Monocytes and NK Cells Modulate Therapeutic Outcomes in Severe COVID-19: A Single-Cell RNA Sequencing Study

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ABSTRACT

Monocytes and NK Cells Modulate Therapeutic Outcomes in Severe COVID-19: A Single-Cell RNA Sequencing Study

Severe COVID-19 infection is characterized by a profoundly dysregulated immune response, including hyperactivation and functional impairment of immune cells along with an excessive inflammatory response. Corticosteroid tocilizumab is used in accordance with guidelines to improve the prognosis of patients with severe COVID-19. However, the differences in drug response among patients remain a challenge to be addressed. This study aims to identify the molecular and cellular determinants that cause differences in treatment outcomes in the combination therapy of tocilizumab, dexamethasone, and remdesivir in patients with severe COVID-19 using single-cell RNA sequencing. From June 2021 to January 2022, blood samples were collected from 17 matched patients with severe COVID-19 at a tertiary hospital according to age, gender, and WHO Clinical Progression Scale. Patients were categorized into two groups: ten recovered without deterioration, and seven required intubations due to respiratory failure. Samples were taken on day 1 and day 7. Dropkick was employed to process and analyze single-cell ribonucleic acid sequencing data derived from Peripheral Blood Mononuclear Cells. Heatmaps of differentially expressed genes were generated using the Complex Heatmap package, while Gene Ontology enrichment analysis was performed using the Enrichr tool from Ma'ayan lab, integrated into Seurat (versions 3.9 and 4). This study compared differences in immune cells between patient groups by examining patients with good prognosis (day 1 [n=13,580] and day 7 [n=14,017]) and patients with poor prognosis (day 1 [n=13,747] and day 7 [n=13,630]) after TDR treatment. In the poor prognosis group, the nucleotide-binding oligomerization domain-like receptor signaling pathway and natural killer (NK) cell-mediated cytotoxicity were downregulated after TDR treatment. However, inflammatory macrophage related lysosome, phagosome, and apoptosis pathways persistently remained upregulated. On the other hand, the good prognosis group showed increased NK cell-mediated cytotoxicity and decreased inflammatory macrophage related pathways after TDR treatment. Additionally, the good prognosis group exhibited elevated expression of genes linked to T cell receptor activation, signaling pathways, and differentiation processes related to immune regulation. In conclusion, the suppression of the hyperinflammatory pathway by macrophages and the enhancement of natural killer cell-mediated cytotoxicity, along with the adaptive immune response induced by T cells, emphasize the importance of maintaining a well-regulated immune balance in the management of severe COVID-19 cases.

Key words : SARS-CoV-2 infection, COVID-19, Monocytes, Killer Cells Natural, Single-Cell Gene

Expression Analysis, Immunomodulation.

I. INTRODUCTION

COVID-19 has emerged a global infectious disease, causing approximately 776,205,140 cumulative cases and 7,064,380 deaths worldwide by September 2024 (1). The clinical spectrum of COVID-19 varies significantly, ranging from asymptomatic to severe illness, with some patients experiencing respiratory failure and fatal outcomes (2). The pathophysiology of COVID-19 involves several mechanisms, including direct viral cytotoxicity, damage to endothelial cells and microvasculature, immune system dysregulation with a heightened proinflammatory response, hypercoagulability resulting in thrombosis, and disruption of the angiotensin-converting enzyme 2 (ACE2) pathway (2, 3). As an RNA virus, COVID-19 is highly prone to mutations during replication, facilitating its evolution into various forms (4). In recent times, its severity in terms of causing critical illness has decreased, while its transmissibility has remained high (4). However, the mortality rate remains elevated in high-risk populations, including individuals with underlying diseases and the elderly, underscoring the need for proactive therapeutic interventions (5).

Severe COVID-19 infection is characterized by a profoundly dysregulated immune response, including hyper activation and functional impairment of immune cells along with an excessive inflammatory response (3, 6, 7). High concentrations of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, and C-C motif chemokine ligand (CCL)2, are characteristic of severe COVID-19 and correlate with poorer clinical outcomes (3, 7). Therefore, in addition to strategies that directly target the virus, additional treatments have been developed that focus on the human immune system using immunomodulators such as corticosteroids or the IL-6 receptor-targeting antibody Tocilizumab. Dexamethasone, employed as an immunomodulatory strategy in the RECOVERY trial, was shown to improve survival in COVID-19 patients dependent on supplemental oxygen (8). Dexamethasone reduces the production of proinflammatory cytokines, such as TNF, IFN-γ, IL-1, IL-2, IL-6, IL-8, and prostaglandins, linked to severe COVID-19, by regulating gene transcription processes (9). Dexamethasone promotes the production of anti-inflammatory cytokines, such as lipocortin-1 and IL-10, by activating the synthesis of glucocorticoid response elements (9). Dexamethasone also impacted circulating neutrophils by reducing interferon-stimulated genes, expanding immunosuppressive immature neutrophils, and modifying cellular interactions (10). However, some reports suggest that dexamethasone is relatively less effective on innate immune cells compared to adaptive immune cells (11). Tocilizumab, a humanized antibody targeting the IL-6 receptor, has shown improved survival outcomes in critically ill COVID-19 patients (12). While each drug has its own advantages and disadvantages, combination therapies have generally demonstrated superior efficacy compared to single agent treatments in severe COVID-19 patients. In the REMAP-CAP trial, the combination of corticosteroids and Tocilizumab reduced in-hospital mortality in COVID-19 patients with respiratory failure (12, 13). However, there are patients who progress to respiratory failure and die

despite immunomodulation with combination therapy, and this variability in drug response remains a significant challenge in the treatment of COVID-19.

Single-cell sequencing analysis is an effective method to further characterize the heterogeneous progression of COVID-19-mediated diseases. A prior study using single-cell sequencing revealed a dominance of HLA-DR^{low} monocytes and immature CD10^{Low}CD101^{-}CXCR4$^{+/-}$ neutrophils with immunosuppressive characteristics in patients with severe COVID-19 (14, 15). Another single-cell sequencing study demonstrated that immune dysfunction, encompassing reduced immunosuppressive activity of blood myeloid cells and the substitution of naïve T cells with pulmonary memory CD8^{+} T cells, is linked to severe symptoms and increased mortality. (3). Most single-cell sequencing studies conducted to date have focused primarily on identifying cell subsets, immunological factors, and pathways associated with COVID-19 disease severity. However, there have been relatively few single-cell sequencing analyses examining the effects of current regimens in clinical practice for severe COVID-19 patients, with limited attention paid to variations in drug response, particularly in the context of immunomodulation.

In this study, we aim to explore the underlying pathways determining the drug response of severe COVID-19 patients to Tocilizumab/dexamethasone/Remdesivir (TDR). Additionally, it seeks to propose a complementary therapeutic strategy for treating COVID-19.

II. MATERIALS AND METHODS

2.1. Patients selection

Blood samples were collected from patients with severe COVID-19 admitted to a 2,400-bed tertiary care hospital between June 2021 and January 2022 (fig S1). Adult patients with a WHO Clinical Progression Scale of 6 at the time of enrollment were included in the study. All participants received dexamethasone, tocilizumab, and remdesivir, accordance with the COVID-19 guideline for the therapeutic management of hospitalized patients. Because the effect of the immunomodulatory agent may not be fully reflected, patients who died within one week and patients who underwent intubation prior to tocilizumab administration were excluded. A total of 17 patients were included after matching for age, sex, and initial ordinal scale. Of the 17 patients, 10 patients recovered without deterioration (good prognosis), and 7 patients were intubated for respiratory failure (poor prognosis) (table S1).

The Institutional Review Board (IRB) of Yonsei University College of Medicine (IRB no. 4-2020-1377) approved this study. Written informed consent was collected from all participants. The

study adhered to the principles of the Declaration of Helsinki and followed Good Clinical Practice standards.

2.2. Sample selection

Blood samples were obtained at two distinct time points: day 1 and day 7 in hospitalized COVID-19 patients who recovered without deterioration (n=10) or in hospitalized COVID-19 patients with respiratory failure (n=7). Peripheral Blood Mononuclear Cells (PBMCs) were extracted from whole blood using a standard density gradient centrifugation method with Ficoll-Paque (GE Healthcare, Uppsala, Sweden) as described in established protocols (7, 16). The 10x Genomics Chromium platform was used to capture and barcode the cells to generate single-cell Gel Beads-in-Emulsion (GEMs). Briefly, along with the reverse transcription master mix, cell suspensions were loaded onto 10x Genomics Single Cell Chips. During this step, cells were partitioned into the GEMs along with gel beads coated with oligonucleotides. These oligonucleotides enable mRNA capture inside the droplets. Following reverse transcription, cDNAs with both barcodes were amplified, and a library was constructed for each sample. The resulting libraries were sequenced on an Illumina NovaSeq 6000 System in a 2 × 150 bp paired-end mode.

2.3. Single cell RNA sequencing analysis

Sample demultiplexing, barcode processing and UMI counting were performed by using the official 10x Genomics pipeline Cell Ranger (v7.1.0) (https://support.10xgenomics.com) (17). Briefly, raw base call files generated by Illumina sequencers were demultiplexed into reads in FASTQ format using the bcl2fastq developed by Illumina. The raw reads were trimmed from the 3' end to get the recommended number of cycles for read pairs. The reads of each library were then processed separately using the "cellranger count" pipeline to generate a gene-barcode matrix for each library. During this step, the reads were aligned to a human reference genome (version: hg38). Cell barcodes and UMIs associated with the aligned reads were subjected to correction and filtering.

We utilized Dropkick, an automated software tool, for the quality control and filtering of scRNA-seq data. Utilizing predictive global heuristics, we employed Dropkick to set initial thresholds and learn a gene-based representation of real cells and ambient barcodes. Our primary objectives included excluding ambient barcodes, often indicative of background noise or non-cellular RNA, while salvaging real cells near the quality threshold. We operated Dropkick on a per-dataset basis, tailoring analysis and filtering parameters to specific dataset characteristics, thereby enhancing robustness and applicability across diverse experimental conditions and sequencing platforms. Overall, Dropkick streamlined preprocessing of scRNA-seq data in our study, automating

quality control measures and ensuring the retention of valuable biological information in downstream analyses (18).

