serious symptoms accordingly. The interaction between blood immune cells plays a vital role in eliciting an efficient immune response to defeat infections. However, immune system alteration might happen in severe COVID-19 cases resulting in life-threatening symptoms.

Therefore, it is hypothesized that single-cell RNA sequencing data could identify blood cell-cell interactions in healthy individuals and the alteration that happened to COVID-19 patients in moderate versus severe cases to identify the proper immune response versus the alteration that happened in severe cases.

2.2 Objectives:

1. To cluster blood cells from healthy and COVID-19 patients and to identify cluster markers.
2. To assign cell types to the identified clusters.
3. To identify incoming and outgoing cell-cell interactions among blood cells.
4. To identify disruption in cell-cell interactions in COVID-19 cases compared to healthy individuals
5. To identify the differentially expressed genes in T cells from healthy and COVID-19 patients
6. To analyze pathways activity in T cells in healthy and COVID-19 patients.

CHAPTER 3: Materials and Methods

3.1 Data source

The data (12 RNA-seq samples) were obtained from the GEO [http://www.ncbi.nlm.nih.gov/geo] under the accession number GSE155673. The data was produced by Arunachalam et al., a research group in the USA, in 2020 (Arunachalam et al. 2020). Their paper is entitled "Systems biological assessment of immunity to mild versus severe COVID-19 infection in humans." The paper was published in Science Journal, Volume 369, Issue 6508, 2020. Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) is a multimodal single-cell phenotyping technique. CITE-seq, besides RNA sequencing, was performed with DNA-barcoded antibodies for protein quantification and immune-phenotyping of cells, benefiting from current advances in single-cell sequencing approaches. (Stoeckius et al. 2017). The authors performed CITE-seq to profile the gene and protein expression in PBMC samples of seven patients with COVID-19 and five healthy controls at both RNA and protein levels. Three out of the seven patients showed moderate disease symptoms, and the rest showed severe symptoms according to the WHO classification.

3.2 Single-Cell RNA-Seq Data Preprocessing

The count matrix of the 12 samples was processed with a quality check, and further downstream analyses were performed using the Seurat package (v.4.0.1) in R (v.4.0.4) (Butler et al. 2018, Stuart and Satija 2019).

3.2.1 Data Integration

Integration of the twelve samples was performed. Data were first normalized to identify and integrate anchors among cells using log transformation of the expression values for each gene and 10,000 molecules for each cell. The standardization value for each gene was calculated, shifting its expression; so the variance and mean were 1 and 0, respectively, across all cells.

Canonical correlational analysis (CCA) and data dimensionality reduction were done on the highly variable 2000 genes in the twelve samples to detect cells with similar biological states. K-nearest neighbors (KNNs) were identified and each pair of cells from the twelve samples represented an anchor. A calculated score for each anchor allowed for anchor filtering using shared nearest neighbor (SNN) graphs. The defined anchors were used to integrate all samples (Haghverdi et al. 2018).

3.2.2 Filtering low-quality cells

Because of the positive correlation between the expression level of detected mitochondrial genes and apoptosis (Detmer and Chan 2007, Galluzzi et al. 2012), the cells that showed "mitochondrial contamination", defined as >25% of transcripts are mitochondrial genes, were excluded. Such cells were considered of low-quality because they may represent apoptotic cells. The cells with abnormal gene counts (more than 30,000 or less than 500) were excluded from downstream analysis to avoid any doublets, multiplets, empty droplets, or premature cell rupture. Finally, cells with a total number of detected genes of less than 200 or more than 6,000 genes were also excluded to avoid false-positive reads from downstream analysis.

3.2.3 Dimensionality Reduction

Principal component analysis (PCA) was employed to emphasize variations among gene sets. Each gene set's information was represented in a corresponding principal component. An elbow plot was then generated using a heuristic method based on the standard deviation of each gene set to estimate the number of principal components used for further analysis. Twenty-five principal components were chosen for the data dimensionality reduction because more principal components added more features and fewer variability among gene sets.

3.3 Clustering of Single Cells

For cell clustering, a graph-based approach was performed using the function *FindClusters* implemented in Seurat with a resolution of 0.3. Based on the cell euclidean distance in the PCA space, cells were embedded in a graph structure (KNN graph). The edge weights between any pair of cells were refined based on their similarity and variability represented by their Jaccard similarity coefficient. Following edge refinement, using the previously determined first 25 PCs, edges were drawn between cells with similar gene expression patterns. Louvain algorithm was applied to group cells together as a modularity optimization technique. Finally, t-Distributed Stochastic Neighbor Embedding (TSNE) was used for cell visualization.

3.4 Identification of cell types

SingleR and Celldex packages implemented in R (Aran et al. 2019) were used to assign a cell type identity to the identified 22 clusters. The algorithm computed the similarity between each identified cluster against a reference dataset: The Human Primary Cell Atlas (HPCA), containing data from 713 microarrays which have been classified into 37 main cell types and 157 subtypes. For gene expression of each cluster and each sample of the reference dataset, the Spearman coefficient was computed. Eighty percent of the correlation values were used to perform correlation analysis, followed by multiple correlation coefficients aggregation per cell type. Another correlation analysis was performed incorporating the top cell types generated from the last step and the variable genes between those cell types. Several repeats of the previous step were performed until the top cell type was assigned to the cluster. Then clusters with the same cell types were merged. To identify whether an increase or a reduction in a specific cell population occurred because of the disease or the disease severity, GraphPad Prism software v6.0 was used for analysis of the percentage of each cell type among other cell population in each sample between healthy individuals, moderate and severe COVID-19 cases. Multiple comparison analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

3.5 Identification of cell type Markers

Wilcoxon Rank-Sum Test was performed to compare gene expression values from each cell type with the rest of the cells to identify the identified cells' types markers. Log fold change cutoff value > 0.5 and an adjusted p-value less than 0.05 were used to identify the cell type positive markers (upregulated genes compared to all other cells). For the gene to be considered in the differential expression analysis in each comparison, the minimum gene expression percentage should be more than 20% in either the cell type or the rest of the cells. Ingenuity pathways analysis (IPA) software was used to retrieve gene's location and family for all the identified genes in the dataset [QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis]. The annotation was merged with the resulting cluster markers using CytoTalk web application "Integrate Excel" [https://cytotalk.com/] as represented in supplementary file 1.

3.6 Cell-cell communication analysis

To infer the intercellular communications between cell types in each condition (healthy individuals, moderate and severe COVID-19 cases), CellChat library in R was used (Jin et al. 2021). CellChat database is a manually curated database of human literature-supported ligand-receptor interactions. It contains (As for March 2021) 1,939 interactions, consists of 61.8%, 21.7% and 16.5% of paracrine/autocrine signaling interactions, extracellular matrix (ECM)-receptor interactions and cell-cell contact interactions, respectively (Jin et al., 2021). All interactions were classified into 229 signaling pathways. Firstly, Wilcoxon rank-sum test was performed to identify each cell type markers in each condition with the significance level of < 0.05. To avoid the noise effect, CellChat used a statistically robust mean method as described in the following equation:

$$EM= (1/2)\ Q_{2}+1/4((Q_{1}+Q_{3}))$$

Where EM refers to the Ensemble mean. Q_1, Q_2, and Q_3 are the first, second, and third quartiles of the expression levels of a signaling gene in a cell group. Then, interactions probabilities were calculated between different cell groups across multiple signaling pathways in each condition. To identify significant interactions with a *p*-value < 0.05, CellChat permuted group labels of cells 100 times and recalculated the interactions probability described in the previous step. It calculates the aggregated cell-cell communication network by summarizing the interactions probability, which means calculating the number of significant interactions between any two cell types. To identify significantly differential pathways between healthy and COVID-19 cases, CellChat calculated the signaling pathways probability score for each condition alone by summarizing all the ligand-receptor interactions probabilities associated with each signaling pathway from the curated CellChat database. Next, the relative contribution of specific ligand-receptor pairs to the overall signaling pathways was calculated for the significantly differential pathways between healthy individuals and severe COVID-19 cases, between healthy individuals and moderate COVID-19 cases and between moderate and severe COVID-19 cases. Next, each signaling pathway between each two conditions was compared based on the information flow which is the sum of communication probability among all pairs of cell groups in the inferred intercellular network. Manifold learning and information flow are used for ranking all the significant signaling pathways based on their differences within the inferred networks between the moderate and severe datasets. The overlapping signaling pathways between each two conditions were ranked based on their pairwise Euclidean distance in the shared two-dimensional manifold. A more considerable distance implies a more significant difference.

3.7 T cells Analysis

T cells were subsetted from the Seurat object, which includes all the cells. The top 2,000 highly variable genes among T cells were identified. Then, PCA was performed, and the first 20 principal components were chosen for the downstream analysis. Three

comparisons were performed to identify the disease effect and its severity effect on T cells: severe COVID-19 cases T cells versus healthy individuals T cells, moderate COVID-19 cases T cells versus healthy individuals T cells, and severe versus moderate COVID-19 cases T cells. All analyses were performed using Wilcoxon Rank Sum Test using adjusted p-value < 0.05 and logFC cutoff ±0.2. The resulting differentially expressed genes among T cells of healthy, moderate and severe COVID-19 cases were used for pathways' enrichment using the Reactome database (Jassal et al. 2020). Furthermore, IFN-γ protein interactors were retrieved from STRING database (Szklarczyk et al. 2019).

CHAPTER 4: RESULTS

The authors in the original study from which the data were retrieved performed CITE-seq using 10x Genomics technology and performed the demultiplex and transcripts quantification using CellRanger software v3.1.0 against the 10x Genomics GRCh38 reference v3.0.0 (Arunachalam et al., 2020). The current study aims to study cell-cell signaling interactions between blood cells in healthy individuals and COVID-19 patients in moderate and severe cases (Figure 3).

