TLR2-mediated innate immune priming boosts lung anti-viral immunity

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Supplementary Materials and Methods

Ethics statements

Primary bronchial epithelial cells were provided by Professor Peter A. B. Wark (The University of Newcastle), obtained from healthy donors or donors with asthma during bronchoscopy, with written informed consent. All experiments were conducted with approval from the University of Newcastle Safety Committee (Safety REF# 25/2016 and R5/2017). Animal experiments were conducted in accordance with the NSW, Australia Animal Research legislation. Experimental protocol A-2016-605 was reviewed and approved by the University of Newcastle Animal Ethics Committee.

TLR2 agonist administration and RV-A1 infection for in vivo mouse models

6-8-week-old female BALB/c mice from Australian Bioresources (ABR) were used for all experiments. INNA-X was obtained from Ena Therapeutics in lyophilized form and resuspended in sterile saline. Mice were treated with INNA-X intranasally (i.n.) or the commercially available Pam2Cys molecule at time points stipulated in figure legends. Mice were infected i.n. with RV-A1 (5×10^6 $TCID_{50}$), as previously described [1, 2]. Bronchoalveolar lavage (BAL) was collected 2 days post infection.

Bronchoalveolar lavage (BAL) cell analysis

Mouse tracheas were cannulated, and lower airways flushed with Hanks Buffered Salt Solution (HBSS) (Hyclone^{TM}, GE Life Sciences). Cells and supernatants were divided, and supernatants stored at -80^{o}C. Cells were RBC-lysed and remaining cell concentrations determined by

hemocytometer count. Cytospins were performed and May-Grunwald + Giemsa staining was used to enumerate neutrophils, lymphocytes and macrophages by microscopy.

HEK-blue screening of TLR2-specificity of INNA-X

Toll-Like Receptor (TLR) stimulation and specificity was tested by assessing NF-κB activation in HEK-blue ™ cells transfected with a given TLR (InvivoGen). The activity of INNA-X was tested on seven different human TLRs (TLR2, 3, 4, 5, 7, 8 and 9) and eight different mouse TLRs (TLR2, 3, 4, 5, 7, 8, 9 and 13) as a potential agonist. INNA-X was tested at one concentration, 10 ng/mL, and compared to control ligands (listed below). Assays were performed in triplicate by InvivoGen.

In the HEK-blue ™ cells, the secreted embryonic alkaline phosphatase (SEAP) reporter is under the control of a promoter inducible by the transcription factor NF-κB. This reporter gene allows the monitoring of signaling through TLRs, based on the activation of NF-κB. In a 96-well plate (200 µL total volume) containing the appropriate cells (50,000 – 75,000 cells/well), 20 µL of INNA-X (10 ng/mL) or the positive control ligand is added to the wells. The media added to the wells is designed for the detection of NF-κB induced SEAP expression. After a 20 hour incubation the optical density (OD) was read at 650 nm on a Molecular Devices SpectraMax 340PC absorbance detector.

Control Ligands

hTLR2: HKLM (heat-killed Listeria monocytogenes) at 1x108 cells/mL

hTLR3: Poly(I:C) HMW at 1 µg/mL

hTLR4: E. coli K12 LPS at 100 ng/mL

hTLR5: S. typhimurium flagellin at 100 ng/mL

hTLR7: CL307 at 1 μg/mL

hTLR8: CL075 at 1 μg/mL

hTLR9: CpG ODN 2006 at 1 μg/mL

mTLR2: HKLM (heat-killed Listeria monocytogenes) at 1x108 cells/mL

mTLR3: Poly(I:C) HMW at 1 μg/mL

mTLR4: E. coli K12 LPS at 100 ng/mL

mTLR5: S. typhimurium flagellin at 100 ng/mL

mTLR7: CL307 at 1 μg/mL

mTLR8: CL075 at 10 μg/mL + Poly(dT) 10 μM

mTLR9: CpG ODN 1826 at 1 μg/mL

mTLR13: ORN Sa19 200 ng/mL

Comparison of potency between INNA-X and Pam2CysSK4

6-8-week-old female BALB/c mice from Australian Bioresources (ABR) were used for all experiments. INNA-X and PamCysSK4 was obtained from Ena Therapeutics in lyophilised form and resuspended in sterile, physiological saline. Mice were treated with INNA-X or Pam2CysSK4 TLR2 agonists intranasally (i.n.) under light isofluorane anesthesia at the times and doses indicated in the figure legends. Mice were infected by i.n. instillation of RV-A1 (5 x 10^6 $TCID_{50}$). At day 2 post-infection, mice were euthanised for sample collection. Lungs were harvested for total RNA to assess viral loads and immune transcriptome response.

