Open Forum Infectious Diseases

MAJOR ARTICLE

Thymosin Alpha 1 Mitigates Cytokine Storm in Blood Cells From Coronavirus Disease 2019 Patients

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Background. Coronavirus disease 2019 (COVID-19) is characterized by immune-mediated lung injury and complex alterations of the immune system, such as lymphopenia and cytokine storm, that have been associated with adverse outcomes underlining a fundamental role of host response in severe acute respiratory syndrome coronavirus 2 infection and the pathogenesis of the disease. Thymosin alpha 1 (Tα1) is one of the molecules used in the management of COVID-19, because it is known to restore the homeostasis of the immune system during infections and cancer.

Methods. In this study, we captured the interconnected biological processes regulated by Tα1 in CD8+ T cells under inflammatory conditions.

Results. Genes associated with cytokine signaling and production were upregulated in blood cells from patients with COVID-19, and the ex vivo treatment with Tα1-mitigated cytokine expression, and inhibited lymphocyte activation in a CD8+ T-cell subset specifically.

Conclusion. These data suggest the potential role of Tα1 in modulating the immune response homeostasis and the cytokine storm in vivo.

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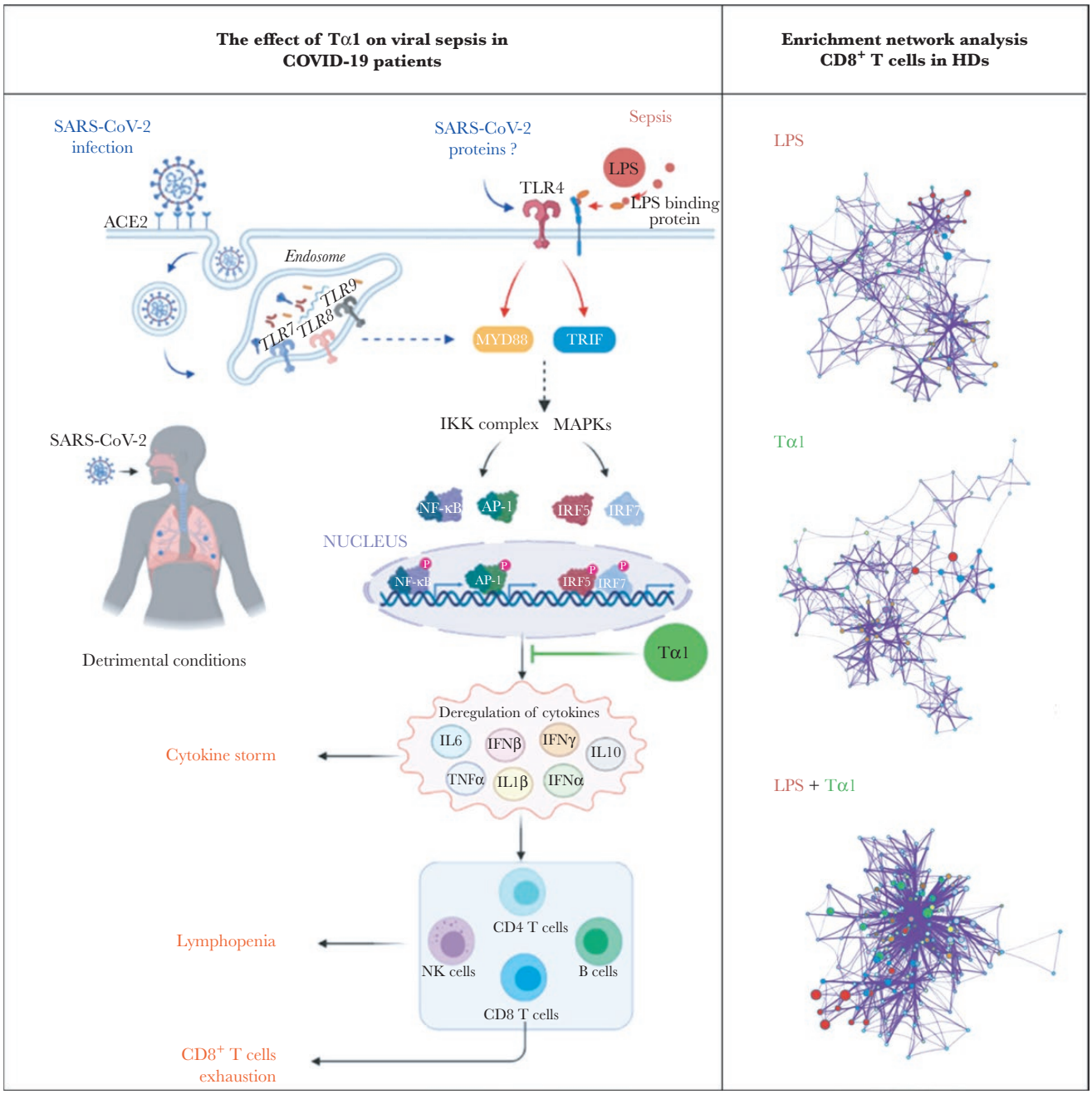
Graphical Abstract



Keywords. cytokine storm; enrichment analysis; immune modulation; SARS-CoV-2; Thymosin alpha 1.

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) represents a potentially fatal disease of great global concern for public health. Immune system dysregulations, such as lymphopenia and cytokine storm, have been associated with the severity of the disease, suggesting a fundamental role of host response in the pathogenesis [1, 2]. Severe acute respiratory syndrome coronavirus 2 uses the angiotensin-converting enzyme-2 to infect target cells, and it determines the activation of Toll-like receptors (TLRs), triggering the production of proinflammatory cytokines and chemokines from epithelial and immune effector cells [3]. Regardless of the activation of the cellular and humoral immune response, several individuals developed persistent inflammation, which can lead to a cytokine storm and diffuse organ involvement and is mainly associated with the severe condition of patients with COVID-19, such as acute respiratory distress syndrome (ARDS) [4]. Moreover, modification of total lymphocytes indicates a potential association between

lymphocyte subsets alteration and viral pathogenic mechanism [5]. The function of natural killer and $CD8^+$ T cells were affected during infection, and the restoring after therapy highlights the association of functional exhaustion of cytotoxic lymphocytes with COVID-19 [6]. Most of the severe cases showed elevated levels of infection-related biomarkers and inflammatory cytokines [7]. Indeed, abnormally high plasma levels of innate or proinflammatory cytokines have been detected, and higher mortality was associated with elevated levels of interleukin (IL)-6 [8]. It is unfortunate that only a small proportion of patients benefit from drugs currently used to manage COVID-19, such as antiviral drugs or drugs aimed at reducing inflammation or blocking a single cytokine. Thus, to date, no univocal standard of care has been defined for COVID-19 patients, and no effective drugs have been identified to mitigate the cytokine storm. Thymosin alpha 1 (Tα1), one of the molecules that has been used in the management of COVID-19 [9], is a thymic peptide endowed with the ability to restore the homeostasis of the immune system [10, 11]. Thymosin alpha 1 has been chemically synthesized and used in diseases with hindered or impaired immune response, particularly infections and cancer [12, 13]. Moreover, Tα1 has been used as an immune enhancer in patients with SARS, demonstrating efficacy in controlling the spread of the disease [14, 15]. Recent data show that Tα1 enhances the number of lymphocytes in patients with COVID-19 with severe and critical disease, reverses T-cell exhaustion, and induces immune reconstitution increasing thymus output [16]. Based on our previous study showing that Tα1 induces the release of antiviral soluble factors in $CD8^+$ T cells stimulated by lipopolysaccharide (LPS) [17], here the biological processes and networks modulated by Tα1 in that condition have been evaluated by an enrichment pathway analysis. The identified genes, including cytokines and immune regulatory factors, have been then analyzed in Tα1-treated blood cells from patients with COVID-19, to investigate the ability of Tα1 to mitigate cytokine dysregulation in SARS-CoV-2 infection.

