Journal of Infection 89 (2024) 106314

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Infectious Disease Practice

Inflammatory cytokine responses in pediatric tuberculosis with or without SARS-CoV-2 seropositivity

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ARTICLE INFO

Article history:
Accepted 8 October 2024
Available online 18 October 2024

Keywords:
Pediatric TB
SARS-CoV2
COVID-19
Cytokines

SUMMARY

Objectives: To characterize the inflammatory cytokine profiles in children with TB in the presence and absence of SARS-CoV2 seropositivity.
Methods: This study evaluated cytokine responses in two groups of children with TB: CoV2+ (TB and SARS-CoV2 seropositive) and CoV2- (TB and SARS-CoV2 seronegative). Each group had 30 children, and cytokine levels were measured at baseline, months 3 and 6.
Results: At baseline, CoV2+ children exhibited significantly elevated levels of cytokines, including IFN-γ, IL-2, TNFα, IL-1α, and IL-6, and reduced levels of IL-1β and IL-18, compared to CoV2- children. No significant differences in cytokine levels between the groups were observed at months 3 and 6. Additionally, a general decline in cytokine levels was noted over the course of treatment in both groups. A positive correlation was found between most cytokines and SARS-CoV2 IgG spike protein levels at baseline and at month 3 in the CoV2+ group.
Conclusions: This study is one of the first studies to characterize the systemic inflammatory responses in SARS-CoV2 seropositive and seronegative children with TB from a TB endemic country. The findings enhance our understanding of the immunopathogenesis of TB and SARS-CoV2 seropositivity in children and may inform future therapeutic strategies

Introduction

From an epidemiological standpoint, tuberculosis (TB) continues to cause significant global mortality annually.[1,2] Recently, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) became one of the deadliest viral pathogens resulting in the coronavirus disease 19 (COVID-19) pandemic with huge mortality.[3,4] The emergence of COVID-19 not only exacerbated socioeconomic challenges but also led to substantial disruptions in public health systems worldwide, including the management of TB.[3,4] Both TB and COVID-19 have been reported to co-infect individuals.[5,6] However, the complex clinical and immunopathological interplay between these two diseases, as well as the factors influencing mortality in cases of co-infection, are not well understood.[5,6] A study by the Global Tuberculosis Network, which analyzed 49 patients co-infected with TB and COVID-19 across eight countries, suggests that TB may develop simultaneously or subsequent to COVID-19 infection, potentially leading to higher case-fatality rates.[7] Current research also indicates that COVID-19 can develop independently of TB, whether before, during, or after TB diagnosis.[6,7] The coexistence of active TB and COVID-19 is particularly concerning in regions with high TB prevalence. Both diseases primarily affect the respiratory system and are known to trigger severe inflammation within the lungs. COVID-19 may possibly accelerate TB progression due to the hyperinflammatory conditions caused by COVID-19.[5,8]

To tailor effective treatment strategies and risk mitigation in areas at high risk for this syndemic, it is crucial to characterize the shared immunopathogenic mechanisms.[9] Therefore, in this study we

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https://doi.org/10.1016/j.jinf.2024.106314

aim to examine and compare the inflammatory responses in seropositive and seronegative SARS-CoV-2 children with TB.

Materials and methods

Ethics statement

Informed consent was obtained from the parents or guardians of all participating children, with assent from the children where appropriate. The study received approval from the Internal Ethics Committee (IEC) of the ICMR-National Institute for Research in Tuberculosis and the collaborating institutions (Institute of Child Health, Chennai and Madurai Medical College, Madurai).

Study population and procedures

This study leveraged from ongoing TB-COVID study and included children aged 2 to 17 years, diagnosed with pulmonary TB (PTB) or extrapulmonary TB (EPTB); both confirmed and clinically diagnosed, from the Institute of Child Health, Chennai and Government Rajaji Hospital, Madurai. Children with CNS TB and disseminated TB or requiring anti-tuberculous therapy for more than 6 months were excluded. All children were SARS-CoV-2 RT-PCR negative at baseline, and we included children for whom samples for immunological assays were available at three time points (at baseline, 3 months and 6 months). Based on the anti-SARS-CoV-2 IgG status children were categorized into two groups: TB+SARS-CoV-2 antibody positive [CoV2+] (n = 30) and TB+SARS-CoV-2 antibody negative [CoV2-] (n = 30). Blood samples were collected at baseline (prior to anti-TB treatment), and at months 3 (mid treatment) and 6 months (end of treatment). The collected blood samples were transported to the ICMR-National Institute for Research in Tuberculosis (NIRT), Chennai and Madurai and processed within 4 h of collection. Samples were centrifuged, and the plasma was stored in −80 °C freezers until analysis.

