bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Classical drug digitoxin inhibits influenza cytokine storm, with implications for COVID-19 therapy.

Harvey B. Pollard *[1] Bette S. Pollard [2] and John R. Pollard [3]

1. Department of Anatomy, Physiology and Genetics (APG), Uniformed Services University School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, 20814

2. Silver Pharmaceuticals, Rockville, MD 20854

3. Department of Neurology, University of Pennsylvania, Philadelphia PA (USA) and Christiana Care Epilepsy Center, Newark, DE (USA), 19173

*Corresponding author

Abstract:

Influenza viruses, corona viruses and related pneumotropic viruses cause sickness and death partly by inducing a hyper-proinflammatory response by immune cells and cytokines in the host airway. Here we show that the cardiac glycoside digitoxin suppresses this response induced by influenza virus strain A/Wuhan/H3N2/359/95 in the cotton rat lung. The cytokines TNFα, GRO/KC, MIP2, MCP1, TGFβ, and IFNγ. are significantly reduced. Since the hyper-proinflammatory overproduction of cytokines is a host response, we suggest that digitoxin may have therapeutic potential for not only influenza and but also for coronovirus infections.

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Introduction:

Influenza viruses, corona viruses 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 cytokine storm [2]. Similarly, 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]. A recent description of COVID-19 infection in humans has stressed that cytokine storm marks the airways of infected patients that were admitted to the Intensive Care Unit (ICU) with more severe disease [5].

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 among the most potent inhibitors of the proinflammatory TNFα/NFκB pathway in the human airway and in other epithelial cells, both *in vitro* [9], and *in vivo* [11-13]. Corroborating this is a screen of 2800 drugs and bioactive compounds which found digitoxin to be the 2nd most potent inhibitor of TNFα/NFκB activity [14]. Digitoxin has been a drug to treat heart failure for decades, and is safe for children and adults with normal hearts [15]. In a clinical trial of digitoxin administered to young adults with the proinflammatory lung disease cystic fibrosis, digitoxin was safe [13.] The study showed "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, IL-6, lung epithelial inflammation, neutrophil recruitment and mucus hypersecretion." [13].

To test the ability of digitoxin to inhibit cytokine storm, we used the cotton rat to investigate the effects of digitoxin on influenza virus strain A/Wuhan/H3N2/359/95. The cotton rat model has the important advantage of susceptibility to influenza infection without engineered adaptation. In addition, it has been shown that the response of the cotton rat to this virus strain evokes a pattern of pulmonary cytokine changes that parallel the human response [16]

Results:

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Digitoxin blocks cytokine storm

Figure 1 shows the changes in cytokine protein in the lung due to digitoxin administration in the cotton rat after intranasally instilling 10^7TCID50/100 gm of influenza strain A/Wuhan/H3N2/359/95 virus. Animals were given four different doses of digitoxin, starting one day prior to virus administration and continuing until sacrifice on day 7. The maximum dose of digitoxin, 30 μg/kg, was calculated to be similar to the human dose routinely used to treat heart failure. As shown in **Figure 1**, protein data were collected for **IFNγ** (Interferon gamma); **GRO/KC** (rodent equivalent of human IL8); **MIP2** (Chemokine (C-X-C motif) ligand 2, CXCL2, Macrophage inflammatory protein 2-alpha); **TNFα** (Tumor Necrosis Factor alpha); **IL-1β** (Interleukin one beta); **MCP1** (Monocyte chemoattractant Protein 1, CCL2); and **TGFβ** (Transforming Growth Factor beta). As summarized in **Table 1**, digitoxin-dependent changes in protein were found to be significant for 6 of the 7 cytokines. The digitoxin-dependent reductions are specific and saturating for each cytokine, but do not reduce any of them to zero.

Digitoxin differentially affects cytokine expression

Table 1 shows that the greatest significant digitoxin-dependent reductions in cytokine proteins were found for **IFNγ** (68.9%), **GRO/KC** (46.6%), and **MCP1** (54.9%). Smaller but still significant reductions in cytokine proteins were found for **MIP2** (32.2%) and **TNFα** (38.4%). As also shown in **Table 1**, a significant reduction of only 15.3% was found for **TGFβ** cytokine protein at a concentration of 3 μg/kg, while only trending significance was noted at higher digitoxin concentrations. In the case of **IL-1β** only the highest digitoxin concentration trended towards significance. Thus digitoxin independently, dose-dependently and significantly lowers the individual concentrations in the lung of at least these six cytokines which have been induced by viral exposure.

