Profiling transcription factor sub-networks in type I interferon signaling and in response to SARS-CoV-2 infection

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Abstract

Type I interferons (IFN α/β) play a central role in innate immunity to respiratory viruses, including coronaviruses. Genetic defects in type I interferon signaling were reported in a significant proportion of critically ill CoOVID-19 patients. Extensive studies on interferon-induced intracellular signal transduction pathways led to the elucidation of the Jak-Stat pathway. Furthermore, advances in gene expression profiling by microarrays have revealed that type I interferon rapidly induced multiple transcription factor mRNA levels. In this study, transcription factor profiling in the transcriptome was used to gain novel insights into the role of inducible transcription factors in response to type I interferon signaling in immune cells and in lung epithelial cells after SARS-CoV-2 infection. Modeling the interferon-inducible transcription factor mRNA data in terms of distinct sub-networks based on biological functions such as antiviral response, immune modulation, and cell growth revealed enrichment of specific transcription factors in mouse and human immune cells. The evolutionarily conserved core type I interferon gene expression consists of the inducible transcriptional factor mRNA of the antiviral response sub-network and enriched in granulocytes. Analysis of the type I interferon-inducible transcription factor sub-networks as distinct protein-protein interaction pathways revealed insights into the role of critical hubs in signaling. Interrogation of multiple microarray datasets revealed that SARS-CoV-2 induced high levels of IFN-beta and interferon-inducible transcription factor mRNA in human lung epithelial cells. Transcription factor mRNA of the three major sub-networks regulating antiviral, immune modulation, and cell growth were differentially regulated in human lung epithelial cell lines after SARS-CoV-2 infection and in the tissue samples of COVID-19 patients. A subset of type I interferon-inducible transcription factors and inflammatory mediators were specifically enriched in the lungs and neutrophils of Covid-19 patients. The emerging complex picture of type I IFN transcriptional regulation consists of a rapid transcriptional switch mediated by the Jak-Stat cascade and a graded output of the inducible transcription factor activation that enables temporal regulation of gene expression.

Key words: Interferon-α/β, Jak-Stat, Transcription factor, Protein-protein interaction, Signal transduction pathway, Coronaviruses, COVID-19.

1 Introduction

Interferons (IFN) are pleiotropic cytokines and exert a wide range of biological activities that include antiviral, antiproliferative, and immunoregulatory effects (1-3). There are 2 major classes of type I interferons consisting of IFN-alpha (IFN-α) represented by 14 isoforms and one IFN-beta (IFN-β). Type I IFN is produced by many cell types, including leukocytes, dendritic cells, and fibroblasts. The amount of IFN-α vs IFN-β produced varies depending on cell type and also on the virus/stimulus (1-4). The biological effects of interferons are mainly mediated by the rapid and dramatic changes in gene expression of several hundred genes (5). The role of the Janus kinase and signal transducers and activators of transcription (Jak-Stat) pathway and transcriptional regulation by interferon-stimulated gene complex (ISGF-3) consisting of Stat1, Stat2 and, Irf-9 in Type I IFN signaling has been well established (1-6). Recent advances in high throughput gene expression profiling in primary immune cells have shown that IFN α/β induces several transcription factor mRNA that sustains secondary and tertiary rounds of transcription (7,8). Several studies had shown that the activation of the canonical Jak-Stat pathway is not sufficient to explain the broad range of biological activities of type I IFN signaling. IFN α/β inhibits the interleukin-7-mediated growth and survival of B lymphoid progenitors by a Stat1-independent pathway (9). This growth inhibitory effect involves cell cycle arrest followed by apoptosis and mediated by Stat1-independent induction of Daxx, a Fas binding protein implicated in apoptosis (10). The most prominent phenotype of the Stat1-deficient mice is their increased susceptibility to microbial and viral infection due to the decreased ability to respond to the antiviral effects of the interferons (11,12). Nevertheless, Stat1-deficient mice mount an IFN-mediated resistance to virus infection. Stat1-null mice are more resistant to challenge with murine cytomegalovirus (MCMV) or Sindbis virus than mice lacking both the type I and type II IFN

receptors (13). There is evidence for differential regulation of type I interferon signaling and interferon regulatory factors in neuronal cells and astrocytes of wild-type and Stat1-deficient mice in the mouse brain (14,15). IFN-α/β regulates dendritic cells (DC) and natural killer (NK) cells that are resident immune cells of the lung and are critical for innate immunity to respiratory viruses (16,17). The availability of gene expression datasets of primary immune cells treated with Interferon α/β for a short period of time facilitated profiling type I interferon-inducible transcription factor mRNA in the transcriptome (7.8). Coronaviruses are RNA viruses of the Coronaviridae family, including Severe Acute Respiratory Syndrome coronaviruses SARS-CoV-1 and SARS CoV-2 (18). The current pandemic of coronavirus disease known as COVID-19 is caused by a highly infectious SARS-CoV-2 that emerged first in Wuhan, China (19,20). The world health organization (WHO) has declared the current pandemic as a global public health emergency because of the rapid spread around the world with high mortality and morbidity. The importance of a functional interferon system for protection against SARS-CoV-2 was demonstrated by the report that 10% of nearly a thousand Covid-19 patients who developed fatal pneumonia had autoantibodies to interferons and an additional 3-5% of critically ill patients had mutations in genes that control interferons (21,22). In addition to the genetic deficiency, genetic variants in the components of type I Interferon signaling such as type I IFN receptor (IFNAR2), protein tyrosine kinase (TYK2) and the antiviral target gene Oligoadenylate synthetase (OAS1) were detected in critically ill COVID19 patients (23). Transcription factor profiling is a powerful technique to gain insights into mammalian signal transduction pathways. (24,25). Transcription factor profiling of genes involved in a biologic function such as innate and adaptive immunity led to the identification of functional connectivity of signaling hubs, critical nodes, and modules (25,26). In this study transcription factor profiling in the transcriptome of immune cells in response to type I Interferon treatment and lung epithelial cells in response to SARS-CoV-2 infection was investigated.

2 Materials and Methods

Gene expression datasets Gene expression profiling in response to Type I interferon treatment in human peripheral blood mononuclear cells (PBMC) and mouse immune cells were published previously (7,8). Gene expression datasets representing human lung cell lines infected with coronaviruses and tissue samples of healthy and COVID-19 patients were also reported (27,28). Supplementary data was downloaded from the Journal publisher websites and from Geo datasets that were archived at Pubmed (NCBI). The identification of the differentially expressed genes in the transcription profile was analyzed using the GEO2R tool and differential expression analysis using DESeq2 using default parameters. Gene expression resources from Immgen RNA seq SKYLINE COVID-19 resources were used (http://rstats.immgen.org/Skyline_COVID-19/skyline.html). Cluster analysis was performed using gene expression software tools (29). Outliers of expression were not included in the analysis. The evaluation of the gene ontology (GO) enrichment and signaling pathway analysis was conducted using DAVID, KEGG, and Metascape software (30-32). Protein interactions were interrogated and visualized in the STRING, WIKI-PI, and BIOGRID databases (33-35) Protein-protein interaction network centrality features were calculated according to https://www.sscnet.ucla.edu/soc/faculty/mcfarland/soc112/cent-ans.htm

3 Results and Discussion

3.1 Functional Annotation of the Inducible transcription factors of Type I Interferon signaling

Gene expression profiling studies in human peripheral blood monocytic cells (PBMC) and mouse immune cells revealed that a large number of transcription factor mRNA were significantly induced in the transcriptome by type I interferons (7,8). A master list of 35 human and 32 mouse interferon-induced transcription factors was subjected to the functional annotation in Database for Annotation, Visualization and Integrated Discovery (DAVID), Kyoto Encyclopedia of Genes and

Genomes (KEGG), and Metascape to investigate underlying biological processes and signaling pathways (30-32). These transcription factors were broadly organized into three major functional categories-antiviral response, immune modulation, and cell growth (Table1). Mapping the list of interferon-induced transcription factors into gene ontogeny (GO) terms in Metascape provided insights into the common functional categories and signal transduction pathways in the human and mouse transcriptomes (Figure 1A and 1B). These GO terms included human papillomavirus infection, Hepatitis B, HTLV-1, response to the virus, interferon-gamma mediated signaling pathway in the antiviral response category. Immune modulation or inflammation category GO terms included regulation of cytokine production, Toll-like receptor signaling pathway, and AP1 pathway. In contrast, cell growth terms included transcriptional mis-regulation in cancer, positive regulation of cell death, and DNA damage response (Figure 1A and 1B). Protein-protein interaction abundance analysis using molecular complex detection (MCODE) algorithm in Metascape revealed three modules consisting of cell proliferation/Ionizing radiation, interferon-γ and interferon-α/β signaling represented by red, blue and green, respectively (Figure 1C). The human type I interferon-induced transcription factor data was obtained from gene expression profiling of a heterogeneous mixture of cells from the PBMC while the mouse list was derived from a purified population of B-lymphocytes, dendritic cells, granulocytes, natural killer cells, macrophages, and T-lymphocytes. Cluster analysis in gene expression profiling is often used to discriminate genes that are co-regulated under the given experimental conditions (29). The grouping of transcription factors into functional sub-categories facilitated the identification of enrichment in distinct cell types. For example, mouse antiviral transcription factors were highly expressed in granulocytes among immune cells (Figure 2A). Granulocytes including neutrophils, eosinophils, and basophils are characterized by the presence of large cytoplasmic granules and essential for the control of infection. Enhanced expression of transcription factors involved in inflammation such as Ahr, Bcl3. and Egr2 in NK cells and cell

growth such as Myc, Max, and Jun in B-cells was observed (Figure 2B and Figure 2C). Furthermore, IFN-α and IFN-β mediated changes in distinct functional sub-categories such as antiviral response, immune modulation and cell growth of human PBMC can be compared in detail (Figure 3).

3.2 Time-course analysis of Type I Interferon signaling

Most of the gene expression profiling studies in response to type I IFN have some technical limitations (5). These studies were performed in transformed cells or cells cultured in the presence of high serum or in a limited number of cell types such as fibroblasts or epithelial cells. In addition, cells were subjected to prolonged interferon treatment of several hours to capture the maximum range of gene expression levels. Furthermore, antiviral response genes were overrepresented while transcription factor and growth-regulated genes were under-represented on many custom-designed arrays of interferon-regulated gene expression. These studies may have missed earlier dynamic changes in transcriptional factor mRNA levels and cell growth-related gene expression. Interrogation of gene expression datasets of early time points after type I Interferon treatment revealed that several transcription factor mRNA were expressed after 1-4 hour treatment of human PBMC and mouse B cells. (Figure 4A and 4B). Transcription factors involved in antiviral response including Stat1, Stat2, Irf1, Irf2, and Irf7 mRNA levels were rapidly induced in both PBMC and B cells (Figure 4). An intact type I IFN response is necessary to inhibit viral replication in cells, control the tissue restriction of virus, and increase the production of type I IFN acting as a feedback loop (4,36). A large number of type I interferon regulated genes like 2,5-oligoadenylate synthetase (OAS), Mx, PKR, and Rnase L implicated in antiviral defense were induced rapidly after infection. Mutations of Stat1 have been characterized in human populations. Patients with heterozygous Stat1 mutations show susceptibility to mycobacteria but not the viral disease. Interferon-induced Stat1 homodimer or gamma-activated factor (GAF) formation was diminished, while the response to the ISGF-3 complex was normal in cells derived from these patients (37). The clinical and cellular phenotypes of these patients suggest

that the response to mycobacteria was mediated by Stat1 homo-dimer and the antiviral immunity is dependent on ISGF-3 formation. Interestingly, transcription factors and growth-related genes such as Myc, Jun, and Schlafen (Sfn) family members were rapidly induced by type I Interferon in B cells (Figure 4B). Schlafens regulate immune cell proliferation, differentiation, and restricting virus replication (38). However, Schlafens has no DNA binding activity or direct role in transcriptional regulation.

