Lung and Blood RNA Expression Analysis to Identify the Potential Drug Target for COVID-19 Patients with Cytokine Storm

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Abstract

The outcome of SARS-CoV-2 infection may differ depending on factors, especially age. The elderly population is more likely to have a severe condition than the younger population. However, several young people suffer from severe lung diseases, leading to multiple organ failure and death caused by dysregulation of cytokine release, known as a "cytokine storm." The cytokine storm was first reported in 1993 in graft versus host disease and many tissue transplantation cases, but the mechanism and specific treatment have not yet been discovered. The transcriptomic analysis of RNA expression data from both lung and blood samples of COVID-19 patients was conducted to gain insight into the potential mechanism of cytokine storm in COVID-19. The data were obtained from the Gene Expression Omnibus database. The RNA reads were processed, aligned to the GRCh38 genome reference, and normalized after batch correction. Differential gene expression analysis was utilized to identify the potential contributors to the cytokine storm. Following this, the gene list was used for the co-expression network analysis and immune cell fraction analysis to identify a potential drug target for COVID-19. The analysis identified five genes, including IGLV5-35, C8B, INHBC, TNFSF11, and AMHR2, as potential contributors to the cytokine storm. Among them, AMHR2 exhibits the highest fold change compared to the other genes and plays a role in the immune system-related pathway indicating that AMHR2 is a prospective drug target. AMHR2 dysregulation might disrupt the equilibrium of the TGF beta signaling pathway, causing pro-inflammatory macrophage activation and enhanced cytokine release. The systemic effect will lead to a cytokine storm. Therefore, modulating TGF beta signaling via AMHR2 can potentially inhibit the cytokine storm in COVID-19 patients.

Keywords: Cytokine storm, Covid 19, Transcriptomic, Drug target

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Introduction

The SARS-CoV-2 virus outbreak in 2019 caused a global pandemic with a profound impact, leading to millions of deaths worldwide (World Health Organization, 2023).In the case of COVID-19, several factors, including sex and age, significantly influence disease severity (Takahashi et al., 2020; Farshbafnadi et al., 2021; Ahmad, 2020). Senior population individuals tend to experience more severe disease outcomes and higher mortality compared to the younger population (Ahmad, 2020; Farshbafnadi et al., 2021). The senior population has potentially had higher disease severity than the younger population due to dysregulation in immune aging. The dysregulation shifts the immune cell responses toward inflammatory states, up-regulates inflammatory gene expression, impairing the adaptive immune system's ability to limit infections and inflammation (Farshbafnadi et al., 2021). However, in some cases, several young people suffer from severe lung diseases indicated by hyperinflammation and low oxygen saturation (Metha et al., 2020; Cao 2020). The condition is triggered by the dysregulation of cytokine, known as a "cytokine storm" (Guo et al., 2021; Rowaiye et al., 2021).

Cytokine storm is a condition that occurs when the immune system fails to eliminate a viral infection and subsequently promotes inflammation along with the elevation of the number of cytokines released in the blood (Guo et al., 2021; Schultze & Aschenbrenner, 2021). Besides SARS-CoV-2 infection, several viruses infections including, H1N1, Mers-CoV, and SARS-CoV, can trigger cytokine storms. However, SARS-CoV-2 induces a more complex immune response compared to other infections. SARS-CoV-2 infection triggers the production of both pro-inflammatory cytokines, including TNF-α, and IL-6, and anti-inflammatory cytokines, including IL-4, and IL-10. This leads to more widespread immune dysregulation. On the other hand, SARS-CoV's Th1-dominated response with lower IL-10 levels and MERS-CoV's higher IL-6 and IL-1β levels with less regulatory T cell involvement, as well as from influenza's Th1 cytokine profile, where SARS-CoV-2 exhibits a more diverse Th1/Th2/Th17 immune activation (Pacheco-Hernández et al., 2022; Liu et al., 2021).

