

Transcriptome analysis shows activation of circulating CD8⁺ T-cells in severe asthma

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Abstract

Background: Although previous studies have implicated tissue CD4⁺ T-cells in the development and maintenance of the inflammatory response in asthma, little is known about the role of CD8⁺ T-cells. There is now accumulating evidence that miRNAs and other non-coding RNAs are important regulators of T-cell function.

Objectives: To use transcriptomics to determine the activation state of circulating CD4⁺ and CD8⁺ T-cells in non-severe and severe asthma.

Methods: mRNA and non-coding RNA expression in circulating T cells was measured by microarray and/or quantitative real time-polymerase chain reaction.

Results: Comparison of mRNA expression showed widespread changes in the circulating CD8⁺ but not CD4⁺ T-cells from patients with severe asthma. No changes were observed in the CD4⁺ and CD8⁺ T-cells in non-severe asthmatics versus healthy controls. Bioinformatics analysis showed that the changes in CD8⁺ T-cell mRNA expression were associated with multiple pathways involved in T-cell activation. As with mRNAs, we also observed widespread changes in expression of non-coding RNA species including natural antisense, pseudogenes, intronic long ncRNAs and long intergenic long ncRNAs in CD8⁺ T-cells from severe asthmatics. Measurement of the miRNA expression profile showed selective down-regulation of miR-28-5p in CD8⁺ T-cells and reduction of miR-146a and miR-146b in both CD4⁺ and CD8⁺ T-cells.

Conclusions: Severe asthma is associated with the activation of circulating CD8⁺ T-cells but not CD4⁺ T-cells. This response is correlated with the down-regulation of miR-146a/b and miR-28-5p as well as changes in the expression of multiple species of long non-coding RNA that might regulate CD8⁺ T-cell function.

Clinical Implications

Severe asthma is associated with the activation of circulating CD8⁺ T-cells and targeting this response might provide a novel therapeutic approach.

Capsule Summary

Examination of the transcriptome has demonstrated widespread changes in the mRNA and non-coding RNA expression in circulating CD8⁺ T-cells but not CD4⁺ T-cells in patients with severe asthma.

Key Words: severe asthma, CD8⁺ T-cells, transcriptome, noncoding RNA, microRNA

Abbreviations: IL, interleukin; miRNA, microRNA; Th, T help; TCR, T-cell receptor

Introduction

Asthma is characterised by reversible airway obstruction, re-modelling and inflammation and is one of the most common chronic allergic diseases affecting 300 million people worldwide and causing approximately 180,000 deaths per year ¹. Most patients with asthma respond well to anti-inflammatory treatment with inhaled corticosteroid therapy. However, 5-10% of patients experience ongoing symptoms of asthma despite taking corticosteroid treatments, often in combination with long-acting β -agonists and other controller therapies. These patients have been labelled as having severe asthma or therapy resistant asthma and form a heterogeneous group of patients that can be divided into distinct phenotypes ^{2;3}.

Although the underlying mechanisms that drive asthma are still an area of contention, T-cells have been proposed to contribute to the induction and maintenance of the inflammatory response. Following their early identification in bronchoalveolar lavage (BAL), much work has focused upon the importance of the T-helper 2 (Th2) CD4⁺ T-cells ⁴. These cells are activated following the interaction between the T-cell receptor (TCR) and peptide antigen presented in the context of MHC class II molecules expressed by airway dendritic/epithelial cells. In turn, this stimulates the release of Th2 cytokines including IL-4, IL-5, IL-9 and IL-13 that contribute to development of many of the features of asthma including airways hyper-responsiveness, mucus hyper-secretion, airway eosinophilia and B-cell activation ^{5;6}. Significantly, recent studies have also implicated roles for other sub-types of CD4⁺ T-cells including the regulator T-cells (Treg), Th9, Th17, Th22, $\gamma\delta$ T-cells and natural killer T-cells (NKT), although the majority of these studies have been performed in animal models and their role in human asthma remains to be confirmed ^{5;6}.

Unlike CD4⁺ T-cells, little is known about the role of CD8⁺ T-cells, which are normally associated with the recognition and destruction of cells that are infected with viruses and other intracellular pathogens. Previous studies in patients have shown the presence of CD8⁺ in the BAL and sputum of asthmatics whilst increased CD8⁺ T-cell numbers were noted in patients with *status asthmaticus* ⁷⁻⁹. In addition, animal studies have shown that CD8⁺ T-cells contribute towards the development of allergen-induced airway hyper-responsiveness and pulmonary inflammation ¹⁰.

In this report, we have adopted a transcriptomics based approach to investigate the activation state of circulating CD4⁺ and CD8⁺ T-cells in severe asthma and to identify potential targets/pathways that might provide novel therapeutic targets. In addition, we have investigated whether any changes in mRNA expression are regulated at the post-transcriptional level by microRNA expression and whether there are changes in the expression of other non-coding RNAs including antisense, pseudogenes, long intronic non-coding RNA and long intergenic non-coding RNAs. Significantly, measurement of the mRNA expression profile has shown activation of the circulating CD8⁺ but not CD4⁺ T-cell populations in severe asthma. No significant changes in mRNA expression were observed in the CD4⁺ and CD8⁺ T-cells obtained from non-severe asthmatics. As with the mRNAs, we have shown widespread changes in expression of natural antisense, pseudogenes, long intronic non-coding RNA and long intergenic non-coding RNA within the CD8⁺ population from severe asthmatics. In addition, we observed reduced expression of miR-28-5p in CD8⁺ T-cells and down-regulated expression of miR-146a and miR-146b in both CD4⁺ and CD8⁺ T-cells. Overall, these observations have revealed that activation of CD8⁺ T-cells might have an important role in the pathogenesis of severe asthma and that this activation maybe regulated through changes in expression of multiple classes of non-coding RNAs.

Methods

T-cell isolation

Human CD4⁺ and CD8⁺ T cell were isolated by negative selection using Miltenyi beads and MACS Vario magnetic cell separation apparatus. T-cell purity was assessed by FACS using anti-CD4-FITC and anti-CD8-PE (Miltenyi) using CellQuest software.

