Classical Drug Digitoxin Inhibits Influenza Cytokine Storm, With Implications for Covid-19 Therapy

BETTE S. POLLARD[1*], JORGE C. BLANCOl[2*] and JOHN R. POLLARD[3]

[1]*Silver Pharmaceuticals, Rockville, MD, U.S.A.;*
[2]*Sigmovir Biosystems, Rockville, MD, U.S.A.;*
[3]*Department of Neurology, University of Pennsylvania,*
Philadelphia PA (USA) and Christiana Care Epilepsy Center, Newark, DE, U.S.A.

Abstract. *Background/Aim: Influenza viruses, corona viruses and related pneumotropic viruses cause sickness and death partly by inducing cytokine storm, a hyper-proinflammatory host response by immune cells and cytokines in the host airway. Based on our in vivo experience with digitoxin as an inhibitor of TNFα-driven NFκB signaling for cytokine expression in prostate cancer in rats and in cystic fibrosis in humans, we hypothesize that this drug will also block a virally-activated cytokine storm. Materials Methods: Digitoxin was administered intraperitoneally to cotton rats, followed by intranasal infection with 107TCID50/100 g of cotton rat with influenza strain A/Wuhan/H3N2/359/95. Daily digitoxin treatment continued until harvest on day 4 of the experiment. Results: The cardiac glycoside digitoxin significantly and differentially suppressed levels of the cytokines TNFα, GRO/KC, MIP2, MCP1, and IFNγ, in the cotton rat lung in the presence of influenza virus. Conclusion: Since cytokine storm is a host response, we suggest that digitoxin may have a therapeutic potential not only for influenza and but also for coronavirus infections.*

Influenza, corona and related pneumotropic viruses cause sickness and death partly by inducing a hyper-proinflammatory immune response in the host airway. This immune overreaction, called a cytokine storm, can lead to

multiorgan failure and death (1). For example, Influenza A (H5N1) has been shown to activate the TNFα-driven NFκB signaling pathway in a mouse host during viral infection, generating a massive overproduction of cytokines, including interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP1), known as cytokine storm (2). As anticipated, inhibitors of NFκB acutely suppress cytokine storm and increase survival in a mouse model of SARS-CoV infection (3). Recent data show that COVID-19 also activates NFκB (4). Cytokine storm marks the airways of SARS-CoV-2-infected patients that were admitted to the Intensive Care Unit (ICU) with more severe disease (5). Since there are multiple strains of influenza as well as coronavirus, there might be an advantage to develop therapies that suppress host-induced cytokine storm, in addition to developing strain-specific vaccines.

The clinical problem is that there are limited options for treating respiratory cytokine storm, most of which are predicated on inhibiting NFκB-activated cytokine expression (6-8). The absence of NFκB inhibitory drugs from the human formulary is due to most candidate drugs being either neurotoxic or nephrotoxic when administered chronically (9). One drug that lacks these toxicities is the cardiac glycoside digitoxin. We have previously shown digitoxin to be amongst the most potent inhibitors of the proinflammatory TNFα/NFκB pathway in the human airway and in other epithelial cells, both *in vitro* (10) and *in vivo* (11-13). Corroborating this discovery is a screen of 2800 drugs and bioactive compounds which found digitoxin to be one of the most potent inhibitors of TNFα-driven–NFκB activity (14). Digitoxin is a drug that has been used to treat heart failure for decades, and a clinical trial (11) demonstrated that in addition to giving it to people with heart failure or heart arrythmias, it is also safe to give digitoxin for diseases like cystic fibrosis to children and adults with normal hearts who need to have reduction of lung inflammation (15). Digitoxin was also recently shown to block MERS-CoV infectivity *in*

vitro (16). The digitoxin analogues digoxin and ouabain also block SARS-CoV-2 infectivity *in vitro* (17). In a clinical trial where digitoxin was administered to young adults with the proinflammatory lung disease cystic fibrosis, it proved safe. This clinical trial also showed that "the mRNAs encoding chemokine/cytokine or cell surface receptors in immune cells were decreased in nasal epithelial cells, leading to pathway-mediated reductions in IL-8 and IL-6 levels, lung epithelial inflammation, neutrophil recruitment and mucus hypersecretion" (11).

