RV Infections in Asthmatics Increase ACE2 Expression and Cytokine Pathways Implicated in COVID-19

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Running title: RV infections increase ACE2 and CV2-associated cytokines

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To the Editor:

SARS-Co-V2 (CV2) is a novel virus first identified in December 2019 in Wuhan, China as causing COVID-19, with over 7.5 million cases currently reported worldwide (1). Angiotensin-converting enzyme 2 (ACE2) is the receptor for CV2 and has recently been identified as an interferon stimulated gene (2). Rhinovirus (RV) infections are potent inducers of interferon stimulated genes and subsequent cytokine production. RV infections are the most frequent virus identified in the common cold, and are responsible for the majority of asthma exacerbations in children and adults (3).Young asthmatics have higher rates of COVID-19, accounting for 27% of hospitalized patients in the United States in the 18-49 yo age group (4). We hypothesized that RV infections could increase expression of ACE2 and subsequently activate cytokine pathways associated with severe COVID-19 infections.

We developed air-liquid-interface (ALI) cultures from nasal tissues biopsied from thirty adults with physician-diagnosed asthma. Subjects averaged 35 years of age, 60% were non-Hispanic whites, and were evenly divided by gender. We infected ALI cultures with common RV strains RV-A16 ($1x10^5$ RNA copies/well), RV-C15 ($1x10^5$ RNA copies/well) or DMEM/F12 media (control) for 4 hrs at 34°C, 5% CO_2. RNA was then extracted from whole-cell lysates, sequenced using KAPA Stranded RNA-Seq libraries on an Illumina HiSeq 3000 for a 1x50 run, demultiplexed with Illumina Bcl2fastq2 (v2.17) and then mapped to the UCSC transcript set using Bowtie2 (v2.1.0). We processed the discovery (n=22) and validation (n=8) cohorts separately through the NOISeq library (5) to filter out genes with low counts (cpm<30), resulting in 7,474 and 7,905 unique genes in the discovery and validation cohorts. We then utilized the function "ARSyNseq" followed by "voomWithQualityWeights" (6) to process RNA counts for downstream statistical analysis with the linear model implemented in the LIMMA R library. We utilized the moderate t-test for paired samples for statistical analyses to prioritize 402

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differentially expressed genes (DEGs) adjusted by false discovery rate (FDR) < 1% and absolute log2 fold-change > 0.5.

When compared to controls, both RV-A16 and RV-C15 infected ALI cultures resulted in a greater than 3-fold increase in ACE2 expression in the discovery and validation cohorts (**Figure 1**). Interestingly, levels of transmembrane serine protease 2 (TMPRSS2), a protease that primes the CV2 virus for cellular entry, were not increased after either RV-A16 or RV-C15 infections. How could RV infections induce ACE2 expression? Ziegler et al. determined that stimulation of primary nasal epithelial cells with interferon increased ACE2 expression. They also identified four potential ACE2 transcription factors located within 2K bp of the ACE2 start site: STAT1, STAT3, IRF8, and IRF1 (2). Of these four transcription factors, only IRF1 was reproducibly differentially expressed in our dataset and showed a significant three-fold increase in expression after RV-A and RV-C infections.

Next, we sought to determine if the patterns observed in nasal cells among patients with asthma were also observed for other viruses in human bronchial epithelial cells (HBECs) unselected for asthma. We analyzed microarray data (GSE32140) to quantify gene expression changes after exposure to influenza A and respiratory syncytial virus (RSV) in ALI cultures of HBECs. Two hours post infection with influenza A or RSV, ACE2 expression levels were 6-fold higher while TMPRSS2 levels were not altered compared to control uninfected cells (data not shown).