METHODS

Subject Details

Fifteen SARS-CoV-2-positive individuals were enrolled in an open study by the Infectious Diseases Clinic, Departments of System Medicine and Experimental Medicine, University of Rome "Tor Vergata." Ethical approval for the collection and use of human samples was obtained from the ethical board of "Tor Vergata" Hospital, COrona VIrus Disease: Safety and Efficacy of Experimental Treatment (COVID_SEET prot.7562/2020, April 9, 2020, experimental register 46.20). Blood cells from 4 healthy donors (HDs) (males, age range 54–70, mean 62) were obtained from individuals attending the local blood transfusion unit of Policlinico "Tor Vergata" in Rome and referred to the Virology Unit for diagnosis. All of the subjects included in the study provided written informed consent. Clinical data were collected and reported in Supplementary Table S1.

Patient Consent Statement

The local institutional review board approved the study protocol. Patients' consents were obtained for all included patients. The study was done in accordance with the ethical principles of the Declaration of Helsinki and the Guidelines for Good Clinical Practice.

Ex Vivo Treatment

Blood samples were diluted (1:2) in Roswell Park Memorial Institute (RPMI) 1640 medium enriched with 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum. Blood samples were exposed for 8 hours at 37°C in 5% CO_2 to 50 μg/mL Tα1 (SciClone Pharmaceuticals). After incubation, samples were recovered and analyzed by flow cytometry and real-time polymerase chain reaction (PCR). Each culture condition was done in duplicate.

Real-Time Analysis

Blood samples were centrifuged and treated twice with red blood lysing buffer to remove red cells. After extraction, 100 ng of DNase-treated ribonucleic acid ([RNA] Total RNA extraction kit blood; GRiSP) was reverse transcribed into complementary deoxyribonucleic acid according to the manufacturer's protocol (ImProm-II Reverse Transcription System; Promega). The gene expression was assessed by real-time PCR in the Bio-Rad instrument CFX96Real-Time System, using SYBR Green (SMOBiO) chemistry. Primer pairs sequences used in the real-time PCR analysis are listed in Supplementary Table S2 [18].

Flow Cytometry Analysis

Blood cells were incubated with VersaLyse Lysing Solution and the monoclonal antibodies of interest for 15 minutes in the dark: antihuman CD38-ECD, CD4-APC, CD8-BV605, and PD1-BV711 (Beckman Coulter); HLA-DR-PE and CD56-PE (BD Biosciences); and CD3-BV510 (BioLegend). Stained cells were then washed with Dulbecco's phosphate-buffered saline, permeabilized with IntraPrep Permeabilization Reagent kit, and stained with anti-IL-6 FITC (ImmunoTools), anti-IFNγ BV650 (BD Bioscences), and anti-TNFα AF700 (Beckman Coulter). All of the stained cells were analyzed via CytoFLEX (Beckman Coulter) and the CytExpert 2.0 software (Beckman Coulter). The gating strategy has been described in Supplementary Figure S1. Results were expressed as percentage of positive cells and median fluorescence intensity (MFI).

Metascape Analysis and Database for Annotation Visualization and Integrated Discovery Tools

For pathway and network analysis, the Metascape online tool (http://metascape.org) was used to identify the predominant biological processes and network that are regulated by Tα1 in

$CD8^+$ T cells stimulated with LPS. In brief, after extracting the expression values from the gene expression data profile analyzed in a previous work [19], gene lists were divided into 4 groups: (1) CD8 T cells, (2) CD8 T cells + Tα1, (3) CD8 T cells + LPS, and CD8 T cells + LPS+Tα1. Subsequently, enrichment analysis was performed using the Metascape online tool using the gene lists to identify the significant biological processes and networks modulated by Tα1. The DAVID online tool (https://david.ncifcrf.gov/) was used to perform functional annotation clustering by reference database of human complex diseases and disorders Genetic Association Database (GAD_DISEASE) at a P value cutoff point ($P < .050$).

Statistical Analysis

The statistical analysis of group-wise expression levels and response to treatment was performed through the nonparametric Kruskal-Wallis test in independent samples and through the Friedman test in dependent samples. Pairwise associations between continuous variables were tested through the Spearman correlation coefficient. In addition, to determine possible interactions between treatment effects and clinical disease score, all biomarkers were analyzed using multivariate linear mixed models with an unstructured estimate of the covariance matrix, which modeled the presence of treatment as a repeated within-subject factor, as well as group and clinical state as between subject factor. When a statistically significant ($P < .050$) overall effect of time was found, pairwise comparisons between factor levels were performed and corrected for multiple comparisons using the Dunn-Šidák correction. Significant differences are shown as $^*P < .050$, $^{**}P < .010$, and $^{***}P < .001$. Data analyses were performed using the SPSS statistical software system (version 23.0 for Windows).

RESULTS

Enrichment Network Analysis Captured the Interconnected Biological Processes Regulated by Thymosin Alpha 1 in Lipopolysaccharide-Stimulated $CD8^+$ T Cells

To identify the biological pathways in which the in vitro effects of Tα1 on peripheral blood cells from patients with COVID-19 should be focused, we performed an in silico enrichment analysis using the Metascape tool in cells treated with Tα1 and stimulated with LPS as proinflammatory condition. To accomplish this, we took advantage of the results of our previous microarray-based study on genes modulation by Tα1 and/or LPS in $CD8^+$ T cells from HDs [17]. Among the different pathways highlighted, several are related to cytokines network and regulation of immune response against pathogens (Figure 1A and Supplementary Figure 2S). Most of the genes were involved in regulating the interaction between "cytokines and their receptors" (n = 78), "cytokine signaling" (n = 45), and "regulation of cytokine production" (n = 32). Other genes regulated inflammatory processes mediated by nuclear factor (NF)-κB (n = 14), IL-17 (n = 17), and IL-12 (n = 12). To better understand the relationship between the various enriched biological pathways that were identified, we performed Metascape network analysis for each treatment (Figure 1B). The analysis enabled us to determine the interconnections between the enriched biological processes by collapsing multiple genes from a multinetwork into a single node intertwined by edges similarly scored, according to their annotation. The single treatment with Tα1 affected the genes associated with immune response, inflammation, and response to infection pathways. The LPS treatment, according to the Metascape analysis, affected several cytokines and inflammation-related pathways, which were strongly regulated in cotreatment with Tα1. These networked cellular responses predominantly reflected that Tα1 differentially regulates biological processes according to the pathophysiological condition analyzed (Table 1). Moreover, the treatment with Tα1 in $CD8^+$ T cells in the presence of LPS modulates several more and different biological processes than Tα1 or LPS alone (Figure 2). It is notable that several pathways that played important roles in infection and inflammation were negatively modulated (Toll-like receptors, IL-17, and IL-23, production of cytokines and chemokines, etc). In $CD8^+$ T cells, we found that LPS modulates 30 genes, Tα1 modulates 39 genes, and combined treatment modulates 41 genes (Supplementary Table S3 and Supplementary Figure 3S). Only 4 genes were expressed and modulated in all 3 groups: *AMHR2*, *CCL22*, *LTB4R2*, and *STAT4*. Nine genes were modulated in the same manner in both Tα1 and LPS+Tα1: upregulated *CCL3*, *CCL4*, and *CD3e*; and downregulated *IL6ST*, *IL7*, *NFATC2*, *TLR9*, *TRAF2*, and *TRAF3*). Note that these genes also play important roles in the regulation of inflammatory processes in COVID-19 disease [7]. The analysis of genes exclusively modulated in the different treatments underscores that 24 of 30 genes are modulated exclusively upon LPS treatment, 24 of 39 genes are modulated exclusively in Tα1 treatment, and 22 of 41 genes are modulated when in combination.

