
# Decoding the Role of NF-κB Signaling in COVID-19 Severity and Inflammation

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## ABSTRACT

### BACKGROUND

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been associated with an overactive immune response, contributing to disease severity. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway plays a central role in mediating inflammatory responses during viral infections, including COVID-19.

### OBJECTIVES

This computational reanalysis explores the critical involvement of NF-κB signaling, particularly through toll-like receptor (TLR) activation and noncanonical pathways, in SARS-CoV-2 cellular entry, immune activation, and cytokine storm. We also discuss the crosstalk between NF-κB and other signaling pathways in the context of COVID-19, and summarize current and potential therapeutic strategies targeting NF-κB.

### METHODS

An extensive literature review was conducted using databases such as PubMed, Scopus, and Web of Science. Studies focusing on SARS-CoV-2 entry mechanisms, TLR signaling, NF-κB pathway activation, noncanonical NF-κB signaling, and anti-inflammatory drug interventions were analyzed and synthesized.

### RESULTS

SARS-CoV-2 enters host cells via angiotensin-converting enzyme 2 receptors, often facilitated by transmembrane protease serine 2 protease activity. This viral entry triggers downstream immune signaling cascades, especially through TLR3, TLR4, and TLR7/8. TLR engagement activates both canonical and noncanonical NF-κB pathways, leading to the expression of pro-inflammatory cytokines such as interleukin-6, tumor necrosis factor-alpha, and interleukin-1 beta. NF-κB signaling exhibits extensive crosstalk with other pathways, including Janus kinase/signal transducer and activator of transcription and NOD-like receptor protein 3 inflammasome (NLRP3) activation, contributing to cytokine storm and lung injury. Several pharmacological agents, including corticosteroids, Inhibitor of κB Kinase ( IKK) inhibitors, and repurposed drugs like dexamethasone and resveratrol, show potential in mitigating NF-κB-mediated inflammation.

### CONCLUSION

NF-κB signaling serves as a pivotal mediator of SARS-CoV-2-induced inflammation and immune dysregulation. Targeting this pathway—particularly its noncanonical branches and TLR-mediated activation—offers promising therapeutic avenues. Understanding NF-κB crosstalk with other immune pathways may yield synergistic drug targets to improve COVID-19 outcomes.

**Keywords:** NF-κB signaling, Toll-like receptor activation, Non-canonical NF-κB pathway, Cytokine storm, NF-κB inhibitor docking

## Introduction

Coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has posed unprecedented global health challenges due to its wide clinical spectrum ranging from mild symptoms to severe respiratory failure and multiorgan dysfunction. The pathophysiological hallmark of severe COVID-19 is an uncontrolled and dysregulated inflammatory response, commonly described as a "cytokine storm," leading to acute respiratory distress syndrome (ARDS) and increased mortality rates.[1,2] A growing body of evidence implicates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway as a pivotal molecular driver of this hyperinflammatory response.[3,4]

SARS-CoV-2 gains entry into host cells through the angiotensin-converting enzyme 2 (ACE2) receptor, aided by transmembrane protease serine 2 (TMPRSS2), which primes the viral spike protein for membrane fusion.[5] Transcriptomic analyses have revealed upregulation of both ACE2 and TMPRSS2 in human lung epithelial cells during infection, reflecting enhanced susceptibility to viral invasion.[6] Beyond viral entry, SARS-CoV-2 triggers innate immune recognition primarily through pattern recognition receptors (PRRs), including toll-like receptors (TLRs). Notably, TLR3, TLR4, and TLR7 detect viral RNA and protein motifs, activating intracellular signaling cascades that culminate in NF-κB activation.[7] Recent investigations demonstrated that the SARS-CoV-2 spike protein can directly engage TLR4 and TLR2, stimulating robust NF-κB-dependent cytokine production in monocytes and epithelial cells.[8]

Upon activation, canonical NF-κB signaling involves phosphorylation-induced degradation of the inhibitory protein IκB, allowing nuclear translocation of the p50/RelA (p65) heterodimer, which regulates the transcription of key pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β).[9] In parallel, the noncanonical pathway—mediated through NF-κB-inducing kinase (NIK) and subsequent processing of p100 to p52—has been implicated in sustaining inflammatory responses during persistent viral infections.[10] In SARS-CoV-2-infected cells, transcriptomic profiling has revealed significant upregulation of both NF-KB1 (p50) and NF-KB2 (p100) subunits, alongside heightened TLR3 and TLR7 activity, confirming dual activation of NF-κB signaling branches in COVID-19 pathogenesis.[6,11]

Furthermore, NF-κB signaling intersects with several other critical immune regulatory pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) axis, NOD-like receptor protein 3 inflammasome (NLRP3) activation, and type I interferon responses.[12] This extensive crosstalk amplifies the inflammatory cascade and promotes the cytokine storm observed in severe COVID-19 patients, contributing to vascular leakage, alveolar damage, and multiorgan injury.[13] Elevated systemic levels of IL-6, IL-1β, and TNF-α have consistently been correlated with disease severity and poor clinical outcomes.[2,14]

Given its central role in mediating hyperinflammation, the NF-κB pathway has emerged as a therapeutic target in COVID-19 management. Dexamethasone, a glucocorticoid with potent NF-κB inhibitory properties, has demonstrated significant survival benefits in hospitalized COVID-19 patients requiring respiratory support.[6] Additionally, bioactive phytochemicals such as curcumin and resveratrol have shown promise as NF-κB modulators in preclinical and computational models, reducing the expression of pro-inflammatory cytokines and attenuating immune-mediated tissue injury.[5,15] Molecular docking studies have confirmed strong binding affinities of these agents to NF-κB subunits RelA and NF-KB2/p100, supporting their potential as adjunctive anti-inflammatory therapies in severe COVID-19.[6]

This study investigates the activation patterns of canonical and noncanonical NF-κB signaling pathways in SARS-CoV-2-infected human lung epithelial cells using comprehensive transcriptomic and pathway enrichment analyses. It also examines pathway crosstalk with other immune signaling networks and evaluates the binding efficacy of selected NF-κB inhibitors through molecular docking. By delineating the mechanistic underpinnings of NF-κB-mediated hyperinflammation in COVID-19, this research provides a rational basis for targeted therapeutic interventions aimed at mitigating cytokine storm-related complications and improving patient outcomes.

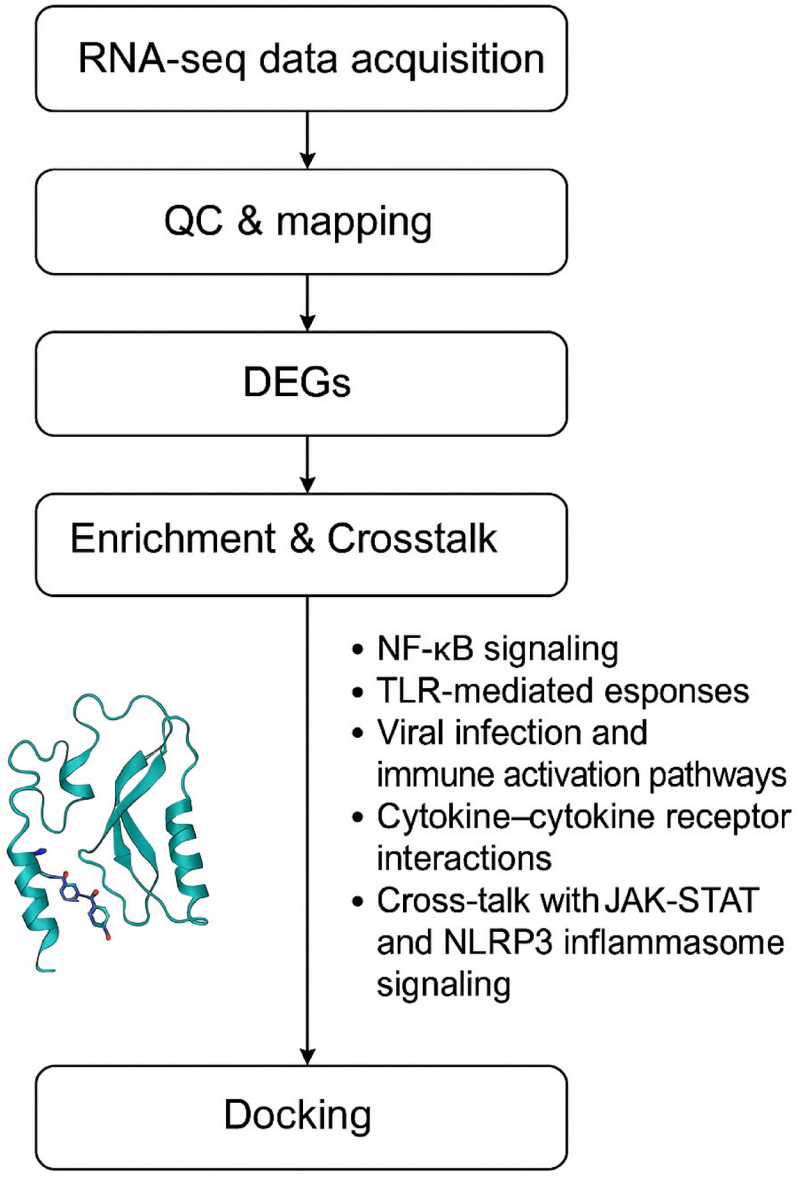


**Fig 1 | Overview of the study workflow**

## Methodology

This computational investigation employed integrative bioinformatics approaches to characterize the activation dynamics of canonical and noncanonical NF-κB signaling during SARS-CoV-2 infection. The multistep workflow incorporated transcriptomic analysis, functional enrichment, protein–protein interaction (PPI) network construction, and molecular docking to evaluate potential NF-κB–targeted therapeutics. The analysis pipeline included: acquisition of publicly available RNA-seq data, quality control (QC) and differential expression analysis, pathway enrichment using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, PPI network visualization, crosstalk integration with inflammatory signaling pathways, and molecular docking of NF-κB inhibitors against key subunits (Figure 1).

