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Research Article

The Immunopathogenesis of COVID-19 and *Streptococcus pneumoniae* Co-infection: A Focus on PMN-Driven Pro-inflammatory Cytokine Dynamics

Muslim Idan Mohsin [1*]

[1] *University of Kufa, Faculty of science, Pathological laboratory analysis Department, Iraq*
[*] *Corresponding author: muslim.aljabri@uokufa.edu.iq*

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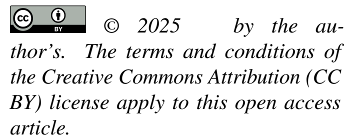

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Abstract

This study explored the immunopathological interplay between COVID-19 and *Streptococcus pneumoniae* co-infection by examining pro-inflammatory gene and protein expression in polymorphonuclear cells (PMNs). PMNs were isolated from healthy controls, COVID-19-positive patients, and COVID-19 patients with *S. pneumoniae* co-infection. Quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) were used to quantify mRNA and protein levels of TNF-α, IFN-γ, and IL-6. Results revealed significant upregulation of TNF-α and IFN-γ mRNA and protein in both COVID-19 and co-infected groups ($p<0.0001$), indicating a robust pro-inflammatory response. Notably, IL-6 mRNA was significantly elevated only in the COVID-19 group, suggesting a distinct role in viral, but not co-infection-associated, inflammation. These findings demonstrate that *S. pneumoniae* co-infection significantly modulates pro-inflammatory responses in PMNs during COVID-19, with a specific focus on TNF-α and IFN-γ, highlighting the potential for exacerbated inflammation in co-infected individuals. This study underscores the importance of considering bacterial co-infections in COVID-19 management and warrants further investigation into targeted therapeutic interventions.

1. Introduction

S. pneumoniae exhibits a broad clinical spectrum, ranging from common, self-limiting infections like pharyngitis and scarlet fever to life-threatening invasive diseases, including necrotizing fasciitis and toxic shock syndrome, both associated with substantial mortality [1]. The rapid progression of these invasive infections can result in fatal outcomes within hours of symptom onset, highlighting the urgency of understanding the underlying mechanisms [1–3].

The observed increase in iGAS morbidity has been attributed, in part, to heightened population susceptibility following COVID-19 pandemic restrictions. Furthermore, the potential synergistic effects of co-infections with respiratory viruses, such as influenza and respiratory syncytial virus (RSV), which compromise respiratory epithelial integrity, are hypothesized to facilitate *S. pneumoniae* colonization and invasion. Notably, current evidence suggests that this surge is not driven by the emergence of novel *S. pneumoniae* strains or a global increase in antibiotic resistance [4, 5]. Genomic analysis conducted by London researchers identified the emm1 strain, particularly the M1UK lineage, as the predominant cause of pediatric iGAS infections in England during the 2022-2023 season, with this lineage demonstrating sustained dominance since 2020 [6].

Effective resolution of extracellular bacterial infections, including those caused by *S. pneumoniae*, relies on a robust innate immune response characterized by neutrophil and macrophage-mediated phagocytosis and intracellular killing [7]. This process is facilitated by

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opsonins, such as natural antibodies and acute-phase proteins (e.g., complement and C-reactive protein), which are rapidly induced upon infection [8]. These innate immune components are, in turn, regulated by a network of proinflammatory cytokines, including interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α), IL-12, and interferon-gamma (IFN-γ) [9, 10]. Subsequent adaptive immunity is primarily mediated through the production of IgG antibodies specific to bacterial protein and polysaccharide antigens [11].

While the critical role of proinflammatory cytokines in initiating innate defense against extracellular bacteria is well-established, the precise cytokine profiles governing the adaptive humoral response remain less defined. Furthermore, the potential interplay between early innate cytokine release and the subsequent development of adaptive humoral immunity warrants further investigation. The recruitment, maturation, and migration of antigen-presenting cells (APCs) in response to proinflammatory mediators suggest a potential mechanistic link [11, 12]. Additionally, the potential involvement of anti-inflammatory cytokines, such as IL-4 and IL-10, in modulating the immune response to extracellular bacteria cannot be overlooked [13].