The complex immune response within cytokine storm caused by SARS-COV-2 infection triggers severe conditions in COVID-19 patients, with the most common symptoms being hypoxemic and respiratory failure, leading the patient to be admitted to the ICU (Hariyanto et al., 2021). Identifying potential drug targets is essential to overcoming cytokine storms in COVID-19 patients and enhancing their survivability. Furthermore, the underlying cytokine storm mechanism still needs to be determined, whether it is the direct effects of the virus or the synergistic effects of both (Meftahi et al., 2020; Wang et al., 2020). Besides causing lung damage, cytokine storm also influences the cytokine level in the blood, which triggers systemic effects, including leucocyte overactivation and recruitment to the infected site that leads to an excessive

pro-inflammatory mediated response (Agüero et al., 2021). Thus, the gene expression analysis should be conducted within the blood and lungs as the systemic effect not only dysregulates cytokine activity in the lungs but also in the blood.

The development of transcriptomics analysis has a potency to reveal the molecular mechanisms underlying complex diseases (Paananen & Fortino, 2019). The transcriptomic analysis was used to identify potential targets for Alzheimer's disease by identifying differentially expressed genes in Alzheimer's disease and normal brain samples (Patel et al., 2019). Furthermore, correlation networks are frequently used to examine gene expression data and collect biologically important data from genes with comparable co-expression patterns. Co-expression analysis has successfully identified drug targets for several infectious diseases, including influenza, tuberculosis, and hepatitis (Hasankhani et al., 2021). This study attempts to identify the potential drug target for COVID-19 patients with cytokine storm through transcriptomic data analysis and network-based analysis. The patient's gene expression data and clinical conditions were collected from the public database and the original publication. Differential gene expression, differential expression at the pathway level, gene co-expression network analysis, and immune cell fraction analysis were used to identify a potential drug target for COVID-19 cytokine storm patients. Potential targets should be upregulated in the lung and blood, which are part of immune-related pathways, and they should be of regulatory importance in network analysis.

Purpose

The research aims to investigate the potential drug targets for Covid 19 patients with cytokines storm according to gene expression in the lung and blood using differential expression analysis and gene co-expression network analysis.

Research Methodology

Data Preparation

Lung and blood Transcriptomic data from COVID-19 patients were collected from the GEO database with identifiers GSE150316, GSE134692, and GSE183533 for lung, GSE155454, and GSE166424 for blood. These datasets included samples from both young and senior populations, with ages ranging from 15 to 77 years, and included both men and women (Desai et al., 2020; Sivakumar et al., 2019; Budhraja et al., 2022; Chan et al., 2021). The data were assigned to normal, non-trigger, and trigger cytokine storms. The normal group comprised samples that did not have an infection of SARS-CoV-2. On the other hand, the COVID-19 sample that tested positive and had one or more clinical conditions such as pneumonia, edema, dyspnea, hypoxemia, and ARDS, was categorized as part of the cytokine storm trigger group since these conditions

are developed by a cytokine storm (Fajgenbaum & June, 2020). The non-trigger group consists of positive COVID-19 samples that do not exhibit any of these clinical criteria.

Blood and lung data were prepared separately during the preparation process. First, the gene with a count lower than ten counts per million (CPM) was filtered out (Lun et al., 2016). Afterward, the data were normalized using the trimmed mean of the M-values method by Robinson and Oshlack (2010). The Gene co-expression network analysis data set was prepared by normalizing the filtered-out data using reads per kilobase per million (RPKM) by EdgeR function. Data preprocessing was done to ensure the data quality for further analysis.

Differential Expression (DE) Gene Analysis

The DE analysis can be employed to investigate genes associated with the disease's condition. The analysis compared the gene expression from the cytokine trigger group sample to the non-trigger cytokine storm and normal groups. The analysis was conducted using the edgeR robust method in the R-based package: edgeR to prevent the interference of the outlier data in the case of the high level of expressed count dispersion (Robinson et al., 2009; Zhou et al., 2014; Raithel et al., 2016). The results with P-value < 0.01, False Discovery Rate (FDR) < 0.05, and the absolute value of Log Fold Change (LFC) > 1 were considered as differentially expressed genes (Sadanandam et al., 2020).

Differential Expression Analysis at Pathway-Level

The differential expression analysis at the pathway level was conducted to gain more insight into the relationship between differentially expressed genes and biological activity. Firstly, the gene set containing a list of genes, and their associated pathway was obtained from the Reactome and the KEGG database (Kanehisa & Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2022; Gillespie et al., 2021; Jassal et al., 2019). Then, the gene set enrichment score calculation was done using the R-based package: GSVA using normalized CPM (Hänzelmann et al., 2013). The hypothesis was that there are no significantly different enriched pathways in cytokine trigger samples compared to negative and non-trigger samples. R-based package limma was used to conduct the analysis. The results with P-value < 0.05, FDR < 0.05, and an absolute value of LFC > 0 were chosen as significantly different enriched pathways limma (Smyth, 2004).