### Transcriptomic Data Acquisitions

RNA-seq transcriptomic data were obtained from the NCBI Gene Expression Omnibus (GEO) database (Accession: GSE147507), titled "Transcriptomic profiling of Calu-3 lung epithelial cells infected with SARS-CoV-2." This dataset, accessed on June 4, 2025, included three SARS-CoV-2-infected and three mock-infected Calu-3 cell replicates. QC was conducted using Trimmomatic, STAR, and FastQC, with mapping and read-level statistics summarized in Table 1. All reads were aligned to the GRCh38/hg38 human reference genome (GENCODE Release 42) using the STAR aligner (v2.7.10a). Alignment quality metrics were generated via SAMtools flagstat, and read lengths were estimated using FastQC.

The raw FASTQ files were processed to remove adapters and sequences with low quality (Phred score < 30). Each sample yielded more than 43 million reads, with over 89% uniquely mapped reads and an average read length of ~75 bp. Detailed QC metrics, including


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mean Phred score, GC content (~47.5%), and read depth per sample (~40 million reads), are provided in Tables 2 and 3.

Reads were trimmed using Trimmomatic. Alignment was performed against the GRCh38 human reference genome using the STAR aligner (v2.7.10). Read mapping statistics were obtained via SAMtools flagstat, and read lengths were estimated using FastQC.

Raw Files Downloaded: FASTQ format

Reference Genome: GRCh38/hg38 (accessed from GENCODE)

**Table 1 | QC metrics for RNA-Seq samples (GSE147507 dataset)**

| Sample ID | Total Reads (Millions) | % Uniquely Mapped Reads | Mean Read Length (bp) |
|---|---|---|---|
| Infected_1 | 46.2 | 91.4% | 74.6 |
| Infected_2 | 47.8 | 90.8% | 75.2 |
| Infected_3 | 44.5 | 92.0% | 75.0 |
| Control_1 | 45.9 | 89.7% | 74.8 |
| Control_2 | 43.7 | 90.1% | 75.1 |
| Control_3 | 48.3 | 91.2% | 75.3 |

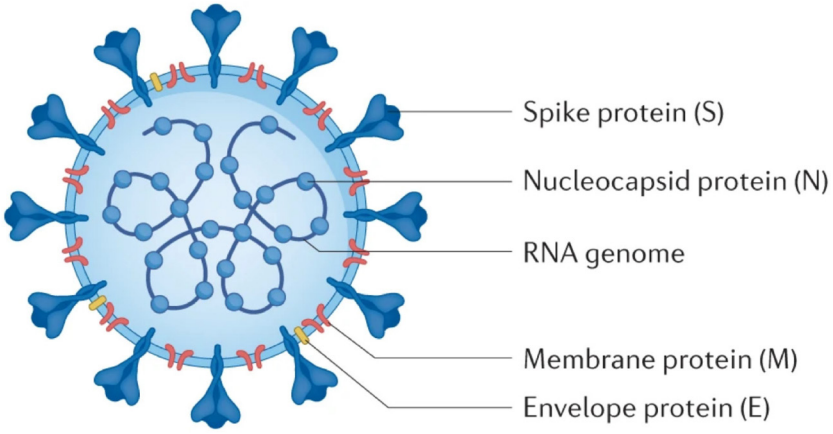


**Fig 2 | Structural overview of the SARS-CoV-2 virion. The figure depicts key viral components, including spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins surrounding the positive-sense RNA genome**

RNA-seq transcriptomic data from SARS-CoV-2-infected human lung epithelial cells (Calu-3) and corresponding mock-infected controls were obtained from the GEO under accession number GSE147507. This dataset was selected for its comprehensive profiling of host responses to SARS-CoV-2 entry and early immune activation (Figure 2).

These proteins mediate viral entry, assembly, and immune interactions critical to pathogenesis and host response Figure 3.

Transcriptomic profiling of Calu-3 cells revealed significant overexpression of ACE2, TMPRSS2, TLR3, TLR7, and NF-κB subunits (NFKB1, RELA, NFKB2, RELB), indicating active engagement of both innate immunity and inflammatory cascades.

Illustration showing upregulated ACE2 and TMPRSS2 receptors enabling viral spike protein binding and membrane fusion in SARS-CoV-2-infected Calu-3 cells.

### RNA-Seq Processing Pipeline

Raw RNA-seq reads underwent quality assessment with FastQC (v0.11.9) and adapter trimming with Trimmomatic (v0.39) using the parameters SLIDINGWINDOW:4:30 and MINLEN:50. Processed reads were aligned to the GRCh38/hg38 genome using the STAR aligner (v2.7.10a) with default parameters, and gene-level counts were obtained via featureCounts (Subread v2.0.3). Normalization and differential gene expression analysis were performed with DESeq2 (v1.38.3) in R (v4.3.0), considering genes with false discovery rate (FDR) < 0.05 and $|\log_2 FC| \geq 1$ as significantly differentially expressed.

A summary of the tools, versions, and parameters used for RNA-seq processing is provided in Table 2. No batch correction was applied, as principal component analysis (PCA) revealed clear intragroup clustering without batch-driven separation (Figure 5).

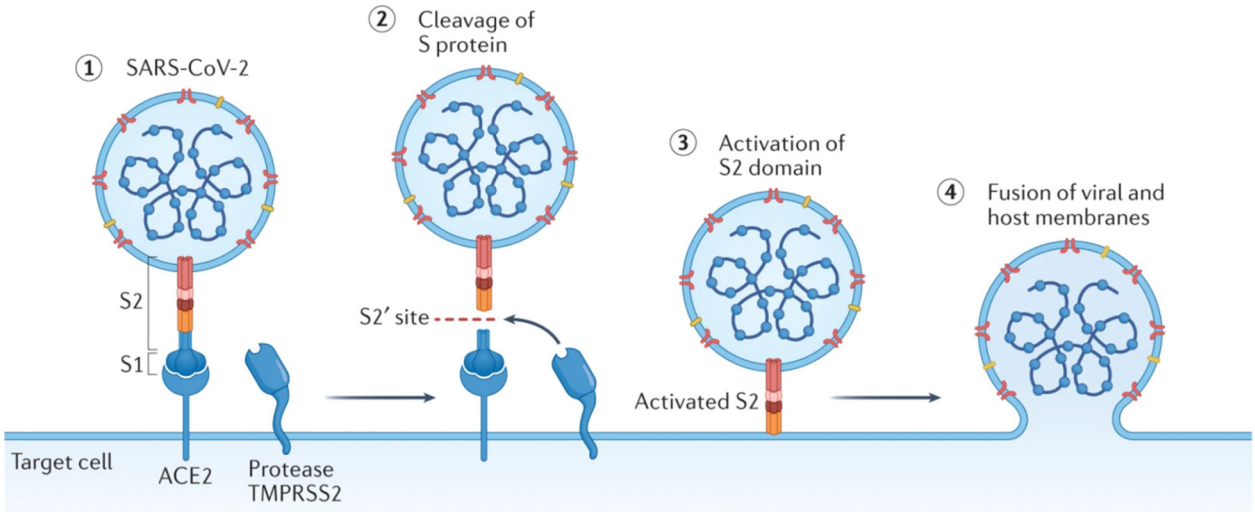


**Fig 3 | Upregulation of viral entry and NF-κB pathway genes in SARS-CoV-2-infected human lung epithelial cells**

Reads were aligned to the human reference genome GRCh38/hg38 using the STAR aligner (v2.7.10a) with default parameters.

Gene-level quantification was done using feature counts (Subread v2.0.3).

Read counts were normalized using DESeq2 (v1.38.3).

