Research article

MicroRNA-125a/b Inhibits A20 and MAVS to Promote Inflammation and Impair Antiviral Responses in Chronic Obstructive Pulmonary Disease

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ONLINE SUPPLEMENTARY MATERIALS AND METHODS

Ex vivo methods

Subject recruitment

Patients with chronic obstructive pulmonary disease (COPD) were recruited; defined by a previous smoking history and fixed airflow limitation on spirometry with an forced expiratory volume in 1 second (FEV_1) / forced vital capacity (FVC) ratio < 70%, and FEV_1 < 80% predicted and classified by the GOLD criteria (1). Those with GOLD Stage III (severe COPD) FEV_1 30 – 50% were included. All COPD subjects were ex-smokers (at least one year abstinent) and none were using inhaled corticosteroids for two weeks before bronchoscopy. Healthy non-smoking controls and current smokers without COPD with no evidence of airflow obstruction, bronchial hyper-responsiveness to hypertonic saline challenge, or chronic respiratory symptoms were also recruited. A clinical history, examination and spirometry were performed on all individuals. At the time of recruitment

no subject had symptoms of an acute respiratory tract infection for the preceding four weeks. None were diagnosed with cancer. All subjects gave written informed consent.

Viruses

Human influenza A viruses (IAVs) A/Wellington/43/2006 (H3N2), A/Auckland/1/2009 (H1N1), were obtained from the WHO Collaborating Centre for Reference and Research on Influenza (Victoria, Australia) (2). Virus stocks were propagated in Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection (ATCC), USA). Virus titers were determined by plaque assay on MDCK cells (3, 4).

Cell culture and IAV infection

Human primary bronchial epithelial cells (pBECs) were obtained by endobronchial brushing during fiber-optic bronchoscopy in accordance with standard guidelines (5). pBEC were cultured as described previously (3, 4, 6, 7). Virus was diluted in serum free medium and added to cells at a multiplicity of infection of five. After 1hr incubation, inocula were removed and replaced with serum-free medium.

A20 plasmid, siRNA and microRNA(miR)-125a and b antagomiRs and mimetics

A20 gene was amplified by PCR using the forward primer containing XbaI (5'-aaat*tctaga*gccgccaccATGGCTGAACAAGTCCTTCCTC-3') and reverse primer containing EcoRI (5'- gcgc*gaattc*TTCTGTCAATGTGAACATGTTCAG -3'). The restriction sites are italic and underlined. The PCR product was cloned into pcDNA3.1 expression vector (pcDNA-A20). The construct was then transfected into pBECs using Lipofectamine 3000 (Life Technologies, USA) according to the manufacturer's instructions. For experiments

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using short interfering (si)RNAs, A20-specific siRNAs (Applied Biosystems, USA) were transfected into cells using siPORT NeoFX transfection agent (Ambion, USA). AllStars Negative controls (Qiagen, USA) were used as siRNA negative controls. For miR-125a/b antagomiR and mimetic experiments, anti-hsa-miR-125a and anti-hsa-miR-125b antagomiRs (Ambion, USA), and miR-125a and miR-125b mimetics (Ambion, USA) were transfected into cells using siPORT NeoFX transfection agent (Ambion, USA) 24hr before infection according to manufacturer's instructions. Anti-miRNA inhibitor negative control (Qiagen, USA) was used.

Cloning and mutagenesis of miR-125a/b binding site in MAVS 3`UTR

The fragment spanning the miR-125a/b putative binding site in 3` untranslated region (UTR) of MAVS was generated by PCR and cloned into a pMIR luciferase vector using MluI and HindIII cloning sites (italic; Life Technologies, USA). Next, site-directed mutagenesis was performed to introduce a mutation (TCA→AGT) into the binding site sequence (shown in bold below). All constructs were sequenced to confirm their identity. The primers used for PCR amplification and mutagenesis are underlined.