After QC, we employed the Seurat R package to address key analytical challenges in scRNA-seq data analysis. Utilizing Seurat's feature selection capabilities, we identified informative genes driving cellular heterogeneity and biological processes. Harmony tool allowed us to harmonize scRNA-seq datasets from multiple experimental conditions, facilitating comparative analysis and identification of common and condition-specific cell populations. Leveraging Seurat's clustering algorithms, we delineated distinct cell clusters based on transcriptional profiles, enabling the characterization of cellular diversity and identification of rare cell types. Furthermore, Seurat's dimensionality reduction techniques, such as principal component analysis (PCA) and uniform manifold approximation and projection (UMAP), enabled visualization of high-dimensional scRNA-seq data in lower-dimensional space, aiding in the interpretation of complex cellular landscapes (19, 20).

2.4. Uniform manifold approximation and projection

We employed the scType computational platform, utilizing the scTypeDB (Human) database, to annotate cell clusters identified in our scRNA-seq data. Leveraging scType's automated and data-driven approach, we assigned cell types to each cluster based on gene expression profiles and the comprehensive cell marker database. This annotation process enabled us to gain insights into the cellular composition of our scRNA-seq dataset, facilitating the interpretation of the biological significance of the identified cell populations (21). Differential cell frequencies (expressed as percentages of total PBMCs) were compared across patient groups for each annotated cell type: good prognosis Day 1 (n=13,580), good prognosis Day 7 (n=14,017), poor prognosis Day 1 (n=13,747), and poor prognosis Day 7 (n=13,630).

2.5. Heatmap visualization

Heatmaps were created using the Complex Heatmap package to display differentially expressed genes (10). Cells were categorized into $CD4^+$ T cells, $CD8^+$ T cells, monocytes, and B cells, with the FindMarkers function identifying genes differentially expressed for two comparisons: (1) day 1 versus day 7 and (2) respiratory failure versus recovery. Genes with an absolute logFC value exceeding 0.5 were selected for inclusion in both the heatmap visualization and EnrichR pathway analysis.

2.6. Gene Ontology enrichement

Neutrophils were extracted from a Seurat object (versions 3.9 and 4) that had been realigned with scVelo and analyzed following the standard and recommended settings outlined in the SCENIC vignette (https://github.com/aertslab/SCENIC) using the hg19 RcisTarget reference (10). Regulon activity scores, generated as part of the SCENIC workflow ('3.4_regulonAUC.Rds'), were incorporated into the scVelo object via the CreateAssayObject function, allowing trajectory data and transcription factor (TF) activity to be simultaneously visualized on UMAP embeddings. The target genes of transcription factors were analyzed using iRegulon, a computational tool available as a Cytoscape plugin that reverse-engineers transcriptional regulatory networks from co-expressed gene sets by integrating cis-regulatory sequence analysis, motif discovery, and regulatory tracks such as ChIP-seq data to identify master regulators and their downstream gene networks (22). Gene Ontology enrichment analysis was performed using the DEenrichRPlot function in Seurat (versions 3.9 and 4), which incorporates the Ma'ayan lab's Enrichr tool (23).

2.7. Statistical analysis

PASW Statistics 23 (SPSS) and GraphPad Prism 7.0 (GraphPad Software) were utilized for statistical analysis. Data are expressed as mean ± standard error. Unpaired Student's t-tests were used to evaluate differences between means, and the log-rank test was applied for additional statistical comparisons. Statistical significance was defined as a *P*-value below 0.05.

III. RESULTS

3.1. Classical monocytes and NK cells exhibit opposite patterns of compositional changes following TDR treatment in groups with poor and good prognoses

To discern the distinctions among COVID-19 patients exhibiting either a poor or good prognosis post-TDR treatment, PBMCs were obtained from three individuals in each category at day 1 (D1) before treatment initiation and at day 7 (D7) post-treatment (fig 1A). The patients had a median age of 71 years (66–75 years), with 52.9% being male (Supplementary Table 1). The median body mass index was 23.5 (22.1-28.0) kg/m^2. All patients were receiving high-flow nasal cannula support, and no notable differences were observed between the two groups in terms of age, sex, body

mass index, or WHO Clinical Progression Scale at the time of enrollment. Subsequently, scRNA-seq was conducted on the collected samples. Given the variability in cell viability across samples, the identified cell numbers in each group were adjusted accordingly, following the dropkick protocol as reported previously (18). The clustering analysis revealed 11 distinct clusters comprising $CD8^{+}$NKT-like cell (NKT), classical monocyte (CM), myeloid dendritic cell, naïve B cell, naïve $CD4^{+}$ T cell, natural killer (NK) cell, non-classical monocyte (NCM), plasma B cell, platelet, progenitor cell, and unknown cell (not classified) (24) (fig 1B and Supplementary fig S1 and S2). Among patients with a poor prognosis, significant changes in cell populations were observed when comparing D1 and D7 samples. Specifically, there was an increase in CM, naïve B cells, and naïve $CD4^{+}$ T cells, along with a decrease in NK cells and NCM during this period (Fig. 1C, left). Conversely, the good prognosis group showed an increase in NKT cells, naïve B cells, naïve $CD4^{+}$ T cells, and NK cells, while showing a decrease in CM (Fig. 1C, left). The inverse correlation between good and poor prognosis patients was particularly notable in CM and NK cells. Additionally, an increase in NKT cells and a decrease in NCM were observed in the good and poor prognosis groups, with no significant changes observed in the counterpart group. Focusing on CM and NK cells, a comparison of D1 samples between the poor and good prognosis groups revealed that their baseline compositions for those were almost identical. Upon comparing the D7 samples from both groups, the results further reinforced the earlier findings, with the poor prognosis group showing a higher quantity of CM and a lower quantity of NK cells (Fig. 1C, right).

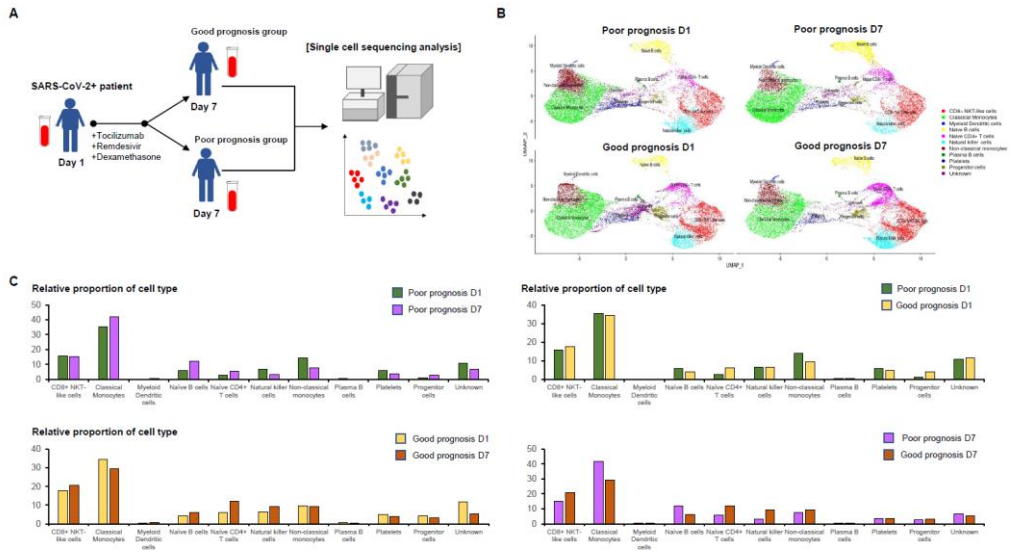


Figure 1. Differential changes in monocyte and natural killer cell composition in COVID-19 patients with poor and good prognosis. (A) Study design: Adult patients with a WHO Clinical Progression Scale of 6 who received tocilizumab, dexamethasone, and remdesivir (TDR) according to COVID-19 guidelines were enrolled. Blood samples were collected at two time points: day 1

(before treatment) and day 7 (after treatment) in hospitalized COVID-19 patients who recovered without deterioration (n=10) or patients with respiratory failure (n=7). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood and analyzed by Single-cell RNA sequencing analysis. (B) Uniform manifold approximation and projection (UMAP) visualization of PBMC of COVID-19 patients following the dropkick protocol (good prognosis day 1 [n=13580], good prognosis day 7 [n=14017], poor prognosis day 1 [n=13747], poor prognosis day 7 [n=13630]) (C) Differential changes in major cell composition before and after TDR treatment.

3.2. The featured genes for clustering cell types primarily belong to innate immunity related pathways

A featured gene set comprising 2000 genes was identified based on standardized variance and average expression values (see material and method). Subsequent KEGG pathway and gene ontology (GO) analyses revealed that these genes are associated with various inflammatory responses. According to the KEGG pathway analysis, viral protein interaction with cytokines and cytokine receptors, antigen processing and presentation, and hematopoietic cell lineage pathways were enriched by the gene set (fig 2A). Moreover, inflammation related GO terms such as cytolysis (Biological Process, BP), chemotaxis (BP), hemostasis (BP), inflammatory responses (BP), innate immunity (BP), and cytokine (Molecular Function, MF) encompass a significant number of the featured genes (fig 2A). The KEGG term 'viral protein interaction with cytokine and cytokine receptor' notably contained a wide array of overlapping genes related to inflammatory activators and suppressors, encompassing chemokines, cytokines, TNF family members, and TGF-β families (fig 2B). Moreover, the identified featured genes included previously recognized crucial pro-inflammatory mediators related to monocyte activation in severe COVID-19, such as IL-6, IL-1β, IFN, TNF, CCL2, CCL3, CCL7, CCL8, and CXCL10, along with other relevant genes (25). GO terms such as ‘chemotaxis’ and ‘innate immunity’ primarily consist of genes associated with anti-viral related innate immune functions. These genes encompass neutrophil and monocyte chemotaxis-related genes such as CCL2, CCL3, CCL8, CCL20, CXCL2, CXCL3, CXCL8, ISG15, S100A8, and S100A9 along with complement activation-related genes such as C1QB, C1QA, and C1QC (fig S3) (26). Within the KEGG term 'hematopoietic cell lineage', essential genes involved in the maturation of NK cells, B cells, and myeloid dendritic cell/macrophage were identified (fig 2B).