To further test the ability of digitoxin to inhibit cytokine storm in related pneumotropic viruses, we used the cotton rat model of influenza infection to investigate the effects of digitoxin in influenza-associated cytokine storm. The cotton rat model has the important advantage of susceptibility to influenza infection without engineered adaptation (18). Based on mRNA changes in an experiment from the same laboratory that we used, when the cotton rats were given only the influenza A H3N2 virus but no drug, the cytokine levels were shown to increase in the cotton rat lung from baseline 10-fold for tumor necrosis factor alpha (TNFα), 40-fold for interferon gamma (IFNγ), 10-fold for growth-regulated oncogene/ keratinocyte chemoattractant (GRO/KC), and 35-fold for macrophage inflammatory protein-2 beta (MIP1β) (19). Consistently, there is a close relationship between mRNA and protein changes for cytokine proteins (20). In addition, it has also been shown *in vivo* that in the presence of lipopolysaccharides (LPS), GRO/KC and MIP2 mRNAs increase 50-fold and 20-fold, respectively (21). Thus, cytokine levels in the absence of virus or other immune stimulant in the cotton rat lung are very low. Furthermore, it has been shown that the cytokine response of the cotton rat to this virus strain evokes a pattern of pulmonary cytokine changes that parallel the human response (19).

Materials and Methods

Animal protocol. Cotton rat experiments were performed as previously described (19). All experiments were performed using protocols that followed federal guidelines and were approved by the Institutional Animal Care and Use Committee. Animals were sacrificed by carbon dioxide inhalation.

Drugs and protocol for drug preparation. Digitoxin was obtained from Sigma-Aldrich (> 95% pure) (St. Louis, MO, USA). The drug was prepared as a stock solution in 95% ethanol. It was diluted into phosphate buffered saline (PBS) as the suspension solution from the stock solution. It was administered in a 200 μl volume. Digitoxin (0, 3, 10 and 30 μg per 100 g of body weight) was administered to 3 cotton rats in one dose intraperitoneally 6.25 h before intranasal infection with 107TCID50/100 g of cotton rat with influenza strain A/Wuhan/H3N2/359/95. This ensured drug availability at the time of virus infection, as distribution is complete within 4 to 6 h (22). Daily digitoxin treatment continued until harvest on day 4 of the experiment.

Tissue preparation and histological analysis. On day 4 post infection the animals were sacrificed by CO_2 inhalation. The left lung was first tied off and reserved for cytokine analysis. These lung samples were then immediately frozen on dry ice, and kept at –80˚C until further processed. The remaining lung tissue was processed for histological analysis. The right lung was inflated with 70% formalin and fixed for histology, as previously described (19). The frozen left lungs were then transferred to author BSP.

Later, sections were stained by Hematoxylin and Eosin (H&E) and immune cell density was automatically analyzed in brightfield mode on a Zeiss Axio-Scan.Z1 digital slide scanner (Carl Zeiss AG, Oberkochen, Germany). Whole lung images (2 sections from each lung) were analyzed using NIH-ImageJ. Briefly, the program was trained to identify immune cells as dark stained objects in the section that were the size of neutrophils. The per cent total area occupied by such objects was measured.

Biochemical analysis. Frozen lung samples were weighed, thawed and minced with scissors in 10% (w/v) ice cold PBS, homogenized with 10 strokes of a Ten Broeck homogenizer (Vavantor, Radnor, PA, USA), and centrifuged at $20,000 \times g$ for 30 min. The supernatant solutions were kept at –80˚C until assay. The supernatant solutions were brought to Bioassay Works (LLC, Ijamsville, MD, USA) by author BSP where they were tested for cytokines and chemokines by ELISA. The samples were then sent for corroboration to Pierce-Thermo (Waltham, MA, USA) for ELISA assay on the Searchlight® ELISA platform. Rat antibody reagents were used in both instances.

Statistics. All data are presented as means ± SEM from three animals for each digitoxin dose. The mean values for each cytokine were analyzed using one-way ANOVA followed by Dunnett's *post-hoc* comparisons of each dose of digitoxin *vs*. vehicle (0 μg). Differences were considered significant when $p<0.05$. Complete statistical data are summarized in Table I. The same method was used to compute the significance of histology-based differences between immune cell density at each dose of digitoxin *vs*. vehicle (0 μg) in Table II.