Discussion:

Administration of digitoxin to the cotton rat inhibits expression of many cytokines in the lung that are induced by influenza strain A/Wuhan/H3N2/359/95, including TNFα, the key

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

activator of the TNFα/NFκB inflammation pathway. With the exception of IFNγ, which is secreted only from activated T lymphocytes and NK cells of the immune system [17], the rest of the cytokines are secreted by epithelial cells in the airway, as well as by endothelial cells, immune cells and others [18,19]. GRO/KC (CXCL1, the rodent equivalent of human IL8), a key target of NFκB signaling, is the most powerful known chemoattractant for drawing neutrophils into the lung. MIP2 and MCP1 induce entry and accumulation of monocytes and macrophages into the lung, and are targets of NFκB. TGFβ drives, and is driven by, NFκB-signaling for inflammation and fibrosis. IL-1β also drives NFκB and is driven by NFκB. It appears that digitoxin-dependent reduction in TNFα/NFκB signaling is sufficient to suppress influenza A-driven cytokine storm.

Digitoxin also blocks IFNγ, which is secreted by NK cells and activated T lymphocytes from the innate and adaptive immune systems [17]. In the cotton rat lung, IFNγ mRNA expression in response to infection is biphasic [16]. There is an early phase, from 6 hours after infection on day 1 to day 6, which may reflect the presence of activated NK cells. The late phase, from day 6 to day 28, may be the product of incoming antigen-specific T cells. Importantly, simply neutralizing INFγ in a mouse model of infection with influenza A virus strain A/California/07/2009 (H1N1v; "Swine Flu") is sufficient to not only alleviate acute lung injury but also to increase weight and survival rate [20]. Reduced IFNγ is associated with reduced TNFα and NFκB activation. The data also show that digitoxin treatment causes profound reduction in INFγ expression. It is further known that IFNγ expression is driven by a combination of both NFκB and NFAT [21]. As summarized in **Figure 2**, digitoxin not only reduces NFκB expression, but also reduces NFAT through digitoxin-dependent activation of Caspase 3 [22].

Finally, since antiviral properties have been reported for digitoxin and other cardiac glycosides, it is limitation of the study that we cannot exclude other antiviral effects by digitoxin from contributing to the reduction in influenza-dependent cytokine concentration [23-25].

In conclusion, these data show that digitoxin blocks the host cytokine storm induced by influenza strain A/Wuhan/H3N2/359/95 in the cotton rat lung. Since digitoxin has already been shown in people to improve respiratory cytokines in human disease, this drug may be a good candidate for further investigation into therapy for influenza and potentially for COVID-19.

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Methods:

Drugs and protocol for preparation

Digitoxin (μg/kg) was obtained from Sigma-Aldrich (> 95% pure). The drug was prepared as a stock solution in 95% ethanol, and further diluted in PBS before administration.

Animals and protocol for drug administration

Cotton rat experiments were managed as previously described [16]. Digitoxin was administered to cotton rats one day before intranasal administration of 10^7TCID50/100 gm cotton rat with influenza strain A/Wuhan/H3N2/359/95. Daily digitoxin treatment continued until harvest on day 7 of the experiment. Lungs were dissected with the lower one-third of the trachea left attached. The left lung was first tied off and reserved for cytokine analysis. Lung samples were then immediately frozen on dry ice, and then kept at -80°C until further processed.

Biochemical analysis

Frozen lung samples were weighed, thawed and then minced with scissors in 10% (v/v) ice cold PBS, homogenized in 10 strokes in a Ten Broeck homogenizer, and centrifuged at 20,000 X g for 30 minutes. The supernatant solution was kept at -80°C until assayed by ELISA. The supernatant solutions were first tested by ELISA at a now-defunct company in Ijamsville, MD. The data were corroborated by the Searchlight® ELISA platform from Pierce-Thermo to assay for cytokines and chemokines. Rat reagents were used in both instances.