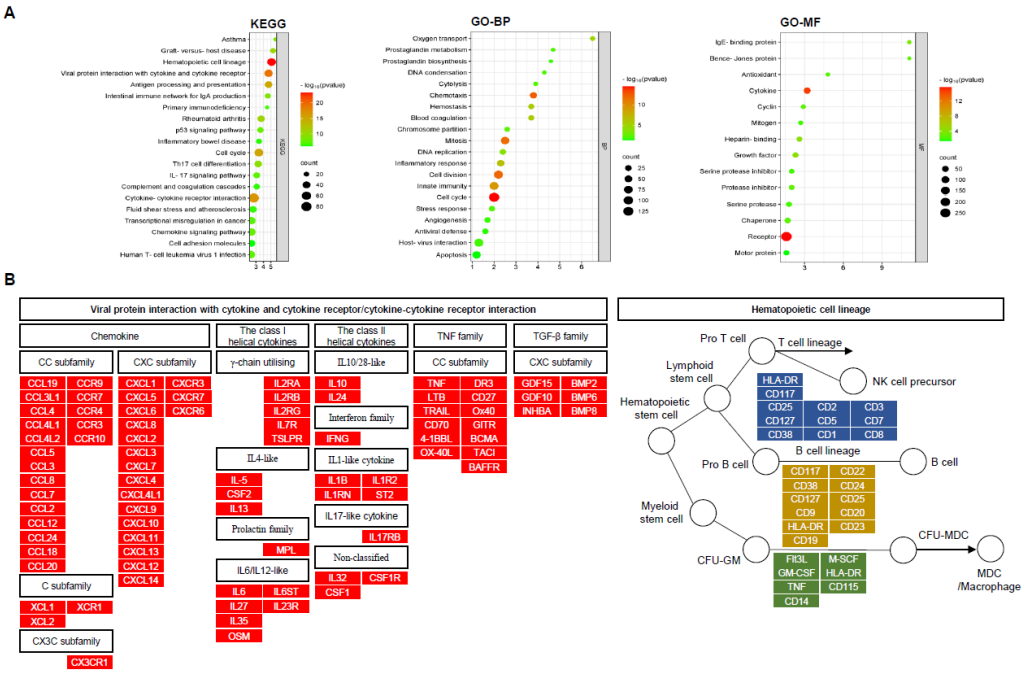
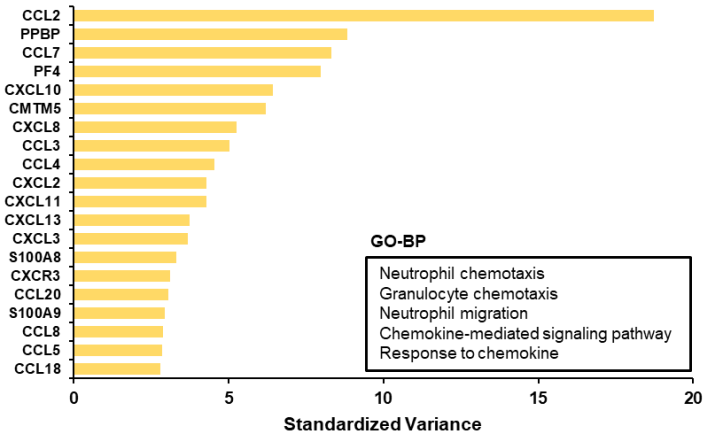


Figure 2. The distinguished marker genes for the clustering of cell types belong to innate immunity related pathways. (A) KEGG pathway and gene ontology (GO) term analysis of 2000-gene set associated with inflammatory responses (B) Genes associated with KEGG term 'viral protein interaction with cytokines and cytokine receptors' and 'hematopoietic cell lineage'.



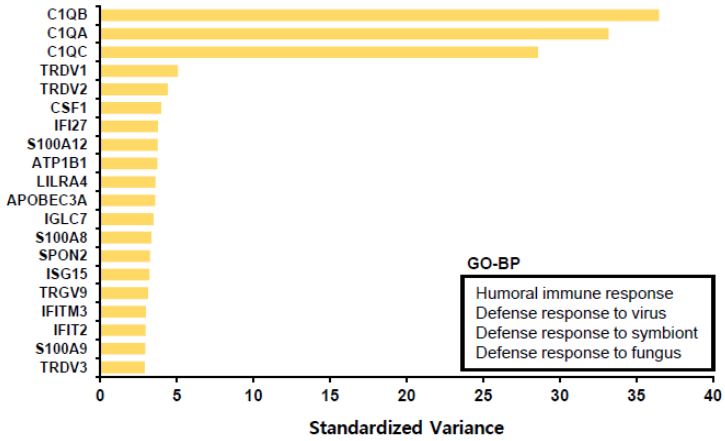


Figure 3. GO terms related to chemotaxis and innate immunity, highlighting genes involved in neutrophil chemotaxis and complement activation.

3.3. Classical monocyte- and NK cell-associated genes exhibit opposite patterns of expressional changes after TDR treatment in groups with good and poor prognoses

As highlighted above, changes in cell numbers of classical monocytes (CM) and natural killer (NK) cells demonstrated opposing trends between COVID-19 patients with good and poor prognoses after TDR treatment. Therefore, genes used to characterize CM and NK in comparison to others were reorganized based on their log2(FC) expression in relation to other cell types. For validation, the top 100 genes were subjected to KEGG and GO analyses. Marker genes for CM indicated involvement of KEGG terms such as antigen processing and presentation, phagosome, reactive oxygen species, and GO term innate immunity (fig S4A). Similarly, marker genes for NK cells were linked to a distinct KEGG term such as NK cell-mediated cytotoxicity and reactive oxygen species, and GO terms including cytolysis and innate immunity (fig S4B).

To further characterize the CM and NK cells that showed distinct changes in their population between patients with poor and good prognoses, the top 20 gene set from the above top 100 was further analyzed through UMAP plot visualization. Notably, S100A9, S100A8, LYZ, S100A12, CD14, FOS, and SPI1 showed relatively selective expression in monocytes. Post-TDR treatment, these genes were either maintained or further upregulated in classical monocytes (CM) of the poor prognosis group. In contrast, in the good prognosis group, expression of these genes decreased to varying extents in CM following treatment. (fig 3A). Other genes, including S100A6, CEBPD, CST3, MAFB, and TSPO, exhibited consistent or increased expression in CM of the poor prognosis group, with partial or no change in the good prognosis group (fig S5A). These genes might represent

an inflammation-related subtype of classical monocytes increased after TDR treatment in the poor prognosis group. Other genes for CM were excluded due to their nonselective expression across cell types and lack of meaningful expression changes following TDR treatment.

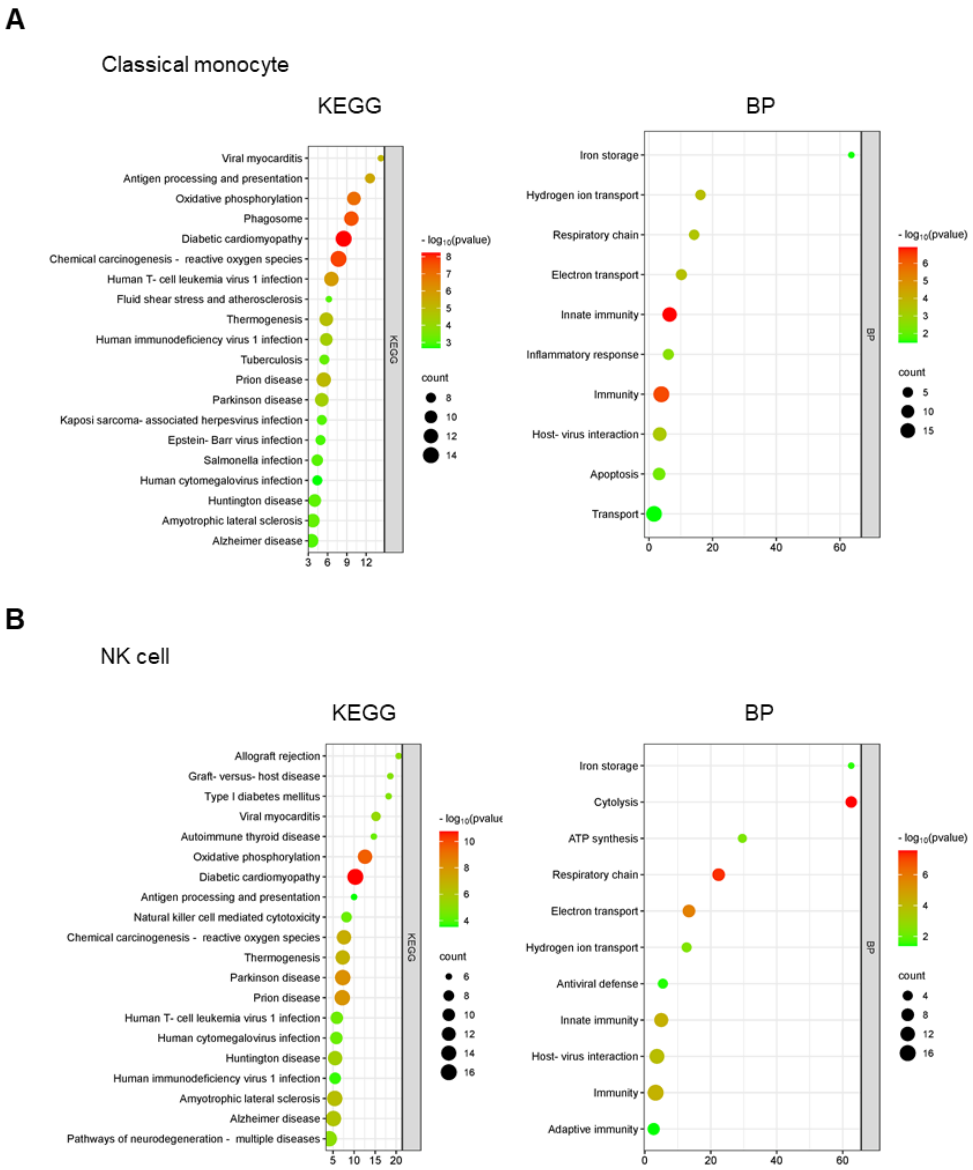


Figure 4. KEGG and GO analysis of Distinct patterns of gene expression in each cell type. (A) Classical monocytes, (B) Natural killer cells

As altered markers in the NK cell population according to TDR treatment, SPON2, PTGDS, IGFBP7, LAIR2, KLRB1, KLRF1, CD247, and CLIC3 showed relatively specific expression in NK cells and exhibited reduced expression after treatment in the poor prognosis group, whereas these genes maintained or increased expression in NK cells of the good prognosis group (fig 3B). Genes such as CTSW, FGFBP2, KLRD1, CST7, GNLY, NKG7, PRF1, GZMA, and GZMM mostly showed a shared downregulation in both NK and NKT cells of the poor prognosis group post-TDR treatment, while displaying a maintained or upregulated expression in these cells among the good prognosis group (fig S5A). Other genes were excluded due to their non-specific distribution across cell types and unexpected expression changes.