Multiplex assay

Cytokine levels were measured using the Magpix multiplex cytokine assay system (Bio-Rad, Hercules, CA). Assays were conducted to quantify cytokines including Interferon-gamma (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), IL-4, IL-5, IL-13, IL-17A, IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-18, and Granulocyte-macrophage colony-stimulating factor (GM-CSF), using Enzyme-Linked Immuno Sorbent Assay (ELISA) kits from R&D Systems (Minneapolis, MN). Assay personnel were blinded to clinical data.

Antibody assays

Serological testing for antibodies targeting the viral Spike protein IgG (S) and membrane protein IgM was performed using YHLO iFlash 1800 Chemiluminescence Immunoassay Analyzer using iFlash-SARS-CoV-2 IgG (S). The cut-off value for SARS-CoV-2 IgG and IgM, according to the manufacturer, IgG and IgM concentrations more than or equal to 10.00 AU/mL was considered as positive.

Statistical analysis

Geometric means (GM) were employed to describe central tendencies. Differences between the CoV2+ and CoV2- groups were analyzed using the Mann-Whitney U test, with $P < 0.05$ considered statistically significant. Cytokine levels across different time points were compared using the Wilcoxon signed-rank test. Data analyses were performed using GraphPad PRISM Version 9.0 (GraphPad Software, CA, USA) and R Studio for principal component analysis (PCA) and heat maps visualization.

Table 1
Demographic data of study population.

S.No	Characteristics of study population	TB+SARS-CoV-2 antibody positive	TB+SARS-CoV-2 antibody negative
1	Age	10.5 (2–16)	6.5 (2–17)
2	Male	12 (30)	13 (30)
3	Female	18 (30)	17 (30)
4	BMI		
5	Weight (median)	25.5 (14.5–73.7)	21.6 (11.6–43.6)
6	Height	136 (104–170)	136 (96–157)
7	MUAC	17.5 (13.5–30)	18 (12.5–24)
8	TB Contact	9 (30%)	21(70%)
	Current TB status		
9	TB (Confirmed/ Unconfirmed)	10(34%) / 20 (66%)	12(40%) / 18 (60%)
10	Type	8 PULMONARY 2 EXTRA PULMONARY	12 PULMONARY
11	**Symptoms**		
12	Fever	12(40%)	14(46.6%)
13	Cough	16(53.3%)	20(66.6%)
14	Shortness of breath	4(13.3%)	8(26.6%)
15	Loss of weight	13(43.3%)	18(60%)
16	Lack of energy	7(23.3%)	13(43.3%)
17	Vomiting	5(16.6%)	11(36.6%)
18	Abdominal pain	6(20%)	7(23.3%)
19	Headache	2(6.66%)	4(13.3%)
20	Loss of appetite	7(23.3%)	11(36.6%)
21	Night sweats	1(3.33%0	2(6.66%)
	Investigations		
22	TST	23(76.6%)	24(80%)
23	HIV Status	0	0
24	CXR	12(40%)	17(56.6%)
25	CBNAAT	4(13.3%)	0
	COVID IgG SPIKE		
26	IgG(S) BL	91.32 (14.7–383.75) 30POSITIVE (100%)	0.65 (0.14–9.69)
27	IgG(S) M3	125.7 (12.59–481.52) 30POSITIVE (100%)	1.7 (0.22–77.36) 4POSITIVE (13.3%)
28	IgG(S) M6	33.84 (2.9–550.05) 20POSITIVE (66.6%)	0.53 (0.25–57.35) 6POSITIVE (20%)

Results

Demographics of study population

A total of 60 children were included in this study, with a median age of 8.5 years. Among them, 23(38%) were males. Of the total cohort, 22 children (36%) were microbiologically confirmed TB cases, comprising of 20 pulmonary TB and 2 extra-pulmonary TB. All children had received BCG vaccination at birth. Tuberculin skin test (TST) was positive in 47 (78%) children (Table 1).