Diseases Association of Thymosin Alpha 1-Modulated Genes in Lipopolysaccharide-Stimulated $CD8^+$ T Cells

There is an increasing interest in the identification of genes network contributing to the etiology of complex diseases for drug-repositioning [19]. The DAVID online tool, which is used to get a functional annotation clustering using reference databases of human complex diseases and disorders, was then applied to the list of total genes modulated by Tα1 in LPS-stimulated $CD8^+$ T cells used for the Metascape analysis. This tool allowed us to identify the potential use of Tα1 treatment to modulate a span of gene sets dysregulated in several diseases. The wide range of disorders evidenced by the disease enrichment analysis is listed in Table 2, and the regulated genes are listed in Supplementary Table S4. Of note, the top identified diseases are "respiratory diseases" such as respiratory syncytial virus

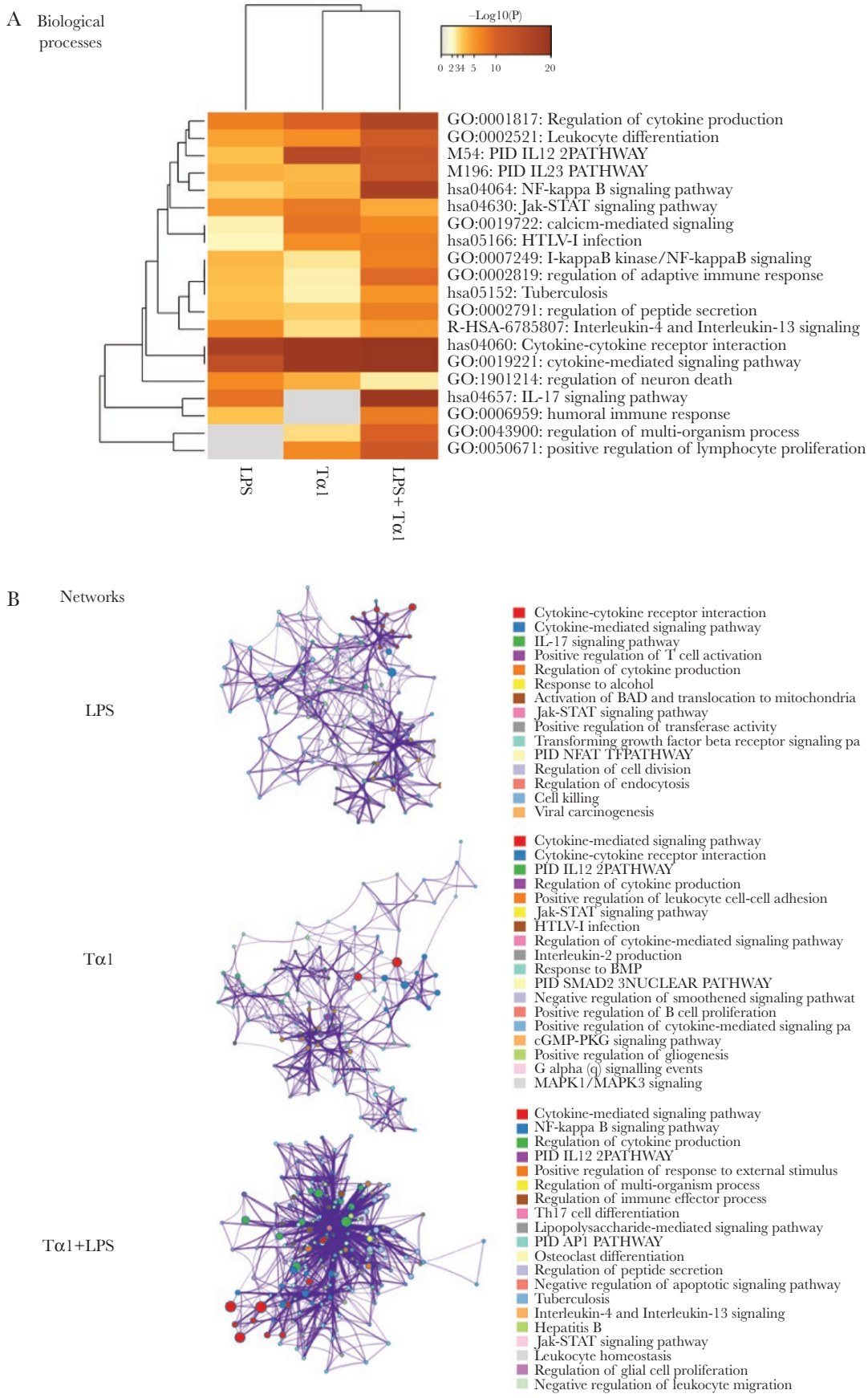


Figure 1. Enrichment analysis (A). Biological processes enrichment analysis of regulated genes in $CD8^+$ T cells after in vitro treatment with lipopolysaccharide (LPS) and/or Thymosin alpha 1 (Tα1). Heatmap of enriched terms across input gene lists, colored by *P* values, related to the regulated genes in $CD8^+$ T cells after in vitro treatments with

infections, bronchiolitis, and asthma—all disorders involved in SARS-CoV-2 infection—as well as rheumatoid arthritis and diabetes type 2, which are also risk factors for COVID-19.

Ex Vivo Thymosin Alpha 1 Treatment Reduces Cytokine-Related Gene Transcriptional Expression in Blood Cells From Coronavirus Disease 2019 Individuals

Based on the enrichment analysis in silico, several selected genes were analyzed by real-time PCR after an ex vivo Tα1 short treatment of blood cells from individuals with COVID-19 and HDs (Figure 3). Patients with COVID-19 have a higher transcriptional expression of several cytokine- and chemokine-related genes—such as *IL-6* ($P = .049$), *TNFα* ($P = .005$), *IL-1β* ($P < .001$), *IL-10* ($P = .003$), *CCL2* ($P < .001$), *LTA* ($P = .013$), and *CXCL6* ($P < .001$), *CXCR1* ($P < .004$), *TRAF2* ($P = .002$), *TRAF3* ($P = .004$), and *IL-17RA* ($P = .003$)—than HDs. The treatment with Tα1 significantly decreased *IL-6* ($P = .050$), *IL-1β* ($P = .001$), *CCL2* (P = .020), *TNFα* ($P = .008$), and *TRAF2* ($P = .005$) expression. In contrast, *IL-10* was significantly upregulated by Tα1 ($P < .001$). Conversely, significant positive modulation of *IL-6* ($P = .043$), *IL-1β* ($P = .021$), *IL-17RA* ($P = .043$), and *TRAF3* ($P = .021$) resulted in blood cells from HDs after treatment with Tα1.