3.3 Protein-protein interaction network and functional connectivity of signaling hubs in type I Interferon signaling

Transcriptional factors bind to regulatory elements such as promoters and enhancers located upstream of the gene transcription start site and act as platforms for the assembly of co-regulators and with general transcriptional factors regulate eukaryotic gene expression (39). Co-operative protein-protein interactions play an important role in the functional diversity of signal transduction pathways and biological functions of multiple cell types (40). Stat1 plays a major role in the transcriptional regulation by type I and type II interferons (1-3). Stat1 is regulated by post-translational modifications such as tyrosine and serine phosphorylation and interacts with a large number of general and specific transcription factors. Stat1 requires distinct components of the co-activator complex, including the CREB-binding protein family of transcriptional activators (CBP/p300), and p300/CBP-associated factor (p/CAF) for distinct platform assembly and histone acetyltransferase (HAT) activity to activate interferon-stimulated gene transcription (41). Two contact regions between Stat1 N-terminal and C-terminal regions and CBP/p300 were identified in interferon signaling (42). RNA polymerase II and associated general transcription factors such as upstream stimulatory factors (USF) and TATA box binding protein (TBP) play an important role in transcriptional initiation (43). In contrast to Stat1, the role of inducible transcription factors in type I interferon signaling is not well understood. It is possible to construct potential protein interaction networks of inducible transcription

factors of distinct functional categories in type I Interferon signaling using the data from protein-interaction databases (33-35,44). As an example, antiviral transcription factor interactions in the STRING database was shown as a graph with proteins or nodes as ovals and protein interactions as edges (Figure 5A). Network analysis in TRUUST database revealed extensive interactions between Stats, IRF, and Sp family of transcription factors (45, data not shown). A major advantage of this approach is that it allows tools developed for social network analysis such as centrality measures to be applied to understand the organization of protein interaction networks and critical nodes. These graph theory algorithms reveal the importance of any particular node to the entire network. Some of the well-known centrality methods include degree centrality that measures the node connectivity or the total number of inbound and outbound links. In contrast, between centrality identifies the nodes that are bridges on the shortest path between other nodes. Furthermore, closeness centrality measures the closeness of any node to other nodes in the network (46). Applying all these measures facilitated the ranking of each node in the antiviral sub-network (Table 2). These calculations have shown that Stat1, Stat2, and Irf1 were the highest-ranked nodes in the antiviral sub-network. In contrast, Irf5 and Sp110 were the lowest-ranked nodes in the sub-network. Furthermore, transcription factors involved in a related biologic function often share common protein interaction partners and target genes as revealed by pairwise analysis of transcription factor hubs involved in innate and adaptive immunity (25). Implementing the pairwise analysis of common protein interaction partners of Stat1 and other hubs in antiviral sub-network in Biogrid database revealed that Rela and Stat3 as additional members of the extended network (data not shown). Although Stat1 was included as a hub in the antiviral sub-network, it is possible to include Stat1 in immune modulation or cell growth sub-network (Figure 5B). Such a hub can be designated as an authority among hubs. In social network analysis hubs and authority designations were developed in the context of hyperlink-induced topic search (47). Type I Interferon is often released simultaneously with pro-inflammatory cytokines such as TNF-α, IL-1.

IL-6 by immune cells in response to respiratory virus infection resulting in the cross-talk between Stat1 and other transcription factors such Stat3, Rela, and Jun and fine-tuning of pathogen response (48). The protein interaction module of Stat1, Stat3, Rela, and Jun was shown to be a characteristic feature of innate and adaptive immunity (25)

3.4 Regulation of Core Type I Interferon signaling in immune cells

Phylogenetic analysis of type I Interferon regulated gene expression across multiple species provided novel insights into the origin and evolution of innate immunity (49). Many components of innate immunity and interferon signaling like pathogen sensors, transducers, transcription factors such as interferon regulatory factors (IRF), and target genes were already present and functional before the origin of the type I interferon signaling system in fishes (50). Microarray analysis of peripheral blood monocytic cells (PBMC) treated with type I interferon across multiple vertebrate species revealed a set of 62 highly conserved interferon-stimulated genes (ISG) designated as core ISG involved in innate immunity (51). The functional sub-categories of these genes include antiviral defense, antigen presentation, the immunoproteasome, ubiquitin-modification, cell signaling, and apoptosis. This list includes RNA-sensing pathogen recognition receptors such as Ifih (Mda5), Dhx58 (Lgp2), Rig-I (Ddx58), and the adaptor molecule Myd88. The highest level of Core ISG expression was observed in granulocytes compared with other immune cells and includes genes involved in antiviral (Oas, Mx, Eif2ak2), cell growth and apoptosis (Tnfsf10, Parp9, Parp14, Casp8), and MHC class I antigen presentation (Figure 6 and data not shown). The group of inducible transcription factor mRNA in core type I Interferon signaling was dominated by mediators of antiviral response such as Stat1, Stat2, Irf1, Irf7, Irf9, Pml, and Sp110. Many of the core antiviral response transcription factors were enriched in granulocytes and interact with each other to form a tight protein interaction network (Figure 7A and 7B). Consistent with the results, promoter scanning of core ISG by P-Scan program revealed a highly significant representation of binding sites for Stat1, Stat2 and IRF family members

(data not shown). These studies revealed that the evolutionarily conserved core ISG primary function was the antiviral response and other biological functions such as immune modulation and cell growth were elaborated later in the evolution. Novel inducible core transcription factors detected in this functional screen include two zinc finger proteins Zcc3hav1, Zcchc2 involved in the regulation of antiviral response and cell growth, respectively (52,53).

3.5 Role of tyrosine kinase Tyk2 and alternative signaling pathways for inducible transcription factors in Type I Interferon signaling

Type I interferon signaling involves the binding of IFN α/β to its receptor (IFNAR) and activation of receptor-associated protein tyrosine kinases Jak1 and Tyk2 by auto and trans-phosphorylation (1,3,54). These kinases are involved in the phosphorylation of the receptor on specific tyrosine residues (Y337, Y512) that serve as docking sites for the recruitment and subsequent activation of Stat1 and Stat2 (55). Tyk2-null cells are impaired in the activation of Stat1 and Stat2, and ISRE-mediated gene expression (56,57). Furthermore, the induction of several transcription factor mRNA by IFN-α was attenuated in Tyk2-null B-cells suggesting that activation of Tyk2 is required for Stat-dependent and –independent pathways of signaling (Figure 8A). In addition to Jak kinases, IFNAR also activates alternative pathways involving multiple kinases in parallel such as extracellular signal-regulated kinases (Erk1/2), p38 mitogen-activated protein kinase (p38 MAPK), phosphatidylinositol-3 kinase (PI-3K), Akt serine kinase, and protein kinase C-delta (3,67,68). Jak1 and Tyk2 interact with a large number of proteins as documented in protein-interaction databases and common protein interaction partners of Jak1 and Tyk2 are potential candidates for alternative pathways of type I interferon signaling (25).

IFN-α/β has been shown to activate multiple Stat members such as Stat3, Stat4, Stat5, and Stat6 in different cell types as demonstrated by tyrosine phosphorylation and DNA binding assays. Activation of these Stat members in the regulating of antiviral response, cell proliferation, and differentiation has

been observed in different cell types (58-63). IFN-α/β activated Stat3 in RAW264.7 macrophages and in primary B-lymphocytes (data not shown). Catalytically active Tyk2 was required for IFN-β mediated tyrosine phosphorylation and activation of Stat3 (64). Stat3 regulates a large number of target genes including Myc, Jun and Schafen2 that are rapidly induced by IFN-α in B cells (Figure 2). Positive and negative regulation of type I interferon signaling by Stat3 was reported. Two patients with a homozygous mutation of the Stat1 gene were identified (65). Both individuals suffered from mycobacterial disease but died of viral disease. IFN-α treatment of B-lymphocyte cell lines derived from these patients resulted in the tyrosine phosphorylation of Stat3, but no activation of ISGF-3 was observed. Furthermore, IFN-α induced Stat3 target genes such as Suppressor of cytokine signaling -3 (SOCS-3) in these Stat1-deficient cell lines indicating that IFN-α/β stimulated Stat1-independent signaling in human cells. Stat3 activation may have a negative role in type I interferon signaling by antagonizing multiple steps in ISGF3 transcriptional activity (66). The physiological significance of activation of additional members of the Stat family in the induction of transcription factor mRNA by IFN α/β remains to be elucidated. A schematic model of type I interferon signaling involving canonical Jak1 and Tyk2 leading to the rapid activation of ISGF3 and additional Stat family members by tyrosine phosphorylation was shown (Figure 8B). The members of the Stat family of transcription factors share structure, activation mechanism and have distinct binding affinities for cis-elements in the gene promoter leading to the regulation of a different set of genes. Different combinations of Stats may be activated in distinct cell types resulting in a variety of biological effects. The cytoplasmic domain of IFNAR contains several tyrosine phosphorylation sites that are not involved in Stat activation (69). The role of these additional IFNAR phosphorylation sites and activation of multiple kinases in type I interferon signaling and inducible transcription factor mRNA expression remains to be elucidated (Figure 9B).