Acknowledgements: The authors thank Val Hemming, M.D. for advice, Jorge C Blanco, Ph.D. for managing the experiment, and Max Tran, MBA, for providing statistical analysis.

Corresponding author:
* Harvey B. Pollard, M.D., Ph.D.
T: 1-301-295-3661; Email: harvey.pollard@usuhs.edu

The opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense, the U.S. Government or the Uniformed Services University of the Health Sciences. The use of trade names does not constitute an official endorsement or approval of the use of such reagents or commercial hardware or software. This document may not be cited for purposes of advertisement.

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

References

1. Tisoncik, J. R. *et al.* Into the Eye of the Cytokine Storm. *Microbiol. Mol. Biol. Rev.* **76**, 16–32 (2012).
2. Schmolke, M., Viemann, D., Roth, J. & Ludwig, S. Essential Impact of NF-κB Signaling on the H5N1 Influenza A Virus-Induced Transcriptome. *J. Immunol.* **183**, 5180–5189 (2009).
3. DeDiego, M. L. *et al.* Inhibition of NF- κB-Mediated Inflammation in Severe Acute Respiratory Syndrome Coronavirus-Infected Mice Increases Survival. *J. Virol.* **8 8**, 913–924 (2014).
4. Guo, T.R., *et al*. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. *Mil. Med. Res.* 7(1):11. doi:10.1186/s40779-020-00240-0 (2020)
5. Huang, C. *et al.* Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. **395**, 497–506 (2020).
6 Teijaro, J.R. *et al.* Mapping the innate signaling cascade essential for cytokine storm during influenza virus infection. *Proc Natl Acad Sci USA.*111:3799-3804 (2014).
7. Yang, M. *et al.* Chloroquine inhibits HMGB1 inflmmatory signaling and protects mice from lethal sepsis. *Biochem Pharmacol.* 86:410-418 (2013).
8. Yang, S. *et al.* NF-kB signaling activation induced by chloroquine requires autophagosome, p62 protein,and c-Jun N-terminal kinase (JNK) signaling and promotes tumor cell resistance. *J Biol Chem.* 292:3379-3388 (2017).
9. Zhang, Q., Lenardo, M. J. & Baltimore, D. 30 years of NF-kB: A blossoming of relevance to human pathophysiology. *Cell* 168: 37–57 (2017).
10. Srivastava, M. *et al.* Digitoxin mimics gene therapy with CFTR and suppresses hypersecretion of IL-8 from cystic fibrosis lung epithelial cells. *Proc Natl Acad Sci U S A*. 101(20):7693-7698 (2004).
11. Yang, Q. *et al.* Gene therapy-emulating small molecule treatments in cystic fibrosis airway epithelial cells and patients. *Respir. Res.* 20:290 doi.org/10.1186/s12931-019-1214-8 (2019).
12. Pollard, B. S. *et al.* Digitoxin Inhibits Epithelial-to-Mesenchymal-Transition in Hereditary Castration Resistant Prostate Cancer. *Front. Oncol.* 9, 9:630. doi: 10.3389/fonc.2019.00630 (2019).

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

13. Zeitlin, P.L., Diener-West, M., Callahan, K.A., Lee, S., Talbot, C., Pollard, B., Boyle, M.P., and Lechtzin, N. Digitoxin for airway inflammation in cystic fibrosis: Preliminary assessment of safety, pharmacokinetics, and dose finding . *AnnalsATS*. 14:220-229 (2017)

14. Miller, S.C. *et al.* Identification of known drugs that act as inhibitors of NF-κB signaling and their mechanism of action. *Biochem Pharmaco*l. 79:1272-1280 (2010).

15. Goodman and Gilman, The Pharmacologic Basis of Therapeutics. Eighth Edition.(Editors, Molinoff P.et al). Chapter 34. Digitalis and Allied Cardiac Glycosides. Hoffman BF and Bigger JT, p833 (1990).

16. Ottolini, M. G. *et al.* The cotton rat provides a useful small-animal model for the study of influenza virus pathogenesis. *J. Gen. Virol.* 86, 2823–2830 (2005).

17. Mah, A. and Cooper M.A. Metabolic regulation of natural killer cell IFN-γ production. *Crit Rev Immunol.* 36(2):13-147 (2016).

