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Retinal pigment epithelium transcriptome analysis in chronic smoking reveals a suppressed innate immune response and activation of differentiation pathways

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Abstract

Cigarette smoking, a powerful mixture of chemical oxidants, is the strongest environmental risk factor for developing age-related macular degeneration (AMD), the most common cause of blindness among the elderly in western societies. Despite intensive study, the full impact of smoking on the retinal pigment epithelium (RPE), a central cell type involved in AMD pathobiology, remains unknown. The relative contribution of the known dysfunctional pathways to AMD, at what stage they are most pathogenic, or whether other processes are relevant, is poorly understood, and furthermore, whether smoking activates them, is unknown. We performed global RNA-sequencing of the RPE from C57BL/6J mice exposed to chronic cigarette smoke for 6 months to identify potential pathogenic and cytoprotective pathways. The RPE transcriptome induced by chronic cigarette smoking exhibited a mixed response of marked suppression of the innate immune response including type I and II interferons and upregulation of cell differentiation and morphogenic gene clusters, suggesting an attempt by the RPE to maintain its differentiated state despite smoke-induced injury. Given that mice exposed to chronic smoke develop early features of AMD, these novel findings are potentially relevant to the transition from aging to AMD.

Graphical Abstract

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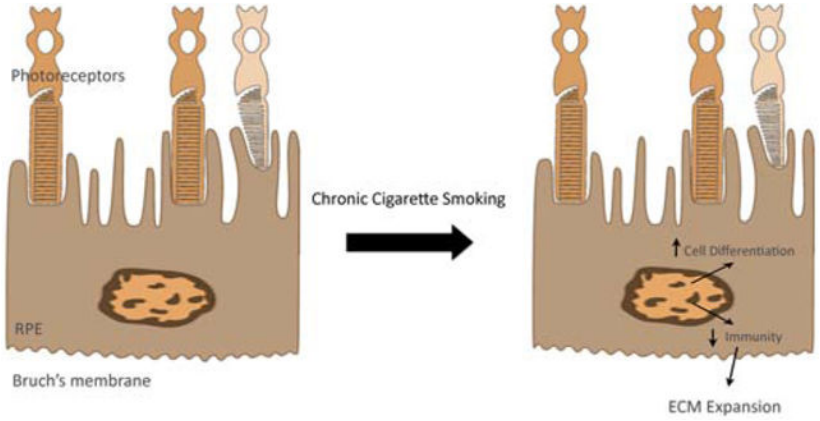

Keywords

aging; age-related macular degeneration; differentiation; innate immunity; RNA sequencing; smoking

Introduction

It is perplexing that people choose to smoke cigarettes, given that it contains nearly 5000 chemical oxidants and toxins[1], and more than 16 million Americans are afflicted with a smoking-induced disease such as cancer, heart disease, stroke, chronic obstructive pulmonary disease, diabetes, and/or age-related macular degeneration (AMD)[2]. The total economic cost of smoking is over $300 billion per year to cover direct medical care and lost work productivity[2]. AMD is the world's leading cause of blindness among the elderly, with 196 million people currently afflicted worldwide, and with the aging population, this number is predicted to expand to 288 million in 2040[3]. In the US alone, 11 million people have AMD, a number similar to those with all invasive cancers combined, and more than double those with Alzheimer's disease[4].