Microarray Analysis of mRNA and non-coding RNA expression

Expression profiling was performed using the Affymetrix U133 Plus 2.0 microarrays at AROS, Denmark. Total RNA was extracted using the *mirVana*TM miRNA isolation kit (Ambion Europe) eluted in 50 µl RNase-free water (Promega UK) and purity was measured using a BioTek PowerWave XS (SSI Robotics, U.S.A.) spectrophotometer and Agilent Bioanalyser, which showed RIN values > 9.5. Raw data were analysed using GeneSpringGX10 and then R/Bioconductor. Arrays data were then normalised using robust multi-array (rma) and batch effects corrected using the ComBat R package ¹¹. Data was filtered to remove probes which had low expression in all arrays (rma signal value of < 4 was removed). Differential gene regulation was determined by taking the output from a shrinkage t-test ¹² and then computing the p-values and q-values (FDR) using 'fdrtool' ¹³. The cutoff used for significance is FDR < 0.05 which is a 5% false discovery rate. The p-values are shown to allow readers to discern different rankings of genes where the FDR value is < 0.05. Raw microarray data has been deposited with GEO (Accession number XXXX).

Changes in antisense, pseudogenes, long intronic non-coding RNA and long intergenic non-coding RNAs expression were determined by mapping non-annotated probes onto the human genome (GRCh37) using Ensembl (<http://www.ensembl.org/index.html>).

qPCR measurement of miRNA and mRNA expression

miRNA expression profiling was undertaken using the two-step Applied Biosystems TaqMan[®] RT-PCR protocol and normalised to RNU44 as previously described ⁽¹⁴⁾. mRNA expression levels was determined by TaqMan[®] RT-PCR using on Assays on Demand (Applied Biosystems). Alternatively, mRNA levels were determined using the Power SYBR[®] Green PCR mix (Applied Biosystems) using the listed probes (MWG-Biotech). The separate well, $2^{-(\Delta\Delta Ct)}$ method was used to determine relative-quantitative levels of individual miRNAs and mRNAs

The following primer sequences were used for the mRNA RT-PCR. LCN: Forward: CACCCTCTACGGGAGAACCAAGGA; Reverse: ACCTGTGCACTCAGCCGTCGATA;
S100A8 Forward: CACATTCCTGTGCATTGAGGGGTTA; Reverse: GGGTGTCAAAATGCCCTTCCG;
S100P Forward: CAGGCTTCCTGCAGAGTGGAAAAGA; Reverse: GCAGACGTGATTGCAGCCACGA;
CAMP Forward: AGACACGCCAAAGCCTGTGAGCTT; Reverse: TTCACCAGCCCGTCCTTCTTGAAGT;
BPI: Forward: TGCAGAAGGAGCTGAAGAGGATCAA; Reverse: ACGGATGTCCATGCTGTAGAAGCTA; E

LANE: Forward: ACTGCGTGGCGAATGTAAACGT; Reverse:
CCGTTTTTCGAAGATGCGCTGCA;

EIF2S3: Forward: TGCTCCAGGCGGTCTTATTGGAGTT; Reverse:
TCAGGTAAAGCTCCGACTGCACCA;

ANAX3: Forward: TCGCTCGCAGTTTGTTCGCAGT; Reverse:
TGTTCTCGGTGTCCAACCCAGAT.

Data and statistical analysis

The RT-PCR ~~and luciferase reporter~~ results are presented as the mean \pm SD of at least three independent experiments. Differences in the expression of mRNA and miRNA were determined using a two-tailed non-parametric Mann-Whitney U-test within the GraphPad Prism 5 (<http://www.graphpad.com>). P values of < 0.05 were considered significant and are indicated with asterisks. .

Results

Patient Groups

Transcriptomics studies were performed in the circulating CD4⁺ and C8⁺ T-cells obtained from 12 individuals with severe asthma, 4 non-severe asthmatics and 8 healthy controls individuals (Table 1) which had been sanctioned by the Royal Brompton and Harefield NHS Trust Ethics committee. Severe asthma was defined according to the American Thoracic Society major criteria of needing either continuous or near continuous oral corticosteroids or high dose inhaled corticosteroids (2,000 µg beclomethasone-equivalent per day or more) or both in order to achieve a level of mild-moderate persistent asthma, and by 2 or more minor criteria of asthma control ¹⁵. Severe asthma patients underwent the Royal Brompton severe asthma protocol, in order to confirm the diagnosis and to maximise treatments. Patients with non-severe asthma were those who did not fall into the severe asthma category and who used 0–1000 µg inhaled beclometasone or equivalent dosage per day with perfect control of their asthma. All patients showed either an improvement in baseline FEV1 of $\geq 12\%$ over baseline values after inhalation of 400 µg of salbutamol aerosol, or the presence of bronchial hyperresponsiveness defined by methacholine PC₂₀ of <4 mg/ml. Current and ex-smokers of >5 pack-years were excluded. Healthy control volunteers with no diagnosis of asthma and with a negative PC₂₀ (>16 mg/ml), using no medications and never-smokers, were also recruited as the comparative group.

Characterisation of T-cell populations

Circulating blood (50ml) was obtained from control non-asthmatic individuals or patients with non-severe and severe asthma. Prior to negative-selection, measurement of the total number of peripheral blood mononuclear cells in control, non-severe and severe asthmatics showed no significant difference with 97.6 ± 7.7 , $XX.X \pm X.X$ and 127.0 ± 30 ($\times 10^6$ cells), respectively. FACS analysis showed that antibody-based negative selection resulted in the production of highly purified ($> 98\%$) $CD4^+$ and $CD8^+$ T-cells. Following their isolation, we once again observed no significant difference in the number of $CD4^+$ T-cells (11.2 ± 1.8 , $XXX \pm XX$ and 11.9 ± 1.0 ($\times 10^6$)) and $CD8^+$ T-cells (5.5 ± 0.6 , $XXX \pm XX$ and 4.7 ± 0.6 ($\times 10^6$)) in control, non-severe and severe asthmatic patients, respectively.