This study aims to investigate the role of key proinflammatory cytokines, specifically IL-1, IL-6, TNF-α, IL-12, and IFN-γ, in patients who experienced concurrent COVID-19 and *S. pneumoniae* infections. By quantifying these cytokines, we seek to elucidate the mechanisms underlying the increased susceptibility to *S. pneumoniae* infections in the post-COVID-19 era and to gain insights into the interplay between viral and bacterial pathogenesis. This research will contribute to a deeper understanding of the complex immune responses associated with co-infections and inform strategies for mitigating the impact of future outbreaks.

2. Methodolgy

2.1. Collection of the Specimens and Bacteriological Processing

A prospective study was conducted between November 14, 2023, and February 15, 2024, to investigate bacterial pathogens associated with lower respiratory tract infections (LRTIs). A total of 220 clinical specimens were collected from patients presenting with clinical signs and symptoms of LRTI. Of these, 30 were confirmed as lower respiratory samples, encompassing diverse age groups and both genders. Specimens were inoculated onto nutrient agar and blood agar plates and incubated aerobically at 37°C for 24-48 hours. Bacterial identification was performed using standard biochemical tests, as described by [10].

2.2. The Identification of Bacterial Isolates

Following the isolation of single colonies from primary positive cultures, a multi-faceted approach was employed for bacterial identification. Initial characterization involved detailed assessment of colony morphology, including shape, size, color, margin, and texture, followed by microscopic examination after Gram staining. A suite of biochemical tests was then conducted to further delineate the isolates. Final identification was confirmed using the VITEK 2 Compact automated system with Gram-positive identification (ID) cards. This system utilizes a sophisticated algorithm that compares the observed biochemical reaction profiles to a comprehensive database, enabling accurate species-level identification [14].

2.3. Patient Cohort and Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from three distinct cohorts: healthy controls, patients with active disease, and patients in clinical remission. Ethical approval was obtained from MOH of ALNajaf hosptils committee name and approval number 349T at 1st Nov 2023, and informed consent was acquired from all participants.

2.4. RNA Extraction

Total RNA was extracted from PBMCs using the Solarbio Life Science RNA extraction kit (Solarbio, Beijing, China), according to the manufacturer's instructions. Briefly, cells were pelleted by centrifugation (200 x g, 5 minutes), lysed, and RNA was purified through sequential washes with RPE buffer followed by two washes with WT buffer. The concentration and purity of the extracted RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was further evaluated using [Specify method, e.g., Agilent Bioanalyzer 2100 with RIN score. Purified RNA was eluted in RNase-free water and stored at -80°C until further use.

2.5. cDNA Synthesis

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the [Specify cDNA synthesis kit Solarbio Life Science according to the manufacturer's protocol. The cDNA was diluted 1:5 with nuclease-free water and stored at -20°C.

2.6. Quantitative Real-Time PCR qPCR

qPCR was performed using the Precision 2x qPCR SYBR Green Master Mix (Solarbio Life Science China) on an Applied Biosystems 7900HT Fast Real-Time PCR System (GeneLine company from China). Pre-designed, validated primer sets (Macrogen, Seoul, South Korea) were used to quantify the expression of TNF-α, IFN-γ, IL-6, and GAPDH. The primer sequences were as follows: TNF-α:
Forward: 5'-GGACCGTATGTCTCCAGTCAC-3',
Reverse: 5'-ATGAGGTACAGGCCCTCTGAT-3'
IFN-γ: Forward: 5'-GAGTGTGGAGACCATCAAGGA-3',
Reverse: 5'-TGGACATTCAAGTCAGTTACCGAA-3'
IL-6: Forward: 5'-TCCGTAGTTTCCTTCTAGCTTCTT-3',
Reverse: 5'-CCTCAGACATCTCCAGTCCTCT-3'

GAPDH: Forward: 5'-TGCACCACCAACTGCTTAGC-3',
Reverse: 5'-GCATGGACTGTGGTCATGAG-3'.
Each reaction mixture (20 μL) contained 10 μL of 2x SYBR Green Master Mix, 1 μL of each primer (10 μM), 2 μL of cDNA, and 6 μL of nuclease-free water. The thermal cycling conditions were: 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. A melting curve analysis was performed to confirm the specificity of the amplification. All samples were run in triplicate.

2.7. Data Analysis

The relative expression levels of TNF-α, IFN-γ, and IL-6 were normalized to the expression of GAPDH, which served as an endogenous control. The cycle threshold (Ct) values were determined using the Applied Biosystems 7900HT software. The $\Delta\Delta$Ct method was used to calculate the relative fold change in gene expression between the experimental groups.