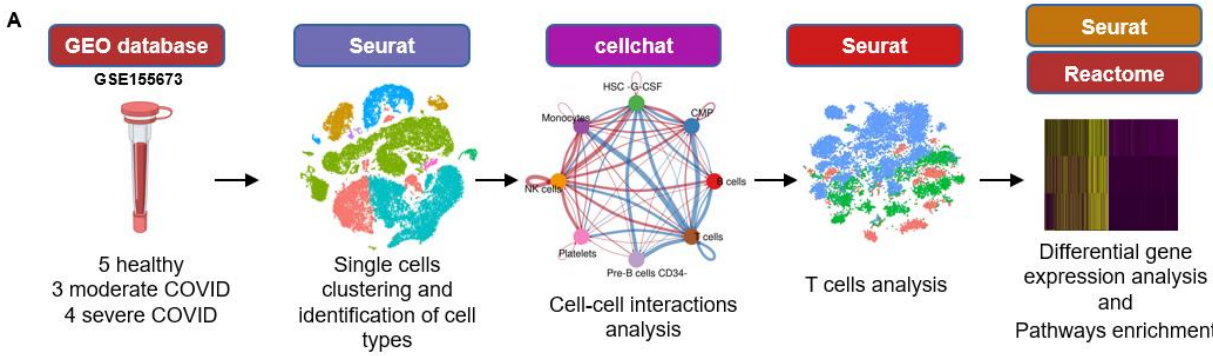


Figure 3. Schematic diagram of the analysis workflow. 12 RNA-seq samples were obtained from GEO database with the accession number GSE155673. Cells were clustered and cell types were assigned to the identified clusters. Cell-cell interactions were inferred. Then, T cells were plotted and differential expression analysis were performed on T cells followed by Reactome pathways enrichment. The above labeling boxes represent the tools used to perform each step in the analysis. (Created with BioRender.com).

4.1 Clustering of cells and identification of cell types markers

A total of 63,469 cells were analyzed from three groups (five healthy samples: 30,870 cells, three moderate COVID-19 cases samples: 13,269 cells, and four severe COVID-19 cases samples: 19,330 cells) as represented in Figure 4A. After excluding low-quality cells (as described in methods section 3.2.2; Figure 4B), 56,381 cells remain for the downstream analysis (five healthy samples: 29,213 cells, three moderate COVID-19 cases samples: 11,083 cells, and four severe COVID-19 cases samples: 16,085 cells). A total of 25 principal components were used to abstract the data (Figure 4C).

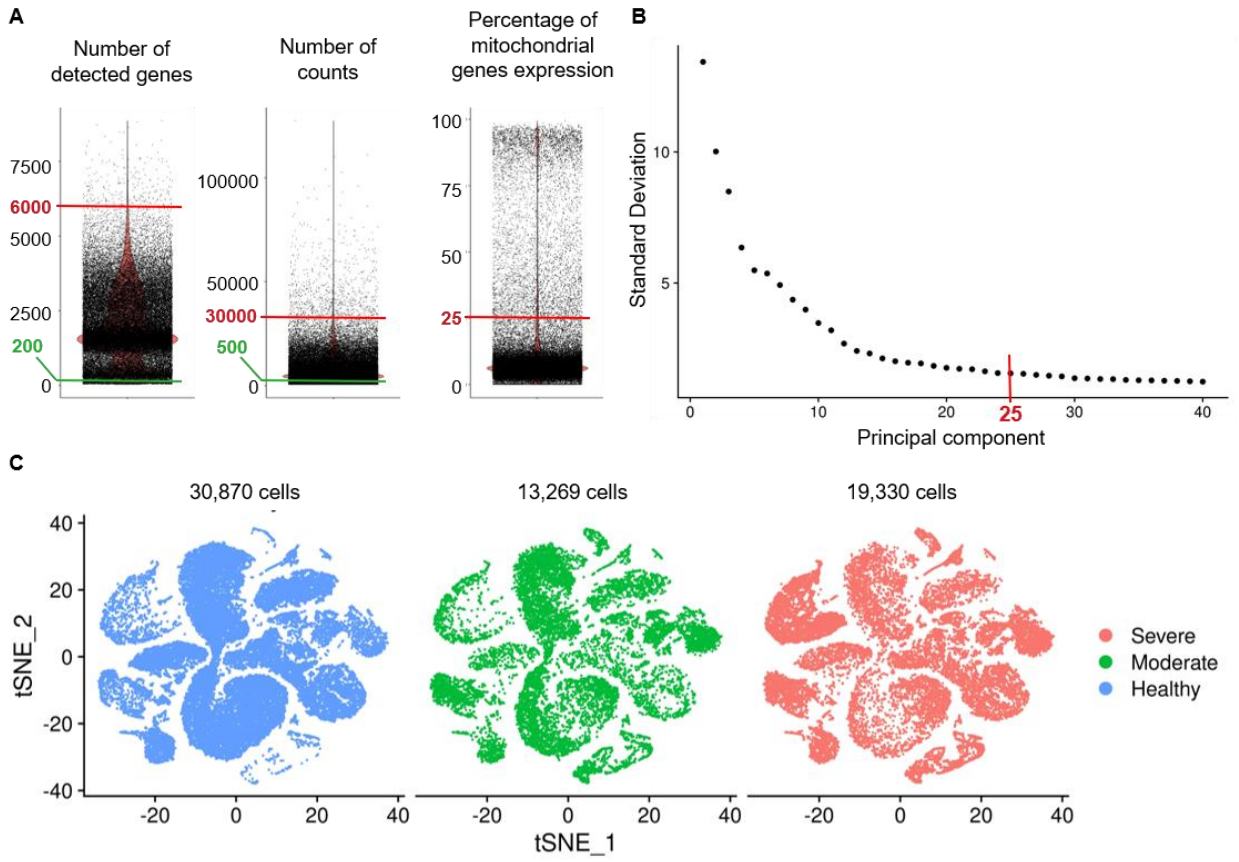


Figure 4. Preprocessing and quality control of single cells. (A) Violin plots representing the total number of detected genes per cell (left panel), total number of counts per cell (middle panel) and the percentage of mitochondrial genes expression each cell (right panel). Cutoffs used for excluding low quality cells are marked with red color (upper limit) and green color (lower limit). **(B)** Elbow plot showing the variance among the first 40 principal components. The first 25 principal components were used for the downstream analysis. **(C)** t-distributed stochastic neighbor embedding (t-SNE) projection of the data before excluding low quality cells (63,469 cells). Each cell is represented as a dot.

Clustering analysis identified 22 clusters, as shown in Figure 5A. The identified 22 clusters were assigned to 8 cell types using SingleR and celldex packages after calculating the similarity between each cell type markers and the human primary cell atlas database (Monocytes: 19,973, T cells: 16,768, NK cells: 8,538, Platelets: 5,332, B cells: 4,193 cells, CMP: 722, Pre-B cells CD34-: 493 and HSC: 362) as shown in Figure 5B.