Elevated cytokine levels in TB children with SARS-CoV2 seropositivity

To explore differences in cytokine responses between TB children with and without SARS-CoV2 seropositivity, we evaluated cytokine levels in plasma. As depicted in Fig. 1A, seropositive SARS-CoV-2 children (CoV2+) exhibited significantly heightened production of IFN-γ, IL-2, TNF-α, IL-6, IL-10, IL-1α, IL-1β, IL-18, and IL-5 at baseline compared seronegative SARS-CoV2 seropositivity. Similarly, elevated levels of IFN-γ, IL-2, TNF-α, IL-6, IL-10, IL-1α, IL-18, and IL-5 were observed in SARS-CoV2 seropositive children compared to seronegative children (CoV2-) at month 3. However, at month 6 (completion of anti-TB treatment), circulating levels of TNF-α, IL-10, and IL-1α alone were increased in SARS-CoV2 seropositive children compared to seronegative children. These findings indicate a hyperinflammatory immune response in children with TB and SARS-CoV2 seropositivity.

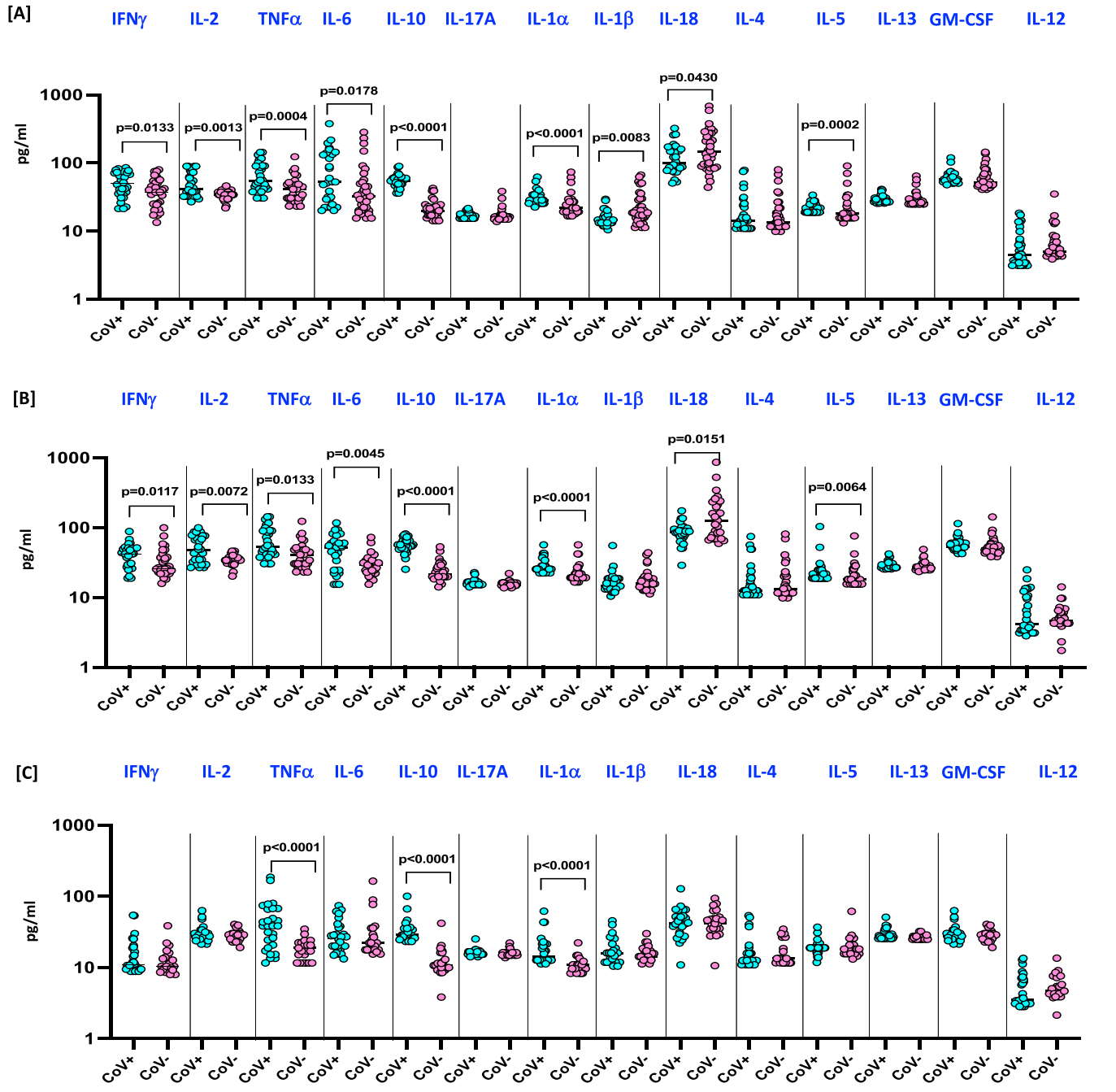

Fig. 1. CoV2+ Results in Elevated Plasma Levels of Pro- and Anti-inflammatory Cytokines. The plasma levels of pro- and anti-inflammatory cytokines were measured in CoV2+ (n = 30) and CoV2- (n = 30) children with TB disease at [A] Baseline, [B] Month 3, and [C] Month 6. Data are presented as scatter plots, with each circle representing a single individual. P values were calculated using the non-parametric Mann-Whitney U test, with Holms correction for multiple comparisons.

Decrease in plasma cytokine levels following anti-TB treatment

We investigated whether the elevated plasma levels of cytokines were directly associated with TB disease by measuring these cytokine levels in both groups at baseline (pre-treatment), month 3 (mid-treatment), and month 6 (after completion of anti-TB treatment). As depicted in Fig. 2, following completion of TB treatment, cytokine levels of IFN-γ, IL-2, IL-10, IL-1α, and GM-CSF significantly decreased in both groups, returning to pre-treatment levels. However, the levels of IL-5 and IL-6 decreased only in the CoV2+ children, while the levels of TNF-α, IL-1β, and IL-18 decreased in CoV2- children when compared to pre-treatment levels.

Plasma inflammatory markers as discriminators of TB children with SARS-CoV-2 seropositivity

We conducted a principal component analysis (PCA) using data on a subset of cytokines to compare and visually represent the grouping between TB children with and without SARS-CoV2 seropositivity. PCA enables the detection of variation and patterns within our dataset. Specifically, we performed PCA of IFN-γ, IL-2, TNF-α, IL-1α, IFN-β, IL-6, IL-18, and IL-10 to assess the discriminatory power of plasma cytokines in distinguishing both groups of children (refer to Fig. 3). Our PCA results clearly demonstrate the ability of these markers to differentiate between the study groups.