2.8. Measurement of Cytokine Levels

Serum levels of IL-6, TNF-α, and IFN-γ were quantified using commercially available ELISA kits (SolarBio company). Briefly, serum samples were centrifuged to remove particulates. Standards were reconstituted to a stock concentration of 1000, for TNF-α and IFN-γ but Standard was reconstituted to a stock concentration 250 pg/mL for IL-6, and serial dilutions were prepared to generate standard curves. 100 μL of standards and serum samples were added to pre-coated microplate wells and incubated for 90 minutes at 37°C. Following three washes with wash buffer, 100 μL of biotin-conjugated anti-human TNF-α, IFN-γ, or IL-6 antibody working solution was added and incubated for 30 minutes at 37°C. After another three washes, 100 μL of HRP-avidin working solution was added and incubated for 30 minutes at 37°C. The plate was washed again three times, and 100 μL of TMB substrate solution was added. The reaction was allowed to develop for 15-20 minutes at 37°C, protected from light, and stopped by the addition of 50 μL of stop solution. The optical density was measured at 450 nm using a microplate reader within 30 minutes. Cytokine concentrations in the samples were determined by interpolation from the standard curves.

2.9. Statistical Analyses

GraphPadd prism. 9 was used to make all graphs and analyse the data statistically. The significant differences between samples were determined using one and two-way ANOVA for multiple comparisons. Results are shown as mean $\pm$ SEM.

3. Results and Discussion

This study investigated the bacterial etiology of lower respiratory tract infections (LRTIs) in patients with and without concurrent COVID-19 infection, utilizing 220 sputum and blood specimens collected over a four-month period (November 14, 2023, to February 15, 2024) from hospitals within Al-Najaf city. Specimens were processed using standard microbiological techniques, including serial dilution with normal saline, inoculation onto a variety of selective and differential growth media, and phenotypic characterization. Presumptive Streptococcus pneumoniae isolates were identified through morphological examination, biochemical testing, and growth characteristics Table 1. Definitive identification was achieved using the Vitek 2 compact system.

The analysis revealed a diverse bacterial profile within the LRTI samples. *Streptococcus pneumoniae* and *Klebsiella pneumoniae* emerged as the most prevalent pathogens, each accounting for 20.27% (n=44) of the total samples. A substantial proportion of samples (35.98%, n=79) yielded "other pathogens," indicative of a polymicrobial or less frequently encountered bacterial community. Notably, 25.25% (n=55) of the samples exhibited no bacterial growth, suggesting potential non-bacterial etiologies, prior antibiotic administration, or other factors influencing bacterial viability. Lower prevalence pathogens included *Pseudomonas aeruginosa* (9.64%, n=21), *Enterobacter* spp. (6.8%, n=15), and *Staphylococcus aureus* (2.06%, n=5). These findings suggest that *Streptococcus pneumoniae* represents a significant bacterial pathogen associated with LRTIs, potentially as a secondary infection in the context of COVID-19. However, the high prevalence of "other pathogens" and the substantial percentage of samples with no bacterial growth underscore the complexity of LRTI etiology and the need for further investigations to fully characterize the microbial landscape in this patient population.

Table 1: Bacterial Pathogen Distribution in Lower Respiratory Tract Infection Samples (n=220).

Bacterial Pathogen	Percentage (%)	Number of Samples (n)
Streptococcus pneumoniae	20.27	44
Klebsiella pneumoniae	20.27	44
Pseudomonas aeruginosa	9.64	21
Enterobacter spp.	6.8	15
Staphylococcus aureus	02.06	5
Other Pathogens	35.98	79
No Bacterial Growth	25.25	55

Our findings, which identified *Streptococcus pneumoniae* and *Klebsiella pneumoniae* as prominent isolates in lower respiratory tract infections, are consistent with the existing body of literature. This concordance with previous reports strengthens the evidence base supporting their clinical relevance. However, further research is warranted to explore potential regional variations in pathogen prevalence, as well as the specific virulence factors contributing to their pathogenicity in this context [15–18]. However, [19, 20] have found that *Klebsiella pneumoniae* was recorded in 15% and 3% respectively [19, 20]. These different results could be because the condition of collection and source of infection.

