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Brief report: Tempol, a novel antioxidant, inhibits both activated T cell and antigen presenting cell derived cytokines in-vitro from COVID-19 patients

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ABSTRACT

COVID-19 is characterized by a dysregulation of inflammatory cytokines ultimately resulting a cytokine storm that can result in significant morbidity and mortality. We developed an in-vitro assay using activated peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide (LPS) or CD3 + CD28 to examine secretion of cytokines from antigen presenting cells (APCs) and T cells, respectively, in donor patients with a history of COVID-19 (convalescent) and uninfected negative controls. We hypothesized that a novel antioxidant called Tempol may decrease cytokines from activated peripheral blood cells from both COVID-19 patients and normal donors. Preincubation of immune cells with Tempol resulted in a significant ($P < 0.05$) decrease in multiple T cell and APC-derived cytokines from both cells of COVID-19 ($n = 7$) and uninfected donors ($n = 7$). These preliminary results suggest that Tempol has strong in-vitro anti-cytokine activity and supports additional studies examining the use of Tempol for the treatment of COVID-19.

1. Introduction

The immunopathogenesis of SARS-CoV-2, the causative agent for COVID-19, remains incompletely understood [1]. The early phases of SARS-CoV-2 infection appear to be characterized by viral replication along with an upregulation of inflammatory cytokines [2]. Indeed, severe inflammation accompanied by systemic markers of inflammation such as C-reactive protein (CRP) along with an upregulation of inflammatory cytokines are poor prognostic indicators of outcome for COVID-19 patients [3,4]. In addition, for those who survive the initial infection, long-term clinical sequelae to COVID-19 have been observed secondary to damage caused by an initial dysfunctional inflammatory response [5,6].

The National Institute of Health has released Coronavirus Disease 2019 Treatment Guidelines, which include information on therapeutic options for COVID-19 [7]. Per the guidelines, the use of the antiviral Remdesivir has been recommended in severe hospitalized cases. Furthermore, dexamethasone, a corticosteroid, is also recommended in patients requiring supplemental or mechanical ventilation but has not been shown to be effective in earlier diseased patients. This recommendation is consistent with the pathogenesis of COVID-19 infection, which involves multi-system inflammation. However, because of its pan-

immunosuppressive effects, corticosteroids would not likely be used in early COVID-19 infection.

Oxidative stress is thought to play a critical role in SARS-CoV infection [8] as well as other respiratory infections, including influenza [9–11]. As with other respiratory pathogens, it has been hypothesized that damage from free radicals due to oxidative stress may result in host systemic injury perpetuated by an aberrant immune response in patients with COVID-19 [12–14]. An antioxidant and anti-inflammatory that protects healthy cells could be hypothesized to prevent both the progression of the disease in high-risk individuals and long-term complications of COVID-19.

Tempol is a redox cycling nitroxide that promotes the metabolism of many harmful reactive oxygen species (ROS) and improves nitric oxide bioavailability [15]. In-vitro and preclinical studies of Tempol have demonstrated an attenuation of LPS induced lung damage in both acute and chronic lung injury in-vivo models in small animals [16–18]. In addition, in these animal models Tempol decreases inflammatory cytokines from a variety of cell types [19]. Tempol can be delivered by different routes including administered orally. To our knowledge, no studies have examined the effects of Tempol on peripheral blood mononuclear cells (PBMCs) from COVID-19 patients.

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COVID-19 remains randomized placebo-controlled trials. However, in-vitro immunologic assays may be informative and provide insights into potential biomarkers and therapeutic options for infection caused by SARS-CoV-2. We developed an in-vitro cytokine assay and examined the effects of Tempol on peripheral blood mononuclear cells (PBMCs) stimulated with LPS or CD3 + CD28 from patients with a history of COVID-19 (convalescent) and uninfected negative controls. Stimulation of PBMCs with LPS in this in-vitro assay induces cytokine secretion primarily from activated innate antigen presenting cells (APCs) such as macrophages/monocytes or dendritic cells. Cytokines from cells stimulated with CD3 + CD28 represent secretion primarily from T cells. We hypothesized that cytokines from activated immune cells from COVID-19 positive and COVID-19 negative donors would be suppressed by preincubation with Tempol.

