**Toll-like receptor 7 is reduced in severe asthma and linked to altered microRNA profile**

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**Online Supplement**

**Supplementary Methods**

**Characterisation of study subjects**

The study was approved by the Southampton and South West Hampshire Local Research Ethics Committee. Healthy volunteers were recruited through local advertising and consented to having characterisation studies and a research bronchoscopy. Baseline spirometry was measured using the Jaeger Masterscreen with Viasys® software. Healthy volunteers underwent methacholine (Stockport Pharmaceuticals, Stockport UK) challenge testing to exclude airway hyperresponsiveness. Skin prick testing was performed using allergen extract from the following: *Aspergillus fumigatus*, *Alternaria tenius*, birch tree pollen, mixed grasses, mixed tree pollen, rape pollen, weed pollen, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, dog fur and cat dander, along with positive (histamine) and negative (saline) control (all from Diagenics Ltd., Milton Keynes UK).

Severe asthma patients filled out the Asthma Control Questionnaire (ACQ) which has been validated as a tool to assess the adequacy of asthma control ([1](#_ENREF_1)). It has 7 questions regarding asthma control during the previous week. The response to all 7 questions is on a 7-point scale and the ACQ score is the mean of the 7 items.

Asthma exacerbations were defined as per the American Thoracic Society/European Respiratory Society guidelines as events clinically identified to be outside the patient’s usual range of day-to-day asthma variation, which are troublesome for the patient and require a change in treatment, such as at least 3 days of oral corticosteroids, an increase from maintenance dose of systemic corticosteroids, or hospitalisations/emergency department visits ([2](#_ENREF_2)). Bronchoscopies were performed at least 6 weeks post recovery from an exacerbation.

**Alveolar macrophages (AM)**

Bronchoalveolar lavage (BAL) was performed by instilling 6x20ml aliquots normal saline into the right upper lobe. BAL was immediately placed on ice and then filtered using a 100µm nylon filter and washed twice in an attempt to remove dead cells and any cell debris. The cells were then re-suspended in RPMI 1640 (Life Technologies Ltd., Paisley UK) at a concentration of 1X106 cells/ml and placed at 37°C in a humidified 5% CO2 incubator for 2 hours to allow AM adherence. The supernatant was discarded and adhered AMs washed with PBS to remove any remaining dead cells.

Due to the number of AMs obtained from each subject, they were either used for baseline RNA and protein analysis or for functional studies. For functional studies, AMs were plated onto 96-well plates at a concentration of 1x106 cells/ml in RPMI 1640+10% FCS and penicillin/streptomycin and incubated with imiquimod (Invivogen, Toulouse France, 5µg/ml) or rhinovirus 16 (RV16; MOI of 0.6). As negative controls, cells were treated with medium alone or UV-inactivated RV16 (UV-RV). Cells and supernatants were harvested at 24 hours.

The amount of RV16 viral RNA (RV load) was measured in healthy and SA-AM 24 hours after exposure to RV16 to ensure that any differences in the induction of IFN and ISGs observed were not due to defective infection or uptake of the virus by AMs. Samples employed in Figure 1A were analysed for HRV16 presence. Figure E2 shows that the amount of RV16 RNA is not statistically significantly different in SA-AM compared to healthy AMs. HRV16 detection was done employing a commercially validated HRV16 Pimer/Probe mix (PrimerdesignTM genesig® Kit for Human Rhinovirus 16, Primerdesign Ltd, UK).

For the transfection studies, AMs were obtained from healthy and severe asthma patients. We felt we could use cells from both sets of subjects because we wanted to demonstrate a mechanism- inhibition of miR-150, miR-152 and miR-375 boosts TLR7 mediated IFN responses. Furthermore, the availability of primary AMs is limited.

Briefly, AMs were plated onto 96-well round bottom plates at a cell concentration of 1x106 cells/ml in RPMI 1640+10% FCS and penicillin/streptomycin. The experimental set-up for these experiments included a blank control (i.e. an untreated sample), scrambled antagomir, scrambled antagomir with stimulant, anti-miRs 150, 152 and 375 together (anti-miR mix) and finally anti-miR mix with stimulant. Anti-miR oligonucleotides were added at a final concentration of 150nM: anti-miR-150, anti-miR-152 and anti-miR-375 together, or anti-miR scrambled control (all Life Technologies Ltd.). After 48h in culture (to give time for the antagomirs to inhibit the specific cellular miRNAs and allow downstream effects on target mRNA) cells were treated with imiquimod (5µg/ml, RV-16 (MOI 0.6) or Poly:IC (Invivogen, 10µg/ml) and 24 h later RNA was collected and subjected to analysis.