Air-liquid interface of airway epithelial cell cultures

Primary BECs obtained from one healthy donor were grown until confluent and differentiated at air liquid-interface (ALI), as previously described [3, 4].

TLR2 agonist dosing and RV-A1 inoculation of human ALI BECs

24 hours prior to infection, the differentiated epithelium was treated apically with INNA-X at the indicated concentrations in BEBM minimal starvation media (Lonza; BEBM + 1% ITS and 0.5 lipoteichoic acid). The basal compartment was refreshed with BEBM minimal starvation media. Healthy donor cultures were inoculated with RV-A1 (MOI 0.1) for 2 hours on the apical culture surface at 35°C. Following infection, the apical compartment was washed twice with PBS. Starvation media containing TLR2 agonist was then placed in the apical compartment (controls received media alone) at 35°C until the harvest time points.

Sample collection from ALI cultures

ALI culture samples were collected at timepoints indicated in text. Half of the transwell was collected in RLT buffer (Qiagen) containing 1% 2-Mercaptoethanol (2ME) for molecular analyses.

RNA isolation and quantitative reverse-transcription (qRT)-PCR

Apical lung lobes were harvested in RNA-Later (Ambion). Tissues were processed by transferring to RLT (Qiagen) containing 1% betamercaptoethanol (RLT/2ME) buffer for tissue

dissociation using a TissueLyser II (Qiagen; 25Hz, twice for 2 minutes). Cell debris was pelleted by centrifugation and supernatant stored at -80^{o}C. ALI membranes were vortexed in RLT/2ME, the membrane was removed and lysates stored at -80^{o}C.

Mouse lung sample RNA was manually extracted using a miRNeasy kit (Qiagen) following the supplier's protocol for extracting total RNA, including miRNA. ALI RNA was extracted on the QiaCube (Qiagen) using the miRNeasy kit (Qiagen). Following extraction, RNA concentrations were determined by spectrophotometry (Nanodrop) and 200ng (human ALI samples) or 1000ng (mouse lung) total RNA was reverse transcribed with random primers. qPCR analysis was performed on an ABI700 or Quant Studio 6 using TaqMan FAM-TAMRA reagents (Life Technologies), mastermix containing ROX (Qiagen), and primer-probe combinations outlined in Table 1 of the main test. Absolute quantification of genes of interest were determined using standards of known concentration. Copy numbers for all genes of interest were normalised to the reference gene 18S.

Immune transcriptome expression analyses

Purified total RNA was hybridized to the human immunology panel version 2 (Nanostring), as per manufacturers' instructions. Raw data was quality control checked and normalized based on positive controls, negative controls and housekeeper gene expression based on most stable housekeeping genes with robust detection, followed by identification of differentially expressed genes (DEGs).

Supplemental Figures:

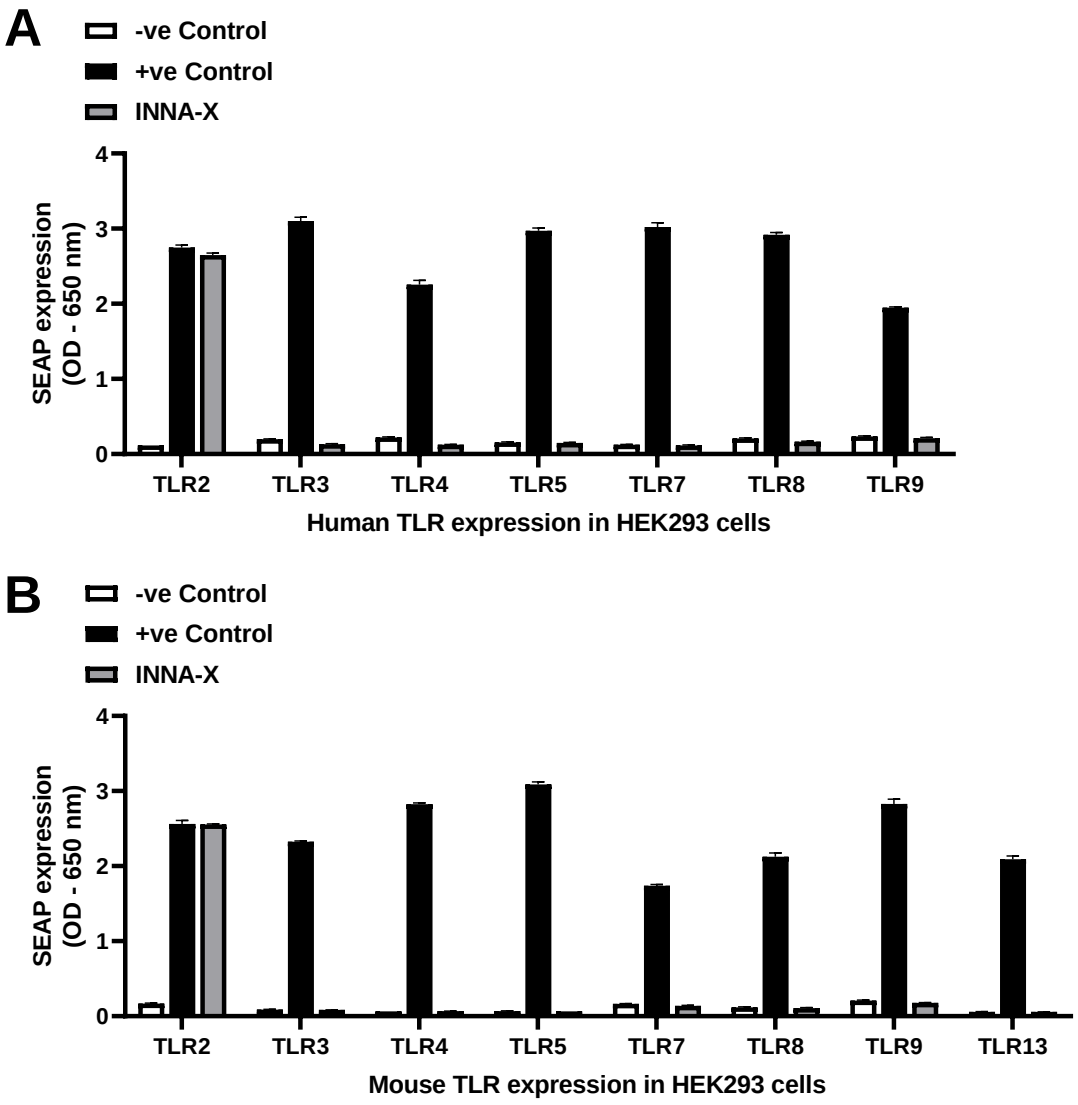

Fig. S1. INNA-X treatment increased NF-κB activity in HEK293 cells expressing human or mouse TLR2. HEK293 cells were transfected with individual **A)** human or **B)** mouse TLRs and stimulated with 10ng/mL of INNA-X or TLR-specific positive control and SEAP production was measured as 650nm OD 20 hours post-treatment. Data shown represents n=3 mean±SD.

