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The Role of TLR2 in Cigarette Smoke-Induced Gene Induction

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Abstract

Background: Previous work form our group has shown that innate immune cells perceive cigarette smoke as an inflammatory and xenobiotic stimulus, which alters the immune response to invading pathogens. It is unclear as to the specific cellular and molecular mechanisms driving cigarette smoke-induced inflammation, but we have demonstrated that this response is, in part, driven by Toll-Like Receptor (TLR) 2 in innate immune cells *in vitro* and in mice *in vivo*.

Methodology/Principle findings: To address the impact TLR2/6, has on cigarette smoke-induced gene induction, HEK293 cells were permanently transfected with TLR2/6 or Null vector, and were stimulated with 10% cigarette smoke extract (CSE) for 8 h. Total RNA was extracted and the transcriptome analysed using Illumina Bead Chip arrays. In HEK293 Null cells, CSE induced 33 genes and down-regulated 41. In HEK293 TLR2/6 cells, CSE induced 23 genes and down-regulated 44. Further analysis revealed that 42 genes were regulated in a TLR2/6-dependent manner. Comparison of these genes with those induced by smoke in human primary monocytes revealed that 5 were mutually regulated. The major pathways affected were those associated with anti-oxidant pathways, tumorgenesis and cell survival.

Conclusions: Our data suggest that the innate immune receptor TLR2/6 has a critical role for the expression of a particular cassette of genes induced by cigarette smoke. Pathway analysis indicates these are related to functions in both cell survival and tumorgenesis. Future validation of the relative importance of these pathways using more complex models is required a may lead to improved understanding of the pathology of cigarette smoke-induced diseases such as COPD, cardiovascular disease and cancer.

Introduction

Cigarette smoke contains in excess of 4,000 compounds [1], many of which are highly toxic to the cell and include many oxidants. The outcomes of cigarette smoke on immunity are broad and complex, with both pro-inflammatory and suppressive effects being reported [2]. Our group and others have shown that exposure of cells to cigarette smoke results in cellular oxidative stress and can result in the damage of proteins, DNA, and lipids [3,4]. It has also been shown that cigarette smoke promotes autoimmunity and the modification of antigen presentation [5], which, in blood from smokers, can manifest itself in the sensitisation of blood to pathogens [6]. The mechanism by which cigarette smoke causes inflammation has been attributed to activation of the transcription factors NF-κβ and AP-1 [7]. Binding of these transcription factors to response elements in DNA leads to *de novo* synthesis and release of pro-inflammatory mediators such as CXCL8 and TNF-α [8-11].

Toll-like receptors (TLRs) are innate immune receptors that are part of a larger family of pattern recognition receptors (PRRs) that recognise conserved sequences known as pathogen-associated molecular patterns (PAMPs), and the more recently identified danger-associated molecular patterns (DAMPs) [12]. DAMPs consist of a growing list of molecules that include hyaluronan, fibrinogen and oxidants that contribute to what is now termed "sterile inflammation" [12]. The inflammation induced by oxidants contained in cigarette smoke has been shown both *in vivo* and in innate immune cells *in vitro* to be sensed, in part, through TLR2 and TLR4 [13,14]. There is also increasing evidence that cigarette smoke detrimentally affects viral TLR signaling on innate immune and lung epithelial cells [15-17].

There have been a number of studies assessing gene changes in smokers compared with healthy non-smokers, typically identifying changes in inflammatory, oxidative stress and xenobiotic genes [18-20]. Moreover, when evaluating changes in gene expression of macrophages and airway epithelial cells in response to cigarette smoke extract (CSE), the major genes altered were also associated with oxidative stress and xenobiotic pathways [21-23]. Previous work in our group used a transcriptomics approach to assess gene expression changes induced by acute cigarette smoke exposure in the human monocytic cell line, THP-1, and in primary human peripheral blood mononuclear cells (PBMCs). It revealed that THP-1 cells and PBMCs respond to cigarette smoke extract by up-regulating oxidative stress response and inflammatory pathways [24]. We have also shown that oxidants present in cigarette smoke induce an inflammatory response *in vivo*, and this is, in part, regulated by TLR2 and TLR4 [14]. Furthermore, in this study we showed that smoke-induced CXCL8 release from human monocytes was TLR2/6 dependent. However, the role of TLR2/6 in smoke-induced transcriptomic changes is not known.

In the current study, therefore, we used basic human cell models to investigate the role of TLR2/6 receptors in transcriptomic effects induced by CSE. To elucidate which genes were controlled in a TLR2/6-dependent and -independent manner, we examined the transcriptome of HEK293 cells that were transfected with TLR2/6, and compared these with control vector-transfected null cells, after stimulation with CSE. These cells offer a stable and well-established method

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for assessing the effects of agents of interest on various responses. Moreover, to contextualize and assess using HEK293 cells as a model for this purpose, we compared our results to previously analyzed data in THP-1 monocytes and PBMCs. This may help us to identify target genes that are TLR2/6-dependent, and can affect inflammation caused by cigarette smoke.

Materials and Methods

Materials

All cell culture plastics and general disposables were obtained from Fisher Scientific (Loughborough, Leicestershire, UK). *Invitro*gen (Paisley, Renfrewshire, UK) supplied all cell culture reagents, unless stated otherwise. General laboratory reagents were purchase from Sigma-Aldrich (Poole, Dorset, UK).

Cell Culture

Stably transfected HEK293 cells with pUNO-mcs (Null control), or pDUO-hTLR2/6, were purchased from *Invivo*Gen (CA, USA) and maintained according to the manufacturer's instructions.

Preparation of Cigarette Smoke Extract (CSE)

To prepare CSE, four full strength Marlboro cigarettes (filters removed) were combusted through a modified 60ml syringe apparatus, and the smoke passed through 100ml of RPMI 1640. Each cigarette yielded 5 draws of the syringe (to 60 ml mark), with each individual draw taking approximately 10 seconds to complete. CSE was then passed through a 0.25 μm filter to sterilise and remove particulate matter and was used immediately at a 10% concentration diluted in control media [25]. As described previously [26] smoke extract "strength" was evaluated by measuring nitrite using the Griess reaction to ensure continuity between batches. In all experiments nitrite levels in 100% cigarette smoke extract was between 12 and 16μM. CSE (1-100%) made using this method does not contain detectable levels of bacterial antigens for TLR4 (endotoxin; measured by the E-toxate™ kit, Sigma, UK) or TLR2 (LTA; measured by in house ELISA).

Treatment of Cells

TLR2/6 cells and nulls were plated at a density of 1×10^6 cells/ml onto 6-well plates and allowed to equilibrate for 16h before being treated for 8h with fresh 10% CSE or DMEM [14,41]. After 8 h, RNA was then extracted using RNeasy kit (Qiagen, Sussex, UK) as described in the manufacturers' handbook. A control and a CSE sample set were prepared on each day for three consecutive days using fresh reagents on each day.

Gene Array

Total RNA was subject to standard microarray procedures. Samples were converted to cDNA, labelled, and hybridized to the Illumina HumanRef-8v3 Bead Chip array (Illumina, UK) at St Bartholomew's and The London Genome Centre (BLGC), Queen Mary, University of London. Quality control and basic interpretation of data was also performed at BLGC before datasets were received for further analysis.

PCR verification of target genes regulated by CSE in a TLR2/6-dependant manner

THP-1 cells and PBMCs were treated with 10% CSE for 8 and 24h. Total RNA from these cells was extracted using a Qiagen RNeasy™ mini kit with a DNAase treatment step as instructed by the manufacturer. The purity of RNA and concentration of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Basingstoke, UK). RNA was converted to cDNA using a qScript™cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, UK) and probed for PPM1F (Protein Phosphatase, Mg2+/Mn2+ Dependent, 1F), SRGN (Serglycin), EIF2C2 (Eukaryotic Translation Initiation Factor 2C, 2) and GAPDH using off the shelf TaqMan® probes under conditions stated by the manufacturer (*Invitro*gen, CA, USA). All levels of genes assessed were normalised against GAPDH and reported as fold change compared with control.