The National Eye Institute's AMD Pathobiology group recently concluded that identifying all of the pathogenic signals, prioritizing their contribution relative to one another, and establishing the disease stage when the predominant signals initiate the disease process, will enable effective treatment design for each stage of AMD[5]. The AREDS2 formulation slows intermediate AMD progression, and anti-VEGF therapies have transformed treatment of exudative AMD[6-8]. Since preventing or curing early/intermediate AMD would eliminate the burdens of advanced AMD and reduce the accompanying financial cost, understanding the pathogenic signals that induce the transition from aging to early AMD will enable targeted treatment for this disease stage. In addition to advanced age and genetic susceptibility[9-11], smoking is a major risk factor for age-related macular degeneration (AMD)[12]. Retinal pigment epithelial (RPE) cell atrophy is a hallmark feature of early AMD, and the RPE, in particular, appears to be a target of cigarette smoke in AMD[13, 14]. Most studies have characterized the acute response to cigarette smoke by the RPE, and these studies have focused on a specific pathway. While valuable, a broad perspective of the most

relevant pathogenic and cytoprotective responses by the RPE to chronic cigarette smoke exposure is still poorly understood.

Rod photoreceptor dysfunction and death in association with RPE morphologic derangement and the appearance of binucleated nuclei in the perifoveal macula are among the earliest changes in AMD[15-18]. With a similar RPE and rod/cone density as the human perifovea[19], mice are a reasonable model for studying early AMD changes. Our lab and others have previously reported that when mice are exposed to cigarette smoke for 6 months, the RPE developed marked ultrastructural derangement with increased apoptosis that is reminiscent of early AMD[20-22]. How the RPE atrophies, what pathogenic pathways are activated, and what cytoprotective responses fail as a consequence of cigarette smoking are not well characterized. Each cell's function is dictated in large part, by its transcriptional program. To identify the key pathogenic pathways and cytoprotective responses by the RPE to chronic cigarette smoke, we exposed C57BL/6J mice to cigarette smoke for a period of 6 months, and evaluated their transcriptomic response by RNA-sequencing.

Materials and Methods

Animals and treatments

All experimental protocols used in this study were in accordance with National Institute Health (NIH) guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee. Briefly, an equal number of 2-month female and male C57BL/6J mice (RD8 negative) were placed in a smoking chamber for 2.5 hours per day, 5 days per week for 6 months, as described previously[20], or raised in a filtered air environment for 6 months.

Tissue preparation

After mice were sacrificed and eyes were enucleated, one eye was dissected to remove the RPE/choroid, which was prepared for RNA or protein extraction. The other eye was fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.08 M cacodylate buffer for transmission electron microscopy (TEM). The central 2x2 mm tissue temporal to the optic nerve was postfixed with 1% osmium tetroxide, dehydrated, and embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, PA).

Ultrastructural analysis

Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-100 CX electron microscope (JEOL, Tokyo, Japan).

RNA extraction and Library Preparation

Total RNA from mouse RPE/choroid using triplicate biological replicates for air or smoking were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA), with on-column DNA digestion by RNase-free DNase, following the manufacturer's instructions. RNA integrity was verified using Agilent 4200 TapeStation (Agilent, Santa Clara, CA). Stranded RNA-seq library construction was carried out using 100 ng of RNA with the TruSeq Stranded mRNA

Sample Preparation Kit (Illumina, San Diego, CA). Libraries were paired-end sequenced to 126 bases using a HiSeq 2500 Sequencing System (Illumina, San Diego, CA).

RNA-Sequencing Analysis

The analysis pipeline has been described previously[23, 24] and was performed using mouse genome GRCm38.p6 with Ensembl v98 annotation. The gene-level differential expression analysis was performed between control (Air) and cigarette smoking (CS) treatment using the exact test from the edgeR v3.26.6[25] package in R (https://r-project.org). Genes were kept for analysis if all replicates of either group expressed at 1.0 count per million (CPM) or higher. Genes were defined as significantly differentially expressed (DEG) between Air and CS, if the absolute fold change exceeded 1.5 and had a Benjamini-Hochberg false discovery rate (FDR) of less than 5%. Functional gene enrichment was performed using gProfileR v0.6.7[26] with Gene Ontology (GO) Biological Process[27] gene sets. Reduced redundancy representation was performed using the most child term of any significant (less than 1% FDR) group of terms. Gene Set Enrichment Analysis (GSEA) was performed using the Hallmark Pathways from the GSEA database [28] using the fgsea v1.10.0 [https://doi.org/10.1101/060012] package in R. Potential protein–protein interactions of differentially expressed genes was analyzed using the STRING database (version 11.0) [29, 30]. The resulting dataframe object was modified to be plotted as enrichment plot with R package DOSE[31].

