Immunomodulation for ARDS
Insights From Proteomics in COVID-19

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BACKGROUND: The success of targeted immunomodulation in COVID-19 underscores its potential for ARDS resulting from other causes. However, it is important to understand both its targeted and broader impacts on the inflammatory host response. To guide future ARDS studies, we explored this in patients with COVID-19 using targeted proteomics.

RESEARCH QUESTION: How do different immune modulators affect the immune profiles of patients who are critically ill with COVID-19-related ARDS?

STUDY DESIGN AND METHODS: In this multicenter cohort study, we used 2 Dutch biorepositories to compare patients with COVID-19 with acute respiratory failure treated with: no immunotherapy (n = 18), corticosteroids (n = 21), anakinra plus corticosteroids (n = 9), or tocilizumab plus corticosteroids (n = 22). Plasma proteins related to inflammation and cardiovascular injury were measured using proximity extension assays on ICU days 0 through 1, ICU days 2 through 4 (T3), and ICU days 6 through 8 (T7) after treatment initiation.

RESULTS: We observed lower expression of inflammatory biomarkers immediately after tocilizumab administration and from T3 onward after anakinra administration. After treatment with corticosteroids alone, fewer inflammatory biomarkers were suppressed, and only at T3. Multivariate analyses at T3 identified tumor necrosis factor-related apoptosis-inducing ligand, IL-1 receptor-like 2, and tumor necrosis factor β as markedly increased and proto-oncogene tyrosine-protein kinase (SRC) and serine/threonine kinase 4 (STK4) as decreased, solely after tocilizumab. At T7, lower concentrations of 2,4-dienoyl-CoA reductase 1, signaling lymphocytic activation molecule family member 7, SRC, and STK4 were observed in patients treated with tocilizumab or anakinra, whereas interferon γ, chemokine (C-X-C motif) ligand 9, and chemokine (C-C motif) ligand 19 were decreased only after anakinra treatment.

INTERPRETATION: In this exploratory study, adding tocilizumab or anakinra to corticosteroids triggered a much broader immunoregulatory response than can be explained by their receptor-specific actions. The response after tocilizumab occurred more rapidly than that after anakinra, offering a potential advantage in the time-sensitive ICU setting. Additionally, tocilizumab preserved the interferon pathway, crucial for antiviral defense, whereas anakinra suppressed it.

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KEY WORDS: anakinra; ARDS; corticosteroids; COVID-19; immunomodulation; inflammation; tocilizumab

ABBREVIATIONS: CCL = chemokine (C-C motif) ligand; MCP = monocyte chemoattractant protein; MMP = matrix metalloproteinase; sPLS-DA = sparse partial least squares discriminant analysis; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; T7 = ICU days 6 through 8; T3 = ICU days 2 through 4; T0 = ICU days 0 through 1; VIP = variable importance in projection

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Take-Home Points

Research Question: How do different immune modulators affect immune profiles in patients with COVID-19-related ARDS who are critically ill?

Results: In a multicenter cohort, patients with COVID-19 with acute respiratory failure were treated with no immunotherapy, corticosteroids alone, anakinra plus corticosteroids, or tocilizumab plus corticosteroids. Tocilizumab rapidly suppressed inflammatory biomarkers, whereas anakinra's effects were delayed, but equally broad. Corticosteroids alone showed a limited impact. Tocilizumab preserved the interferon pathway, vital for antiviral defense, whereas anakinra suppressed it.

Interpretation: Our results show that tocilizumab and anakinra, added to corticosteroids, triggered broader immunoregulation than expected from their receptor-specific actions. Our findings provide in-depth insights into the immunomodulatory effects of tocilizumab and anakinra in patients with COVID-19 who are critically ill and may inform future research on targeted immunomodulation strategies in patients with ARDS.

ARDS is a clinical syndrome characterized by acute hypoxic respiratory failure, often requiring ICU admission.[1] The pathophysiologic characteristics are complex, involving dysregulation of the inflammatory host response, endothelial dysfunction, and activation of coagulation pathways.[2] The diverse causes and complex pathophysiologic mechanisms underlying ARDS lead to substantial clinical and biological heterogeneity among patients.[3] Therefore, a one-size-fits-all immunomodulation approach for ARDS is unlikely to be effective.[4-6] Several studies have tried to identify subgroups of patients with ARDS who may benefit from specific interventions, yielding—among others—a hypoinflammatory and hyperinflammatory subphenotype.[7,8] Yet, available data are difficult to interpret. For instance, secondary analyses of randomized controlled trials showed the effectiveness of simvastatin in patients with ARDS with a hyperinflammatory subphenotype, but not in patients with a hypoinflammatory subphenotype.[8,9] However, simvastatin was effective in patients with COVID-19 who are critically ill, despite 98.8% of these patients demonstrating the hypoinflammatory subphenotype.[10] Personalizing immunotherapy requires detailed knowledge of the effects of immunomodulation on the pathophysiologic pathways involved in ARDS.