Data analysis

Analysis of datasets was performed using GeneSpring GX 11.3 (Agilent Technologies). Raw data were pre-processed to remove variability across and within array samples. To minimize non-biological variability across arrays, raw data were first $\log_2$ transformed and then quantile normalized [27], which is the recommended normalization algorithm for Illumina Bead Chip array analyses [28]. Normalization at the level of genes was performed on GeneSpring GX 11.3.

Samples were sorted into conditions based on the treatment applied and the cell type: HEK293 null controls (1-3) and HEK293 null 10% CSE (1-3) and HEK293 TLR2/6 controls (1-3) and HEK293 TLR2/6 10% CSE (1-3). A very stringent filtering of the dataset was performed, by selecting only the genes that had detectable signal intensity value in all samples (filter by flags present in all samples). Data from the THP-1 cells and PBMCs were previously published [24], but were reanalysed for this study using the moderated *t*-test. Fold change differences between control- and CSE-treated samples were calculated using cut-offs of 1.5-fold for statistically significant genes. These genes were identified using moderated unpaired *t*-test ($p<0.05$) with Benjamini-Hochberg False Discovery Rate (FDR) correction for single group comparisons [29]. Where more than one group were analysed, a one-way analysis of variance followed by a Tukey's post-hoc test and Benjamini-Hochberg FDR correction was used. Significantly differentially expressed genes at each fold-change cut-off were used to generate hierarchical clustering plots using Pearson's centred correlation and Ward's linkage rule, and were displayed as heat maps.

Data sets consisting of genes significantly altered by ≥1.5-fold (compiled on GeneSpring GX11.3) were uploaded onto Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) and mapped to Ingenuity's Knowledge Base. The significance of the association between the dataset and the canonical pathways were measured using a ratio of number of genes from the dataset that mapped to the pathway divided by the total number of genes in that pathway, and p-value was generated using Fisher's exact test. A threshold of 0.10 was used to indicate canonical pathways that are significantly represented by genes in a gene list.

Database searches using PubMed, GeneCards and other sources were performed to identify differentially expressed genes from the current study that were also found to be differentially expressed or important in the response to cigarette smoke in previous studies.

Results

Pre-Processing and analysis of raw and normalised global gene expression data

A single time point of 8 h was chosen based on previously published data showing this time point as appropriate to demonstrate both transcriptomic changes [24] and expression of inflammatory and anti-oxidant proteins of interest [7]. HEK293 Null and TLR2/6 positive cells

were treated for 8 h with RPMI medium (n=3) ± 10% CSE-conditioned medium. A 10% CSE concentration was chosen because a 3% solution failed to induce CXCL8 protein release from cells, and a 20% solution resulted in >50% cell death. Extracted RNA was hybridised onto Illumina HumanRef-8v3 Bead Chip arrays and analysed for global changes in the expression of genes in the transcriptome.

Box-whisker plots (not shown) of raw signal intensity values for all samples revealed that the overall distribution of intensities was similar in both HEK293 Null and HEK293 TLR2/6 cells. Quantile normalization of the dataset made the distribution profiles of all samples identical (data not shown). Stringent filtering was used to select only probe sets that were measured as "present" or "marginal" in all samples. This resulted in 22837 probe sets for HEK293 Nulls and 21372 probe sets for HEK293 TLR2/6 cells, which were suitable for further analysis. All data are MIAME compliant and all results have been deposited on the Array Express database (http://www.ebi.ac.uk/cgi-bin/microarray/magetab.cgi) under the experimental title "inflammatory transcriptome profiling of human monocytes exposed acutely to cigarette smoke" as an additional data set named "HEK293 data".

Gene expression patterns in HEK293 Nulls and HEK293 cells treated with 10% CSE or control media

Unsupervised principal component analysis was performed; using 3 principal components selected using the elbow method and is represented as a 3D scatter plot (Figure 1).The samples clearly stratify between cell type and treatment, indicating detectable differences in the total gene transcription patterns of these two groups. The influence of individual components on global transcriptome gene expression is as follows: X-axis, component 1 (cell treatment) is 59.5% Y-axis, component 2 (cell type) is 18.5% Z-axis, component 3 (n number) is 12.1%.

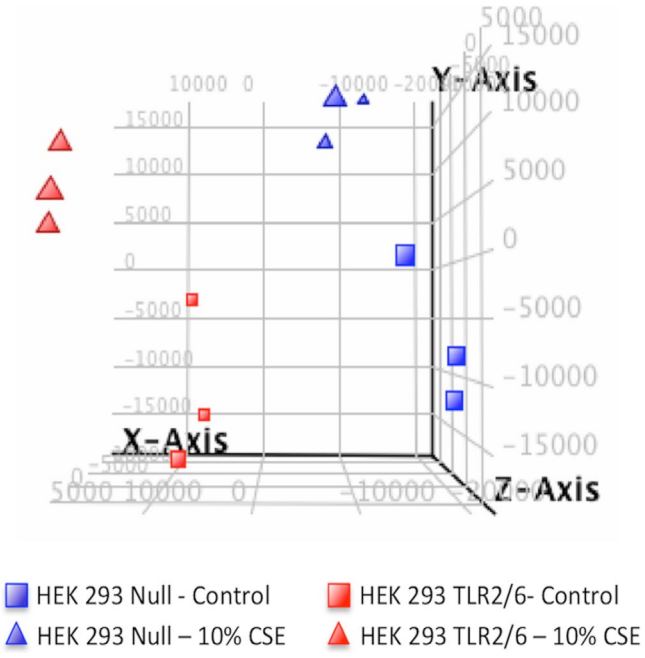


Figure 1: HEK293 cells treated for 8h with control media or 10% CSE cluster according to treatment conditions. Principal Component Analysis (PCA) plot on HEK293 Null (blue) and TLR2/6 (red) samples treated for 8h with control media (squares) or 10% CSE (triangles). Distinct clustering by cell type/treatment group indicates that global gene expression is altered by transfection with TLR2/6 and by stimulation with cigarette smoke. Data are n=3, and percentage influence of each component was 59.5% (PC1), 18.5% (PC2), 12.1% PC3.

Effect of 10% CSE on the expression of genes in HEK293 Null cells

Fold change analysis followed by a moderated student *t*-test and Benjamini-Hochberg FDR correction identified 74 genes that were significantly altered by ≥ 1.5-fold in HEK293 Null cells treated with CSE versus control media. 41 of these genes were downregulated and 33 up regulated (Figure 2A). Gene and conditions are displayed in order of how they cluster using Pearson's centred rank correlation distance metric and Ward's linkage rule on the entire 22837 probes gene sets. Using this analysis, there was a clear distinction between controls and smoke-treated samples. Gene symbols displayed more than once represent transcript variants of the same gene.

Effect of 10% CSE on the expression of genes in HEK293 TLR2/6 cells

Analysis of the data identified 67 genes that were significantly altered by ≥1.5-fold in HEK293 TLR2/6 cells treated with CSE versus controls. 43 of these genes were downregulated and 24 up regulated (Figure 2B). As was observed in the HEK293 Null cells, the HEK293 TLR2/6 cells also showed a clear distinction in their hierarchical clustering between controls and smoke-treated samples. Gene symbols displayed more than once represent transcript variants of the gene.