Effects of Thymosin Alpha 1 Treatment in $CD8^+$ T Cells From Coronavirus Disease 2019 Individuals

Flow cytometry analysis confirmed a higher expression of IL-6 ($P = .045$), CD38 ($P = .048$), and HLA-DR ($P = .001$) in $CD8^+$ T cells from patients with COVID-19 than HDs (Figure 4A–C) as well as in CD4+ T cells (IL-6, $P = .036$; CD38, $P = .050$) (Figure 4D–F). The treatment with Tα1 significantly attenuates the percentage of cells expressing IL-6 ($P = .050$) and CD38 ($P = .044$), as well as the relative MFI of IL-6 ($P = .050$) in $CD8^+$ T cells from patients with COVID-19 (Figure 4A and B). In contrast, the percentage of $CD8^+$ T cells expressing CD38 increased in HDs after treatment (Figure 4B), whereas no difference was observed in CD38 MFI. A significant decrease in the percentage of $HLA\text{-}DR^+CD8^+$ T cells ($P = .001$) and in the HLA-DR MFI ($P = .001$) was found after Tα1 treatment in COVID-19 blood cells, as well as in HDs (Figure 4C). In blood cells from patients with COVID-19, the percentage of $CD4^+$ T cells expressing IL-6 was higher than that from HDs ($P = .047$), and the IL-6 MFI was downmodulated in both patients with COVID-19 ($P = .039$) and HDs ($P = .031$) after Tα1 treatment (Figure 4D). In addition, the percentage of $CD4^+$ T cells expressing CD38, HLA-DR, and the MFI of CD38 was higher in patients with COVID-19 than in HDs (Figure 4E and F). Moreover, the treatment with Tα1 significantly decreased the percentage of CD38 ($P = .041$) and HLA-DR MFI ($P < .001$) in COVID-19 and in HDs (MFI CD38, $P = .048$ and HLA-DR, $P = .041$); the percentage of $CD4^+$ T cells expressing HLA-DR in patients with COVID-19 also decreased ($P < .001$) (Figure 4E and F).

Cytokines and Immune Activation Markers Correlate in Coronavirus Disease 2019 Patients and Are Disrupted by Thymosin Alpha 1

Spearman's correlation analysis has shown that there is a significant positive correlation between the expression of CD38 in $CD8^+$ T cells detected by flow cytometry and messenger RNA (mRNA) blood levels of *IFNγ* (rho 589, $P = .021$), *TNFα* (rho 0.598, $P = .019$), and *IL-10* (rho 0.550, $P = .033$) in patients with COVID-19 (Figure 5A–C). Likewise, the expression of HLA-DR in $CD8^+$ T cells correlates positively with mRNA levels of *TNFα* (rho 0.591, $P = .020$), *IL-10* (rho 0.688, $P = .050$), and *IL-17RA* (rho 0.779, $P = .001$) in patients with COVID-19 (Figure 5D–F). All of these statistically significant correlations were lost after in vitro treatment with Tα1 (Figure 5A–F). Moreover, in the $CD8^+$ T-cell subset, the percentage of CD38, intracellular IL-6, and IFNγ after Tα1 treatment changes with the severity of the disease (Figure 5G–I).

DISCUSSION

In the last few months, several studies characterized COVID-19 as a disease distinguishable by hyperinflammation and immune-mediated lung injury. These features have been associated with adverse outcomes in patients and suggested the potential advantage of anti-inflammatory drugs [20]. However, COVID-19 actually resembles detrimental sepsis [21], with complex alterations of the immune system ranging from inhibition to activation and exhaustion [22]. In this context, and while seeking effective and specific drugs to inhibit the SARS-CoV-2 infection, researchers have studied several molecules with inhibitory and immunomodulating activity [23, 24]. Nevertheless, the controversy in the scientific community concerning their effectiveness is ongoing, and only a portion of the patients benefitted from anti-inflammatory drugs such as glucocorticoids [25].

Thymosin alpha 1 has been proposed for immunomodulation in COVID-19 [26, 27]. Indeed, Tα1 has already been used in China during the SARS-CoV-2 outbreak [16] for its known ability to restore homeostasis of the immune system during infections from different kinds of pathogens [11, 12]. Moreover, Tα1 has already been shown to reduce mortality

LPS (left column), LPS+Tα1 (middle), and Tα1 (right), all normalized to the untreated samples. Up to the top 20 enriched clusters are shown. All of the statistically enriched terms were identified (GO/KEGG terms, canonical pathways, hallmark gene sets, etc), and accumulative hypergeometric *P* values and enrichment factors were calculated and used for filtering. Then, a 0.3 kappa score was applied as the threshold to cast the tree into term clusters. (B) Enrichment network analysis for Tα1 treatment. Networks layout of the clusters generated with the list of the genes regulated by LPS, Tα1, and LPS+Tα1 in $CD8^+$ T cells. Each circle node represents 1 enriched term, where its size is proportional to the number of input genes falling into that term, and its color represents its cluster identity (ie, nodes of the same color belong to the same cluster). All similar terms with a Kappa similarity score >0.3 are connected by edges (the thicker the edge, the higher the similarity). One term from each cluster is selected to have its term description shown as label. Created by Metascape (http://metascape.org).

Table 1. Number of Genes Involved in Biological Processes Regulated Genes in CD8$^+$ T Cells After In Vitro Treatment With LPS and/or Tα1

GO	Category	Description	Count
LPS Treatment in CD8$^+$ T Cells			
hsa04060	KEGG Pathway	Cytokine-cytokine receptor interaction	13
GO:0019221	GO Biological Processes	Cytokine-mediated signaling pathway	15
hsa04657	KEGG Pathway	IL-17 signaling pathway	6
GO:0050870	GO Biological Processes	Positive regulation of T-cell activation	7
GO:0001817	GO Biological Processes	Regulation of cytokine production	10
GO:0097305	GO Biological Processes	Response to alcohol	6
R-HSA-111447	Reactome Gene Sets	Activation of BAD and translocation to mitochondria	3
hsa04630	KEGG Pathway	Jak-STAT signaling pathway	5
GO:0051347	GO Biological Processes	Positive regulation of transferase activity	8
GO:0007179	GO Biological Processes	Transforming growth factor beta receptor signaling pathway	5
M60	Canonical Pathways	PID NFAT TFPATHWAY	3
GO:0051302	GO Biological Processes	Regulation of cell division	4
GO:0030100	GO Biological Processes	Regulation of endocytosis	4
GO:0001906	GO Biological Processes	Cell killing	3
hsa05203	KEGG Pathway	Viral carcinogenesis	3
Tα1 Treatment in CD8$^+$ T Cells			
GO:0019221	GO Biological Processes	Cytokine-mediated signaling pathway	20
hsa04060	KEGG Pathway	Cytokine-cytokine receptor interaction	15
M54	Canonical Pathways	PID IL12 2PATHWAY	9
GO:0001817	GO Biological Processes	Regulation of cytokine production	13
GO:1903039	GO Biological Processes	Positive regulation of leukocyte cell-cell adhesion	8
hsa04630	KEGG Pathway	Jak-STAT signaling pathway	7
hsa05166	KEGG Pathway	HTLV-I infection	7
GO:0001959	GO Biological Processes	Regulation of cytokine-mediated signaling pathway	6
GO:0032623	GO Biological Processes	Interleukin-2 production	4
GO:0071772	GO Biological Processes	Response to BMP	5
M2	Canonical Pathways	PID SMAD2 3NUCLEAR PATHWAY	4
GO:0045879	GO Biological Processes	Negative regulation of smoothened signaling pathway	3
GO:0030890	GO Biological Processes	Positive regulation of B cell proliferation	3
GO:0001961	GO Biological Processes	Positive regulation of cytokine-mediated signaling pathway	3
hsa04022	KEGG Pathway	cGMP-PKG signaling pathway	4
GO:0014015	GO Biological Processes	Positive regulation of gliogenesis	3
R-HSA-416476	Reactome Gene Sets	G alpha (q) signaling events	4
R-HSA-5684996	Reactome Gene Sets	MAPK1/MAPK3 signaling	3
LPS+Tα1 Treatment in CD8$^+$ T Cells			
GO:0019221	GO Biological Processes	Cytokine-mediated signaling pathway	24
hsa04064	KEGG Pathway	NF-kappaB signaling pathway	11
GO:0001817	GO Biological Processes	Regulation of cytokine production	18
M54	Canonical Pathways	PID IL12 2PATHWAY	8
GO:0032103	GO Biological Processes	Positive regulation of response to external stimulus	12
GO:0043900	GO Biological Processes	Regulation of multiorganism process	11
GO:0002697	GO Biological Processes	Regulation of immune effector process	11
hsa04659	KEGG Pathway	Th17 cell differentiation	7
GO:0031663	GO Biological Processes	Lipopolysaccharide-mediated signaling pathway	6
M167	Canonical Pathways	PID AP1 PATHWAY	6
hsa04380	KEGG Pathway	Osteoclast differentiation	7
GO:0002791	GO Biological Processes	Regulation of peptide secretion	10
GO:2001234	GO Biological Processes	Negative regulation of apoptotic signaling pathway	7
hsa05152	KEGG Pathway	Tuberculosis	6
R-HSA-6785807	Reactome Gene Sets	Interleukin-4 and interleukin-13 signaling	5
hsa05161	KEGG Pathway	Hepatitis B	5
hsa04630	KEGG Pathway	Jak-STAT signaling pathway	5
GO:0001776	GO Biological Processes	Leukocyte homeostasis	4
GO:0060251	GO Biological Processes	Regulation of glial cell proliferation	3
GO:0002686	GO Biological Processes	Negative regulation of leukocyte migration	3