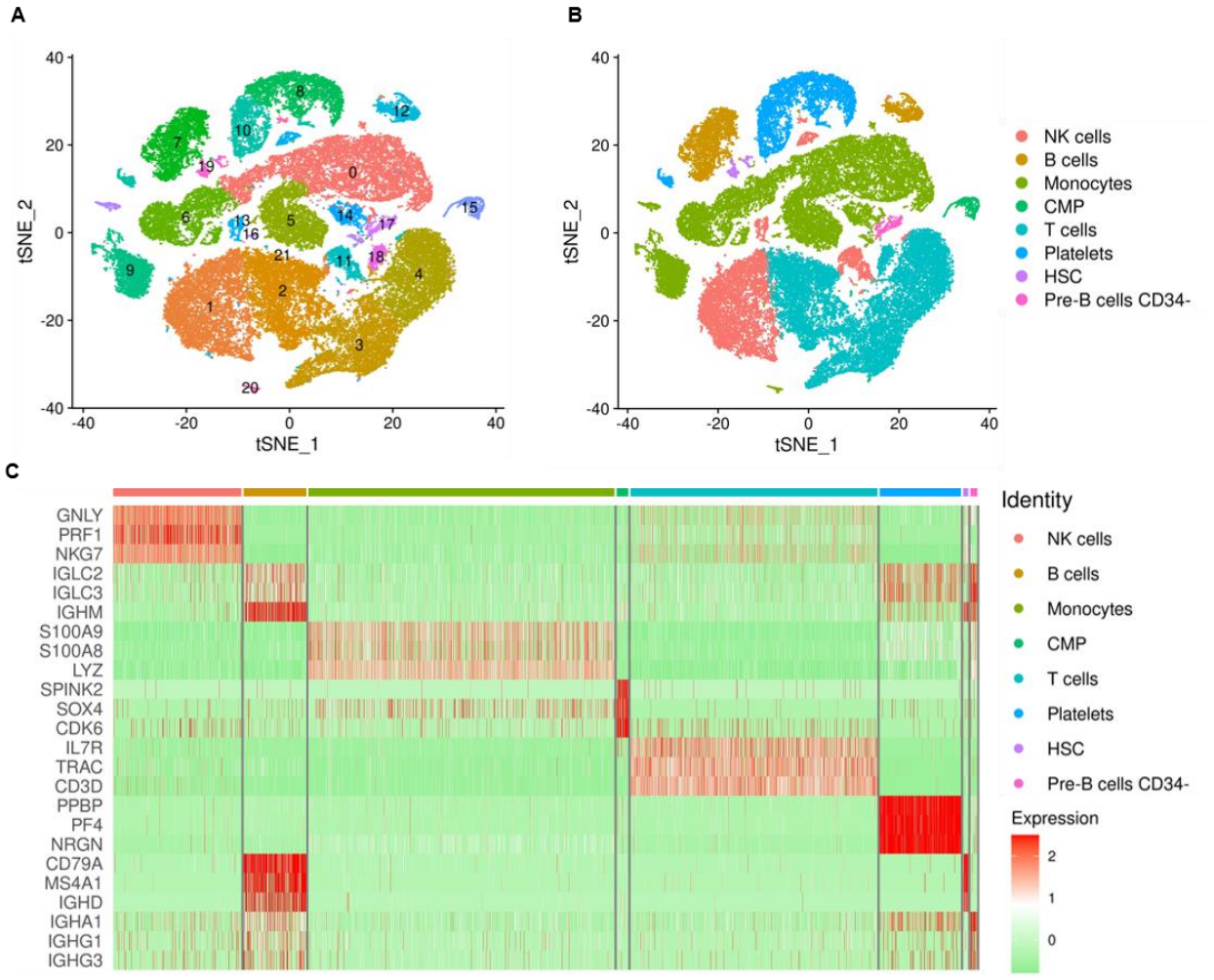


Figure 5. Identification of eight cell types among blood samples. (A) t-SNE projection of the filtered data (56,381 cells) clustered into 22 clusters. Each cell is represented as a dot. **(B)** t-SNE projection of the cells color coded with the assigned cell types. **(C)** Heatmap representing the top 3 markers relative gene expression for each cell type. Red color denotes higher expression, while green color denotes lower expression. Each row represents one gene and each column represents one cell.

Differential expression analysis identified markers (genes) to be upregulated in each cell type with logFC > 0.5 and adjusted p-value < 0.05 as follows: 137 markers for NK cells, 117 markers for B cells, 296 markers for monocytes, 115 markers for CMP, 117 markers for T cells, 459 markers for platelets, 17 markers for HSC and 28 markers for pre-B cells CD34-. Top three markers identified for each cell type were represented in Figure 5C and all cell types markers were provided in supplementary file 1. Compositional

analysis identified that percentage of T cells significantly decreased from 41.5% in moderate COVID-19 cases to 16% in severe COVID-19 cases. On the other hand, cell percentage in all identified eight cell types were represented in supplementary file 2.

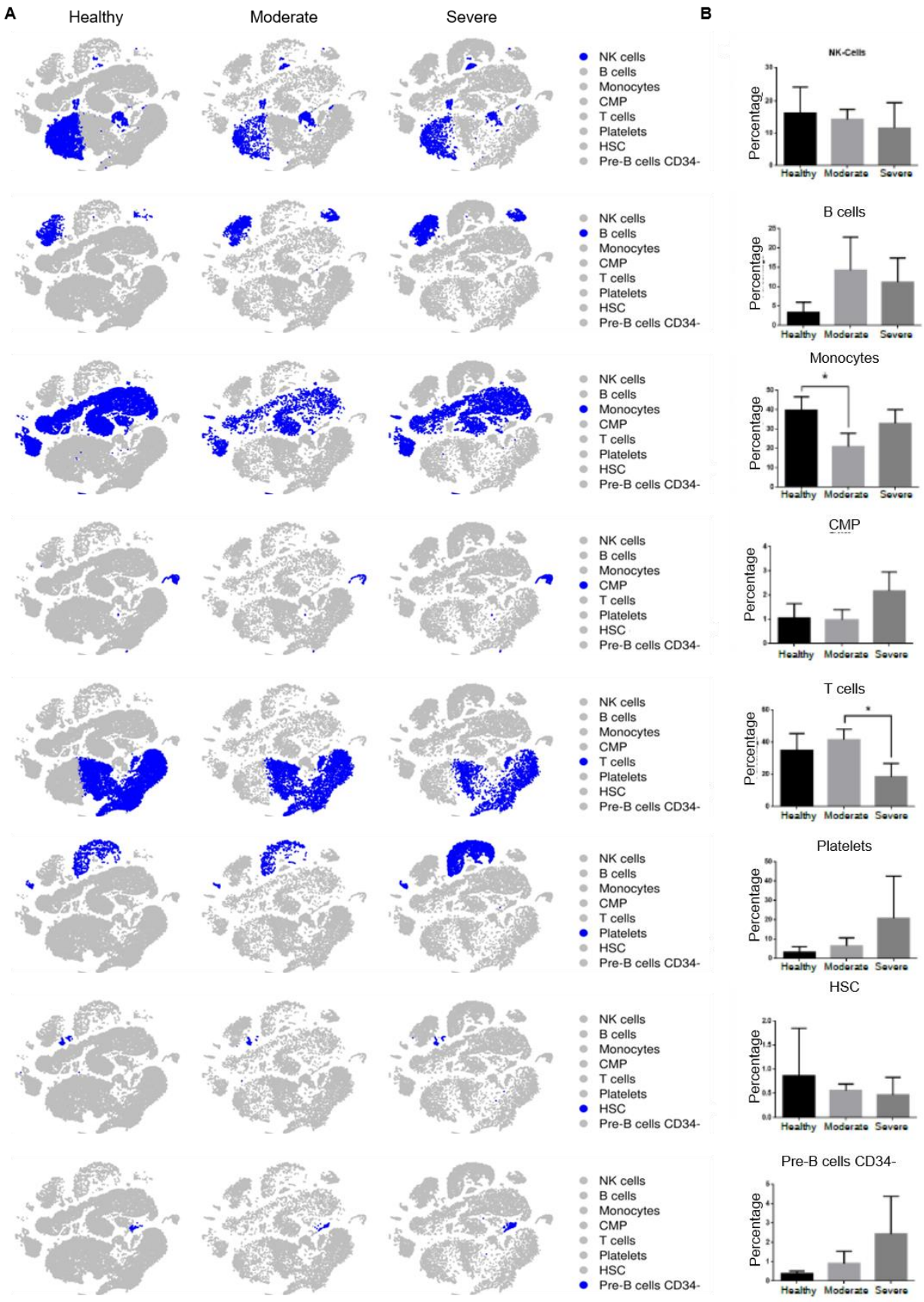

A
Healthy
Moderate
Severe
B
NK cells
B cells
Monocytes
CMP
T cells
Platelets
HSC
Pre-B cells CD34-
NK-Cells
B cells
Monocytes
CMP
T cells
Platelets
HSC
Pre-B cells CD34-
Percentage
Healthy
Moderate
Severe
*

Figure 6. Compositional analysis. (A) tSNE projection representing each cell types in blue color and the rest of the cells in grey color. **(B)** Barplots representing percentage of each cell types among other cell types of each condition. Stars denote significant adjusted p value.

4.2 Signaling pathways' alterations through cell-cell interaction analysis

A total of 586 interactions in healthy, 603 in moderate COVID-19 cases, and 606 in severe COVID-19 cases were inferred using the CellChat package in R (as described in methods section 3.2.2). When comparing the total strength of incoming signaling patterns in moderate COVID-19 cases versus healthy individuals, there was an increase in T cells and a reduction in NK cells' incoming signaling. On the other hand, when comparing the total strength of incoming signaling patterns in severe COVID-19 cases versus healthy individuals, there was a reduction in T cells and an increase in NK cells incoming signaling as shown in Figure 7A, B and C and Figure 8A, B and C. Additionally, incoming signaling to platelets increases in severe COVID-19 cases when compared to healthy or moderate COVID-19 cases. Analysis of relative information flow at pathways level identified pathways to be found in healthy individuals, not in COVID-19 moderate or severe cases such as TNF pathway. On the other hand, pathways identified to be present in COVID-19 cases and not found in healthy individuals included SN, IL16, and JAM signaling pathways. Surprisingly, CD6 and ALCAM were found to be present in moderate COVID-19 cases, but not in severe ones. Furthermore, Resistin. THBS and MPZ signaling were identified in severe COVID-19 cases, but not in moderate ones.

