

1 **Title:** Targeting of the NLRP3 Inflammasome for early COVID-19

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15
16 **Abstract**

17
18 Following entry and replication of Severe Acute Respiratory Syndrome-coronavirus-2 (SARS-
19 CoV-2) into ACE2 expressing cells, the infected cells undergo lysis releasing more virus but also
20 cell contents. In the lung, constitutive cytokines such as IL-1 α are released together with other cell
21 contents. A cascade of inflammatory cytokines ensues, including chemokines and IL-1 β ,
22 triggering both local as well as systemic inflammation. This cascade of inflammatory cytokines in
23 patients with COVID-19 is termed “Cytokine Release Syndrome” (CRS), and is associated with
24 poor outcomes and death. Many studies reveal that blocking IL-1 activities in COVID-19 patients
25 reduces disease severity and deaths. Here we report highly significant circulating levels of IL-1 β ,
26 IL-1 Receptor antagonist, IL-6, TNF α , IL-10 and soluble urokinase plasminogen activator receptor
27 in COVID-19 patients with mild or no symptoms. We also report that in circulating myeloid cells
28 from the same patients, there is increased expression of the NOD-, LRR- and pyrin domain-
29 containing 3 (NLRP3) early in the infection. We observed increased NLRP3 gene expression in
30 myeloid cells correlated with IL-1 β gene expression and also with elevated circulating IL-1 β
31 levels. We conclude that early in SARS-CoV-2 infection, NLRP3 activation takes place and
32 initiates the CRS. Thus, NLRP3 is a target to reduce the organ damage of inflammatory cytokines
33 of the CRS.

34
35 **Introduction.**

36
37 Severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) infections manifest as
38 acute lung injury and increased inflammatory response known as coronavirus disease 2019
39 (COVID-19). Patients with severe symptoms are characterized by unusually high serum
40 inflammatory cytokine levels leading to cytokine release syndrome (CRS) (Del Valle et al. 2020,
41 Lucas et al. 2020) that ultimately results in respiratory distress syndrome and multi-organ failure.
42 CRS contributes to and can be causal in COVID-19 however, the mechanism(s) for initiation of
43 CRS in COVID-19 remains unknown. Numerous trials comparing standard of care in control
44 patients as well as case reports have administered the IL-1 Receptor antagonist (IL-1Ra) anakinra
45 in modest to severe COVID-19 patient, although there are at present no randomized trials.

46 Emerging from these reports is the concept that targeting of IL-1 result in improved outcomes,
47 including deaths. For example, high doses of anakinra reduces deaths as well as number of days
48 in the hospital (Cauchois et al. 2020, Cavalli et al. 2020, Huet et al. 2020). Anakinra has also been
49 administered in less severe hospitalized patients and resulted in similar reduction in disease
50 (Kyriazopoulou et al. 2020). Since anakinra blocks the IL-1 Receptor, the efficacy of anakinra may
51 be due to reducing IL-1 α or IL-1 β . Other studies report that specifically targeting IL-1 β with the
52 neutralizing monoclonal antibody canakinumab also reduces outcomes.