2. Methods

Healthy and COVID-19 convalescent PBMCs were isolated by density-gradient centrifugation and cryopreserved [20]. Blood samples were collected under Stanford University IRB protocol #55689, approved 6/8/2021. On Day 1, PBMCs were thawed, washed, and plated 100,000 cells per well in a 96-well plate. PBMCs were pre-incubated with three different concentrations (1, 3 and 5 mM) of Tempol (EMD Millipore Corporation, Temecula CA), as per the company's template. Cells were rested overnight in the incubator at 37 °C. On day2, PBMCs in the pre-incubated wells with Tempol were either left unstimulated or stimulated with LPS (200 ng/ml) and Human T-Activator CD3 + CD28 activation beads (as per manufacturer's instructions) for 6 h at 37 °C. After 6 h, the 96 well plate with PBMCs were centrifuged to transfer 75 ul of supernatants to a fresh 96 well plate. The supernatants (25 ul) were analyzed by Luminex EMD Millipore Human High Sensitivity T Cell Panel (Catalog # HSTCMAG28SPMX21) on a FlexMap 3D instrument. The following analytes were quantified: Fractalkine, GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17A, IL-21, IL-23, I-TAC, MIP-1 α , MIP-1 β , MIP-3 α and TNF- α along with Assay Chex control beads: CHEX1, CHEX2, CHEX3, CHEX4 (Radix Bio-Solutions, Georgetown, Texas). After removal of the supernatants, cells remaining in the 96 well plate were checked for viability using a TC20 cell counter with trypan blue to assess total cell count in each well. Two tailed, non-parametric t-tests (GraphPad Prism, version 9.1.0) were used for statistical comparisons for cytokine measurements with and without Tempol. Graphs depict the mean MFI of duplicate wells and the standard error of the mean (SEM). $P < 0.05$ was considered significant. The severity of COVID-19 donors is based on NIH Treatment Guidelines [7,21].

3. Results

Demographics and disease characteristics of the donor PBMCs from 7 convalescent COVID-19 patients and 7 healthy controls are shown in Table 1. Donors were roughly matched for age and sex, and COVID-19 convalescent donors ranged from mild to severe disease.

The effect of increasing in-vitro doses of Tempol on T cell secreted cytokines (CD3 + CD28) is shown in Fig. 1a from both convalescent COVID donors (red) and normal donors (blue) for each donor. Statistical analysis revealed significant decreases ($P < 0.05$) in the secretion of cytokines from stimulated T cells (CD3 + CD28 stimulated) with increasing doses of Tempol with at least two significant concentrations of Tempol compared to no Tempol included GM-CSF, IFN- γ , MIP-3 α , IL-13, IL-10, IL-17A, IL-4, IL-2, IL-1beta, IL-6 and TNF- α . The effect of increasing in-vitro doses of Tempol on APC secreted cytokines (LPS) is shown in Fig. 1b from both convalescent COVID donors (red) and normal donors (blue). Statistical analysis revealed significant decreases ($P < 0.05$) in the secretion of multiple cytokines from stimulated APCs (LPS stimulated) with increasing doses of Tempol. Cytokines for which at least two concentrations of Tempol were significantly decreased from

Table 1

Demographic and disease characteristics data of the fourteen PBMC donors: seven convalescent COVID-19 patients and seven healthy controls, NA- Not applicable.