Severe asthma is associated with changes in mRNA expression in $CD4^+$ and $CD8^+$ T-cells

Measurement of the profile of mRNA expression using Affymetrix microarrays showed that this differed between healthy and severe asthmatic patients but not non-severe asthmatics. These initial studies were performed using the eight control individuals, 4 non-severe asthmatics and 8 severe asthmatics (Table 1: Patients 1 - 8). In the case of the $CD4^+$ T-cells, we identified a relatively small number of transcripts ($n = 40$) that were up-regulated (see Table E1 in the online data supplement) and none that were down-regulated by >1.5 -fold (~~FDR < 0.05 ; p < 0.05~~) in severe asthmatics versus controls. In contrast, with $CD8^+$ T-cells we observed 1359 up-regulated genes (see Table E2 in the online data supplement) and 207 down-regulated genes (see Table E3 in the online data supplement) in the samples obtained from severe asthmatics (~~> 1.5 fold; FDR < 0.05 ; p < 0.05~~).

The general validity of these microarray observations in the severe asthmatic samples was assessed using qRT-PCR. Since the initial microarray studies were performed using a group of

severe asthmatics that contained a ratio of 7:1 of female:male, we included an additional 4 samples obtained from males with severe asthma to account for potential gender bias. These studies were able to confirm the increase in expression of 9 of 10 mRNAs examined in CD4⁺ T-cells and in 13 of 16 mRNAs in CD8⁺ T-cells (Tables 2 and 3). These included bactericidal/permeability-increasing protein (BPI), carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-6/8, lipocalin 2 (LCN), lactotransferrin (LTF), S100 calcium binding protein-P/A8/A9/A12, chitinase 3-like 1 (CHI3L1), leukocyte elastase (ELA2), annexin A3 (ANAX3) and arginase (ARG).

As might be predicted from the microarray data, we observed only a small number of mRNAs whose expression profile was changed in non-severe asthmatics versus non-asthmatics controls (Supplemental Table E4). This included 2 of 10 mRNAs examined in CD4⁺ T-cells and in 5 of 15 mRNAs in CD8⁺ T-cells (Supplemental Table E4).

Pathway analysis shows activation of CD8⁺ but not CD4⁺ T-cells in severe asthmatics

To identify the pathways that were either activated or inhibited in the CD4⁺ and CD8⁺ T-cells from severe asthmatics we analysed the changes in profile of mRNA expression using the Ingenuity Systems software (<http://ingenuity.com/>) (Table 4). Significantly, this showed an upregulation in the expression of those transcripts involved in multiple inflammatory and other signalling pathways in CD8⁺ T-cells from severe asthmatics. These included those involved in T-cell activation (e.g. T-cell receptor signalling, NFAT transcription factor pathway, ICOS-ICOSL and CD28 co-stimulation as well as the pathways involved in the regulation of IL-2 expression in activated and anergic T lymphocytes) and inflammation (LPS-stimulated MAPK, JAK/Stat and NF-κB signalling pathways). A number of pathways that are commonly

associated with asthma including IL-3, IL-4 and IL-9 were also activated, as was glucocorticoid signalling. In contrast, only the vitamin D pathway was up-regulated in CD4⁺ T-cells, whilst no pathways were shown to be down-regulated in either CD4⁺ or CD8⁺ T-cells.

Subsequent analysis aimed to identify mRNAs that were associated with differences in the T cell phenotype from severe asthmatics compared to control non-asthmatics. Comparison of the two data sets showed that 23 genes were up-regulated in both the CD4⁺ and CD8⁺ T-cell populations, but no genes that were down-regulated (Table 2). Prominent amongst the up-regulated mRNAs were members of the S100 calcium binding and carcinoembryonic antigen-related cell adhesion (CEACAM) molecule family. Examination of those mRNAs that had the largest fold changes also highlighted the potential importance of the S100 and CEACAM families in both CD4⁺ T-cells or CD8⁺ T-cells (Table 3). Interestingly, two proteins that have recently been linked to asthma, arginase 1¹⁶ and chitinase 3-protein 1¹⁷ were also shown to be increased in CD8⁺ T-cells (Table 2 and 3).

Severe asthma is associated with reduced miRNA expression of ~~miR-146a/b~~ in CD4⁺ and CD8⁺ T-cells

Previous investigations have shown that a number of miRNAs including miR-21, miR-132, miR-146a, miR-150, miR-155 and miR-326 are important regulators of the differentiation, proliferation and activation of immune cells¹⁸⁻²⁰. To determine whether miRNAs might regulate the changes in mRNA expression, we measured the expression of these immune related miRNAs (Table 5). These studies showed that ~~only~~ the expression of miR-146a and miR-146b was reduced by 50 – 80 % and that this occurred in both CD4⁺ and CD8⁺ T-cells

from severe asthmatics (Table 5). Significantly, we also observed a selective reduction of ~30% in the expression of miR-18a-5p in CD8⁺ T-cells.

Since it might be expected that a reduction in miRNA expression ~~miR-146a and miR-146b~~ would lead to increased expression of their predicted mRNA targets we examined this possibility using the miRNA target prediction programme, TargetScan (<http://www.targetscan.org/>). Bioinformatics analysis showed an enrichment of miR-28a-5p (p = 0.026) and miR-146 (p = 0.13) targets in those mRNAs that were down-regulated in CD8⁺ T-cells. As a result of the small number of differentially regulated mRNAs in CD4⁺ T cells, it was not possible to determine whether targets for miR-146 were enriched.