Figure 5. UMAP visualization of differentially expressed genes in Classical monocytes.

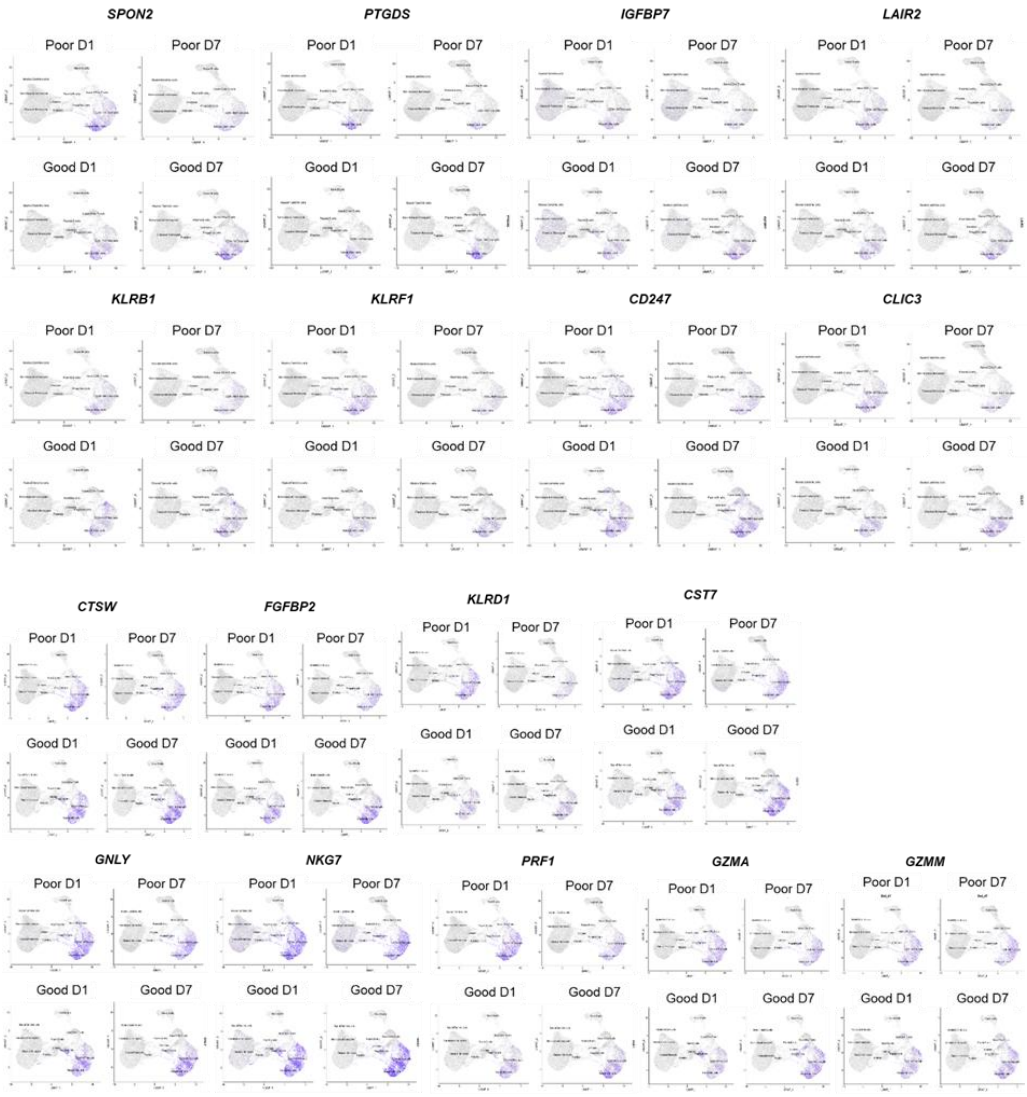


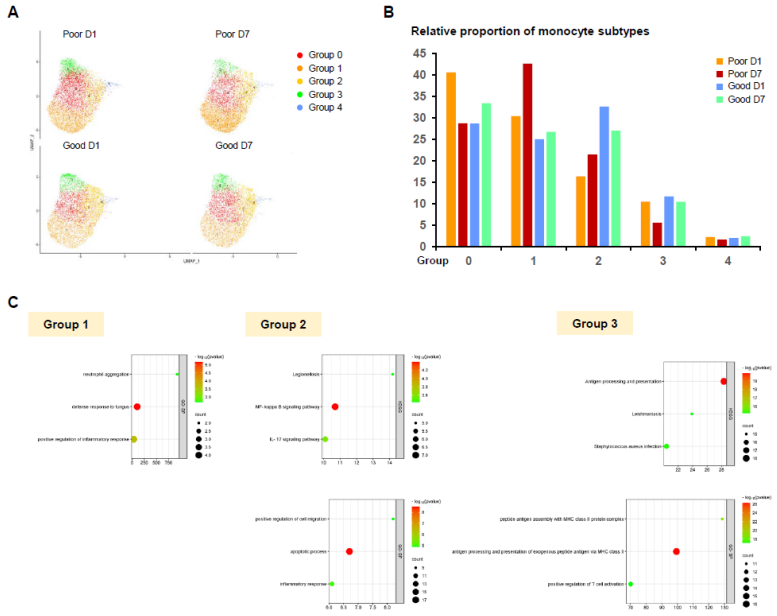
Figure 6. UMAP visualization of differentially expressed genes in Natural killer cells.

3.4. The poor and good prognosis groups display different subtypes of classical monocytes according to TDR treatment

Classical monocytes, exhibiting noticeable increase in the proportion of the cell numbers post-treatment in the poor prognosis group, were subjected to further analysis. Cells annotated as

monocytes were sub-clustered according to gene expression profiles, resulting in the identification of five distinct clusters (groups 0 to 4) (fig 4A). Group 0, which emerged as a distinct cluster due to a lack of enriched genes, was excluded from further examination. In the poor prognosis group, a notable decrease in cell populations was observed in group 3 after treatment, whereas an increase was detected in the group 1 and 2 populations. Conversely, in the good prognosis group, group 2 cell populations diminished, while group 1 and 3 remained barely changed after treatment (fig. 4B).

Subsequent Gene Ontology (GO) analysis of genes distinguishing groups 1, 2, and 3 revealed distinct functional involvements (fig. 4C). Genes in group 1 primarily related to defense responses to fungi and positive regulation of inflammatory responses. Group 2 genes were associated with the NF-kappa B signaling pathway and apoptotic processes. Group 3 genes primarily involved antigen processing and presentation of exogenous peptide antigen presentation via MHC class II. Groups 1 and 2 predominantly related to macrophage mediated inflammatory responses and tissue damage, while group 3 was linked to regular functions of macrophages and subsequent adaptive immune system activation. Key representative genes for each group were identified (fig 4D): proinflammatory S100 protein families such as S100A12, S100A8, and S100A9 for group 1; proinflammatory CXCL chemokines, NFKBIA, IL1B, CCR1, and CLEC7A for group 2; and genes primarily related with antigen processing and presentation for group 3. Although group 4 also exhibited treatment-related changes in cell populations, it was deemed less relevant due to its comparatively low cell populations.



D

Group 1	Group 2	Group 3
S100A12	CXCL2	CD74
S100A8	CXCL3	IFI30
S100A9	CXCL8	B2M
	NFKBIA	CTSB
	IL1B	CTSS
	CCR1	HSPA6
	CLEC7A	LGMN
		FCER1G
		HLA-DMA
		HLA-DMB
		HLA-DPA1
		HLA-DPB1
		HLA-DQA1
		HLA-DQA2
		HLA-DQB1
		HLA-DRA
		HLA-DRB1
		HLA-DRB5

Figure 7. Functional Analysis of Monocyte subpopulations in poor and good prognosis groups. (A) Uniform manifold approximation and projection (UMAP) visualization of monocyte sub-clustering. (B) Sub-clustering of monocytes based on gene expression profiles. (C) Gene ontology analysis of Groups 1, 2, and 3. (D) Representation of key expressed genes of groups 1, 2 and 3.

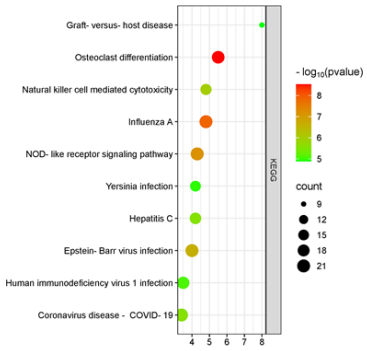
3.5. Impaired NK cell-mediated cytotoxicity ameliorates defense system to virus, accompanying upregulation of ROS burden in the poor prognosis group

To determine the most significantly impacted KEGG pathways and GO terms following TDR treatment in the poor prognosis group, we examined differentially expressed genes (DEGs) with a fold change greater than ⌊1.5⌋. DAVID analysis of the DEG set revealed that downregulated genes on D7 were enriched in KEGG pathways such as NK cell-mediated cytotoxicity and the NOD-like receptor signaling pathway, as well as in GO-BP terms like response to viruses and innate immune response (Fig. 5A). The KEGG term 'NK cell-mediated cytotoxicity' encompasses genes critical for NK cell function, including FCGR3A, KLRD1, and KLRK1 for target cell recognition; CD247, ZAP70, LCP2, and TYROBP for activation and signaling; and GZMB, PRF1, and TNFSF10 for cytotoxicity. The 'NOD-like receptor signaling pathway' includes genes essential for inflammasome-mediated antiviral defense and NK cell activation. The GO-BP terms additionally highlight genes essential for interferon related anti-viral responses (OAS1, OAS2, OAS3, STAT1, STAT2, IRF7, and IRF9), inflammasome formation (GBP1, NLRP3, PYCARD, and CASP1), and inflammatory regulation (IL-18 and NFKBIA) (Fig. 5B).