Dissecting the Modulation of Pro-inflammatory Gene Expression in Polymorphonuclear Cells during COVID-19 and Streptococcus pneumoniae Co-infection: A Combined qPCR and ELISA Approach.

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The interplay between viral and bacterial infections in the respiratory tract presents a complex immunopathological landscape. This study aimed to elucidate the dynamic changes in pro-inflammatory gene and protein expression within polymorphonuclear cells (PMNs) during COVID-19 infection, specifically focusing on the impact of co-infection with *Streptococcus pneumoniae*, a prevalent pathogen in lower respiratory tract infections. We hypothesized that *S. pneumoniae* co-infection would significantly alter the expression profile of key pro-inflammatory mediators, potentially exacerbating the inflammatory response observed in COVID-19. In berif, Peripheral blood PMNs were isolated from patients stratified into three groups: (1) healthy controls, (2) COVID-19 positive, and (3) COVID-19 positive with confirmed *S. pneumoniae* co-infection. Total RNA was extracted, and cDNA was synthesized using standard protocols. Quantitative real-time PCR (qPCR) was employed to quantify mRNA levels of TNF-α, IFN-γ, and IL-6, with GAPDH serving as an internal control. Primer specificity was validated through melting curve analysis Figure 1. Relative gene expression was calculated using the 2-ΔΔCt method. Protein levels of TNF-α were subsequently measured using enzyme-linked immunosorbent assay (ELISA) Figure 2. Statistical significance was determined using one-way ANOVA, with $p < 0.0001$ considered highly significant. All experiments were conducted in triplicate (qPCR) or with 145 samples per group (ELISA). The results showd that TNF-α and IFN-γ: Significant upregulation of both TNF-α and IFN-γ mRNA was observed in PMNs from COVID-19 patients and those with COVID-19 and *S. pneumoniae* co-infection compared to healthy controls (****p<0.0001). This suggests a heightened pro-inflammatory state driven by these cytokines in both viral and bacterial contexts. However, IL-6: While IL-6 mRNA was detectable in all groups, a significant increase was observed exclusively in the COVID-19 group (****p<0.0001). This indicates a specific role for IL-6 in the inflammatory response to COVID-19, which is not further augmented by *S. pneumoniae* co-infection in this set of data. Internstnly, the INF-gama at serum level Consistent with the qPCR findings, a significant increase in TNF-α protein levels was observed in both COVID-19 and COVID-19 with *S. pneumoniae* groups compared to controls (****p<0.0001). This confirms the translational relevance of the observed mRNA upregulation. This study meticulously examined the intricate interplay between COVID-19 and *Streptococcus pneumoniae* co-infection, focusing on the pro-inflammatory responses within polymorphonuclear cells (PMNs). The research revealed a striking upregulation of TNF-α and IFN-γ mRNA in PMNs from both COVID-19 patients and those with co-infection, indicating a robust pro-inflammatory state in both scenarios. This significant increase, observed at both the gene and protein levels for TNF-α, underscores its pivotal role in the host's response to both the viral and bacterial challenges. The consistent elevation of TNF-α protein, validated through ELISA, solidifies the translational relevance of the mRNA findings, confirming that the observed gene expression translates into a tangible protein response.

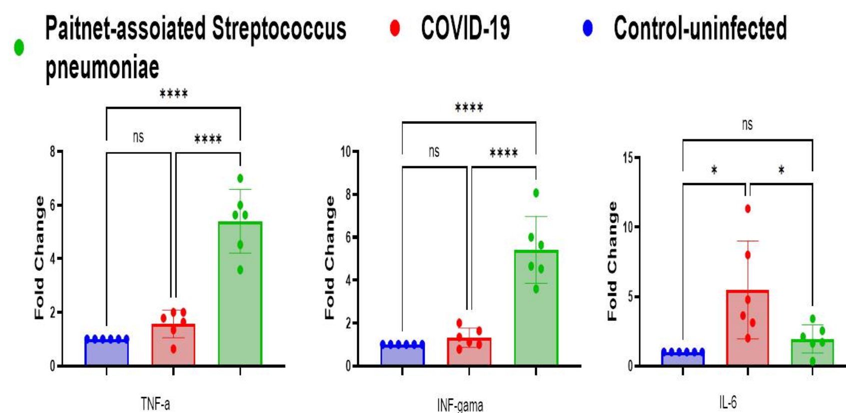

Figure 1: The TNF-a, INF gama and IL-6 genes expression have changed in response to infection