Currently, the only potential immunotherapeutic intervention in ARDS is early administration of corticosteroids, offering broad, nonspecific immunomodulation. Early administration of dexamethasone has been shown to reduce both the duration of mechanical ventilation and overall mortality in ARDS,[11] although study outcomes vary and optimal timing, duration, and dosing remain uncertain.[12] In patients with ARDS caused by COVID-19 who are critically ill, corticosteroids also significantly reduce mortality.[13-15] Although targeted immune modulation has yet to gain a foothold in the treatment of ARDS,[4] its success in COVID-19-related ARDS suggests potential for treating ARDS resulting from other causes.[4,16] Targeted immunomodulation with IL-6 receptor blockade (with tocilizumab or sarilumab) in addition to corticosteroids was shown to reduce mortality further in patients with severe COVID-19, as well as reducing the need for organ support and duration of ICU stay.[17] However, other targeted immunomodulators, such as the IL-1 receptor antagonist anakinra, did not improve outcomes in these patients.[18] Although the variable efficacy of these immunotherapeutic strategies is apparent from clinical studies, the underlying pathophysiologic reasons for these differential effects remain poorly understood.[19,20]

In this study, we used targeted proteomics to uncover the host response pathways differentially affected by various immunomodulation strategies in patients with COVID-19 with respiratory failure who are critically ill. Our goal was to identify key differences in protein expression among immunomodulatory treatments, providing a foundation for developing more effective, personalized immunotherapeutic strategies for ARDS.

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Study Design and Methods

Study Design and Population

This exploratory multicenter study used clinical data and leftover blood samples that were sourced from the biorepositories of 2 Dutch academic hospitals: the Amsterdam University Medical Center and the University Medical Center Utrecht. Ethical approval was obtained from the relevant committees of both hospitals (Amsterdam University Medical Center biobank ethics committee protocol number, 2020.182; and University Medical Center Utrecht TCBio protocol number, 22-483).

We included adult patients with severe COVID-19 pneumonia treated in the ICU. Patients were eligible if they had a plasma sample drawn within 24 hours of treatment initiation (ICU days 0-1 [T0]) and at least 1 additional sample 2 to 4 days later (ICU days 2-4 [T3]) or 6 to 8 days later (ICU days 6-8 [T7]). Patients not receiving immunomodulatory treatment were included if a sample was drawn on ICU admission (T0) and at T3 or T7. Patients with severe immunosuppression (eg, advanced HIV disease, organ or bone marrow transplantation) were excluded. We categorized patients into 4 groups based on the immunotherapy received in the ICU: (1) no immunotherapy, (2) corticosteroids only, (3) anakinra plus corticosteroids, and (4) tocilizumab plus corticosteroids. Corticosteroid regimens included hydrocortisone 200 mg/d for 7 days or dexamethasone 6 mg/d for 10 days. Anakinra was given as a 300-mg loading dose, followed by 100 mg every 6 hours until extubation or up to 14 days. Tocilizumab was given as a single 8-mg/kg dose (maximum, 800 mg), with an optional second dose after 12 to 24 hours if clinical improvement was judged insufficient by the treating clinician.

Data collection

Clinical data were retrieved from electronic health records, including patient demographics, severity of disease scores, laboratory results, duration of mechanical ventilation, and mortality.

Sample Preparation and Protein Assays

Leftover daily ethylenediaminetetraacetic acid plasma was stored at −80 °C until assay. Proximity Extension Assays (Olink; service provider: Arcadia, University Medical Center Utrecht) were used to measure relative protein concentrations, expressed in normalized protein expression ratios.[21,22] Specifically, the Target 96 inflammation and Target 96 Cardiovascular II panels were selected to capture key biomarkers relevant to ARDS pathophysiologic features, including inflammation, endothelial activation, and coagulation. Values less than the detection limit were imputed as the lower limit of detection. Proteins with $>$ 80% of measurements that were less than the lower limit of detection were excluded from the analyses.

Statistical Analysis

We used Wilcoxon rank-sum tests for between-group comparisons of protein expression and visualized the results with volcano plots after applying multiple testing correction (Benjamini-Hochberg procedure). We used multivariate analyses to identify differentially expressed proteins between groups. First, principal component analysis was performed for each time point separately. Then, sparse partial least squares discriminant analysis (sPLS-DA) on z scores of protein expression at T0, T3, and T7 was used to identify proteins that differed among the 4 groups. A variable importance in projection (VIP) score of $>$ 1 was used as a cutoff for importance. The sPLS-DA model was fitted with and without IL-6 as a covariate separately, because IL-6 effectively distinguished among groups (because of tocilizumab's direct action[23,24]) and excluded other informative biomarkers. Details are available in e-Appendix 1.

To assess treatment effects on protein levels over time, we used generalized linear mixed-effects models. Proteins with VIP scores of $>$ 1 from sPLS-DA and a predetermined subset of 28 ARDS-related proteins were modeled independently. Treatment groups were included as fixed effects, with no immune modulation as the reference group. Linear time was added to all models and quadratic time, random intercepts, and slopes were added depending on model fit, based on the lowest Akaike information criterion. Coefficients were estimated using restricted maximum likelihood estimation.[23] All analyses were performed in R version 4.3.1 software (R Foundation for Statistical Computing). P values of $<$ .05 were considered statistically significant.