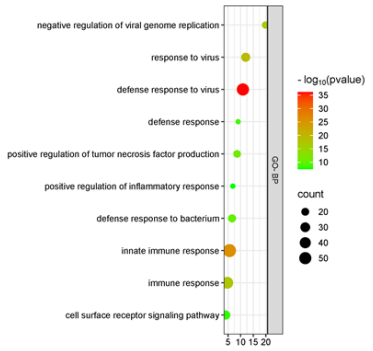
Conversely, genes upregulated on D7 were associated with KEGG pathways such as reactive oxygen species (ROS) and diabetic cardiomyopathy, a reported COVID-19-related complication (27). GO-BP terms for these genes were primarily associated with cellular respiration (Fig. 5C). The GO-BP term ‘ROS’ encompasses genes involved in mitochondrial function and ROS generation, including MT-ATP6, MT-ATP8, MT-ND4L, MT-ND5, MT-CYB, MT-CO1, MT-CO2, MT-CO3, ATP5F1A, SLC25A5, and SLC25A6 for ATP synthesis and ROS generation, as well as JUN, NCF1, GRB2, and SOD2 for ROS generation and protection. JUN functions as a key proinflammatory mediator in response to ROS presence.

A

KEGG



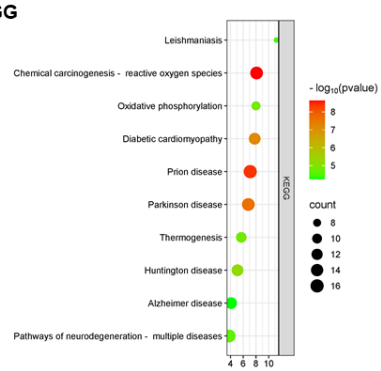
GO-BP



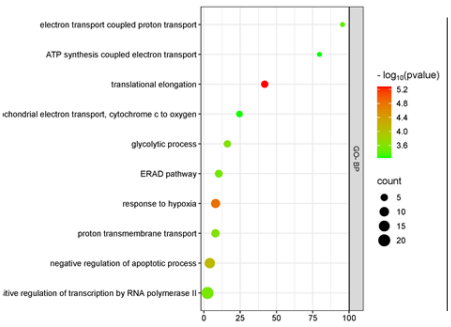
Poor D7 DN

B

KEGG



GO-BP



Poor D7 UP

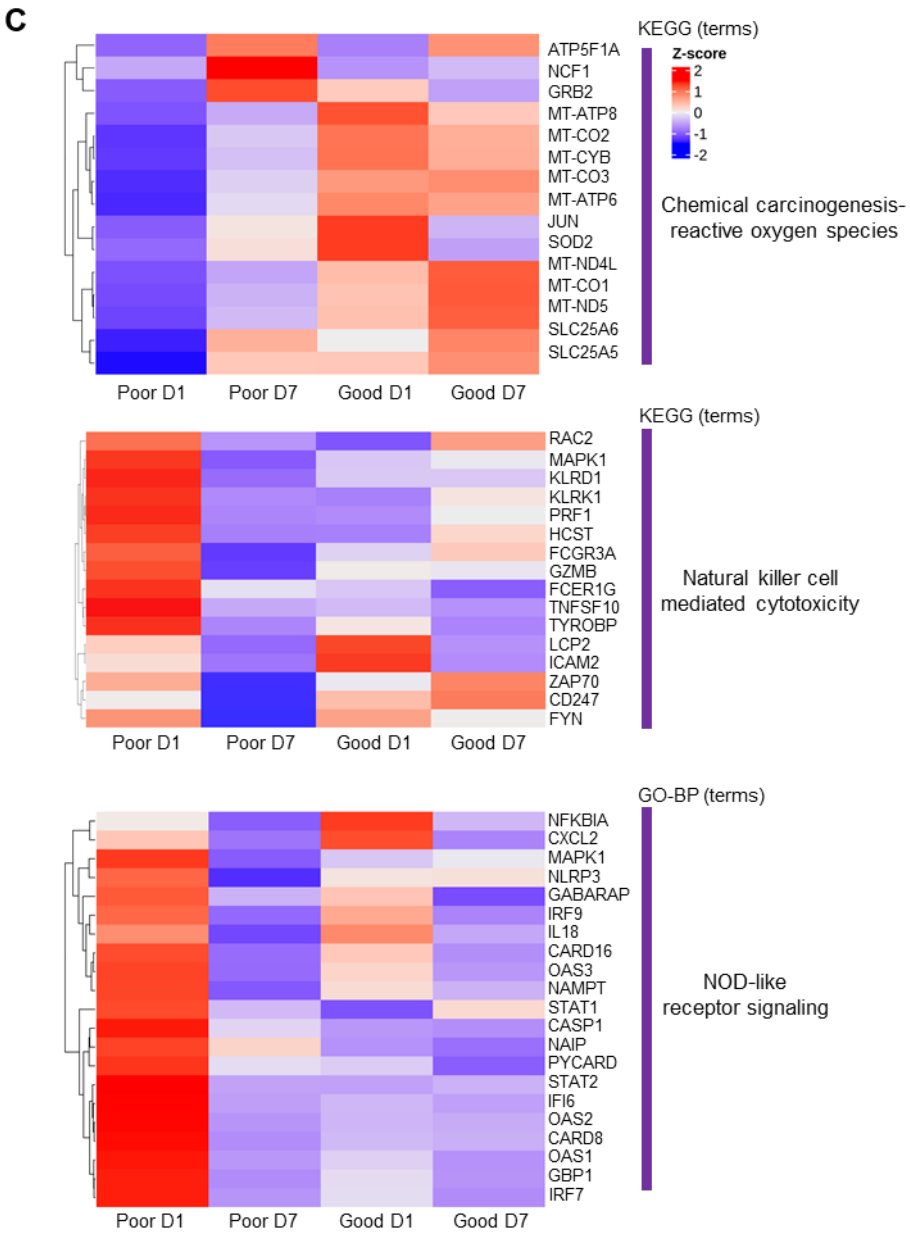
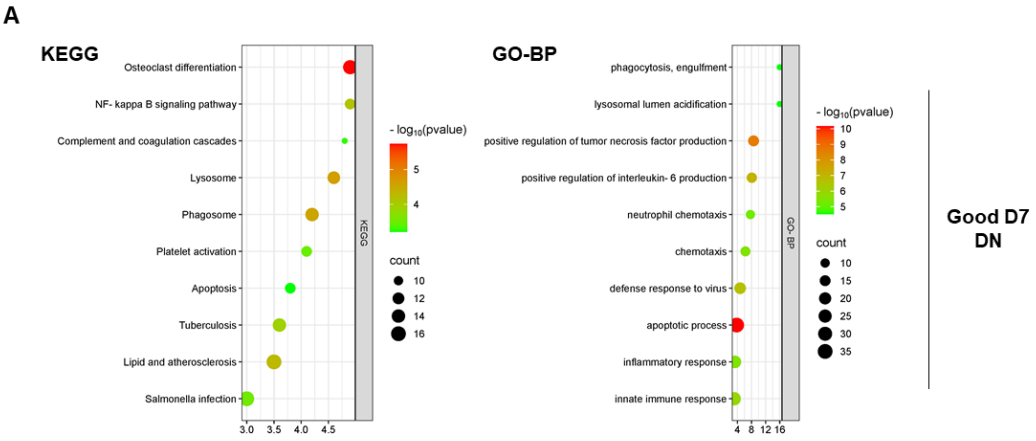


Figure 8. Impaired NK cell activity, increased ROS burden, and macrophage mediated antiviral defense system are associated with the poor prognosis. (A) Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis for downregulated genes in the poor prognosis group. (B) DAVID analysis for upregulated genes in the poor prognosis group. (C) Heatmap of Differential expression of key genes in the poor prognosis group.

3.6. TDR treatment reduces macrophage-mediated inflammatory responses and promotes T cell maturation and activation in the good prognosis group

In the good prognosis group, DAVID analysis showed post-treatment downregulation in KEGG pathways such as lysosome and phagosome, as well as GO-BP terms including the positive regulation of TNF-α, IL-6 production, chemotaxis, and innate immune response. The KEGG pathway term "phagosome" includes CLEC7A, CD14, CD36, MARCO, FCGR2A, and FCGR1A, which facilitate recognition and phagocytosis by macrophages and neutrophils, and ATP6V0B, ATP6V0C, ATP6V1F, and CTSL, which contribute to degradation and digestion by phagocytes. The GO-BP terms "TNF-α and IL-6 production" contain many of the same genes, such as CLEC7A, CD14, CD36, FCGR2A, LILRA2, LILRA5, and LILRB2, which are involved in macrophage and neutrophil phagocytosis, while BTK, TYROBP, and PYCARD promote pro-inflammatory cytokine release in these cells. For the GO term "chemotaxis," CCR1, CXCL16, CXCL2, and CXCL8 are key genes for neutrophil recruitment to sites of inflammation. The term "innate immune response" encompasses genes associated exclusively with macrophage- and neutrophil-mediated activation and inflammatory responses. Notably, S100A8, S100A9, and S100A12 were decreased after treatment; these genes play crucial roles in recruiting phagocytes and facilitating ROS production.

Conversely, genes upregulated after treatment were associated with KEGG pathways including Th1 and Th2 cell differentiation, Th17 cell differentiation, and T cell receptor signaling, sharing many genes critical for T cell functions. CD3D, CD3E, CD3G, LCK, IL2RG, and PTPRC are involved in T cell activation and signaling. STAT1, HIF1A, and PPP3CA are linked to T cell-mediated intracellular activation processes. Additionally, CD8B, a cytotoxic T cell marker, was upregulated post-treatment.