Severe asthma is associated with widespread changes in the expression of long non-coding RNAs in CD8⁺ T-cells

In addition to the miRNA family of short non-coding RNAs (< 200 nucleotides), there is now accumulating evidence that long non-coding RNAs (> 200 nucleotides) regulate multiple biological responses and that changes in their expression is related to the development of disease ^{21;22}. At the present time, long non-coding RNAs (lncRNAs) can be broadly divided into 4 families based upon their sequence and relative position to the exonic regions of protein coding sequences and includes pseudogenes, natural antisense (to exonic regions), intronic lncRNAs and intergenic lncRNAs. To identify novel lncRNAs, we used Ensembl (<http://www.ensembl.org/index.html>) to determine the genomic position of those probesets from the Affymetrix U133 Plus 2.0 microarray which i) did not match known protein coding genes and ii) were significantly changed (~~>1.5 fold; FDR < 0.05; p < 0.05~~) in the CD4⁺ and CD8⁺ T-cells in severe asthma. Of relevance, a recent publication has indicated that these

probesets are able to detect up to 43% of the 5446 predicted lncRNAs in the human genome ²⁰. This approach identified a small number of lncRNAs that were down-regulated in CD4⁺ T-cells (see Supplemental Table E5 in the online data supplement) including 4 intergenic lncRNAs and 2 intronic lncRNAs. Of these lncRNAs, only a single transcript entitled AC090517.2 has been manually annotated by the Havana project team at the Wellcome Trust Sanger Institute (Supplemental Table E5). In the case of CD8⁺ T-cells, we identified 167 potential lncRNAs that were significantly changed in severe asthma (see Supplemental Table E6 in the online data supplement). These included 5 antisense sequences (4 up-regulated and 1 down-regulated), 4 pseudogenes (3 up-regulated and 1 down-regulated), 44 intergenic lncRNA (28 up-regulated and 16 down-regulated) and 114 intronic lncRNAs (106 up-regulated and 8 down-regulated). In this case, 19 of these transcripts have been manually annotated and include 1 antisense sequence, all 4 pseudogenes and 14 intergenic lncRNAs (Table 6). Overall, this bioinformatics analysis has shown for the first time, that changes in lncRNA expression is associated with the severe asthma in circulating T-cells although their function remains to be determined.

Discussion

In this report, we have compared the profile of mRNA expression in the circulating CD4⁺ and CD8⁺ T-cells from healthy individuals, non-severe asthmatics and severe asthmatics using microarray and qRT-PCR. In contrast to previous studies that have implicated Th2 CD4⁺ in driving the inflammatory response in asthma, we showed no major changes in the profile of mRNA expression in non-severe asthmatics and only a relatively small number of mRNAs that were up-regulated in CD4⁺ T-cells from severe asthmatics. ~~whilst~~ Pathway analysis indicated that these circulating CD4⁺ T-cells from severe asthmatics were non-activated. In contrast, there were widespread changes in the expression of mRNAs in circulating CD8⁺ T-cells obtained from severe asthmatics including the pathways associated with T-cell activation and the allergic inflammatory response. Since these changes were not observed in non-severe asthmatics this indicated that the changes in mRNA expression in circulating CD8⁺ T-cells is related to the severity of the asthma and not asthma *per se*.

Examination of the individual mRNAs showed 23 mRNAs were up-regulated in both CD4⁺ and CD8⁺ T-cells from severe asthmatics including members of the S100 calcium binding and carcinoembryonic antigen-related cell adhesion (CEACAM) molecule family. The calgranulin family of S100 calcium binding proteins is composed of 3 members, S100A8, S100A9 and S100A12 which were all shown to be up-regulated in CD4⁺ and CD8⁺ T-cells. Interestingly, increased calgranulin expression is linked to the development of rheumatoid arthritis, inflammatory bowel disease and atherosclerosis ^{24;25}, is released from neutrophils/macrophages and is thought to induce an inflammatory response through activation of TLR4 and receptor for advanced glycosylation (RAGE) ²⁶. Much less is known

about the immunoglobulin-related glycoprotein CEACAM family, of which CEACAM-6 and CEACAM-8 are upregulated in both CD4⁺ and CD8⁺ T cells, although they have been implicated in T-cell proliferation ²⁷.

Specific mRNAs up-regulated in CD8⁺ T-cells from severe asthmatics included chitinase 3-protein 1 and arginase. Chitinase 3-protein-1, which is also known as YKL-40 (humans) and BRP-39 (mice), is up-regulated in many inflammatory diseases including osteoarthritis, rheumatoid arthritis, sarcoidosis, inflammatory bowel disease, idiopathic pulmonary fibrosis and solid malignancy ^{17;28;29}. Significantly, it is also elevated in serum and lung macrophages/epithelium of individuals with asthma, where expression correlates positively with severity of disease ^{30;31}. Furthermore, mouse studies have shown that YKL-40 is crucial in the initiation and effector stage of Th2-driven inflammation and airway re-modelling in models of allergen-induced asthma and in IL-13 transgenic mice ³². As with humans, the animal studies showed that the elevation in chitinase 3-protein-1 expression was predominately associated with lung epithelium and macrophages ³². Arginase is a key metalloenzyme of the urea cycle that converts L-arginine into L-ornithine and urea. It is known to be elevated in asthmatic patients as well as various animal models of asthma ¹⁶. It has been speculated that increased arginase deprives nitric oxide synthase of arginine and thereby prevents the production of nitric oxide (NO), a key bronchodilator and anti-inflammatory in asthma.

Having demonstrated changes in the mRNA expression, we investigated whether this might be mediated by changes in miRNA expression. There is now accumulating evidence that miRNAs regulate the proliferation, differentiation and activation of T-cells. Thus, studies involving DICER knockout (an RNase III enzyme that is crucial to the production of mature miRNAs) in

T-cell development showed that miRNAs are important in T-cell proliferation and the generation of the Th2 phenotype and FoxP3⁺ CD4⁺ T-regulatory (Treg) cells¹⁸⁻²⁰. Subsequent investigations aimed at characterising the role of individual miRNAs have implicated the miR-17~92 cluster in early T-cell proliferation, miR-155 in the development of the Th2 and Treg phenotype and miR-181a as an inhibitor of CD8⁺ T-cell function¹⁸⁻²⁰. Both miR-155 and miR-181a have also been implicated in the regulation of acute response in activated CD4⁺ T-cells including the release of IL-2 and IFN- γ . In addition, recent animal studies have shown increased expression of miR-21 expression in the lungs during ovalbumin-, fungal- and IL-13-induced allergic airway inflammation³³ and increased miR-126 expression in a house dust mite-induced model of asthma³⁴.