3.6 Profiling inducible transcription factors of Type I Interferon signaling in lung epithelial cells after SARS-CoV-2 infection and in the tissues of COVID-19 patients

Respiratory virus infection of lung epithelial cells results in type I interferon production, which then acts in an autocrine or paracrine manner to stimulate interferon-stimulated gene expression (16,17). Genomic analysis of critically ill COVID-19 patients revealed deficiency or alterations in type I interferon signaling (21-23). Imbalanced cytokine and interferon responses in COVID-19 patients were described (27,70,71). Autoantibodies to type I interferon and bacterial products were also detected in the tissue samples of COVID-19 patients (22,70). Furthermore, the differential expression of ISG was correlated with lung damage and survival in COVID -19 patients (72). Respiratory virus infection leads to the immunopathology of the lung mediated by the direct effect of the virus as well as imbalanced host immune response (19,73,74). Gene expression data of human lung epithelial cells infected with SARS-CoV-2 as well as healthy and COVID 19 tissue samples were published recently, making it possible to study type I interferon regulation of inducible transcription factor gene expression (27,28). Interrogation of microarray datasets revealed that interferon beta (IFN-β) and STAT1 mRNA levels were significantly increased 24 hours after infection with SARS-CoV-1 or SARS-CoV-2 in human Calu-3 lung epithelial cells (Figure 9). Consistent with these results, type I interferon-inducible transcription factor mRNA levels involved in biological functions such as antiviral response, inflammation, and cell growth were significantly enhanced in Calu-3 cells infected with SARS-CoV-1 and SARS-CoV-2 (Figure 10). In contrast, SARS-CoV-2 infection of human A549 lung type II cells did not induce IFN-β mRNA (Figure 11A). It has been shown that SARS-CoV-2 entry into lung epithelial cells required the expression of virus entry receptor Angiotensin-converting enzyme 2 or ACE2 (75). Infection of A549 cells expressing ACE2 with SARS-CoV-2 rescued in the inducible expression of IFN-β and STAT1 mRNA within 24 hours (Figure 11A and Figure 11B). Furthermore, expression of type I interferon-inducible transcription factor mRNA

levels involved in biological functions such as antiviral response, immune modulation, and cell growth were significantly enhanced in A549 cells expressing ACE2 receptor (Figure 12). A high level of interferon-stimulated gene expression (ISG-high) in patients was correlated with high viral titers and high levels of cytokines and limited lung damage. In contrast, a low level of interferon-stimulated gene expression (ISG-low) in COVID-19 patients was correlated with lower viral loads, extensive lung damage with the presence of activated $CD8^+$ T cells, and macrophages (72). ISG-high expressing patients died significantly earlier after hospitalization than ISG-low expressing patients suggesting that type I interferon signaling intensity may be used as a prognostic feature of survival in COVID-19 patients (72). Stat1 mRNA levels were increased in COVID 19 lung compared with healthy lung tissue samples (Figure 13A). Furthermore, inducible transcription factors involved in antiviral response were up-regulated and the transcription factors involved in inflammation and cell growth were down-regulated in COVID 19 lung compared with healthy lung samples (Figure 13B). The peripheral blood monocytic cells (PBMC) composed of several distinct cell types and the distribution of changes in gene expression varied significantly in different cell types, in response to type I interferon (Figures 2 and 3). Expression of the inducible transcription factors of the antiviral, cell growth, and inflammation sub-networks were specifically enhanced in neutrophils of COVID-19 patients, compared with healthy subjects (Figure 14A-C). High-level expression of a subset of type I interferon-stimulated genes in tissue samples of COVID-19 patients was observed including a gene signature of STAT1, STAT2, TNFSF10, S100A8, S100A9, and S100A12. This gene signature was similar to the up-regulated gene expression profile observed in patients with the autoimmune disease known as Sjogren's syndrome, characterized by systemic inflammation (76,77). This gene expression signature was highly expressed in both the lungs and PBMC of COVID19 patients (Figure 13C and Figure 14B). Elevated expression of inflammatory markers such as EGR1, TNFSF10, TNFSF14, S100A8, and S100A9 was reported in COVID-19 patients (26,70,78). Members of the

TNF superfamily such as TNFSF10 (TRAIL) and TNFSF14 (LIGHT) were implicated in apoptosis and inflammation (79). The S100 family members include S100A8. S100A9 and S100A12 are intracellular calcium-binding proteins that are released into extracellular space and function as damage-associated molecular pattern molecules (DAMPS) involved in tissue repair (80). These studies demonstrate that IFN-a/β mediated induction of three distinct transcription factor sub-networks in human and mouse immune cells. Furthermore, differential regulation of these transcription factor sub-networks was observed in human lung epithelial cell lines in response to SARS-CoV-2 infection, and in the tissue samples of healthy and COVID-19 patients.

4 Conclusion

Genetic variants or deficiency in components of type I interferon signaling was reported in a significant proportion of intensive care COVID-19 patients (21-23). In contrast, enhanced inflammatory response mediated by interferons and cytokines was also reported ln COVID-19 patients leading to an unbalanced cytokine response (27,70-72). Transcriptional factor profiling revealed that there are two distinct steps in the transcriptional regulation by type I interferons- a fast-acting tyrosine phosphorylation switch leading to the activation of the ISGF-3 complex and a gradual increase of inducible transcription factors that regulate the secondary and tertiary transcription programs. These two pathways overlap and co-regulate distinct sets of genes to confer temporal regulation of the type I interferon response. Distinct sub-networks of transcription factors including antiviral response, immune modulation, and cell growth mediate the biological effects in type I interferon signaling. Differential regulation of sub-networks was observed in human lung epithelial cells after SARS-CoV-2 infection and in COVID-19 patients. Furthermore, a gene signature of type I interferon signaling transcription factors and inflammatory mediators was observed in the lungs and peripheral blood of COVID-19 patients. The topology of the interferon regulated transcription factor sub-networks and their target gene expression may be critically assessed using software tools

developed for social network analysis. Type I interferons have pleiotropic effects and are promising therapeutic agents in the treatment of different classes of diseases such as viral infection, autoimmune disease, and cancer. A detailed understanding of the IFN α/β signaling mechanisms is important for the effectiveness of the therapies. Transcription factor profiling in type I interferon signaling may have therapeutic implications for treating COVID-19 patients by targeting selective inflammatory pathways such as MAP kinase pathway and Early growth response 1 (Egr-1) involved in the regulation of chemokines and cell growth (26). In addition, transcription factor profiling of target genes involved in innate and adaptive immunity revealed that critical nodes such as BRCA1 and GILZ involved in regulating multiple cytokine signaling pathways can be simultaneously targeted with small molecules such as dexamethasone to attenuate inflammation in lung airway epithelial cells (25). Furthermore, COVID-19 patients with high interferon-stimulated gene expression (ISG^{high}) in the lungs die significantly earlier than patients with low ISG expression (ISG^{low}) after hospitalization (72). In this report, high-levels of expression of a subset of type I interferon-inducible transcription factors and genes involved in cell apoptosis and inflammation were observed in the lungs and neutrophils in COVID-19 patients. The use of Jak1 or Tyk2 selective inhibitors to block type I interferon signaling in the periphery and lungs or locally to lower ISG expression may have therapeutic applications in the treatment of critically ill COVID-19 patients (81,82).

Acknowledgements I would like to thank Dr. Donald Forsdyke (Queen's University, Canada) for a careful reading of the manuscript and N.S. Sastry (Chelmsford, MA, USA) for continuous support.

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Figure Legends

Figure 1. Annotation of biological functions and signal transduction pathways in human PBMC and mouse immune cells using Metascape software tools (A) Biological functions and signaling pathway gene ontology (GO) terms associated with transcription factors of human PBMC were ranked by significance (B) Biological functions and signaling pathway gene ontology (GO) terms associated with transcription factors of mouse immune cells were ranked by significance (C) Molecular complex detection (MCODE) algorithm detection of modules associated with transcription factors induced by IFN-β in human PBMC.

Figure 2. Functional organization of transcription factor gene expression in the transcriptome induced by IFN-α in mouse immune cell types (A-C) Transcription factor sub-networks of antiviral, immune modulation, and cell growth were retrieved from microarray datasets of mouse immune cells treated with IFN-α for two hours. Expression in B-lymphocytes (B), dendritic cells (DC), granulocytes (GN), macrophage (MF), natural killer cells (NK), and T-lymphocytes (T) were shown. Cluster analysis was performed using Heatmapper software tools.

Figure 3. Functional organization of transcription factors induced by IFN-α or IFN-β in the human PBMC (A-C) Transcription factor sub-networks of antiviral, immune modulation, and cell growth was retrieved from microarray dataset of human PBMC treated with IFN-α or IFN-β for 4 hours. Cluster analysis was performed using Heatmapper software tools.

Figure 4. Time-course analysis of type I interferon-induced transcription factor (TF) mRNA levels in human and mouse immune cells (A) Human PBMC treated with IFN-β for 0.5-4 hours (B) Mouse B lymphocyte cells treated with IFN-α for 0.25 to 6 hours. The gene expression data were retrieved from different sources as described in materials and methods section. Cluster analysis of gene expression across the arrays was accomplished using the Heatmapper software.

Figure 5. Protein-protein interactions of transcription factor sub-networks induced by type I interferon in mouse and human immune cells represented in the STRING database (A) Antiviral sub-network of mouse immune cells induced by type I interferon (B) Cell growth sub-network of human PBMC induced by type I interferon. Transcription factors were represented by ovals and protein interactions by edges, respectively.

Figure 6. Core interferon-stimulated gene expression (core ISG) in mouse immune cells. Cluster analysis of core ISG expression levels in B-lymphocytes (B), dendritic cells (DC), granulocytes (GN), macrophage cells (MF), natural killer cells (NK), and T-lymphocytes (T) were shown.

Figure 7. Expression levels of core interferon-inducible transcription factor (TF) mRNA and protein-protein interaction network in mouse immune cells (A) Cluster analysis of interferon-inducible transcription factor expression levels in B-lymphocytes (B), dendritic cells (DC), granulocytes (GN), macrophage cells (MF), natural killer cells (NK), and T-lymphocytes (T) were shown. (B) Protein interaction network of transcription factors in STRING database was shown.

Figure 8. Role of tyrosine kinase Tyk2 and signaling pathways in phosphorylation–dependent and inducible transcription factors in Type I Interferon signaling (A) Expression levels of transcription factor mRNA in wild-type B cells untreated (control), wild-type B cells (Wt), and Tyk2 knock-out B cells (KO) treated with IFN-α for 2 hours (B) A model for type I interferon signaling pathways involving rapid phosphorylation-dependent activation of Stat transcription factors (0.5 hr) and gradual increase of inducible transcription factors (1-4 hr) in transcriptional regulation

Figure 9. Regulation of IFNB1 and STAT1 mRNA levels by corona viruses in Calu-3 lung epithelial cells (A) Calu-3 cells were mock treated or infected with SARS-CoV-1 or SARS-COV-2 for 24 hours. IFNB1 mRNA expression values normalized by DEseq2 were shown (B) STAT1 mRNA expression values normalized by DEseq2 under the same experimental conditions were shown. Data from two independent samples for each condition were shown.

Figure 10. Regulation of transcription factor mRNA levels of type I interferon sub-networks by coronaviruses in Calu-3 lung epithelial cells. Calu-3 cells were mock treated or infected with SARS-CoV-1 or SARS-CoV-2 for 24 hours (A) Transcription factors of the antiviral sub-network were shown (B) Transcription factors of the immune modulation sub-network were shown (C) Transcription factors of the cell growth sub-network were shown. Gene expression data from two independent samples for each condition were shown.

Figure 11. Regulation of IFNB1 and STAT1 mRNA levels by SARS-CoV-2 in A549 lung type II cells (A) A549 or A549 cells expressing ACE2 receptor were mock treated or infected with SARS-CoV-2 virus for 24 hours. IFNB1 mRNA expression values were normalized by DEseq2 (B) STAT1 mRNA expression values were normalized by DEseq2 under the same conditions were shown. Data from three independent samples for each condition were shown.

Figure 12. Regulation of transcription factor mRNA of type I interferon sub-networks by SARS CoV-2 in A549 cells expressing ACE2 receptor (A) A549 cells expressing virus entry receptor ACE2 were mock infected or infected with the virus SARS-CoV-2 for 24 hours. Transcription factors of the antiviral sub-network were shown (B) Transcription factors of the immune modulation network under the same conditions were shown (C) Transcription factors of the cell growth sub-network under the same conditions were shown. Three independent samples for each condition were shown.