No transfection reagent was used as AMs take up antimiR oligonucleotides spontaneously. Confocal imaging (described in detail below) was used to show the presence of antagomirs within the cytoplast of AMs after transfection (Figure E1). The blue stain is DAPI (representing the nucleus) and the magenta stain is Cy3TM Dye-Labelled Anti-miRTM Negative Control #1 (ThermoFisher Scientific). This confirms that antagomirs rapidly enter AMs without the use of a transfection reagent. The use of the scrambled antagomir was to account for possible side effects of the antagomirs themselves and our analyses showed that IFN expression was similar in the blank control, scrambled antagomir control and anti-miR mix control samples (data not shown).

To study the effects of steroids on miRNA expression, healthy AMs were treated with 10nM, 100nM and 1000nM of dexamethasone (Sigma Aldrich, Dorset UK) for 24 and 48 hours. To study the effects of cytokines on miRNA expression, healthy AMs were treated with the following cytokines (each at 10ng/ml) to reflect Th1 and Th2 stimulation: IL-4, IL-13, TNFα and IFNγ (all from R&D Systems, Abingdon UK).

**Confocal microscopy**

AMs were transfected with 150nM Cy3TM Dye-Labelled Anti-miRTM Negative Control #1 (ThermoFisher Scientific). 24 hours post-transfection cells were washed with cold phosphate buffered saline (PBS) and fixed with PFA 3.5% in cold PBS for 10 minutes at 4°C. Cells were then washed 3 times with ice cold PBS and stained with DAPI (Sigma Aldrich, Dorset UK) for 15 minutes on ice, washed and imaged immediately using a Leica SP2 upright confocal/multiphoton microscope.

**Viral culture**

Human rhinovirus 16 (RV16; a gift from Dr Jens Madsen, University of Southampton, Southampton UK) was amplified in HeLa cells, as previously described ([3](#_ENREF_3)). Infectivity was determined with a HeLa titration assay as 50% tissue culture infective dose (TCID50)/ml.

**Reverse transcription and Quantitative PCR (qPCR) and microRNA array**

For miRNA analysis 5ng of RNA was reverse transcribed with specific stem loop primers for each miRNA while for mRNA analysis 200ng of RNA was used. qPCR was performed using Applied Biosystems TaqMan® Assays and the internal controls used were RNU44 and GAPDH for miRNA and mRNA data respectively. Changes in the expression of miRNA and mRNA were expressed as fold change relative to the control sample.

MiRNA profiling was performed using Taqman® Low Density Array System (Life Technologies) following manufacturer’s instructions. 500ng of total cellular RNA was reverse transcribed using Megaplex RT primers and the cDNA was then loaded onto the array card for PCR amplification. Arrays were carried out on AMs from 4 healthy and 4 subjects with mild steroid naïve asthma subjects. Mild asthma subjects were used for the array due to the greater heterogeneity within the severe asthma population and use of high doses of steroids, which could confound the results in a small number of samples. Results were displayed as a Heat map using MultiExperiment Viewer (MeV: <http://www.tm4.org/mev.html>).

**Bioinformatics**

MiRNA targets were predicted using Targetscan 4.2 (http://www.targetscan.org/vert\_42/) with default options. miRanda (<http://www.microrna.org/microrna/getMirnaForm.do>) was also used to ensure target predictions were present in more than one database.

**Protein expression analysis**

Antibodies employed for western blotting were: anti-TLR7 (1:500, Abcam, Cambridge UK), anti-mouseIgG-HRP (Fisher Scientific UK Ltd., Loughborough UK) and anti-β-actin peroxidase (1:25,000, Sigma Aldrich).

Meso Scale Discovery (MSD, Rockville, MD) singleplex kits were used for evaluation of IFNα (IFNα1, subtypes 1, 6 and 13; IFNα2, subtypes 2, 3, 5, 8, 10, 14 and 17) and IFNβ protein measurement following manufacturer’s instructions. Briefly, like a standard ELISA, MSD employs a sandwich immunoassay format. The cytokine in the sample binds to capture antibody immobilised at the bottom of the plate. The labelled detection antibody binds to the cytokine to complete the sandwich and when the read buffer is added, an electrochemiluminescence signal is emitted which can be read by a Sector Imager. The lower limit of detection was 0.7pg/ml and 34pg/ml for IFNα and IFNβ respectively.