18. Mills, P.R., Davies, R.J., and Devalia, J.L. Airway epithelial cells, cytokines and pollutants. *Am J Crit Care Med*. 160:S38-S43(1999).

19. Liu, T. *et al.* NF-κB signaling in inflammation. *Sig.Transduc Targ Ther.* 2,e7023;DOI:10.1038/sigtrans.2017.23 (2018).

20. Liu, B, *et al.* Anti-IFN-γ therapy alleviates acute lung injury induced by severe Influenza A (H1N1) pdm09 infection in mice. *J Microb Immun Infec.* doi.org/10.1016/j.jmii.2019.07.009 (2019).

21. Sica, A, *et al.* Interaction of NF-kB and NFAT with the interferon-γ promoter. *J Biol Chem.* 272:30412-30420 (1996).

22. Yang, Q, *et al.* Digitoxin induces apoptosis in cancer cells by inhibiting Nuclear Factor of activated T cell-driven c-Myc expression. *J Carcinogenesis.* doi: 10.4103/1477-3163.112268.(2013).

23. Amarelle, L, and Lecuona, E, The antiviral effects of Na,K-ATPase inhibition: A minireview. *Int J Mol Sc*i. 19, 2154; doi:10.3390/ijms19072154 (2018).

24. Wei, T. *et al*. *in silico* screening of potential spike glycoprotein inhibitors of SARS-CoV-2 with drug repurposing strategy. *Research Square.* DOI: 10.21203/rs.3.rs-17720/v1 (2020).

25. Burkard, C, *et al.* ATP1A1-Mediated Src Signaling Inhibits Coronavirus Entry into Host Cells. *J. Virol.* **89**, 4434–4448 (2015).

bioRxiv preprint doi: https://doi.org/10.1101/2020.04.09.034983. this version posted April 13, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