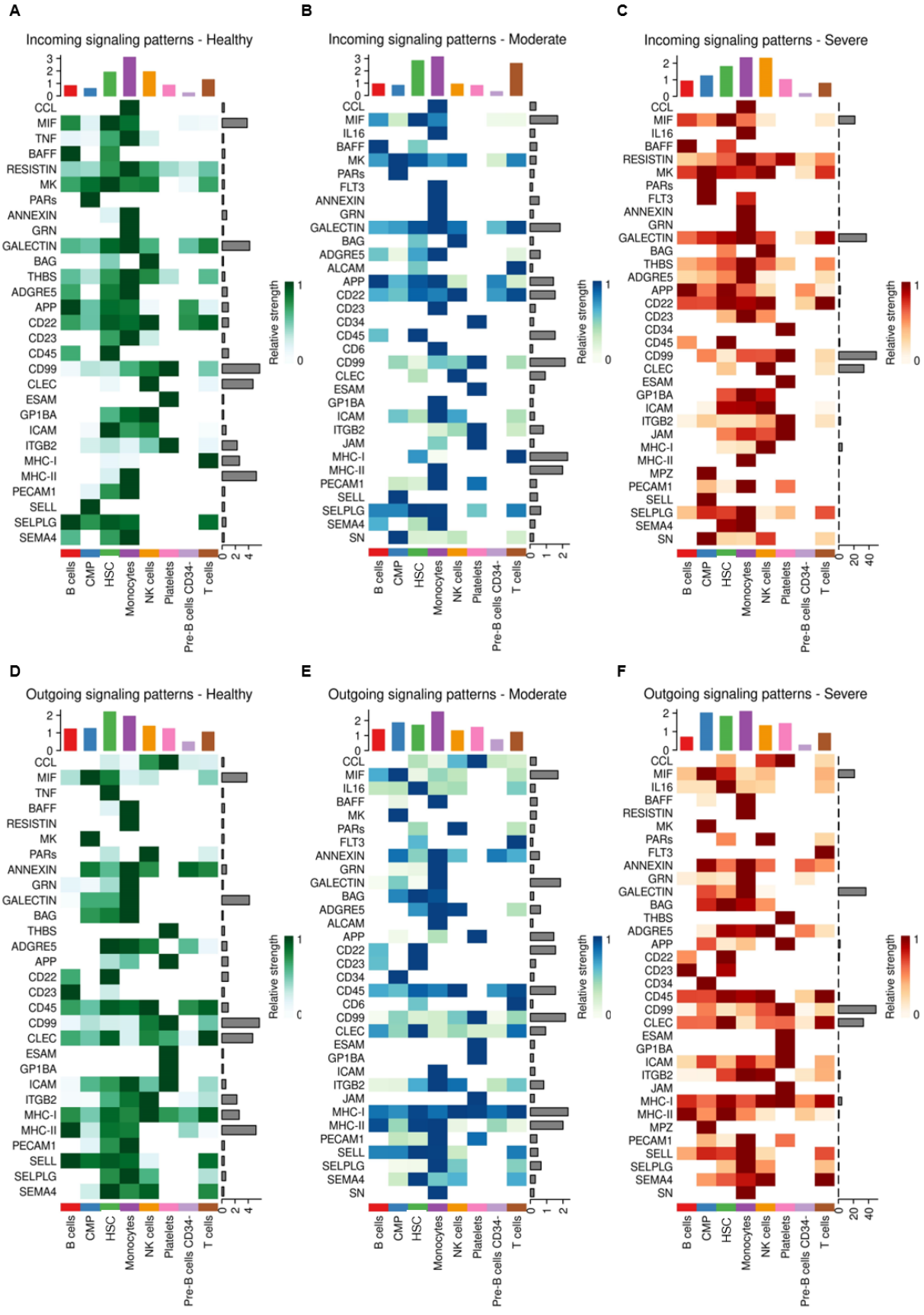

A
Incoming signaling patterns - Healthy
B
Incoming signaling patterns - Moderate
C
Incoming signaling patterns - Severe
D
Outgoing signaling patterns - Healthy
E
Outgoing signaling patterns - Moderate
F
Outgoing signaling patterns - Severe
Relative strength
B cells
CMP
HSC
Monocytes
NK cells
Platelets
Pre-B cells CD34-
T cells

Figure 7. Interaction signaling patterns in blood cells of healthy and COVID-19 patients. (A, B and C) Heatmaps representing probabilities of incoming signaling interactions in healthy, moderate and severe cases respectively. **(D, E and F)** Heatmaps represent outgoing signaling interactions in healthy, moderate and severe cases respectively. Rows represent signaling pathway and columns represent cell types. X-axis bar-plots represent sum of signaling interactions probabilities in each cell for all inferred signaling pathways. Y-axis bar plots represent sum of signaling interactions probabilities in each signaling pathway in all cell types.

The relative information flow between severe COVID-19 cases versus healthy individuals, moderate COVID-19 cases versus healthy individuals, and severe versus moderate COVID-19 cases were represented in Figure 8D, E and F.

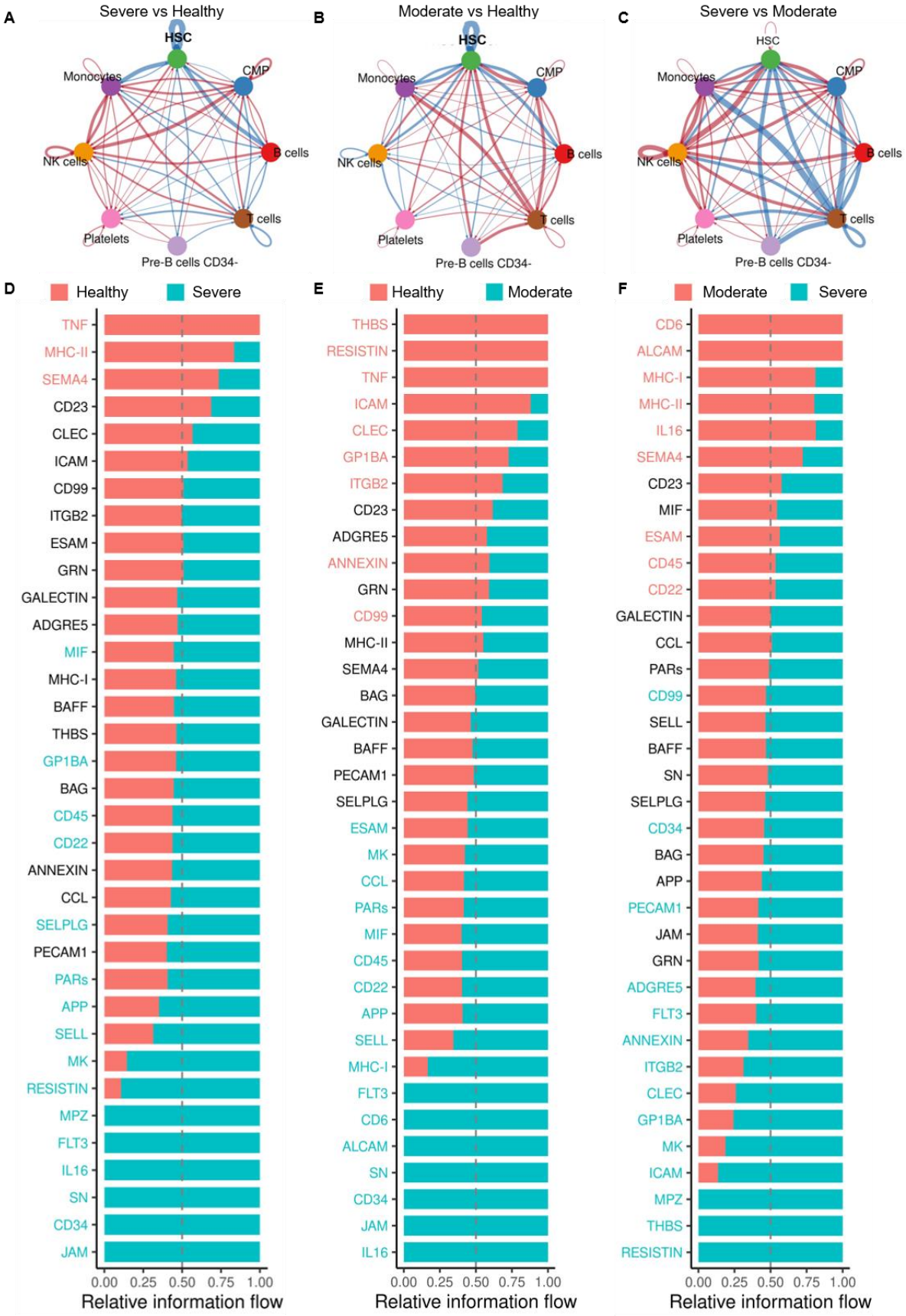

A
Severe vs Healthy
B
Moderate vs Healthy
C
Severe vs Moderate
HSC
CMP
Monocytes
B cells
NK cells
T cells
Platelets
Pre-B cells CD34-
D
Healthy
Severe
E
Healthy
Moderate
F
Moderate
Severe
TNF
MHC-II
SEMA4
CD23
CLEC
ICAM
CD99
ITGB2
ESAM
GRN
GALECTIN
ADGRE5
MIF
MHC-I
BAFF
THBS
GP1BA
BAG
CD45
CD22
ANNEXIN
CCL
SELPLG
PECAM1
PARs
APP
SELL
MK
RESISTIN
MPZ
FLT3
IL16
SN
CD34
JAM
0.00 0.25 0.50 0.75 1.00
Relative information flow

Figure 8. Differential interactions among blood cells between healthy, moderate and severe COVID-19 cases. (A, B and C) Differential number of the overall interactions between severe cases and healthy (left), moderate cases and healthy (middle) and severe cases and moderate ones (right). Red color denotes a greater number of interactions respectively. While blue color denotes a smaller number of interactions. **(D, E and F)** Relative information flow of inferred signaling interactions in severe cases versus healthy (left), moderate cases versus healthy (middle) and severe cases versus moderate ones (right) respectively.

SN signaling pathway, which was identified to be present in moderate and severe COVID-19 cases only, included the interaction between the pair SIGLEC1- SPN as shown in Figure 9. SIGLEC1 was found to be expressed in monocytes of moderate and severe COVID-19 cases. SIGLEC1- SPN signaling was identified to be directed from monocytes towards monocytes, T cells, NK cells, multipotent common myeloid progenitor (CMP) cells and hematopoietic stem cells (HSC) (Figure 9D).