Cell Fraction Analysis

Cell fraction analysis provides information on the relative distribution of 22 immune cell types and subtypes within samples. The samples' relative percentage of immune cell fraction was identified using Cibersortx. Cibersortx identifies the cell abundance by comparing the normalized CPM value of genes from

the data set to the single-cell gene signature matrix (Newman et al., 2019). Analysis using Cibersortx was conducted on the website interface (CIBERSORTx, n.d.) using the LM22 signature matrix and batch correction removal running parameters. Since the number of samples in each group is unequal, the mean comparison of relative fractions from several immunological subtypes is compared using Welch's t-test (Zhenqiu & Ke-Hai, 2010).

Gene Co-expression Network Analysis

A gene co-expression network is utilized to identify the crucial genes in a specific biological system under certain conditions. The network construction began by computing the correlation coefficient between genes within the gene set using the RPKM normalized data of the samples. Each gene is represented by a node, while the co-expressed genes are connected by edges. Prior to network construction, C2 curated gene sets were imported as a gene set reference using the GSVAdata library on R studio. Afterward, the prepared data sets from the primary data set preparation were imported to the R studio. The gene co-expression network was calculated using Gene Set Net Correlation Analysis (GSNCA) by "GSNCAtest" and "plotMST2.pathway function ()". GSNCA introduced weight to each gene according to its cross-correlation with other genes. The essential gene in the network tends to have a high weight (Rahmatallah et al., 2013; Liu et al., 2019). The top 10 highly connected genes are considered the hub genes.

Results

Data Preparation

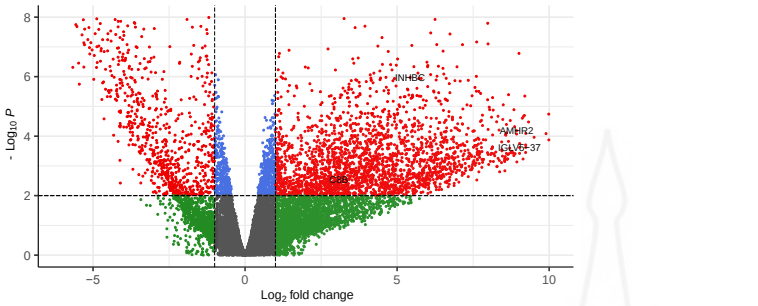
The gene count of RNA expression in lung tissue was retrieved from the GEO database, accession numbers GSE150316, GSE134692, and GSE183533, whereas the data for blood was obtained from GSE155454 and GSE166424. Based on the clinical condition of the samples, 80 lung samples and 96 blood samples are eligible to be assigned to the groups. Table 1 of the supplementary materials presents the subject information. After batch correction, the data showed a more dispersed distribution instead of being clustered according to its batch. Supplementary Figure 1 visualizes the result of batch correction.

Differential Gene Expression analysis

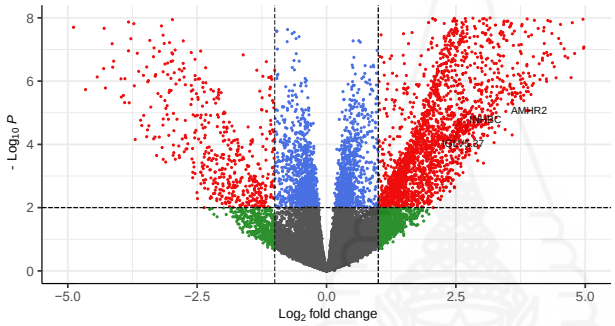
The differentially expressed (DE) genes analysis approach is used with the prepared data set to identify the significantly changed genes for the cytokine storm condition in Covid 19 patients. Out of 25,541 genes in lung samples, 2188 genes were detected as upregulated genes, and 493 genes were downregulated, as shown in Figure 1A. In addition, the blood DE analysis reveals that 3,337 genes are

upregulated, while 523 genes are downregulated. The DE genes from the lung and blood are compared to find the upregulated genes in both tissues. As shown in Figure 1 C, there are 290 intersection genes between the lung and blood. Gene set from the KEGG dan Reactome database was utilized to observe the biological relevance of the 290 upregulated genes. According to the KEGG and Reactome database, the 290 intersection genes are involved in 62 known biological pathways with at least two genes as members of the pathway gene set (Kanehisa & Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2022; Gillespie et al., 2021; Jassal et al., 2019).