Abbreviations: BMP, bone morphogenetic protein; cGMP-PKG, cyclic guanosine monophosphate-protein kinase G; GO, Gene Ontology; HTLV, human T-lymphotropic virus; IL, interleukin; LPS, lipopolysaccharide; Tα1, Thymosin alpha 1.

and improve immune responses in patients with sepsis [28]. In this study, we demonstrated that Tα1 restored gene expression in CD8$^+$ T cells under proinflammatory condition such as LPS stimulation. Indeed, in presence of LPS stimulation, Tα1 downregulated biological processes related to inflammatory response such as "IL-17 signaling pathways," cytokine and chemokine production, and "TLRs cascade," but, in contrast, it upregulated "IL-10 signaling" and gene pathways related to "response to virus" and "aging." It has been demonstrated that Tα1 interacts with TLRs signaling and activates intracellular signaling pathways such as NF-κB, p38 MAPK, and the MyD88-dependent [29, 30]. Toll-like receptors are the crucial sensors of bacterial and viral pathogen-associated molecular patterns [31]. Other than bacterial LPS, TLR4 can bind proteins of several viruses, and in silico, ex vivo, and in vitro analyses demonstrate that TLR4-related pathways are involved in recognizing molecular patterns from SARS-CoV-2 to induce

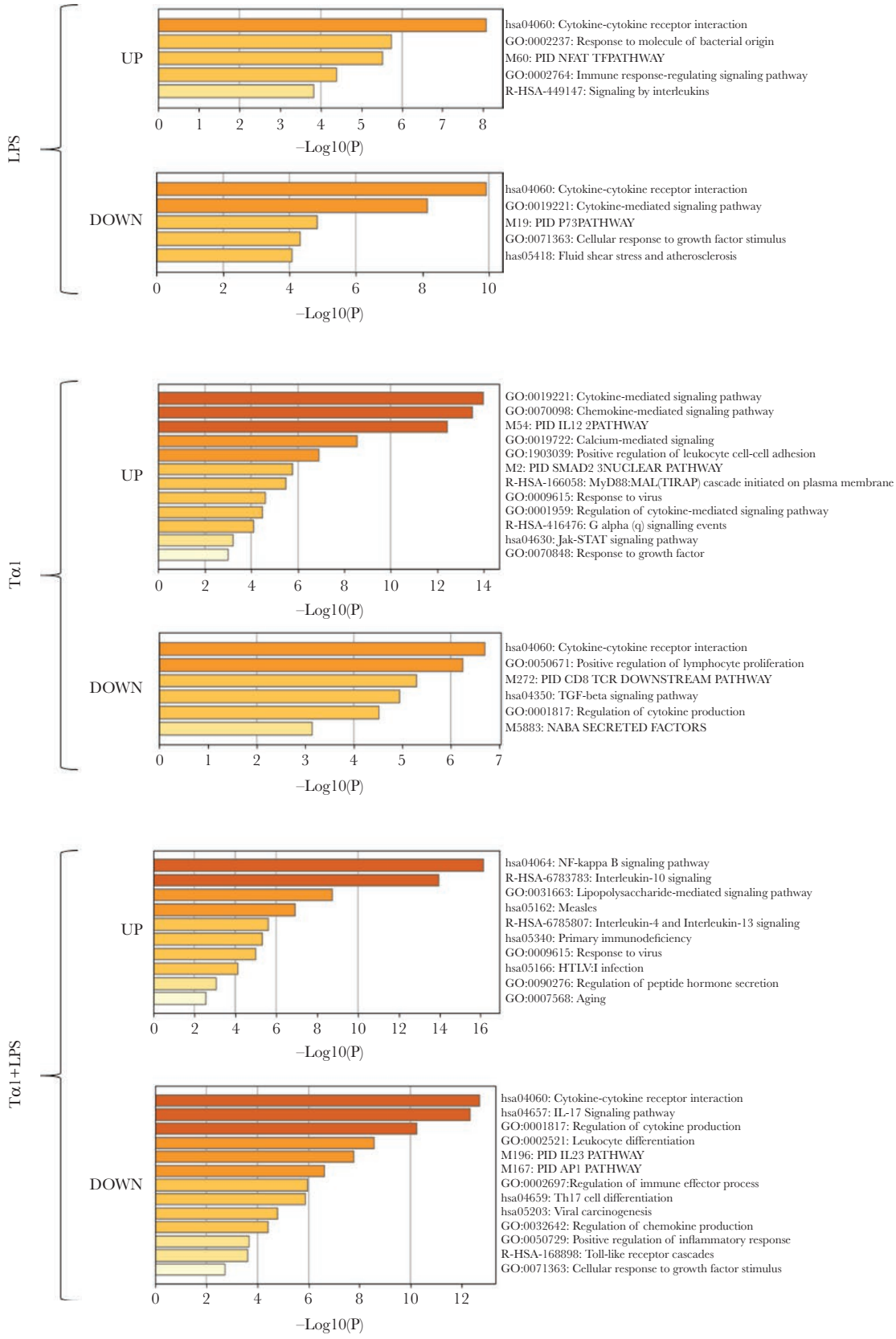


Figure 2. Biological processes enrichment analysis related to the up- and downregulated genes in $CD8^+$ T cells after in vitro treatment with lipopolysaccharide (LPS) and/or Thymosin alpha 1 (Tα1). Biological processes enrichment analysis related to the up- and downregulated genes after LPS, Tα1, and LPS+Tα1 treatments. All of the statistically enriched terms were identified, and accumulative hypergeometric *P* values and enrichment factors were calculated and used for filtering. Then, a 0.3 kappa score was applied as the threshold to cast the tree into term clusters. Created by Metascape (http://metascape.org).