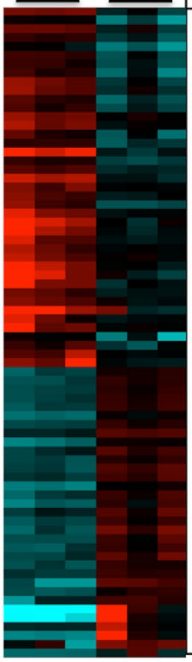
Genes altered by 10% CSE in a TLR2/6-dependent manner in HEK293 cells

A Venn diagram was constructed to assess which genes were differentially altered by 10% CSE in a TLR2/6-dependent manner (Figure 3). This consisted of a comparison of the mutual genes that were: 1) significantly altered by ≥1.5-fold in HEK293 TLR2/6 cells after treatment with 10% CSE (67 entities), 2) not altered in these cells under control conditions (18377 entities), and 3) not altered by 10% CSE in HEK293 Null cells (18556 entities). This resulted in the identification of 42 TLR2/6-dependent genes altered by cigarette smoke. 29 of these genes were downregulated and 13 upregulated (Figure 3). Ingenuity pathway analysis of these 42 TLR2/6-dependent CSE-regulated genes highlighted a significant role in cancer, cardiovascular disease, developmental disorders, hereditary disorder and infectious diseases. These genes play a significant role in the following canonical pathways retinoate biosynthesis, aryl hydrocarbon receptor signaling, diacylglycerol biosynthesis, the visual cycle and phosphatidyl glycerol biosynthesis (Figure 4A). When looking at the top network functions, 34 of the 42 genes that were reliant on TLR2/6, NOTCH 1 and the micro RNA activator EIF2C2 and NURP1 seemed to form central hubs (Figure 4B). Network analysis of these genes showed a significant association with connective tissue disorder, inflammatory disease and the inflammatory response.

Comparison of genes altered by 10% CSE in HEK293 cells and THP-1 cells

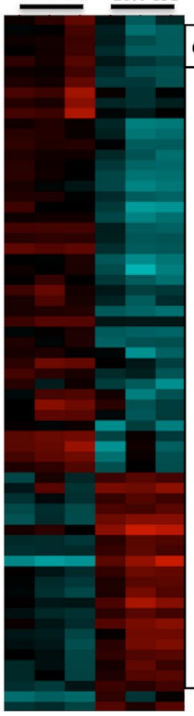
To find TLR2/6 independent genes, a Venn diagram comparison was made between HEK293 Null cells (74 entities) and THP-1 cells (282 entities),after both sets of cells were exposed to 10% CSE for 8 hours, to identify mutual genes that were significantly altered by ≥ 1.5-fold. 26 genes were identified of which 14 were down regulated and 12 up regulated (Figure 5A). When a similar comparison was made between

A



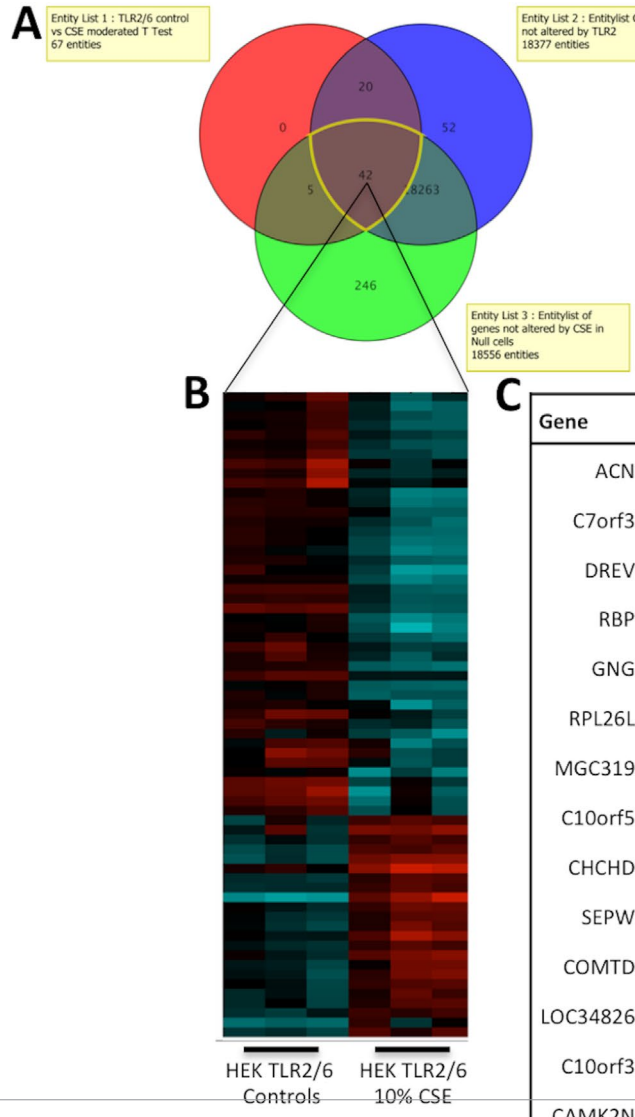
Gene	FC	p	Gene	FC	p	Gene	FC	p	Gene	FC	p	Gene	FC	p
ANAX1	-1.9	$5x10^{-5}$	METTL5	-1.5	$2x10^{-4}$	MT1G	-3.1	0.004	REX04	1.6	$1x10^{-4}$	KHSPR	1.6	$6X10^{-4}$
LMO4	-1.6	$7x10^{-4}$	BGN	-3.2	$5x10^{-5}$	MGP	-2.1	$9x10^{-4}$	NELF	1.6	$4x10^{-5}$	FLJ21742	1.6	$5X10^{-5}$
WBP2	-1.6	$6x10^{-4}$	SLC40A1	-1.5	$5x10^{-5}$	KIDINS220	-1.5	$5x10^{-4}$	ZFAND2A	1.5	$6x10^{-5}$	SPRYD3	1.7	$4X10^{-4}$
MTCP1	-1.5	$5x10^{-4}$	C5orf 13	-1.6	$4x10^{-5}$	IGFBP7	-1.5	$3x10^{-4}$	PPP1R15A	1.6	$2x10^{-5}$	LRRC41	1.5	$4X10^{-4}$
NRF2	-1.6	$4x10^{-4}$	HLA-DRA	-2.3	$2x10^{-5}$	MT1E	-3.3	0.007	SH3GL1	1.7	$4x10^{-5}$	CBX61	1.5	$4X10^{-5}$
LITAF	-1.6	0.001	DCN	-1.5	$5x10^{-4}$	MT1M	-1.7	0.021	TSPAN33	1.5	$4x10^{-4}$	HMOX1	2.3	$6X10^{-5}$
YAP1	-1.5	$9x10^{-4}$	CDH5	-1.5	0.001	HBB	-2.2	0.022	RFWD3	1.5	$1x10^{-4}$	CP1A1	1.6	$5X10^{-5}$
FLJ34969	-1.7	0.002	EFEMP1	-1.9	$4x10^{-4}$	PABPC1	-1.6	0.014	TIPARP	1.8	$4x10^{-5}$	SQSTM1	1.7	0.003
C20orf108	-1.9	$3x10^{-4}$	MT2A	-1.9	$6x10^{-4}$	STC1	-1.5	0.002	SRXN1	1.6	$1x10^{-4}$	CTSG	6.7	0.004
LDOC1	-1.7	$6x10^{-4}$	SERPINE1	-2.7	$5x10^{-4}$	COL1A2	-1.6	0.003	SLC25A19	1.7	$2x10^{-5}$	ELA2	4.9	0.007
HOXA4	-1.7	$5x10^{-4}$	THBS1	-2.4	$9x10^{-4}$	COL3A1	-2.2	0.002	CDT1	1.6	$5x10^{-4}$	PRTN3	1.5	0.042
C20orf29	-1.5	$6x10^{-4}$	MT1A	-1.8	0.003	ARAF	1.5	$1x10^{-4}$	FAM53C	1.7	$5x10^{-4}$	RNASE2	3.0	0.021
HTRA1	-1.6	$2x10^{-4}$	ESM1	-1.6	0.004	FOXD4L1	1.6	$7x10^{-5}$	VCP	1.5	0.001	CTGF	1.8	0.015
VWF	-1.7	$2x10^{-4}$	SRPX	-1.6	0.002	GRAMD1A	1.5	$1x10^{-6}$	PRDM4	1.6	$3x10^{-4}$	GCLM	1.5	0.009
SC65	-1.6	0.005	TM4SF1	-2.4	0.001	RNPEP	1.5	$1x10^{-4}$	TXNRD1	2.0	$2X10^{-4}$			