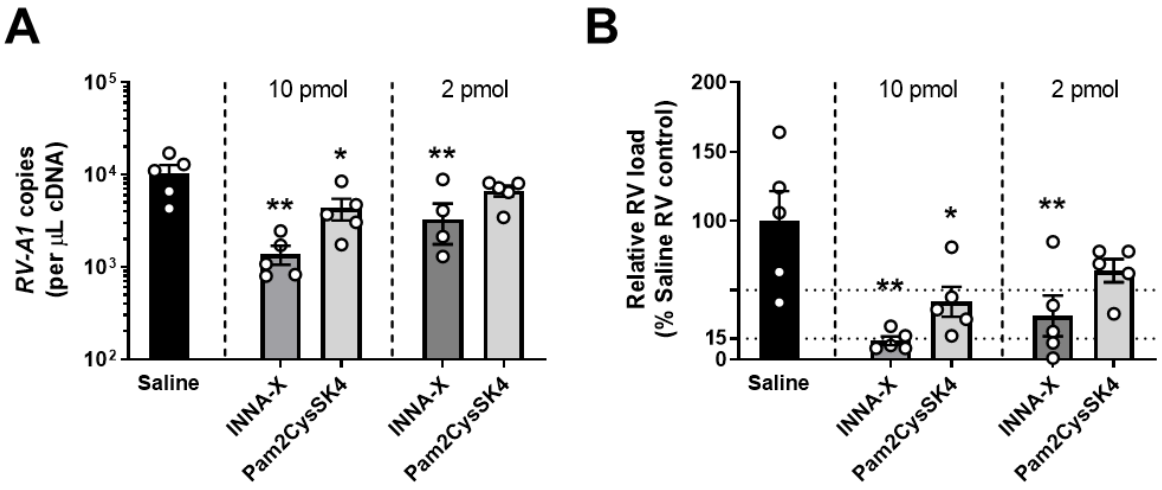

Fig. S2 INNA-X suppressed viral load at lower doses than Pam2CysSK4. Mice were treated via intranasal administration of INNA-X (10 pmol or 2 pmol), Pam2CysSK4 (10 pmol or 2 pmol) or saline control on day -7 (d-7) before infection. On day 0, mice were infected with RV-A1 or mock (PBS vehicle). Samples were harvested on day 2 (48 hours post-infection) to measure RV-A1 levels in apical lung samples, as determined by **A)** qPCR normalised to 18S expression and expressed as copy number per µL cDNA or **B)** relative viral load compare to Saline treated controls. Data represent mean±SEM, n=5 mice per group, *p<0.05, **p<0.01 one-way ANOVA with Holm-Sidak's correction for multiple comparisons, compared to Saline RV-A1 control.

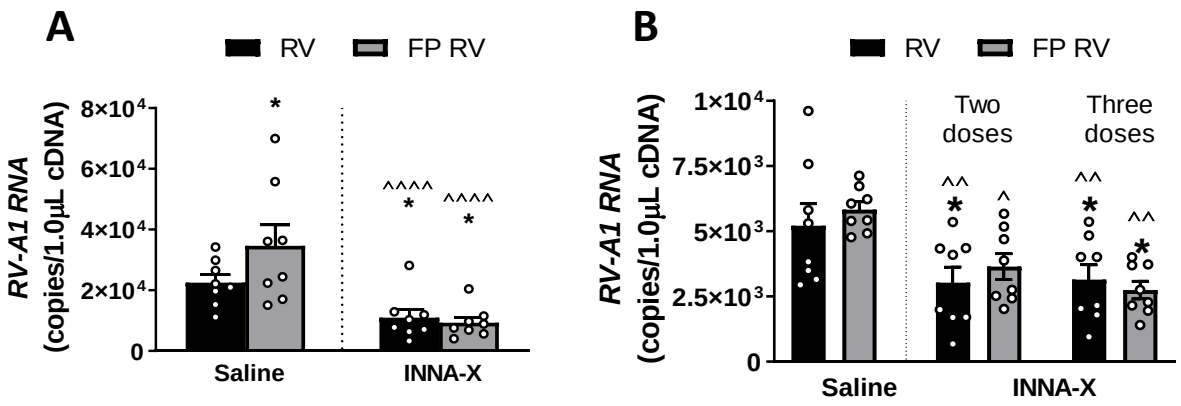

Fig. S3 INNA-X suppressed viral copy numbers with single or multiple weekly doses, and in combination with fluticasone propionate treatment. Mice were treated via intranasal administration of INNA-X (2 pmol) or saline control on day -7 (d-7) before infection, or were treated with once weekly INNA-X (2pmol) dosing for either two or three weeks prior to infection. On day 0, mice were treated with FP/vehicle treatment and sequently RV-A1/mock infected as previous and samples were harvested 2 days after infection to determine levels of RV-A1 RNA in the apical lung lobe. qPCR normalised to 18S expression and expressed as copy number per µL cDNA. Data show mean±SEM. *p<0.05 ANOVA with Holm-Sidak's correction for multiple comparisons, compared to Saline RV-A1 control.