The intersection of genes associated with immune system signaling and cytokine signaling was observed to narrow down the gene candidates, as immune system signaling and cytokine signaling dysregulation might trigger the cytokine storm (Guo et al., 2021; Yang et al., 2021). According to the Reactome immune system signaling gene set, intersection genes associated with the immune system signaling are IGLV5-35 and C8B. On the other hand, The KEGG cytokine-cytokine receptor signaling pathway reveals that INHBC, TNFSF11, and AMHR2 are associated with cytokine signaling activity. In addition, the log-fold change (logFC) of AMHR2 is consistently high compared to the other four genes.



(A)



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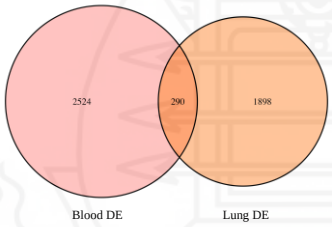
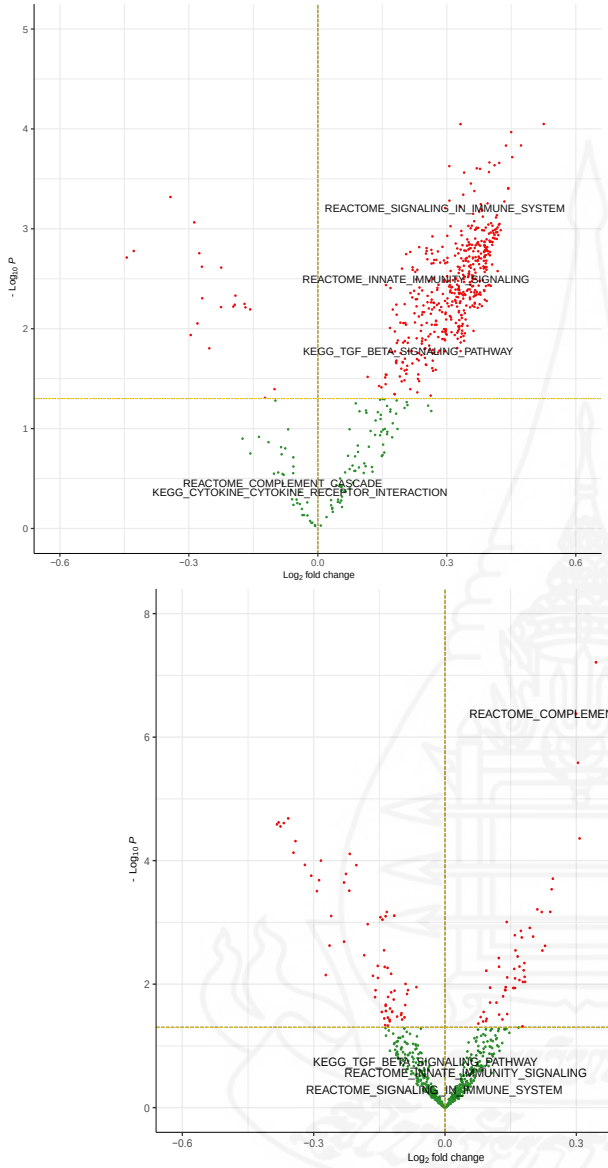


(C)

Figure 1A. Visualization of lung DE result, 1B. Visualization of blood DE. DE genes that exceeded -1 and 1 log fold change thresholds are displayed in red, while those that did not are shown in blue. Non-DE genes with a p-value above 0.01 are below the black dashed line. Figure 1C illustrates the number of upregulated genes in the lung, blood, and intersection genes. There are 290 genes upregulated in lung and blood samples, whereas 1,898 genes are only upregulated in the lungs, and 2,524 genes are only upregulated in the blood.