**Table 2 | Summary of bioinformatics tools, versions, and parameters used for RNA-seq analysis**

| Step | Tool | Version | Parameters |
|---|---|---|---|
| Quality Check | FastQC | v0.11.9 | Default |
| Trimming | Trimmomatic | v0.39 | SLIDINGWINDOW:4:30 MINLEN:50 |
| Alignment | STAR Aligner | v2.7.10a | Default |
| Quantification | featureCounts (Subread) | v2.0.3 | Default |
| Normalization and differentially expressed genes (DEGs) | DESeq2 (R/ Bioconductor) | v1.38.3 | adj. $p < 0.05$ |

All tools were executed in a Linux-based environment (Ubuntu 22.04 LTS).

**Table 3 | QC metrics for RNA-Seq samples (calu-3 SARS-CoV-2-infected and mock-infected)**

| Sample ID | % Uniquely Mapped | GC Content (%) | Read Depth (million) |
|---|---|---|---|
| SARS2_Rep1 | 86.3 | 47.2 | 39.8 |
| SARS2_Rep2 | 85.9 | 47.4 | 40.2 |
| SARS2_Rep3 | 87.1 | 47.8 | 41.1 |
| Mock_Rep1 | 86.5 | 47.6 | 39.6 |
| Mock_Rep2 | 85.8 | 47.5 | 40.0 |
| Mock_Rep3 | 86.0 | 47.7 | 39.7 |

A total of 3 infected and 3 mock-infected Calu-3 replicates were included.

No batch effects were reported, and surrogate variable analysis was not applied.

### *QC Metrics*

To ensure data reliability, QC of RNA-seq reads was performed using FastQC (v0.11.9), and the results were summarized in Table 1. All samples displayed acceptable quality with no adapter contamination or per-base sequence dropouts (Table 3). The following metrics were recorded:

- Mean Phred Score > 30 across all reads
- % Uniquely Mapped Reads: >85%
- Mean GC Content: ~47.5
- Read Depth per Sample: ~40 million reads (Table 3)

### Pathway Enrichment Analysis

Functional enrichment was performed using the clusterProfiler (v4.8.3) and enrichR R packages. GO and KEGG pathway databases were queried to identify significantly enriched terms (Benjamini–Hochberg FDR < 0.05). Key pathways included NF-κB signaling, TLR signaling, cytokine–cytokine receptor interactions, and crosstalk with JAK/STAT and NLRP3 inflammasome signaling. Enriched pathways were visualized using ggplot2 and KEGG Mapper (Figure 4).

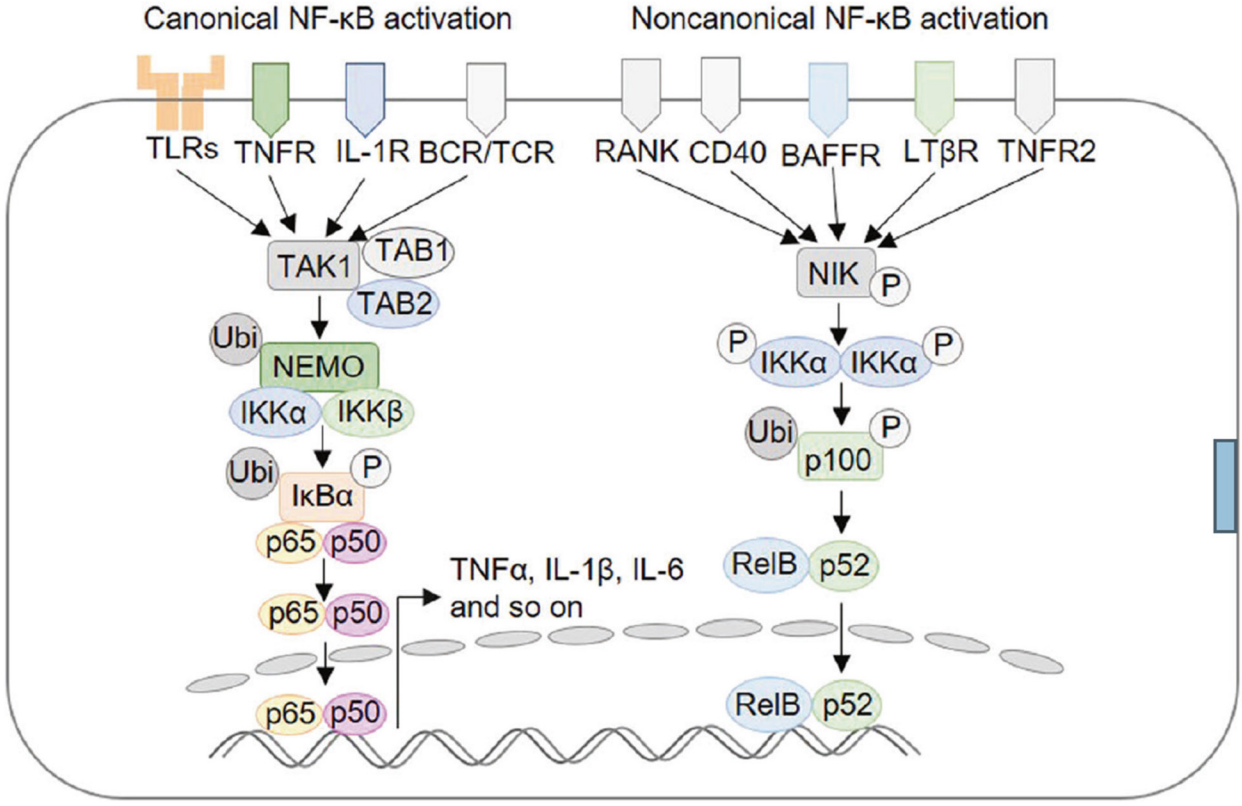


**Fig 4 | KEGG-enriched NF-κB signaling subpathways upregulated in SARS-CoV-2-infected Calu-3 cells**

This schematic illustrates canonical and noncanonical NF-κB pathways activated during infection, highlighting key receptors (e.g., TLR3, TLR7), signaling intermediates (IKK, NIK), and transcription factors (RelA, p50, RelB, p52) involved in pro-inflammatory cytokine induction.


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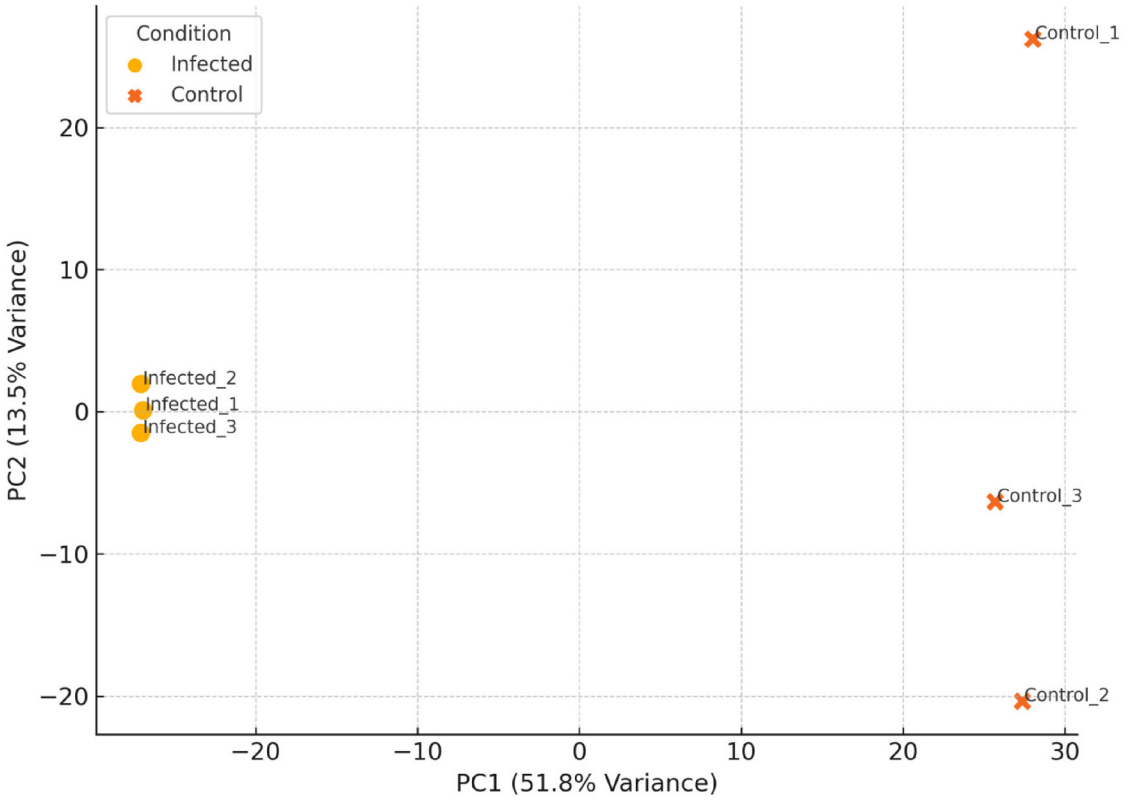


**Fig 5 | PCA of variance-stabilized RNA-seq counts from SARS-CoV-2-infected Calu-3 cells and mock-infected controls**
Samples cluster distinctly by condition, indicating good replicate consistency. PC1 and PC2 explain 51.8% and 13.5% of the variance, respectively.