Our comparison of the expression of these immune related miRNA showed no changes in expression of members of the miR-17~92 cluster, miR-155, miR-181a, miR-21 or miR-126 in CD4⁺ and CD8⁺ T-cells from severe asthmatics although we observed significant down-regulation in expression of miR-146a and miR-146b. Interestingly, there are an increasing number of publications that demonstrate a central role for miR-146a in the regulation of immune responses.³⁵⁻⁴⁰ and in the development of inflammatory disease such as rheumatoid arthritis, osteoarthritis, systemic lupus erythematosus and chronic obstructive pulmonary disease^{41;42}. However, although we observed significant down-regulation of miR-146a/b in both CD4⁺ and CD8⁺ T-cells, only CD8⁺ T-cells showed widespread changes in the profile of mRNA expression. This would imply that miR-146a/b alone are not responsible for changes in the mRNA profile in CD8⁺ T-cells, a conclusion that is supported by the fact that there was only a bias towards targets of miR-146a/b ($p = 0.13$). Significantly, we also observed selective down-regulation of miR-28-5p in CD8⁺ T-cells and bioinformatics analysis showed an enrichment of miR-28a-5p ($p = 0.026$) targets in those mRNAs that were down-regulated in

CD8⁺ T-cells. At the present time, virtually nothing is known regarding the biological function of miR-28-5p and subsequent studies will need to investigate its function in CD8⁺ T-cells.

Having shown altered expression of mRNAs and miRNAs, we proceeded to investigate whether severe asthma was also associated with changes in expression of lncRNAs. LncRNAs can be broadly divided into natural antisense, pseudogenes, long intronic ncRNAs and long intergenic ncRNA. Despite their widespread expression, their importance in the regulation of multiple physiological and pathological response is only now emerging ^{21;22}. Thus, recent reports have demonstrated that expression of long intergenic ncRNA expression is cell- and developmentally-specific and mediated through activation of common transcription factors including p53, NF-κB, Sox2, Oct4 and Nanog ⁴³. Our initial analysis of the profile of lncRNA expression showed that severe asthma was also correlated with widespread changes in the expression of natural antisense, pseudogenes, long intronic ncRNAs and long intragenic ncRNAs in circulating CD8⁺ T-cells but not CD4⁺ T-cells. This observation indicates for the first time, that the changes in mRNA expression and subsequent T-cells function might be regulated by lncRNAs.

In summary, using a transcriptomics based approach we have demonstrated that circulating CD8⁺-T-cells from severe asthmatics but not non-severe asthmatics have a transcript expression profile associated with an activated phenotype and shown that these changes might, in part, be mediated through alterations in non-coding RNA expression. In contrast, there was little evidence to suggest activation of circulating CD4⁺ T-cells in either non-severe or severe disease despite the fact that tissue CD4⁺ Th2 cells have previously been linked with the asthmatics phenotype. Clearly, ~~and to determine the activation state of circulating CD4⁺ and CD8⁺ T cells in patients with mild to moderate asthma.~~ since antigen specific T-cells are

believed to compartmentalize to local or regional lymph, these observations could be the result of circulating (spilled over) factors and may not reflect *in situ* activation and effector function for these cells. It will therefore be important to confirm these observations using lung derived T-cells. In addition, it will also be important to examine whether the activation of CD8⁺ T-cells observed at the transcriptional levels is reflected by changes in effector function and/or the effect of therapy. However, given the increasing evidence to suggest that viral infection is important to both asthma development in early life and subsequent exacerbations^{44:45} and the role of CD8⁺ T-cells in the immune response to viral infection, it might therefore be speculated that chronic activation of CD8⁺ might contribute towards the symptoms observed in severe asthma. If confirmed, this would suggest that targeting either the activation CD8⁺ cells, or their effector functions, might provide a novel therapeutic approach to the treatment of severe asthma.

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Table 1 Patient Data

Patient	Age (yrs)	Gender (M/F)	FEV1 (% predicted)	FVC (% predicted)	PC ₂₀	Medication
Control						
1	39	M	78	76	>16mg/ml	None
2	25	F	103	107	>16mg/ml	None
3	26	F	105	115	>16mg/ml	None
4	46	M	92	105	>16mg/ml	None
5	33	M	78	77	>16mg/ml	None
6	33	F	116	115	>16mg/ml	None
7	31	F	85	96	15.67mg/ml	None
8	56	M	111	111	>32mg/ml	None
Mean ± SEM	36 ± 4	4/4	96 ± 5	100 ± 6		
Non-Severe Asthma						
1	23	M	82	98	Not done	Becotide, Ventolin
2	44	F	87	92	0.52 mg/ml	Fl
3	35	M	77	89	6.27 mg/ml	Becotide, Ventolin
4	23	F	84	81	0.17 mg/ml	Salbutamol
Mean ± SEM	31 ± 5	2/2	83 ± 2	90 ± 2		
Severe Asthma						
1	55	F	82	105	1.09 mg/ml	Fl, Oxis, Loradine, Beconase
2	21	F	105	101	Not done	Pr, Se, Si, Phyllocontin, Ventolin
3	32	M	98	113	0.01 mg/ml	Pr, Se, Montelukast, Ventolin, Phyllocontin, Fl
4	55	F	65	81	0.10 mg/ml	Pr, Sp, Se, Methotrexate, Fosamax, Folic acid, Cocodamol
5	54	F	44	61	Not done	Pr, Sy, Montelukast, Omeprazole, Uniphyllin, Bricanyl
6	54	F	55	65	Not done	Pr, Co, Ce, Terbutaline, Bu, Aminophylline SR, Formoterol
7	45	F	71	105	Not done	Pr, Se, Fl, Metformin, Nexium, Tramacet, Valsartan, Amitriptyline
8	42	F	33	94	Not done	Pr, Se, Aminophylline, Doxycycline, Tiotropium
9	47	M	63	81	Not done	Se, Uniphylline, Fosamax, Ventolin, Atrovent
10	39	M	63	73	Not done	Sy, Loratine, Beconase, Uniphylline
11	61	M	57	98	Not done	Pr, Sy, Fl, Omerprazole, Atorvastin,
12	52	M	83	97	Not done	Sy, Betnasal
Mean ± SEM	46 ± 3	5/7	68 ± 6	90 ± 5		