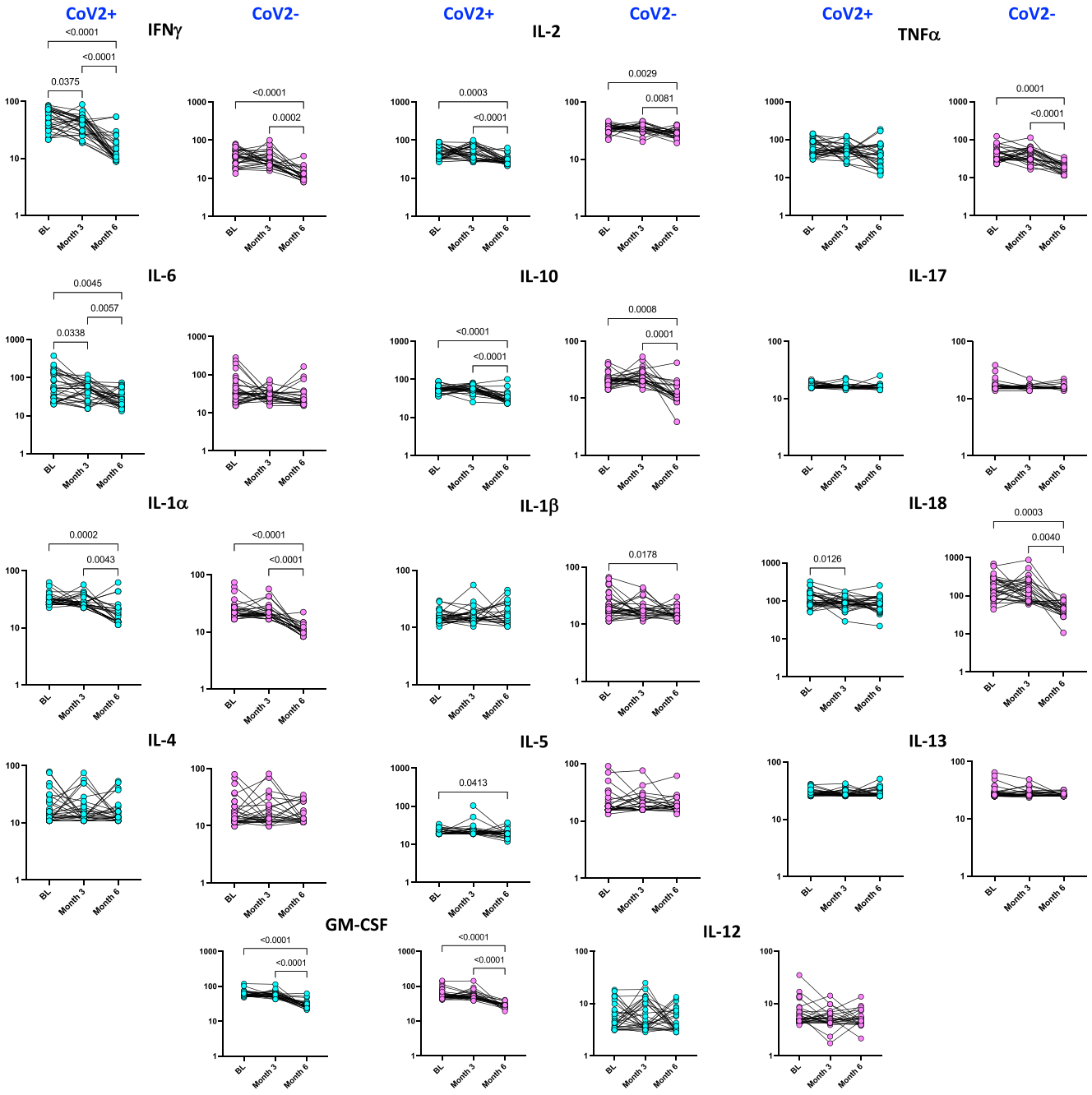

Fig. 2. CoV2+ and CoV2- Children Show Diminished Plasma Levels of Pro- and Anti-inflammatory Cytokines Following Treatment. The plasma levels of pro- and anti-inflammatory cytokines in CoV2+ and CoV2- children at [M0] (n = 30), [M3] (n = 30), and after [M6] (n = 30) are shown. Data are represented as line diagrams, with each line representing a single individual. For the analysis of M0 vs M3 vs M6, P values were calculated using the Wilcoxon matched-pair tests.

Canonical correlation analysis (CCA) as a tool for evaluating TB treatment response

CCA, a multivariate statistical model, facilitates the analysis of linear interrelationships between two sets of variables: one set serving as independent variables and the other as dependent variables. It forms composite scores for each set, developing a canonical function that maximizes the correlation between the two composite variables [40]. As illustrated in Fig. 4, the utilization of CCL1 comprising IFN-γ, IL-2, TNF-α, IL-17A, IL-4, IL-5, IL-13, and CCL2 including IL-6, IL-10, IL-1α, IL-1β, IL-18, IL-12, and GM-CSF, clearly discriminate between responses at baseline versus at months 3 and 6. Consequently, this set of cytokines demonstrates substantial capability in delineating the evolution of immune responses before and after the completion of anti-TB treatment in both groups of children.

Correlation analysis of cytokine levels over time in TB children with and without SARS-CoV2 seropositivity

We aimed to identify correlations between cytokine responses at baseline, month 3 (M3), and month 6 (M6) in CoV2+ and CoV2- children, respectively. Spearman's correlation coefficients were employed to determine the correlation effect, and the data were visualized using heat map color intensity, with variables ordered by hierarchical clustering. As depicted in Fig. 5, significant correlations were observed between SARS-CoV-2 IgG Spike protein levels and