Differential Pathway Expression Analysis

Differential pathway expression analysis compares gene sets to identify a highly active pathway in cytokine storm trigger samples. The investigation included 604 pathways, for which information was acquired from the databases of the KEGG and Reactome gene sets (Kanehisa & Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2022; Gillespie et al., 2021; Jassal et al., 2019). There are 469 and 54 significantly upregulated pathways at 0.05 significant levels in lung and blood, respectively. As focusing on the immune-related pathways, the KEGG TGF beta signaling pathway, Reactome signaling in the immune system, and Reactome innate immunity signaling are significantly upregulated in cytokine storm lung samples, while only the Reactome complement cascade is significantly upregulated in cytokine storm blood samples, as illustrated in Figure 2A and B. Furthermore, no upregulated pathway in the lung has been identified as an upregulated pathway in the blood.



(A) (B)

Figure 2. Volcano plot of differential pathway expression analysis: A.Lung, B.Blood. The red dot reflects significantly different expression pathways, while the green dots represent non-significant pathways. The red dots with a Log2 fold change greater than zero show enhanced expression in cytokine trigger samples compared to negative and non-trigger data.

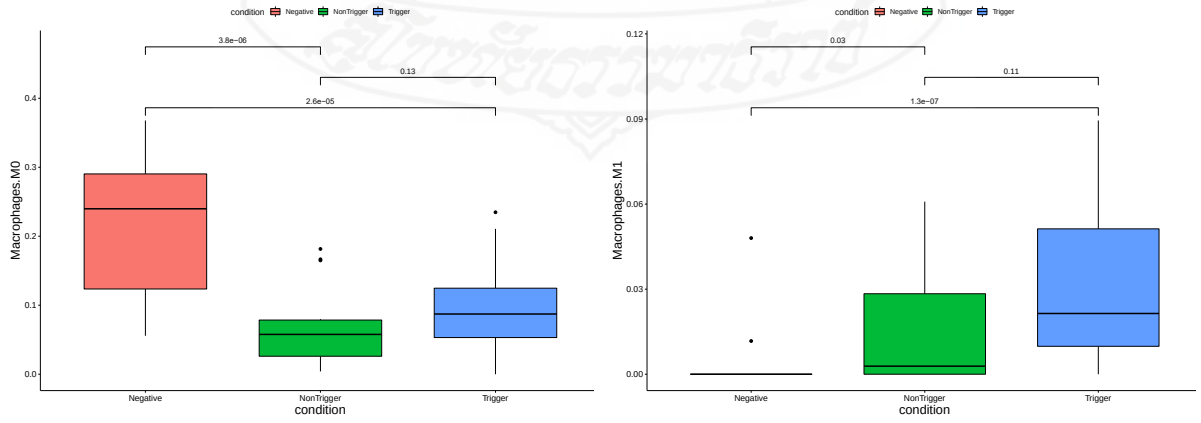
Table 1. Gene weight within the gene co-expression network

Pathways	Genes	GSCNA weight	Rank
Reactome Immune system signaling	IGLV5-37	1.005	149 of 344
	C8B	0.917	224 of 344
KEGG TGF beta signaling pathway	INHBC	0.885	55 of 82
	AMHR2	1.059	27 of 82

Gene co-expression network

The gene co-expression network was built using gene set information from the Reactome and KEGG databases (Kanehisa & Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2022; Gillespie et al., 2021; Jassal et al., 2019). The Reactome immune system signaling pathway co-expression network is constructed from 344 genes, while the KEGG TGF beta signaling pathway has 85 genes, as illustrated in Supplementary Figure 2. The analysis demonstrates that the co-expression pattern of the KEGG TGF beta signaling pathway in lung cytokine storm trigger samples is not significantly different from co-expressed compared to the non-trigger samples. A similar result was obtained for the Reactome immune system signaling pathway co-expression network. Besides, the analysis provides a net gene correlation value term as a weight for each gene in the network, with AMHR2 having the highest weight and the lowest rank compared to INHBC, C8B, and IGLV5-37, as described in Table 1. Since the TNFSF11 is not part of any of those pathways then it was not included in Table 1.