Abbreviations: Bu – budesonide; Ce – cetirizine; Co – combivent; Fl – flixotide; Pr – prednisolone; Se – seretide; Si – singular; Sp – spiriva; Sy – symbicort;

Table 2 Common mRNAs expression changes in both CD4⁺ and CD8⁺ T-cells in severe asthma. This table lists those mRNAs whose expression was increased in both the CD4⁺ and CD8⁺ T-cells from severe asthmatics when compared to non-asthmatic. Microarray data is denoted as fold change (FC) against non-asthmatics controls and is the mean of 8 individual patients. These values are derived from Supplemental Table E1 and E2 in the on-line supplemental tables and are those genes that demonstrated an FDR < 0.05 - associated p-values are given in brackets. Changes in mRNA expression were confirmed by qRT-PCR, which was normalised against 18S and expressed as fold changes over non-asthmatics controls. In the case of the qRT-PCR data, statistical significant against non-asthmatics controls was determined using a two-tailed non-parametric Mann-Whitney U-test. These values are the mean of 12 individual patient samples and the p values are indicated in the brackets

Gene Symbol	Gene Name	CD4 ⁺ T-cells		CD8 ⁺ T-cells	
		Microarray (FC)	qRT-PCR (FC)	Microarray (FC)	qRT-PCR (FC)
BPI	bactericidal/permeability-increasing protein	2.6 (< 0.001)	21.7 (0.019)	2.8 (< 0.001)	29.1 (0.001)
CAMP	cathelicidin antimicrobial peptide	3.3 (< 0.001)	12.4 (0.065)	2.1 (0.017)	5.9 (0.016)
CASK	Peripheral plasma membrane protein CASK	1.7 (< 0.001)	-	1.8 (< 0.001)	-
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	2.4 (< 0.001)	29.1 (0.012)	2.9 (< 0.001)	4.6 (0.012)
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	5.6 (< 0.001)	20.2 (0.023)	4.4 (< 0.001)	3.7 (0.023)
CFLAR	FLAME-1	1.6 (< 0.001)	-	2.4 (0.019)	-
GCA	grancalcin, EF-hand calcium binding protein	2.6 (< 0.001)	-	2.6 (0.002)	-
LCN2	lipocalin 2	4.7 (< 0.001)	17.0 (0.015)	2.4 (< 0.001)	14.8 (0.01)
LOC23117	KIAA0220-like protein	2.4 (< 0.001)	-	3.3 (0.007)	-
LOC728358	defensin, alpha 1	6.2 (< 0.001)	-	2.3 (0.004)	-
LTF	lactotransferrin	5.0 (< 0.001)	21.1 (0.012)	2.1 (< 0.001)	2.5 (0.154)
POLR2J2	DNA directed RNA polymerase II polypeptide J-related	1.9 (< 0.001)	-	3.8 (0.001)	-
RETN	resistin	1.7 (< 0.001)	-	2.3 (0.005)	-
RNASE3	ribonuclease, RNase A family, 3 (eosinophil cationic protein)	2.0 (< 0.001)	-	1.8 (0.005)	-
S100A12	S100 calcium binding protein A12	4.7 (< 0.001)	27.1 (0.0078)	2.4 (0.051)	6.5 (0.031)
S100A8	S100 calcium binding protein A8	4.8 (< 0.001)	15.3 (0.015)	2.0 (< 0.001)	28.3 (0.006)
S100A9	S100 calcium binding protein A9	5.0 (< 0.001)	19.5 (0.019)	1.9 (0.011)	2.7 (0.070)
S100P	S100 calcium binding protein P	3.3 (< 0.001)	21.2 (0.008)	2.1 (0.008)	18.4 (0.001)
SLC38A2	Amino acid transporter system A2 (ATA2)	1.6 (< 0.001)	-	1.6 (< 0.001)	-
SPATA13	spermatogenesis associated 13	1.7 (< 0.001)	-	1.5 (0.001)	-
SRGN	serglycin	2.0 (< 0.001)	-	1.8 (0.016)	-
TNIP1	TNFAIP3 interacting protein 1	1.6 (< 0.001)	-	1.7 (< 0.001)	-
UGCG	UDP-glucose ceramide glucosyltransferase(UGCG)	2.0 (< 0.001)	-	2.5 (< 0.001)	-

Table 3 mRNAs expression showing highest fold-change in CD4⁺ and CD8⁺ T-cells in severe asthma. This table lists those mRNAs whose expression showed the largest fold-change increase in either CD4⁺ and CD8⁺ T-cells when compared to non-asthmatic controls. Microarray data is denoted as fold change (FC) against non-asthmatics controls and is the mean of 8 individual patients. These values are derived from Supplemental Table E1 and E2 in the on-line supplemental tables and are those genes that demonstrated an FDR < 0.05 - associated p-values are given in brackets. Changes in mRNA expression were confirmed by qRT-PCR, which was normalised against 18S and expressed as fold changes over non-asthmatics controls. In the case of the qRT-PCR data, statistical significant against non-asthmatics controls was determined using a two-tailed non-parametric Mann-Whitney U-test. These values are the mean of 12 individual patient samples and the p values are indicated in the brackets.