**Generation of vectors and in vitro Luciferase assays**

The 3’UTR of TLR7, containing the predicted miR-150, miR-152 and miR-375 seed sequences was amplified from human genomic DNA and cloned into XbaI and NotI sites of the pRLTK vector (Promega) and called pRLTK\_WT\_3’UTR\_TLR7:



Site-directed mutagenesis was performed on this vector to create vectors containing mutations in the seed sequence(s) for miR-150 (pRLTK\_MUT\_3’UTR\_TLR7\_150\_1, pRLTK\_MUT\_3’UTR\_TLR7\_150\_2), miR-152 (pRLTK\_MUT\_3’UTR\_TLR7\_152) and mir375 (pRLTK\_MUT\_3’UTR\_TLR7\_375). For pCDNA3.1\_150, pCDNA3.1\_152 and pCDNA3.1\_375 genomic DNA was amplified by PCR and DNA fragments cut with different pairs of enzymes were cloned into BamHI/XhoI, XhoI/KpnI and XbaI/BamHI pCDNA3.1 multi-cloning sites respectively. In order to generate pCDNA3.1\_MIX, these fragments were cloned simultaneously in pCDNA3.1(-) using XbaI/KpnI (order of cloning from promoter: miR-375, miR-150 and miR152).

Primers used to generate the vectors:

|  |  |  |
| --- | --- | --- |
| pCDNA3.1\_150 | For | 5'-GGATCCTGGGTATAAGGCAGGGACTGGG-3' |
|  | Rev | 5'-CTCGAGAGCAGAGATGGGAGTACAGGG-3' |
| pCDNA3.1\_152 | For | 5'-CTCGAGCCGGCCAGGGATCAGCTGG-3' |
|  | Rev | 5'-GGTACCACGCGTGAGTGGGCGCTGTGCCCGTTGGG-3' |
| pCDNA3.1\_375 | For | 5'-AAGCTTTCTAGAGACCAGGAGATCACCGAGGG-3' |
|  | Rev | 5'-GGATCCGGTGCCTGCGTGGCGATCAGGC-3' |
| pRLTK\_WT\_3’UTR\_TLR7 | For | 5'-TCTAGACCATATTTCAGGGGAGCCACCAA-3' |
|  | Rev | 5'-GCGGCCGCGGAAAATACGACATCGCCAATCTAA-3' |
| pRLTR\_MUT\_3’UTR\_TLR7\_150\_1 | For | 5'-CTTGTAATCCCAGCACTCTCGAGGGCCGAGGCAGGTGGAT-3' |
|  | Rev | 5'-ATCCACCTGCCTCGGCCCTCGAGAGTGCTGGGATTA-3' |
| pRLTR\_MUT\_3’UTR\_TLR7\_150\_2 | For | 5'-CCTGTAATCCCAGCTACTTCTAGAGCTGAGGCAGGAGAATCGC-3' |
|  | Rev | 5'-GCGATTCTCCTGCCTCAGCTCTAGAAGTAGCTGGGATTACAGG-3' |
| pRLTR\_MUT\_3’UTR\_TLR7\_152 | For | 5'-CCTGCTTAAATGTTTTTATCCTCGAGGCAAAGTACTGTATCC-3' |
|  | Rev | 5'-GGATACAGTACTTTGCCTCGAGGATAAAAACATTTAAGCAGG-3' |
| pRLTR\_MUT\_3’UTR\_TLR7\_375 | For | 5'-CAGAGCTAGACTGTCTCAAAACTCGAGAAAAAAAAAAACAC-3' |
|  | Rev | 5'-GTGTTTTTTTTTTTCTCGAGTTTTGAGACAGTCTAGCTCTG-3' |

Transfections were performed 3 times in duplicate. HeLa cells were co-transfected with the luciferase reporter constructs containing either the wild-type or 3’UTR mutants and 800ng of pCDNA3.1\_150 or pCDNA3.1\_152 or pCDNA3.1\_375. Transfections were carried out using Superfect (Qiagen, Manchester UK) and normalization was performed using pGL3 (25ng/well, Promega, Madison WI). Transfected cells were cultured for 24 hours and measurements were made using the Dual-Luciferase® Reporter Assay System (Promega).

**Supplementary Figures and Tables**

**Figure E1:**

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**Figure E1. Anti-miRs are taken up by macrophages after 24 hours and distributed in the cytoplasm.** AMs were exposed to 150nM Cy3-Anti-miR control (magenta) and stained with DAPI (blue, nuclear staining). Panels A-E depict a series of images taken using confocal microscopy corresponding to sections (depth of slice/plane 1µm) of AMs and demonstrate the internal localisation of the transfected anti-miR oligonucleotides.