Cell fraction analysis



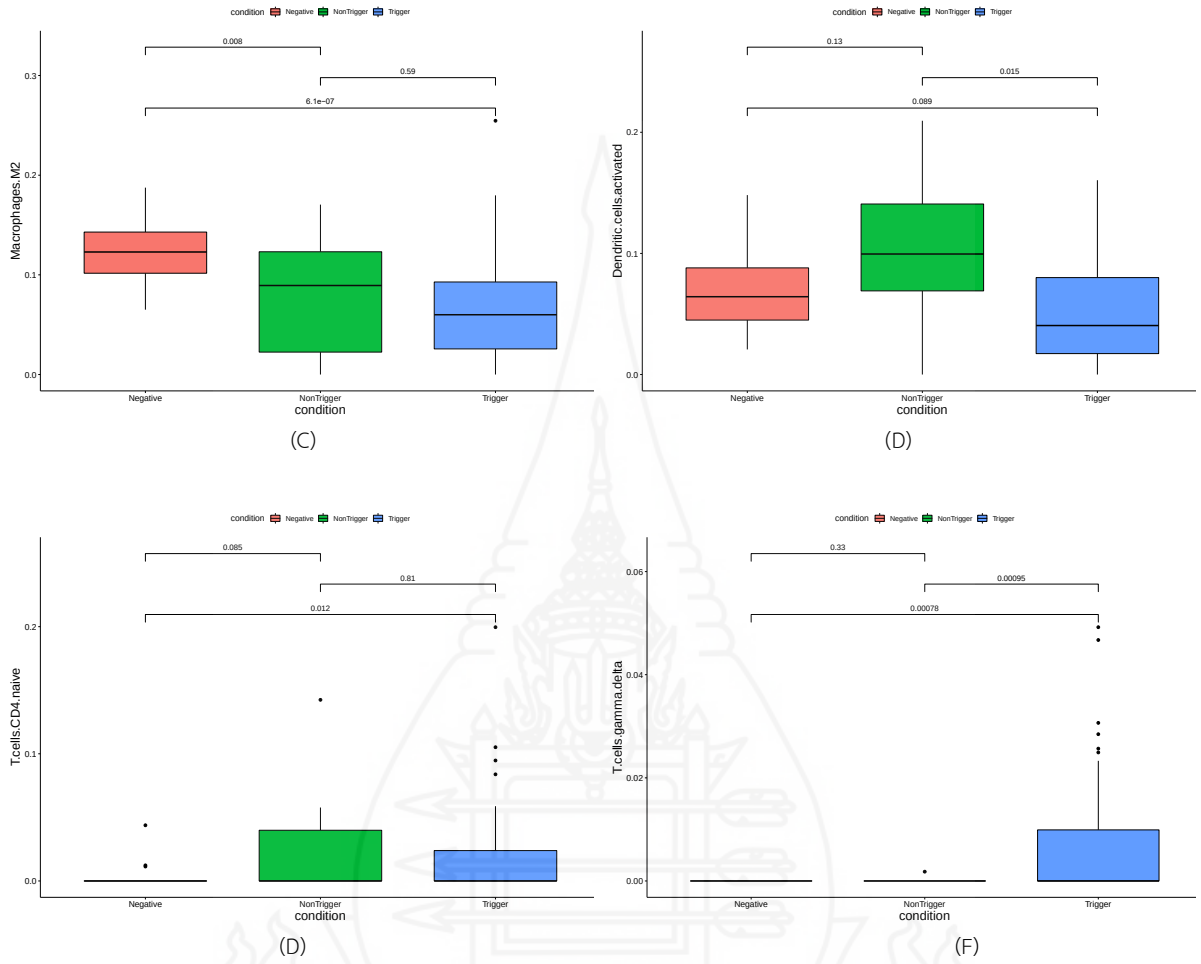


Figure 3. Lung immune cell fraction means comparison: A. Macrophages M0, B. Macrophages M1, C. Macrophages M2, D. Activated dendritic cell, E. naive CD 4 T cell, F. T cell gamma delta.

The analysis attempts to observe the relative immune cells of lung cytokine storm trigger samples, lung cytokine storm non-trigger samples, and lung negative samples. Under the P value 0.05 cutoff, lung cytokine storm trigger samples had a significantly smaller proportion of macrophage M0 and M2 than negative samples. In contrast, their macrophage M1, T cell gamma delta, and naive CD 4 T cell fractions were significantly higher compared to the negative group. Furthermore, the lung cytokine storm trigger group has a significantly lower activated dendritic cell fraction and higher T cell gamma delta than the cytokine storm non-trigger group. The comparison is illustrated in Figure 3.