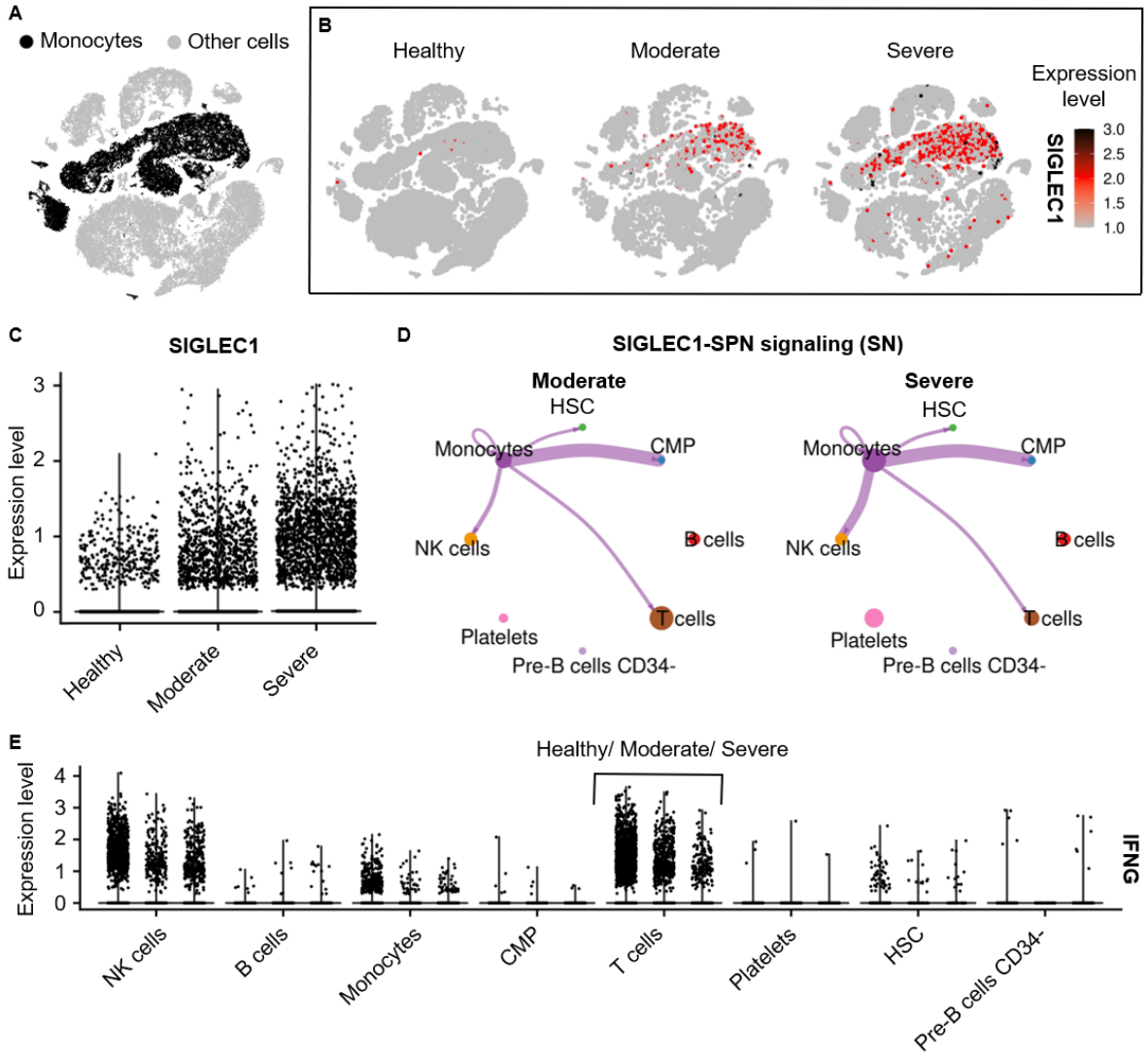


Figure 9. SN signaling pathway induction in moderate and severe COVID-19 cases. (A) tSNE projection representing monocytes as black dots and other cell types as grey dots. **(B) E**xpression level of SIGLEC1 in different conditions (healthy, moderate and severe cases). **(C)** Violin plot representing SIGLEC1 expression level in all cells from healthy, moderate and severe COVID-19 cases. **(D)** Network representing SIGLEC1-SPN signaling in moderate cases (left panel) and in severe cases (right panel). Signaling are outgoing from monocytes to monocytes, NK cells, T cells, CMP and HSC cells. **(E)** Violin plot representing IFNG expression level in different cell types in healthy (left), moderate (middle) and severe cases (right).

4.3 T cells differential expression analysis

Clustering of T cells identified different distribution of cells on the tSNE plot based on the disease condition (Figure 10A and B). The differential expression analysis identified

201 upregulated genes and 76 downregulated genes when comparing severe COVID-19 cases T cells versus healthy individuals T cells. When moderate COVID-19 T cells were compared to healthy ones, 181 genes were found to be upregulated, and 78 genes were found to be downregulated. Finally, comparing T cells in severe versus moderate COVID-19 cases identified 34 upregulated genes and 39 downregulated genes (Figure 10C). Top 15 upregulated and downregulated genes in each comparison were represented in Figure 10D, E, and F and all differentially expressed genes resulting from the three comparisons were represented in supplementary file 3. The top 25 genes identified to be upregulated in T cells of moderate COVID-19 cases compared to healthy individuals were represented in table 2 one along with their logFC in severe versus moderate COVID-19 T cells. Interestingly, some of the genes upregulated in moderate COVID-19 T cells when compared to healthy individuals were found to be downregulated in severe COVID-19 T cells when compared to moderate ones such as IRF9, STAT1, HLA-C, IFITM, IFI6 and ITGA4.

Table 2: Top 25 upregulated differentially expressed genes in moderate COVID-19 cases compared to healthy individuals and their logFC in severe vs moderate cases.

Gene	Description	LogFC in Moderate vs Healthy	LogFC in Severe vs Moderate
IGKC	Immunoglobulin kappa constant	1.90	0.81
IGLC2	Immunoglobulin lambda constant 2	1.36	0.32
IGLC3	Immunoglobulin lambda constant 3 (Kern-Oz+ marker)	1.14	0.47
IFI44L	Interferon induced protein 44 like	1.13	
ISG15	ISG15 ubiquitin like modifier	1.07	
IGHA1	Immunoglobulin heavy constant alpha 1	0.93	0.68
IFI6	Interferon alpha inducible protein 6	0.91	-0.25
XAF1	XIAP associated factor 1	0.90	
MX1	MX dynamin like GTPase 1	0.80	
JCHAIN	Joining chain of multimeric IgA and IgM	0.72	0.41
IGHM	Immunoglobulin heavy constant mu	0.65	
IFITM1	Interferon induced transmembrane protein 1	0.62	-0.36
XIST	X inactive specific transcript	0.59	-0.74
EIF2AK2	Eukaryotic translation initiation factor 2 alpha kinase 2	0.58	
MTRNR2L8	MT-RNR2 like 8	0.57	-1.34
LY6E	Lymphocyte antigen 6 family member E	0.57	
IRF7	Interferon regulatory factor 7	0.48	
SP100	SP100 nuclear antigen	0.47	
EPSTI1	Epithelial stromal interaction 1	0.47	
SMCHD1	Structural maintenance of chromosomes flexible hinge domain containing 1	0.47	
RNF213	Ring finger protein 213	0.46	
MTRNR2L12	MT-RNR2 like 12	0.45	
STAT1	Signal transducer and activator of transcription 1	0.43	-0.23
ITGA4	Integrin subunit alpha 4	0.41	-0.23
OAS1	2'-5'-Oligoadenylate Synthetase 1	0.40	

4.4 Pathways activity analysis in T cells

Pathways enrichment analysis using Reactome identified seven upregulated and 48 downregulated pathways in severe versus healthy comparison. Twenty-two upregulated and 40 downregulated pathways in the moderate versus healthy comparison, while 7 upregulated and 48 downregulated in severe COVID-19 cases compared to healthy and 24 upregulated and 20 downregulated pathways in the severe versus moderate comparison using false discover rate < 0.001 (tables 3,4 and 4; supplementary file 3). The top two pathways resulted from each comparison were represented in Figure 10G and the top ten upregulated and downregulated pathways were represented in tables three, four and five for severe COVID-19 compared to healthy individuals, moderate COVID-19 compared to healthy individuals and severe compared to moderate COVID-19 cases respectively. Interferon-gamma signaling and ISG15 antiviral mechanism were upregulated in T cells of moderate Covid-19 patients compared to healthy ones. However, the interferon signaling pathway was downregulated in severe cases T cells compared to moderate ones.

Table 3: Top 10 upregulated and downregulated pathways in severe COVID-19 cases compared to healthy individuals.