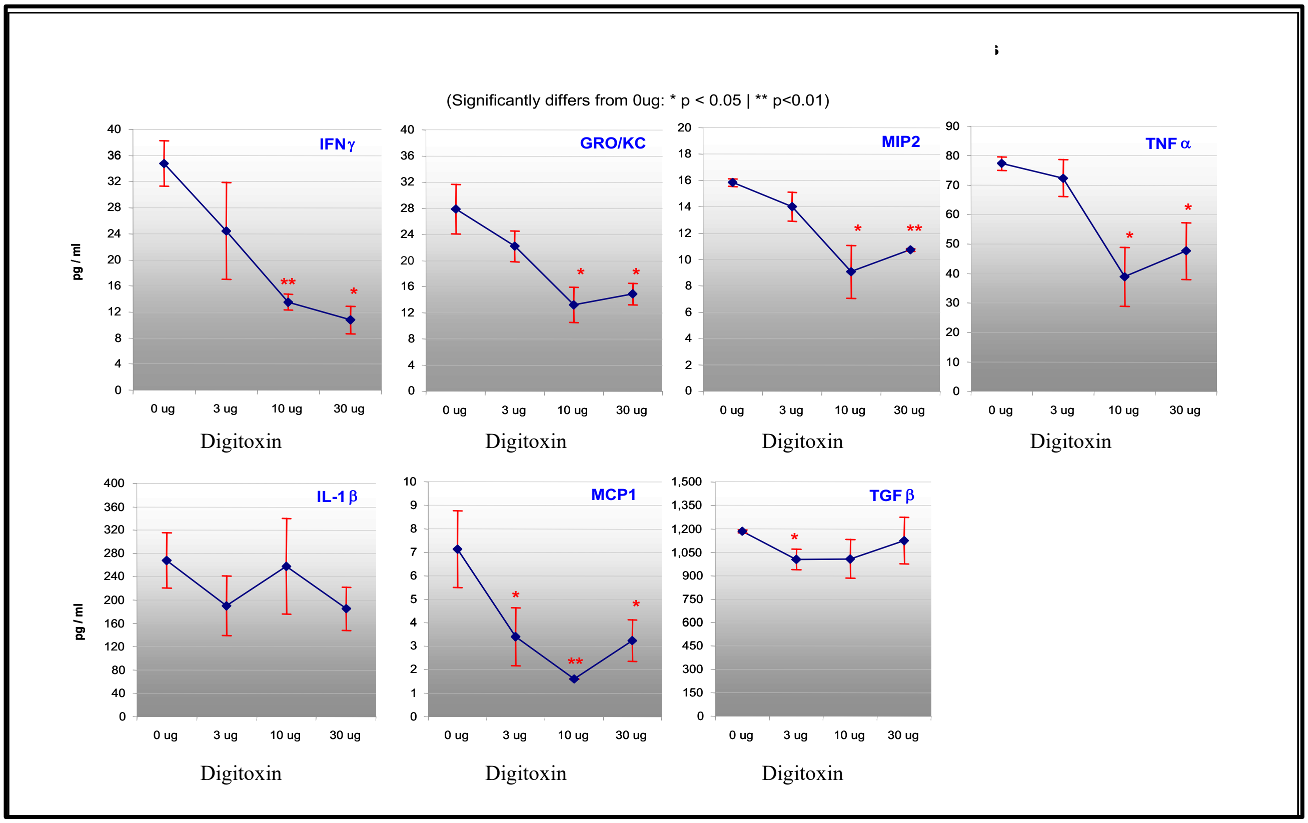

Figure 1. **Cotton rats treated with digitoxin and influenza strain A/Wuhan/H3N2/359/95 virus**. Animals were treated with different concentrations of digitoxin one day before intranasal virus administration and thereafter for 7 days. Samples assayed were lung tissue. Digitoxin dose is in units of μg/kg. Abbreviations are **INFγ** (interferon gamma, IFNγ); **GRO/KC** (Chemokine (C-X-C motif) ligand 1, CXCL1); **MIP2** (Chemokine (C-X-C motif) ligand 2, CXCL2, macrophage inflammatory protein 2-alpha); **TNFα** (TNFalpha, TNFA, tumor necrosis factor alpha); **IL-1β** (IL1B, interleukin 1 beta); **MCP1** (Monocyte chemoattractant Protein 1, CCL2); **TGFβ** (TGFB, transforming growth factor beta). Significance: * ($p < 0.05$); ** ($p < 0.01$); N = 3.

