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.
Graphical Abstract

The effect of Tα1 on viral sepsis in COVID-19 patients

Enrichment network analysis CD8+ T cells in HDs

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% CO₂ 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-ECRD, 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 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 Biosciences), 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 S3). 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, CCLA, 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 Ta1 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), TNFa (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 Ta1 significantly decreased IL-6 (P = .050), IL-1β (P = .001), CCL2 (P = .020), TNFa (P = .008), and TRAF2 (P = .005) expression. In contrast, IL-10 was significantly upregulated by Ta1 (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 Ta1.

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 Ta1 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-DR–CD8+ T cells (P = .001) and in the HLA-DR MFI (P = .001) was found after Ta1 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 Ta1 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 Ta1 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), TNFa (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 TNFa (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 Ta1 (Figure 5A–F). Moreover, in the CD8+ T-cell subset, the percentage of CD38, intracellular IL-6, and IFNγ after Ta1 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 immunemediated 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, Ta1 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, Ta1 has already been shown to reduce mortality

LPS (left column), LPS+Ta1 (middle), and Ta1 (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 Ta1 treatment. Networks layout of the clusters generated with the list of the genes regulated by LPS, Ta1, and LPS+Ta1 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 in CD8+ T Cells After In Vitro Treatment With LPS and/or Tα1

| GO Category | Description | Count |
|-------------|-------------|-------|
| hsa04060 KE Galaxy Pathway | Cytokine-cytokine receptor interaction | 13 |
| GO:0019221 GO Biological Processes | Cytokine-mediated signaling pathway | 15 |
| hsa04657 KE Galaxy 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 KE Galaxy Pathway | Jak-STAT signaling pathway | 5 |
| GO:0051334 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 TPATHWAY | 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 KE Galaxy Pathway | Viral carcinogenesis | 3 |
| GO:0019221 GO Biological Processes | Cytokine-mediated signaling pathway | 20 |
| hsa04060 KE Galaxy 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 KE Galaxy Pathway | Jak-STAT signaling pathway | 7 |
| hsa05168 KE Galaxy Pathway | HTLV 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:0001981 GO Biological Processes | Positive regulation of cytokine-mediated signaling pathway | 3 |
| hsa04022 KE Galaxy 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 pathway | 4 |
| R-HSA-5684996 Reactome Gene Sets | MAPK1/MAPK3 signaling | 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.

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-kB, 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 and improve immune responses in patients with sepsis [28].
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).
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, TNFa, 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, TNFa, 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 TNFa 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+ T cells.
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 human 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.
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...
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 demonstrated.

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.
during administration of Ta1 in patients with COVID-19 [16]. Moreover, Ta1 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 Ta1 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 Ta1 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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References