Figure 13. Regulation of transcription factor and inflammatory biomarker mRNA levels by type I interferon signaling in the lung biopsies of healthy and COVID19 patients (A) Relative mRNA expression levels of STAT1 in the lung biopsies of healthy and COVID19 patients (B) Transcription factors of the antiviral, immune modulation and cell growth sub-networks in the lung biopsies of healthy and COVID19 patients (C) Relative mRNA expression levels of inflammatory biomarkers and transcription factors in the lung biopsies of healthy and COVID19 patients. Two independent samples for each condition were shown.

Figure 14. Regulation of transcription factor and inflammatory biomarker mRNA levels by type I interferon signaling in the PBMC of healthy and COVID19 patients (A) Expression levels of transcription factors of the antiviral, immune modulation, and cell growth sub-networks in the PBMC of healthy and COVID19 patients (B) Expression levels of inflammatory biomarkers in the PBMC of healthy and COVID19 patients (C) Cluster analysis of transcription factors and effectors of antiviral sub-network in the PBMC of healthy and COVID19 patients. Natural killer cells (NK), macrophages (MAC, CD16 positive), dendritic cells (pDC), and neutrophils (Neu) in Healthy (H) and COVID19 patients (C) were shown.

Table 1. Inducible transcription factors in Type I Interferon signaling in human and mouse immune cells

Function	Mouse	Human	Function	Human	Mouse
Antiviral	Stat1	STAT1	**Cell growth**	E2F1	Myc
	Stat2	STAT2		TFDP2	Max
	Irf1	IRF1		NMI	Nmi
	Irf2	IRF2		NOTCH1	Myb
	Irf5	IRF4		FOXO1A	Id2
	Irf7	IRF7	**Differentiation**	PAX8	Jun
	Irf8	IRF8		ETV4	Ets2
	Irf9	IRF9		ETV6	Etv6
	Sp100	SP100		ELF1	Elf1
	Sp110	SP140		ELF4	Ell3
	Pml	PML		HHEX	Maf
	Zc3hav1	ZC3HAV1		WT1	Mafk
	Zcchc2	ZCCHC2		KLF2	Klf6
Immune	Atf3	ATF3		BRCA1	Kif9
modulation	Litaf	ATF5	**DNA repair**	ATM	Atm
	Nr4a2	NFE2L3		GADD45B	Gadd45b
	Nr4a3	NR4A3		BRCA2	Gadd45g
	Bcl3	BCL3			
	Creb5	NR3C1			
	Ahr	BLGF1			
	EGR1	Egr2			

Table 2. Centrality measures for ranking nodes in antiviral sub-network

RANK	DEGREE CENTRALITY	CLOSENESS CENTRALITY	BETWEEN CENTRALITY
1	Stat1	Stat1	Stat1
2	Stat2	Stat2	Stat2
3	Irf1	Irf1	Irf1
4	Irf7	Irf9	Irf9
5	Irf9	Sp100	Irf7
6	Sp100	Irf2	Irf5
7	Irf8	Irf8	Sp100
8	Irf2	Sp110	Irf5
9	Irf5	Irf5	Irf2
10	Sp110	Irf7	Sp110

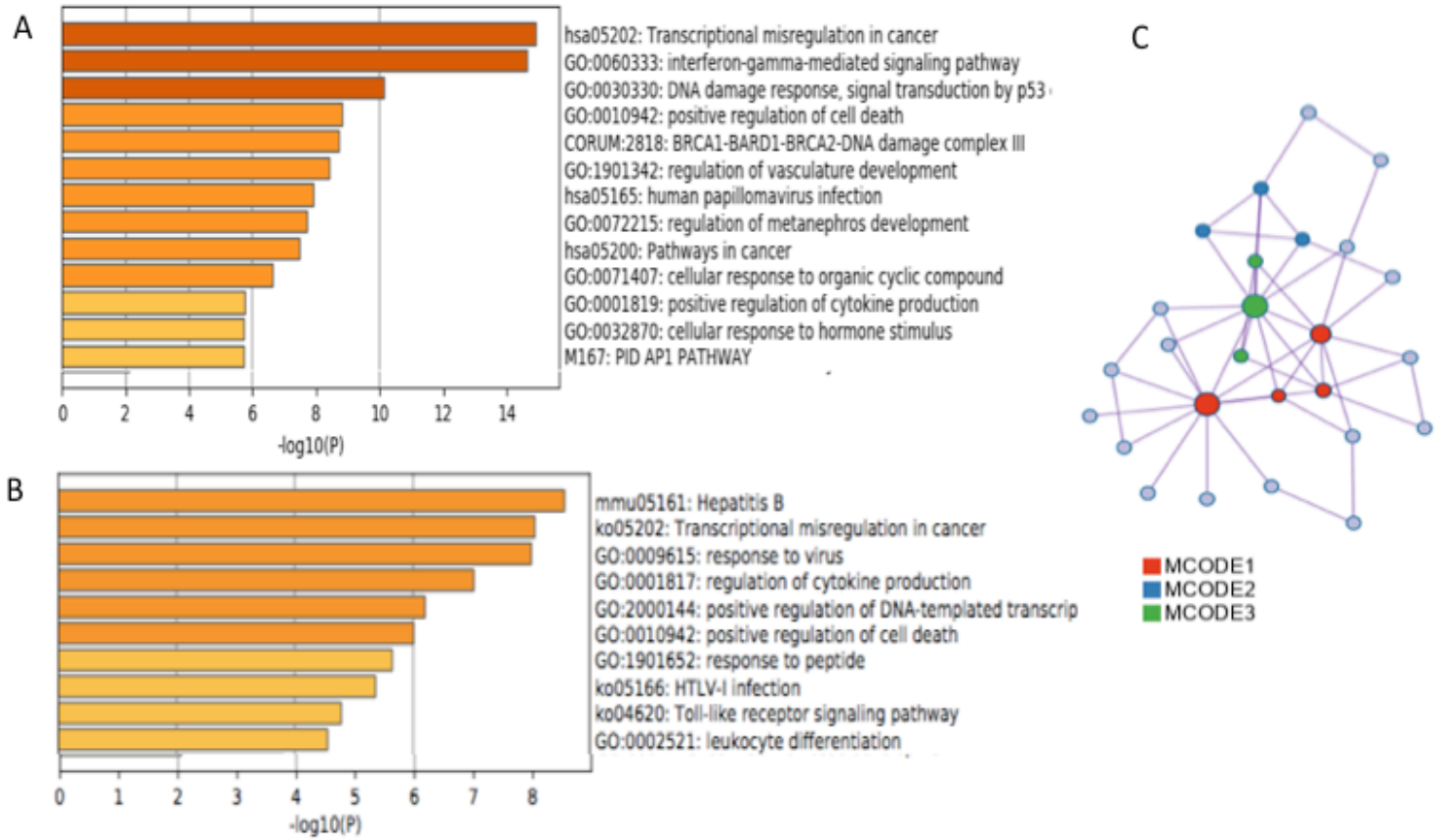
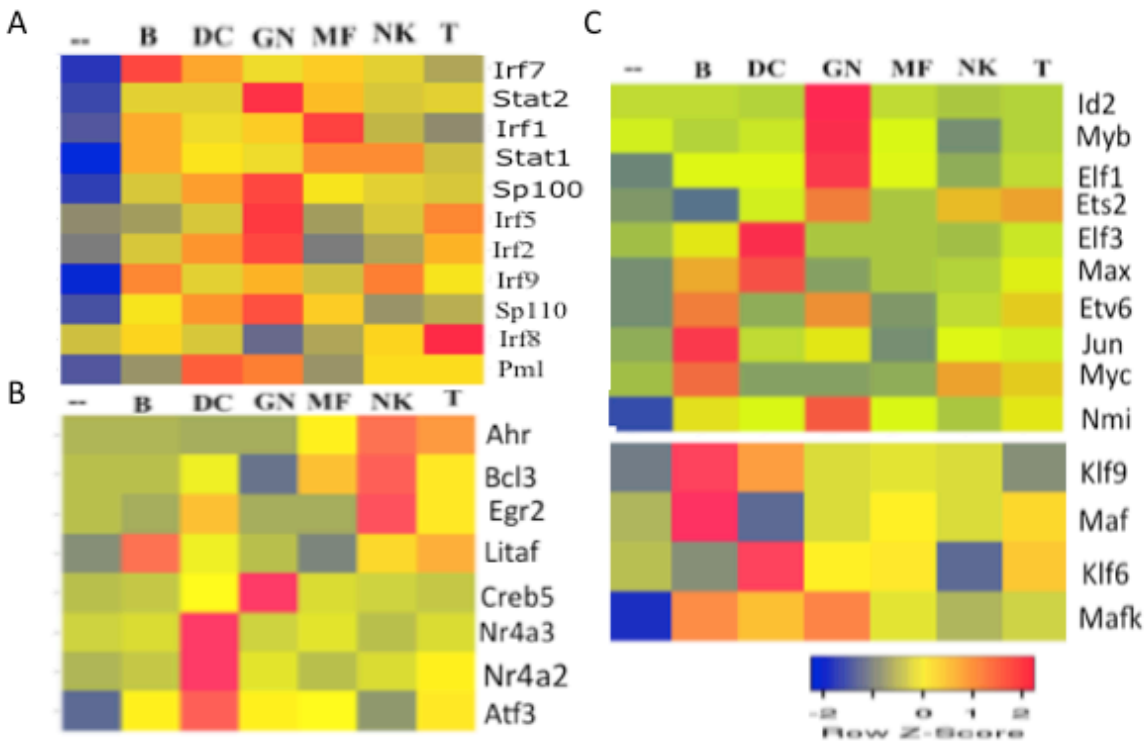

A
hsa05202: Transcriptional misregulation in cancer
GO:0060333: interferon-gamma-mediated signaling pathway
GO:0030330: DNA damage response, signal transduction by p53
GO:0010942: positive regulation of cell death
CORUM:2818: BRCA1-BARD1-BRCA2-DNA damage complex III
GO:1901342: regulation of vasculature development
hsa05165: human papillomavirus infection
GO:0072215: regulation of metanephros development
hsa05200: Pathways in cancer
GO:0071407: cellular response to organic cyclic compound
GO:0001819: positive regulation of cytokine production
GO:0032870: cellular response to hormone stimulus
M167: PID AP1 PATHWAY
0 2 4 6 8 10 12 14
-log10(P)
B
mmu05161: Hepatitis B
ko05202: Transcriptional misregulation in cancer
GO:0009615: response to virus
GO:0001817: regulation of cytokine production
GO:2000144: positive regulation of DNA-templated transcrip
GO:0010942: positive regulation of cell death
GO:1901652: response to peptide
ko05166: HTLV-I infection
ko04620: Toll-like receptor signaling pathway
GO:0002521: leukocyte differentiation
0 1 2 3 4 5 6 7 8
-log10(P)
C
MCODE1
MCODE2
MCODE3