The role of ACE2 overexpression on the cytokine surge, which has been shown to be clinically relevant in the severity of COVID-19, is unknown. Huang et al. recently reported that critically ill COVID-19 patients had high serum levels of IL-1β, IL-1RA, IL-2, IL-4, IL-7, IL-8, IL-9, IL-10, IL-13, IL-17, G-CSF, IFN-γ, IP-10, MCP-1, MIP-1A, and TNF-α (CV2-associated cytokine surge) (7). Using our *in vitro* model, we sought to identify DEGs associated with RV-induced

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ACE2 overexpression and with CV2 cytokine regulation. 63 DEGs were correlated to RV-induced ACE2 overexpression and overrepresented in the “Regulation of cytokine production” gene ontology (GO) set (GO:0001817). We then identified 34 GO annotations correlated to the regulation and production of the CV2-associated cytokine surge (8, 9). 29 of these 63 DEGs were annotated in 7 GO annotations and several of these genes have also been implicated in the aberrant anti-viral response in asthma (Figure 2).

Here, we present novel findings suggesting that (1) RV infections are potential mechanisms of ACE2 overexpression in patients with asthma and (2) ACE2 activation regulates multiple cytokine anti-viral responses. These results suggest that viral infections associated with asthma exacerbations exhibit synergistic biomolecular interactions with CV2 infection. Therefore, co-infections with RV and CV2 may pose significant risks for patients with asthma. One limitation of this study was that we did not evaluate the surface protein expression of ACE2 after RV infection. Unfortunately, testing of current available ACE2 antibodies have been non-specific or inconclusive (2). We also were unable to directly infect our ALI cultures with CV2 due to safety concerns. However, the recent availability of pseudo-typed viral models expressing the CV2 spike protein will be invaluable to assess differences in CV2 binding in correlation to ACE2 expression. Although we used RV infection as a model of ACE2 activation and cytokine induction, it is not known if similar findings are found in the cytokine surge in severe CV2 infections seen in ICU patients. Are there potential therapies that could downregulate ACE2 expression to decrease SARS-CoV-2 susceptibility? Zaheer et al. found that knockdown of IRF-1 abrogated the production of antiviral cytokines after RV infections (3). Further studies are required to determine if IRF-1 blockade also affects ACE2 expression. Peters et al. also identified that the use of inhaled corticosteroids in asthmatics was associated with lower ACE2 expression levels, suggesting that nasal or inhaled corticosteroid use could be a potential therapy in ACE2 downregulation (10). Our study suggests that common viral infections may

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prime the host to respond excessively to COVID-19 infections, and potentially correspond to an increase in disease severity when multiple respiratory viruses are circulating.