Results

Between March 2020 and September 2021, 109 patients with COVID-19 fulfilled the eligibility criteria. As a result of clinical trials on the effectiveness of corticosteroids, and later tocilizumab, most eligible patients received both drugs.[13-15] To ensure a balanced

representation of all treatment groups, we selected all available patients who received no immune modulation, corticosteroids alone, or anakinra plus corticosteroids. We then included an equally large random sample of the patients who received tocilizumab plus corticosteroids, bringing the total to 69 patients. Among these, 18 patients did not receive immunotherapy (26%), 20 patients received only corticosteroids (29%), 9 patients received anakinra plus corticosteroids (13%), and 22 patients received tocilizumab plus corticosteroids (32%) (Table 1). All T0 samples were collected between −24 hours and +24 hours of the first treatment dose, with 76% of the T0 samples being a sample obtained after treatment. The duration of immunomodulation before sample collection was similar across the treatment groups on each time point (e-Table 1). Two samples were discarded because of insufficient quality. Thirteen of 184 biomarkers were excluded before of > 80% of values being less than detection limits (e-Table 2).

Rapid Decline in Protein Expression After Tocilizumab, Occurring Later After Anakinra

Univariate analyses with no immunotherapy as the reference group showed decreasing expression of several inflammation-related proteins (discussed herein) within 24 hours of treatment with tocilizumab, which persisted through T7 (Fig 1). In contrast, after anakinra treatment, inflammation-related protein concentrations began decreasing only from T3 onward and continued to decrease progressively through T7, ultimately reaching an immunosuppressive response similar to the response after tocilizumab administration (Fig 1). Treatment with corticosteroids alone was associated with a far less extensive decrease of inflammation-related proteins, and only at T3.

TABLE 1] Patient Characteristics

Variable	No Immune Modulation (n = 18)	Corticosteroids Only (n = 20)	Anakinra Plus Corticosteroids (n = 9)	Tocilizumab Plus Corticosteroids (n = 22)
Age, y	62 (53-72)	62 (58-66)	59 (56-72)	64 (58-73)
Sex				
Male	11 (61)	16 (80)	7 (78)	12 (55)
Female	7 (39)	4 (20)	2 (22)	10 (45)
BMI, kg/m^2	27.2 (24.6-29.7)	27.3 (24.7-30.3)	28.7 (24.4-30.9)	30.9 (27.6-37.1)
ICU severity scores				
SOFA score at inclusion	7 (5-8)	5 (4-6)	6 (3-7)	6 (4-7)
APACHE II score	12 (10-22)	12 (10-13)	13 (5-17)	17 (14-21)
Days to enrollment				
Since onset of symptoms	10 (7-15)	9 (7-12)	12 (11-13)	10 (8-14)
Since hospital admission	1 (1-4)	2 (1-5)	4 (4-7)	3 (2-4)
Since ICU admission	0 (0-1)	1 (1-1)	1 (0-1)	0 (0-1)
Comorbidities[a]				
Diabetes mellitus	3 (17)	7 (35)	2 (22)	7 (32)
Immune deficiency[b]	0 (0)	2 (10)	2 (22)	2 (9)
Autoimmune diseases[b]	2 (11)	2 (10)	1 (11)	2 (9)
Home medication[a]				
Corticosteroids[c]	0 (0)	1 (5)	1 (11)	0 (0)
Anakinra or tocilizumab	0 (0)	0 (0)	0 (0)	0 (0)
Anticoagulation	1 (6)	4 (19)	3 (33)	11 (50)
Direct oral anticoagulants	0 (0)	0 (0)	1 (11)	1 (4)
Vitamin K antagonists	1 (6)	1 (5)	0 (0)	2 (9)
Antiplatelet drugs	0 (0)	3 (15)	2 (22)	8 (36)
Respiratory support at baseline				
None or supplemental oxygen only	0 (0)	1 (5)	0 (0)	2 (9)

(Continued)

TABLE 1] *(Continued)*

Variable	No Immune Modulation (n = 18)	Corticosteroids Only (n = 20)	Anakinra Plus Corticosteroids (n = 9)	Tocilizumab Plus Corticosteroids (n = 22)
High-flow nasal canula	1 (6)	8 (40)	5 (56)	11 (50)
Noninvasive ventilation	0 (0)	1 (5)	1 (11)	4 (18)
Invasive mechanical ventilation	17 (94)	10 (50)	3 (33)	5 (23)
Cardiovascular support at baseline				
Vasopressor support[d]	14 (78)	10 (50)	3 (33)	4 (18)
Laboratory values				
Serum creatinine, μmol/L	65 (59-90)	81 (59-111)	68 (54-87)	67 (58-92)
Serum bilirubin, μmol/L	9 (6.5-11.5)	7 (6-10)	10 (8-10)	6.5 (5-9)
C-reactive protein, mg/L[e]	205 (177-269)	112 (57-262)	65 (47-133)	106 (76-173)
Pao_2 to FIo_2 ratio[f]	90 (74-105)	78 (67-104)	80 (65-81)	69 (63-76)
Outcome				
Length of ICU stay in survivors	12 (8-17)	19 (7-40)	7 (5-15)	16 (8-33)
Length of hospital stay, d	24 (18-42)	39 (24-60)	18 (13-28)	27 (20-43)
21-d all-cause mortality	4 (22)	2 (10)	2 (22)	4 (18)
In-hospital mortality	5 (28)	5 (25)	2 (22)	6 (27)
ICU mortality	4 (22)	5 (25)	2 (22)	5 (23)

Data are presented as No. (%) or median (interquartile range). APACHE = Acute Physiology and Chronic Health Evaluation; SOFA = Sequential Organ Failure Assessment.
[a]As documented in medical history.
[b]See e-Appendix 1 for definition.
[c]Any systemic corticosteroid, documented as chronic use in medical history.
[d]Any of the following medications: norepinephrine, phenylephrine, dobutamine, or dopamine.
[e]C-reactive protein at baseline was missing in 8 individuals who received no immune modulation.
[f]For patients with invasive mechanical ventilation, noninvasive ventilation, or high-flow nasal cannula, Pao_2 to FIo_2 ratio was calculated using FIo_2, which was directly extracted from the device settings. For patients receiving supplemental oxygen only, for FIo_2, an estimation table was used.