**Figure E2:**

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**Figure E2. Alveolar macrophages from severe asthmatics and healthy subjects contain comparable amounts of RV16 viral RNA.** Alveolar macrophages from healthy (n=10) and severe asthma (n=10) subjects were exposed to RV16 *ex vivo* for 24 hours and viral RNA was quantified using quantitative reverse-transcriptase polymerase chain reaction. Data is plotted on a log scale. ns=not significant.

**Figure E3:**



**Figure E3. TLR7 expression is similar in alveolar macrophages from atopic and non-atopic severe asthma subjects.** Expression of TLR7 mRNA is reduced in alveolar macrophages (AMs) from atopic (n=15) and non-atopic (n=8) subjects with severe asthma, compared to healthy AMs (n=23) while there is no difference between atopic and non-atopic individuals. Quantified using quantitative reverse-transcriptase polymerase chain reaction and is normalised to glyceraldehyde phosphate dehydrogenase. \*\*\*p<0.001, \*\*\*\*p<0.001, ns=not significant.

**Figure E4:**



**Figure E4. Expression of MyD88 mRNA is not reduced in severe asthmatic AMs compared to healthy AMs.** Expression of MyD88 mRNA was evaluated in healthy (n=11) and SA-AM (n=8) using quantitative reverse-transcriptase polymerase chain reaction and normalised to glyceraldehyde phosphate dehydrogenase. ns=not significant.

**Figure E5**



**Figure E5. Poly:IC induced production of IFNβ is comparable in healthy and SA-AM.** AMs from healthy (n=4) and SA subjects (n=4) were exposed to Poly:IC for 24 hours and IFNβ mRNA quantified using quantitative reverse-transcriptase polymerase chain reaction and normalised to glyceraldehyde phosphate dehydrogenase. Fold induction was calculated based on the expression of IFNβ by the untreated (control) sample in each experiment. ns=not significant.

**Figure E6:**

3' gugaccauguucccAACCCUCu 5'   
 |||||||   
 5' guaaucccagcacuUUGGGAGg 3'

guaaucccagcacu**C**U**C**G**AG**Gg MUT

3' gugaccauguucccAACCCUCu 5'   
 |||||||   
 5' guaaucccagcuacUUGGGAGg 3’

guaaucccagcuacUU**CUAGA**g MUT

3' gugaccAUGUUC-CCAACCCUCu 5'   
 |:| :| || ||||||   
 5' auuuuuUGCUGGAGGAUGGGAGa 3'

3' ggUUCAAGACAGUACGUGACu 5'   
 | |||:| : |||||||   
 5' aaAUGUUUUUAUCUGCACUGc 3'

aaAUGUUUUUAUC**CU**C**GAG**Gc MUT

3' agUGCGCUCGGCUUGCUUGUUu 5'   
 || :| :| || ||||||   
 5' agAC-UGUCUCAAAAGAACAAa 3'

agAC-UGUCUCAAAA**CUCG**A**G**a MUT

**Hsa-miR-150**

Position 484 3’UTR

(7mer-m8)

**Hsa-miR-150**

Position 617 3’UTR

(7mer-m8)

**Hsa-miR-150**

Position 1272 3’UTR

(7mer-1A, not studied)

**Hsa-miR-152**

Position 1036 3’UTR

(7mer-m8)

**Hsa-miR-375**

Position 728 3’UTR

(7mer-1A)

MiRanda-generated alignments of target binding sites on the 3’UTR of TLR7 with seed sequences of miR-150, miR-152 and miR-375. Also shown are sequences of mutants generated (MUT). The mutated nucleotides in the target sites are underlined.

**Figure E7:**



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**Figure E7. MicroRNAs -15a, -19b, -101 and -301 are not up-regulated in severe asthma AMs compared to healthy AMs.** Expression of miR-15a, miR-19b, miR-101 and miR-301 was determined in healthy (n=15) and SA-AM (n=15). The expression of miR-144 was undetectable in both healthy and SA-AM. miRNA expression evaluated by quantitative reverse-transcriptase polymerase chain reaction and normalised to RNU44. ns= not significant, \*p<0.05

**Figure E8:**



**Figure E8. Transfection of alveolar macrophages with anti-miR-150, anti-miR-152 and anti-miR-375 leads to a significant reduction in the expression of the corresponding miRNA.** AMs from SA and healthy subjects were transfected with anti-miR oligonucleotides or scrambled anti-miR control. miRNA expression was evaluated by quantitative reverse-transcriptase polymerase chain reaction and is normalised to RNU44. Data is from 5 independent experiments (5 donors) and is expressed as a fold induction to the control transfected with scrambled anti-miR. \*\*p<0.01, \*\*\*p<0.001 compared to control.