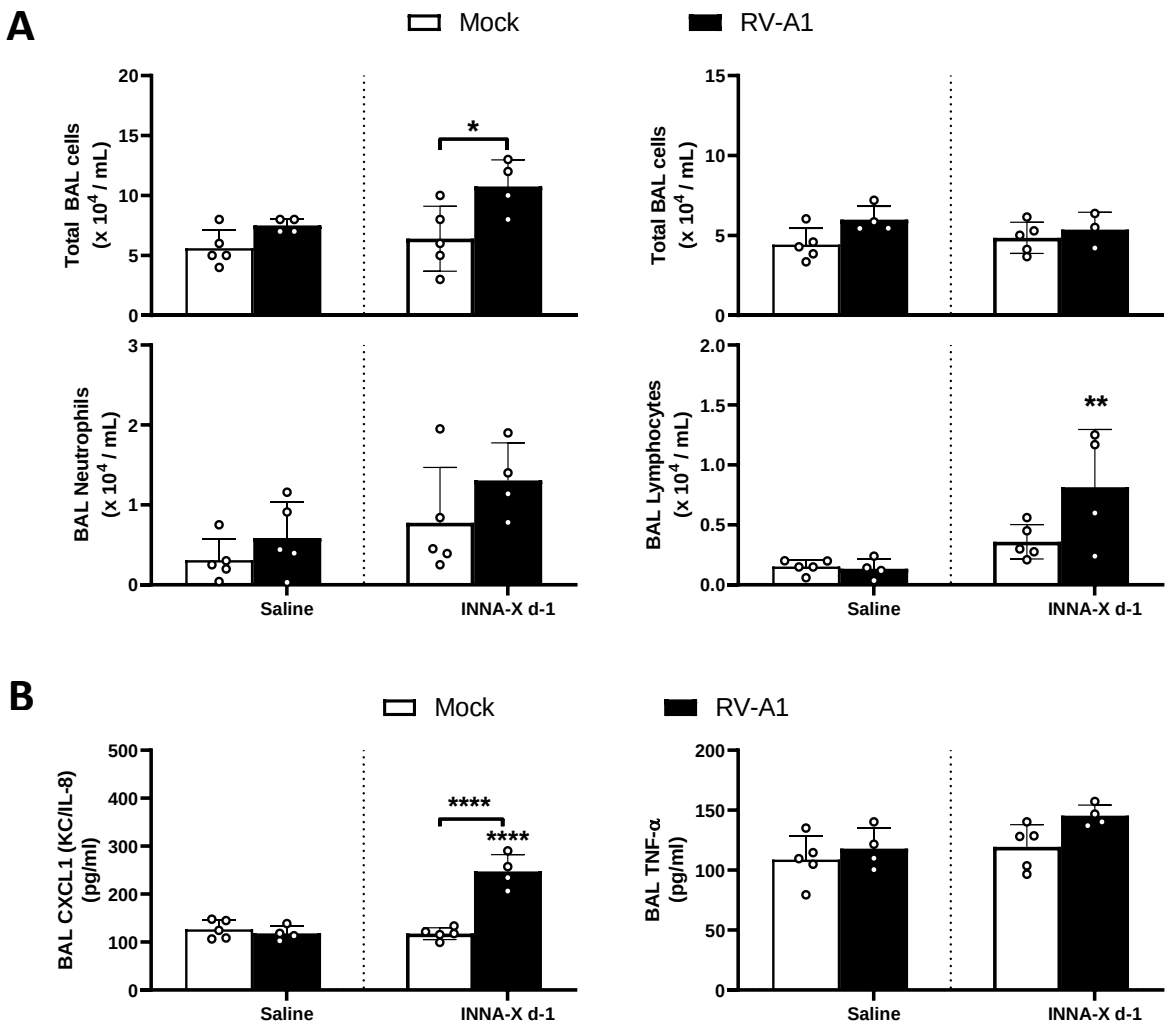

Fig. S4 INNA-X treatment on day -1 results in increased lymphocyte recruitment and enhanced neutrophil chemokine production. Mice were treated via intranasal administration of INNA-X (2 pmol), or saline control on day -1 (d-1). On day 0, mice were infected with RV-A1 or mock (PBS vehicle). Samples were harvested on day 2 (48 hours post-infection) to measure A) total cell numbers and differential, macrophage, neutrophil and lymphocyte counts in bronchoalveolar lavage (BAL) and B) Protein levels of BAL cytokines CXCL1 and TNF-α. Data represent mean±SEM, n=4-5 mice per group, *p<0.05, **p<0.01,

****p<0.0001 one-way ANOVA with Holm-Sidak's correction for multiple comparisons, compared to Saline RV-A1 control unless shown otherwise.