Assay ID	Age	Gender	Race	Ethnicity	Time since initial diagnosis (days)	Severity at diagnosis
163	27	Female	White	Hispanic or Latino	146	Mild
170	74	Male	White	Not Hispanic or Latino	99	Mild
174	26	Female	White	Not Hispanic or Latino	64	Mild
147	55	Male	White	Hispanic or Latino	105	Moderate
153	57	Male	White	Not Hispanic or Latino	166	Mild
158	50	Female	White	Not Hispanic or Latino	76	Mild
149	64	Male	White	Not Hispanic or Latino	41	Mild
88		Female			NA	Healthy
63		Male			NA	Healthy
2236	30–50	Male	Race or ethnic group	was not reported by	NA	Healthy
20	age	Female		Stanford Blood Bank	NA	Healthy
01	group	Male			NA	Healthy
243		Female			NA	Healthy
2095		Male			NA	Healthy

no Tempol included IL-1beta, GM-CSF, MIP-3 α , IL-10, IL-2, IL-6, and IL-21. Other cytokines measured in this assay appeared to be unaffected by pre-treatment with Tempol or were not stimulated in this assay (Fractalkine, IL-5, IL-7, IL-8, IL-12, IL-21, IL-23, I-TAC, and MIP-1beta). In general, the suppression of cytokines by Tempol was restricted to activated cells except for IL-2, IL-10, IL-13, IFN- γ and GM-CSF, which showed some decrease of low concentrations of cytokines with Tempol even in the absence of stimulation (Fig. 2). No appreciable differences were observed for the effect of Tempol on activated PBMCs comparing those from convalescent COVID-19 patients (red) versus normal (blue) donors, although the numbers of subjects make statistical comparison difficult.

To ensure that decreases in cytokine production observed with Tempol were not simply due to toxicity, we checked cell viability at the end of the incubation period. Differences in cell viability with increasing doses of Tempol are shown in Fig. 3. While some decrease in viability was seen at the highest Tempol dose, the viability changes did not appear sufficient to account for the level of cytokine effect. A summary of the effects of Tempol on activated APCs and T cells as well as unstimulated cells is shown in Table 2. There is a trend for COVID-19 convalescent subjects to have lower cytokine production in the presence and absence of Tempol. This was statistically significant ($p < 0.05$, unpaired Mann-Whitney test) for certain cytokines with either LPS or CD3/CD28 stimulation (Supplementary Table 1). This difference was independent of the suppressive effect of Tempol, which was seen in both control and COVID-19 convalescent subjects.

4. Discussion

Cytokines play an important role in the immunopathogenesis of COVID-19 and the level of inflammatory cytokines in the plasma have been shown to be independent predictors of mortality and morbidity (2). Indeed, the measurement of cytokines in-vitro may have a role as important prognostic biomarkers. The cytokine release syndrome also known as cytokine storm observed with COVID-19 has also been