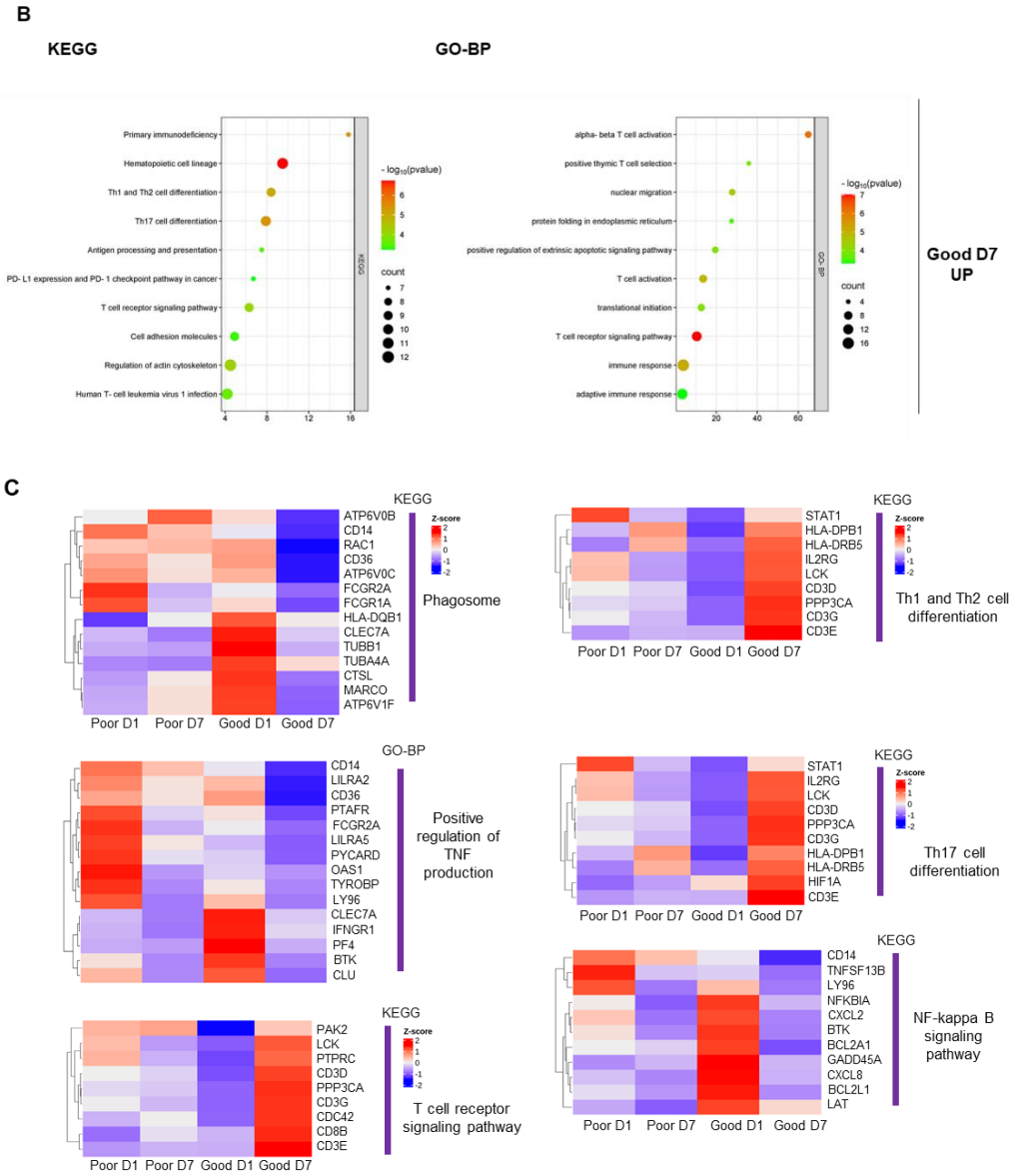
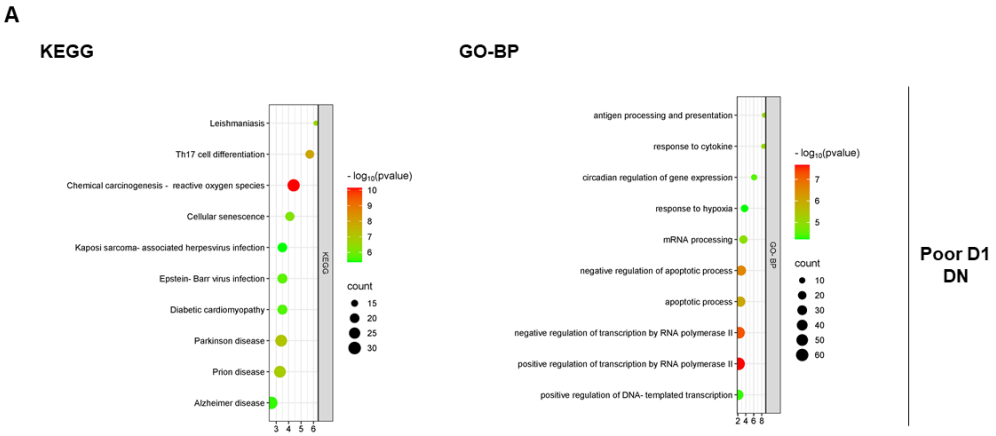


Figure 9. TDR treatment reduces macrophage-mediated inflammatory responses and promotes T cell maturation and activation in the good prognosis group. (A) Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis for downregulated genes in the good prognosis group. (B) DAVID analysis for upregulated genes in the good prognosis group. (C) Heatmap of Differential expression of key genes in the good prognosis group.

3.7. Antiviral response pathways are paradoxically enriched in the poor prognosis group compared to the good prognosis group

We further analyzed DEGs before treatment between the good and poor prognosis groups. To compare the baseline differences between these groups, we performed DAVID analysis, as our cohorts were generated with patients who had relatively similar baseline backgrounds. This analysis revealed that the KEGG pathway "Th17 cell differentiation" was upregulated in the good prognosis group, while notably, "ROS" was enriched in both the poor and good prognosis groups. The genes associated with Th17 cell differentiation in the KEGG pathway include NFKBIA, NFKB1, SMAD3, TGFB1, and IL6ST, which play critical roles in Th17 cell differentiation and activation. The ROS-related genes enriched in the good prognosis group include NFE2L2 (NRF2), AHR, PPIF, SOD2, GSTO1, and CYP1B1, which are involved in ROS sensing, removal, and stress response. In contrast, ROS-related genes enriched in the poor prognosis group include NCF2, CYBA, NDUFA3, NDUFA7, NDUFB1, NDUFB2, NDUFB3, NDUFS6, UQCRQ, UQCRC1, UQCR10, and UQCR11, which are directly or indirectly involved in ROS generation. In the GO-BP terms, the pathway "negative regulation of apoptotic process" with genes such as BCL2A1, MCL1, PPIF, FOXO1, HSP90AB1, HSPB1, SOD2, and PIK3R1 was enriched in the good prognosis group, whereas "defense response to virus" and "innate immunity" were enriched in the poor prognosis group. The innate immunity genes included OAS1, OAS2, ISG15, MX1, MX2, IFI27, IFI6, IFI16, and IRF7 as interferon-related genes; CYBA, TLR4, and MYD88 as macrophage-related genes; and PYCARD, SAMHD1, CASP1, HERC5, and PARP9 as anti-viral response genes. These results suggest that patients in the poor prognosis group exhibit pre-existing inflammatory responses and an elevated ROS production status compared to those in the good prognosis group.



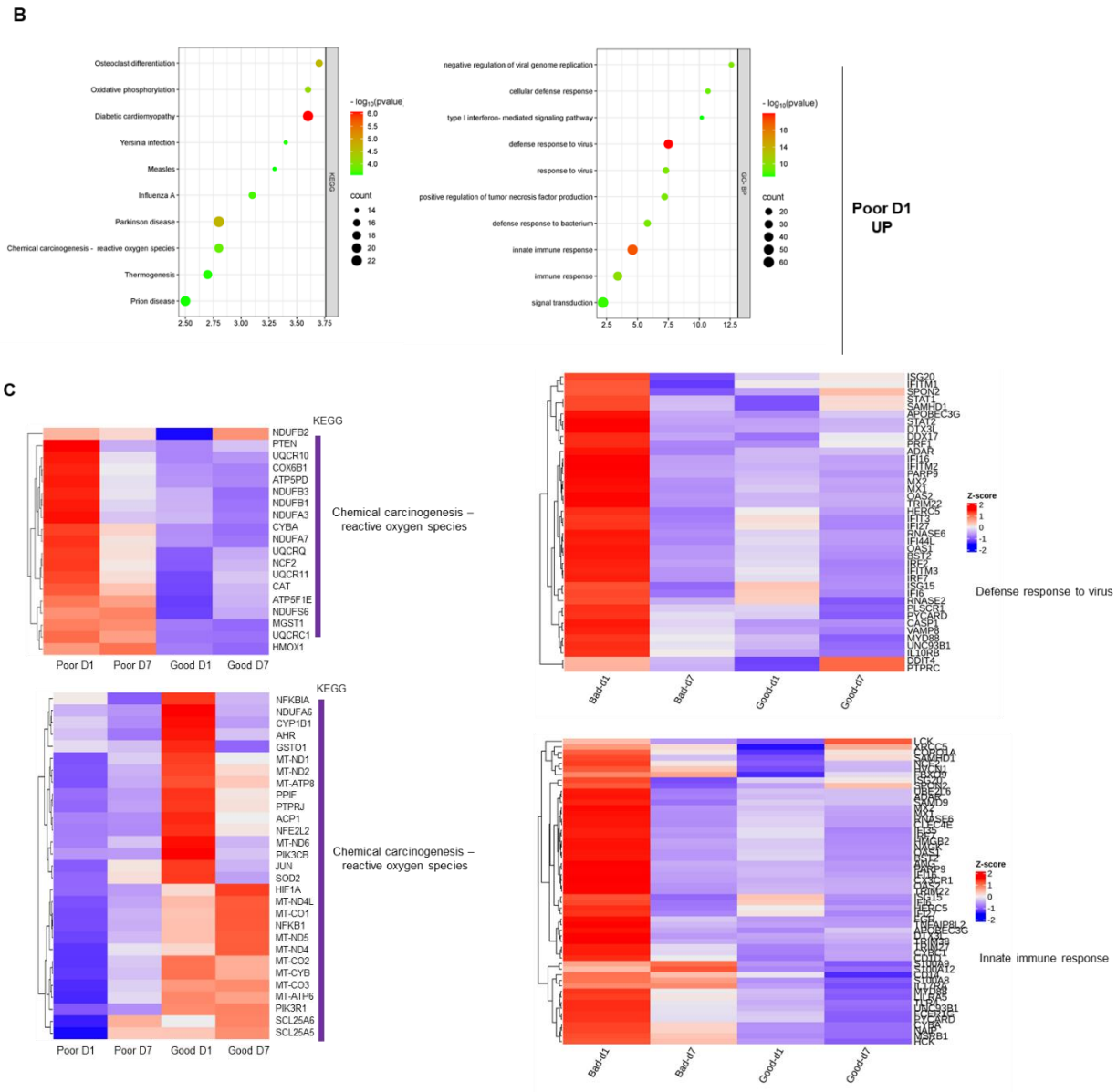
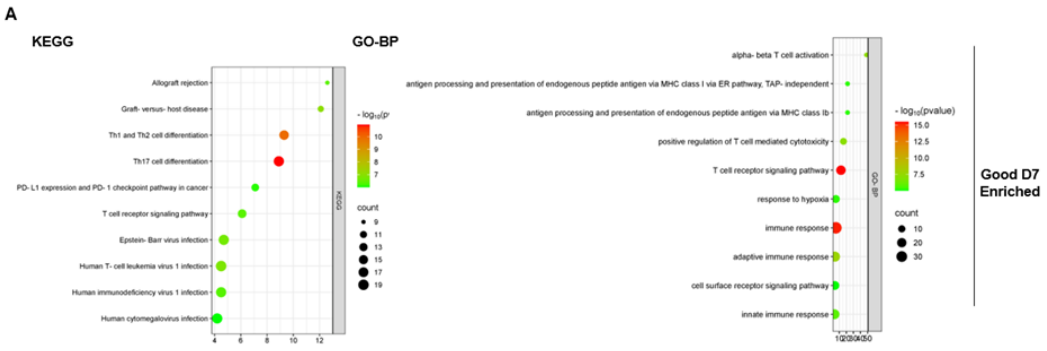


Figure 10. Antiviral response pathways are paradoxically enriched in the poor prognosis group compared to the good prognosis group. (A) Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis for downregulated genes in the poor prognosis group. (B) DAVID analysis for upregulated genes in the poor prognosis group. (C) Heatmap of Differential expression of key genes in the poor prognosis group.