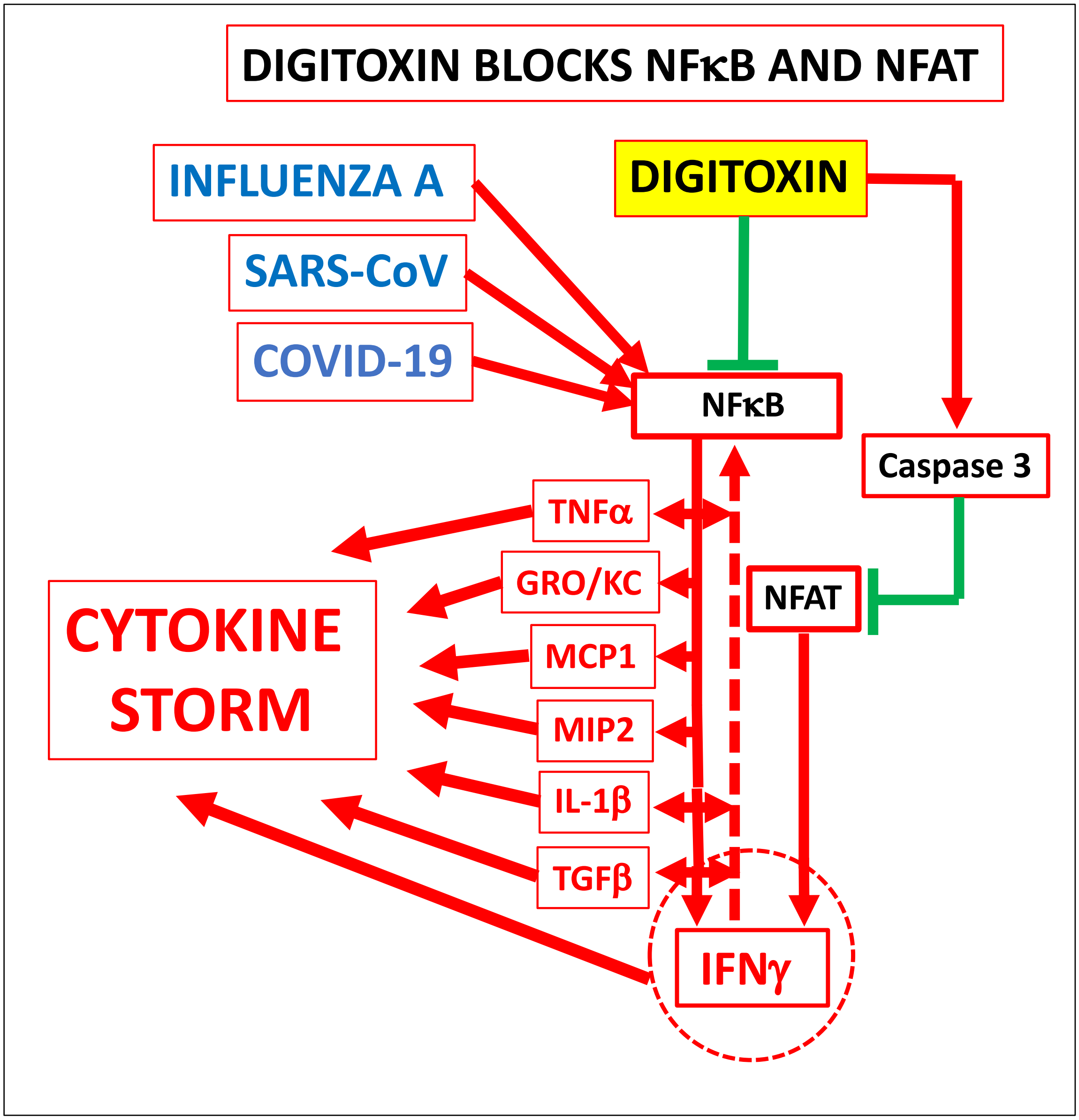

Figure 2. **Digitoxin blocks cytokine storm, and interferon gamma**. Influenza A virus, SARS-CoV and COVID-19 induce cytokine storm in the host. Present data show that digitoxin blocks cytokine storm when cotton rats are challenged with influenza strain A/Wuhan/H3N2/359/95. Expression of these cytokines and chemokines depend on NFκB, which is blocked by digitoxin. TNFα, IL-1β and TGFβ also activate NFκB. In addition, interferon gamma (INFγ) is blocked by digitoxin. INFγ also indirectly activates NFκB. Digitoxin activates caspase 3, which, proteolyzes NFAT. Experiments with SARS-CoV show that inhibition of NFκB suppresses cytokine storm and enhances survival in a mouse model (see text) [3]. COVID-19 activates NFκB [4]. Cytokine storm induced by COVID-19 has been observed in severely affected patients [5]. Color code: red (activation); green (inhibition); See cytokine abbreviations in Figure 1.

Table 1. Analysis of Digitoxin Suppression of Cytokine Expression in Cotton Rat Lung Following Nasal Installation of Influenza Strain A/Wuhan/H3N2/359/95 Virus