Cell culture

The established human ARPE-19 cell line[32] was maintained in Dulbecco's Modified Eagle Medium:F12 50/50 mix, supplemented with 10% inactivated fetal bovine serum and 2 mM L-glutamine, at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded at 50,000 cells/cm^2 in 12-well plates for 2 days followed by 1 day of serum starvation. Cells were treated with cigarette smoke extract (CSE) for up to 24 hr, and RNA was isolated for RT-qPCR, or protein was extracted for immunoblotting.

RT-PCR

RT-qPCR was performed as previously described[33](Applied Biosystems, Foster City, CA) on a StepOne-Plus Real-Time PCR system (Applied Biosystems) using Primer sequences (Applied Biosystems). Data were analyzed by the comparative threshold cycle method, with Cyclophilin A as an internal control.

Immunoblot analysis

Western blot analysis was performed as previously described[33]. Briefly, RPE/choroid, whole cell lysates, or supernatant were prepared using RIPA buffer (Sigma, Inc., St. Louis, MO). Proteins were separated by 4–12% Bis-Tris sodium dodecyl sulphate polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with primary antibodies and then secondary antibodies (Table S5).

Statistical analysis

Statistical analysis was carried out using the unpaired t-test, with GraphPad software (GraphPad Software, Inc., San Diego, CA). Significance is indicated by *p<0.05, **p<0.01, and ***p<0.001. Each experiment was repeated at least three times. Blots are selected as the representative one of specific group of experiments, and graphs represent the mean ± SEM of at least three independent experiments.

Results

The RPE undergoes morphologic and ultrastructural derangement with chronic cigarette smoke exposure

Mice exposed to chronic cigarette smoke develop significant oxidative injury and morphologic derangement to the RPE, as observed in early AMD[20-22]. To verify that our model reproduced these essential changes, 2-month old C57BL/6J mice were exposed to either cigarette smoke (CS) or air for 6 months, and the RPE/choroid was examined by TEM. Figure 1 shows the marked ultrastructural derangement to the RPE that includes mesenchymal cell shape, loss of apical microvilli and basal infoldings, and development of intracellular vacuoles, all of which are seen in human AMD[34-37].

Global transcriptional response by the RPE/choroid to chronic cigarette smoke

To gain an understanding of the prominent cellular pathways that are impacted by chronic smoke exposure, we performed RNA-seq of the RPE/choroid from mice exposed to either CS or air for 6 months. Samples were sequenced to a mean depth of 15.1 ± 1.1 million fragments per sample, of which 86.1 ± 1.2 million fragments aligned to known gene annotations that were used for quantitation (Figure S1A).

Gene expression values (CPM) were examined by principal component analysis (PCA) to evaluate replication of samples within the two groups (Figure S1B). Sample air.3 was determined to have a high level of retina contamination (Figure S1C) and was eliminated from the analysis (Figure 2A). As an initial characterization of the molecular alterations induced by chronic smoke exposure, we identified 558 differently expressed genes (DEGs) in the RPE/choroid between the CS and air treated groups, as represented by the Volcano plot (Figure 2B). The complete DEG results are listed in Table S1.