Proteins that decreased after tocilizumab or anakinra administration included cytokines involved in immune signaling (IL-4 receptor α, IL-1 receptor antagonist, IL-12B, tumor necrosis factor superfamily member 14, tumor necrosis factor receptor superfamily member 10A, nuclear factor κB essential modulator), chemotaxis-related proteins (IL-8, monocyte chemoattractant protein [MCP] 1, MCP-3, MCP-4, chemokine [C-X-C motif] ligand 1, chemokine [C-X-C motif] ligand 11, chemokine [C-C motif] ligand [CCL] 11, CCL-19, and CCL-20), and matrix metalloproteinases (MMPs) involved in tissue remodeling (MMP-1, MMP-10, and MMP-12) (Fig 1B, 1C; e-Fig 1). In addition, in the tocilizumab group, several biomarkers of coagulation were expressed differentially. Tissue factor and thrombomodulin expression increased, whereas thrombopoietin and serpin family A member 12 expression decreased (Fig 1C). This pattern was not observed after anakinra administration. Conversely, in the anakinra group, interferon γ was lowered markedly, but it remained unchanged in the tocilizumab group. Finally, from T3 onward, IL-6 dynamics differed markedly among treatment groups: IL-6 levels declined in patients receiving corticosteroids with or without anakinra, yet IL-6 levels increased in patients who had received tocilizumab. This likely reflects a downstream inhibition of broader inflammatory pathways in the former groups and compensatory upregulation of IL-6 production resulting from IL-6 receptor blockade in the latter group.[24]

Supervised Analysis Reveals Treatment-Specific Signatures of Inflammation-Related and Cardiovascular Injury-Related Protein Profiles

Because of the large number of proteins measured, we applied dimensionality reduction techniques (both unsupervised and supervised) to identify treatment-specific protein signatures. A total of 13, 14, and 12 principal components explained 80% of the variance in the data at T0, T3, and T7, respectively. The first 2 principal components accounted for 48% of the variance at T0 and for 49% and 51% of the variance for T3 and T7, respectively. However, at none of the time points was specific clustering per treatment group observed (e-Fig 2).