Table 2. Disease-Enriched Analysis of Genes Modulated by Tα1 in LPS-CD8⁺ T Cells (DAVID Tool)

Diseases	Count	Bonferroni *P* Value	Benjamini *P* Value
Viral Respiratory Syncytial Virus Infections, Bronchiolitis, Asthma	**19**	1.35E-03	1.35E-03
Rheumatoid Arthritis	**15**	3.49E+07	1.16E+07
HIV	11	5.12E+08	1.28E+08
Hodgkin Disease Leukemia, Lymphocytic, Lymphoproliferative	9	5.85E+09	1.17E+10
Tuberculosis	8	2.01E+10	2.51E+10
Chorioamnionitis, Fetal Membranes, Infection of Amniotic sac	9	8.10E+09	9.00E+09
Pre-Eclampsia, Premature Birth	9	8.44E+09	8.44E+09
Inflammation, Premature Birth	8	1.11E+11	9.29E+09
Leukemia, Lymphocytic, Chronic, B Cell	9	2.22E+12	1.71E+11
Asthma Bronchial, Hyperreactivity, Hypersensitivity	7	3.24E+11	2.32E+10
Celiac Disease	8	3.95E+12	2.63E+11
Alzheimer's Disease	10	5.97E+11	3.74E+10
Multiple Sclerosis	12	9.48E+10	5.58E+11
Atherosclerosis	10	0.00101936447	5.67E+10
Benzene Hematotoxicity	9	0.00110884633	5.84E+10
Asthma|	7	0.001507127158	7.54E+09
Diabetes, Type 2	**21**	0.001531703595	7.30E+10
Diabetes, Type 1	8	0.001625875505	7.40E+09
Sarcoidosis	6	0.002121145763	9.23E+10
Sclerosis, Systemic	5	0.005365580531	2.15E+12
Psoriasis	7	0.012823557119	4.78E+11
Ovarian Cancer	9	0.014147999533	5.09E+10
Duodenal Ulcer, *Helicobacter* Infections	4	0.018016866577	6.27E+11
Precursor Cell Lymphoblastic Leukemia-Lymphoma	6	0.0237326830433	8.00E+11

Bold indicates the most important features or risk factors associated to COVID-19.

Abbreviations: HIV, human immunodeficiency virus; LPS, lipopolysaccharide; Tα1, Thymosin alpha 1.

inflammatory response similar to bacterial sepsis [32, 33]. Moreover, the TLR4 signaling pathway has been connected to inflammatory diseases that are seen as risk factors for COVID-19 [34].

In the present study, the Tα1-related enrichment network analysis allowed us to identify cytokine signaling and production-related genes, evocating the cytokine storm already described in COVID-19 [1, 35], which we found were indeed upregulated in the blood cells from patients with COVID-19. We observed the overexpression of cytokines such as *IL-6*, *TNFα*, *LTA*, *IL-1β*, and *IL-10* and chemokines such as *CCL2* (also known as *MCP1*) and *CXCL6* in blood cells from patients with COVID-19 compared with HDs. It is peculiar that the ex vivo treatment with Tα1 significantly downregulates the transcriptional expression of *IL-6*, *TNFα*, and *IL-1β* in COVID-19 blood cells, whereas it upregulates the same cytokines in HDs, confirming the ability of Tα1 to regulate biological processes according to the cellular state of activation and to the physiopathological condition. It is also peculiar that, in LPS-stimulated CD8⁺ T cells, Tα1 inhibits the expression of important lymphocyte activation factors such as *NFATC2*, which is associated with the production of IL-6 [36]. It is important to emphasize that high levels of IL-6 and IL-10 have already been recognized as disease severity predictors in COVID-19 [37]. In this study, we show that the Tα1-mediated inhibition of *IL-6* is accompanied by the induction and maintenance of high levels of *IL-10*, a cytokine that is well known as a master regulator of immune responses [38], and demonstrate that Tα1 differentially modulates functional genes to control immune response homeostasis. In addition, the proinflammatory cytokines *IL-1β* and *TNFα* were downregulated by Tα1 in COVID-19 blood cells. Tumor necrosis factor α and IL-1β are important in acute inflammatory reactions, because they act as an amplifier of inflammation, and anti-TNFα or anti-IL-1β therapy have been evaluated in patients with COVID-19 [39, 40]. It is interesting to note that we found a strong downregulation of the TNF receptor-associated factor *TRAF2*, but not of *TRAF3*, suggesting a fine regulation of the TNF signaling by Tα1 [30].

Furthermore, several modulated chemokines are associated with pulmonary inflammatory processes. In LPS-stimulated CD8⁺ T cells, Tα1 downregulated *LTB4R2* and *CCL2*, which play a pivotal role in the pathogenesis of airway inflammation [41] and ARDS [42]. In COVID-19 blood cells, Tα1 downmodulated the transcriptional expression of *CCL2* and slightly downmodulated *CXCL6*, chemokines involved in fibrosis and leucocytes recruitment [41–43].

The immunophenotyping analysis revealed a strong effect of Tα1 in T-cell subsets. In a recent study, researchers demonstrated that Tα1 administration could increase lymphocytes count, and this could be a potential approach to protect effector T cells during COVID-19 [44]. Thymosin alpha 1 significantly decreases the expression of the intracellular IL-6 and the activation markers CD38 and HLA-DR in CD4⁺ T cells in both patients with COVID-19 and HDs. It is notable that a specific response to Tα1 appeared in CD8⁺ T cells. Indeed, the treatment with Tα1 determined a significant decrease of intracellular IL-6, as well as of CD38 and HLA-DR in CD8⁺ T cells from patients with COVID-19 but not from HDs. It is interesting to note that the decrease of CD38 in CD8⁺ T cells was also significantly associated with the clinical status of the patients. It is worth mentioning that CD38 is a crucial marker of inflammation in CD8⁺ T cells and is overexpressed during viral infections [45]. CD38 and HLA-DR were upregulated in a CD8⁺ subset of patients with COVID-19 [46], even if different expression was related to the stage of the disease or was the result of drug treatment [47]. It is interesting to note that our study also specified evidence in CD8⁺ T cells, but not in CD4⁺