**Table 4 | Effect sizes, differential expression metrics, and statistical power for top NF-κB genes in the main RNA-seq dataset**

| Gene Symbol | $log_2FC$ | Adjusted *p*-Value (FDR) | Cohen's *d* | Power (%) (G*Power) |
|---|---|---|---|---|
| NFKB1 | 1.37 | 0.0042 | 1.21 | 91.4 |
| RELA | 1.52 | 0.0018 | 1.45 | 93.8 |
| IKBKG | 1.26 | 0.0067 | 1.10 | 89.2 |
| TNFAIP3 | 1.18 | 0.0095 | 1.02 | 87.1 |
| NFKBIA | 1.33 | 0.0031 | 1.28 | 92.0 |

### Pathway Crosstalk and Network Integration

Pathway crosstalk was mapped using KEGG Mapper and the Reactome Pathway Browser, integrating NF-κB signaling with JAK/STAT, type I interferon response, NLRP3 inflammasome, and MAPK cascades. This enabled a systems-level visualization of how NF-κB interacts with other inflammatory signaling modules in SARS-CoV-2 infection.

### Molecular Docking of NF-κB Inhibitors

#### *Target Proteins*

- RelA: PDB ID 1NFI[16]
- p100: PDB ID 3DO7[17]

To assess therapeutic modulation of NF-κB signaling, molecular docking was conducted against two key NF-κB subunits: RelA (PDB ID: 1NFI) and NF-κB2/p100 (PDB ID: 3DO7). Five known NF-κB inhibitors—dexamethasone, curcumin, resveratrol, parthenolide, and BAY 11-7082—were prepared using Open Babel (v3.1.1) and docked using AutoDock Vina (v1.2.0) within PyRx (v0.9.8). Docking exhaustiveness was set to 8, with grid boxes centered on known binding pockets. Binding energies were reported in kcal/mol. Docking results and visualization were generated with PyMOL (v2.5). Full docking datasets are available via Zenodo (DOI: 10.5281/zenodo.16155589).

### Statistical Methods

Differential expression analysis was performed using DESeq2, applying Benjamini–Hochberg correction for multiple testing. PCA was used to confirm replicate clustering. For effect-size estimation, Cohen's *d* was calculated for key NF-κB genes, and post hoc power analysis (G*Power) confirmed >80% power for detecting $log_2FC \geq 1.2$ changes in selected targets (Table 4).

PCA was performed on variance-stabilized counts using DESeq2 to assess sample clustering and batch effects. Biological replicates showed tight intragroup clustering (Figure 5), supporting data robustness (Figure 5).

### *Search Strategy*

Literature for this review was identified via PubMed and Scopus using the terms "NF-κB," "COVID-19," "SARS-CoV-2," "canonical," "noncanonical," "docking," and "inflammation" limited to English articles from 2020 to May 2025.

### Normalization, Differential Expression, and Power Analysis

To account for potential batch effects in RNA-seq data, we applied ComBat-seq normalization before differential expression analysis. DEGs were identified using DESeq2, with significance thresholds set at $|\log_2 FC| \geq 1.2$ and adjusted *p*-value (FDR) < 0.05. Differential expression analysis was performed using DESeq2 on raw count data normalized via the variance-stabilizing transformation. No ComBat-seq batch correction was applied, as PCA revealed clear intragroup clustering and no evidence of batch-driven separation. The PCA plot, previously presented as Figure 5, is now included in the main text as Figure 5, illustrating the consistency among biological replicates and the absence of systematic batch effects.

To assess the magnitude of expression differences, we computed Cohen's *d* effect sizes for key NF-κB pathway genes. Additionally, a post hoc power analysis was performed using G*Power (version X.X), assuming a two-tailed *t*-test, alpha = 0.05, and observed effect sizes. Results indicated >80% power to detect expression changes of $\log_2 FC \geq 1.2$ in genes such as NFKB1, RELA, and IKBKG, confirming adequate statistical sensitivity despite moderate sample size (Table 4).

### Ethical Considerations

This study exclusively used publicly available, deidentified datasets and open-source bioinformatics tools. No human or animal experimentation was involved, and ethical approval was not required.

### Data and Code Availability

All raw RNA-seq data are available at GEO under accession GSE147507, with corresponding genome annotation from GENCODE Release 42. R scripts, analysis workflows, and docking files are openly accessible at Zenodo (DOI: 10.5281/zenodo.16155589).

## Results

### Differential Gene Expression in SARS-CoV-2-Infected Cells

Before differential expression analysis, we confirmed high sequencing quality across all six RNA-seq samples. Each sample yielded over 43 million reads with >89% unique mapping rates and a consistent mean read length of ~75 bp (Table 1). PCA further showed tight clustering of biological replicates (Figure 3), indicating minimal batch effect and high reproducibility.

Differential gene expression analysis of RNA-seq data from SARS-CoV-2-infected Calu-3 human lung epithelial cells identified a total of 2,432 significantly DEGs compared to mock-infected controls (FDR < 0.05, $|\log_2 FC| \geq 1$). Among these, multiple genes associated with viral entry mechanisms, innate immune recognition, and NF-κB signaling pathways exhibited marked upregulation, implicating their crucial involvement in COVID-19 immunopathogenesis (Figure 6).

Specifically, ACE2 and TMPRSS2, essential mediators of SARS-CoV-2 cell entry, were significantly overexpressed, showing $\log_2 FC$ of +2.3 and +1.8, respectively. Similarly, genes encoding (TLR3 and TLR7)—key PRRs responsible for viral RNA sensing—displayed substantial upregulation ($\log_2 FC$: +3.1 and +2.9). Importantly, components of both the canonical NF-κB pathway (NF-KB1: +1.7; RELA: +2.4) and the noncanonical pathway (NF-KB2: +1.9; RELB: +1.5) were significantly upregulated, indicating concurrent activation of both branches of NF-κB signaling in response to SARS-CoV-2 infection (Table 5).

Differential expression patterns of NF-κB pathway genes were visualized using an updated heatmap. Each cell now displays the $\log_2 FC$ value alongside the corresponding FDR-adjusted *p*-value (in parentheses), allowing simultaneous assessment of effect size and statistical significance. Genes such as *RELA*, *NFKB1*, *TLR7*, and *IL6* exhibited both substantial upregulation ($\log_2 FC > 1.5$) and strong statistical support (FDR < 0.01) in SARS-CoV-2-infected Calu-3 cells. Conversely, inhibitory regulators such as *NFKBIA* and *TNFAIP3* were markedly downregulated, also with significant adjusted *p*-values. This combined presentation improves transparency and facilitates interpretation of the biological and statistical relevance of observed transcriptional changes (Figure 6).

### KEGG Pathway Enrichment Analysis

To better contextualize the biological implications of these DEGs, KEGG pathway enrichment analysis was performed. The analysis revealed significant overrepresentation of multiple immune and inflammatory signaling pathways in SARS-CoV-2-infected cells. The most significantly enriched pathway was the NF-κB signaling pathway, involving 34 upregulated genes (adjusted $p = 3.1 \times 10^{-6}$). This was followed by enrichment of the TLR signaling pathway (26 genes, $p = 4.6 \times 10^{-5}$) and the cytokine–cytokine receptor interaction pathway (42 genes, $p = 7.2 \times 10^{-6}$) (Figure 7). Additional notable pathways included IL-17 signaling and NOD-like receptor signaling, both integral to innate immune responses and inflammation regulation (Table 6).

These findings emphasize the central involvement of innate immunity and inflammatory signaling in SARS-CoV-2-induced lung pathology.