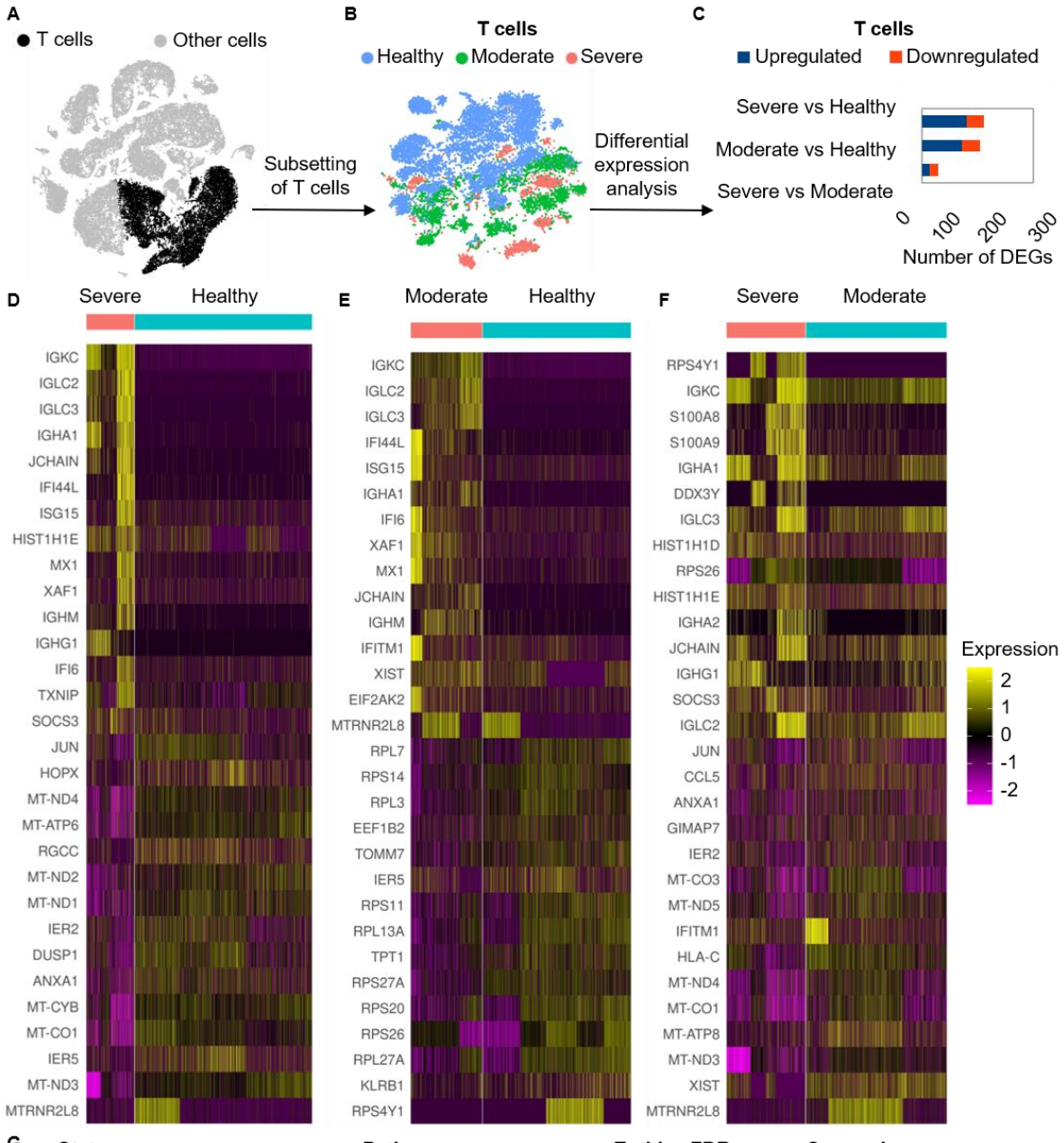
Pathway name	Status	Reactions found/ Total reactions	FDR Adjusted P-Value
Cytokine Signaling in Immune system	Upregulated	218/708	2.14021E-10
Interferon alpha/beta signaling	Upregulated	4/22	4.23205E-10
Interferon Signaling	Upregulated	24/69	2.52897E-09
Immune System	Upregulated	395/1621	6.39654E-07
Interleukin-4 and Interleukin-13 signaling	Upregulated	25/47	5.30247E-05
ATF6 (ATF6-alpha) activates chaperone genes	Upregulated	3/5	5.30247E-05
ATF6 (ATF6-alpha) activates chaperones	Upregulated	4/10	9.3349E-05
Interleukin-6 signaling	Upregulated	17/20	0.001647834
Signaling by Interleukins	Upregulated	173/493	0.004010453
Apoptosis induced DNA fragmentation	Upregulated	3/12	0.007979309
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	Downregulated	1/1	2.44249E-15
Formation of a pool of free 40S subunits	Downregulated	2/2	2.44249E-15
Viral mRNA Translation	Downregulated	2/2	2.44249E-15
SRP-dependent cotranslational protein targeting to membrane	Downregulated	5/5	2.44249E-15
L13a-mediated translational silencing of Ceruloplasmin expression	Downregulated	3/3	2.44249E-15
GTP hydrolysis and joining of the 60S ribosomal subunit	Downregulated	3/3	2.44249E-15
Major pathway of rRNA processing in the nucleolus and cytosol	Downregulated	6/7	2.44249E-15
Nonsense-Mediated Decay (NMD)	Downregulated	5/6	2.44249E-15
Peptide chain elongation	Downregulated	4/5	2.44249E-15
Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)	Downregulated	4/5	2.44249E-15

Table 4: Top 10 upregulated and downregulated pathways in moderate COVID-19 cases compared to healthy individuals.

Pathway name	Status	Reactions found/ Total reactions	FDR Adjusted P-Value
Interferon gamma signaling	Upregulated	11/16	1.4988E-14
Interferon Signaling	Upregulated	46/69	1.4988E-14
Interferon alpha/beta signaling	Upregulated	11/22	1.4988E-14
Cytokine Signaling in Immune system	Upregulated	182/708	1.4988E-14
Immune System	Upregulated	411/1621	1.4988E-14
Phosphorylation of CD3 and TCR zeta chains	Upregulated	5/7	1.66103E-11
Generation of second messenger molecules	Upregulated	6/17	3.28804E-11
Translocation of ZAP-70 to Immunological synapse	Upregulated	4/4	9.85558E-11
PD-1 signaling	Upregulated	1/5	1.89771E-10
Endosomal/Vacuolar pathway	Upregulated	3/4	1.89771E-10
Formation of a pool of free 40S subunits	Downregulated	2/2	1.66533E-15
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	Downregulated	1/1	1.66533E-15
L13a-mediated translational silencing of Ceruloplasmin expression	Downregulated	3/3	1.66533E-15
GTP hydrolysis and joining of the 60S ribosomal subunit	Downregulated	3/3	1.66533E-15
Viral mRNA Translation	Downregulated	2/2	1.66533E-15
SRP-dependent cotranslational protein targeting to membrane	Downregulated	5/5	1.66533E-15
Translation initiation complex formation	Downregulated	2/2	1.66533E-15
Ribosomal scanning and start codon recognition	Downregulated	2/2	1.66533E-15
Eukaryotic Translation Elongation	Downregulated	8/9	1.66533E-15
Major pathway of rRNA processing in the nucleolus and cytosol	Downregulated	6/7	1.66533E-15

Table 5: Top 10 upregulated and downregulated pathways in severe COVID-19 cases compared to moderate ones.

Pathway name	Status	Reactions found/ Total reactions	FDR Adjusted P-Value
Scavenging of heme from plasma	Upregulated	1/12	0.00015359
Apoptosis induced DNA fragmentation	Upregulated	2/12	0.000483111
Binding and Uptake of Ligands by Scavenger Receptors	Upregulated	1/33	0.000483111
Classical antibody-mediated complement activation	Upregulated	2/2	0.000483111
Eukaryotic Translation Elongation	Upregulated	6/9	0.000483111
FCGR activation	Upregulated	6/6	0.000483111
Formation of Senescence-Associated Heterochromatin Foci	Upregulated	1/2	0.000483289
Creation of C4 and C2 activators	Upregulated	2/8	0.000483289
Formation of the ternary complex, and subsequently, the 43S	Upregulated	1/3	0.000483289
Initial triggering of complement	Upregulated	4/21	0.000599555
Endosomal/Vacuolar pathway	Downregulated	3/4	7.99361E-15
Interferon gamma signaling	Downregulated	9/16	7.99361E-15
Interferon alpha/beta signaling	Downregulated	9/22	7.99361E-15
Interferon Signaling	Downregulated	20/69	7.99361E-15
Cytokine Signaling in Immune system	Downregulated	94/708	7.99361E-15
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	Downregulated	13/16	2.26485E-14
rRNA processing in the mitochondrion	Downregulated	1/6	2.26485E-14
tRNA processing in the mitochondrion	Downregulated	1/3	6.4948E-14
ER-Phagosome pathway	Downregulated	4/10	9.72555E-13
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	Downregulated	14/44	2.06635E-12



G

Status	Pathway	Entities FDR	Comparison
Upregulated	Cytokine Signaling in Immune system	2.14e-10	Severe cases versus Healthy
	Interferon alpha/ beta signaling	4.23e-10	
Downregulated	NMD independent of the Exon Junction Complex	2.44e-15	
	Formation of a pool of free 40S subunits	2.44e-15	
Upregulated	Interferon gamma signaling	1.49e-14	Moderate cases versus Healthy
	Interferon signaling	1.49e-14	
Downregulated	Formation of a pool of free 40S subunits	1.66e-15	
	NMD independent of the Exon Junction Complex	1.66e-15	
Upregulated	Scavenging of heme from plasma	0.00015	Severe versus Moderate cases
	Apoptosis induced DNA fragmentation	0.00048	
Downregulated	Endosomal/ Vacuolar pathway	7.99e-15	
	Interferon gamma signaling	7.99e-15	

Figure 10. T cells differential expression analysis. (A) tSNE projection representing monocytes as black dots and other cell types as grey dots. **(B)** tSNE projection of T cells alone from healthy and COVID-19 cases. **(C)** Bar plot representing number of differentially expressed genes in T cells from 3 comparisons: severe COVID-19 cases versus healthy, moderate COVID-19 cases versus healthy and Severe versus moderate COVID-19 cases. **(D, E and F)** Heatmaps representing the top 15 upregulated and downregulated genes from the three comparisons mentioned in A respectively. **(G)** The top 2 upregulated and downregulated pathways identified in the three comparisons mentioned in A resulting from Reactome enrichment analysis. Genes used for enrichment were chose based on the following criteria: adjusted p value < 0.05 and logFC +-0.2.

4.5 Interferon-gamma protein-protein interaction

Protein-protein interactions of IFN-γ was retrieved from STRING database including interactions with SOCS1, SOCS3, STAT1, STAT4, IFNGR1, IFNGR2, TNF, FOXP3, IRF1 and RELA as shown in Figure 11A. SOCS3, the negative regulator of IFN-γ showed upregulation in both moderate and severe COVID-19 cases, while the downstream of IFN-γ signaling such as STAT1 and IRF1 had lower level of expression in severe COVID-19 cases and in both moderate and severe COVID-19 cases respectively (Figure 11C).