Results

Digitoxin blocks cytokine storm. As shown in Figure 1, protein data were collected for IFNγ, GRO/KC as the rodent equivalent of human IL-8, MIP2, chemokine (C-X-C motif) ligand 2 (CXCL2), TNFα, IL-1β, MCP1, and transforming growth factor beta (TGFβ). Figure 1 also shows the changes in cytokine protein in the lung due to digitoxin administration in the cotton rat following intranasal (IN) infection with a dose of 107TCID50/100 g of animal with influenza strain A/Wuhan/H3N2/359/95 virus. Animals were given three different doses of digitoxin, starting 6.25 h prior to virus administration and continuing with a daily dose until sacrifice on day 4. The dose range of digitoxin, 0-30 μg/100g cotton rat, was calculated (23) to approximate the human dose routinely used to treat heart failure (0.1 mg digitoxin, Merck KGaA, Darmstadt, Germany). As shown in Figure 1 and summarized in Table I, significant digitoxin-dependent reductions were found at >10 μg doses for 5 of the 7 cytokines. The digitoxin-

Table I. *Reduction of cytokine expression by digitoxin in cotton rat lungs*.

Cytokine	Digitoxin Dose, μg/100 g	N	Cytokine Concentrations, pg/ml			Mean	Std. Deviation	*p*-Value (Dunnett's)
IFNγ	0	3	**29.1**	**37.1**	**38.1**	**34.77**	**4.93**	
	3	3	13.7	25.0	34.6	24.43	10.46	0.159
	10	3	12.5	12.5	15.5	13.50	1.73	**0.007**
	30	3	7.3	12.5	12.5	10.77	3.00	**0.003**
GRO/KC	0	3	**22.0**	**29.2**	**32.4**	**27.87**	**5.33**	
	3	3	18.4	23.4	24.7	22.17	3.88	0.238
	10	3	8.9	14.6	16.1	13.20	3.80	**0.004**
	30	3	12.2	16.0	16.4	14.87	2.32	**0.008**
MIP2	0	3	**15.6**	**15.6**	**17.6**	**15.83**	**0.40**	
	3	3	12.4	14.1	15.5	14.00	1.55	0.421
	10	3	7.1	7.8	12.3	9.07	2.82	**0.002**
	30	3	10.6	10.7	10.9	10.73	0.15	**0.012**
TNFα	0	3	**74.6**	**76.5**	**80.9**	**77.33**	**3.23**	
	3	3	62.4	75.4	79.4	72.40	8.89	0.899
	10	3	28.2	33.4	54.9	38.83	14.15	**0.006**
	30	3	35.6	44.9	62.3	47.60	13.55	**0.025**
IL1β	0	3	**194.0**	**285.8**	**324.0**	**267.93**	**66.82**	
	3	3	106.7	224.4	239.3	190.13	72.64	0.534
	10	3	123.6	317.7	332.7	258.00	116.64	0.997
	30	3	125.2	203.5	226.2	184.97	52.99	0.489
MCP1	0	3	**4.5**	**8.1**	**8.8**	**7.13**	**2.31**	
	3	3	2.2	2.6	5.4	3.40	1.74	**0.049**
	10	3	1.6	1.6	1.6	1.60	0.00	**0.007**
	30	3	1.9	3.4	4.4	3.23	1.26	**0.040**
TGFβ	0	3	**1,167.1**	**1,192.5**	**1,192.5**	**1,184.07**	**14.61**	
	3	3	901.3	1,029.9	1,079.9	1,003.70	92.14	0.350
	10	3	806.1	1,095.2	1,122.1	1,007.80	175.19	0.366
	30	3	969.9	1,041.1	1364.0	1,125.00	210.02	0.923

Bold *p*-Values indicate statistical significance.

dependent reductions were specific and saturating for each cytokine, but did not reduce any of them to zero.