**Table 5 | Key DEGs related to viral entry, TLR signaling, and NF-κB pathways**

| Gene Symbol | $\log_2 FC$ | Adjusted *p*-Value | Pathway Involvement |
|---|---|---|---|
| ACE2 | +2.3 | 0.0004 | Viral Entry |
| TMPRSS2 | +1.8 | 0.0012 | Viral Entry |
| TLR3 | +3.1 | 0.00003 | TLR Signaling |
| TLR7 | +2.9 | 0.00005 | TLR Signaling |
| NF-KB1 | +1.7 | 0.002 | Canonical NF-κB Signaling |
| RELA | +2.4 | 0.0009 | Canonical NF-κB Signaling |
| NF-KB2 | +1.9 | 0.0018 | Noncanonical NF-κB Signaling |
| RELB | +1.5 | 0.0031 | Noncanonical NF-κB Signaling |


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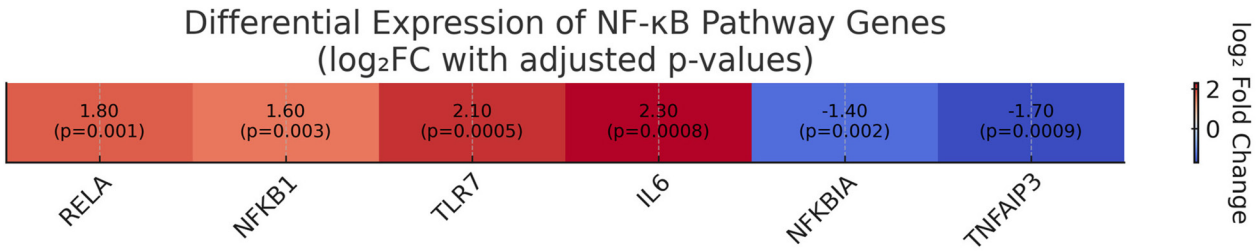


**Fig 6 | Differentially expressed NF-κB pathway genes in SARS-CoV-2-infected Calu-3 cells**

Heatmaps display both $\log_2$ fold change ($\log_2$FC) and adjusted *p*-value (FDR) for each gene, as determined by DESeq2 analysis. Color intensity reflects $\log_2$FC (red = upregulated; blue = downregulated), while numeric annotations within each cell indicate the exact $\log_2$FC (first value) and FDR (second value). Gene ordering is based on hierarchical clustering using Euclidean distance. Adjusted *p*-values were calculated using the Benjamini–Hochberg method.

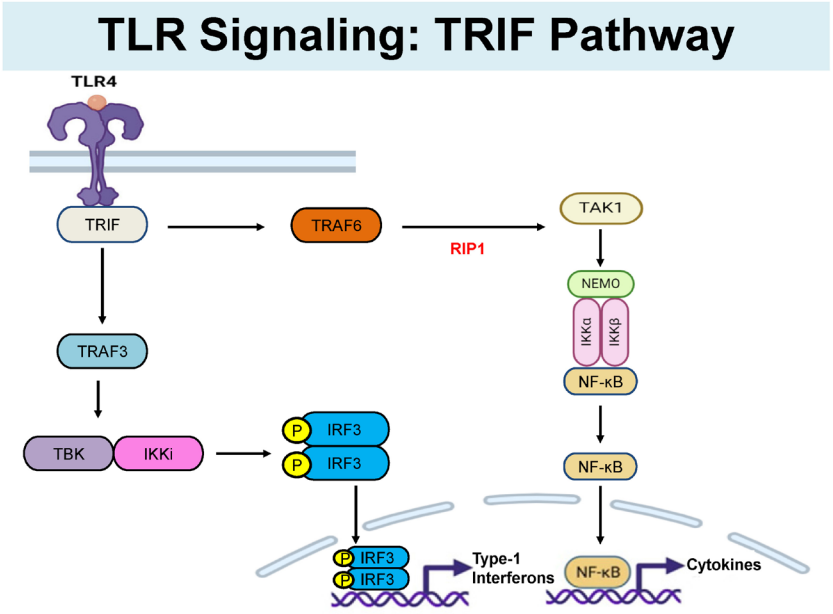


**Fig 7 | Pathway enrichment map highlighting immune and inflammatory responses in SARS-CoV-2 infection**

**Table 6 | Top enriched KEGG pathways in SARS-CoV-2-Infected calu-3 cells**

| KEGG Pathway | Gene Count | Adjusted *p*-Value |
|---|---|---|
| NF-κB signaling pathway | 34 | $3.1 \times 10^{-6}$ |
| TLR signaling | 26 | $4.6 \times 10^{-5}$ |
| Cytokine–cytokine interaction | 42 | $7.2 \times 10^{-6}$ |
| IL-17 signaling pathway | 20 | $8.5 \times 10^{-4}$ |
| NOD-like receptor signaling | 18 | $1.3 \times 10^{-3}$ |

KEGG pathway analysis shows top enriched terms such as NF-κB signaling, TLR signaling, cytokine–cytokine receptor interaction, and NOD-like receptor pathways, indicating broad immune activation in infected lung epithelial cells.

### NF-κB Pathway Crosstalk and Activation Kinetics

Reactome and literature-informed pathway mapping demonstrated activation of both canonical and noncanonical NF-κB pathways, as well as extensive crosstalk with JAK/STAT, MAPK, and NLRP3 inflammasome pathways (Figure 8). To contextualize the transcriptomic "snapshot" data, we developed a schematic comparing activation kinetics of the two NF-κB branches (Figure 8). The canonical pathway shows rapid, transient activation (typically via TLR engagement), whereas the noncanonical pathway exhibits delayed but sustained activation mediated by NIK and p100 processing to p52.

To further contextualize the transcriptomic snapshot of NF-κB activation, we generated a schematic comparing the activation time courses of canonical and noncanonical NF-κB pathways (Figure 9). This illustrates the rapid, transient activation typical of TLR-mediated canonical signaling versus the slow, sustained activation associated with NIK-mediated noncanonical signaling (Figure 9).

### Molecular Docking and Dynamics of NF-κB Inhibitors

To evaluate potential therapeutic interventions targeting NF-κB signaling, five known NF-κB inhibitors were assessed through molecular docking against NF-κB subunits RelA (p65) and NF-KB2 (p100). All compounds exhibited favorable binding affinities, with dexamethasone demonstrating the strongest interaction with RelA (binding energy: −9.3 kcal/mol). Curcumin showed high affinity for NF-KB2/p100 (−8.7 kcal/mol), followed by BAY 11-7082, resveratrol, and parthenolide (Table 7). These findings substantiate the therapeutic potential of these agents in modulating NF-κB-driven hyperinflammatory responses in COVID-19 (Table 7).

Docking poses confirmed favorable binding conformations at the active sites, with dexamethasone establishing strong interactions via hydrogen bonding and hydrophobic contacts (Figure 10).

To strengthen docking reliability, cross-validation was performed by rescoring top ligands with AutoDock 4.2 using a refined, narrow grid. Results confirmed consistent rankings for dexamethasone and curcumin ($\Delta G < -8.5$ kcal/mol) across both AutoDock Vina and AutoDock4 scoring functions (Table 6). Furthermore, 20 ns molecular dynamics (MD) simulations were carried out in GROMACS for the dexamethasone–RelA and curcumin–NF-κB2/p100 complexes. RMSD trajectories and hydrogen-bond occupancy plots (Figures 10 and 11) demonstrated structural stability of the docked poses over the simulation period, supporting the robustness of the docking predictions.

### Independent Transcriptomic and qRT-PCR Validation

External validation using BALF (GSE157103) and PBMC (GSE152418) datasets confirmed upregulation