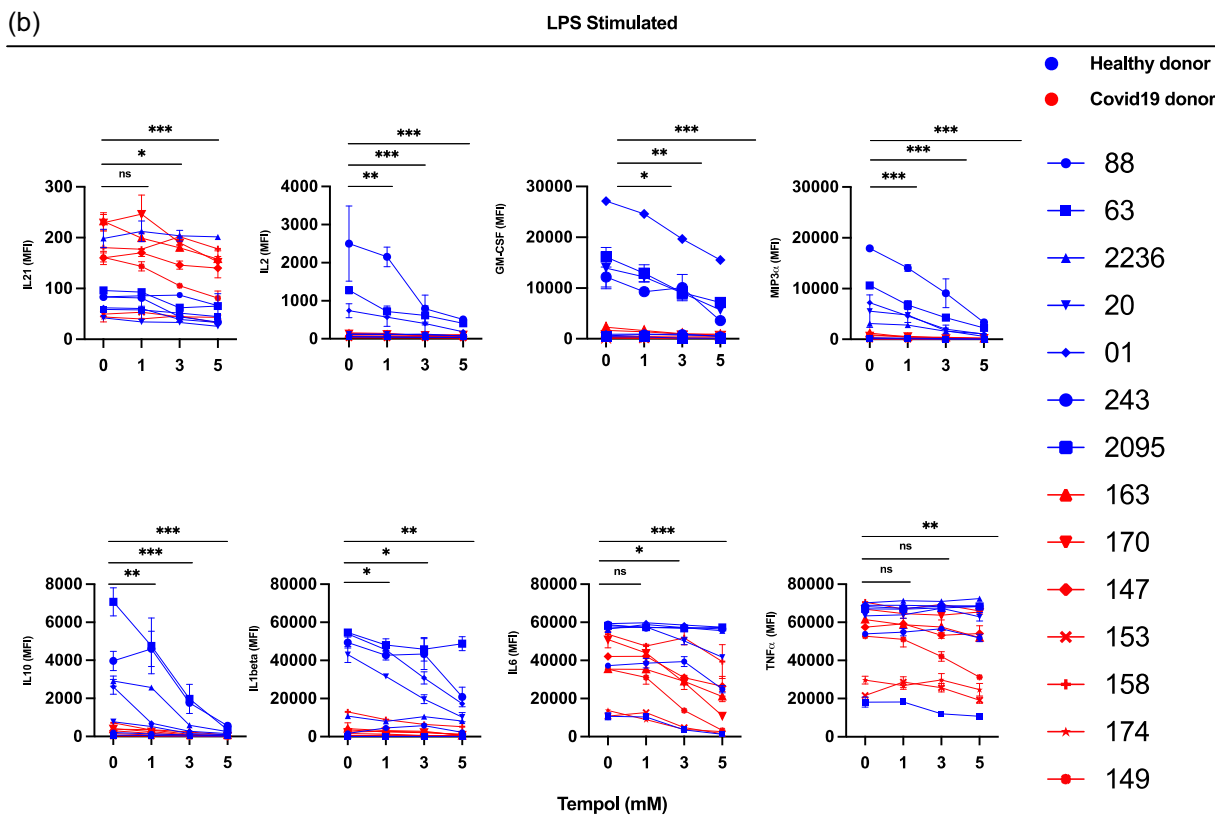