**Figure E9:**



**Figure E9. Transfection of alveolar macrophages with anti-miR-150, anti-miR-152 and anti-miR-375 does not increase Poly:IC induced IFNβ production.** Control=alveolar macrophages transfected with an anti-miR scrambled control, Anti-miR Mix=alveolar macrophages transfected with anti-miR-150, anti-miR-152 and anti-miR-375. Quantified using quantitative reverse-transcriptase polymerase chain reaction and normalised to glyceraldehyde phosphate dehydrogenase. ns= not significant.

**Figure E10:**

**Figure E10. Expression of TLR7 is not reduced in moderate asthma alveolar macrophages or mild asthma alveolar macrophages compared to healthy alveolar macrophages**. TLR7 expression was quantified in AMs from moderate asthma subjects (n=8), mild asthma subjects (n=17) and healthy donors (n=23) using quantitative reverse-transcriptase polymerase chain reaction and normalised to glyceraldehyde phosphate dehydrogenase. ns= not significant.

Moderate asthma subjects were well controlled (ACQ <1 and no disease exacerbations in previous year) and on inhaled steroids (mean dose 700 mcg, range 400-100mcg). Mild asthma subjects were steroid naïve.

**Table E1:**

|  |  |  |
| --- | --- | --- |
|  | **TLR7 mRNA expression in severe asthma subjects** | |
| Spearman r | *p* value |
| Age of patients | -0.06134 | 0.78 |
| BMI | 0.02174 | 0.92 |
| % predicted FEV1 | -0.08801 | 0.69 |
| ICS dose (BDP equivalent) | -0.1438 | 0.51 |
| BAL macrophages | -0.2956 | 0.17 |
| BAL neutrophils | 0.03560 | 0.87 |
| BAL eosinophils | -0.03667 | 0.87 |

**Table E1 Correlation between TLR7 mRNA expression and other clinical parameters.** TLR7 expression in SA-AM (n=23) does not correlate with age, BMI, lung function, ICS dose and inflammatory cells in BAL. BAL, bronchoalveolar lavage; BDP, beclomethasone; BMI, body mass index; FEV1, forced expiratory volume in one second; ICS, inhaled corticosteroid.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Fold** | **p-value** |  | **Potential TLR Target** |
| **hsa-miR-146b-3p** | 2.16 | 0.0139 | \* |  |
| **hsa-miR-149** | 5.48 | 0.4775 |  |  |
| **hsa-miR-150** | 2.35 | 0.0159 | \* | TLR7 |
| **hsa-miR-152** | 2.64 | 0.1441 |  | TLR7 |
| **hsa-miR-18b** | 3.15 | 0.1477 |  |  |
| **hsa-miR-193b** | 21.60 | 0.047 | \* |  |
| **hsa-miR-20b** | 4.99 | 0.2025 |  |  |
| **hsa-miR-218** | 2.86 | 0.06 |  |  |
| **hsa-miR-224** | 2.06 | 0.4171 |  | TLR3 |
| **hsa-miR-24** | 2.78 | 0.0196 | \* |  |
| **hsa-miR-328** | 2.10 | 0.126 |  |  |
| **hsa-miR-339-3p** | 2.06 | 0.0031 | \*\* |  |
| **hsa-miR-375** | 4.22 | 0.1441 |  | TLR7 |
| **hsa-miR-484** | 2.00 | 0.1772 |  |  |
| **hsa-miR-886-3p** | 4.24 | 0.1682 |  |  |
| **hsa-miR-886-5p** | 2.48 | 0.2836 |  |  |

**Table E2. Low density microRNA arrays.** List of the 16 microRNAs highlighted by the microarray as being up-regulated at least 2-fold in asthmatic alveolar macrophages compared to healthy. Only one miRNA- miR-451- was down-regulated at least 2-fold in asthmatic AM compared to healthy. Putative toll-like receptor (TLR) targets for the microRNAs highlighted in the microarray with a context score ≤-0.4 (<http://www.targetscan.org/vert_42/>) are also listed. Ct values of microRNAs were normalised using RNU44, and fold changes were calculated employing the delta delta Ct method. Data were normalised by log transformation and parametric t-test performed.\*: p-value≤0.05, \*\*: p-value≤0.01

**References for Online Supplement**

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