B



Gene	FC	p	Gene	FC	p	Gene	FC	p	Gene	FC	p	Gene	FC	p
ACN9	-1.5	0.005	LITAF	-1.5	$7x10^{-4}$	C5orf13	-1.7	$3x10^{-5}$	ELA2	-1.9	0.001	NOTCH1	1.8	$9x10^{-4}$
C7orf36	-1.5	0.006	ZFPM1	-1.6	$4x10^{-4}$	COMTD1	-1.5	$9x10^{-5}$	SRGN	-1.5	0.003	PPM1F	1.5	$4x10^{-4}$
DREV1	-1.5	0.001	NIPSNAP1	-1.6	0.001	IGFBP7	-1.5	$2x10^{-4}$	AGPAT5	1.5	0.003	FAM53C	1.7	$4x10^{-4}$
RBP7	-1.5	0.001	TSGA14	-1.5	$5x10^{-5}$	LOC348262	-1.5	$3x10^{-4}$	UHRF1	1.6	0.008	LOC389286	1.6	0.004
GNG5	-1.5	0.001	NFIX	1.8	0.001	IGF2BP3	-1.5	0.011	CYP1B1	1.5	$3x10^{-4}$	VCP	1.7	0.001
RPL26L1	-1.5	0.001	C7orf21	-1.7	$3x10^{-4}$	PCYOX1	-1.6	0.002	SRXN1	1.8	$5x10^{-5}$	FTHL7	1.5	0.002
MGC3196	-1.6	0.001	IL27RA	-1.8	$3x10^{-4}$	C20orf29	-1.5	0.001	TXNRD1	2.2	$3x10^{-5}$	FLJ1006	1.5	0.002
C10orf53	-1.6	0.006	OAF	-1.6	$4x10^{-4}$	PEX14	-1.6	0.003	FOSB	1.9	$3x10^{-4}$	FEM1A	1.5	0.002
CHCHD1	-1.6	0.006	HOXA4	-2-.0	$6x10^{-5}$	LRRFIP1	-1.6	0.026	TIPARP	1.6	$2x10^{-4}$	FOXD4L1	1.5	$3X10^{-4}$
SEPW1	-1.6	0.006	LDOC1	-1-.7	$3x10^{-4}$	SEMA3E	-1.6	0.030	TFRC	1.6	$9x10^{-5}$	PSMD3	1.5	0.003
C10orf38	-1.6	0.002	SC65	-1.8	0.001	PLCXD1	-1.6	0.005	HMOX1	3.4	$6x10^{-5}$	SQSTM1	1.7	$3X10^{-4}$
FLJ34969	-1.6	0.002	C20orf108	-1.5	0.001	ARHGDIB	-1.6	0.001	EIF2C2	1.5	0.001			
CAMK2N1	-1.5	0.003	NF1B	-1.6	0.002	RNASE2	-1.8	$5x10^{-4}$	PTCD1	1.5	0.001			
OPRS1	-1.5	0.002	RKHD3	-1.5	0.002	CTSG	-2.2	0.002	SAFB	1.6	0.002			

Figure 2: Expression of genes that were significantly altered in HEK293 Null and TLR2/6 cells after treatment with cigarette smoke extract (CSE) for 8h. (A) Heat map representation of normalized signal intensity values for genes altered by ≥ 1.5-fold by 8h exposure to 10% CSE in HEK293 Null cells. Red denotes high expression and turquoise denotes low expression. Order of samples was dictated by hierarchical clustering and genes represented are listed in the accompanying table in the order that they appear. **(B)** Heat map representation of normalized signal intensity values for genes altered by ≥ 1.5-fold by 8h exposure to 10% CSE in HEK293 TLR2/6 cells. Red denotes high expression and turquoise denotes low expression. Order of samples was dictated by hierarchical clustering and genes represented are listed in the accompanying table in the order that they appear. Statistical significance ($p<0.05$) was calculated using moderated student's t-test followed by Benjamini-Hochberg false discovery rate correction on GeneSpring GX11.0.2 software. Fold change represents a comparison between mean normalised signal intensity between treatment groups.



C

Gene	FC	p	Gene	FC	p	Gene	FC	p
ACN9	-1.5	0.005	C7orf21	-1.6	$3x10^{-4}$	SRGN	-1.5	0.002
C7orf36	-1.5	0.005	IL27RA	-1.8	$3x10^{-4}$	AGPATS	1.5	0.003
DREV1	-1.5	0.002	OAF	-1.6	$3x10^{-4}$	CYP1B1	1.5	$3x10^{-4}$
RBP7	-1.5	0.001	OPRS1	-1.5	0.001	TFRC	1.6	$9x10^{-5}$
GNG5	-1.5	0.001	TSGA14	-1.5	0.001	EIF2C2	1.5	0.001
RPL26L1	-1.5	0.001	ZFPM1	-1.6	$4x10^{-4}$	PTCD1	1.5	0.001
MGC3196	-1.6	0.001	IGF2BP33	-1.5	0. 011	SAFB	1.6	0.002
C10orf53	-1.6	0.005	NFIB	-1.6	0.001	NOTCH1	1.7	$9x10^{-4}$
CHCHD1	-1.6	0.005	RKHD3	-1.5	0.002	PPM1F	1.5	$4x10^{-4}$
SEPW1	-1.5	0.005	PCYOX1	-1.6	0.001	LOC389286	1.6	0.004
COMTD1	-1.5	$9x10^{-5}$	PEX14	-1.6	0.003	FTHL7	1.5	0.001
LOC348262	-1.5	$3x10^{-4}$	LRRFIP1	-1.6	0.026	FEM1A	1.5	0.001
C10orf38	-1.6	0.002	SEMA3E	-1.6	0.030	FLJ1006	1.5	0.001
CAMK2N1	-1.5	0.003	PLCXD1	-1.6	0.005	PSMD3	-1.5	0.003

Figure 3: Expression of genes that were significantly altered in a TLR2/6-dependent manner after treatment with 10% CSE for 8h. (A) Venn diagram constituting mutual genes differentially expressed between; 1) genes not altered by 10% CSE in HEK293 Null cells (green circle), 2) genes not altered by transfection with TLR2/6 (blue circle) and 3) genes significantly upregulated by ≥ 1.5-fold in HEK293 TLR2/6 cells after treatment with 10% CSE for 8 hours (red circle). **(B)** Heat map representation of normalized signal intensity values for genes altered by 10% CSE in a TLR2/6-dependent manner. Red denotes high expression and turquoise denotes low expression. Order of samples was dictated by hierarchical clustering, and genes represented are listed in the accompanying table in the order that they appear. Data represent n=3 and statistical significance ($p<0.05$) was calculated using moderated student's t-test followed by Benjamini-Hochberg false discovery rate correction on GeneSpring GX11.0.2 software.

THP-1 cells (282 entities) and HEK293 TLR2/6 cells (67 entities) treated with 10% CSE, to identify TLR2/6 dependent genes, 18 genes were mutually expressed, 7 were downregulated and 11 upregulated (Figure 5B). When genes that were significantly modulated by 10% CSE in THP-1 cells (282 entities) were compared with genes significantly regulated by 10% CSE in a TLR2/6 dependent manner from HEK293 cells (42 entities), only 1 gene was up regulated and this was SRGN (Figure 5C).