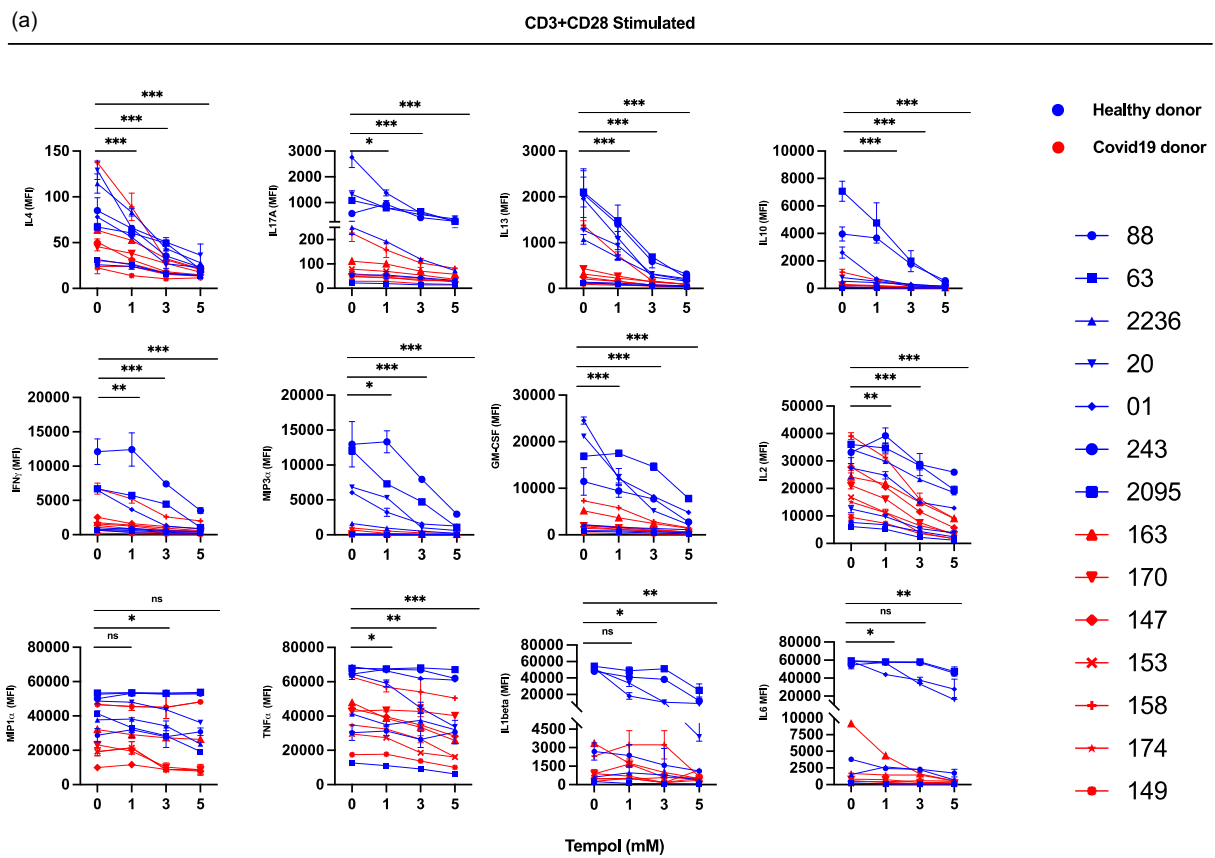