3.8. The poor prognosis group displays sustained activation of CM-related inflammatory responses and the good prognosis group exhibits sustained cell-mediated cytotoxicity after TDR treatment

Although major pathways and ontology terms were identified before and after treatment in both the poor and good prognosis groups, additional DAVID analysis was performed to pinpoint differences in adaptation after treatment between the two groups. This analysis revealed that KEGG terms such as Th1, Th2, and Th17 cell differentiation, T cell receptor signaling pathway, and GO terms related to the positive regulation of T cell-mediated cytotoxicity were more enriched in the good prognosis group after treatment. The genes associated with these pathways include CD247, CD3D, CD3E, CD3G, ZAP70, LAT, LCK, FYN, SKAP1, and LIME1, which are involved in antigen recognition and TCR activation; IL2RB, IL2RG, IFNGR1, and JAK1, which are essential for T cell proliferation and activation; GATA3, RUNX3, RORA, and TGFB1, which play a role in balanced T helper cell differentiation; and CD8B, TRBC1, and TRBC2, which are related to CD8 T cell activation and cytotoxicity. Although it was not among the top 10 representative pathways, natural killer cell-mediated cytotoxicity was also significantly enriched in the good prognosis group. In contrast, KEGG pathways such as lysosome and phagosome, as well as GO terms related to the positive regulation of inflammatory response and innate immune response, were more enriched in the poor prognosis group after treatment. Genes associated with these pathways include CD14, S100A8, S100A9, and S100A12, which are linked to the activation of macrophages and neutrophils; CYBA and NCF1, which contribute to ROS production; and PYCARD and NAIP, which are involved in inflammatory cell death. These findings suggest that the D7 sample comparison between the two groups consistently supports our previous results obtained by comparing D1 and D7 samples within the same group. Specifically, NK and T cell activity is reduced, while macrophage activity is upregulated in the poor prognosis group after treatment, compared to the good prognosis group.



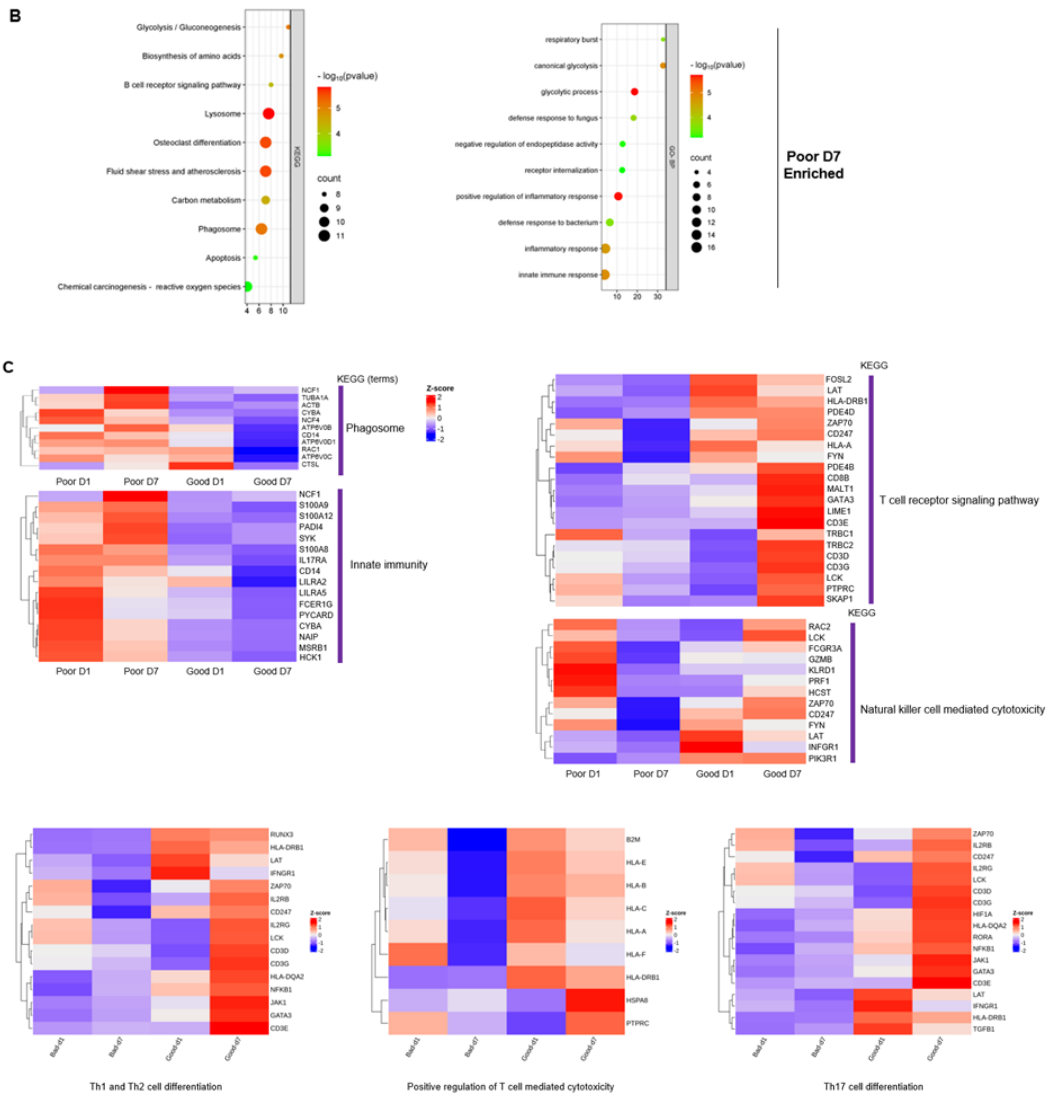


Figure 11. The poor prognosis group displays sustained activation of CM-related inflammatory responses and the good prognosis group exhibits sustained cell-mediated cytotoxicity after TDR treatment. (A) Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis for upregulated genes in the good prognosis group. (B) DAVID analysis for upregulated genes in the poor prognosis group. (C) Heatmap of Differential expression of key genes in the good prognosis group and the poor prognosis group.

IV. DISCUSSION

Severe COVID-19 infection is characterized by a profoundly dysregulated immune response, including hyper activation and functional impairment of immune cells along with an excessive inflammatory response. REMAP-CAP and RECOVERY clinical trials have shown that corticosteroids and tocilizumab can reduce mortality from severe COVID-19 infection by reducing the excessive inflammatory response. However, in some patient groups, the use of immunomodulatory strategies does not improve prognosis. In this study, we found that NOD-like receptor signaling pathways and NK cell-mediated cytotoxicity were significantly downregulated in patients with poor prognosis, while macrophage-mediated inflammatory pathways remained high even after treatment. On the other hand, in patients with a good prognosis, the T-cell-mediated adaptive immune response was strengthened to relieve excessive inflammation while effectively eliminating pathogens.

In a prior single-cell RNA sequencing study, dexamethasone was observed to alter neutrophil states by suppressing interferon-active pathways while simultaneously expanding immunosuppressive ARG1+ immature neutrophils (10). Additionally, dexamethasone has been demonstrated to reverse the dysfunctional HLA-DRloS100Ahi monocyte phenotype, suppressing proinflammatory genes such as CCL3 and S100A8/9 while simultaneously upregulating regulatory genes like IL1R2 (28). Moreover, recent proteomic and transcriptomic studies have demonstrated that tocilizumab reduces excessive inflammation by rapidly resolving lymphopenia and myeloid dysregulation, as well as downregulation of IL-6-mediated inflammatory responses (29). Our study showed that a downregulation of GO-BP terms including inflammatory response, IL-6 production, TNF-α production and cytokine production was observed in the entire patient population after treatment, as in the previous study. However, despite a reduction in the excessive inflammatory response, some patients developed a poor prognosis.