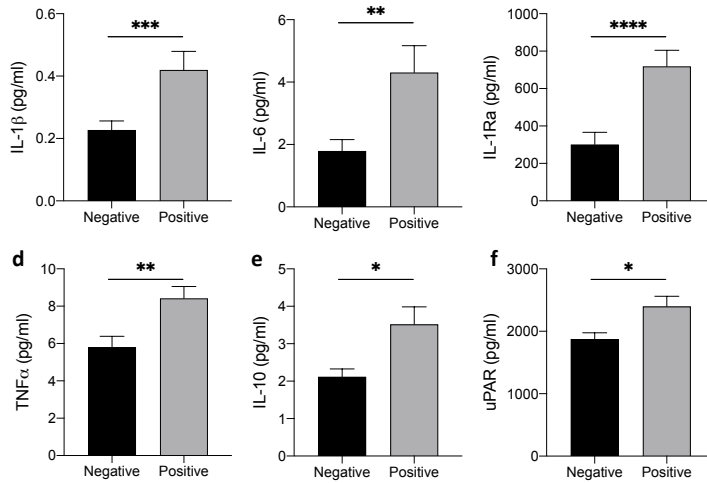
53 The intracellular processing of IL-1 β into its biologically active form is largely governed
54 by cytosolic macromolecular complexes termed inflammasomes (Franchi et al. 2009, Marchetti
55 2019). Notably, it has been observed that viral proteins of the SARS-CoV virus ORF3a, ORF8b
56 and Viroporin 3a activate the NLRP3 inflammasome (Chen et al. 2019, Shi et al. 2019, Siu et al.
57 2019). More recently, *in vitro* studies showed that also SARS-CoV-2 induces the NLRP3
58 inflammasome formation (Xu et al. 2020). Presence of NLRP3 inflammasome aggregates has also
59 been shown in the lungs of fatal COVID-19 pneumonia and in PBMCs and tissues of COVID-19
60 positive post-mortem patients upon autopsy (Rodrigues et al. 2021, Toldo et al. 2021). Notably,
61 *Rodrigues et al.* have been shown that SARS-CoV-2 virus can infect monocytes leading to the
62 NLRP3 inflammasome formation in these cells (Rodrigues et al. 2021). These studies confirm
63 activation of the NLRP3 inflammasome in COVID-19 in moderate to severe cases. The use of an
64 early treatment for non-hospitalized COVID-19 subjects in order to reduce the burden of
65 hospitalizations and intensive care units is clear (Kim, Read and Fauci 2020). Here we show
66 increased NLRP3 in non-hospitalized SARS-CoV-2 positive subjects. These data support the
67 rationale for early inhibition of NLRP3 to prevent inflammasome formation and the release of
68 IL-1 β in SARS-CoV-2 infection.

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71 **Results and Discussion.**

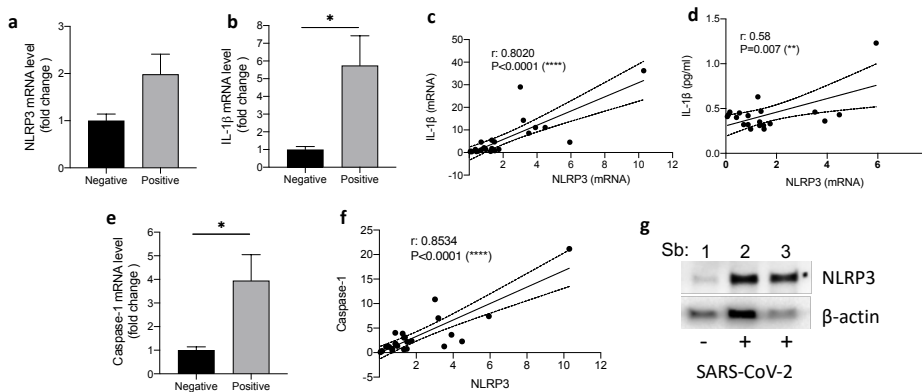
72 As shown in Figure 1a, compared to healthy controls, circulating IL-1 β is elevated in
73 ambulatory subjects positive for SARS-CoV-2. The mean level in 39 healthy subjects (0.23 pg/mL
74 \pm 0.03) matches the mean IL-1 β (0.33 pg/mL) in 500 healthy Dutch subjects (Ter Horst et al. 2016)
75 whereas in 24 COVID-19 subjects the mean level is 2-fold greater (0.42 \pm 0.06; p <0.001). Similarly,
76 mean IL-6 level in infected subjects is greater than 2-fold higher (Figure 1b, p <0.01). The naturally
77 occurring IL-1 Receptor antagonist (IL-1Ra) is 2.5-fold higher in the infected individuals
78 compared to healthy subjects (Figure 1c, p <0.0001). These data are consistent with those of non-
79 infectious autoinflammatory diseases where IL-6 and IL-1Ra are induced by IL-1 β and commonly
80 used as surrogate cytokines when circulating levels of IL-1 β are exceedingly below most detection
81 assays. Also shown in Figure 1 are significantly elevated levels of tumor necrosis factor alpha
82 (TNF α), IL-10 and urokinase plasminogen activator receptor (uPAR). Plasma uPAR is elevated

83 in COVID-19 patients and levels positively correlate with poor outcomes (Kyriazopoulou et al.
84 2020).



85 **Figure 1.** Increased circulating cytokines in early COVID-19. (a-f) Mean \pm SEM of plasma IL-1 β
86 (a), IL-6 (b), IL-1Ra (c), TNF α (d), IL-10 (e) and uPAR (urokinase plasminogen activator receptor) in
87 SARS-CoV-2 positive patients (N=39) compared to SARS-CoV-2 negative (N=24). * p <0.05; ** p <0.01;
88 *** p <0.001; **** p <0.0001.