Digitoxin	IFNγ			n	Average	STDEV	SE	Variance	Sp	SED	actual p Value **	Signif Level
0 μg	29.1	38.1	37.1	3	34.8	4.9	3.49	24.33	n/a	n/a	n/a	n/a
3 μg	34.6	25	13.7	3	24.4	10.5	7.40	109.44	8.1785288	6.677741	0.098335067	—
10 μg	15.5	12.5	12.5	3	13.5	1.7	1.22	3.00	3.6968455	3.018462	0.001069744	p < 0.01
30 μg	12.5	7.3	12.5	3	10.8	3.0	2.12	9.01	4.0832993	3.334	0.00098681	p < 0.01
Digitoxin	GRO/KC			n	Average	STDEV	SE		Sp	SED	actual p Value **	Signif Level
0 μg	22	32.4	29.2	3	27.87	5.3	3.77	28.37	n/a	n/a	n/a	n/a
3 μg	23.4	24.7	18.4	3	22.17	3.3	2.35	11.06	4.440533	3.62568	0.095512211	—
10 μg	14.6	16.1	8.9	3	13.20	3.8	2.69	14.43	4.6261935	3.777271	0.008898912	p < 0.01
30 μg	12.2	16.4	16	3	14.87	2.3	1.64	5.37	4.1077163	3.353936	0.008950663	p < 0.01
Digitoxin]	MIP2			n	Average	STDEV	SE		Sp	SED	actual p Value **	Signif Level
0 μg	15.6	16.3	15.6	3	15.8	0.4	0.29	0.16	n/a	n/a	n/a	n/a
3 μg	14.1	15.5	12.4	3	14.0	1.6	1.10	2.41	1.1343133	0.926163	0.059435249	—
10 μg	7.1	12.3	7.8	3	9.1	2.8	2.00	7.96	2.0157712	1.64587	0.007358259	p < 0.01
30 μg	10.9	10.7	10.6	3	10.7	0.2	0.11	0.02	0.305505	0.249444	1.68981E-05	p < 0.01
Digitoxin	TNFα			n	Average	STDEV	SE		Sp	SED	actual p Value **	Signif Level
0 μg	80.9	76.5	74.6	3	77.3	3.2	2.3	10.44	n/a	n/a	n/a	n/a
3 μg	79.4	75.4	62.4	3	72.4	8.9	6.3	79.00	6.687426	5.46026	0.208676907	—
10 μg	33.4	54.9	28.2	3	38.8	14.2	10.0	200.36	10.266613	8.382654	0.00504268	p < 0.01
30 μg	35.6	44.9	62.3	3	47.6	13.6	9.6	183.69	9.8522417	8.044322	0.01045253	p < 0.05
Digitoxin	IL-1β			n	Average	STDEV	SE		Sp	SED	actual p Value **	Signif Level
0 μg	285.8	324	194	3	267.9	66.8	47.2	4464.41	n/a	n/a	n/a	n/a
3 μg	106.7	239.3	224.4	3	190.1	72.6	51.4	5276.34	69.788096	56.98174	0.121943761	—
10 μg	123.6	317.7	332.7	3	258.0	116.6	82.5	13603.77	95.047839	77.60624	0.452164405	—
30 μg	226.2	203.5	125.2	3	185.0	53.0	37.5	2807.86	60.300401	49.23507	0.083624334	—
Digitoxin	MCP1			n	Average	STDEV	SE		Sp	SED	actual p Value **	Signif Level
0 μg	8.1	8.8	4.5	3	7.1	2.3	1.6	5.32	n/a	n/a	n/a	n/a
3 μg	2.6	5.4	2.2	3	3.4	1.7	1.2	3.04	2.0449124	1.669664	0.044509073	p < 0.05
10 μg	1.6	1.6	1.6	3	1.6	0.0	0.0	0.00	1.6314615	1.332083	0.007107652	p < 0.01
30 μg	1.9	4.4	3.4	3	3.2	1.3	0.9	1.58	1.8583146	1.517308	0.030978174	p < 0.05
Digitoxin	TGFβ			n	Average	STDEV	SE		Sp	SED	actual p Value **	Signif Level
0 μg	1192.5	1167.2	1192.5	3	1184.1	14.6	10.3	213.36	n/a	n/a	n/a	n/a
3 μg	901.3	1079.9	1029.9	3	1003.7	92.1	65.2	8489.32	65.9647	53.85995	0.014300249	p < 0.05
10 μg	806.1	1095.2	1122.1	3	1007.8	175.2	123.9	30693.07	124.31097	101.4995	0.078728888	—
30 μg	969.9	1364	1041.1	3	1125.0	210.0	148.5	44108.11	148.86483	121.5476	0.326209764	—

** Calculation Explanation:

1. pooled 2-tail t-Test because … Alternate Hypothesis H1: Y_{dose} not equal to Y_{0ug} (or, H0 Null Hypothesis: $Y_{dose} = Y_{0ug}$), where Y_{dose} refers to the pg/ml values of 3ug, 10ug, 30ug
2. degrees of freedom (df) = 4 = $n_{dose} + n_{0ug}$ -2 = 3 + 3 - 2
3. Sp = square root [[(n_{dose} - 1)$variance_{dose}$ + (n_{0ug} - 1)$variance_{0ug}$] / df]]
4. SED = Sp x square root [1 / n_{dose} + 1 / n_{0ug}]
5. t statistic = ($average_{dose}$ - $average_{0ug}$) / SED

Cytokine units are in pg/ml.

Digitoxin doses are in units of μg/kg.