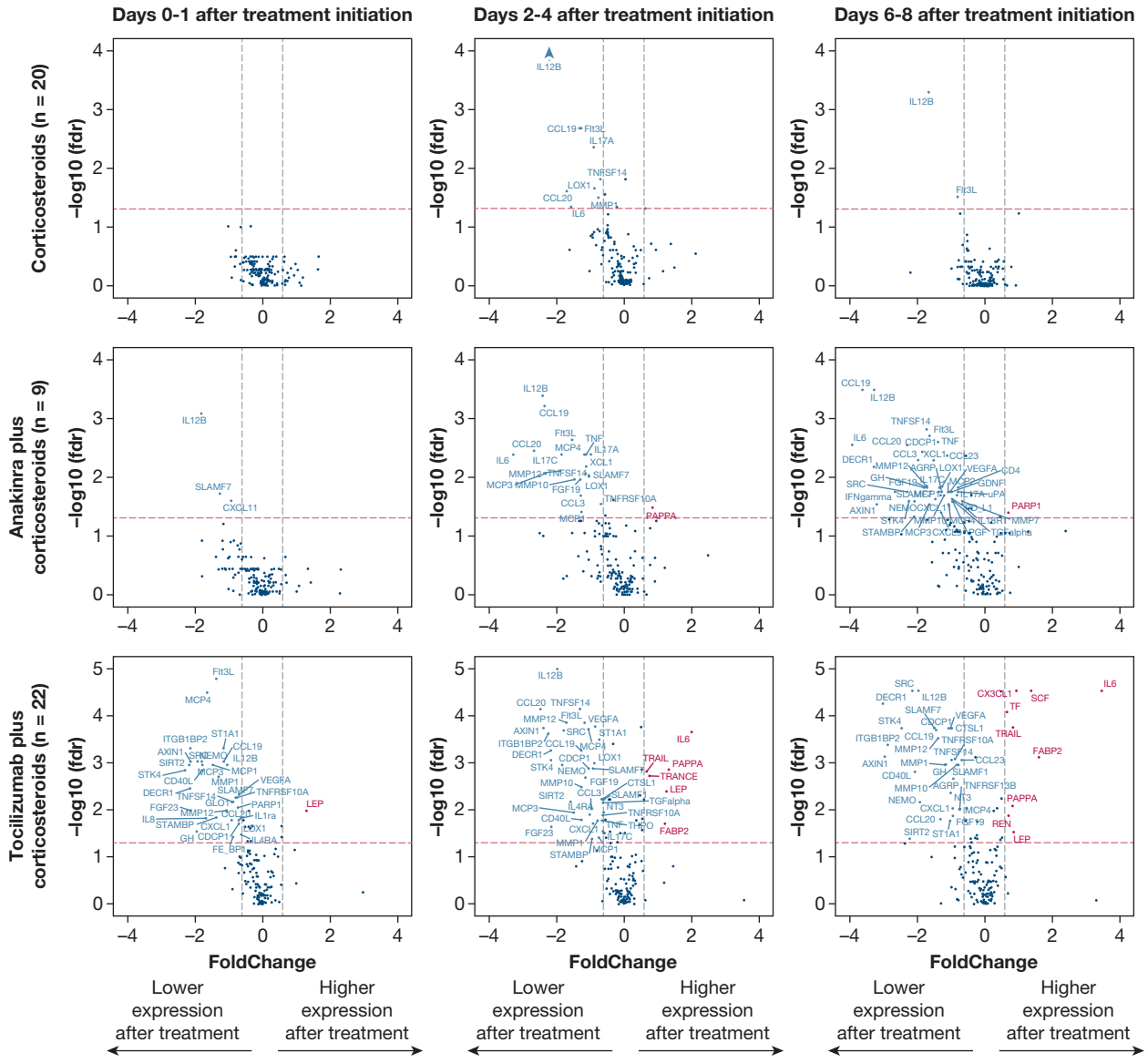

Figure 1 – *Volcano plots showing temporal protein expression changes after immunomodulation. Differences were calculated using the no immune modulation group as a reference, with statistical significance assessed via the Wilcoxon rank-sum test and adjusted for multiple comparisons using the Benjamini-Hochberg procedure. Proteins are labeled in blue if their expression was reduced significantly (< –0.6-fold) and in red if significantly elevated (> 0.6-fold) in the treated group. fdr = false discovery rate; T7 = ICU days 6 through 8; T3 = ICU days 2 through 4; T0 = ICU day 0 through 1. Abbreviations of proteins are listed in e-Table 4.*

Because the unsupervised approach could not differentiate sufficiently among treatment groups, we subsequently used sPLS-DA as a supervised method to identify the proteins that varied most among the treatment groups (Fig 2A). At T0, the sPLS-DA model was unable to separate the groups effectively, as evidenced by high classification errors across all groups (balanced error rate, 0.61; 95% quantile range, 0.54-.68). At T3 and T7, however, the tocilizumab group could be distinguished effectively from all other groups, with classification errors nearing 0 (Table 2). Although the no immunomodulation group could be separated from the corticosteroids group at T3, classification errors increased at T7 (Table 2). This reflects that protein levels in patients treated with corticosteroids alone had reverted to levels comparable with those in the no immunomodulation group at T7. The anakinra plus corticosteroids group displayed a clear decrease in the concentrations of several proteins across subsequent time points, but the sPLS-DA only partially separated this group, possibly because of the small sample size (Fig 2A; e-Fig 3).