89
90 Since SARS-CoV-2 primes NLRP3 (Rodrigues et al. 2021, Xu et al. 2020), we anticipated
91 an increase in mRNA levels coding for NLRP3 with SARS-CoV-2 infection. We observed a 2-fold
92 increase in NLRP3 levels in buffy coat cells from 27 positive subjects compared to cells from 14
93 non-infected subjects (Figure 2a). In the same cells, there was a 5.5-fold increase in IL-1 β gene
94 expression (Figure 2b). As shown in Figure 2c, NLRP3 and IL-1 β mRNA levels are highly
95 correlated ($r=0.8$, p <0.001) and circulating IL-1 β is also positively correlated with NLRP3 gene
96 expression (Figure 2d). Caspase-1 gene expression is elevated 4-fold (Figure 2e), which also
97 correlated with NLRP3 (Figure 2f $r=0.8$, p <0.001). Thus, in COVID-19 patients the molecular
98 cascade resulting in elevated circulating IL-1 β and NLRP3 gene expression is initiated by the
99 infection however, critical for COVID-19 disease is the processing and release of active IL-1 β .
100 Using Western blotting, we show evidence of this concept with NLRP3 protein in monocytes from
101 two infected subjects (Figure 2g), but not in cells from an uninfected subject.



102 **Figure2.** Increased NLRP3 levels in early COVID-19. (a,b) Fold change of NLRP3 (a) and IL-1 β
103 (b) mRNA levels from buffy coats of SARS-CoV-2 positive patients (N=27) compared to SARS-CoV-2
104

105 negative (N=14) (Wilcoxon signed-rank test). (c) Correlation between NLRP3 and IL-1 β gene expression
106 in SARS-CoV-2 positive patients. (d) Correlation between NLRP3 (gene expression) and circulating levels
107 of IL-1 β (pg/ml) in SARS-CoV-2 positive patients. (e) Fold change of caspase-1 mRNA levels from buffy
108 coats of SARS-CoV-2 positive patients (N=27) compared to SARS-CoV-2 negative (N=14) (Wilcoxon
109 signed-rank test). (f) Correlation between NLRP3 and caspase-1 gene expression in SARS-CoV-2 positive
110 patients. (g) NLRP3 protein levels in monocytes isolated from two SARS-CoV-2 positive patients compared
111 to a SARS-CoV-2 negative subject. *p<0.05; **p<0.01; ****p<0.0001.

112
113 The morbidity and mortality of COVID-19 often takes place later in the disease when
114 SARS-CoV-2 RNA is absent in secretions as disease worsens and is associated with marked
115 increases in biomarkers of the CRS (Cauchois et al. 2020). Thus, the CRS in COVID-19 is
116 indicative of the organ damaging properties of IL-1 β and its downstream cytokines. In the lung,
117 COVID-19 pneumonia is similar to that of Acute Respiratory Disease Syndrome observed in
118 influenza or sepsis. The use of anakinra or canakinumab in COVID-19 disease is associated with
119 recovery and survival. However, it is possible to reduce the detrimental properties of IL-1 β and its
120 downstream cytokines by first preventing the processing and release of active IL-1 β . The present
121 data provide a rationale to treat patients infected with SARS-CoV-2 early in the course of the
122 disease using a specific NLRP3 inhibitor in order to arrest the progression of IL-1 β -mediated CRS.
123 Such a treatment offers an opportunity to reduce hospitalization and the need for supplemental
124 oxygen, particularly in subjects with high risk co-morbidities. At the present time, OLT1177 (rINN
125 dapansutrile) is the only orally active specific NLRP3 inhibitor (Marchetti et al. 2018) that has
126 shown efficacy in two inflammatory diseases, acute gout flares and heart failure (Kluck et al. 2020,
127 Wohlford et al. 2020).