MAVS 3`UTR

c*cacgcgt*TGTGAACCACAGCTTATCACATGTCTGGAGTTAGGGACCCCACTTAAA
GTGAGATTTTGGCTGGAGGTGGTGGATCATACCTATAATCCCAGCACTTTGGGA
GACCAAGGCAGAAGGACTGCTTGAGGCCAGGAGTTCAAAACCAGTGTAGGTA
ACAGCTAGACCCTATCTCTACAAAAAATTTAAAAATTAGCTGGGTGTGGTGGT
ATGTGCCTCAAGTTCCAGCTACTCAGGAGGCTGAGGTGGGAGGATCACTTGAG
CACAGGAGTTTGAAGTTACAGTGAGCTATGATGGCACCACTGCACTTCAGCCT
AGGCAACAGAGGGAGACCCTGTCTTTAAAGTACATAGAGGTTTTTCACACCAA

CACATCTCTGCCCAGTGTGCCAACATCTGCCACCTGCTATAATAGTACTATAAC
ACTCAATATGTAATTAATGTAGTC**TCA**GGGATGTTATGACAATATGATTACAAC
TATCACGTGTGTGCCCAGCCAGGCTCAATGCCCCAGGCTGGGCGAGGTGGGGC
AGGGGACACAGCCTAAAATGCCAGGCCTCAGGAAGCCATTTGGTTTAGCAGAC
ATTGTTTATTAAAGGAGTTACCTATGCCAGATCGAAGGCCTAAGATGATTAAG
ACACTATGAGTGCCTTCAAGTGGTTGGGGACGTTCATGATTGTGGTACAGACA
AATAGGCTTTCACATCATTCTTTTATGTAATCATACAACAGATATTTGCACCTA
CATG*aagctt*cg

Luciferase reporter assay

The constructs described above were co-transfected into HEK293 cells with mimetic pre-miR-125a, pre-miR-125b, or pre-miR scrambled control (Applied Biosystems, USA) using Lipofectamine 3000 (Life Technologies, USA). pRL *Renilla* control vector (Promega, USA) was also used. Cells were harvested 48hr after transfection and luciferase activity was measured using a luminometer (Fluostar Optima, BMG Labtech BMG, Germany). The luciferase reading of pMIR and mimetics was normalized to the *Renilla* control for each sample, and expressed as percentage reduction from miR-scrambled control (8).

Immunoblotting and cytometric bead array

Infected pBECs were lysed in ice-cold RIPA buffer containing protease inhibitor cocktail (Roche, UK). Proteins of lysed pBECs and supernatants (5µg) were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes for detection of A20, and phospho-p65 at Ser536 in the cell lysates using anti-A20 (ab74037, Abcam, UK) and anti-phospho-p65 antibodies (#3031L, Cell Signaling Technology, USA). Glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) was detected as a loading control in cell lysates using specific antibody (ab181602, Abcam, UK). Interferon (IFN)-β was detected in the supernatants using specific antibody (ab85803, Abcam, UK). Proteins on membranes were then visualized by chemiluminescence (Bio-Rad ChemiDoc MP System, CA, USA). All blots were probed for proteins of interests and then stripped and re-probed for loading control. The densitometric value of IFN-β was normalized to un-infected media control. For other intracellular proteins, the densitometric values of all lanes in a blot were first normalized to the loading control. Values were then expressed as fold change from healthy media control if comparing between healthy, COPD, and smoker pBECs, or un-treated control if cells were treated with siRNAs, pcDNA-A20, antagomiR or mimetics. Blots were run according to the comparisons being made. If comparing between disease groups (healthy vs COPD vs smoker), then samples from one subject from each group were run on the same blot and compared with or without infection and between the disease group (ie fig. S1). The control group was the healthy controls. These were then run for all subjects from each group. If comparing between experimental conditions such as siRNAs or antagomiR treatment, samples from one subject with all experimental controls were run on the same blot (fig. S2B/C/D). The experimental control was non-silenced or non-treated (pcDNA-A20). Some blots were cut at appropriate protein molecular weights so that multiple proteins of interests could be detected at the same time. Blots were stripped and re-probed only once to avoid high backgrounds. Human IL-6, CXCL-8, TNF-α, and IL-1β concentrations were determined by cytometric bead array using a FACSCanto II flow cytometer (BD Biosciences, USA) according to the manufacturer's instructions. IFN-λ1 was measured by ELISA according to the manufacturer's instructions (R&D Systems, USA).