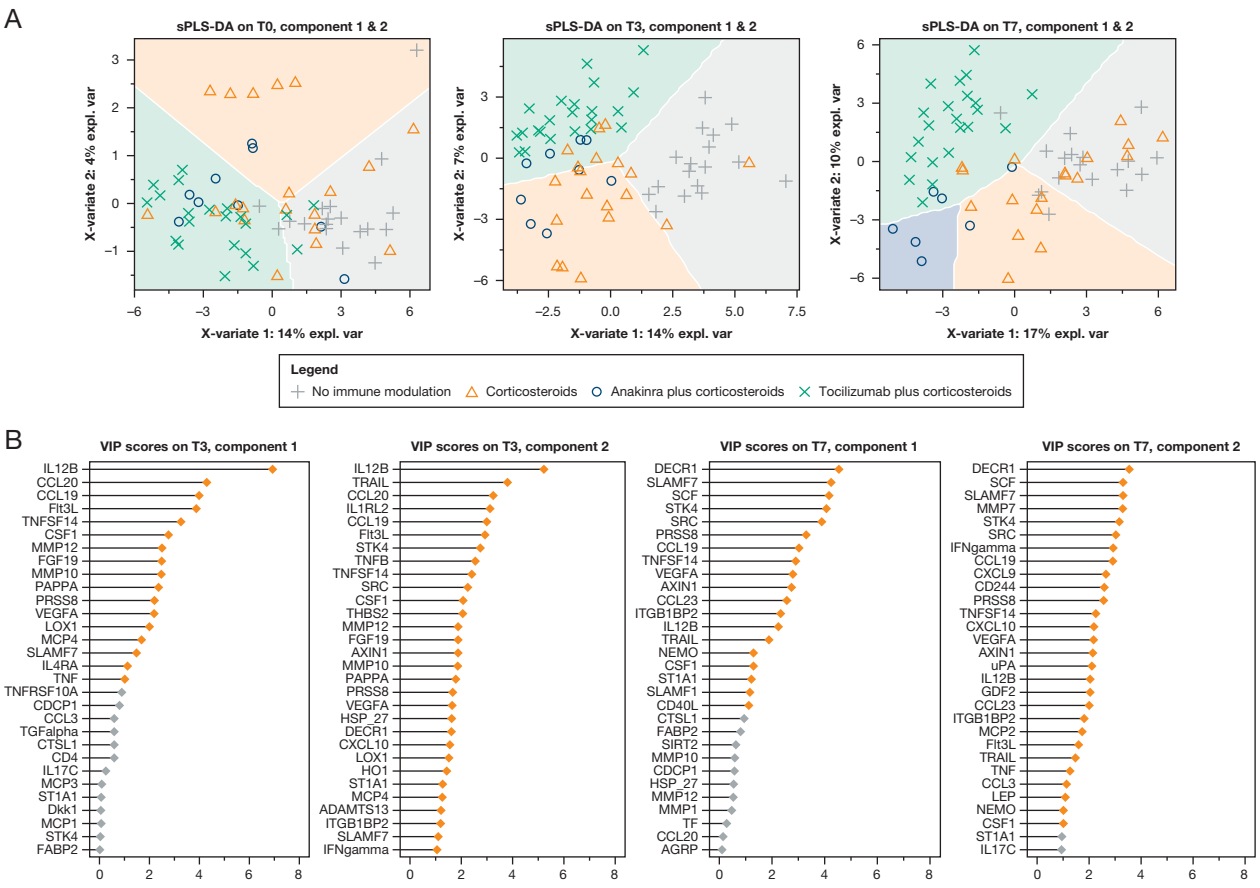

Figure 2 – *Results of the sPLS-DA analysis of 163 inflammation, endothelial damage, and coagulation-related proteins measured at T0, T3, and T7, with normalized protein expression transformed into z scores. A, Sample plots illustrating the model's classification performance. X-variate 1 and X-variate 2 represent the 2 components computed by the model from the protein expression data to distinguish the 4 treatment groups optimally, with symbols denoting the positioning of individual patients across these components. Colored backgrounds indicate the decision boundaries of the model, whereas misclassification is visualized by patient symbols misaligned across these boundaries. B, VIP scores of proteins from the sPLS-DA models using protein expression data from T3 and T7. Higher VIP scores indicate greater differences in protein expression between groups, and thus higher relevance for group separation. These figures should be interpreted in conjunction with the correlation circle plots (e-Fig 3), which illustrate the direction of protein expression differences between groups. sPLS-DA = sparse partial least squares discriminant analysis; T0 = ICU day 0 through 1; T3 = ICU days 2 through 4; T7 = ICU days 6 through 8; VIP = variable importance of projection. Abbreviations of proteins are listed in e-Table 4.*

So, although sPLS-DA did not identify distinct expression profiles for each treatment at T0, it did reveal proteins that differed markedly among the treatment groups at T3 and T7. The relative importance of each protein for distinguishing between treatment groups was quantified by VIP scores (ie, proteins with larger differences in concentration between groups have higher VIP scores) (Fig 2B). The direction of protein contributions to group separation are visualized in a correlation circle plot (e-Fig 3). At T3, IL-12B, CCL-19, CCL-20, FMS-related tyrosine kinase 3 ligand, tumor necrosis factor superfamily member 14, and colony stimulating factor 1 demonstrated high VIP scores because their concentration declined in all immunotherapy groups (Fig 2B, e-Fig 3). Additionally, pregnancy-associated plasma protein A and protease serine 8 showed high VIP scores as their concentrations increased in all treatment groups (Fig 2B, e-Fig 3). At T3, for the tocilizumab group, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), IL-1 receptor-like 2, and tumor necrosis factor β expression increased compared with the other immunotherapy groups, whereas proto-oncogene tyrosine-protein kinase and serine/threonine kinase 4 expression declined (Fig 2B, e-Fig 3). At T7, the model identified 2,4-dienoyl-CoA reductase 1, signaling lymphocytic activation molecule family member 7, and serine/threonine kinase 4 as prominent biomarkers with lower concentrations in both the tocilizumab and anakinra groups. Finally, SCF and protease serine 8 expression was increased only in the tocilizumab group, whereas interferon-γ, CCL-19, and chemokine (C-X-C motif) ligand 9 expression decreased exclusively in the anakinra group (Fig 2B, e-Fig 3). These findings suggest that, in

TABLE 2] Performance of Sparse Partial Least-Squares Discriminant Analyses

Variable	No. (%)	Not Including IL-6			Including IL-6		
		Classification Error per Class[a] (2.5th-97.5th Percentile)	AUC[a,b]	Balanced Error Rate[a] (2.5th-97.5th Percentile)	Classification Error per Class[a] (2.5th-97.5th Percentile)	AUC[a,b]	Balanced Error Rate[a] (2.5th-97.5th Percentile)
Model days 0-1 (T0)							0.56 (0.49-0.63)
No immunomodulation	18 (26)	0.33 (0.17-0.56)	0.80 (0.73-0.85)	0.61 (0.54-0.68)	0.33 (0.17-0.50)	0.80 (0.74-0.84)	
Corticosteroids alone	20 (29)	0.85 (0.70-1.00)	0.43 (0.30-0.55)	NA	0.75 (0.60-0.90)	0.51 (0.36-0.63)	
Anakinra plus corticosteroids	9 (13)	1.00 (1.00-1.00)	0.49 (0.34-0.60)	NA	1.00 (1.00-1.00)	0.58 (0.43-0.69)	
Tocilizumab plus corticosteroids	22 (32)	0.23 (0.14-0.36)	0.77 (0.69-0.82)	NA	0.14 (0.05-0.32)	0.82 (0.75-0.88)	
Model days 2-4 (T3)							0.36 (0.30-0.42)
No immunomodulation	18 (26)	0.12 (0.00-0.29)	0.95 (0.92-0.97)	0.43 (0.38-0.48)	0.06 (0.00-0.17)	0.95 (0.91-0.97)	
Corticosteroids alone	19 (28)	0.58 (0.42-0.68)	0.66 (0.58-0.72)	NA	0.47 (0.32-0.63)	0.72 (0.66-0.74)	
Anakinra plus corticosteroids	9 (13)	1.00 (1.00-1.00)	0.72 (0.65-0.78)	NA	0.89 (0.67-1.00)	0.92 (0.84-0.94)	
Tocilizumab plus corticosteroids	22 (32)	0.00 (0.00-0.09)	0.92 (0.86-0.96)	NA	0.00 (0.00-0.05)	0.99 (0.98-1.00)	
Model days 6-8 (T7)							0.30 (0.26-0.37)
No immunomodulation	18 (27)	0.28 (0.11-0.44)	0.85 (0.81-0.87)	0.49 (0.41-0.57)	0.22 (0.17-0.39)	0.85 (0.82-0.87)	
Corticosteroids alone	19 (29)	0.83 (0.67-0.94)	0.65 (0.53-0.72)	NA	0.78 (0.67-0.94)	0.66 (0.61-0.70)	
Anakinra plus corticosteroids	7 (11)	0.86 (0.57-1.00)	0.80 (0.74-0.84)	NA	0.14 (0.14-0.43)	0.97 (0.96-0.99)	
Tocilizumab plus corticosteroids	22 (33)	0.04 (0.00-0.09)	0.93 (0.90-0.96)	NA	0.00 (0.00-0.00)	0.99 (0.99-1.00)	