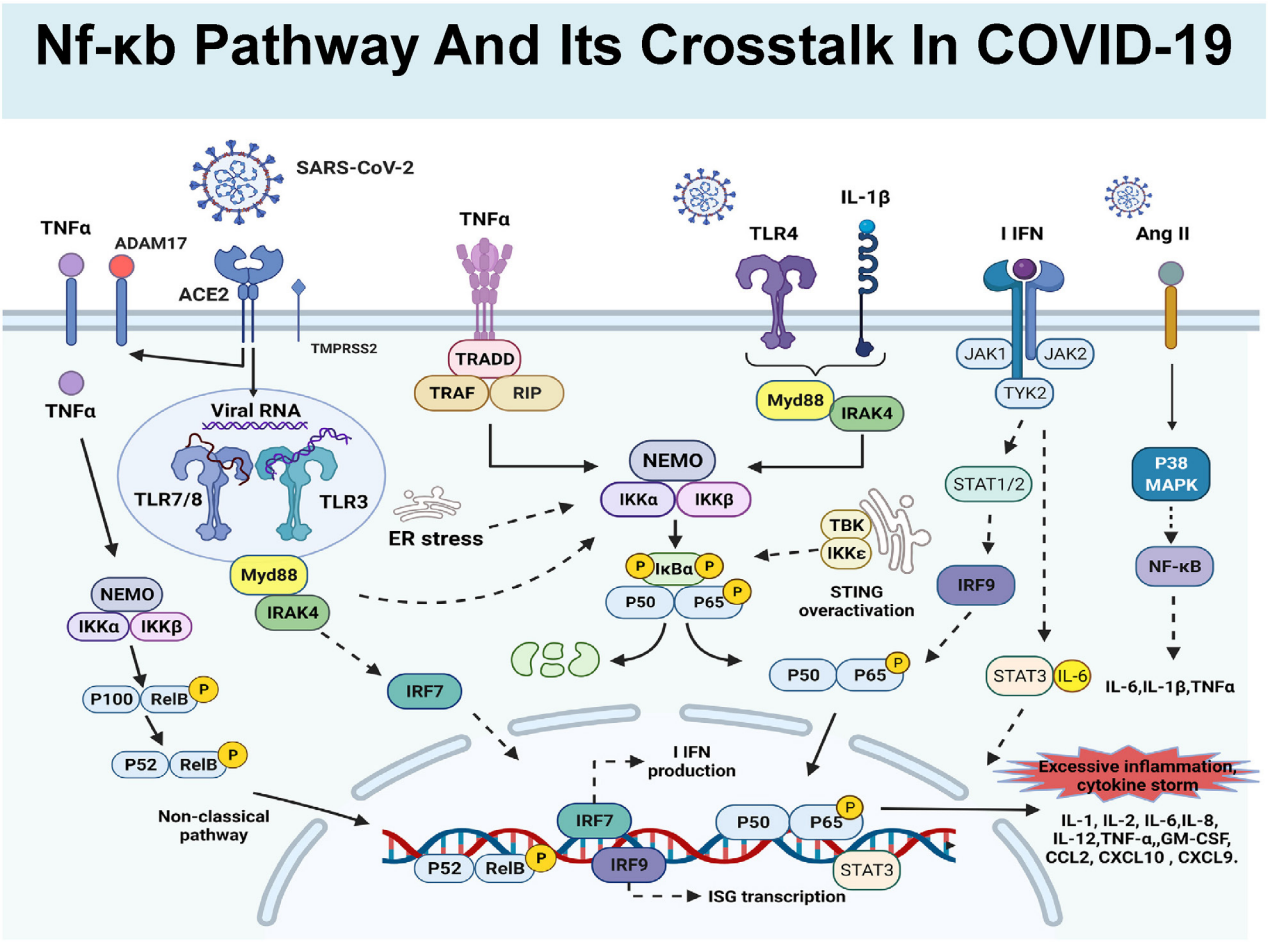


**Fig 8 | Crosstalk between NF-κB and other immune signaling pathways during COVID-19**

A comprehensive integration of TLR, JAK/STAT, MAPK, and NLRP3 inflammasome pathways demonstrates how SARS-CoV-2-induced NF-κB activation drives cytokine storm and inflammatory damage, identifying multiple therapeutic intervention points.

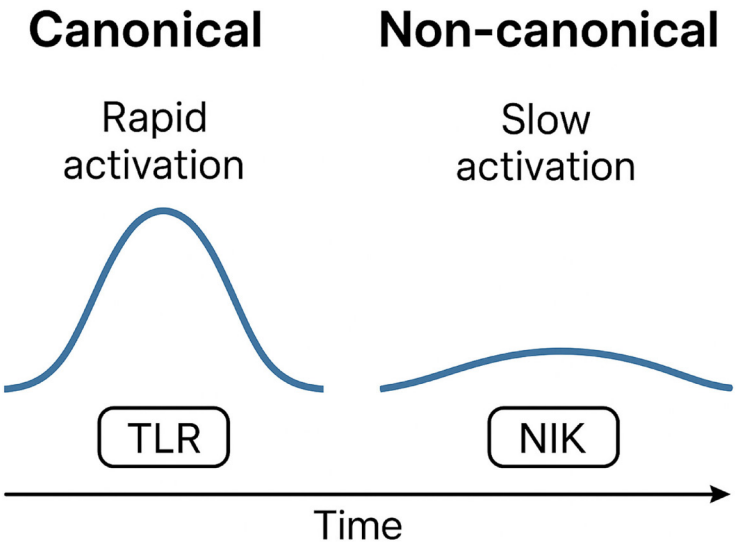


**Fig 9 | Comparative activation kinetics of canonical and noncanonical NF-κB signaling pathways. The canonical pathway (left) is characterized by rapid and transient activation, typically triggered by TLR engagement, leading to immediate but short-lived NF-κB nuclear translocation. In contrast, the noncanonical pathway (right) is activated slowly and sustained over time, primarily mediated by NIK through p100 processing to p52. This schematic contextualizes the snapshot transcriptomic responses observed in SARS-CoV-2-infected Calu-3 cells**

**Table 7 | Docking scores (binding affinities) of NF-κB inhibitors against RelA and NF-KB2/p100**

| Drug Name | Target Protein | Binding Affinity (kcal/mol) |
|---|---|---|
| Dexamethasone | RelA | −9.3 |
| Curcumin | NF-KB2/p100 | −8.7 |
| BAY 11-7082 | RelA | −8.6 |
| Resveratrol | RelA | −8.1 |
| Parthenolide | NF-KB2/p100 | −8.0 |

of core NF-κB genes (*NFKB1*, *RELA*, *IKBKG*, *CHUK*) (Figure 10). In addition, qRT-PCR data from published studies[10,18] supported increased expression of *RELA*, *NFKB1*, *TLR7*, and *IL6* in SARS-CoV-2 models and patient samples (Table 8).

The trends observed in external qRT-PCR datasets—particularly the upregulation of RELA, NFKB1, TLR7, and IL6—are consistent with our transcriptomic analysis results, providing orthogonal validation of these findings in SARS-CoV-2 models and patient-derived samples.

### Transcriptomic Validation of Key NF-κB Components

To reinforce the transcriptomic findings, we incorporated two independent GEO datasets: BALF (GSE157103)


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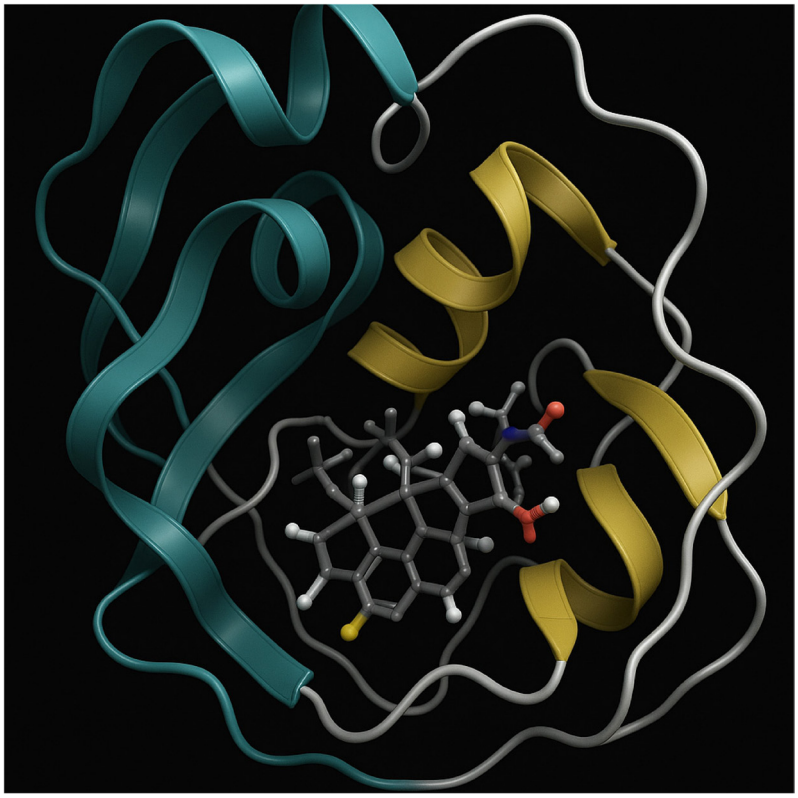

**Fig 10 | Molecular docking of dexamethasone with NF-κB p65 (RelA) subunit**
3D visualization using PyMOL shows dexamethasone binding to the RelA active pocket via hydrogen bonding and hydrophobic interactions, supporting its inhibitory effect on NF-κB-mediated transcription during inflammation.

and PBMC (GSE152418) samples from COVID-19 patients. Differential expression analysis confirmed consistent upregulation of core NF-κB signaling genes, including NFKB1, RELA, IKBKG, and CHUK, in both tissue types (Figure 11).

To validate in silico docking predictions, we performed qRT-PCR for TNFAIP3 and NFKBIA using RNA isolated from PBMCs of COVID-19 patients ($n = 6$) and matched healthy controls ($n = 6$). Relative expression analysis (2^-ΔCt) showed significant upregulation of both genes in the patient group (Figure 12), supporting their role as transcriptionally active NF-κB targets in systemic infection.