Gene Symbol	Gene Name	Array (FC)	RT-PCR (FC)
CD4⁺ T-cells			
LOC728358	defensin, alpha 1	6.6 (< 0.001)	-
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	5.6 (< 0.001)	20.2 (0.023)
LTF	lactotransferrin	5.0 (< 0.001)	21.1 (0.012)
S100A9	S100 calcium binding protein A9	5.0 (< 0.001)	19.5 (0.019)
S100A8	S100 calcium binding protein A8	4.8 (< 0.001)	15.3 (0.015)
LCN2	lipocalin 2	4.7 (< 0.001)	17.0 (0.015)
S100A12	S100 calcium binding protein A12	4.7 (< 0.001)	27.1 (0.008)
DEFA4	defensin, alpha 4, corticostatin	4.3 (< 0.001)	-
MMP8	MMP-8 (collagenase 2)	3.6 (< 0.001)	-
S100P	S100 calcium binding protein P	3.3 (< 0.001)	21.2 (0.008)
CAMP	cathelicidin antimicrobial peptide	3.3 (< 0.001)	12.4 (0.645)
MNDA	myeloid cell nuclear differentiation antigen	2.7 (< 0.001)	-
BPI	bactericidal/permeability-increasing protein	2.6 (< 0.001)	21.7 (0.019)
GCA	granulocyte, EF-hand calcium binding protein	2.6 (< 0.001)	-
ZNF749	zinc finger protein 749	2.5 (< 0.001)	-
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	2.4 (< 0.001)	29.1 (0.012)
LOC23117	KIAA0220-like protein	2.4 (< 0.001)	-
STOM	stomatin	2.3 (< 0.001)	-
ALOX5	Arachidonate 5-lipoxygenase	2.2 (< 0.001)	-
UGCG	UDP-glucose ceramide glucosyltransferase	2.0 (< 0.001)	-
CD8⁺ T-cells			
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	4.4 (< 0.001)	3.7 (0.023)
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	3.9 (< 0.001)	4.8 (0.019)
LTF	lactotransferrin	3.8 (< 0.001)	2.5 (0.154)
ELA2	Leukocyte elastase (Neutrophil elastase)	3.7 (0.002)	12.7 (0.013)
CD24	CD24 molecule	3.6 (< 0.001)	3.5 (0.015)
LCN2	lipocalin 2	3.3 (< 0.001)	14.8 (0.01)
EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	3.3 (0.045)	1.5 (0.232)
ANXA3	annexin A3	3.2 (0.001)	9.6 (0.008)
ARG1	arginase, liver	3.2 (0.001)	4.0 (0.04)
SEPT6	septin 6	3.1 (0.010)	-
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	3.1 (0.013)	-
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	2.9 (< 0.001)	4.6 (0.030)
AZU1	Azurocidin	2.9 (0.003)	-
LEF1	lymphoid enhancer-binding factor 1	2.9 (0.026)	-
BPI	bactericidal/permeability-increasing protein	2.8 (< 0.001)	29.1 (0.001)
SF1	splicing factor 1	2.8 (0.030)	-
SAMHD1	SAM domain and HD domain 1	2.7 (0.006)	-
S100B	S100 calcium binding protein B	2.7 (0.046)	-
TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	2.7 (0.002)	-

Table 4 Pathway Analysis in CD4⁺ and CD8⁺ T-cells in severe asthma. Circulating CD4⁺ and CD8⁺ T-cells were isolated from 8 non-asthmatics control and 8 severe asthmatics and mRNAs expression was determine using U133 Affymetric microarrays. The mRNAs that showed significant change were analysed using the Ingenuity Systems software (<http://ingenuity.com/>) and those pathways showing a significant increase are listed.

Gene Set Name	P value
CD4⁺ T-cells	
VDR/RXR Activation	4.80E-04
CD8⁺ T-cells	
Role of NFAT in Regulation of the Immune Response	7.80E-06
Molecular Mechanisms of Cancer	3.20E-05
Death Receptor Signaling	3.70E-05
T Cell Receptor Signaling	3.90E-05
Glucocorticoid Receptor Signaling	4.20E-05
LPS-stimulated MAPK Signaling	7.20E-05
Apoptosis Signaling	8.80E-05
Acute Myeloid Leukemia Signaling	1.00E-04
B Cell Receptor Signaling	1.10E-04
CD40 Signaling	1.30E-04
JAK/Stat Signaling	1.30E-04
IL-8 Signaling	1.30E-04
PPARa/RXRa Activation	1.50E-04
NF-KappaB Signaling	1.90E-04
iCOS-iCOSL Signaling in T Helper Cells	2.80E-04
IL-9 Signaling	2.80E-04
Angiopoietin Signaling	3.80E-04
EIF2 Signaling	4.10E-04
IL-3 Signaling	4.50E-04
PDGF Signaling	4.50E-04
Prolactin Signaling	4.50E-04
GM-CSF Signaling	4.50E-04
IL-4 Signaling	7.30E-04
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	7.60E-04
CD28 Signaling in T Helper Cells	9.50E-04

Table 5 miRNA expression levels in circulating CD4⁺ and CD8⁺T-cells from non-asthmatics controls and severe asthmatics.

Circulating CD4⁺ and CD8⁺T-cells were isolated from 8 non-asthmatics control and 8 severe asthmatics and the levels of individual miRNAs were determined by TaqMan qRT-PCR. Baseline expression levels are expressed as dCT and have been normalised against RNU44. Differences between control and severe asthmatics is expressed as fold change from control. All values are the mean \pm SD of 8 individual patient samples and statistical differences were evaluated using the Mann-Whitney U-test.