Results of the sparse partial least-squares discriminant analyses of 164 (not including IL-6) and 165 (including IL-6) biomarkers. AUC = area under the receiver operating characteristics curve; T0 = ICU days 0 through 1; T3 = ICU days 2 through 4; T7 = ICU days 6 through 8.

[a]Ranges are derived from 5-fold cross-validation repeated over 1,000 iterations,.

[b]For the specified group vs all other groups.

addition to shared changes in protein expression, treatment-specific alterations occur over time, notably involving the tumor necrosis factor and interferon pathways. Stability plots of proteins included in the sPLS-DA are available in e-Figure 4.

IL-6 was excluded from the primary models because its significant upregulation in patients treated with tocilizumab made it the sole predictor of treatment allocation in the sPLS-DA analysis, hindering the identification of other relevant biomarkers. Although including IL-6 in the models reduced classification errors across all groups (Table 2), the focus of the analysis was to identify key biomarkers, not to achieve optimal classification.

Longitudinal Analyses of the Effect of Immunomodulation on Protein Expressions

We aimed to assess temporal trends in each treatment group relative to the reference group by measuring protein levels over time. Using mixed-effects models, we analyzed 67 proteins: 28 key proteins identified in ARDS literature and 39 additional proteins based on sPLS-DA VIP scores of > 1. Herein, we describe a selection of significant longitudinal findings. Detailed mixed-model outputs, including visual plots per marker, coefficients, and significance levels, are available in e-Table 3 and e-Fig 5. In the tocilizumab group, a significant increase over time was observed in the concentrations of 11 proteins compared with no immune modulation, including angiopoietin 1 ($P = 0.035$), CCL-11 ($P < 0.001$), IL-6 ($P = .023$), MCP-4 ($P = .015$), tissue factor ($P = .013$), thrombomodulin ($P = .021$), and TRAIL ($P = .021$), whereas CCL-23, IL-12B, and signaling lymphocytic activation molecule family member 7 concentrations declined at a faster rate ($P = .001$, $P = .002$, and $P = .029$, respectively). Treatment with anakinra was associated with a significant increase in IL-1 receptor antagonist concentration ($P = .046$), whereas for 15 proteins, we observed a greater decrease over time compared with no immune modulation, including CCL-19 ($P = .001$), 2,4-dienoyl-CoA reductase 1 ($P = .008$), fibroblast growth factor 19 ($P \leq .001$), MMP-10 ($P = .016$), serine/threonine kinase 4 ($P = .016$), and urokinase plasminogen activator ($P = .019$). For the group treated with corticosteroids alone, a greater increase over time was observed in the concentrations of angiopoietin 1 and thrombomodulin compared with the group receiving no immune modulation ($P = .044$ and $P = .048$, respectively), whereas for 5 proteins, we observed a greater decrease in concentration over time, especially for IL-12B ($P \leq .001$). In conclusion, our longitudinal analysis revealed distinct patterns of protein expression changes in response to different immunomodulatory treatments. Both tocilizumab and corticosteroids alone were associated with increasing levels of key biomarkers involved in angiogenesis and coagulation, such as angiopoietin 1 and thrombomodulin. In contrast, treatment with anakinra showed marked declines across a broad spectrum of proteins.

Discussion

In this exploratory study, we used targeted proteomics to compare the effects of different immunomodulation strategies on various pathways related to ARDS pathophysiologic characteristics. Tocilizumab and anakinra broadly suppressed immune signaling and chemotaxis pathways, whereas corticosteroids alone reduced a smaller set of biomarkers, suggesting that tocilizumab and anakinra have wide-ranging downstream effects, despite targeting specific receptors. Additionally, in tocilizumab-treated patients, broad immunosuppression occurred within 24 hours, whereas anakinra's effect appeared only from day 3 onward. This difference in onset may explain tocilizumab's effectiveness in patients hospitalized with COVID-19, in contrast to anakinra.