A number of pathologic pathways are impaired with smoking; these include oxidative stress response, mitochondrial function, innate immune response, and extracellular matrix regulation[5]. Many of the significant DEGs are associated with these pathways. For example, *Cyp1a1* is induced by chronic smoking and likely represents a protective response to smoking since it is involved in xenobiotic metabolism, specifically degrades nicotine[38], and is linked to Aryl Hydrocarbon Receptor Signaling, which is also associated with xenobiotic metabolism and detoxification[39]. *Nnt* couples the hydride transfer between NAD(H) and NADP(+) to proton translocation across the inner mitochondrial membrane, using energy from the mitochondrial proton gradient to produce high concentrations of NADPH that is used for free radical neutralization[40]. *Adamts4*, which was increased 4.0-fold by smoking relative to air, is a metalloproteinase that specifically degrades extracellular

matrix proteins *Aggrecan* and *Bcan*, which were also increased 3.4 fold [41]. Interestingly, mice with an *Adamts4* mutation exhibit RPE dedifferentiation with reduced pigmentation and RPE-specific gene expression[42].

Several of the most downregulated genes from chronic smoking are related to the immune response. For example, *Ngp*, a cystatin superfamily member, regulates inflammatory responses through TLR-4 and phagocytosis[43]. *Npsr1* induces the production of pro-inflammatory cytokines TNF-α and interferon-γ[44]. *Crhbp* activates NF-kB activation to promote the inflammatory response[45]. *Lcn2*, an adipokine, and *S100A8*, a calcium binding protein of the S100 family, are both elevated during acute inflammation as a protective response, and recruit leukocytes and pro-inflammatory cytokines[46, 47]. The downregulation of these genes could contribute to an impaired protective immune response following chronic smoke exposure.

Gene enrichment analysis reveals impaired innate immune and induction of differentiation response after chronic smoking

To define how the RPE responds to chronic cigarette smoke exposure, an unbiased evaluation of the transcriptome was performed using gene ontology and gene set enrichment analysis to identify specific biological processes that were over-represented in DEGs (Figure 2C, D). The top suppressed processes are associated with defense response, including the anti-viral response from type I and II interferon (IFN). The specific genes in these categories are listed in Table S2. On the other hand, the top activated processes include morphogenesis, differentiation, and development genes. The over-representation of these genes is indicative of a transcriptional response by the RPE/choroid to recover essential functions that were impaired by chronic smoke exposure.

We recently reported that in early AMD, some RPE cells have entered epithelial mesenchymal transition (EMT), an adaptive transcriptional process that allows cells to survive a harsh microenvironment[48-51]. The transcriptional responses related to cell proliferation, cell migration, cell-cell adhesion in response to chronic smoking suggest prompted us to assess whether the RPE are entering EMT. The DEGs were ranked by their degree of differential expression in smoke relative to air control, and compared to the EMT gene set from GSEA database[28]. As illustrated in Figure 3, a set of DEGs was significantly related to the EMT gene signature, with a majority of these genes downregulated by chronic smoking (normalized enrichment score (NES) =−1.68, FDR = 0.0178). However, the expression pattern induced by smoking was for the most part, indicative of MET rather than EMT (Table S3). This expression pattern compliments the upregulation of genes related to differentiation and suggests that the RPE is attempting to maintain its epithelial state.

String analysis reveals the influence of multiple signaling pathways after chronic smoking

To identify signaling transduction pathways involved in the major processes identified by GO analysis, we performed String analysis of DEGs followed by enrichment analysis, selected terms with the keyword “pathway”, and identified the corresponding genes. Next, selecting the keyword “pathway” to find corresponding genes, which were intersected with

the first neighborhood genes, performed enrichment analysis. Figure 4 shows the over-represented DEGs and their probability of being assigned to the specific signaling pathway. The cell surface receptor signaling, enzyme linked receptor protein signaling, transmembrane receptor protein tyrosine kinase signaling, regulation of apoptotic signaling, and the Wnt signaling pathway were the top five most likely signaling pathways involved after chronic smoke exposure.