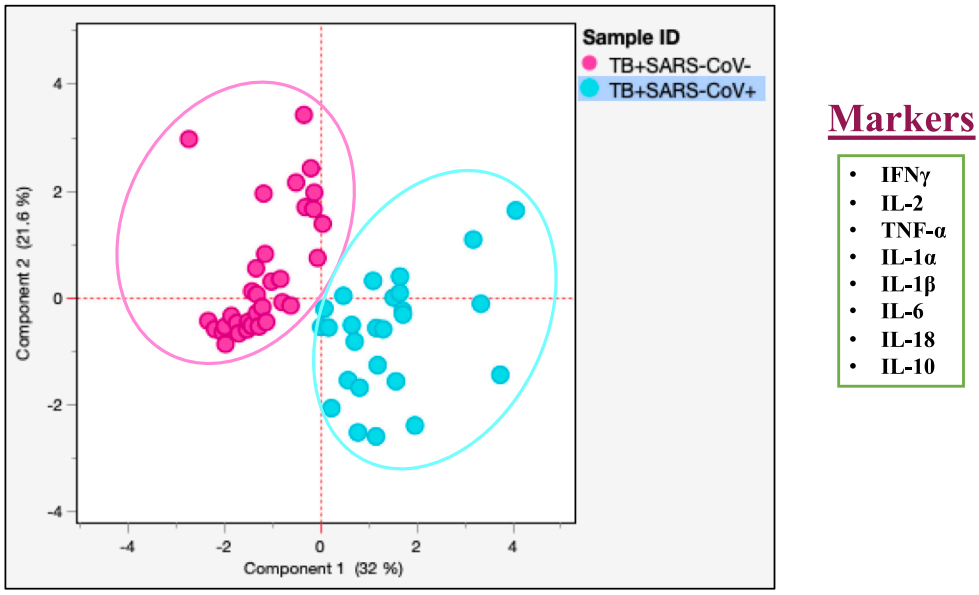

Fig. 3. Principal Component Analysis (PCA) Reveals Patterns in Cytokines. PCA was conducted to demonstrate the dispersion of data from the combination of two groups: CoV2+ (blue circles) and CoV2- (red circles). The PCA illustrates the two principal components of variation.

IFN-γ, TNF-α, IL-18, and IL-1β in both the CoV2+ and CoV2- groups. This analysis highlights potential associations between SARS-CoV2 seropositivity and specific cytokine responses over the course of TB treatment.

Discussion

The global spread of SARS-CoV2, leading to COVID-19 disease, has emerged as a significant public health concern.[10] Evidence suggests that COVID-19 can induce various pathological events by altering individuals' immune status.[11] Concurrently, TB continues to exert a substantial toll on global health annually. Poor outcomes in both TB and COVID-19 may be linked to risk factors such as age extremes and comorbidities like diabetes, hypertension, and chronic respiratory disorders.[7,12] Recent research underscores TB as a significant risk factor for the severity and mortality associated with COVID-19,[13] with the Centers for Disease Control and Prevention (CDC) identifying TB as a risk factor for severe COVID-19, alongside other non-communicable illnesses.[14]

It is well known that in adults, the symptoms of COVID-19 and TB exhibit notable similarities,[15,16] but in children, COVID-19 affects differently from adults, with the disease being mild to moderate presentation. However, COVID-19 had a significant impact on childhood TB services, particularly affecting the diagnosis due to delayed presentation.[17] It is also not known whether previous SARS-COV-2 exposure (SARS-CoV-2 seropositive – IgG positive) would have an influence on TB disease in children.

There are various studies describing how viral diseases like measles or influenza have been found to adversely affect TB, leading to an increased incidence of TB disease. This is mainly due to temporary immune system suppression.[18,19] Furthermore, the current understanding of the pathogenic pathways of COVID-19 is limited, and there is a scarcity of research that has shown the coexistence of TB or the impact of COVID-19 in individuals with TB.[20,21]