Digitoxin differentially affects cytokine expression. Table I shows that the most significant digitoxin-dependent reductions of cytokine proteins were found in IFNγ (68.9%), GRO/KC (46.6%), and MCP1 (54.9%). Smaller but still significant reductions in cytokine proteins were found in MIP2 (32.2%) and TNFα (38.4%). In the cases of IL-1β and TGFβ changes were not significant. Taken together, digitoxin, dose-dependently and significantly lowers the individual concentrations in the lung of at least five cytokines that had been induced by viral infection.

Digitoxin leaves immune cell density intact in virus-infected lung. Figure 2a and Figure 2b show low-power views of cotton rat lung sections, taken 4 days after intranasal virus administration (no digitoxin and 10 μg digitoxin/100 g, respectively). Changes in cytokines appear to saturate at a dose of 10 μg/100 g cotton rat. Regions of heavy hematoxylin staining, representing infiltration foci of

Table II. *Effect of digitoxin on immune cell density in cotton rats treated with influenza virus strain A/Wuhan/H3N2(359/95)**.

Digitoxin dose, μg/100g Cotton Rat	Total positive area (Sq μm)	% Positive area	*p*-Value (Dunnett's)
0 (n=6)	251,575,564.80	8.59	-
3 (n=6)	138,565,605.10	7.72	0.764
10 (n=6)	132,292,021.0	9.54	0.716
30 (n=5)	132,292,021.10	10.02	0.481

*Two sections from each of 3 Cotton Rat lungs were analyzed. One of the 2 sections from one lung treated with 30 μg/100 g digitoxin was damaged.

immune cells, are distributed in the lung. As previously described, pulmonary inflammatory changes can be seen in terms of peribronchitis (inflammatory cells clustered around the periphery of small airways), interstitial pneumonia (inflammatory cell infiltration and thickening of alveolar walls) and alveolitis (immune cells within the alveolar spaces) (19, 24).
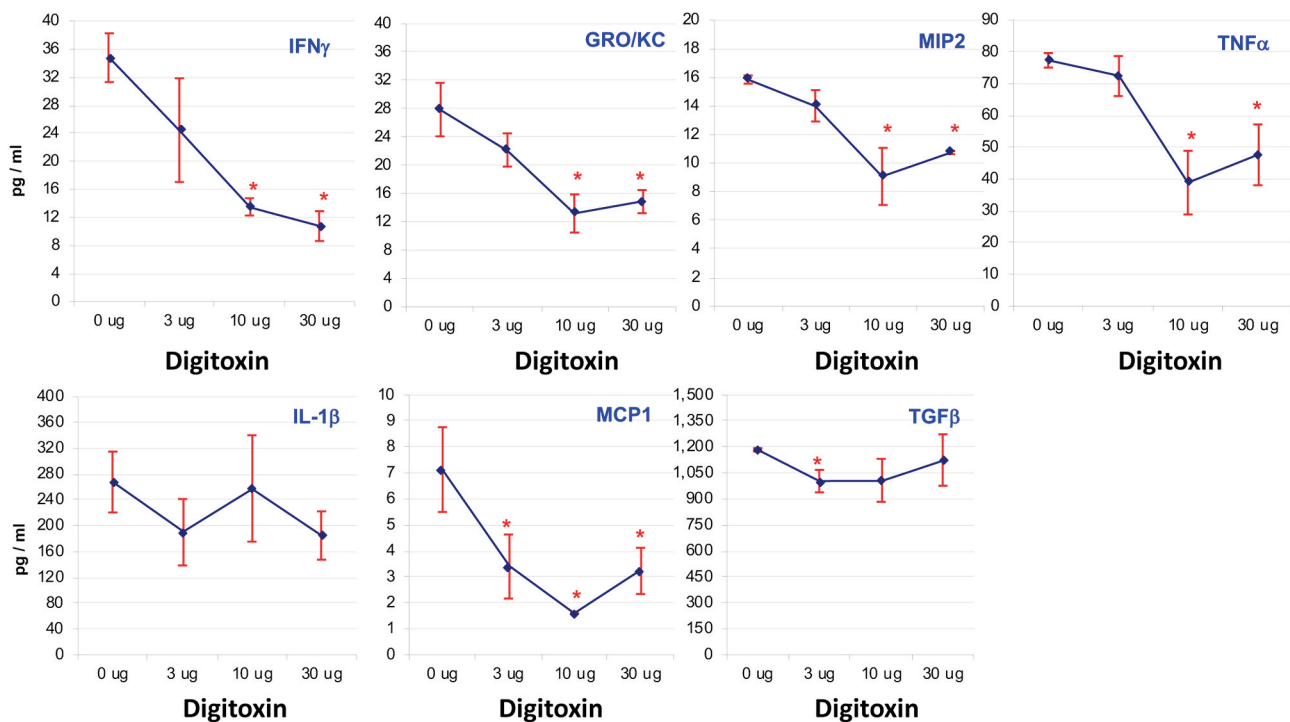
Figure 1. *Cytokine concentrations in lungs of cotton rats treated with digitoxin and influenza strain A/Wuhan/H3N2/359/95 virus. Animals were treated with different concentrations of digitoxin 6.25 hours before intranasal virus administration and daily thereafter for 4 days. Samples assayed were lung tissue. Digitoxin dose is in units of µg/100g. IFNγ: Interferon gamma; GRO/KC: growth-regulated oncogene/ keratinocyte chemoattractant; MIP2: macrophage inflammatory protein-2 ; CXCL2: chemokine (C-X-C motif) ligand 2; TNFα: tumor necrosis factor alpha; IL-1β: interleukin 1 beta; MCP1: monocyte chemoattractant Protein 1 (or CCL2); TGFβ: transforming growth factor beta. *p<0.05, N=3.*