The top suppressed responses to chronic smoking, as noted above, included type I and II interferons (IFN), which are among the cell surface receptor signaling DEGs. Type I IFN responses can dampen type II IFN responses, and type II can reduce type I IFN immunity through STAT1 and STAT3 signaling[52, 53]. STAT1 and STAT3 have a reciprocal relationship in neurodegenerative diseases and tumor metastasis[54-56]. For example, STAT1 causes cell death signals, while STAT3 induces protective responses[54-56]. Because of the influence of STAT1 and STAT3 on IFN responses, we conducted String analysis to identify any potential functional interactions between these two pathways. Of the 1203 downregulated genes that were submitted for String analysis, 13 genes have close connectivity (combined probability score >0.7) and 31 genes have medium connectivity (combined probability score <0.7 but >0.4) with STAT1[57] (Figure 5A). The most connected genes, with their annotated gene function, are listed in Table S4.

Unlike STAT1, STAT3 mRNA levels in the RPE/choroid did not change after chronic smoke exposure. However, ontology enrichment analysis showed that a number of genes downstream of STAT3, such as extracellular matrix molecules, and known STAT3 inhibitors including SOCS1 and TMF1, were up regulated. We then performed String analysis of STAT3 with the 942 genes upregulated with smoke exposure. Of the 556 genes that were identified in the String database, 20 genes show strong (combined probability score >0.8) and 17 genes weak connection (combined probability score <0.5) with STAT3 (Figure 5B). The 20 most connected genes, with their annotated gene function, are listed in Table S4. Of these 20 genes, EGFR and PDGFRA are upstream of STAT3, suggesting that STAT3 signaling could be triggered by different cytokines (EGF/PDGF vs. IFNs) during smoking, and could activate downstream targets such as MMP2, MMP3, and MMP14[58, 59]. Furthermore, MITF, a transcription factor that cooperatively induces cellular transformation with STAT3[60, 61], was upregulated 1.85 fold in the RPE/choroid of mice exposed to chronic smoking. In addition, SOX10 and PAX3, which synergistically activate MITF, were also upregulated by smoking 1.5-fold and 2.0-fold, respectively. While STAT3 itself was not differentially expressed in the RPE/choroid with smoking, this transcriptional pattern suggests that a PDGFR/EGFR-STAT3 signaling pathway was activated, and this could be enhanced by MITF, SOX10, and PAX3. Taken together, the RNA-Seq data suggest that STAT1 signaling is suppressed while STAT3 signaling is increased.

STAT1 and STAT3 are reciprocally activated in the RPE/choroid after smoking

We next determined whether STAT1 and STAT3 signaling were indeed activated by measuring their phosphorylated forms. Following the trend of STAT1 mRNA, both STAT1 and pSTAT1 were reduced in the RPE/choroid of mice exposed to 6 months of smoking (Figure 6A). Likewise, STAT3 in the RPE was unchanged. However, pSTAT3 was increased

in the RPE/choroid of mice with chronic smoking (Figure 6B), suggesting that STAT3 activity is increased and likely post-translationally regulated. To further assess activation of STAT3 signaling, we examined the downstream production of the ECM molecules MMP2 and MMP14 and detected their upregulation in the RPE/choroid of mice after 6 months of smoking (Figure 6C).

STAT1 and STAT3 signaling responses were also similar in human RPE cells treated with cigarette smoke extract (CSE). Basal STAT1 activity is low in RPE cells and was enhanced with 100 ng/ml IFNα. Under these conditions, STAT1 signaling was reduced by CSE relative to controls (Figure 7A).

As in the RPE/choroid from mice exposed to chronic smoke, STAT3 was unchanged, and pSTAT3 was increased with CSE treatment compared to vehicle controls (Figure 7B). The pSTAT3 increase by CSE induced MMP3 and MMP14 production compared to vehicle control cells. This induction could be abrogated by silencing of STAT3 with either an siRNA against STAT3 (Figure 7C) or 1 uM of the STAT3 inhibitor Stattic (Figure 7D).