Comparison of genes altered by 10% CSE in HEK293 cells and PBMCs

A Venn diagram comparison was made between HEK293 Null cells (74 entities) and PBMCs (683 entities), after cells were exposed to 10% CSE for 8 h, to identify mutual genes that were significantly altered by ≥ 1.5-fold. 13 genes were identified; 1 was downregulated and 12 upregulated (Figure 6A). When a similar comparison was made between PBMCs (683 entities) and HEK293 TLR2/6 cells (67 entities) treated with 10% CSE, 12 genes were mutually expressed. Of these, 2 were downregulated and 10 upregulated (Figure 6B). When genes that were significantly modulated by 10% CSE in PBMCs (683 entities) were compared with genes significantly regulated by 10% CSE in a TLR2/6 dependent manner from HEK293 cells, 5 gene were identified (Figure 6C).

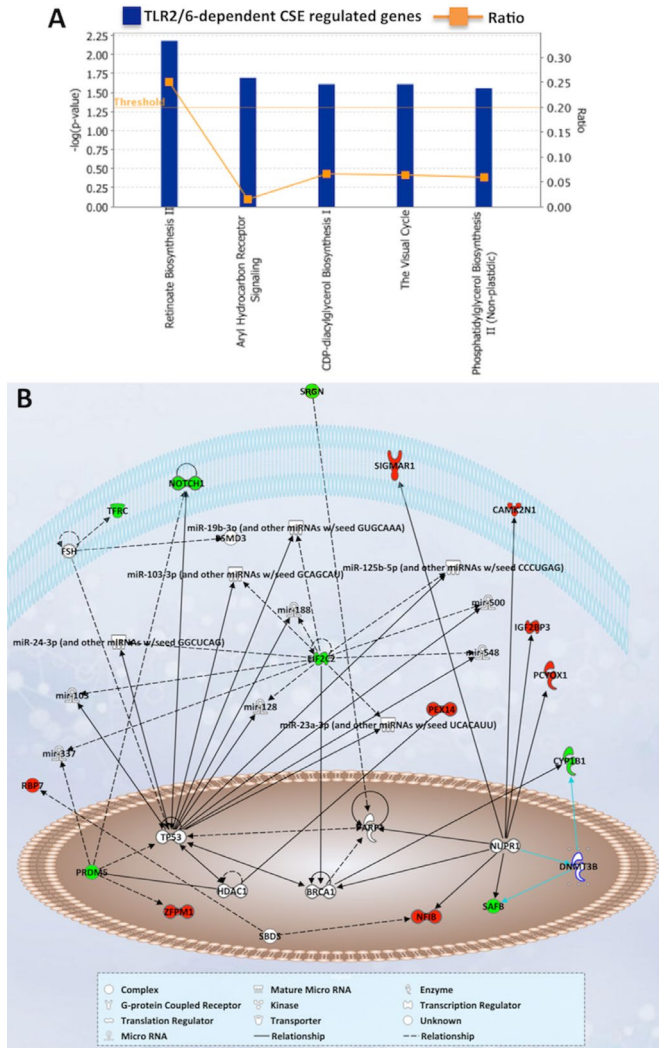


Figure 4: Ingenuity pathway analysis of genes that were significantly altered in a TLR2/6-dependent manner after treatment with 10% CSE for 8 h. (A) Shows the top five canonical pathways that the TLR2/6 dependent genes are significantly involved in. **(B)** Top associated network functions of the TLR2/6-dependent genes and their association with other key genes in these networks. Genes that were upregulated are in green and downregulated are in red. Genes added in due to pathway association are represented in white.

Verification of gene modified by CSE in THP-1 cells and PBMCs

A number of genes that were modified by CSE were confirmed as being altered using qPCR. The genes chosen were taken from the list generated in Figure 6C, from genes altered by CSE in PBMCs in a TLR2/6 manner. PPM1F, SGRN and EIF2C2 were chosen as PPMIF is a known negative regulator of cellular stress and SGRN and EIF2C2 are regulators of the immune function. In THP-1 cells no increase in these genes was observed at 8h, however by 24h there was an observed fold change of 1.48 ± 0.22 (PPM1F), 1.96 ± 0.08 (SRGN) and 1.41 ± 0.06 (EIF2C2). By contrast these genes were significantly regulated in PBMCs at 8h, with a reduction in mRNA levels for PPM1F (0.62 ± 0.23) and an induction in SRGN (2.50 ± 0.27) and EIF2C2 (2.09 ± 0.69). Similar results were obtained in PBMCs treated with CSE for 24 h.

Comparison of genes altered by 10% CSE in HEK293 cells, THP-1 cells and PBMCs

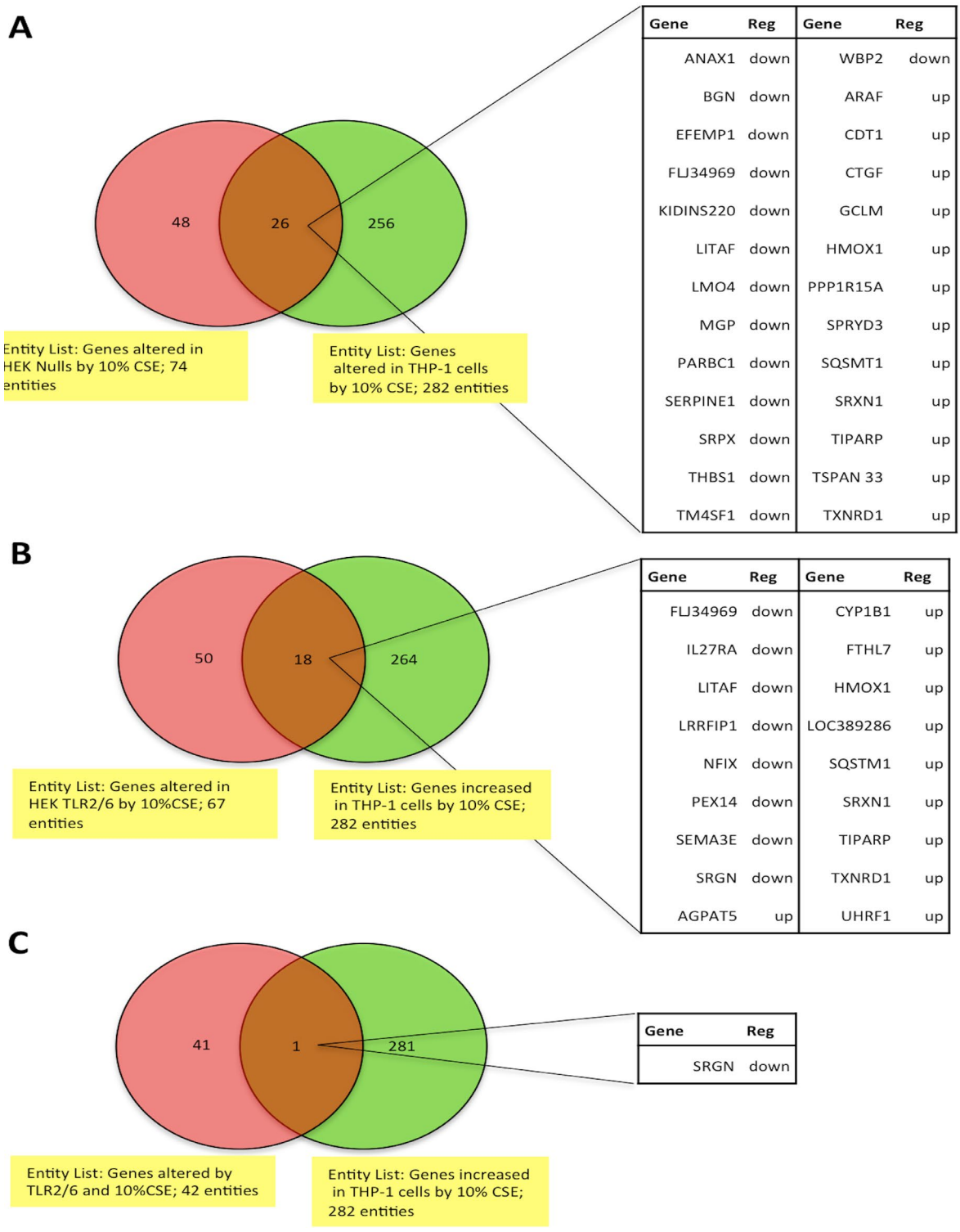
A Venn diagram comparison was made between HEK293 Null cells (74 entities), THP-1 cells (282 entities) and PBMCs (683 entities), after all cells were treated with 10% CSE for 8 h, to identify mutual genes that were significantly altered by ≥1.5-fold. 10 genes were identified of which 1 was downregulated and 9 upregulated (Figure 7A). When a similar comparison was made between PBMCs (683 entities), THP-1 cells (282 entities) modulated by 10% CSE and TLR2/6-dependent CSE-regulated genes (42 entities) only one gene was mutually expressed and this once again was an upregulation of SRGN (Figure 7B). Ingenuity pathway analysis of the 10 CSE-regulated genes in all three-cell types once again highlighted a significant role in very similar bio functions; these were cancer, cardiovascular disease, developmental disorder, hematological disease and hereditary disorder. Although the canonical pathways that these genes were involved with were slightly different: Nrf2 signaling, antioxidant actions of vitamin C, glutathione biosynthesis, hemedegradation and thioredoxin pathway (Figure 8A). When looking at the top network functions, all 10 of the genes analyzed, formed central hubs around HMOX1 and TNFα (Figure 8B). Network analysis of these genes showed a significant association with cell death and survival, cellular movement, hematological system development and function, free radical scavenging, small molecule biochemistry (Figure 9).