Patients with poor prognosis exhibited a pronounced increase in CMs with a decrease in NCMs and NK cells after treatment. This group also demonstrated downregulation of NLR signaling pathway and NK cell-mediated cytotoxicity. NK cells are an essential part of the innate immune system, tasked with recognizing and eliminating virus-infected cells while regulating the immune response through the release of cytokines such as IFNγ and TNF (30). Additionally, NK cells influence both innate and adaptive immunity by releasing

chemokines and cytokines and engaging in cooperative interactions with other immune cells, such as dendritic cells, monocytes, neutrophils, and macrophages (31). NLRs, abundantly expressed in monocytes and macrophages, recognize pathogen- or damage-associated molecular patterns during SARS-CoV-2 infection, facilitating type I IFN and pro-inflammatory cytokine production to drive innate immunity (32). These findings suggest a compromised innate immune system and impaired transition to adaptive immunity. Monocytes are important components of the innate immune response by processing and presenting antigens to T cells, and producing cytokines that modulate immune responses (33, 34). In COVID-19 infection, CMs are known to produce pro-inflammatory cytokines and have been linked to disease severity and the development of ARDS (35, 36). Conversely, NCMs are essential for pathogen recognition and clearance, vascular endothelial homeostasis, and resolution of inflammation (37). Alterations in monocyte and NK cell populations, therefore, lead to immune imbalances that hinder pathogen elimination and cause persistent inflammation. In subgroup analysis of monocytes, group 1 such as S100A8, S100A9, and S100A12 were notably overexpressed in patients with poor prognosis. S100A8/A9, a calcium-binding heterodimer, undergoes conformational changes to regulate leukocyte migration and inflammatory responses. By engaging Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE), the NF-κB signaling pathway is activated, driving cytokine storms in severe COVID-19 through the induction of emergency myelopoiesis and the production of atypical immature neutrophil subsets (38). Furthermore, in subgroup analysis of monocytes, group 2 genes like CXCL2, CXCL3, CXCL8, NFKBIA, IL1B, CCR1, and CLEC7A were upregulated in poor prognosis patients and downregulated in good prognosis patients after treatment. CXCL2 is crucial for the innate immune defense against COVID-19, facilitating the recruitment of neutrophils, monocytes, and mononuclear phagocytes to the infection site (39). CXCL8, secreted by monocytes, macrophages, and alveolar epithelial cells, is an important factor in the progression of lung disease in COVID-19. It drives neutrophil recruitment and activation, promotes the formation of neutrophil extracellular traps (NETs) that induce inflammation and cell damage, and triggers oxidative bursts involving hydrogen peroxide and superoxide from neutrophils (39). CXCL3 or IL1B can also significantly induce or enhance the inflammatory response of COVID-19 infection, leading to additional pulmonary inflammation and tissue damage (40). These findings underscore the role of aberrant immature neutrophils and monocytes in sustaining inflammation and inducing cell death via oxidative stress. In addition, dysregulation of antigen processing and presentation impairs the transition to appropriate adaptive immunity, further

contributing to excessive tissue damage and fibrosis (7). Notably, macrophage- and neutrophil-driven inflammatory pathways remained activated even after TDR treatment by upregulating genes such as S100A8, S100A9, S100A12, CYBA, NCF1, PYCARD, and NAIP. These genes are implicated in macrophage and neutrophil activation, ROS production, and inflammatory cell death. The elevated expression of PYCARD and NLRP3, associated with NET formation and inflammasome activation, suggests persistent inflammation that disrupts immune regulation and hinders normal restoration process. Additionally, upregulation of genes like JUN, NCF1, SOD2, and GRB2 highlights oxidative stress and mitochondrial dysfunction as major contributors to ongoing inflammation and tissue damage.

Patients with a good prognosis showed a marked increase in NK cells and $CD8^{+}$NKT-like cells, along with a simultaneous reduction in CM, after TDR treatment. This immune shift was characterized by a reduction in TNF, IL-6, chemotaxis and inflammatory responses, alongside decreased activity in pathways associated with the apoptotic process, lysosomes and phagosomes, which contribute to the phagocytosis of macrophages and neutrophils. Additionally, the differentiation of Th1, Th2, and Th17 cells and the T cell receptor signaling pathway were more enriched in the good prognosis group after treatment. CD8+NKT-like cells, known for their diverse TCR repertoire and high levels of IFN-γ secretion, play a crucial role in preventing excessive immune responses by suppressing T-cell responses through the antigen-specific elimination of dendritic cells (41). CD3D and CD3G, integral components of the TCR-CD3 complex on T lymphocytes, are phosphorylated by Src family protein tyrosine kinases such as LCK and FYN (42). This phosphorylation triggers downstream signaling pathways essential for an appropriate adaptive immune response (43). The upregulation of GATA3, RUNX3, RORA, and TGFB1 promotes balanced differentiation of Th1, Th2, and Th17 cells, enabling the effective elimination of pathogens while mitigating excessive inflammation. T-cell-mediated adaptive immunity plays a vital role in both the sustained control of viral infections and the successful management of respiratory viral diseases (44). In conclusion, the good prognosis group exhibited a well-regulated and effective immune response, highlighted by improved NK and NKT cell activity and robust T cell-mediated immunity. The downregulation of genes linked to excessive inflammatory responses and tissue damage further emphasized the role of controlled immune modulation in overcoming COVID-19. This comprehensive analysis highlights the mechanisms underlying favorable clinical outcomes in COVID-19 and underscores the potential of targeted therapies like TDR in fine-tuning the immune response for improved patient recovery.

We also propose JUN as a key gene to determine the drug response of COVID-19 patients. The JUN gene, a key component of the AP-1 transcription factor complex, is involved in numerous cellular functions such as proliferation, differentiation, apoptosis, and the response to infections (45). The Jun N-terminal kinase (JNK) signaling pathway facilitates viral infection and replication in infections caused by varicella-zoster virus, herpes simplex virus type 1, dengue virus, and influenza virus (46). The JNK signaling pathway plays a role in virus-triggered cell death processes, including apoptosis and autophagy, which are essential for preserving cellular homeostasis and combating viral infections (46). The JNK signaling pathway modulates apoptosis by inducing c-Jun and Fos or by suppressing the cell survival pathway of STATs and CREB (47). The JNK downstream molecule c-Jun is also associated with viral replication and upregulation of pro-inflammatory cytokines such as TNF-α, IFN-β, and IL-6 in patients with H5N1 influenza virus infection (48). In COVID-19 patients, JUN is involved in activating the NF-κB signaling pathway, which serves as a crucial regulator of the immune response to the infection (49). Activation of the JNK and JAK-STAT pathways lead to increased cytokines, inflammation, and eventually, pulmonary fibrosis in COVID-19 infection (47). In poor prognosis patients of our study, JUN was upregulated, promoting mitochondrial ROS production and apoptosis, which contributed to sustained inflammation and tissue damage. On the other hand, in patients with good prognosis, downregulation of JUN appeared to correlate with reduced tissue damage and immune modulation involving the T cell differentiation pathway. JNK inhibitors have been shown to prevent pulmonary fibrosis in preclinical models and in Phase I and II IPF studies, and to attenuate sepsis-induced lung injury in experimental animal models (50, 51). These findings suggest that JUN could be a key biomarker for determining drug response and prognosis in COVID-19 patients.

Our study has several limitations. Firstly, the relatively small sample size may be viewed as a limitation. However, the sample size was reduced by adjusting for baseline characteristics that could influence prognosis, such as age, male sex, BMI, and the initial ordinal scale. By matching these baseline characteristics, we enhanced the study's reliability by minimizing variability. Secondly, the samples were collected during a period when the Omicron variant of COVID-19 was predominant, which might not accurately represent the broader range of COVID-19 strains. Nevertheless, this approach has the advantage of eliminating differences in immune responses that could arise from variations between different COVID-19 variants. Furthermore, we standardized the timing of drug administration and blood sample collection to control for potential confounding factors related to differences in timing.

V. CONCLUSION

In conclusion, adaptive immune responses driven by T cells, along with suppression of macrophage-driven hyperinflammatory pathways and enhancement of NK cell-mediated cytotoxicity, highlight the importance of maintaining a well-regulated immune balance in managing severe COVID-19 infection.

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Abstract in Korean

단핵구와 자연살해세포에 의한 중증 코로나19 감염의 치료 효과 조절: 단일 세포 RNA 염기서열 분석 연구

중증 코로나19 감염은 과도한 염증 반응과 함께 면역 세포의 과잉 활성화 및 기능 장애를 포함한 심각한 면역 반응 조절 장애가 특징입니다. 코르티코스테로이드와 토실리주맙은 중증 코로나19 환자의 예후를 개선하는 데 도움이 될 수 있습니다. 그러나 환자마다 약물 반응의 차이가 존재한다는 것은 여전히 해결해야 할 과제입니다. 이 연구는 단일 세포 유전체 분석을 활용하여 중증 코로나19 환자에서 토실리주맙, 덱사메타손, 렘데시비르의 병용 요법에 대한 치료 결과의 차이를 유발하는 분자 및 세포 결정 요인을 밝혀내고자 합니다. 연령, 성별, 세계보건기구 임상 진행 척도의 유의미한 차이가 없는 17명의 중증코로나19 환자의 혈액 샘플을 2021년 6월부터 2022년 1월까지 3차 병원에서 수집했습니다. 환자는 두 그룹으로 분류되었습니다: 10명은 악화 없이 회복되었고, 7명은 호흡부전으로 인해 기도 삽관이 필요했습니다. 샘플은 1일차와 7일차에 채취했습니다. Dropkick 을 사용하여 말초혈액 단핵세포에서 추출한 단일세포 리보핵산 시퀀싱 데이터를 처리하고 분석했습니다. 차등 발현된 유전자의 히트맵은 Complex Heatmap 패키지를 사용하여 생성되었으며, 유전자 온톨로지 농축 분석은 Seurat(버전 3.9 및 4)에 통합된 Ma'ayan 연구소의 Enrichr 도구를 사용하여 수행되었습니다. 이 연구는 토실리주맙, 덱사메타손, 렘데시비르 치료 후 좋은 예후(1일째[n=13,580]와 7일째[n=14,017])와 나쁜 예후 환자(1일째[n=13,747]와 7일째[n=13,630])를 조사하여 환자 그룹 간의 면역 세포 구성 차이를 비교했습니다. 예후가 좋지 않은 그룹에서는 치료 후 뉴클레오타이드 결합 올리고머화 도메인 유사 수용체 신호 전달 경로와 자연살해 세포 매개 세포 독성이 하향 조절되었습니다. 그러나 염증성 대식세포 관련 리소좀, 식세포, 세포자멸 경로가 지속적으로 상향 조절되었습니다. 반면, 예후가 좋은 그룹에서는 치료 후 자연살해 세포 매개 세포 독성이 증가하고 염증성 대식세포 관련 경로가 감소했습니다. 또한, 예후가 좋은 그룹은 T 세포 수용체 활성화, 신호 전달 경로, 면역 조절과 관련된 분화 과정과 관련된 유전자의 발현이 증가했습니다. 결론적으로, T 세포에 의해 유도된 적응성 면역 반응과 함께 대식세포에 의한 과염증 경로 억제 및 자연살해세포 매개 세포 독성 강화는 중증 코로나19 사례 관리에 있어 잘 조절된 면역 균형을 유지하는 것이 중요하다는 것을 강조합니다.

핵심되는 말: 코로나19 감염, 단핵구, 자연살해세포, 단일 세포 유전자 발현 분석, 면역 조절.