Discussion

The relative contribution of the dysfunctional pathways that are influenced by smoking to early AMD is not clear. Here, we report the global expression profile of the RPE/choroid from mice exposed to chronic smoking, which develop features of early AMD. We show that the overall RPE transcriptome after smoking exhibits marked suppression of the innate immune response including the antiviral response with type I and II interferons and an upregulation of cell differentiation and morphogenesis gene clusters, which indicates an attempt by the RPE to maintain its function despite smoke-induced injury. These novel changes are distinct from the previously acknowledged pathways involved in AMD pathobiology, such as mitochondrial dysfunction, autophagy, antioxidant response, and complement activation, and may represent events preceding the onset of established dysfunctions. These transcriptome changes are relevant because they are both in response to chronic smoking, and in a model that simulates RPE changes seen in early AMD. Since mice have a similar RPE and rod/cone density as the human perifovea[19], a site of early AMD changes, these alterations may be relevant to the perifovea.

Smoking induces the formation of danger associated molecular patterns (DAMPS), including oxidation-specific epitopes (OSEs), or oxidatively modified nucleic acids, proteins, and lipids that form when antioxidant systems inadequately neutralize reactive oxygen species[62]. DAMPS are recognized by pattern recognition receptors (PRRs) that activate the innate immune response. Relevant to AMD, we previously identified complement factor H and lipoprotein(a) as PRRs that bind to malondialdehyde and oxidized phospholipids, respectively, to induce an innate immune response[63, 64]. PRRs can activate type I and II IFNs in order to neutralize these potentially disease-causing molecules[65, 66]. The suppressed response by the RPE/choroid suggests that the immune response to DAMPs that are generated from smoking was inadequate and could lead to RPE dysfunction. The correlation of this impaired IFN response with an early AMD phenotype suggests that this failed arm of the innate immune response might be an early event in AMD pathobiology. A

suppressed immune response is in contrast to work suggesting an overactive innate immune response, especially complement and the inflammasome. We note that our findings are from a model of early AMD, whereas the genetic link of complement factors with AMD risk was conducted in patients with advanced AMD[67-70]. In addition, our findings are at the transcriptional level. Since a pathogenic role for complement is best studied by determining both its activity and the extent that this enhanced activity damages tissue, we are not able to rule out a role for complement despite minimal transcriptional changes. Likewise, we note that the best evidence of a role for the inflammasome is in late disease, such as in geographic atrophy or neovascular AMD[71, 72]. The innate immune response is complex, cell type specific, and situational. The AMD stage and the specific arm of immunity are fundamental requirements needed in any study design to fully decipher the role of innate immunity on AMD pathobiology. Future investigations will focus on the IFN response on RPE function and the degree of tissue injury induced by smoking induced suppression of this response in early AMD.

The over-representation of upregulated genes involved in morphogenesis, differentiation, and development suggest a transcriptional response by the RPE intended to maintain or re-establish its epithelial state to compensate for injury caused by chronic smoke exposure. We had previously observed that some RPE cells enter EMT in human AMD samples[51]. Our RNA-seq analysis uncovered DEGs related to EMT, but the expression pattern was indicative of mesenchymal epithelial transition, which is consistent with the cell's attempt to differentiate. The RPE has a heterogeneous morphology with a spectrum of normal appearing RPE to marked mesenchymal morphology in both AMD and this model as suggested in Fig. 1[20-22, 73, 74]. It is likely that each RPE cell's attempt to recover from smoking related injury is mosaic. Future studies might benefit from single cell RNA-sequencing to enable characterization of the RPE's heterogeneous response to smoking.