Discussion

There is a clear and unquestionable link between smoking cigarettes and the pathogenesis of many diseases, in particular COPD and cardiovascular disease. We have previously shown that, in an acute exposure model using monocytes, cigarette smoke activates gene networks involved with oxidative stress, xenobiotic metabolism and inflammation [24]. We have also described the dependency of pro-inflammatory gene and protein production on TLR2 and its heterodimer complexes in monocytes [7]. In the current study, a transcriptomic approach was adopted to give an insight into TLR2/6's function in cigarette smoke-induced cellular responses at the level of the transcriptome.

CSE significantly altered 42 genes by greater than 1.5-fold in a TLR2/6-dependent manner in HEK293 cells. Further analysis of these 42 genes identified pathways relating to retinoate biosynthesis, aryl hydrocarbon receptor signaling and CDP-diacylglycerol biosynthesis, with genes encoding for the proteins argonaute-2 (EIF2C2) and p53 (TP53) being central to the gene networks involved. Only 1 of these 42 genes was altered by cigarette smoke in THP-1 monocytes. When the 42 TLR2/6-dependent genes were compared with the genes changed by cigarette smoke in PBMCs, 5 were induced: ACN9, PCYOX1, SRGN, EIF2C2 and PPM1F.

The identification of genes altered by cigarette smoke in a TLR2/6-dependent manner is supported by our previous findings, which proposed a role for TLR2/6 in the acute cellular response to cigarette smoke. One of the genes, EIF2C2, is central to the above described gene networks and encodes for the protein argonaute-2. This protein has endonuclease activity required for RNA-mediated gene silencing, and is found in high concentrations in the processing bodies present in the cytoplasm [15,30]. Data directly linking EIF2C2 to cellular processes involved in inflammation caused by cigarette smoke is extremely limited [31]. However, a recent review highlighted the potential importance of smoke-induced alterations in non-coding



Gene	Reg	Gene	Reg
ANAX1	down	WBP2	down
BGN	down	ARAF	up
EFEMP1	down	CDT1	up
FLJ34969	down	CTGF	up
KIDINS220	down	GCLM	up
LITAF	down	HMOX1	up
LMO4	down	PPP1R15A	up
MGP	down	SPRYD3	up
PARBC1	down	SQSMT1	up
SERPINE1	down	SRXN1	up
SRPX	down	TIPARP	up
THBS1	down	TSPAN 33	up
TM4SF1	down	TXNRD1	up

Gene	Reg	Gene	Reg
FLJ34969	down	CYP1B1	up
IL27RA	down	FTHL7	up
LITAF	down	HMOX1	up
LRRFIP1	down	LOC389286	up
NFIX	down	SQSTM1	up
PEX14	down	SRXN1	up
SEMA3E	down	TIPARP	up
SRGN	down	TXNRD1	up
AGPAT5	up	UHRF1	up

Gene	Reg
SRGN	down

Figure 5: Expression of genes that were mutually altered by 10% CSE in HEK293 cells and THP-1 monocytes after 8h. (A) Venn Venn diagram and gene list of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 Null cells (red circle) and THP-1 monocytes (green circle) after treatment with 10% CSE for 8 h. **(B)** Venn diagram and gene list of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 TLR2/6 cells (red circle) and THP-1 monocytes (green circle) after treatment with 10% CSE for 8 hours. **(C)** Venn diagram and gene list of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 cells in a TLR2/6 dependent manner (red circle) and THP-1 monocytes (green circle) after treatment with 10% CSE for 8 h. Statistical significance ($p<0.05$) was calculated using moderated student's t-test followed by Benjamini-Hochberg false discovery rate correction on GeneSpring GX11.0.2 software.HEK293 Null and TLR2/6 cells have an n=3 and THP-1 cells have an n=6.