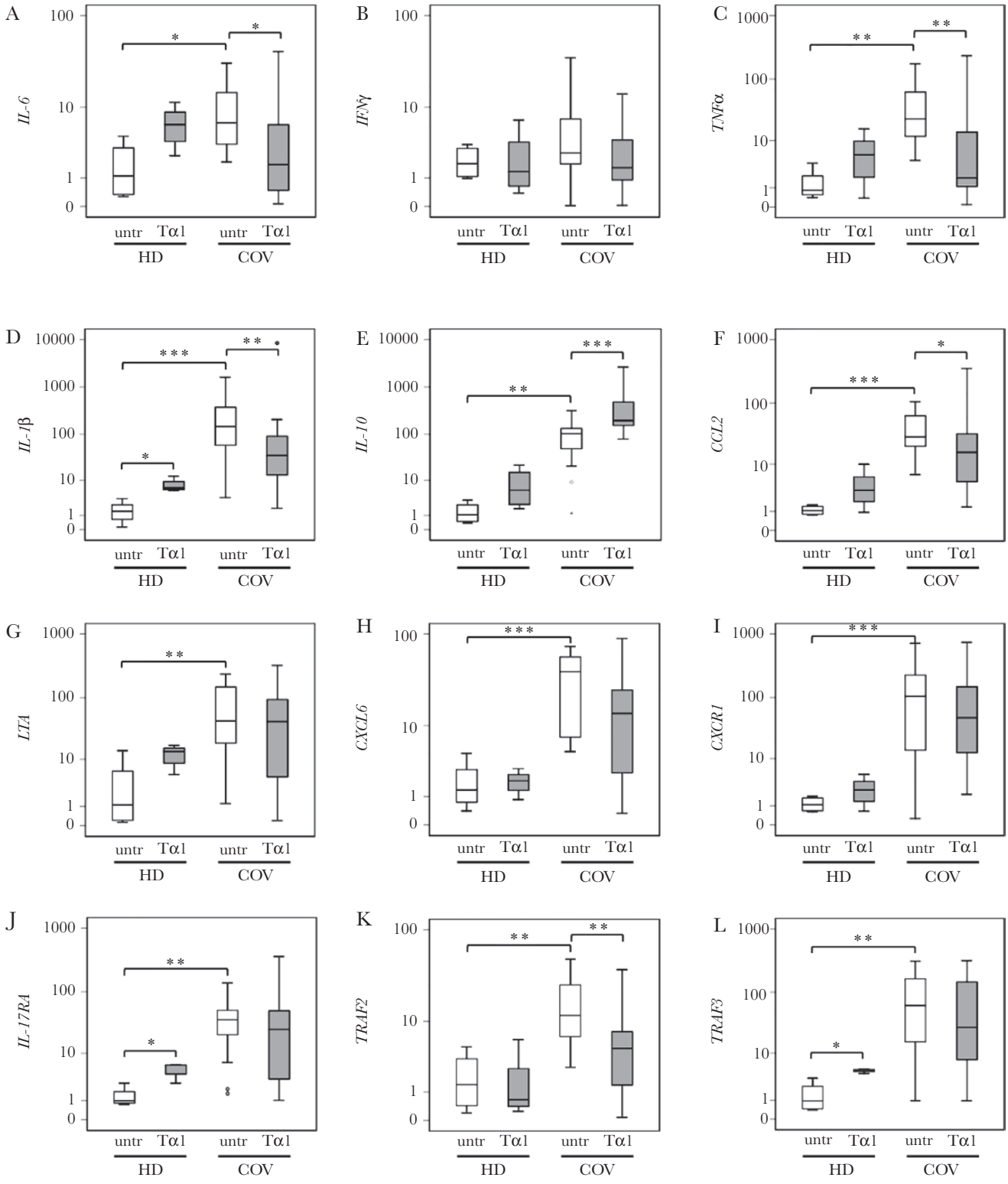


Figure 3. Effects on immune regulation by Thymosin alpha 1 (Tα1) treatment in blood cells of coronavirus (COV) disease 2019 (COVID-19) individuals. *A–L*, Transcriptional Expression of Cytokine-Related Gene in humand blood samples. Transcriptional levels in human blood samples from COVID-19 and healthy donor (HD) individuals. Data are represented as box plot, depicting mild (gray or white dot) and extreme outliers (point). Relative levels were analyzed by real-time polymerase chain reaction and represented in logarithmic scale. Statistical significant values were considered when $P < .050$ (*), $P < .010$ (**), or $P < .001$ (***). Nonparametric Kruskal-Wallis test in the case of independent samples and through the Friedman test in the case of dependent samples.

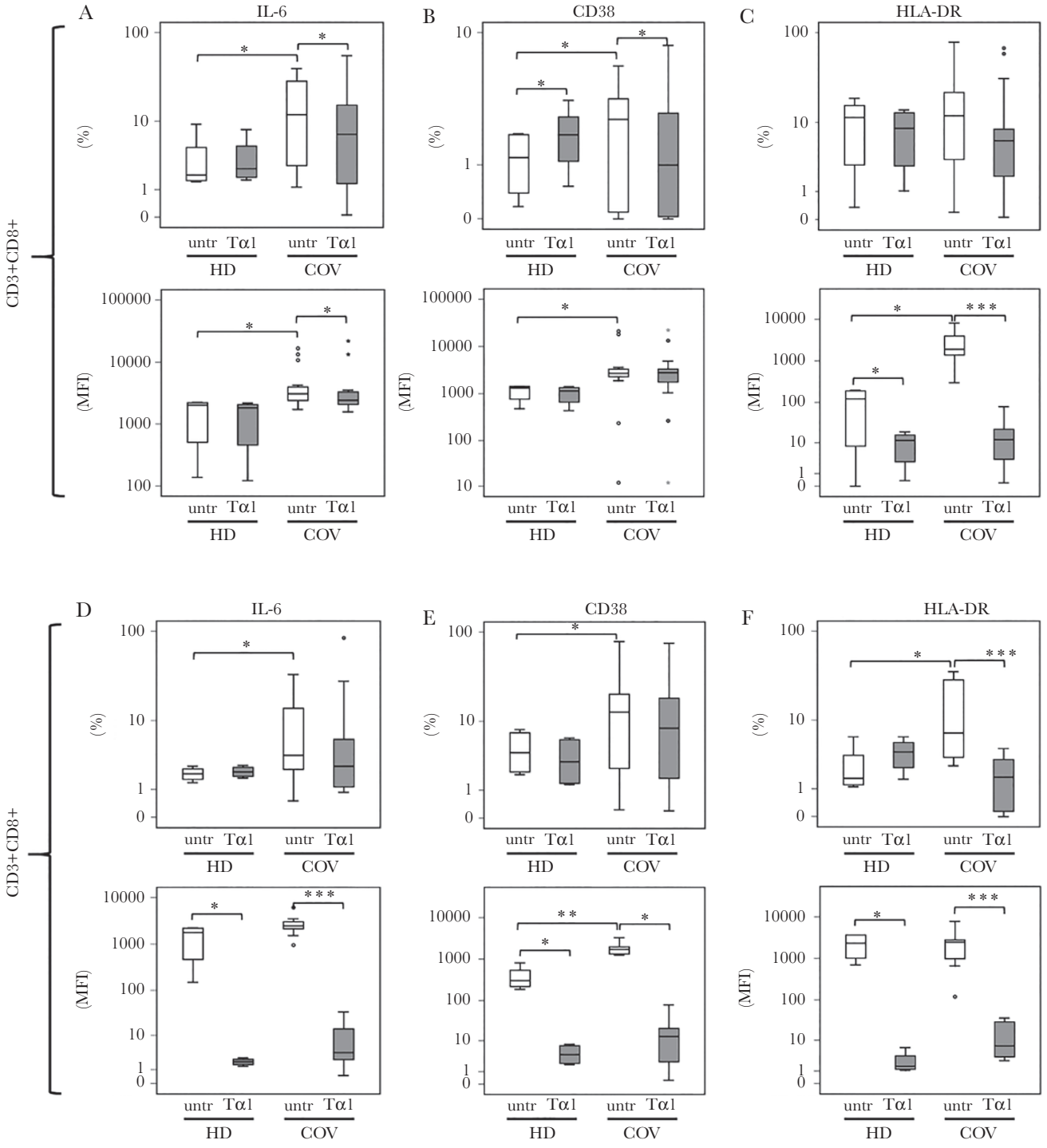


Figure 4. Effects of Thymosin alpha 1 (Tα1) treatment in CD8 and CD4 T cells from coronavirus (COV) disease 2019 (COVID-19) individuals. Flow cytometry analysis of IL-6, CD38, and HLA-DR in $CD8^+$ (A–C) and $CD4^+$ (D–F) T cells from COVID-19 individuals and healthy donors (HD). Data are represented as box plot, depicting mild (gray or white dot) and extreme outliers (point). Statistical significant values were considered when $P < .050$ (*), $P < .010$ (**), or $P < .001$ (***). Nonparametric Kruskal-Wallis test in the case of independent samples and through the Friedman test in the case of dependent samples.

from patients with COVID-19, a significant positive correlation of CD38 detected by flow cytometry with the transcriptional expression of cytokines in blood, such as *TNFα*, *IL-10,* and *IFNγ*, and a significant positive correlation of HLA-DR with the transcriptional expression in blood of *TNFα*, *IL-10* ,and *IL-17RA*. It is worth noting that the correlations were lost by in vitro Tα1 treatment.