Immunoprecipitation

Ago2 was immunoprecipitated from transfected HEK293 cells using anti-Ago2 antibody (ab32381, Abcam, UK) protein-A conjugated Dynabeads (Life Technologies, USA) according to the manufacturer's instructions.

miRNA extraction and analysis

Extraction of total RNA from infected pBECs was performed with miRNeasy Mini Kits (Qiagen, USA) according to the manufacturer's instructions. Total RNAs (200ng) were reverse transcribed to cDNA and amplified using miR-125a or b specific primers (Qiagen, USA) and qPCR. RNU6B was used as the reference gene. Expression levels of miRNAs were calculated relative to RNU6B using the $2^{-\Delta\Delta Ct}$ method, and were analyzed as fold change induction over media controls.

In vivo methods

Experimental mice

Six to eight-week old female BALB/c mice were used in all the experiments. Animals were obtained from The University of Newcastle Animal Services Unit and were given access to food and water *ad libitum*. Animals were housed in a specific pathogen-free facility with controlled environment of 14h/10h light/dark cycles.

Cigarette smoke exposure

Mice were exposed to the smoke from 12x3R4F reference cigarettes (University of Kentucky, USA) twice per day, five times per week, for eight weeks using a custom-

designed and purpose-built specialized nose-only, directed flow inhalation and smoke-exposure system contained in a laminar flow and smoke-extraction unit (CH Technologies) **(9-15)**. Non-smoking control mice were exposed to normal air for the same period of time.

IAV infection and antagomiR treatment

On the last day of smoke exposure, mice were anesthetized with isoflurane and infected intranasally with eight plaque forming units (PFUs) of the mouse-adapted A/PR/8/34 in 50µl of media (UltraMDCK, Lonza) (16, 17). Controls were sham-inoculated with media. The miR-125a and b sequences were downloaded from miRBase University of Manchester, UK (http://www.mirbase.org/). miR-125a and b and scrambled antagomiR control (nonspecific RNA VIII, BLAST searched against the mouse genome) were designed and purchased from Sigma-Aldrich. The sequences of the antagomiRs were: 5'mU.*.mC.*.mA.mA.mC.mA.mU.mC.mA.mG.mU.mC.mU.mG.mA.mU.mA.mA.mG.*. mC.*.mU.*.mA.*.3'-Chl, where (m) denotes 2'-O-methyl-modified nucleotides, (*) denotes phosphorothioate linkages, and (–Chl) denotes hydroxyprolinol-linked cholesterol. Mice were treated with antagomiR (50µg delivered in 50µL sterile saline i.n.) or an equivalent amount of scrambled control as described previously (18, 19). In each experiment, following IAV inoculation, smoking was discontinued to remove the effects of acute smoke exposure, and mice were sacrificed at 7 days post infection.

Histopathology and immunohistochemistry

Mice lungs were perfused, inflated, formalin-fixed, paraffin-embedded and sectioned (4-6µm). Sections were then stained with hematoxylin and eosin and histopathology score was performed as previously described (20-24). Sections were also stained with anti-A20

antibody (Abcam, UK) and followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (R&D Systems). The images were viewed under a light microscope (Olympus, Japan). To assess A20 in mouse lung sections, slides were incubated with Anti-A20 antibody (ab74037, Abcam, UK) at 4°C overnight, followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (VC002-025, R&D systems, 37°C, 30min). Diaminobenzidine (DAB, DAKO) was applied on slides and hematoxylin was used as a counterstain. Photomicrographs were taken and images evaluated with image J (version 1.47) **(25, 26)**. Briefly, airways were divided into three categories according to the perimeter of their basement membrane (Pbm): Pbm ≤1 mm (small), Pbm ≤2 mm (medium) and Pbm > 2mm (large) (27). At least 6 small airways per mouse were blind-selected and examined with a light microscope (BX41, Olympus). The A20 area and width of Pbm were manually measured using Image J. The A20 area was normalized to the Pbm.

Immunoblotting and cytometric bead array

Whole lung tissues were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche). The supernatants containing the protein fraction were collected. Proteins (40µg) were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes for detection of A20, phospho-p65, and IFN-β. β-actin was detected as a loading control. Mouse IL-6, KC, TNF-α, and IL-1β concentrations were determined by cytometric bead array using a FACSCanto II flow cytometer (BD Biosciences, USA) according to the manufacturer's instructions.