The TNF-a, INF gama and IL-6 expression. Has been measured by qPCR in patients have infection with COVID-19 only and with COVID-19 asscoiation *Klebsiella pneumoniae var pneumoniae*. Expression of these Pro-inflammatory in non-infected and infected Polymorphonuclear cells was calculated using the 2-ΔΔCt method following estimation of the house keeping gene **GAPDH. The TNF-a, INF gama and IL-6** showed changes in Polymorphonuclear cells. The significance of differences has been tested by one-way ANOVA, where **** p<0.0001 significant; ns=non-significant. The data are the means of 3 separate experiments with duplicate.

The bars showed changes TNF-α in serum. The significance of differences was tested by one-way ANOVA, where **** p<0.0001 significant; ns=non-significant. The data are the means of 44 samples of each panels.

Intriguingly, the study highlighted a distinct IL-6 response. While detectable across all groups, a significant surge in IL-6 mRNA was exclusively observed in COVID-19 patients without co-infection. This suggests a specific role for IL-6 in the inflammatory cascade triggered by COVID-19 alone, a response that was not further intensified by the presence of *S. pneumoniae.* This nuanced role of IL-6 warrants further exploration, as it implies a potentially different regulatory pathway compared to TNF-α and IFN-γ. The finding aligns with existing knowledge regarding the pleiotropic nature of IL-6 and its involvement in diverse inflammatory processes, particularly in viral infections. The significant increase in TNF-α and IFN-γ during co-infection points towards a potential exacerbation of the inflammatory response. This observation is consistent with previous research demonstrating the critical role of these cytokines in both bacterial clearance and immunopathology [1, 21]. The study also draws parallels to existing literature on TLR signaling, emphasizing the importance of these pathways in orchestrating immune responses to bacterial infections. *Streptococcus sp* appears to trigger autophagy, a cellular self-cleaning process, evidenced by fluctuating levels of autophagic markers alongside inflammatory cytokines IL-6, TNF-α, and IFN-γ. Initially, these cytokines rise during autophagy flux formation, then decrease during induction. Critically, blocking autophagy worsens inflammation and bacterial load, highlighting its protective function. This indicates that *S. pneumoniae* induces autophagy in cells, with IL-6, TNF-α, and IFN-γ playing key roles in both starting and controlling this process [22].

In conclusion, this study provides compelling evidence that *S. pneumoniae* co-infection significantly modulates pro-inflammatory gene

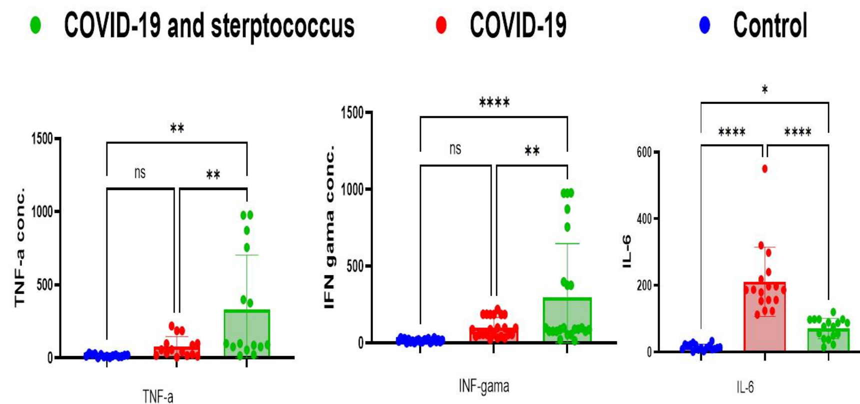

Figure 2: The TNF-a, INF gama and IL-6 protein levels in the patients with normal , COVID-19 and COVID-19 with bacterial infection.

and protein expression in PMNs during COVID-19. The marked upregulation of TNF-α and IFN-γ suggests a potential for heightened inflammatory responses in co-infected individuals. These findings emphasize the importance of considering bacterial co-infections in the clinical management of COVID-19. Future research should delve deeper into the functional consequences of these cytokine changes and explore potential therapeutic interventions targeting these pathways.

Article Information

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