By the functional annotation clustering of human complex diseases and disorders, we demonstrated that Tα1 is able to modulate genes related to respiratory diseases, such respiratory

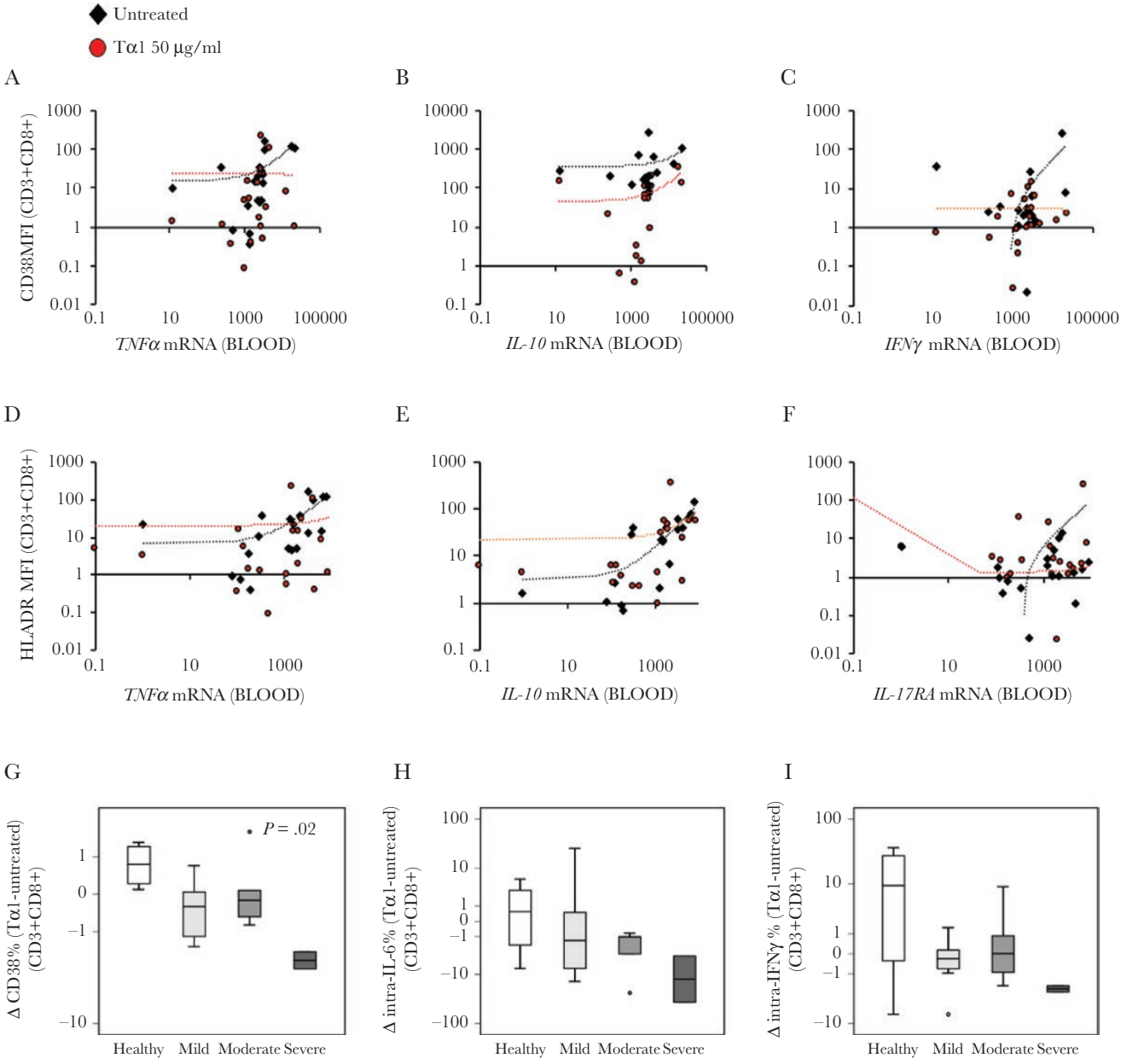


Figure 5. Correlations between immunophenotyping and cytokines transcriptional activity in coronavirus disease 2019 (COVID-19) individuals and modulation by Thymosin alpha 1 (Tα1) in vitro treatment. Correlations analysis between CD38 or HLA-DR protein expression (flow cytometry) in $CD8^+$ T cells and cytokine messenger ribonucleic acid (mRNA) transcriptional levels in blood (real-time polymerase chain reaction) in presence (red dots) or absence of Tα1 (black dots). (A–C) CD38 median fluorescence intensity (MFI) in $CD8^+$ T cells and *TNFα*, *IL-10*, and *IFNγ* mRNA levels in blood; (D–F) HLA-DR MFI in $CD8^+$ T cells and *TNFα*, *IL-10*, and *IL-17RA* mRNA levels; (G–I) effects of Tα1 on CD38, intracellular IL-6, and IFNγ positive percentage in $CD8^+$ T cells from COVID-19 patients according to disease score. Pairwise associations between continuous variables was tested through the Spearman correlation coefficient to determine possible interactions between treatment effects and clinical disease score, all biomarkers were analyzed using multivariate linear mixed models as described in Methods.

syncytial virus infections, bronchiolitis, and asthma, and autoimmune diseases, such as rheumatoid arthritis and diabetes type 2, that are risk factors for COVID-19. Thymosin alpha 1 has already shown remarkable effects in the treatment of respiratory distress syndrome (ARDS) [48]. It was also demonstrated that $CD8^+$ T cells are critical modulators of the rheumatoid arthritis disease, and LPS engagement of the functional TLR4 activates these lymphocytes, contributing to the maintenance of chronic inflammatory processes in the disease [49], and furthermore suggesting a specific role of Tα1 in $CD8^+$ T-cell regulation. Currently, COVID-19 is more severe in elderly patients, especially in those with different comorbidities [50]. Compared with children and adults, elderly patients can be affected by thymus involution and dysfunctions. The promotion of thymus output, in parallel with the restoration of lymphocytopenia and acute exhaustion of cells, have been recently demostrated

during administration of Tα1 in patients with COVID-19 [16]. Moreover, Tα1 has also been used as an adjuvant in vaccinations, showing an increase of antibody response in influenza vaccination in the elderly [51] and increased expression of major histocompatibility complex class I surface molecules in cells of different origin including primary cultures of human macrophages essential for antigen presentation [52].

CONCLUSIONS

Optimized therapeutic strategies to inhibit SARS-CoV-2 and effective drugs to modulate hyperinflammation are urgently needed for patients with COVID-19. In this study, we demonstrated that Tα1 mitigates peripheral blood cytokine expression and inhibits lymphocyte activation specifically in a $CD8^+$ T-cell subset from patients with COVID-19, suggesting the potential to modulate immune response homeostasis and the cytokine storm in vivo. Although the limitation of the study is due to the small sample size, the results we obtained, along with the promising reports on recent trials on Tα1 administration in patients with COVID-19, offer new insights of intervention, and additional blinded randomized clinical trials should also be encouraged in combination with antiviral drugs.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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