Densities of immune cells were not affected by digitoxin. Table II shows that the positive area (%) occupied by immune cells in treated animals was not statistically different from the area occupied by immune cells in untreated, virus-infected animals. Thus, digitoxin appears to inhibit the cytokine storm host response to influenza A infection but does not significantly affect the density and distribution of immune cells as seen in the microscope on the 4th-day after infection.

Discussion

Our experimental results in Figure 1 and Table 1 show that administration of digitoxin to the cotton rat inhibits expression of five cytokines in the lung in the presence of influenza strain A/Wuhan/H3N2/359/95. These cytokines include TNFα, the key activator of the TNFα–driven NFκB inflammation pathway. The data also show that digitoxin inhibits cytokine storm, but does not appear to significantly affect the density of immune cells in the lung four days after viral infection. The data further suggest that digitoxin acts on multiple cell types (25). For example, IFNγ is secreted only from activated T lymphocytes and NK cells of the

immune system 5). The remainder of the cytokines are secreted by epithelial cells in the airway, as well as by endothelial cells, immune cells and others (26, 27). GRO/KC [CXCL1, the rodent equivalent of human IL-8 (28)], and MIP2 are a key target of NFκB signaling and are major chemoattractants for neutrophils (29). MIP1 induces entry and accumulation of monocytes and macrophages into the lung, and are targets of NFκB (29). TGFβ is indirectly dependent on NFκB-signaling and indirectly drives NFκB (12). IL-1β also drives NFκB, albeit not through TNFα. The data from Figure 1 and Table I support the interpretation that digitoxin-dependent reduction in TNFα-driven NFκB signaling may be sufficient to suppress influenza A-associated cytokine storm.