FIGURE 1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C
B DC GN MF NK T
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Stat2
Irf1
Stat1
Sp100
Irf5
Irf2
Irf9
Sp110
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Pml
Ahr
Bcl3
Egr2
Litaf
Creb5
Nr4a3
Nr4a2
Atf3
Id2
Myb
Elf1
Ets2
Elf3
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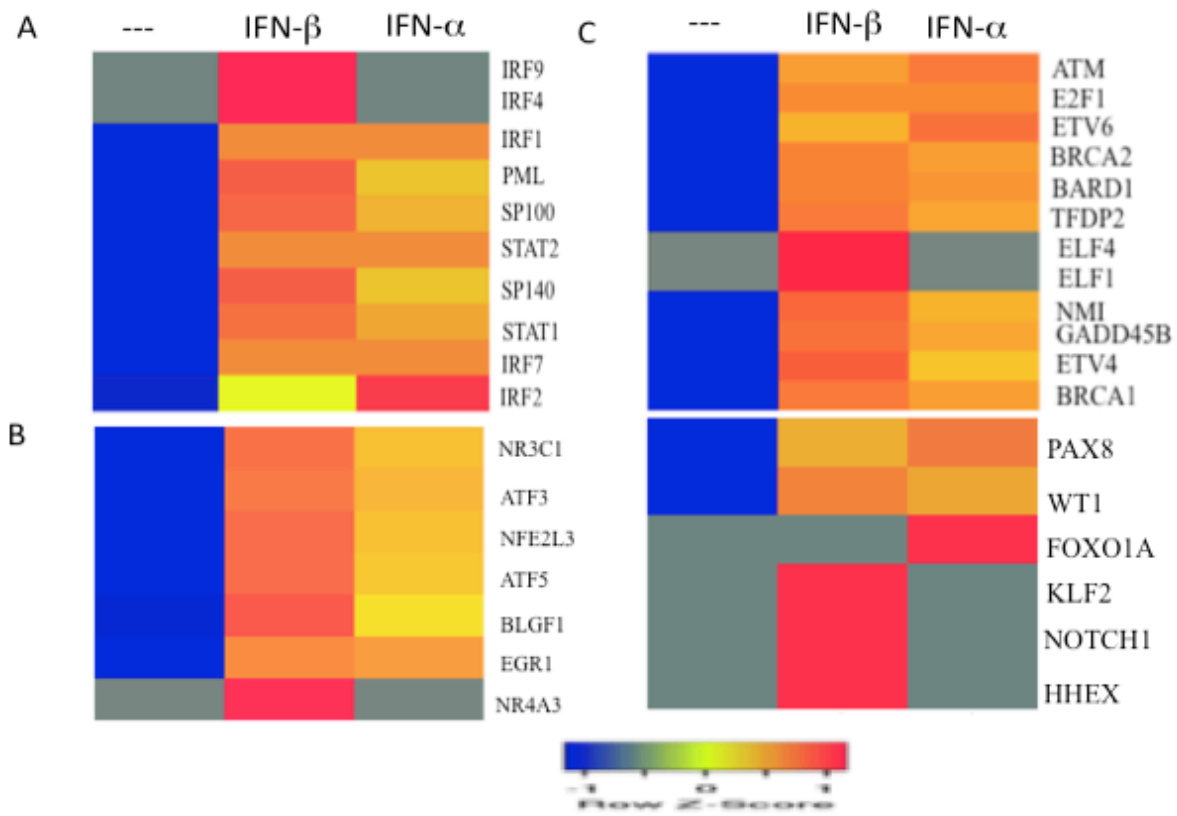
Jun
Myc
Nmi
Klf9
Maf
Klf6
Mafk
-2 -1 0 1 2
Row Z-Score

FIGURE 2


A

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IFN-α
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IRF4
IRF1
PML
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STAT2
SP140
STAT1
IRF7
IRF2
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ATF3
NFE2L3
ATF5
BLGF1
EGR1
NR4A3
C

IFN-β
IFN-α
ATM
E2F1
ETV6
BRCA2
BARD1
TFDP2
ELF4
ELF1
NMI
GADD45B
ETV4
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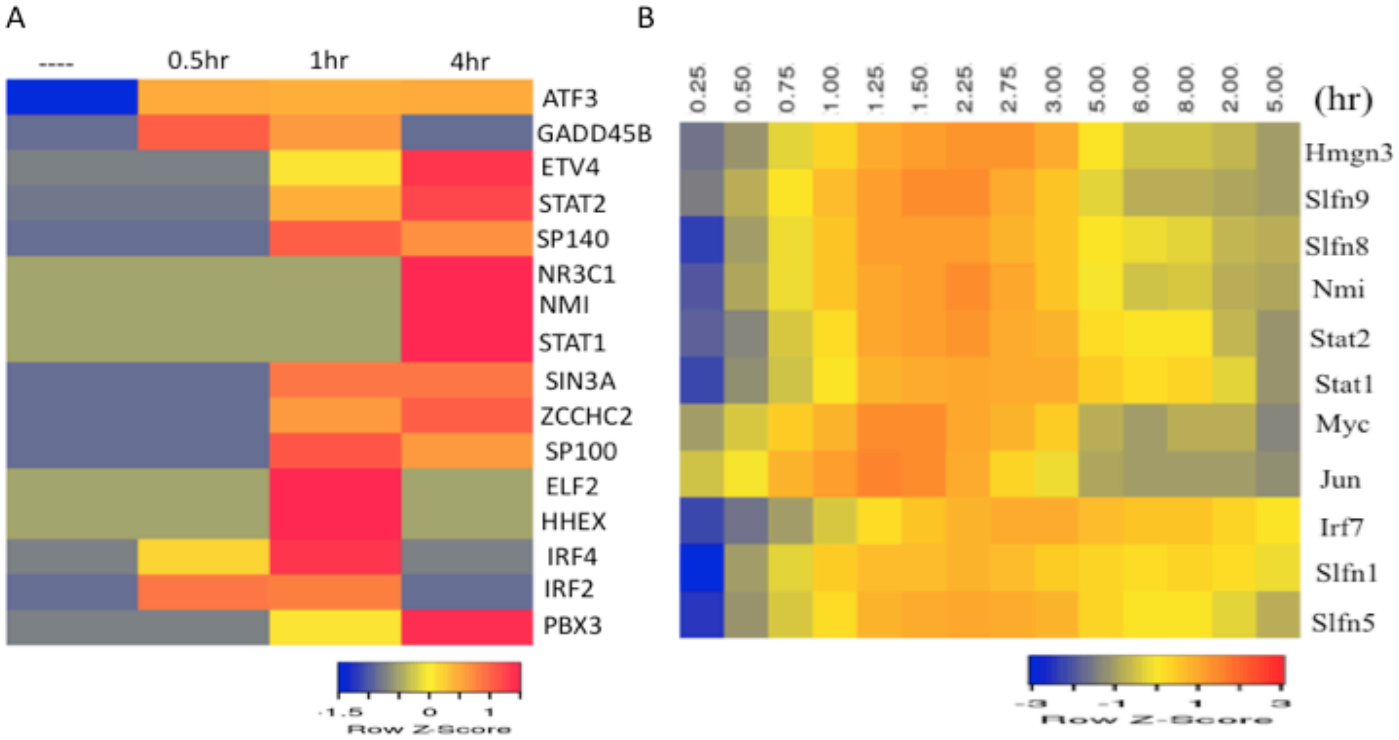
WT1
FOXO1A
KLF2
NOTCH1
HHEX
-1
0
1
Row Z-Score

FIGURE 3


A

0.5hr
1hr
4hr
ATF3
GADD45B
ETV4
STAT2
SP140
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NMI
STAT1
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ZCCHC2
SP100
ELF2
HHEX
IRF4
IRF2
PBX3
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0
1
Row Z-Score
B
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0.50
0.75
1.00
1.25
1.50
2.25
2.75
3.00
5.00
6.00
8.00
2.00
5.00
(hr)
Hmgn3
Slfn9
Slfn8
Nmi
Stat2
Stat1
Myc
Jun
Irf7
Slfn1
Slfn5
-3
-1
1
3
Row Z-Score

FIGURE 4

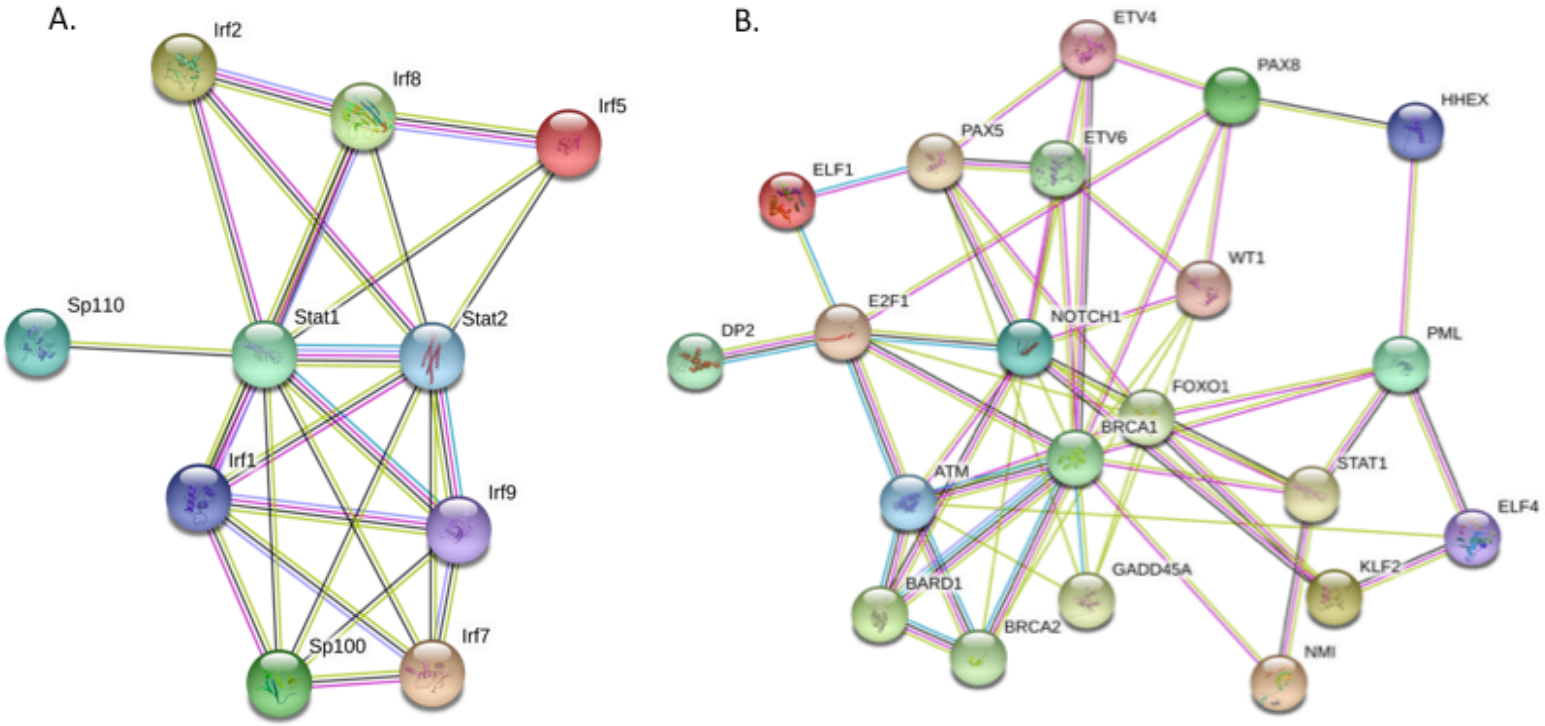

A.
Irf2
Irf8
Irf5
Sp110
Stat1
Stat2
Irf1
Irf9
Sp100
Irf7
B.
ETV4
PAX8
HHEX
PAX5
ETV6
ELF1
WT1
E2F1
NOTCH1
DP2
PML
FOXO1
BRCA1
STAT1
ATM
ELF4
GADD45A
KLF2
BARD1
BRCA2
NMI