Fig. 1. a. T cells secreted cytokines (CD3 + CD28 stimulated) from both convalescent COVID-19 donors (red) and normal donors (blue). Results are depicted as the mean of duplicates with standard error of the mean error bars. * = $p < 0.05$, ** = $P < 0.01$, *** $p \leq 0.001$. Fig. 1b APC secreted cytokines with LPS stimulation from convalescent COVID-19 donors (red) and normal donors (blue). Results are depicted as the mean duplicate with standard error of the mean error bars. * = $p < 0.05$, ** = $P < 0.01$, *** $p \leq 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

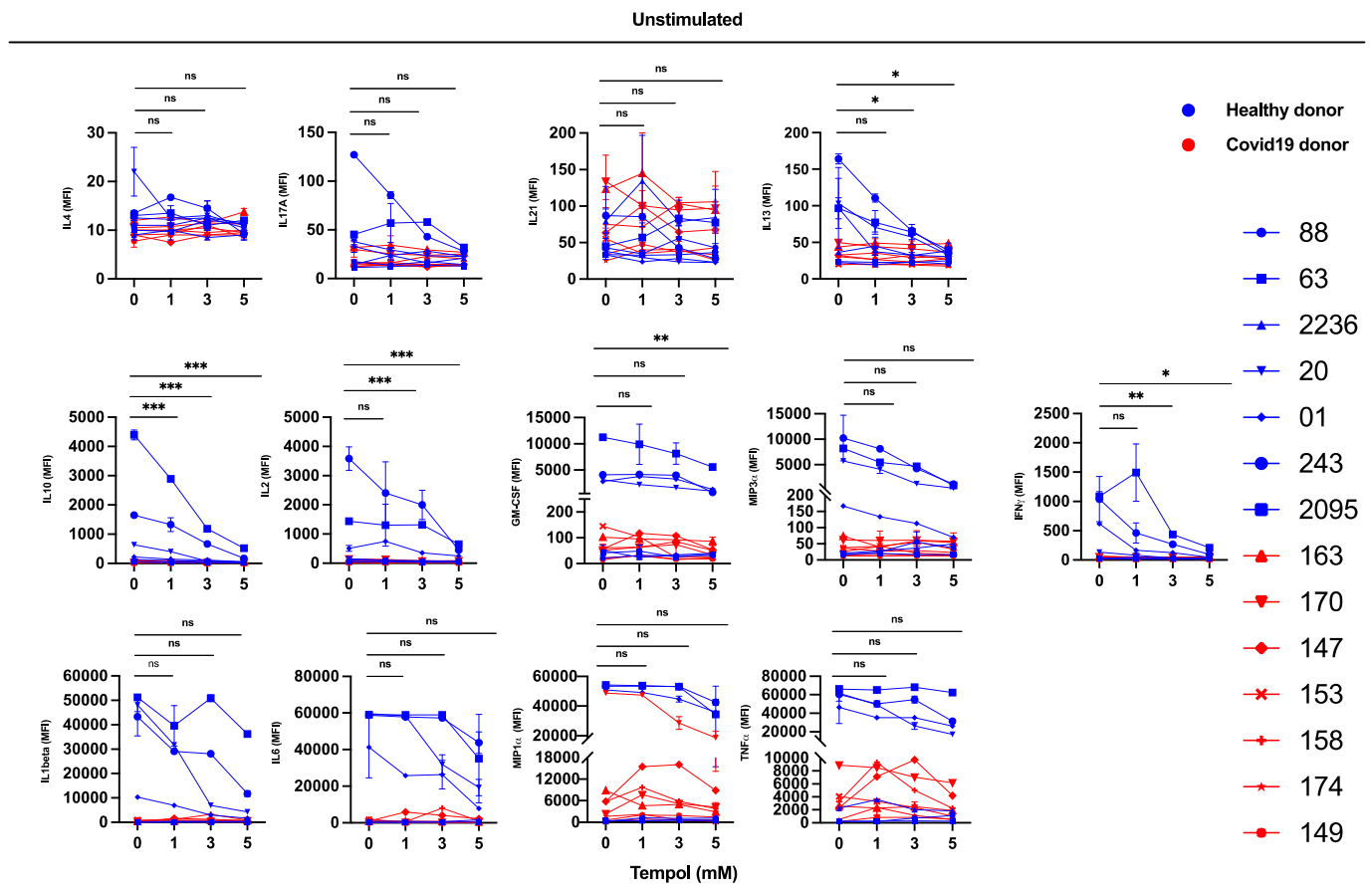


Fig. 2. Secretion of cytokines from unstimulated cytokines. Results are depicted as the mean of duplicate with standard error of the mean error bars. * = $p < 0.05$, ** = $P < 0.01$, *** $p \leq 0.001$.

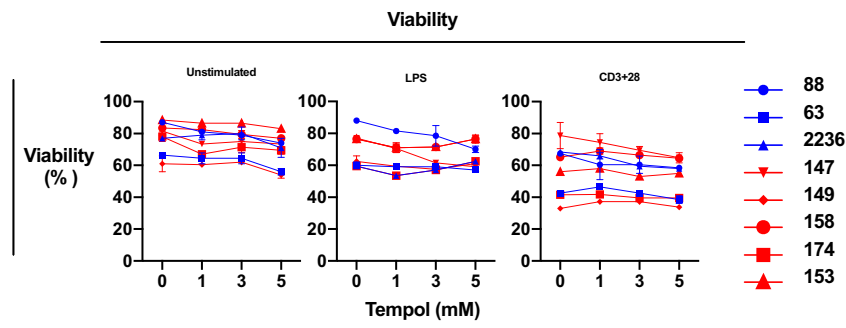


Fig. 3. Percent cell viability observed with increasing doses of Tempol. Counts were obtained using a TC20 (BioRad) cell counter.

observed with immune therapies for cancer including chimeric antigen receptor (CAR) therapy and after infusion of muromonab-CD3 therapy (OKT3) [22]. Thus, the use of the in-vitro immune assay developed in this study may be useful biomarkers to examine cytokine release for cancer immunotherapy as well as other anti-cytokines therapies for COVID-19.

In this cross-sectional study, Tempol, a unique antioxidant, was examined in-vitro with activated immune cells from both COVID positive and COVID negative donors. The results suggest that pre-incubation with Tempol with activated T cell or APCs inhibited the release of multiple cytokines in both donor populations. The broad immune suppressive activity of Tempol appeared to target mostly activated rather than un-activated cells.