Statistical analysis. When data were normally distributed they were expressed as mean ± standard error of mean (SEM). Data were analyzed using nonparametric equivalents and

summarized using the median and inter-quartile range (IQR) when non-normally distributed. Multiple comparisons were first analysed by the Kruskal Wallis test and then by individual testing if significant. A p-value of < 0.05 was considered significant. The study was approved by the University of Newcastle Human and Animal Research Ethics Committee.

References

1. Pauwels RA, A.S. Buist, P.M. Calverley, C.R. Jenkins, and S.S. Hurd. NHLBI/WHO Global initiative for chronic obstructive lung disease (GOLD) Workshp summary. *American journal of respiratory and critical care medicine.* 2001;163(1256-76.
2. Hurt AC, Hansbro PM, Selleck P, Olsen B, Minton C, Hampson AW, and Barr IG. Isolation of avian influenza viruses from two different transhemispheric migratory shorebird species in Australia. *Archives of virology.* 2006;151(11):2301-9.
3. Hsu AC, Parsons K, Barr I, Lowther S, Middleton D, Hansbro P, and Wark P. Critical role of type I interferon response in bronchial epithelial cell to influenza infection. *PLoS ONE.* 2012;7(3):e32947.
4. Hsu AC, Barr I, Hansbro PM, and Wark PA. Human influenza is more effective than avian influenza at antiviral suppression in airway cells. *Am J Respir Cell Mol Biol.* 2011;44(6):906-13.
5. Hurd SZ. Workshop summary and guidelines: investigative use of bronchoscopy. *J Allergy Clin immunol* 1991;88(808-14.
6. Baines KJ, Hsu AC, Tooze M, Gunawardhana LP, Gibson PG, and Wark PA. Novel immune genes associated with excessive inflammatory and antiviral responses to rhinovirus in COPD. *Respiratory research.* 2013;14(15.
7. Conickx G, Mestdagh P, Cobos FA, Verhamme FM, Maes T, Vanaudenaerde BM, Seys LJM, Lahousse L, Kim RY, Hsu AC, et al. MicroRNA profiling reveals a role for microRNA-218-5p in the pathogenesis of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine.* 2016;In Press(

8. Kim SW, Ramasamy K, Bouamar H, Lin AP, Jiang D, and Aguiar RC. MicroRNAs miR-125a and miR-125b constitutively activate the NF-kappaB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). *Proceedings of the National Academy of Sciences of the United States of America.* 2012;109(20):7865-70.

9. Horvat JC, Starkey MR, Kim RY, Beagley KW, Preston JA, Gibson PG, Foster PS, and Hansbro PM. Chlamydial respiratory infection during allergen sensitization drives neutrophilic allergic airways disease. *J Immunol.* 2010;184(8):4159-69.

10. Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, Engels G, Brenker C, Nordhoff M, Mirandola SR, Al-Amoudi A, et al. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nature immunology.* 2014;15(8):727-37.

11. Thorburn AN, Foster PS, Gibson PG, and Hansbro PM. Components of Streptococcus pneumoniae suppress allergic airways disease and NKT cells by inducing regulatory T cells. *J Immunol.* 2012;188(9):4611-20.

12. Haw TJ, Starkey MR, Nair PM, Pavlidis S, Liu G, Nguyen DH, Hsu AC, Hanish I, Kim RY, Collison AM, et al. A pathogenic role for tumor necrosis factor-related apoptosis-inducing ligand in chronic obstructive pulmonary disease. *Mucosal Immunol.* 2015;Advance online publication. 10.1038/mi.2015.111(

13. Essilfie AT, Horvat JC, Kim RY, Mayall JR, Pinkerton JW, Beckett EL, Starkey MR, Simpson JL, Foster PS, Gibson PG, et al. Macrolide therapy suppresses key features of experimental steroid-sensitive and steroid-insensitive asthma. *Thorax.* 2015;70(5):458-67.