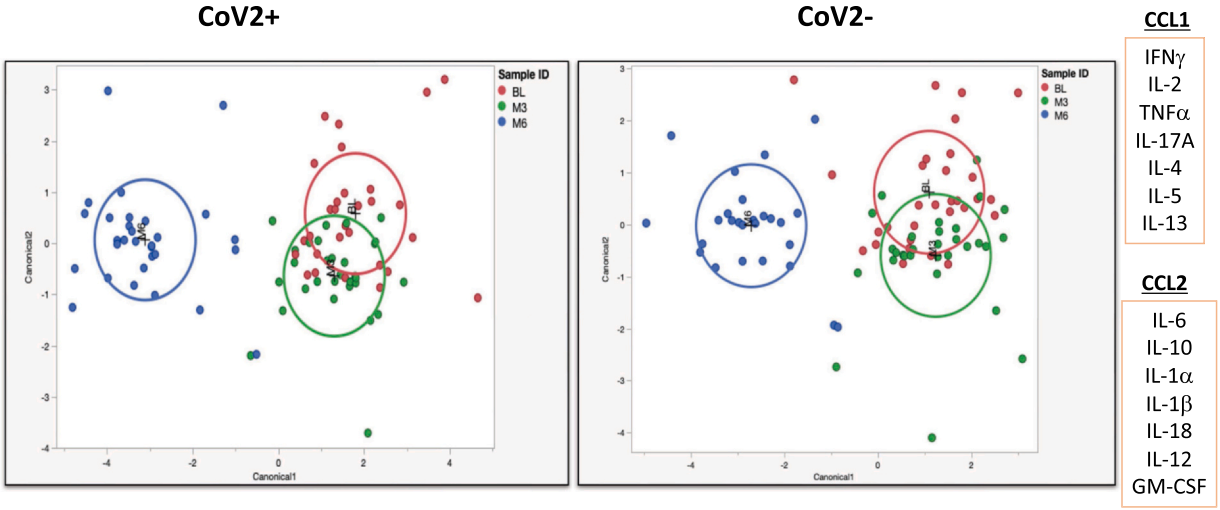

Fig. 4. Cytokines Are Engaged in TB Children with and without CoV2 antibody positivity During Baseline and Post-Treatment. Canonical correlation analysis (CCA) was performed to assess whether a combination of cytokines (CCL1) can differentiate between baseline (M0) and Month 3 (M3) from Month 6 (M6). Data are represented as dot plots, with each dot representing a single individual.