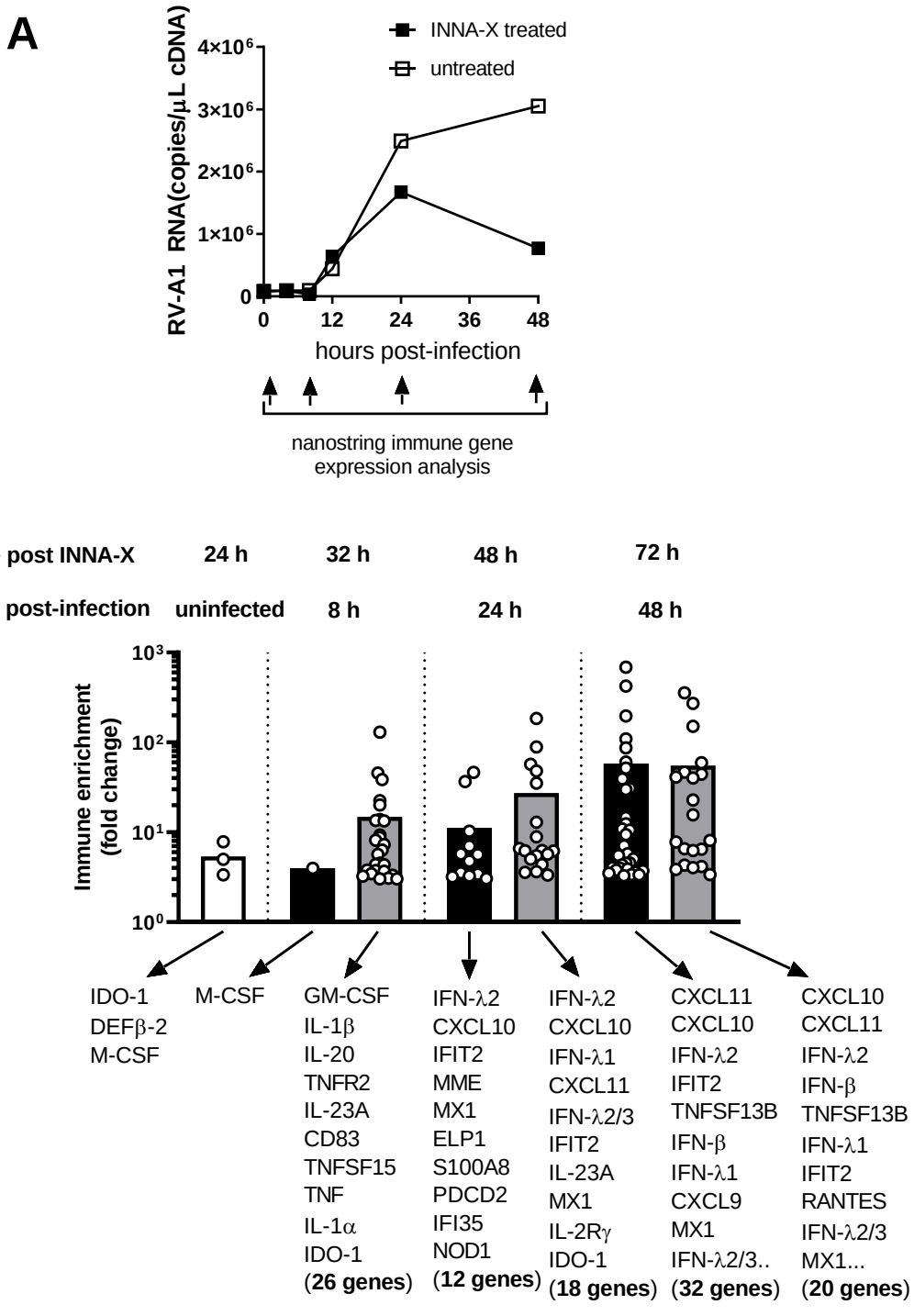

Fig. S5. INNA-X treatment reduced viral load in healthy BEC by priming innate immunity. Primary BECs from a healthy donor were cultured at air-liquid interface, and were treated with INNA-X 24 h prior to infection with RV-A1 and **A)** viral load data was assessed over time by qPCR and **B)** immune enrichment was assessed by Nanostring immune transcriptome data showing >3 fold up regulated genes. Data show n=1 per condition (A), bars represent extent of immune enrichment determined by number and magnitude of upregulated genes (B).