Targeted immunomodulation may benefit patients with ARDS by reducing immune cell recruitment, proinflammatory signaling, and tissue remodeling.[25] After tocilizumab or anakinra administration, we observed reduced expression of more chemokines (including MCP-1 after both anakinra and tocilizumab treatment and IL-8 after tocilizumab treatment) compared with corticosteroids alone, suggesting stronger suppression of cell recruitment. These findings align with proteomics data from the Study to Evaluate the Safety and Efficacy of Tocilizumab in Patients With Severe COVID-19 Pneumonia (COVACTA) trial, in which hospitalized patients with COVID-19 were allocated to receive tocilizumab or placebo before corticosteroids were used widely, showing lower levels of CCL-20 and CCL-23 after tocilizumab administration.[26] In the same trial, alterations in proinflammatory signaling after tocilizumab administration were observed that mostly were consistent with our findings. Interestingly, using sPLS-DA, we identified a decrease in

IL-12B and CCL-20 across all immunotherapy groups, whereas interferon γ and TRAIL (a product of interferon signaling)[27] differed. Specifically, interferon γ concentration declined after anakinra administration, but remained unchanged after tocilizumab administration, whereas TRAIL concentrations increased only after tocilizumab administration. Also, endogenous IL-1 receptor antagonist, which inhibits the antiviral activity of interferon γ,[28] was lower after tocilizumab administration. This suggests that tocilizumab, unlike anakinra, preserves the interferon pathway, which is central to viral defense.[26,29] The effectiveness of tocilizumab, but not anakinra, in hospitalized patients with COVID-19 indicates that although excessive interferon γ can drive hyperinflammation and cause damage,[30] suppressing a physiologic I interferon γ response may be harmful. Moreover, low interferon α levels previously were found to be associated with disease progression in patients with COVID-19.[31]

Inflammation in ARDS triggers important coagulation pathways, resulting in tissue factor release,[32] a procoagulant state, microvascular thrombosis, and increased dead space ventilation.[3,32,33] Mendelian randomization studies suggest that IL-6 receptor signaling causes atherothrombosis[34,35] and canakinumab—targeting IL-1β—reduces the risk of cardiovascular events.[36] Indeed, in previous research in COVID-19, tocilizumab enhanced anticoagulatory gene expression pathways[26] and increased the anticoagulation markers activated partial thrombin time and prothrombin time.[37] Using mixed models, we observed differences in procoagulant and anticoagulant effects among immunotherapeutic strategies over time. Specifically, after tocilizumab administration, both tissue factor and thrombomodulin increased, whereas after anakinra administration, we observed a decrease in urokinase plasminogen activator. Given the nonspecific nature of our findings, more research is required to explore and verify these interactions.

Early elevated plasma MMP levels are associated with adverse clinical outcomes in adults and children with ARDS.[38-41] MMP-3-deficient mice were affected less severely in models of acute lung injury.[42] Tocilizumab reduced MMP-8 in patients with COVID-19[26] and MMP-12 in patients with giant cell arteritis.[43] In our study, decreased levels of MMPs were observed for tocilizumab and anakinra. However, only tocilizumab reduced MMPs within 24 hours, mirroring the detrimental impact of early elevated plasma MMP levels. We observed this effect only beyond 3 days after anakinra administration.

We were able to identify differentially regulated biomarkers for different immunomodulatory strategies. Given the small sample size and observational study design, our findings should be interpreted with caution, particularly in attributing direct cause-and-effect relationships. As such, our results primarily generate hypotheses and provide insights into potential mechanisms and should be regarded as exploratory. Most tocilizumab-treated patients were included after this became standard of care in January 2021, whereas all patients without any immunomodulatory treatment were admitted before June 2020. Changes in circulating SARS-CoV-2 variants, vaccination rates, and the proportion of COVID-19-naïve patients during this period may have introduced bias. Furthermore, some baseline imbalances were present across treatment groups. The use of mechanical ventilation and vasopressors at baseline was considerably higher in the group not receiving immunomodulation. This likely reflects a shift in clinical practice: earlier in the pandemic, patients were intubated on ICU admission more frequently, whereas later admissions were managed with noninvasive strategies. However, Pa_{O_2} to F_{IO_2} ratios at baseline, Sequential Organ Failure Assessment scores, and mortality rates were similar for all groups, suggesting comparable overall disease severity. Although all T0 samples were collected within 24 hours after the start of immunomodulation, we were unable to obtain pretreatment samples for 76% of treated patients, limiting our ability to rule out baseline differences in protein expression and potentially affecting the mixed models' ability to detect longitudinal trends, because treatment effects already may have been present at baseline. Yet, this cohort, uniquely receiving different immunomodulation strategies for the same disease, showed a sufficiently homogeneous endogenous host response, enabling head-to-head treatment comparisons. Finally, data were missing at T7 because of early discharge across treatment groups, but because the number of missingness was small and the bias was likely toward underestimation of the immunosuppressive effects of the treatment, this likely did not alter our overall conclusions.

Interpretation

In patients with COVID-19-related ARDS, the addition of either tocilizumab or anakinra to corticosteroid treatment resulted in a broader immunosuppressive response than could be explained by the receptor-specific actions of either drug alone. Tocilizumab induced an earlier response and did not suppress the interferon pathway, whereas the response to anakinra began later and included a marked decrease in interferon γ expression. These exploratory findings provide in-depth insights into the immunomodulatory effects of tocilizumab and anakinra in patients with COVID-19 who are critically ill and may inform future research on targeted immunomodulation strategies in patients with ARDS.

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Additional information: The e-Appendix, e-Figures, and e-Tables are available online under "Supplementary Data."

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