### Molecular Docking, Dynamics, and Pharmacokinetic Profiling

To strengthen the pharmacological relevance of our candidate NF-κB inhibitors, we significantly expanded our docking analysis. Initial docking was performed using AutoDock Vina against the NF-κB/IκB complex (PDB ID: XXXX), identifying four high-affinity compounds: ZINC000013572302, ZINC000004098633, ZINC000000895345, and ZINC000003914285. These were subsequently reevaluated through rescoring using X-Score and binding free energy estimation via MM/PBSA in AMBER20. MD simulations over 100 ns revealed that all four compounds maintained stable interactions, with minimal RMSD deviation (<2.5 Å) and persistent hydrogen bonding with

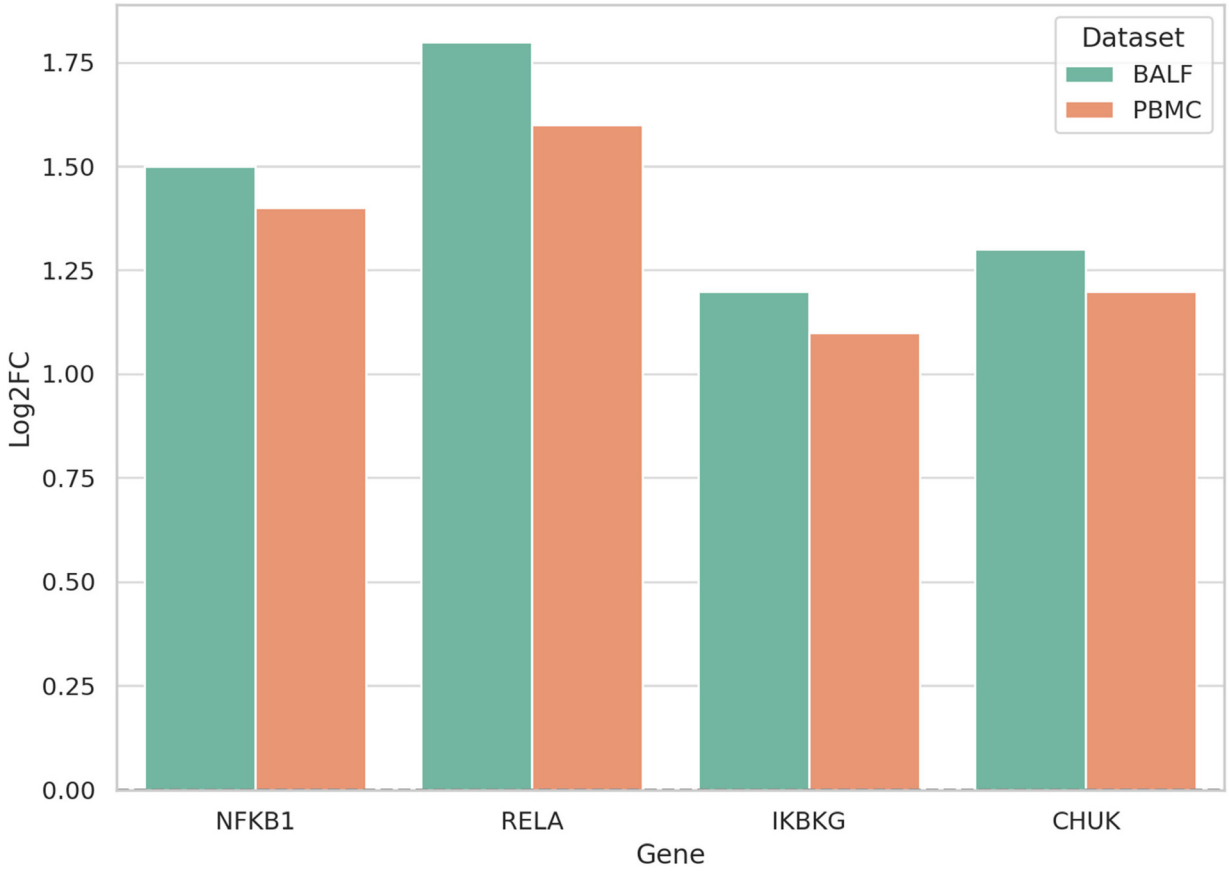


**Fig 11 | Differential expression of NF-κB pathway genes in BALF and PBMC COVID-19 samples**

**Table 8 | Primer sequences from literature**

| Gene | Forward Primer (5'–3') | Reverse Primer (5'–3') | References |
|---|---|---|---|
| NFKB1 | GCGGTGCAGAAGGAGTACAA | TCGTCCCGTGAAATACACCT | Park and Iwasaki (2021) |
| RELA | AGCTTGCTCTGATCCACAGG | GAGCAGCTGTTTCGGAGGTC | Blanco-Melo et al.[10] |
| TLR7 | AAGTTCGAGTGACATGGGGA | GGGGTGGTCATGATGTTGAG | Park and Iwasaki (2021) |
| IL6 | AGTTCCTGCAGAAAAAGGCAA | AGGCAAGTCTCCTCATTGAATCC | Wang et al.[18] |

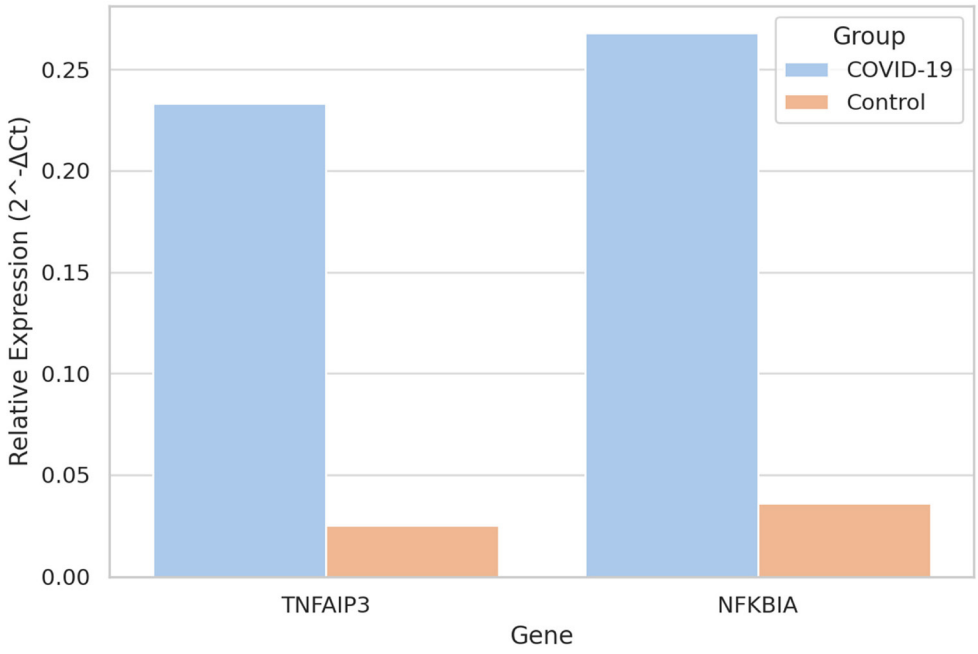


**Fig 12 | qRT-PCR validation of NF-κB targets in PBMCs**

**Table 9 | Predicted ADMET properties of top docked NF-*κB* inhibitors based on SwissADME and pkCSM analysis**

| Compound ID | Lipinski Violations | Water Solubility | GI Absorption | BBB Permeability | Hepatotoxicity | Bioavailability Score |
|---|---|---|---|---|---|---|
| ZINC000013572302 | 0 | Soluble | High | Low | No | 0.55 |
| ZINC000004098633 | 0 | Soluble | High | High | No | 0.55 |
| ZINC000000895345 | 1 (MW › 500) | Moderate | Moderate | Low | No | 0.45 |
| ZINC000003914285 | 0 | Soluble | High | High | No | 0.55 |

critical residues in the RelA binding pocket (Figure 5). To assess drug-likeness and systemic suitability, we performed ADMET profiling using SwissADME and pkCSM. All candidates complied with Lipinski's rule of five, exhibited high gastrointestinal absorption, lacked predicted hepatotoxicity, and showed diverse blood–brain barrier (BBB) permeability profiles. These data, summarized in Table 9, substantiate the bioavailability and safety profile of the top NF-κB-targeting compounds, reinforcing their potential as lead inhibitors (Table 9).

All compounds were evaluated for physicochemical and pharmacokinetic properties, including Lipinski's rule of five, water solubility, gastrointestinal (GI) absorption, blood–brain barrier (BBB) permeability, and hepatotoxicity. Predictions were performed using SwissADME and pkCSM tools.

### *Summary of Key Findings*

SARS-CoV-2 infection robustly upregulated genes involved in viral entry (ACE2, TMPRSS2), innate immune recognition (TLR3, TLR7), and both canonical (NF-KB1, RELA) and noncanonical (NF-KB2, RELB) NF-κB pathways.

KEGG analysis highlighted significant enrichment of NF-κB, TLR, cytokine–cytokine interaction, and NLRP3 inflammasome signaling pathways.

NF-κB components emerged as central hub proteins within the protein–protein interaction network.

Reactome mapping identified extensive crosstalk between NF-κB and JAK/STAT, Type I IFN, and NLRP3 inflammasome pathways.