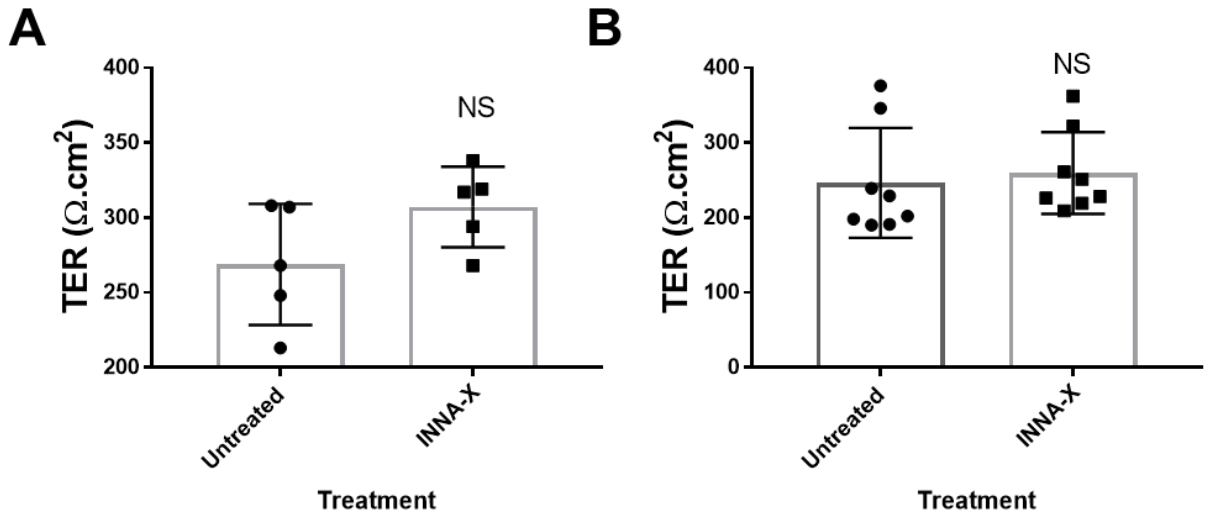

Fig. S6. INNA-X treatment does not alter barrier function of epithelial cells *in vitro*. Transepithelial resistance was measured across differentiated epithelium from **A)** one healthy donor (n=5 repeats) and **B)** asthmatic donors (n=8) without treatment or with INNA-X after 24 hours. Data shown represent mean ± SD, non-parametric Wilcoxon matched-pairs signed rank test.

Supplemental Tables:

Table S1: Bronchial epithelial cell donor characteristics. Donor characteristics including asthma severity (GINA stage) or healthy control status (as relevant), age, sex, smoking status, exacerbation frequency, lung function, atopic status and neutrophil, eosinophil and macrophage counts from bronchial lavage.

Patient Number	**GINA stage**	**Age**	**Sex**	**Smoker**	**Non-smoker**	**Ex-smoker**	**Exac 12mths**	**FEV1%**	**FVC%**	**FEV1 / FVC**	**Atopic**	**Neut (%)**	**Eos (%)**	**Mac (%)**
AS265	Moderate	70	M		Y		1	75	75	77	N	48	14.75	16.75
AS286	Severe	73	M			Y	2	58	88	55	N	83.5	3.5	11.75
AS288	Severe	54	M		Y		14	82	105	64	N	73.5	2.5	9.25
AS293	Severe	28	F	Y			3	79	80	87	N	13.75	7.25	73.75
AS270	Severe	22	F			Y	2	91	104	76	Y	2.25	6	91.75
AS289	Severe	56	F		Y		6	42	NA	NA	Y	51.5	0.5	13
AS291	Severe	45	F			Y	3	53	83	51	Y	96.5	0.5	2.75
AS295	Severe	42	F		Y		3	64	106	49	N	28.5	1	32.5
HC188		52	F					73	81	71				
HC192		89	M					84	92	74				

Table S2: Gene lists included in Nanostring advanced analysis platform for pathways analyses. Each gene in the Nanostring human immunology (version 2) code set are annotated with pathway names each gene is involved with. nSolver advanced analysis software scores pathway enrichment by z score as well as directed and undirected t stat for all annotated genes from each pathway based on magnitude of expression and p values for each gene.