FIGURE 5

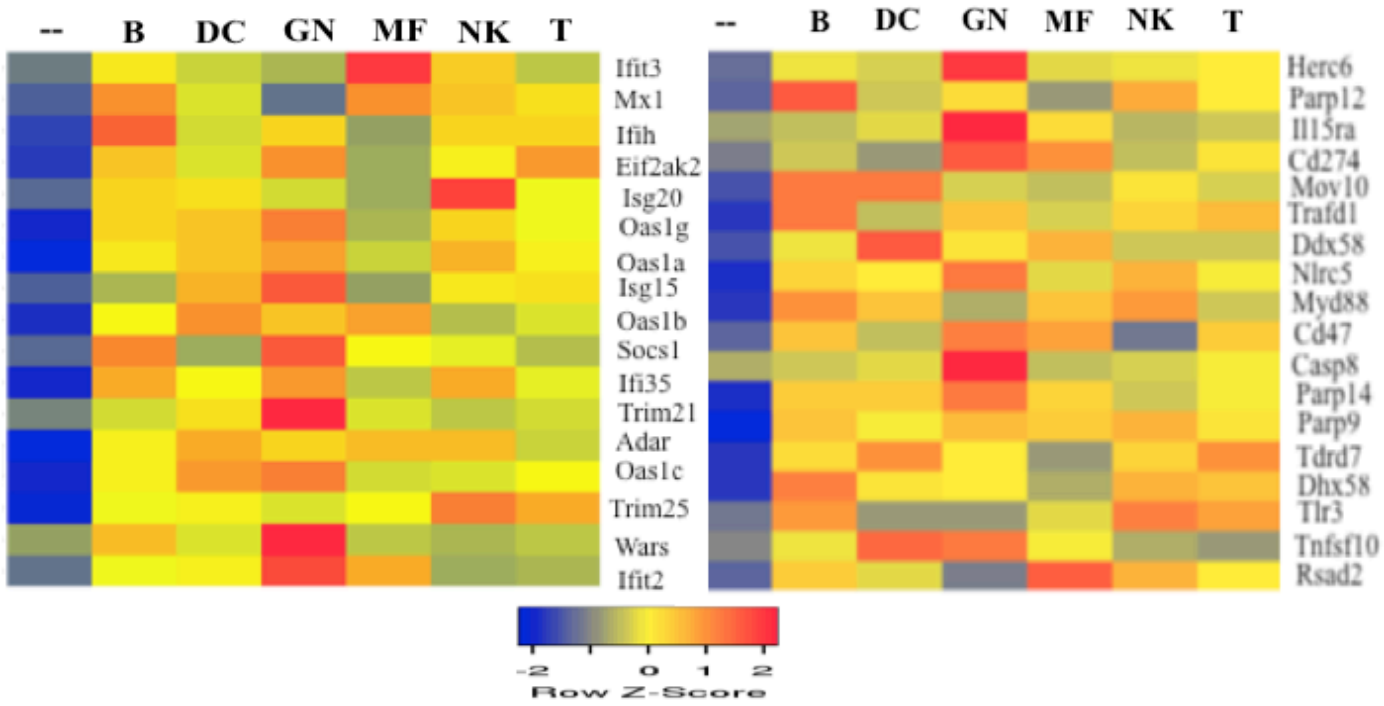
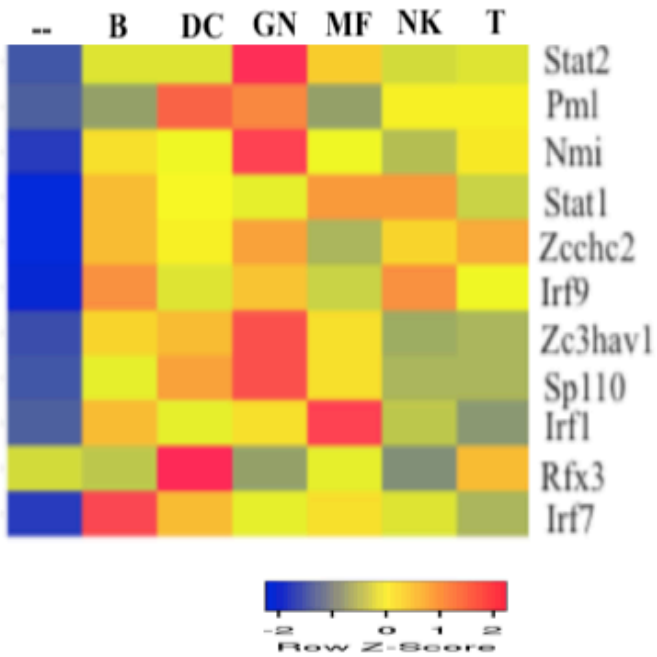

--
B
DC
GN
MF
NK
T
Ifit3
Mx1
Ifih
Eif2ak2
Isg20
Oas1g
Oas1a
Isg15
Oas1b
Socs1
Ifi35
Trim21
Adar
Oas1c
Trim25
Wars
Ifit2
Herc6
Parp12
Il15ra
Cd274
Mov10
Trafd1
Ddx58
Nlrc5
Myd88
Cd47
Casp8
Parp14
Parp9
Tdrd7
Dhx58
Tlr3
Tnfsf10
Rsad2
-2
0
1
2
Row Z-Score

FIGURE 6

A.


--
B
DC
GN
MF
NK
T
Stat2
Pml
Nmi
Stat1
Zcchc2
Irf9
Zc3hav1
Sp110
Irf1
Rfx3
Irf7
-2
0
1
2
Row Z-Score

B.

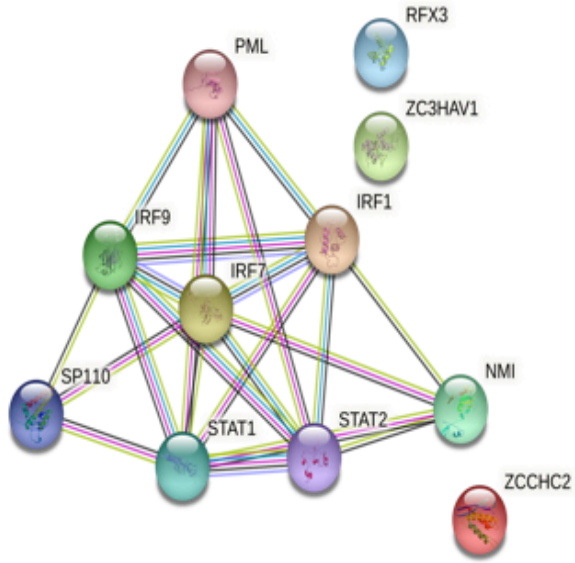

RFX3
PML
ZC3HAV1
IRF1
IRF9
IRF7
SP110
NMI
STAT1
STAT2
ZCCHC2

Figure 7

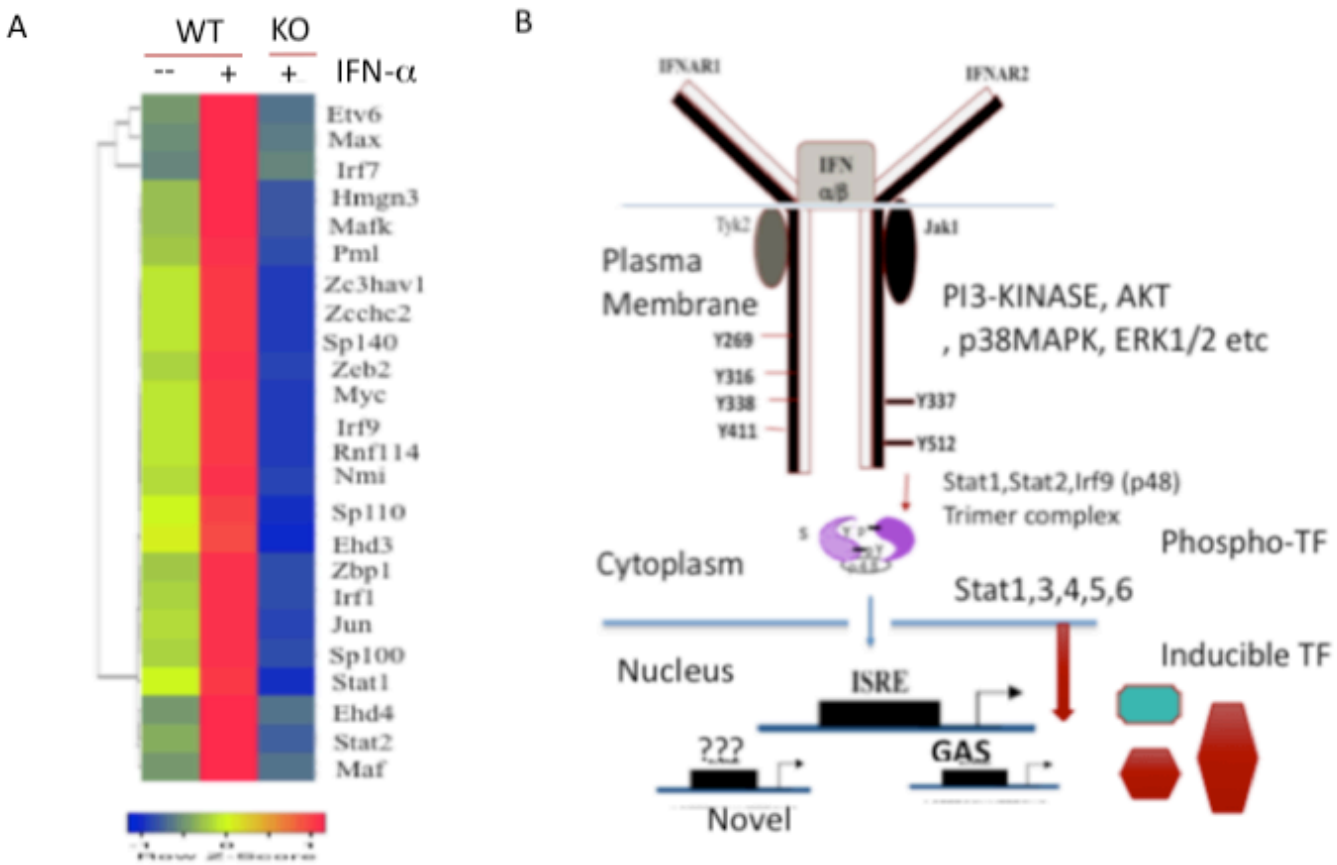

A
WT
KO
-- + + IFN-α
Etv6
Max
Irf7
Hmgn3
Mafk
Pml
Zc3hav1
Zcchc2
Sp140
Zeb2
Myc
Irf9
Rnf114
Nmi
Sp110
Ehd3
Zbp1
Irf1
Jun
Sp100
Stat1
Ehd4
Stat2
Maf
B
IFNAR1
IFNAR2
IFN αβ
Tyk2
Jak1
Plasma Membrane
PI3-KINASE, AKT
, p38MAPK, ERK1/2 etc
Y269
Y316
Y338
Y411
Y337
Y512
Stat1,Stat2,Irf9 (p48)
Trimer complex
Phospho-TF
Cytoplasm
Stat1,3,4,5,6
Nucleus
Inducible TF
ISRE
???
GAS
Novel