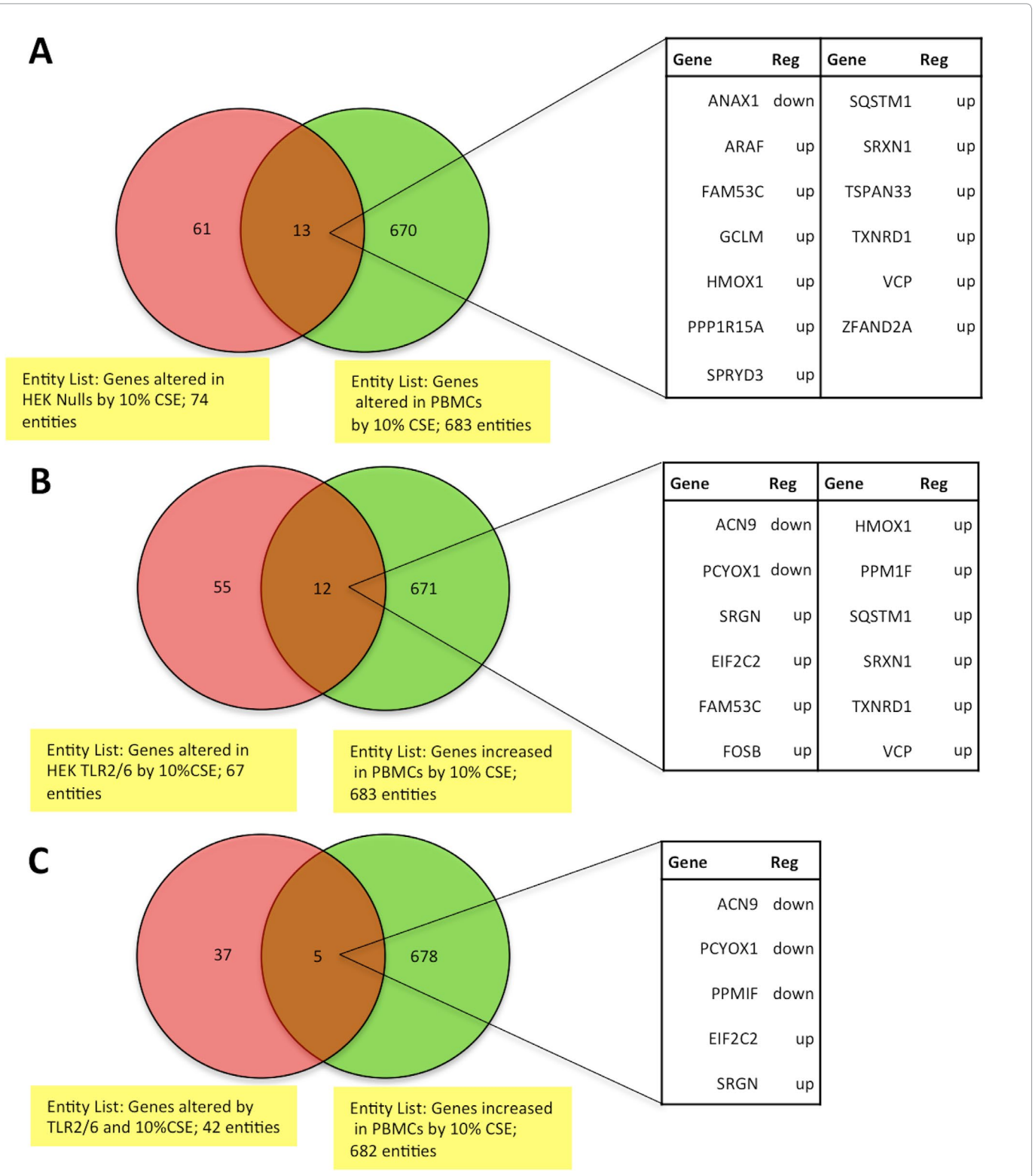


Figure 6: Expression of genes that were mutually altered by CSE in HEK293 cells and PBMCs after 8h. (A) Venn diagram of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 Null cells (red circle) and PBMCs (green circle) after treatment with 10% CSE for 8 hours. **(B)** Venn diagram of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 TLR2/6 cells (red circle) and PBMCs (green circle) after treatment with 10% CSE for 8 hours. **(C)** Venn diagram of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 cells in a TLR2/6 dependent manner (red circle) and PBMCs (green circle) after treatment with 10% CSE for 8 h. Statistical significance ($p<0.05$) was calculated using moderated student's *t*-test followed by Benjamini-Hochberg false discovery rate correction on GeneSpring GX11.0.2 software. HEK293 Null, HEK293 TLR2/6 and PBMCs cells all have an n=3.

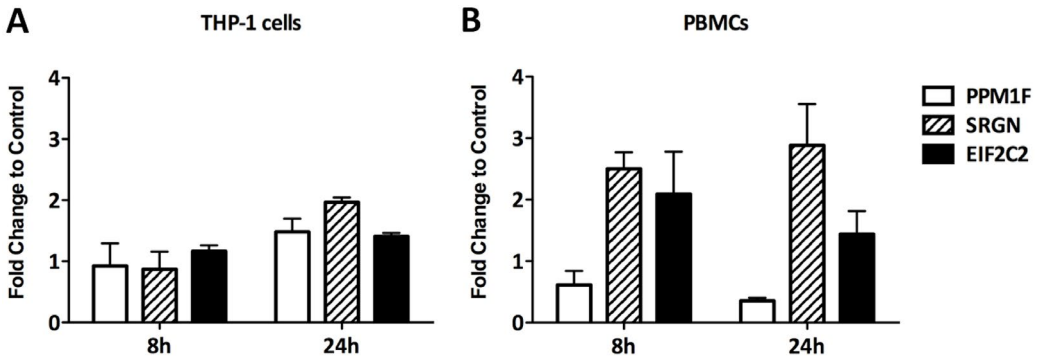


Figure 7: Conformation of the expression of TLR2/6-dependant that were altered by CSE in THP-1 monocyte and PBMCs after 8 and 24h. Cells were treated with cigarette smoke for 8 and 24h, mRNA extracted and the expression of PPMIF, SRGN and EIF2C2 were assessed by qPCR in (A) THP-1 monocytes and (B) PBMCs. THP-1 data represented is from n=3 separate experiments and PBMC data is from n=3 individual donors.

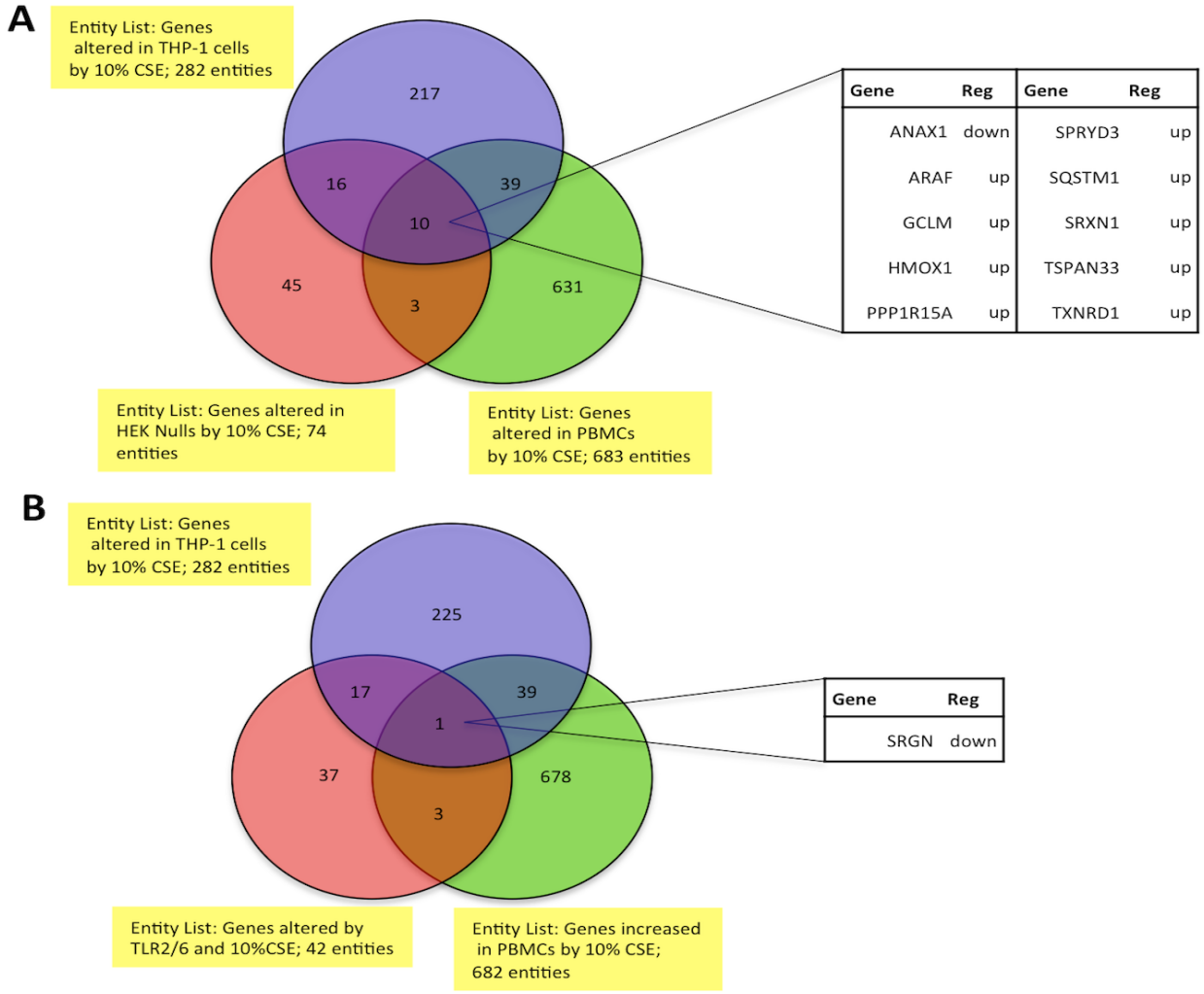


Figure 8: Expression of genes that were mutually altered by CSE in HEK293 cells, THP-1 monocytes and PBMCsafter 8 h. (A) Venn diagram of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 Null cells (red circle), THP-1 monocytes (blue circle) and PBMCs (green circle) after treatment with 10% CSE for 8 h. **(B)** Venn diagram of mutual genes that were significantly modulated by ≥ 1.5-fold in HEK293 cells in a TLR2/6 dependent manner (red circle), THP-1 monocytes (blue circle) and PBMCs (blue circle) after treatment with 10% CSE for 8 h. Statistical significance ($p<0.05$) was calculated using moderated student's t-test followed by Benjamini-Hochberg false discovery rate correction on GeneSpring GX11.0.2 software.HEK293 Null, HEK293 TLR2/6 and PBMCs cells all have an n=3 and THP-1 cells have an n=6.