The reduction of influenza A-driven TNFα expression by digitoxin is specifically relevant to what is known regarding the mechanism of influenza A virus RNA production and propagation (30). For example, TNFα drives NFκB activation and signaling by host NFκB, which has been shown to be a prerequisite for influenza virus infection (30-32). Knockdown of host NFκB_p65 has also been found to reduce influenza virus replication and vRNA synthesis (30).
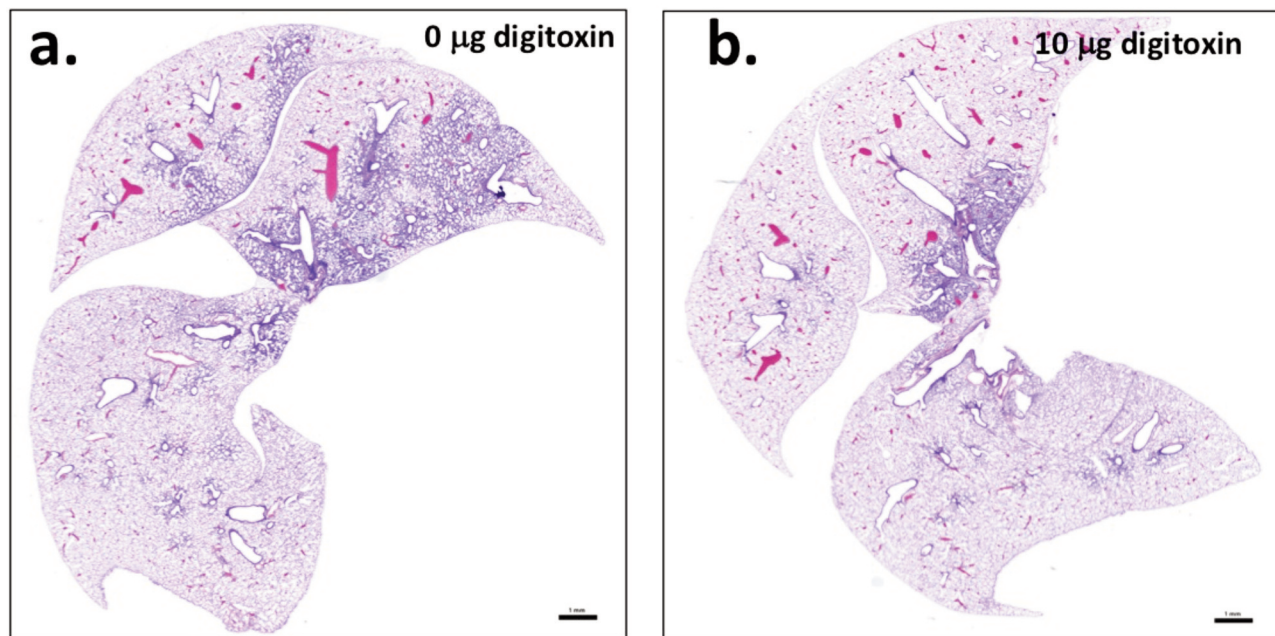
Figure 2. *Histology of cotton rat lungs following infection with influenza strain A/Wuhan/H3N2/359/95 and treatment for 4 days with digitoxin. (a) Low power image of infected, control cotton rat lung (magnification 10×). (b) Low power image of infected cotton rat lung treated with 10 µg/100g digitoxin for 4 days. Analysis shows that the density of immune cells, marked by the NIH ImageJ program, is not statistically different when comparing all treatment conditions from control (also see Table II). Scale bar=1 mm.*

This relationship appears to be dependent on viral genes because mouse-adapted descendants of the avian Influenza A strain H7N7 can be genetically engineered to function independently of NFκB, using CRISPR-Cas9 editing (33). More recently, it was shown that cirsimaritin, a pure flavonoid from Chinese medicine, blocks NFκB signaling induced in MDCK and THP-1 cells by influenza A strains H1N1 and H3N2, and suppresses virally activated expression of TNFα, IL-8 and other cytokines (34). In prospect, our present work shows that digitoxin, a specific blocker of TNFα-driven NFκB signaling, achieves an analogous result *in vivo* with influenza A strain H3N2.

The decision to analyze the response to digitoxin on the 4th day after infection was based on the observation that in the cotton rat lung, mRNA expression for many cytokines reaches maximal level on that day in response to A/Wuhan/H3N2/359/95 infection (19). Our data show that digitoxin treatment causes the most profound reduction in INFγ expression relative to the other cytokines. The potential importance of digitoxin-dependent INFγ reduction may be manifest by a recent report, where simply neutralizing INFγ in a mouse model of infection with influenza A virus strain A/California/07/2009 (H1N1v; "Swine Flu") was sufficient not only to alleviate acute lung injury but also to increase weight and survival rate (35). Why digitoxin is so powerful

a suppressor of INFγ is not immediately obvious. However, it is known that IFNγ expression is driven by a combination of both NFκB and NFAT acting on the IFNγ promoter (36). We have previously reported that digitoxin not only reduces NFκB, but also reduces NFAT expression (37). It is, therefore, possible that the suppression of both of these transcription factors may contribute to digitoxin's potent suppression of virally-induced INFγ expression.