Figure 8

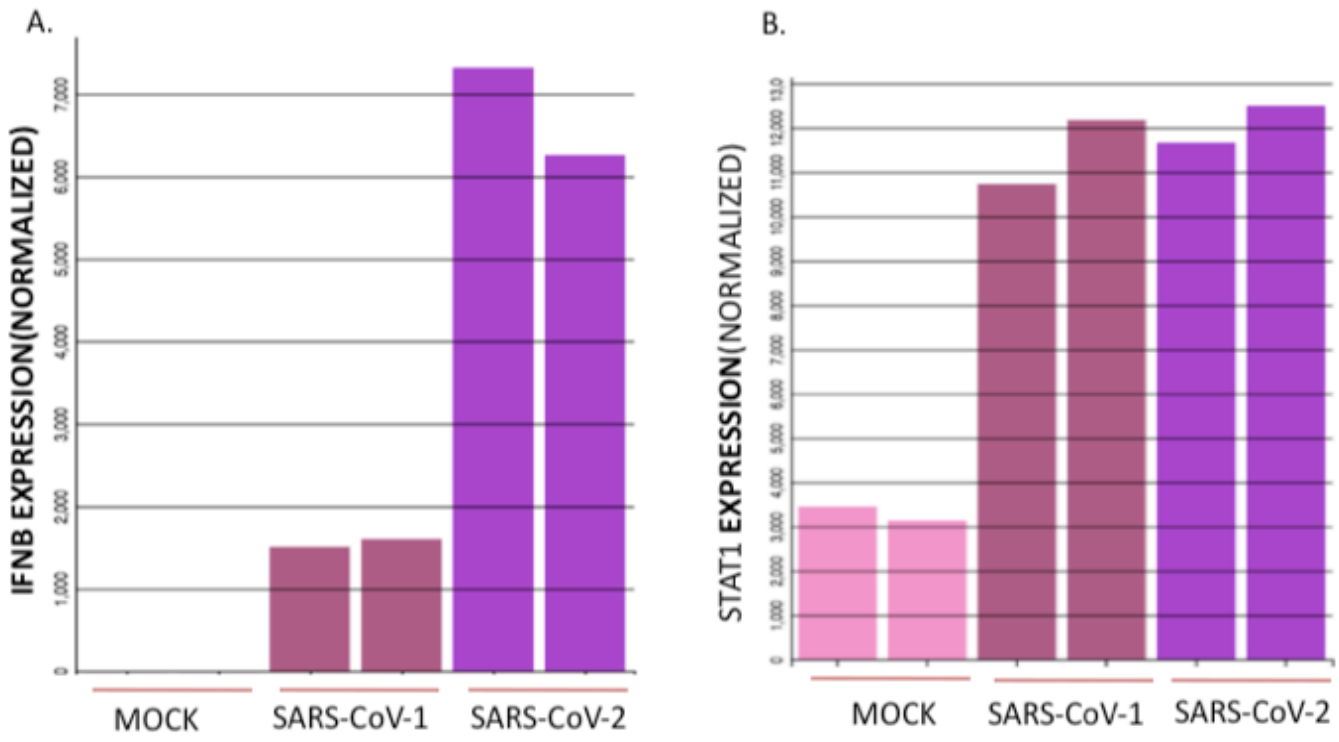

A.
IFNB EXPRESSION(NORMALIZED)
0
1,000
2,000
3,000
4,000
5,000
6,000
7,000
MOCK
SARS-CoV-1
SARS-CoV-2
B.
STAT1 EXPRESSION(NORMALIZED)
0
1,000
2,000
3,000
4,000
5,000
6,000
7,000
8,000
9,000
10,000
11,000
12,000
13,0
MOCK
SARS-CoV-1
SARS-CoV-2

Figure 9

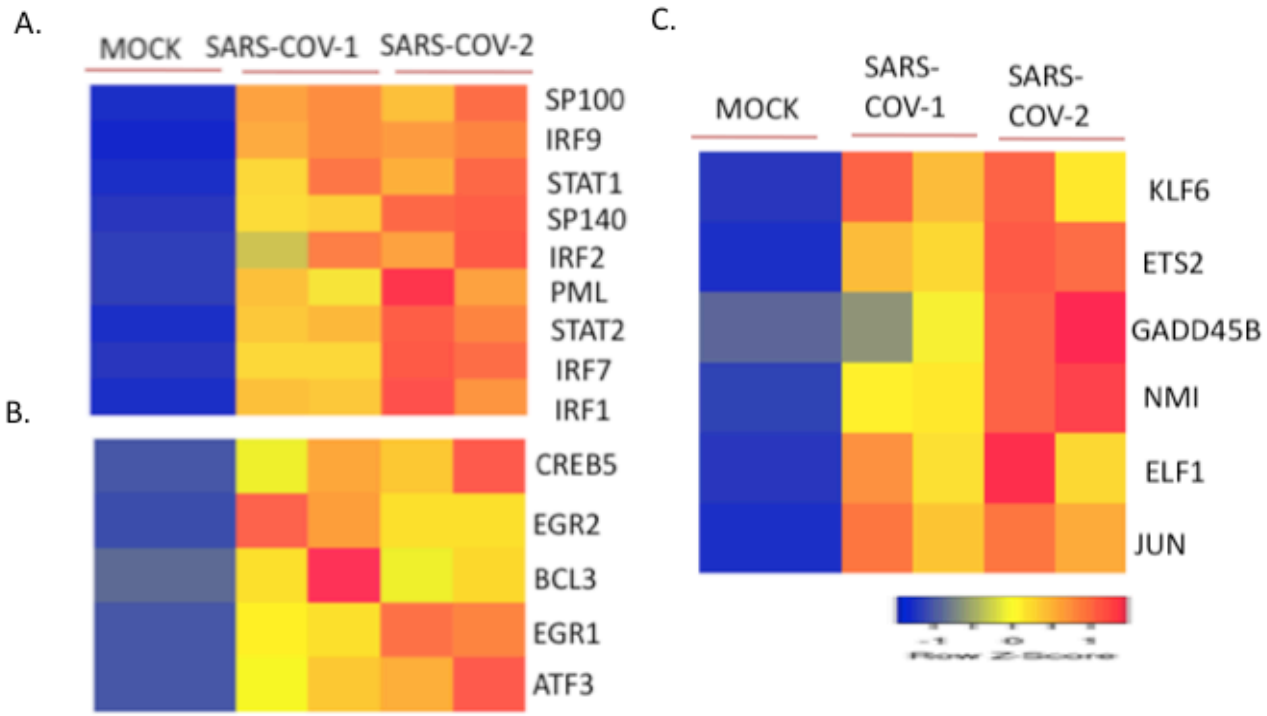

A.
MOCK
SARS-COV-1
SARS-COV-2
SP100
IRF9
STAT1
SP140
IRF2
PML
STAT2
IRF7
IRF1
B.
CREB5
EGR2
BCL3
EGR1
ATF3
C.
MOCK
SARS-COV-1
SARS-COV-2
KLF6
ETS2
GADD45B
NMI
ELF1
JUN

Figure 10

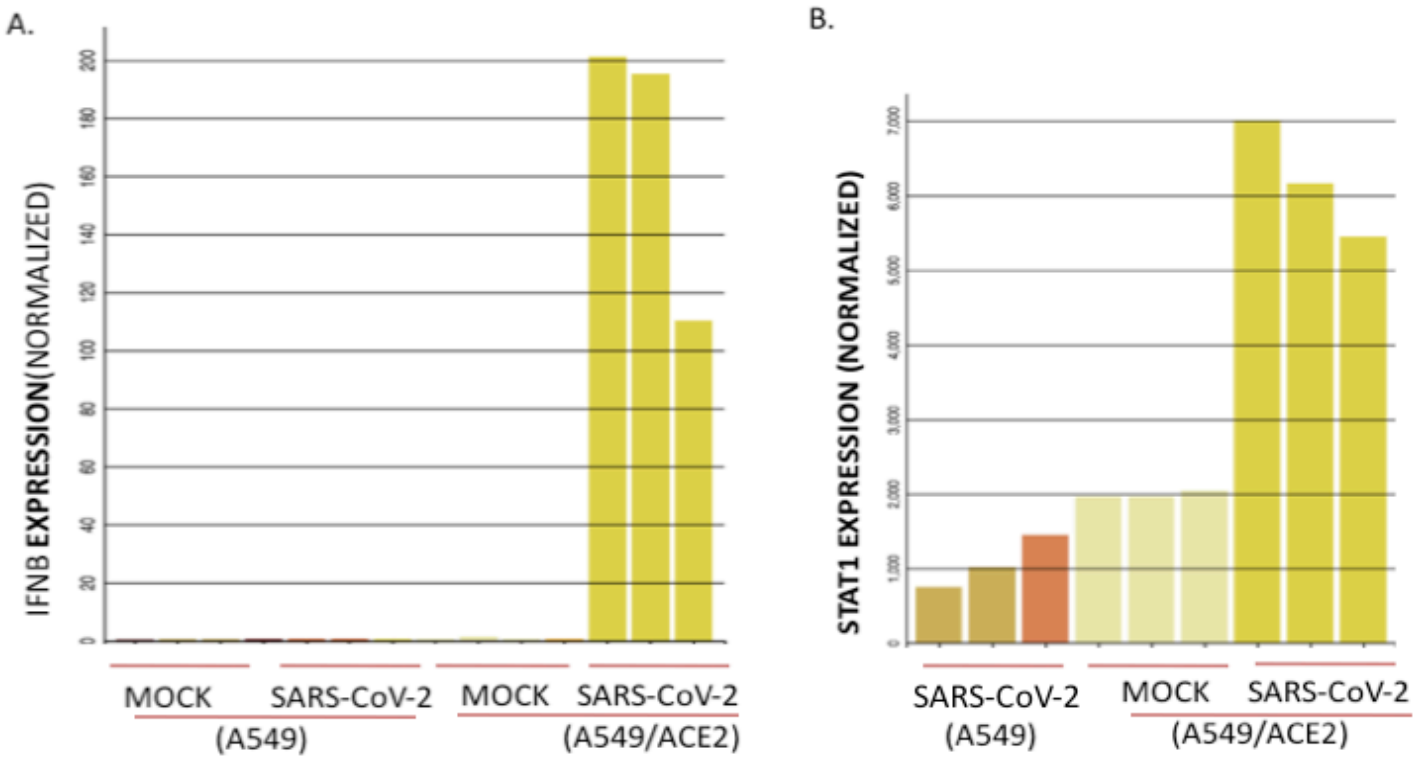

A.
IFNB EXPRESSION(NORMALIZED)
0
20
40
60
80
100
120
140
160
180
200
MOCK
SARS-CoV-2
(A549)
MOCK
SARS-CoV-2
(A549/ACE2)
B.
STAT1 EXPRESSION (NORMALIZED)
0
1,000
2,000
3,000
4,000
5,000
6,000
7,000
SARS-CoV-2
(A549)
MOCK
SARS-CoV-2
(A549/ACE2)