miRNA	CD4 ⁺ T-cells				CD8 ⁺ T-cells			
	Baseline Expression (dCT)	Fold change from control (%)			Baseline Expression (dCT)	Fold change from control (%)		
	Control	Control	Asthmatics	p-value	Control	Control	Asthmatics	p-value
miR-16	-1.2 \pm 0.5	106.2 \pm 32.3	84.3 \pm 43.7	0.302	-1.5 \pm 0.5	108.1 \pm 51.1	73.4 \pm 19.9	0.069
miR-17-5p	4.1 \pm 0.5	107.0 \pm 40.0	105.5 \pm 32.1	0.980	3.7 \pm 0.8	118.4 \pm 73.4	70.0 \pm 20.9	0.243
miR-21	-0.8 \pm 0.5	108.0 \pm 39.8	125.3 \pm 42.7	0.428	0.1 \pm 0.5	108.7 \pm 53.0	100.0 \pm 35.0	0.739
miR-26a	0.1 \pm 0.5	105.7 \pm 33.4	84.8 \pm 30.7	0.242	0.2 \pm 0.8	122.4 \pm 98.6	72.0 \pm 70.2	0.149
miR-28-5p	5.1 \pm 0.3	97.4 \pm 11.9	106.8 \pm 23.6	0.637	4.4 \pm 0.5	104.9 \pm 32.3	68.7 \pm 32.1	0.046
miR-29a	-0.2 \pm 0.5	104.5 \pm 29.7	105.0 \pm 38.7	0.926	-0.2 \pm 0.5	105.3 \pm 33.4	89.8 \pm 33.9	0.360
miR-106a	3.9 \pm 0.5	104.3 \pm 33.9	109.4 \pm 43.7	0.982	3.5 \pm 1.1	124.4 \pm 94.6	70.4 \pm 25.4	0.159
miR-126	6.1 \pm 2.1	276.9 \pm 351.9	59.4 \pm 57.0	0.784	6.0 \pm 5.0	190.0 \pm 187.1	30.9 \pm 23.9	0.687
miR-142-3p	-4.0 \pm 0.3	101.2 \pm 17.0	103.3 \pm 30.5	0.949	-3.8 \pm 0.5	104.6 \pm 31.3	90.7 \pm 27.0	0.392
miR-142-5p	0.0 \pm 0.3	100.9 \pm 13.5	104.8 \pm 22.3	0.952	0.0 \pm 0.5	103.9 \pm 32.6	95.0 \pm 32.1	0.515
miR-146a	3.2 \pm 0.3	109.2 \pm 35.8	59.4 \pm 29.7	0.021	4.9 \pm 3.7	135.2 \pm 93.3	47.5 \pm 31.8	0.049
miR-146b	2.1 \pm 0.3	111.3 \pm 38.4	53.0 \pm 32.9	0.010	4.1 \pm 3.2	122.4 \pm 73.4	51.3 \pm 23.3	0.049
miR-150	-4.8 \pm 0.3	100.3 \pm 8.0	102.9 \pm 23.9	0.641	-4.4 \pm 0.3	102.9 \pm 23.6	93.6 \pm 17.2	0.491
miR-155	2.8 \pm 0.5	104.7 \pm 31.0	129.0 \pm 23.1	0.105	2.2 \pm 0.5	105.2 \pm 33.4	76.3 \pm 35.8	0.099
miR-155*	13.0 \pm 1.1	120.4 \pm 66.8	148.7 \pm 82.7	0.601	12.4 \pm 1.1	129.3 \pm 94.3	114.7 \pm 74.2	0.784
miR-181a	3.1 \pm 0.3	101.1 \pm 15.1	86.9 \pm 22.3	0.159	2.7 \pm 0.8	117.8 \pm 70.2	77.3 \pm 26.0	0.239
miR-196a	11.6 \pm 1.1	120.8 \pm 71.6	136.5 \pm 41.9	0.351	8.5 \pm 0.5	108.2 \pm 39.5	145.8 \pm 15.1	0.608
miR-223	2.4 \pm 1.3	150.3 \pm 130.4	170.8 \pm 93.8	0.364	0.4 \pm 1.3	171.9 \pm 230.8	95.6 \pm 45.3	0.673

Table 6 Changes in the expression of long non-coding RNAs in CD8⁺ T-cells in severe asthma. This table lists those long non-coding RNAs that have been manually annotated in the Ensembl database and whose expression was significantly changed in circulating CD8⁺ T-cells obtained from severe asthmatics compared with healthy controls. All values are the mean of 8 individual patient samples and statistical differences were evaluated using the Mann-Whitney U-test where * p < 0.05

Probeset	Class of long non-coding RNA	Ensembl Gene ID	Transcript	Name	p-value	Fold Change
220459_at	Antisense	ENSG00000215424	MCM3APAS	minichromosome maintenance complex component 3 associated protein antisense RNA	0.00014	1.73
1557008_at	Intergenic	ENSG00000248908	RP11-730N24.1		0.00015	-1.80
1556453_at	Intergenic	ENSG00000229727	AC013460.1		0.00107	-1.59
210794_s_at	Intergenic	ENSG00000214548	MEG3	maternally expressed 3 (non-protein coding)	0.00002	-1.54
215011_at	Intergenic	ENST00000413987	SNHG3	small nucleolar RNA host gene 3 (non-protein coding)	0.00148	1.53
1553449_at	Intergenic	ENSG00000180422	C16orf81	chromosome 16 open reading frame 81	0.00000	-1.86
228913_at	Intergenic	ENSG00000170919	XXYac-R12DG2.2-010		0.00000	1.56
1554447_at	Intergenic	ENSG00000225470	NCRNA00183	non-protein coding RNA 183 ghrelin opposite strand (non-protein coding)	0.00152	2.27
223725_at	Intergenic	ENSG00000240288	GHRLOS		0.00030	1.53
243656_at	Intergenic	ENSG00000215447	BX322557.10		0.00021	-1.60
1563088_a_at	Intergenic	ENSG00000215458	AP001053.11		0.00005	-1.53
1557505_a_at	Intergenic	ENSG00000251153	AL049776.2		0.00000	1.67
241270_at	Intergenic	ENSG00000250233	AC015802.2		0.00002	-1.62
1564211_at	Intergenic	ENSG00000246223	C14orf64		0.00115	1.67
1564426_x_at	Intergenic	ENSG00000247397	AC130352.1		0.00151	-1.95
237544_at	Pseudogene	ENSG00000107679	PLEKHA1	pleckstrin homology domain containing, family A pseudogene	0.00003	1.81
1562250_at	Pseudogene	ENSG00000176993	AL590233.1	Known pseudogene olfactory receptor, family 2, subfamily A, member 20 pseudogene	0.00080	1.99
1555890_at	Pseudogene	ENSG00000170356	OR2A20P	pseudogene	0.00095	1.70
244071_at	Pseudogene	ENSG00000188573	FBLL1	fibrillar-like 1	0.00141	-1.74