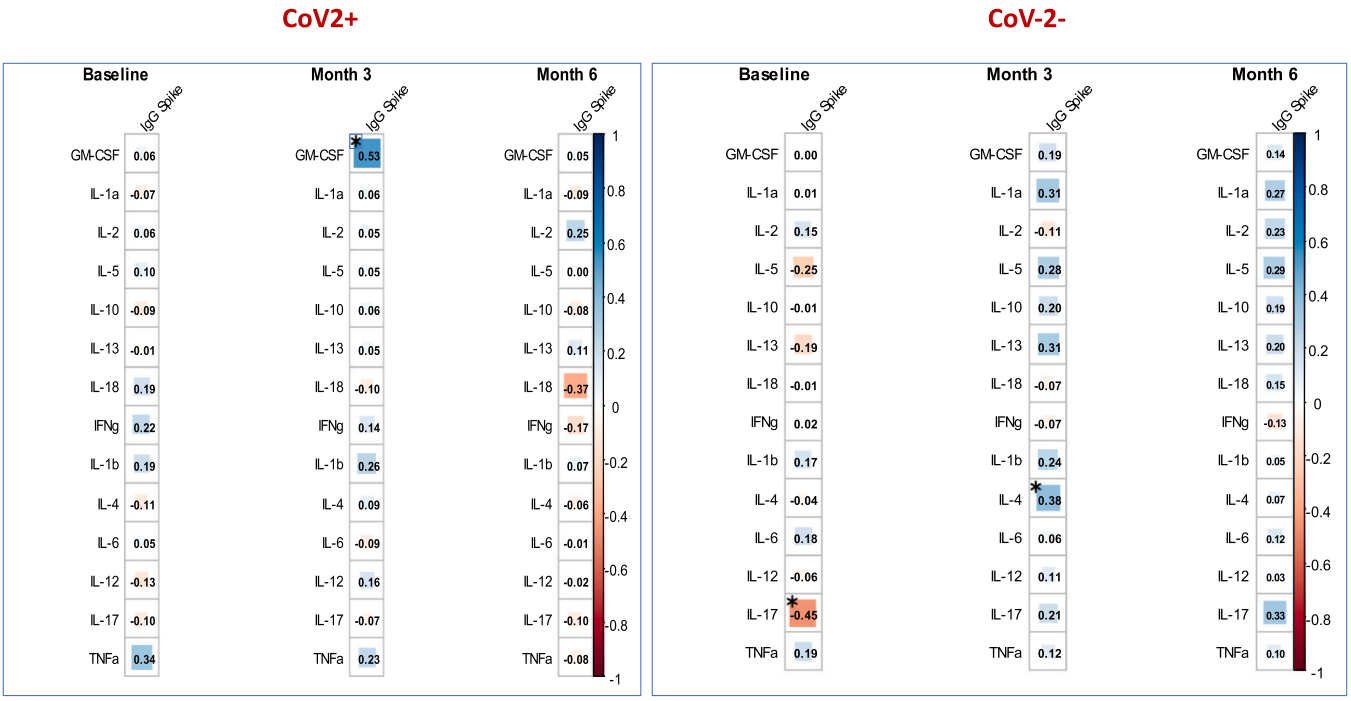

Fig. 5. Relationship Between Cytokine and IgG Spike Protein Levels. A multiparametric matrix correlation plot of cytokines and IgG spike protein levels in all children of CoV2+ and CoV2- at baseline (M0), Month 3 (M3), and Month 6 (M6). Spearman's correlation coefficients are visualized by color intensity. P values and Spearman r values are ordered by hierarchical clustering. In the correlation plot, the box size represents the correlation value ranging from −1 to +1; larger sizes indicate stronger correlations. Blue color represents positive correlation, while red color represents negative correlation. Statistically significant p-values are denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Our study, therefore, focused on the impact of SARS-CoV-2 seropositive on TB disease in the pediatric population. The results presented herein provide compelling evidence that children with TB and SARS-CoV-2 seropositive exhibit heightened levels of inflammatory cytokines. Cytokines play a crucial role in enhancing the host's resistance against TB infection and disease.[22] Proinflammatory cytokines, produced by T cells, macrophages, and other immune cells, serve as signaling molecules to stimulate inflammation and enhance immunity. Their involvement in host resistance to TB is supported by research conducted on animal models.[23,24] Moreover, our previous study on pediatric TB revealed that children with active TB exhibit higher levels of type 1, type 17, and other proinflammatory cytokines compared to children without TB.[25] Similar to TB, cytokines play a pivotal role in COVID-19, with their plasma levels often associated with disease severity.[24] Cytokine release syndrome, characterized by significantly elevated inflammatory and proinflammatory cytokines such as IL-6, is a hallmark of severe COVID-19 cases.[26] This systemic hyperinflammation is commonly observed in children with COVID-19.[27,28] Our previous study on cytokine responses in children with acute COVID-19 and SARS-CoV-2 seropositivity showed that children with COVID-19 or seropositive status exhibited heightened levels of inflammatory cytokines compared to healthy controls.[29] These findings collectively underscore the significant impact of cytokines on host immunological responses in both TB and SARS-CoV-2 seropositive children.