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FIGURE CAPTIONS

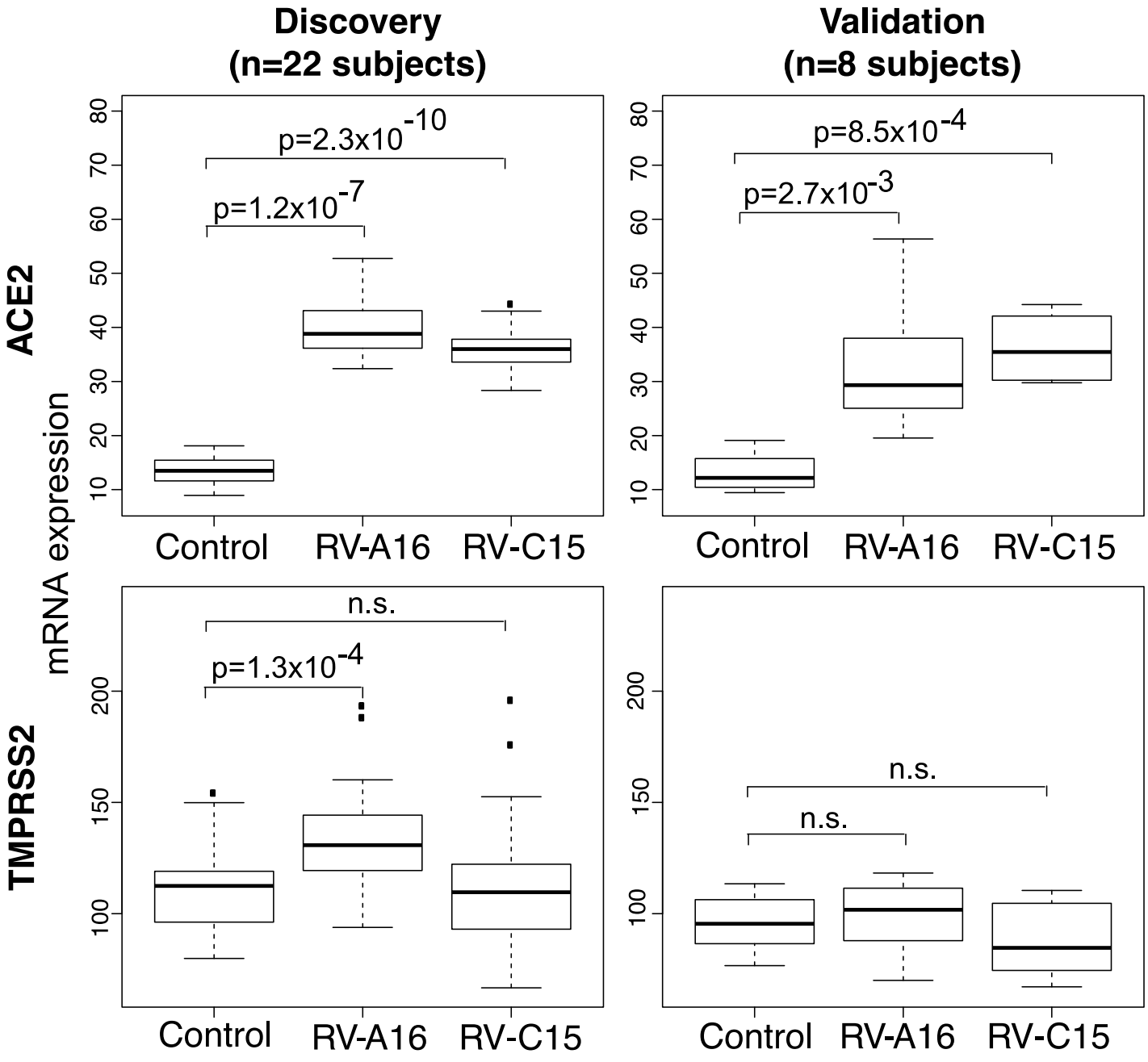

Figure 1. ACE2 is overexpressed in human rhinovirus-infected human nasal tissue cultures. ACE2 fold change (FC) of expression varies from 3.2 to 3.6 in the discovery and validation cohorts after RV-A and RV-C infection. TMPRSS2 is not reproducibly altered by rhinovirus infections. The mRNA expression was calculated by normalization of voom counts.