Pathway	**Defense Response**
Species	Human
	BLNK-mRNA
	C2-mRNA
	CCL20-mRNA
	CD19-mRNA
	CD40-mRNA
	CD97-mRNA
	CEBPB-mRNA
	CX3CL1-mRNA
	CXCL10-mRNA
	CXCL1-mRNA
	CXCL2-mRNA
	HLA-B-mRNA
	IL1A-mRNA
	IL1RAP-mRNA
	IL32-mRNA
	IRAK2-mRNA
	ITGB1-mRNA
	LTB4R-mRNA
	MX1-mRNA
	NFATC3-mRNA
	NFKB1-mRNA
	PTAFR-mRNA
	S100A8-mRNA
	S100A9-mRNA

Table S3: Nucleotide sequences of TaqMan primers and probes

Gene	Sequence (5'-3')		
	Forward	**Reverse**	**Probe**
18S	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG	FAM-ACCGGCGCAAGACGGACCAGA-TAMRA
Rhinovirus	GTGAAGAGCCsCrTGTGCT	GCTsCAGGGTTAAGGTTAGCC	FAM-TGAGTCCTCCGGCCCCTGAATG-TAMRA
Ifn-β	CGCCGCATTGACCATCTA	TTAGCCAGGAGGTTCTCAACAATAGTCTCA	FAM-TCAGACAAGATTCATCTAGCACTGGCTGGA-TAMRA
Ifn-λ1	GGACGCCTTGGAAGAGTCACT	AGAAGCCTCAGGTCCCAATTC	FAM-AGTTGCAGCTCTCCTGTCTTCCCCG-TAMRA
Ifn-λ2/3	CTGCCACATAGCCCAGTTCA	AGAAGCGACTCTTCTAAGGCATCTT	FAM-TCTCCACAGGAGCTGCAGGCCTTTA-TAMRA
Viperin	CACAAAGAAGTGTCCTGCTTGGT	AAGCGCATATATTCATCCAGAATAAG	FAM-CCTGAATCTAACCAGAAGATGAAAGACTCC-TAMRA
OAS1	CTGACGCTGACCTGGTTGTCT	CCCCGGCGATTTAACTGAT	FAM-CCTCAGTCCTCTCACCACTTTTCA-TAMRA
PKR	AAGGGAACTTTGCGATACATGAG	GCGTAGAGGTCCACTTCCTTTC	FAM-CCAGAACAGATTTCTTCGCAAGACTAT-TAMRA

Supplementary Materials References:

1. Bartlett NW, Walton RP, Edwards MR, Aniscenko J, Caramori G, Zhu J, Glanville N, Choy KJ, Jourdan P, Burnet J, Tuthill TJ, Pedrick MS, Hurle MJ, Plumpton C, Sharp NA, Bussell JN, Swallow DM, Schwarze J, Guy B, Almond JW, Jeffery PK, Lloyd CM, Papi A, Killington RA, Rowlands DJ, Blair ED, Clarke NJ, Johnston SL. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. *Nat Med* 2008: 14(2): 199-204.
2. Bartlett NW, Singanayagam A, Johnston SL. Mouse models of rhinovirus infection and airways disease. *Methods Mol Biol* 2015: 1221: 181-188.
3. Stewart CE, Torr EE, Mohd Jamili NH, Bosquillon C, Sayers I. Evaluation of Differentiated Human Bronchial Epithelial Cell Culture Systems for Asthma Research. *Journal of Allergy* 2012: 2012: 11.
4. Hackett T-L, Singhera GK, Shaheen F, Hayden P, Jackson GR, Hegele RG, Van Eeden S, Bai TR, Dorscheid DR, Knight DA. Intrinsic Phenotypic Differences of Asthmatic Epithelium and Its Inflammatory Responses to Respiratory Syncytial Virus and Air Pollution. *American Journal of Respiratory Cell and Molecular Biology* 2011: 45(5): 1090-1100.