Figure 11

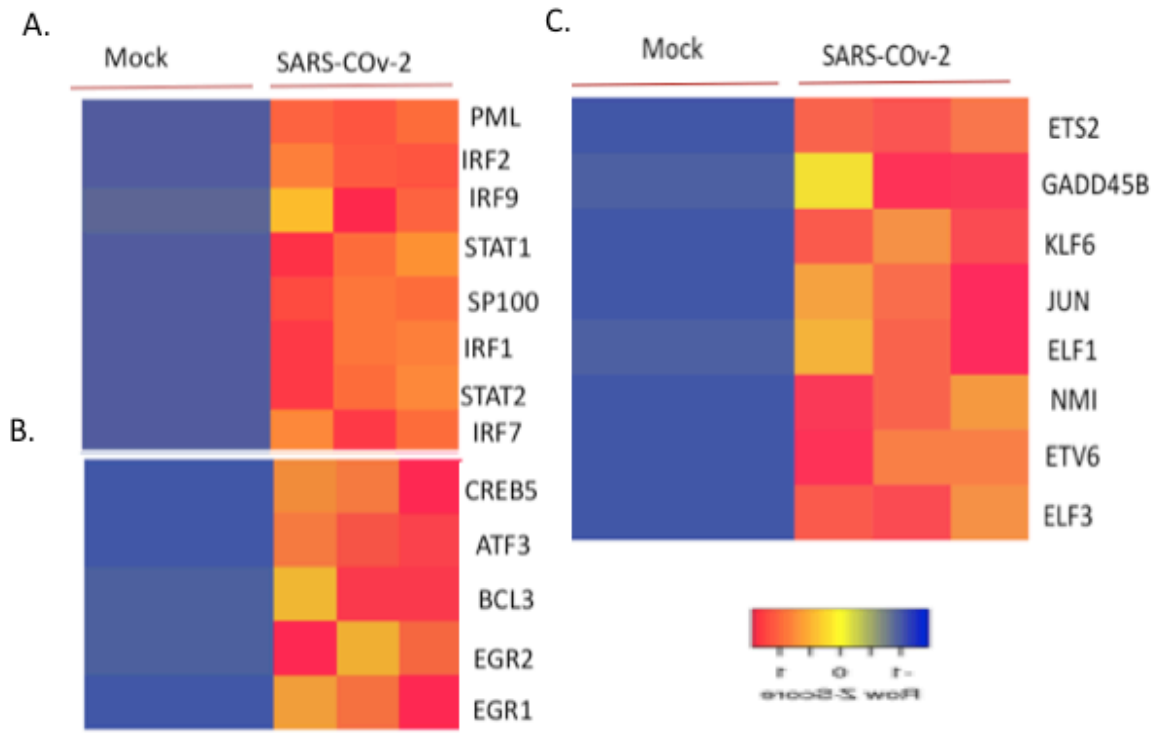

A.
Mock
SARS-COv-2
PML
IRF2
IRF9
STAT1
SP100
IRF1
STAT2
IRF7
B.
CREB5
ATF3
BCL3
EGR2
EGR1
C.
Mock
SARS-COv-2
ETS2
GADD45B
KLF6
JUN
ELF1
NMI
ETV6
ELF3

Figure 12

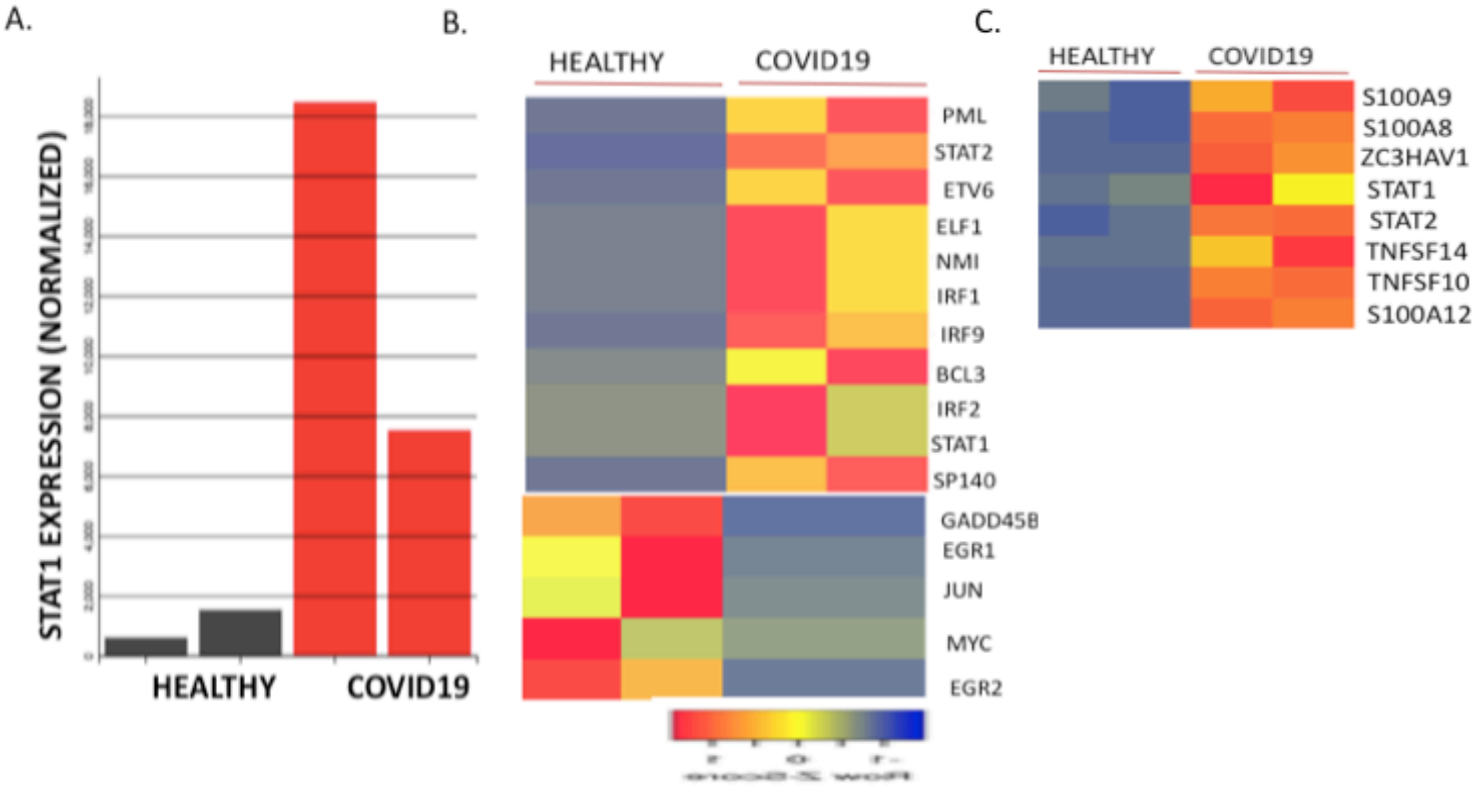

A.
STAT1 EXPRESSION (NORMALIZED)
HEALTHY
COVID19
B.
HEALTHY
COVID19
PML
STAT2
ETV6
ELF1
NMI
IRF1
IRF9
BCL3
IRF2
STAT1
SP140
GADD45B
EGR1
JUN
MYC
EGR2
C.
HEALTHY
COVID19
S100A9
S100A8
ZC3HAV1
STAT1
STAT2
TNFSF14
TNFSF10
S100A12

Figure 13

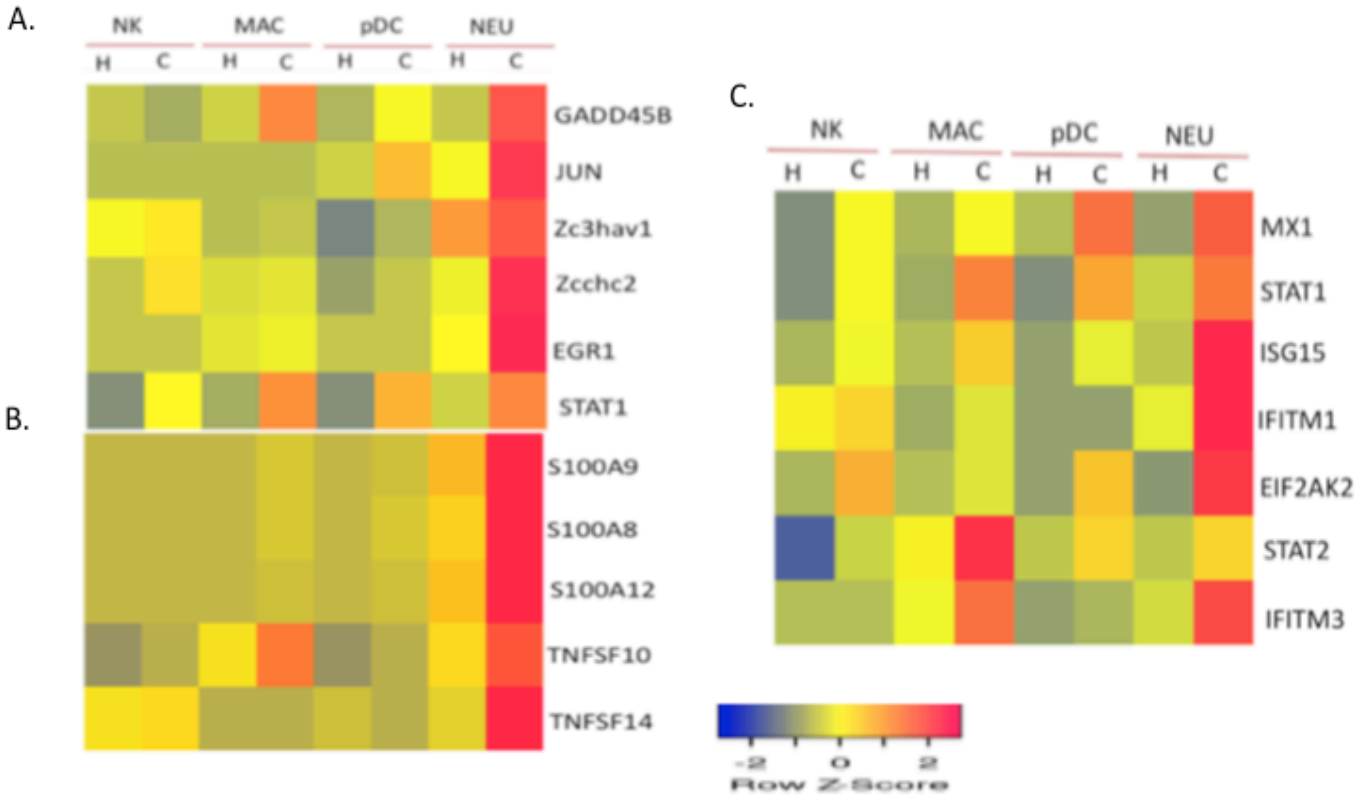

A.
NK
MAC
pDC
NEU
H
C
GADD45B
JUN
Zc3hav1
Zcchc2
EGR1
STAT1
B.
S100A9
S100A8
S100A12
TNFSF10
TNFSF14
C.
MX1
STAT1
ISG15
IFITM1
EIF2AK2
STAT2
IFITM3
-2
0
2
Row Z-Score

Figure 14