1. Wen W, Su W, Tang H, et al. Immune cell profiling of COVID-19 patients in the recovery stage by single-cell sequencing. Cell Discov 2020; 6:31.
2. Costela Ruiz VJ, Illescas-Montes R, Puerta-Puerta JM, et al. SARS-CoV-2 infection: the role of cytokines in COVID-19 disease. Cytokine Growth Factor Rev 2020; 54:62–75.
3. Zhou Z, Ren L, Zhang L, et al. Heightened innate immune responses in the respiratory tract of COVID-19 patients. Cell Host Microbe 2020; 27:883–90.e2.
4. Tay MZ, Poh CM, Rénia L, et al. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol 2020; 20:363–74.
5. Li T, Qiu Z, Zhang L, et al. Significant changes of peripheral T lymphocyte subsets in patients with severe acute respiratory syndrome. J Infect Dis 2004; 189:648–51.
6. Zheng M, Gao Y, Wang G, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. Cell Mol Immunol 2020; 17:533–5.
7. Qin C, Zhou L, Hu Z, et al. Dysregulation of immune response in patients with coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis 2020; 71:762–6.
8. Ruan Q, Yang K, Wang W, et al. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Med 2020; 46:846–8.
9. Zhang L, Liu Y. Potential interventions for novel coronavirus in China: a systematic review. J Med Virol 2020; 92:479–90.
10. Matteucci C, Minutolo A, Sinibaldi-Vallebona P, et al. Transcription profile of human lymphocytes following in vitro treatment with thymosin alfa-1. Ann NY Acad Sci 2010; 1194:6–19.
11. Matteucci C, Grelli S, Balestrieri E, et al. Thymosin alpha 1 and HIV-1: recent advances and future perspectives. Future Microbiol 2017; 12:141–55.
12. Camerini R, Garaci E. Historical review of thymosin alfa 1 in infectious diseases. Expert Opin Biol Ther 2015; 15 (Suppl 1):S117–27.
13. Garaci E, Pica F, Matteucci C, et al. Historical review on thymosin alfa in oncology: preclinical and clinical experiences. Expert Opin Biol Ther 2015; 15:31–9.
14. Gao ZC, Zhu JH, Sun Y, et al. Clinical investigation of outbreak of nosocomial severe acute respiratory syndrome. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 2003; 15:332–5.
15. Kumar V, Jung YS, Liang PH. Anti-SARS coronavirus agents: a patent review (2008 - present). Expert Opin Ther Pat 2013; 23:1337–48.
16. Liu Y, Pang Y, Hu Z, et al. Thymosin alpha 1 (Ta1) reduces the mortality of severe COVID-19 by restoration of lymphocytopenia and reversion of exhausted T cells. Clin Infect Dis 2020; 22:e1aa180.
17. Matteucci C, Minutolo A, Pollicuta M, et al. Thymosin alphai 1 potentiates the release by CD8(+ ) cells of soluble factors able to inhibit HIV-1 and human T lymphotropic virus 1 infection in vitro. Expert Opin Biol Ther 2015; 15 (Suppl 1):S83–100.
18. Balestrieri E, Caprioni C, Matteucci C, et al. Children with autism spectrum disorder and their mothers share abnormal expression of selected endogenous retroviruses families and cytokines. Front Immunol 2019; 10:2244.
19. Matteucci C, Argaw-Denboba A, Balestrieri E, et al. Deciphering cellular biological processes to clinical application: a new perspective for Ta1 treatment targeting multiple diseases. Expert Opin Biol Ther 2018; 18:23–31.
20. Mehta P, McAuley DF, Brown M, et al.; HLH Across Speciality Collaboration, UK. COVID-19: consider cytokine storm syndromes and immunosuppression. Lancet 2020; 395:1033–4.
21. Li H, Liu L, Zhang D, et al. SARS-CoV-2 and viral sepsis: observations and hypotheses. Lancet 2020; 395:1517–20.
22. De Biasi S, Meschiari M, Gibellini L, et al. Marked T cell activation, senescence, exhaustion and skewing towards TH17 in patients with COVID-19 pneumonia. Nat Commun 2020; 11:3434.
23. Wang M, Cao R, Zhang L, et al. Remdesivir and chloroquine effectively inhibit SARS-CoV-2 in cell culture. Nat Med 2020; 26:1567–71.
24. Guaraldi G, Meschiari M, Cozzi-Lepri A, et al. Tocilizumab in patients with severe COVID-19: a retrospective cohort study. Lancet Rheumatol 2020; 2:e474–84.
25. RECOVERY Collaborative Group, Horby P, Lim WS, et al. Dexamethasone in hospitalized patients with covid-19 - preliminary report. N Engl J Med 2020; doi: 10.1056/NEJMoa2021436.