14. Liu G, Cooley MA, Jarnicki AG, Hsu ACY, Nair PM, Haw TJ, Fricker M, Gellatly SL, Kim RY, Inman MD, et al. Fibulin-1 regulates the pathogenesis of tissue remodeling in respiratory diseases. *JCI Insight.* 2016;1(9):e86380(

15. Beckett EL, Stevens RL, Jarnicki AG, Kim RY, Hanish I, Hansbro NG, Deane A, Keely S, Horvat JC, Yang M, et al. A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. *J Allergy Clin Immunol.* 2013;131(3):752-62.

16. Beckett E, Stevens R, Jarnicki A, Kim R, Hanish I, Hansbro N, Deane A, Keely S, Horvat J, Yang M, et al. A short-term model of COPD identifies a role for mast cell tryptase. . *J Allergy Clin Immunol.* 2013;Accepted 28.11.12(

17. Hsu AC, Starkey MR, Hanish I, Parsons K, Haw TJ, Howland LJ, Barr I, Mahony JB, Foster PS, Knight DA, et al. Targeting PI3K-p110alpha Suppresses Influenza Virus Infection in Chronic Obstructive Pulmonary Disease. *American journal of respiratory and critical care medicine.* 2015;191(9):1012-23.

18. Plank MW, Maltby S, Tay HL, Stewart J, Eyers F, Hansbro PM, and Foster PS. MicroRNA Expression Is Altered in an Ovalbumin-Induced Asthma Model and Targeting miR-155 with Antagomirs Reveals Cellular Specificity. *PLoS One.* 2015;10(12):e0144810.

19. Kim RY, Horvat JC, Pinkerton JW, Starkey MR, Essilfie AT, Mayall JR, Nair PM, Hansbro NG, Jones B, Haw TJ, et al. MicroRNA-21 drives severe, steroid-insensitive experimental asthma by amplifying PI3K-mediated suppression of HDAC2. *J Allergy Clin Immunol.*Accepted 30 Apr 2016(

20. Asquith KL, Horvat JC, Kaiko GE, Carey AJ, Beagley KW, Hansbro PM, and Foster PS. Interleukin-13 promotes susceptibility to chlamydial infection of the respiratory and genital tracts. *PLoS pathogens.* 2011;7(5):e1001339.

21. Horvat JC, Beagley KW, Wade MA, Preston JA, Hansbro NG, Hickey DK, Kaiko GE, Gibson PG, Foster PS, and Hansbro PM. Neonatal chlamydial infection induces mixed T-cell responses that drive allergic airway disease. *American journal of respiratory and critical care medicine.* 2007;176(6):556-64.

22. Starkey MR, Essilfie AT, Horvat JC, Kim RY, Nguyen DH, Beagley KW, Mattes J, Foster PS, and Hansbro PM. Constitutive production of IL-13 promotes early-life Chlamydia respiratory infection and allergic airway disease. *Mucosal Immunol.* 2013;6(3):569-79.

23. Starkey MR, Nguyen DH, Essilfie AT, Kim RY, Hatchwell LM, Collison AM, Yagita H, Foster PS, Horvat JC, Mattes J, et al. Tumor necrosis factor-related apoptosis-inducing ligand translates neonatal respiratory infection into chronic lung disease. *Mucosal Immunol.* 2014;7(3):478-88.

24. Preston JA, Essilfie AT, Horvat JC, Wade MA, Beagley KW, Gibson PG, Foster PS, and Hansbro PM. Inhibition of allergic airways disease by immunomodulatory therapy with whole killed Streptococcus pneumoniae. *Vaccine.* 2007;25(48):8154-62.

25. Liu G, Cooley MA, Jarnicki AG, Hsu ACY, Nair PM, Haw TJ, Fricker M, Gellatly SL, Kim RY, Inman MD, et al. Fibulin-1 regulates the pathogenesis of tissue remodeling in respiratory diseases. *JCI Insight.* 2016;1(9).

26. Haw TJ, Starkey MR, Nair PM, Pavlidis S, Liu G, Nguyen DH, Hsu AC, Hanish I, Kim RY, Collison AM, et al. A pathogenic role for tumor necrosis factor-related

apoptosis-inducing ligand in chronic obstructive pulmonary disease. *Mucosal Immunol.* 2015.

27. Palmans E, Kips JC, and Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med.* 2000;161(2 Pt 1):627-35.