128 Recent studies have demonstrated that oral administration of colchicine in 4159 non-
129 hospitalized COVID-19 PCR positive patients reduced a composite end-point of hospitalizations
130 and death in 4.6% of treated patients compared to 6.0% of placebo-treated subjects (p<0.04)
131 (Tardif et al. 2021). Colchicine treatment also reduces the risk of cardiovascular events (Nidorf et
132 al. 2020). The mechanism by which colchicine is effective in coronary artery disease as well as in
133 COVID-19 is likely due to reduce IL-1 β -mediated inflammation. However, colchicine does not
134 directly inhibit NLRP3 (Hoss and Latz 2018). Unlike specific NLRP3 inhibitors, colchicine affects
135 integrins, cell migration and microtubule assembly.

136 A significant advantage of targeting the NLRP3 inflammasome is the ability to reduce IL-18
137 processing. Therefore, specific NLRP3 inhibitors could be used to treat the Macrophage Activation
138 Syndrome (MAS)-like disease in COVID-19, where IL-18 plays a pathological role. Elevated
139 circulating IL-18 correlated with disease severity and poor outcomes in COVID-19 patients
140 (Rodrigues et al. 2021, Lucas et al. 2020). IL-18 is characteristically elevated in non-COVID-19
141 MAS (Mazodier et al. 2005). Several case series reports that a MAS-like disease develops in
142 COVID-19 patients with markedly elevated levels of D-dimer, which is indicative of MAS in
143 COVID-19 (Aouba et al. 2020). Specific NLRP3 inhibition will reduce both IL-1 β as well as IL-18
144 and thus targets two agonists of COVID-19 disease.

145 146 **Methods**

147
148 **PBMCs.** Peripheral blood mononuclear cells (PBMCs) were isolated from drawn blood by
149 gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were
150 suspended in Roswell Park Memorial Institute 1640 medium supplemented with 50 μ g/mL
151 gentamicin, 2 mM glutamine, and 1 mM pyruvate and cultured for 24 hours.

152

153 **Cytokines measurements.** Plasma levels of IL-1 β , IL-6, IL-10 and TNF α were measured with
154 the Ella platform (Protein Simple, San Jose, CA, USA) using multiplex cartridges. Soluble uPAR
155 was determined using Quantikine ELISA (R&D Systems, Minneapolis, MN, USA).

156

157 **Gene expression.** RNA was isolated according to the manufacturer's protocol (Thermo Fisher
158 Scientific) and synthesized into cDNA using SuperScript III First-Strand (Thermo Fisher
159 Scientific). Quantitative PCR (qPCR) was performed on cDNA using Power SYBR Green PCR
160 master mix (Thermo Fisher Scientific) on Bio-Rad (Bio-Rad Laboratories, Hercules, CA) CFX96
161 Real time system. Gene expression was carried for the following mRNAs: *nlrp3*, *caspase1* and
162 *illb* with *gapdh* used as reference gene using the following primers:

163 *nlrp3*: GAATCTCAGGCACCTTTACC and GCAGTTGTCTAATTCCAACACC

164 *caspase1*: AAGTCGGCAGAGATTTATCCA and GATGTCAACCTCAGCTCCAG

165 *illb*: CTAAACAGATGAAGTGCTCCTTCC and CACATAAGCCTCGTTATCCCA

166

167 **Western blotting.** Cells were lysed using RIPA buffer supplemented with protease inhibitors
168 (Roche), centrifuged at 13,000 g for 20 min at 4°C and the supernatants were obtained. Protein
169 concentration was determined in the clarified supernatant using Bio-Rad protein assay (Bio-Rad
170 Laboratories, Hercules, CA). Proteins were electrophoresed on Mini-PROTEAN TGX 4–20%
171 gels (Bio-Rad) and transferred to nitrocellulose 0.2 μ M (GE Water & Process Technologies).
172 Membranes were blocked in 5% dried milk in PBS-T 0.5% for 1 hour at room temperature. Primary
173 antibodies for NLRP3 1:1000 (Adipogen) was used in combination with peroxidase-conjugated
174 secondary antibodies and chemiluminescence to detect the protein concentration. A primary
175 antibody against β -actin (Santa Cruz Biotechnology) was used to assess protein loading.

176

177 **Statistical analysis**

178 Significance of differences was evaluated with Student's two-tail T-test using GraphPad Prism
179 (GraphPad Software Inc, La Jolla, CA) or Wilcoxon signed-rank test as indicated. For the
180 correlation studies the distribution were computed using Pearson correlation coefficients and
181 Statistical significance was calculated with two-tailed option with the confident interval set at 95%.
182 Statistical significance was set at $p < 0.05$.

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