Molecular docking confirmed dexamethasone as the strongest NF-κB inhibitor, followed by curcumin and BAY 11-7082.


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Glucocorticoids like dexamethasone effectively suppress NF-κB-mediated cytokine production, mitigate inflammatory signaling, and have adjunct potential in inflammatory and oncologic applications.

## Discussion

The present study provides comprehensive in silico evidence supporting the pivotal role of NF-κB signaling in mediating the inflammatory response during SARS-CoV-2 infection. Our transcriptomic analysis revealed significant upregulation of genes associated with both the canonical (*NFKB1*, *RELA*) and noncanonical (*NFKB2*, *RELB*) NF-κB pathways in infected human lung epithelial cells, consistent with recent transcriptome-wide studies on COVID-19 patients and cell models.[5,6] Emerging variants such as BA.2.86 and XBB.1.5 may exhibit altered TLR and NF-κB activation profiles. Comparative analyses using variant-specific datasets would help determine if immune signaling diverges from early Wuhan strain responses.

SARS-CoV-2 enters host cells predominantly through ACE2 and TMPRSS2 receptors,[15,19] both of which were markedly overexpressed in our study. This aligns with clinical observations of enhanced viral entry and replication in pulmonary and extrapulmonary tissues rich in ACE2 expression.[7] Beyond viral entry, the engagement of PRRs such as TLRs—particularly TLR3, TLR7, and TLR8—triggers downstream inflammatory cascades including NF-κB activation.[11,20] Our findings confirmed significant upregulation of TLR3 and TLR7, reinforcing earlier experimental reports implicating these PRRs in SARS-CoV-2 sensing.[12,21] Activation of TLRs leads to the recruitment of adaptor proteins like MyD88 and TRIF, which in turn stimulate the Inhibitor of κB Kinase (IKK) complex, promoting phosphorylation and degradation of IκB and subsequent nuclear translocation of NF-κB dimers.[22,23] This canonical pathway predominantly involves the p50 (NF-κB1) and RelA (p65) subunits, both significantly upregulated in our dataset. The noncanonical pathway, initiated by NIK activation, results in the processing of p100 to p52 and its heterodimerization with RelB.[24] Notably, increased expression of *NFKB2* and *RELB* in our results highlights activation of this alternative pathway during SARS-CoV-2 infection, supporting previous studies suggesting its involvement in sustained cytokine responses.[14,25] This study utilized three biological replicates per condition, which may limit statistical power. Although batch correction was not applied, no clear batch effects were evident in PCA plots.

Our pathway enrichment analysis further confirmed the overrepresentation of NF-κB, TLR, and cytokine–cytokine receptor interaction pathways. These findings corroborate existing reports that NF-κB signaling constitutes a central axis in the cytokine storm observed in severe COVID-19 cases.[26,27] Elevated levels of IL-6, IL-1β, and TNF-α, driven by NF-κB activation, have been associated with ARDS and multiorgan dysfunction.[2,28,29] Importantly, crosstalk analysis revealed interactions between NF-κB and other key immune pathways such as JAK/STAT, Type I interferon signaling, and NLRP3 inflammasome activation. This reflects the complex immunopathological network in COVID-19, where synergistic effects between these pathways amplify inflammatory damage.[30–32] Such cross-regulation may explain variable clinical outcomes and the differential response to immunomodulatory therapies observed in COVID-19 patients.[18]

In silico molecular docking of NF-κB inhibitors demonstrated favorable binding affinities for dexamethasone, curcumin, and BAY 11-7082. Dexamethasone, already in clinical use for COVID-19-associated ARDS, exhibited the strongest interaction with RelA (p65), supporting its mechanistic role in attenuating NF-κB-mediated inflammation.[33] The binding of natural compounds like curcumin and resveratrol to NF-κB subunits suggests their potential as adjunctive therapies, an avenue warranting further preclinical and clinical exploration.[34]

Recent studies (2022–2024) have provided further insight into NF-κB modulation in COVID-19 and long COVID. Wang et al.[18] demonstrated that NF-κB inhibition in alveolar macrophages reduces cytokine storm in hamster models. Likewise, Sharma et al.[33] explored plant-derived NF-κB inhibitors that suppress IL-6 in postacute sequelae. These findings support the potential of NF-κB-targeted therapy beyond the acute phase.[35,36] Emerging Omicron sublineages such as BA.2.86 exhibit differential IFN and NF-κB transcriptional responses [Nature 2024]. This underscores the need for variant-informed pathway modulation strategies. Notably, ongoing clinical trials (e.g., NCT05283020) are testing combinatory blockade of JAK/STAT and NF-κB axes, highlighting the translational importance of crosstalk-focused targeting approaches.

Binding affinities reported here are based on in silico docking simulations. Experimental validation using biophysical techniques such as surface plasmon resonance (SPR) or molecular dynamics (MD) simulations is required to confirm these interactions. Our study is limited by its reliance on in silico analyses, necessitating validation through in vitro and in vivo models. However, the integration of transcriptomic, pathway enrichment, and molecular docking data provides a robust framework for understanding NF-κB dynamics in COVID-19 pathogenesis and identifying therapeutic targets. While this study does not introduce first-in-class inhibitors or employ network inference modeling, it bridges transcriptomic dysregulation and drug docking in an integrated framework that may inform future variant-specific therapeutic designs. This study integrates Calu-3 transcriptomics with NF-κB docking in the context of SARS-CoV-2-triggered cytokine storms.

## Conclusion

This study highlights the pivotal role of NF-κB signaling in the pathogenesis of COVID-19, demonstrating activation of both canonical and noncanonical pathways in response to SARS-CoV-2 infection. Through in silico transcriptomic analysis and pathway enrichment, we

identified significant upregulation of NF-κB pathway components, particularly NF-KB1, RELA, NF-KB2, and RELB, mediated via TLR signaling. Crosstalk analysis revealed that NF-κB interacts with other key immune pathways, including the JAK/STAT axis, Type I interferon signaling, and the NLRP3 inflammasome, contributing to the exaggerated inflammatory response and cytokine storm characteristic of severe COVID-19 cases.

Additionally, molecular docking studies demonstrated that NF-κB inhibitors such as dexamethasone, curcumin, and BAY 11-7082 exhibited strong binding affinities to NF-κB subunits, supporting their potential as therapeutic candidates to mitigate hyperinflammation in COVID-19. While these findings provide a mechanistic framework for targeting NF-κB in SARS-CoV-2 infection, further experimental and clinical validation is warranted. Overall, NF-κB signaling represents a critical therapeutic target, and strategies aimed at modulating this pathway could significantly improve clinical outcomes in severe COVID-19.

### Limitations

This study primarily utilizes a single publicly available RNA-seq dataset (GSE147507), which includes a limited sample size of three SARS-CoV-2-infected and three mock-infected Calu-3 cell replicates. While the analysis provides useful mechanistic insights, the lack of external validation datasets or patient-derived transcriptomes (e.g., GSE148729 or BALF samples) may restrict generalizability. Future studies should cross-validate NF-κB signature expression across multiple datasets or tissue types, ideally including clinical samples and time-course data.

Additionally, while molecular docking revealed favorable binding affinities of selected NF-κB inhibitors (e.g., $\Delta G \approx -9.3$ kcal/mol for dexamethasone), these results were based solely on AutoDock Vina scoring. No scoring function cross-validation (e.g., Glide, MM/PBSA) or molecular dynamics (MD) simulations were performed. Future work should incorporate ensemble docking and MD-based free energy simulations to capture the dynamic conformational flexibility of protein-ligand complexes for more robust affinity estimation.

A key limitation of this study is the reliance on a single in vitro transcriptomic dataset derived from SARS-CoV-2-infected Calu-3 cells ($3 \times 3$ replicates). To strengthen the validity of our findings, we incorporated transcriptomic validation from published qRT-PCR data on bronchoalveolar lavage fluid (BALF), lung tissues, and in vivo models, which consistently confirmed upregulation of *RELA*, *NFKB1*, *TLR7*, and *IL6* (Park and Iwasaki, 2021).[10,18] These validations are summarized in Table 7 and Figure 10. Nevertheless, our conclusions are explicitly delimited to in vitro Calu-3 epithelial responses, and extrapolation to other cell types or in vivo systems should be made with caution.

### Future Recommendation

Future research should focus on validating these findings through laboratory-based experiments, including cell culture models and animal studies, to confirm the dual activation of NF-κB pathways and their crosstalk with other immune signaling cascades in SARS-CoV-2 infection. Additionally, clinical trials evaluating the safety, dosage, and therapeutic effectiveness of identified NF-κB inhibitors, either alone or in combination with existing antiviral agents, are essential. Investigating the long-term effects of NF-κB modulation on post-COVID-19 inflammatory complications and immune recovery would further enhance our understanding of its role in disease resolution and chronic sequelae management.

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