Discussion

Prior to analysis, the low-count and zero-count data were excluded since the genes may not achieve a biologically meaningful level (Bourgon et al., 2010). Both typical gene expression data with values less than 10 are technically noise and filtering them will improve the statistical power of the analysis (Law et al., 2018; Lun et al. 2016). The batch correction was necessary since the data came from different technical processing batches and were prone to data variance caused by the experimental batch rather than by the biological condition (Zhang et al., 2020).

The cytokine storm in COVID-19 disturbs lung and blood hemostasis (Fajgenbaum & June, 2020; Shen et al., 2021). Hence, the primary criterion for examining the potential therapeutic target for cytokine storm in COVID-19 patients is that the genes must exhibit increased expression in both the lungs and blood. Besides, the upregulated genes can potentially be drug target candidates, as it is relatively more convenient to inhibit the target activity than to activate it (Sloris et al., 2018). Moreover, as the cytokine storm arises from the deregulation of the immune system (Fajgenbaum & June, 2020; Shen et al., 2021), the specific target should also be linked to pathways relevant to the immune system.

In comparison with other coronavirus infections and influenza, SARS-CoV infection initiates the release of chemokines such as CCL2, CCL3, CCL5, and CXCL10. The chemokine will recruit the immune cells to the infection site. The recruited cells, including dendritic cells and macrophages, produce an excessive number of cytokines like TNF-α, IL-6, and interferons which can lead to cytokine storms. Meanwhile, MERS-CoV infection induces cytokines production, including IL-6, IL-1β, IL-8, and IFN-γ, but with a slower response compared to SARS-CoV (Liu et al., 2021). Unlike both coronaviruses, severe influenza infections are often associated with high levels of both pro-inflammatory cytokines, including IFN-γ and IL-6, and anti-inflammatory mediators like IL-10 and TGF beta (Pacheco-Hernández et al., 2022). The increasing production of both pro-inflammatory and anti-inflammatory cytokines is also observed in cytokine storms caused by SARS-CoV-2 infection, which leads to a complex immune profile (Liu et al., 2021).

Based on the findings, AMHR2 could be a prospective target as its expression changes are relatively high among the other prospected intersection genes related to immune system activity. A study by Matteucci et al.'s (2020) utilized the CD8+ T from peripheral blood cells of patients with COVID-19 cells treated with Tα1 and LPS to observe how Tα1 might modulate or counterbalance the strong inflammatory response

induced by LPS. The study demonstrates that AMHR2 was consistently expressed and regulated, suggesting a possible regulatory role of AMHR2 in cytokine signaling and immune response pathways. The increasing activity of AMHR2 may trigger the increasing activity of associated pathways, such as the KEGG TGF beta signaling pathway (Kanehisa & Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2022; Gillespie et al., 2021; Jassal et al., 2019). As demonstrated in differential pathway expression analysis, the KEGG TGF beta signaling pathway was positively enriched, indicating that the overactivity of AMHR2 might potentially enhance KEGG TGF beta signaling pathway activity. The findings are consistent with a study conducted by Shen et al.'s (2021), which argues that the cytokine storm observed in COVID-19 is a consequence of the imbalanced cytokine activity induced by the heightened TGF beta activity. Increased TGF beta activity inhibits the process of lymphocyte differentiation and proliferation, resulting in a decrease in the number of lymphocytes in circulation. Consequently, it will delay the adaptive immune response and induce cytokine storms in patients with Covid 19 (Gillespie et al., 2021; Shen et al., 2021). In addition, the activity of AMHR2 might have an essential role within the gene network as its net correlation value is relatively high compared to the four other intersection genes.

The immune cell fraction was analyzed to gather additional data regarding the potential impact of the highly active AMHR2 and KEGG TGF beta signaling pathway, which might potentially initiate the cytokine storm. The upregulation of AMHR2 has been reported to cause the M1/M2 disequilibrium through the activity of the TGF-signaling pathway. The M1 macrophage is an activated macrophage with pro-inflammatory properties that is responsible for eliminating pathogens and infected cells. On the other hand, the M2 macrophage plays a role in promoting tissue healing and cell proliferation. These distinctions have been supported by studies (Orecchioni et al., 2019; Italiani & Boraschi, 2014, Beck et al., 2016). The polarization of M1 macrophages correlates positively with lung injury and TGF beta activity (Wang et al., 2021). As AMHR2 was highly upregulated in lung and blood samples, its expression may disturb the equilibrium of the TGF beta signaling pathway, resulting in the M1 macrophage fraction being higher than the M2 macrophage fractions in lung trigger samples. As a result, the excessive production of AMHR2 can stimulate the function of TGF beta signaling, leading to the differentiation of M0 macrophages into M1 and subsequently triggering inflammation in the lung.