The findings from the adult study showed elevated levels of inflammatory cytokines (such as TNFα, IL-1β, IL-17A, IL-5, and GM-CSF) in those with TB-COVID-19 co-infection compared to those with TB alone.[30] Similarly, in our study, we observe elevated levels of inflammatory cytokines in CoV2+ children compared to those with TB alone (CoV2-) suggesting SARS-COV-2 exposure may alter immune responses in active TB. Our data reveals three main findings. Firstly, SARS-COV-2 seropositivity in children with TB amplifies the baseline production of proinflammatory cytokines (such as IFNγ, IL-2, TNFα, IL-1α, IL-6, and IL-10) compared to TB alone. Secondly, following completion of anti-TB treatment, seropositive SARS-COV-2 children display a decline in inflammatory responses. Lastly, the induction of immune responses is positively correlated with SARS-CoV-2 IgG (S) levels at baseline not after completion of TB treatment, indicating a significant alteration in immune response in children with CoV2+ children.

Our study is one of the first comprehensive investigation into the immune profile of children with TB and SARS-CoV-2 seropositivity from India, which has a high burden to TB. The emergence of conflicting clinical data underscores the complex interplay between tuberculosis (TB) and COVID-19, where TB patients exhibit decreased pulmonary function and an elevated risk of adverse outcomes from COVID-19. Significantly, our findings highlight overlapping immune responses in COVID-19 and TB disease, characterized by aberrant pro-inflammatory cytokine production. Both SARS-CoV2 and Mtb induce dysregulated immune responses, which may elevate the risk of TB infection, TB disease and its severe course.

We found that SARS-CoV-2 seropositivity exacerbates immunological responses specific to tuberculosis, delineating a distinct immunopathogenic profile from TB alone children. This nuanced understanding of the immune dysregulation provides crucial insights into the pathogenesis of these dual infections and may inform clinicians in the assessment and management of pediatric patients affected by TB and COVID-19. By elucidating the intricate immune mechanisms underlying SARS-CoV-2 seropositivity and TB, our study offers a foundation for the development of targeted therapeutic interventions aimed at mitigating the adverse outcomes associated with these intertwined diseases.

Financial support

This work was supported by the ICMR-NIRT intramural funds as well as by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases (NIAID) and NIRT-ICER funds.

Data availability

All the reported data are available within the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank the NIRT Director for the constant support throughout the study; we also thank all the Pediatric Consultants and infection control nurses at Institute of Child Health Hospital, Chennai, India for their support and contributions. We also sincerely thank the NIRT-ICER staff for technical support and staff of Department of Clinical Research for their support in participant enrolment process.

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