Influenza A is known to drive activation of IL-1β and TGFβ, but in these experiments digitoxin did not significantly change the expression of these cytokines, as it did to the others. The importance of IL-1β for influenza A is that it is synthesized by alveolar macrophages and dendritic cells in response to viral infection (38). Its role is to drive neutrophilic inflammation in a manner unrelated to the levels of GRO/KC or MIP-2α in the virus-infected mouse lung (39). We conclude that further understanding of this complexity will depend on additional investigation. TGFβ expression is also driven by influenza A in response to viral infection (40); however, the regulation of TGFβ expression itself remains poorly understood, and is also not directly dependent on NFκB (12). It is possible that the difference may lie in the fact that digitoxin acts directly on the TNFα-driven NFκB pathway, but that IL-1β and TGFβ act on NFκB indirectly or by alternative pathways. For example, digitoxin acts directly to suppress

TNFα-driven NFκB signaling by blocking the binding of the TNFα/TNFR1 to TRADD (41, 42). Tumor necrosis factor receptor type 1-associated death domain (TRADD) is the first intracellular adaptor for the TNFα/TNFR1 complex, and the resulting ternary complex directly drives the downstream activation of IKKα, β, & γ, phosphorylation of IκBα, and, thus, activation of NFκB. Increased cytokine expression follows NFκB activation.

Finally, it is a limitation of this study that there may be antiviral effects of digitoxin that may contribute to reduction in host-driven cytokine storm, and may also have implications for COVID-19 therapy. This is because digitoxin and the other approved cardiac glycosides, digoxin and ouabain have been shown to have inhibitory properties for coronaviruses and other viruses (43). With respect to COVID-19, digoxin and ouabain have been shown to block cell penetration and infectivity when tested against SARS-CoV-2 (17). Furthermore, digitoxin itself has been shown to block Middle East respiratory syndrome, MERS CoV penetration into target cells and subsequent infectivity (16). Previously, digoxin was shown to block MERS-CoV penetration and infectivity (44). In silico molecular docking analysis based on CRYO-EM structures has shown that digitoxin, out of 15,000 molecular candidates, binds best to the receptor binding domain (RBD) of the SARS-CoV-2 Spike (45). A similar study by others has come to the same conclusion (46). Both the latter authors suggested that digitoxin may block the interaction of SARS-CoV-2 with the receptor ACE2. Using the same in silico screening approach, but also followed by an in-vitro test, ouabain was shown to dock optimally to the SARS-CoV-2 Main protease (Mpro), and to block viral penetration and infectivity (47). Consistently, digitoxigenin, a digitoxin without the three sugars at the 3'OH position, has also been shown to dock optimally to the Mpro (48). With respect to the activation of the cytokine storm, we have already noted that TNFα-driven NFκB activation by the host drives cytokine storm for Influenza A (2), for SARS-CoV (3), and for SARS-CoV-2 (4). Consistently, digitoxin potently blocks this host-response process, independent of viral activation, at low nM concentrations (10, 11, 13, 37, 41). Since the cardiac glycosides digitoxin, digoxin and ouabain are approved drugs, we conjecture that the suppressive effects of digitoxin on influenza A cytokine storm shown here could be quite relevant to future tests of cardiac glycoside-based therapies for COVID-19.

In conclusion, these data show that digitoxin blocks the host over-production of cytokines raised by influenza strain A/Wuhan/H3N2/359/95 in the cotton rat lung. Since digitoxin has already been shown to be safe in an FDA Phase II clinical trial with cystic fibrosis patients with pulmonary disease and a normal heart, and has been shown to cause a similar reduction in NFκB driven cytokine expression, this drug may be a good candidate for further investigation as a therapy for influenza and potentially for COVID-19.

Conflicts of Interest

Authors' Contributions

BSP requested the experiment, conceived and designed the experiment, analyzed the data, and realized that additional analysis was necessary after histology did not show an effect, and wrote the manuscript. GAP was the pathologist and analyzed the data. JCB designed the experiment, provided the virus, and performed the experiment. JRP analyzed the data and wrote the manuscript. All authors approved the manuscript for publication.

Acknowledgements

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