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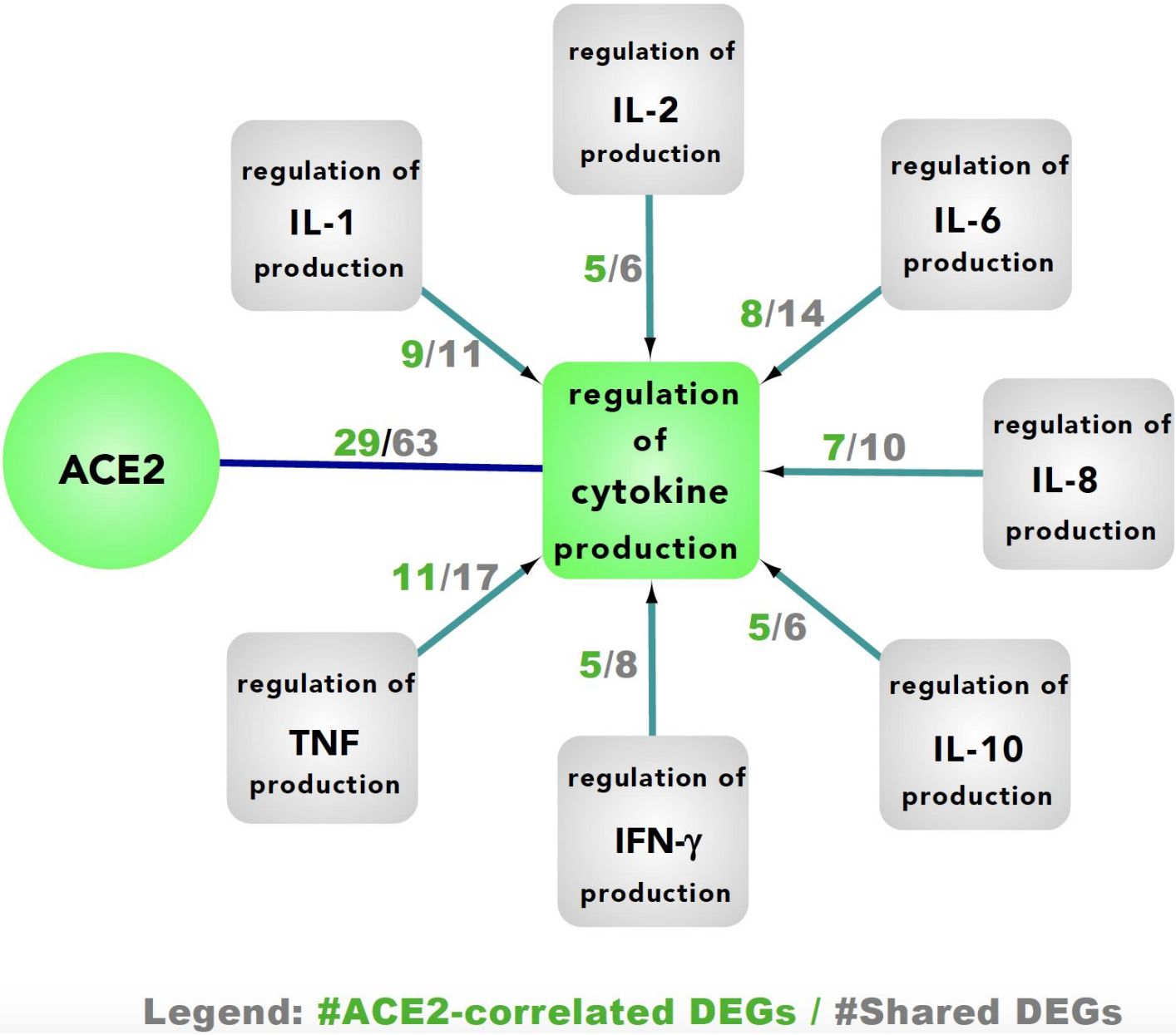

Figure 2. Biomolecular mechanisms of response to rhinovirus infection in 30 asthmatic cultures that are both correlated with ACE2 overexpression and over-represented in COVID-19 cytokine surge pathways. 29 of the 63 Differentially expressed genes (DEGs) in response to RV-A and RV-C infections compared to controls (n=22 asthmatic patients in the discovery cohort, n=8 in the validation cohort; adjusted with false discovery <1% in the

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discovery cohort and Bonferroni adjustment <5% in the validation cohort) were: (i) reproducibly correlated with ACE2 expression and the Gene Ontology mechanism (GO:0001817): regulation of cytokine production (Bonferroni-adjusted p<5%) (green square), and (ii) also over-represented in the GO mechanisms associated with the cytokine surge in ICU-admitted COVID-19 subjects (grey squares). 12 of these DEGs (*CASP1,* ***CEACAM1****, EREG, GBP1, HLA-E, IFI16,* ***ISG15****, KLF4,* ***MYD88****, PML, TRIB2,* and *VTCN1*) were associated with the regulation of a single cytokine and the remainder of the genes (***CD274****, DDX58, F2R, FZD5, IDO1, IFIH1, IRAK3,* ***JAK2****, LGALS9, PRKD2, RIPK1, TICAM1,* ***TLR2****,* ***TLR3****, TNFAIP3, ZC3H12A,* and *ZFP36*) were associated with the regulation of multiple cytokines. Genes in **bold** have been implicated in aberrant anti-viral responses in asthma (references not shown).

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