A number of single anti-cytokine agents are currently in clinical development for the treatment of COVID-19 including anti-IL6 and anti-TNF [23,24]. Of note, we observed that pre-incubation with Tempol decreased the secretion of multiple cytokines secreted from activated immune cells. The inhibition of secreted cytokines from pre-incubation with Tempol was broad, dose-dependent, and included cytokines such as IL-6, IL-1beta, IFN- γ , IL-2, beta chemokines, IL 17, IL-13, IL-10, IL-4, and GM-CSF. In addition, it appears that Tempol in-vitro inhibited release of cytokines from both APCs and T cells, both of which play a role in the immunopathogenesis of COVID19. Thus, the ability to inhibit broader cytokines may have additional therapeutic utility compared to treatment with a single anti-cytokine in COVID-19 patients. Interestingly, except for four cytokines (IL2, IL-10, IL-13, IFN- γ and GM-CSF), Tempol

Table 2

The suppressive effect of Tempol on stimulated T cell cytokines (CD3 + CD28 stimulated), stimulated APC cytokines (LPS Stimulated), and unstimulated cytokines. ✓ = response and ✗ = No response.

Cytokine	LPS	CD3 + CD28	Unstimulated
GM-CSF	✓	✓	✗
IL-1beta	✓	✓	✗
IL-2	✓	✓	✓
IL-6	✓	✓	✗
IL-10	✓	✓	✓
IL-21	✓	✗	✗
TNF-α	✓	✓	✗
MIP-3α	✓	✓	✗
MIP-1α	✗	✓	✗
IFN-γ	✗	✓	✗
IL-4	✗	✓	✗
IL-13	✗	✓	✗
IL-17A	✗	✓	✗
IL-5	✗	✗	✗
IL-7	✗	✗	✗
IL-8	✗	✗	✗
IL-12 (p70)	✗	✗	✗
IL-23	✗	✗	✗
I-TAC	✗	✗	✗
MIP-1beta	✗	✗	✗
Fractalkine	✗	✗	✗

did not significantly suppress cytokines from unstimulated cells.

This is the first published study that we know of that has examined the effects of Tempol in-vitro on immune cells from COVID patients. Although the patient's cells examined herein were convalescing from SARS-CoV-2 infection, long-term effects of COVID-19 have now been described [25]. Other studies are warranted to examine the in-vitro effects of Tempol in patients with active COVID-19 infection. A recent study examined the effects of Tempol on iron-cofactors required for replication of SARS-CoV-2 [25]. Tempol was shown to disassemble iron cofactors sites, inhibiting the RNA dependent RNA polymerase and blocked virus replication in cell culture. Therefore, it may be speculated that Tempol may have both antiviral and anti-inflammatory activity against COVID-19. Furthermore, clinical studies examining the potential protective effects of oral tempol in patients with early COVID-19 infection are planned and should provide some insights into the utility of Tempol to limit cytokine release in the aberrant immune response to SARS-CoV-2 as well as antiviral activity.

There are many limitations of this study including the small sample size of COVID-19 patients and normal donors and limited clinical information on donors. Most of the donors in this study had mild COVID-19 infection. A longitudinal study is warranted to examine the effects of Tempol over time in different stages of COVID-19 infection. However, these results suggest that Tempol has strong, broad in-vitro anti-cytokine activity. Suppression of inflammatory cytokines with an antioxidant may be a beneficial strategy in earlier COVID-19 infection. These preliminary results support additional studies of Tempol in COVID-19 as well as other inflammatory conditions.

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Disclosures

Kavita Mathi, Yael Rosenberg-Hasson and Holden Maecker have no conflict of interest.

Dennis J Carlo and Ronald B Moss are employees and shareholders or own stock of Adamis Pharmaceuticals

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2021.108828>.

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