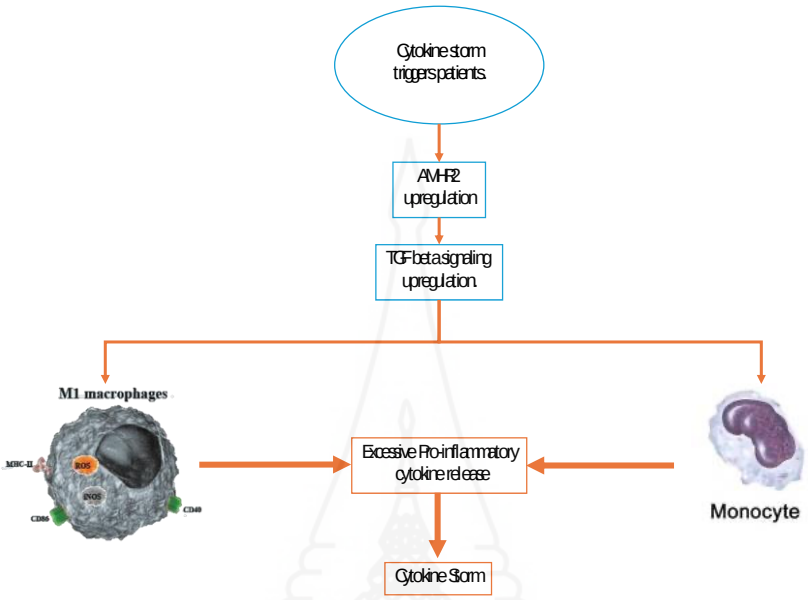


Figure 4. Cytokine storm potential development. The upregulation of AMHR2, a member of the TGF beta signaling pathway, may induce the TGF beta signaling pathway. It may induce the upregulation of the TGF beta signaling pathway and might trigger M1 macrophages, and T cell gamma delta proliferation. Following this, an excessive amount of proinflammatory cytokine will be released.

Further evidence of TGF beta signaling activity causing a cytokine storm can be seen in the elevated proportion of T gamma delta cells in samples that generate a lung cytokine storm. T gamma delta cells are a type of T cell that has a T-cell receptor (TCR) with gamma and delta chains. It is present in various tissues, including the lungs, for maintaining tissue homeostasis by producing cytokines to overcome viral infection, and its activity is enhanced by the TGF beta activity (Cheng & Hu, 2017; Peters et al., 2018). Therefore, the study of differential gene and pathway expression showed that increased AMHR2 expression and enhanced TGF beta pathway activity may lead to the production of excessive cytokines by T gamma delta cells. The potential cytokine storm development is illustrated in Figure 4. Overall, the examination of immune cell infiltration shows that the increased expression of AMHR2 may trigger the upregulation of the TGF beta signaling pathway, which in turn activates pro-inflammatory macrophages and enhances the release of cytokines by T gamma delta cells.

Suggestion

In summary, the analysis demonstrates that 290 genes are upregulated in the lungs and blood of COVID-19 patients with cytokine storm. Among these genes, there are five that are linked to pathways relevant to the immune system: TNFSF11, IGLV5-35, C8B, INHBC, and AMHR2. AMHR2 exhibits the highest fold change compared to the other genes, suggesting that AMHR2 is more active. The increased expression of AMHR2 may result in excessive activation of the KEGG TGF beta signaling pathway, leading to the augmentation of pro-inflammatory macrophages and T gamma delta cells' production of cytokines. This systemic action can lead to the occurrence of a cytokine storm. Thus, AMRH2 can serve as a promising target for alleviating cytokine storms in Covid 19 patients through the inhibition of the KEGG TGF beta signaling pathway. However, additional research is required to confirm the effectiveness of AMHR2 as a therapeutic target for suppressing cytokine storm activity in patients with COVID-19.

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