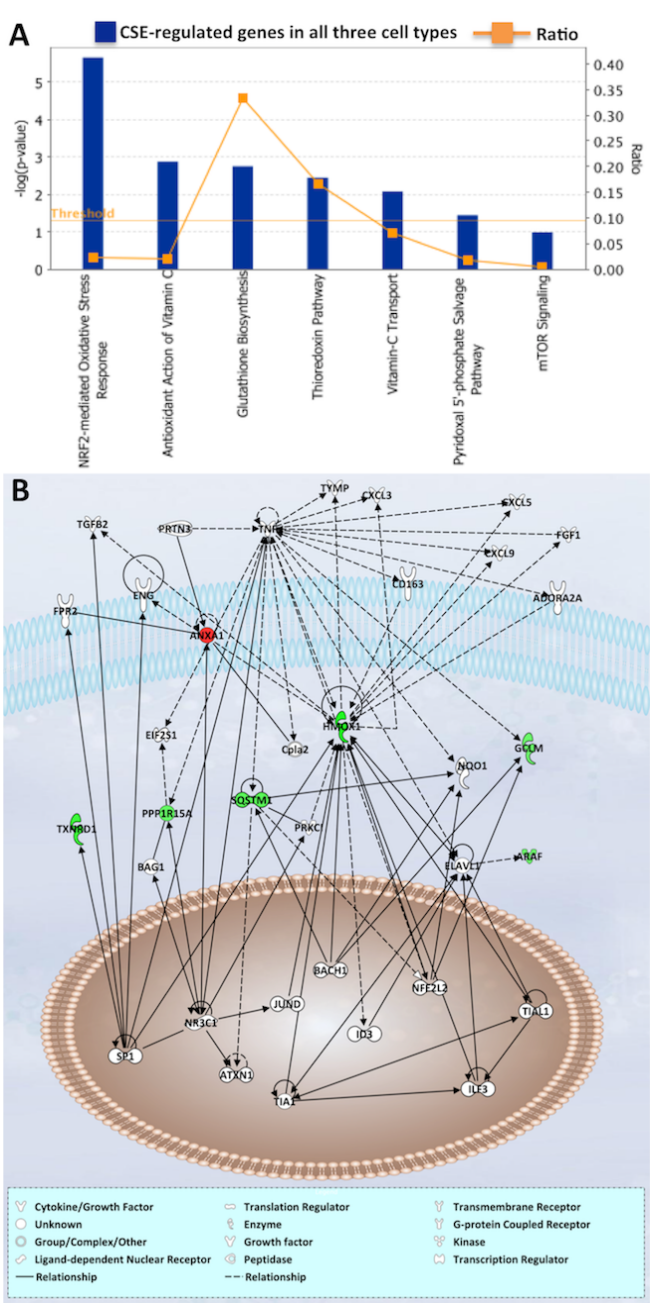


Figure 9: Ingenuity pathway analysis of common genes that were significantly altered by CSE in HEK293 cells, THP-1 monocytes and PBMCs after 8 h. (A) Shows the top seven canonical pathways that were altered in all three cell types. **(B)** Top associated network functions of common genes in all three cell types and their association with other key genes in the networks. Genes that were upregulated are in green and downregulated are in red. Genes added in due to pathway association are represented in white.

RNA, for which argonaute activity is important due to its role in micro-RNA-associated gene silencing [32]. Although the direct link of this gene to acute inflammation caused by cigarette smoke is sparse, the over expression of EIF2C2 was found in precursor lesions of lung adenocarcinomas [33]. Our data implicates an increased expression of EIF2C2 in cigarette smoke-induced inflammation, and highlights a possible mechanism for its link to lung adenocarcinoma development.

Aryl hydrocarbon receptor (AhR) signaling was highlighted as an important pathway affected by TLR 2/6-dependent CSE-induced cellular responses and has been implicated in adenocarcinoma formation moted by cigarette smoke [34,35]. An overexpression of AhR is found lung cancer cells and this can promote cancer cell invasion by increasing the release of matrix metalloproteinase from these cells [36]. Elevated levels of AhR signaling also promotes DNA strand breaks [37], and stimulates clonogenicity of cancer cells [38].The oxidative stress sensitive transcription factor Nrf2, is critically involved in protecting the liver and gastrointestinal tract against disease by regulating a multifaceted cellular antioxidant defence. Its major target, NQO1, was shown to be insensitive to AhR expression level. However, the induction of AhR expression caused a concurrent increase in mRNA expression of xenobiotic-metabolizing gene, CYP1B1 [39] a gene that we identified as being TLR2/6-dependent and others have shown to be involved in cytogenetic damage [34].

CYP1B1 itself has been implicated in the carcinogenicity of cigarette smoke. It has been directly linked to NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides) serum levels, which is a prognostic factor for lung cancer risk. In fact, a single nucleotide polymorphism in CY1PB1 was the only polymorphism out of 11 common polymorphisms on four genes known to be sensitive to tobacco carcinogenesis [40].

Four more TLR2/6-dependent genes were also altered by CSE in PBMCs. As mentioned, EIF2C2 may have a link to smoke-related disease pathogenesis, while PCYOX1 is associated with the release of bound cysteine, which is essential in antioxidant activity. PPM1F is associated with cell recovery, and it is of interest to note that breast cancer cell migration is repressed by microRNA-200c targeted repression of PPM1F [41]. This may be a direct mechanism behind the positive association of cigarette smoke and breast cancer, and may also be a novel mechanism by which smoke-induced PPM1F could lead to cancer progression [42].

When we compared TLR2/6-dependent CSE-regulated genes with CSE-regulated genes in both THP-1 monocytes and PBMCs, there was just one gene found as a match. SRGN, the gene encoding serglycin was upregulated by CSE in all three cell types. Serglycin is essential for the maturation of mast cell secretory granules, and is also the major proteoglycan secreted by peritoneal macrophages [43] and control TNF-α release from peritoneal macrophages [43]. Thus, in the setting of cigarette smoking, up regulated SRGN expression in PBMCs and monocytes may provide an explanation for increased cytokine release, which may, if the observation translates to people, impact on susceptibility to infection in smokers.

In the current, using HEK293 cells, we identify a list of genes that are altered by CSE in a TLR2/6-dependent and –independent manner. We have previously used HEK293 cells to show that oxidants can be perceived through a TLR2-dependent mechanism, in these studies HEK293 cells had a similar response to oxidants as THP-1 cells and monocytes [44]. To compensate for the HEK293 cells being embryonic cell line, we have cross-referenced these genes with CSE-induced genes present in monocytes. The potential importance of our findings have been given further validity, since a recent review has highlighted the importance of the proteoglycan serglycin on immune cell function [45]. The fact that this gene may be controlled through a TLR2-dependent mechanism is potentially important to immunologists.

In this study we have identified both TLR2/6 dependent and TLR-independent genes altered by CSE. This study provides a unifying

answer for others in the literature, where either TLR-dependent or -independent smoke induced inflammation has been reported. The TLR2/6-dependent genes identified seem to be involved in cancer cells development and therefore warrant further investigation to facilitate the development of novel therapies for smoking-related diseases.

Acknowledgement

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