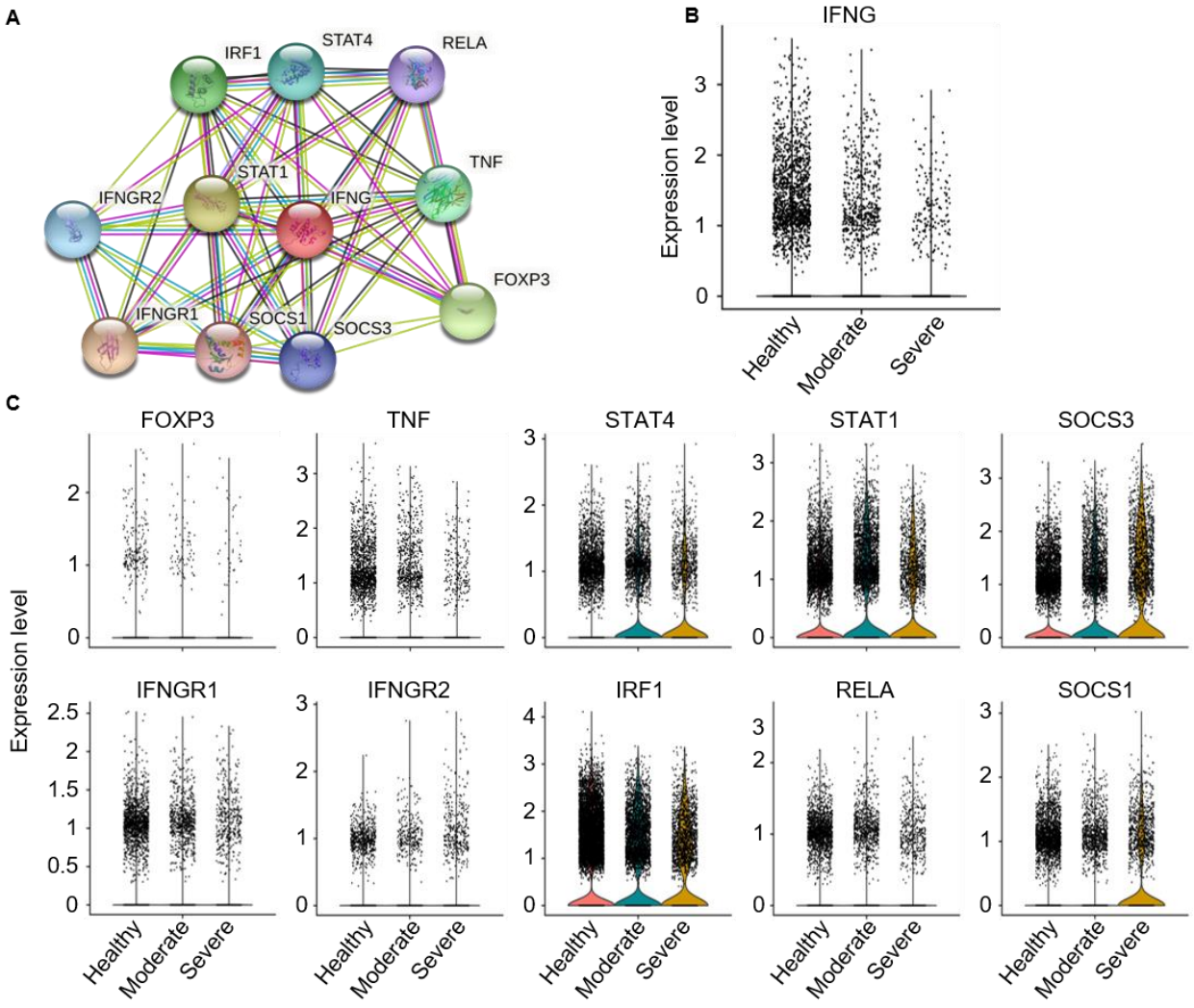


Figure 11. Interferon gamma protein-protein interactions. (A) Interferon gamma and its protein-protein interactions retrieved from STRING database. (B and C) Violin plots representing IFNG and its protein-protein interactors expression level in T cells of healthy, moderate COVID cases and severe COVID cases.

CHAPTER 5: DISCUSSION

Intensive care and hospitalization may be a necessity for some COVID-19 patients. The more the number of hospitalized patients, the more the strain on health care systems. So, understanding the critical activators of the disease severity could reduce hospitalization rates and an economic burden worldwide (Laforge et al. 2020, Sayan et al. 2021). An in-depth evaluation of the hospitalized patients' immune systems and illustrating differences between mild, moderate, and severe COVID-19 cases help identify such activators (Tang et al. 2021). Previous studies described differences between COVID-19 disease stages in peripheral immune cells (Su et al. 2020, Ren et al. 2021). Such differences revealed novel biological signatures of disease severity, such as dysregulation of JAK/STAT, MAPK/mTOR, and NF-B immune signaling networks (Feyaerts et al. 2021). The current study aims to address the alteration of interactions between blood cells in moderate and severe COVID-19 cases.

5.1 COVID-19 Severity influence on cell counts

Clustering of scRNA-seq data of blood samples from five healthy, three moderate, and four severe COVID-19 cases resulted in identification of eight blood cell types: monocytes, T cells, NK cells, platelets, B cells, CMP, Pre-B cells CD34-and HSC (Figure 5B). T cells percentage among all population in severe COVID-19 cases was found to be significantly reduced. T cells percentage decreased from 32.76% in healthy individuals to 16% in severe cases as shown in Figure 6A and B. T cells were reported previously to be significantly reduced in COVID-19 patients with correlation to disease severity (Diao et al. 2020b, Huang et al. 2020b, Zhang et al. 2020b). A previous study on CD3+ T-cells' response to COVID-19 revealed suppression in their counts, which may be an underlying mechanism for disease progression and fatality (Xu et al. 2020). Both CD4+ and CD8+ T-cells are lower in severe COVID-19 patients than in mild and moderate cases (Song et al. 2020, Zhang et al. 2020c). Additionally, in a study by Song et al., CD3+ T-cells involved

in activating CD4+ and CD8+ T-cells were decreased in the severe group compared to a mild clinical presentation (Song et al. 2020). A retrospective review of the T-cells and serum cytokines concentration of 522 COVID-19 confirmed cases and 40 healthy controls also revealed a dramatic reduction of T-cells, especially in the intensive care unit admitted cases. Counts of total T cells, CD4+ T or CD8+ T cells lower than expected, were positively correlated with disease severity (Diao et al. 2020b). The definite mechanism by which some patients develop severe immune reactions to SARS-CoV2, like an exhausted phenotype and T-cell reduction, is still not fully understood.

5.2 Cell-cell interactions alteration in COVID-19 cases

In addition to detecting lower count of T cells in severe COVID-19 cases, analysis of cell-cell interactions identified less overall incoming signaling to T cells in severe cases when compared to healthy individuals and when compared to moderate COVID-19 cases as shown in Figure 7. On the other hand, more incoming signaling was identified to occur to NK cells in severe COVID-19 cases compared to moderate cases (Figure 7). Comparing signaling interactions between the three groups (healthy, moderate and severe COVID-19 cases) identified alteration of signaling between healthy and disease states and between moderate and severe COVID-19 cases.

Our results inferred CD6 signaling to occur among cells from moderate COVID-19 cases, but not in severe ones through CD6-ALCAM interaction. CD6 and ALCAM interaction was the first described example of an interaction between a scavenger receptor cysteine-rich domain and an immunoglobulin-like domain (Hassan et al. 2004). The CD6/ALCAM interaction is involved in T-cell activation and proliferation and is essential for optimal immune response (Chappell et al. 2015). This indicates lack of T cells activation in severe COVID-19 cases that might be related to the observed clinical picture.

Resistin signaling through RETN-CAP1 and RETN-TLR4 was inferred to occur in severe COVID-19 cases, but not in moderate ones (Figure 8F). RETN is one member of the RELMs (Resistin-Like Molecules) molecules expressed by macrophages, monocytes, and neutrophils (Jang et al. 2015). Resistin-Like Molecules (RELM) are mainly associated with metabolic disorders like diabetes and are also expressed in a wide range of microbial and inflammatory diseases (Pine et al. 2018). Our results identified RETN to have logFC of 1.44 when comparing T cells from severe COVID-19 cases with moderate ones. However, RETN role during inflammatory response is not clear. A study reported that RETN binds to the Toll-like receptor 4 (TLR4) to trigger a switch from proinflammatory to anti-inflammatory responses through STAT3 signaling induction (Jang et al. 2017). Other studies reported that Resistin has an inflammation promotion role through release of cytokines like IL-6, TNFα, and IL-1β in human monocytes (Jiang et al. 2014, Lee et al. 2014).

Interestingly SN signaling through SPN-SIGLEC1 was identified to occur in both moderate and severe COVID-19 cases, but not in healthy individuals. Sialic acid-binding immunoglobulin Ig lectin-1 (SIGLEC1) is a sialoadhesion macrophage receptor that modulates interactions with some immune cells, hemopoietic, and T-cells (Jans et al. 2018). Our results showed overexpression of SIGLEC1 in monocytes as shown in Figure 9B and C. This increase in its expression level allowed SPN signaling to occur through SPN-SIGLEC1 from monocytes to monocytes, NK cells, T cells, CMP and HSC cells (Figure 9D). Previously, it was reported that upregulation of SIGLEC1 expression through macrophages had negative correlation with IFN-γ production from T cells (Zheng et al. 2015, Jans et al. 2018). Another microarray study reported that IFN-γ inhibited production by CD4+T cells was due to RSV-induced SIGLEC1 upregulation in human adult RSV infected monocytes (Jans et al. 2018). Our results showed a reduction in IFN-γ in some cell types including T cells as shown in Figure 9E. Although the SPN signaling promotes the expression of IFN-γ by CD4+T cells during TCR activation (Ramírez-Pliego et al. 2007),

there is a possibility that the Siglec-1 dependent reduction of IFN-γ is due to Siglec-SPN cell-cell adhesion and communication (Kirchberger et al. 2005, Jans et al. 2018).