26. Li L, Li R, Wu Z, et al. Therapeutic strategies for critically ill patients with COVID-19. Ann Intensive Care 2020; 10:45.
27. Romani L, Tomino C, Puccetti P, Garaci E. Off-label therapy targeting pathogenic inflammation in COVID-19. Cell Death Discov 2020; 6:49.
28. Wu J, Zhou L, Liu J, et al. The efficacy of thymosin alpha 1 for severe sepsis (ETASS): a multicenter, single-blind, randomized and controlled trial. Crit Care 2013; 17:R8.
29. Romani L, Bistoni F, Gaziano R, et al. Thymosin alpha 1 activates dendritic cells for antifungal Th1 resistance through Toll-like receptor signaling. Blood 2004; 103:4232–9.
30. Zhang P, Wong C, Choy WW, et al. Thymosin alfa 1 decreases the mortality of severe COVID-19 patients. Cell Mol Immunol 2020; 17:112–21.
31. Lester SN, Li K. Toll-like receptors in antiviral innate immunity. J Mol Biol 2014; 426:1246–64.
32. Choudhury A, Mukherjee S. In silico studies on the comparative characterization of the interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. J Med Virol 2020; 10.1002/jmv.25987.
33. Sohn KM, Lee SG, Kim HJ, et al. COVID-19 patients upregulate Toll-like receptor 4-mediated inflammatory signaling that mimics bacterial sepsis. J Korean Med Sci 2020; 35:e343.
34. Brandão SCS, Ramos JOX, Dompieri LT, et al. Is Toll-like receptor 4 involved in the severity of COVID-19 pathology in patients with cardiometabolic comorbidities? Cytokine Growth Factor Rev 2020; S1359-6101(20)30205-7.
35. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the ‘Cytokine Storm’ in COVID-19. J Infect 2020; 80:607–13.
36. Weigmann B, Lehr HA, Yancopoulos G, et al. The transcription factor NFATc2 controls IL-6-dependent T cell activation in experimental colitis. J Exp Med 2008; 205:2099–110.
37. Han H, Ma Q, Li C, et al. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. Emerg Microbes Infect 2020; 9:1123–30.
38. Rojas JM, Avia M, Martin V, Sevilla N. IL-10: a multifunctional cytokine in viral infections. J Immunol Res 2020; 2017:6104054.
39. Feldmann M, Mami RN, Woody JN, et al. Trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed. Lancet 2020; 395:1407–9.
40. Huet T, Beaussier H, Voisin O, et al. Anakinra for severe forms of COVID-19: a cohort study. Lancet Rheumatol 2020; 2:e393–400.
41. Kwon SY, Kim JH. Role of Leukotriene B4 receptor-2 in mast cells in allergic airway inflammation. Int J Mol Sci 2019; 20:2897.
42. Liu X, Das AM, Seideman J, et al. The CC chemokine ligand 2 (CCL2) mediates fibroblast survival through IL-6. Am J Respir Cell Mol Biol 2007; 37:121–8.
43. Besnard AG, Struyf S, Guabraha R, et al. CXCL6 antibody neutralization prevents lung inflammation and fibrosis in mice in the bleomycin model. J Leukoc Biol 2013; 94:1317–23.
44. Yu K, He J, Wu Y, et al. Dysregulated adaptive immune response contributes to severe COVID-19. Cell Res 2020; 30:814–6.
45. Glaria E, Valledor AE. Roles of CD38 in the immune response to infection. Cells 2020; 9:228.
46. Jiang Y, Wei X, Guan J, et al. COVID-19 pneumonia: CD8+T and NK cells are decreased in number but compensatory increased in cytotoxic potential. Clin Immunol 2020; 218:108516.
47. Mathew D, Giles JR, Baxter AE, et al. Deep immune profiling of COVID-19 patients reveals distinct immunotypes with therapeutic implications. Science 2020; 369:15eabc8511.
48. Ji SM, Li LS, Sun QQ, et al. Immunoregulation of thymosin alpha 1 treatment of cytomegalovirus infection accompanied with acute respiratory distress syndrome after renal transplantation. Transplant Proc 2007; 39:115–9.
49. Tripathy A, Khanna S, Padhan P, et al. Direct recognition of LPS drive TLR4 expressing CD8+ T cell activation in patients with rheumatoid arthritis. Sci Rep 2017; 7:933.
50. Liu PP, Blet A, Smyth D, Li H. The science underlying COVID-19: implications for the cardiovascular system. Circulation 2020; 142:68–78.
51. Ershler WB, Gravenstein S, Geloo ZS. Thymosin alpha 1 as an adjunct to influenza vaccination in the elderly: rationale and trial summaries. Ann N Y Acad Sci 2007; 1112:375–84.
52. Giuliani C, Napolitano G, Mastino A, et al. Thymosin-alpha1 regulates MHC class I expression in FRTL-5 cells at transcriptional level. Eur J Immunol 2000; 30:778–86.