5.3 Gene expression alteration in T cells of COVID-19 cases

Genes identified to be downregulated in severe COVID-19 cases' T cells compared to moderate ones and at same time were found upregulated in moderate COVID-19 cases' T cells compared to healthy individuals might play a role in the altered immune response in severe COVID-19 cases. They included STAT1, IRF9, HLA-C, IFITM, IFI6 and ITGA4. Phosphorylated STAT1 translocates to the nucleus, binds to a regulatory DNA element termed gamma-activated sequence (GAS), then induces transcription of interferon-stimulated genes (ISGs). The ISGs include several other transcription factors such as interferon response factor 9 (IRF9) and others. The IRFs have a role in recognizing pathogens, the expression of IFNs, and pro-inflammatory cytokines (Honda and Taniguchi 2006). Moreover, IFN-γ is involved in the upregulation of MHC-I molecules, contributing to the APCs in promoting the NK cells activity, Th cells regulation, expression of MHC-II molecules, and B cells function antiviral immune response (Schroder et al. 2004). The interferon-inducible transmembrane protein1 (IFITM1), which was also downregulated in T cells of severe cases compared to moderate ones, inhibits the viral invasion of some viruses that enter via the host cell plasma membrane (Smith et al. 2019). Furthermore, the downregulation of Interferon-inducible protein 6 (IFI6) might also contribute to the severity of the infection. Ectopic expression of IFI6 has shown to impair CD81/CLDN1 interaction and significantly reduced HCV viral entry (Meyer et al. 2015). Moreover, IFI6 was reported to reduce apoptosis in HBV infected cells caused by type 1 interferon (Park et al. 2013). Additionally, Integrins alpha-4 (ITGA4) receptor was reported to have a role in cytotoxic T-cell interaction with target cells (Fujita et al. 2015). It is also expressed on immune cells and showed a role in T cells migration in different organs (Glatigny et al. 2011). The relationship between those altered genes in severe COVID-19 cases might need further investigation.

5.4 IFN-γ signaling alteration in severe COVID-19 cases

Comparing T cells pathways activity in healthy and disease statuses identified IFN-γ (interferon gamma) signaling pathway to be upregulated in moderate COVID-19 cases when compared to healthy individuals. However, IFN-γ signaling pathway was downregulated in severe COVID-19 cases when compared to moderate ones, and not significantly detected when compared to healthy individuals (Figure 10G). IFN-γ receptor (IFNGR) complex consists of two distinct chains, high-affinity IFNGR1 (alpha) and a low-affinity IFNGR2 (beta) (Pestka et al. 2004). Once The IFNGR complex binds to IFN-γ, it undergoes a conformational change that recruits the receptor-associated Janus protein tyrosine kinases 1 (JAK1) and JAK2 to the complex. IFN-γ binding to IFNGR receptors induces JAK2 phosphorylation, which in turn transphosphorylates JAK1 (Lasfar et al. 2014). IFN-γ can exert direct (Hwang et al. 2012) and indirect antiviral effects on infected cells (He et al. 2004). Direct activation of neighboring immune cells like natural killer cells and macrophages triggers proinflammatory and antiviral activities of those cells (Lee et al. 2000). Moreover, IFN-γ modulates the differentiation and maturation of T-cells and B-cells (Vazquez et al. 2015). The indirect effect includes induction of the expression of HLA-DR, ICAM-1, IL-18BP, and other genes that mediates the antiviral on an IL-1 expression dependent manner (Hurgin et al. 2007). IFN-γ inhibits viral invasion and replication of a variety of viruses including HIV type-1 virus, hepatitis C virus, the porcine reproductive and respiratory syndrome virus (PRRSV), BK virus (BKV), herpes simplex virus type 1 (HSV-1) and EBOV virus (Dhawan et al. 1995, Rowland et al. 2001, Pierce et al. 2005, Abend et al. 2007, Wei et al. 2009, Rhein et al. 2015).

However, viruses may inhibit IFNs production through preventing the entry of IFN-regulating transcription factors (IRFs) to the nucleus (Devasthanam 2014). COVID-19 has recently been reported to interfere with TBK1 and RNF41 proteins, which are mediators of the IFNs' activities (Gordon et al. 2020). Furthermore, insufficient activation time of IFN signaling was reported to contributes to COVID-19 disease progression or even

lethality (Rao et al. 2020). The reduction in IFN-γ signaling in severe COVID-19 cases compared to moderate ones (Figure 10G) indicates improper immune response activation in T cells from severe COVID-19 patients.

IFN-γ have protein-protein interactions with the following molecules (retrieved from STRING database): IRF1, SOCS1, SOCS3, STAT1, STAT4, RELA, TNF, IFNGR1, IFNGR2 and FOXP3 (Figure 11A). Interestingly, SOCS3 (The suppressor of cytokine signaling 3) level was found to be upregulated in moderate and severe COVID-19 cases compared to healthy individuals with logFC of 0.28 and 0.63; respectively as shown in Figure 11C, while SOCS1 expression level was not significantly altered. SOCS1 and SOCS3 down-modulate the response of the immune cells to IFN-γ during the innate immune response (Jager et al. 2011). For example, the lymphocytic choriomeningitis virus (LCMV) induces expression of SOCS3 in T-cells, resulting in impaired antiviral response and viral resistance. Moreover, LCMV-infected mice were treated with IL-7 repressed SOCS3 expression and supported T-cells antiviral functions via mediating T-cells' survival and differentiation (Pellegrini et al. 2011). In the same context, the infection of human cells by either Epstein–Barr virus or Herpes simplex virus (HSV) stimulated SOCS3 expression and IFN-I production and function (Yokota et al. 2005). Additionally, SOCS3 expression may have a role in HIV-1 evasion to the innate immune response in the central nervous system by preventing the effect of IFN-β on HIV-1 replication within the macrophages (Akhtar et al. 2010). SOCS protein family members inhibit the activation of STAT proteins by either binding JAK kinases and inhibit their phosphorylation activities or by inhibiting STAT proteins' recruitment to the cytokine receptor complex (Liau et al. 2018). STAT1 was found in our results to be downregulated in severe COVID-19 T cells when compared to moderate ones (Figure 11C).

Our results showed also that IRF1 (interferon regulatory factor 1) was downregulated in T cells in severe COVID-19 cases when compared to healthy individuals (Figure 11C). IRF1 was reported to be highly correlated to IFN-γ exposure more than other

IFNs (Garcia-Diaz et al. 2017). Also, IRF1 restricts the chikungunya virus (CHIKV) and Ross River virus (RRV) infections in stromal cells, especially muscle cells (Nair et al. 2017). IRF1 downregulation in severe COVID-19 cases' T cells might also have a contribution to the elicited improper immune response compared to moderate COVID-19 cases.

The tumor necrosis factor-alpha (TNF- α) enhances IFN-γ activity by stimulating Jak2 kinase activity, which triggers STAT1 phosphorylation (Kim et al. 2015). Increased Jak2 and STAT1 phosphorylation was observed within minutes upon TNF- α treatment as well as (IFNGR1) tyrosine phosphorylation (Kim et al. 2015). Also, the *IRF*1 and *IRF*8 transcription factors overexpression by IFN-γ induces the transcriptional activity of the human TNF-α in mouse macrophages (Sol et al. 2008). TNF expression level showed a little reduction in severe COVID-19 cases (Figure 11C) and TNF signaling was not identified to occur in moderate nor severe COVID-19 cases as shown in Figure 8.

CHAPTER 6: CONCLUSION

Some COVID-19 patients might have mild symptoms or even be asymptomatic, while others may suffer from severe symptoms and irreversible complications resulting in life threatening conditions. More than three million deaths were reported till April 2021 worldwide with no effective treatment available. Understanding how patients' immune system responds to the infection in moderate versus severe COVID-19 cases might identify new therapeutic targets. ScRNA-seq analysis of blood samples from five healthy individuals, three moderate and four severe COVID-19 cases was performed to identify cell-cell interactions alteration between blood cell types and to identify differentially expressed genes and pathways in T cells of the three groups. T cells percentage among other identified eight cell types decreased from 32.76% in healthy individuals to only 16% in severe COVID-19 cases. Cell-cell interactions analysis identified a reduction in the overall T cells incoming signaling as well. Besides, activation of SN pathway through SPN-SIGLEC1 interaction resulted in reduction of IFN-γ gene expression level. At pathways level, IFN-γ signaling was identified to be upregulated in moderate COVID-19 cases' T cells, but was downregulated in severe cases' T cells. Downstream targets of IFN-γ such as STAT1 and IRF1, which play critical roles in antiviral activity, showed also downregulation in their gene expression level. On the other hand, the negative regulator of IFN-γ signaling -SOCS3- showed significant increase in severe COVID-19 cases compared to healthy individuals and moderate COVID-19 cases. Altogether, COVID-19 severe cases showed improper antiviral activity from T cells number reduction and T cells incoming signaling decrease to alteration of IFN-γ response, which might contribute in the observed symptoms severity compared to moderate COVID-19 patients.

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