STAT1 and STAT3 are essential signaling components of type I and II IFN responses, which was verified by String analysis. In response to cigarette smoke, STAT1 was decreased while STAT3 was increased. Smoking is known to suppress type I and II IFN responses through several mechanisms. Upon interaction of IFNα with its IFN receptor, JAK1 and TYK2 are phosphorylated, which then phosphorylate STAT1 and STAT2 to enable heterodimerization. The heterodimer translocates into the nucleus and binds with nuclear p48/IRF-9 proteins to form the ISGF-3 complex, which binds to interferon stimulated response element in the promoters of IFNα-stimulated genes to induce their expression[75-77]. Cigarette smoke can up-regulate the catalytic activity of serine/threonine phosphatases[78], inhibit IRF-3[79], or reduce STAT1 phosphorylation[80]. With type II IFN signaling, smoking can decrease the expression of IFN-γR, which decreases STAT1 phosphorylation[81].

In contrast, STAT3 is induced by cigarette smoke as a protective response. In the lung, STAT3 is activated by cigarette smoke to regulate key inflammatory, proteolytic and apoptotic responses. At the same time, STAT3 modulates the anti-inflammatory response by increasing SOCS3 and IL-10 expression to prevent tissue injury[82]. When STAT3 is lacking, as in *Stat3*$^{-/-}$ mice, smoke exposure enhances inflammatory, proteolytic, and apoptotic responses, but with a deficient anti-inflammatory response that results in tissue injury. Likewise, STAT3 suppression by siRNA severely damages DNA to induce cell death

after cigarette smoke exposure[83]. Consistent with these studies, the STAT3 induction is a protective response.

We identified ECM regulation by STAT3 signaling, and that smoking induced MMPs. The RPE is attached to Bruch's membrane, a pentalaminar matrix. Normally, Bruch's membrane undergoes constant remodeling by MMPs that are modulated by TIMPs, which are in part, produced by the RPE[84-86]. With aging, Bruch's membrane thickens due to matrix protein accumulation, lipid deposition, and oxidative modification including advanced glycation end product formation that decreases hydraulic conductivity and nutrient transport across Bruch's membrane[74, 87-92]. These changes can enable the accumulation of cellular fragments, lipoproteins, and inflammatory debris during the formation of basal deposits and drusen, hallmark lesions of AMD. As an early event, altered MMP activity by the RPE may contribute to age-related Bruch's membrane thickening[86, 93]. The induction of MMPs through STAT3 by smoking both *in vitro* and *in vivo* suggests that the normal remodeling function of MMPs is intact. With further smoke exposure, it is possible that this process fails and could contribute to basal deposit formation.

Some limitations of this study are recognized. The transcriptomes that we identified were from bulk RNA-seq. While the physiological impact may be similar, we acknowledge that some of the expression profiles could originate from choroidal cells. Single cell RNA-seq would be a valuable approach to separate the transcriptional response of the RPE from choroidal cells. ARPE-19 cells were chosen to study the effects in human cells and did confirm the findings of our mouse studies. However, ARPE-19 cells have their shortcomings, and do other in vitro systems such as human fetal RPE cells or RPE cells from donor globes.

Conclusions

The unbiased global RNA-seq analysis of the RPE/choroid after chronic smoking uncovered an unexpected decline in the innate immune response that coincided with a transcriptional attempt to maintain its epithelial state in a model that simulates early AMD. This work provides insight into the early events caused by smoking that could lead to the conversion to early AMD. While valuable, the RPE response is unlikely to be uniform given its known heterogeneity in AMD. The role of the processes identified in this investigation might be enhanced by implementing single cell RNA-sequencing to identify the subgroups of RPE that may be expressing these potentially pathogenic and protective signals identified in this investigation. Future investigations to evaluate the relationship of smoking-induced transcriptional changes in RPE (reported here) to epigenomic alterations in aging (Corso Diaz et al. Cell Reports in press) and AMD-associated expression quantitative trait loci from human retina and RPE[94, 95] will be helpful in formulating a coherent and comprehensive platform for analyses of interactions among distinct susceptibility factors leading to AMD pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Chronic smoking suppressed the immune response including interferons in the RPE
- Cell differentiation genes were also upregulated as a compensatory response
- STAT1 and STAT3 were reciprocally activated to regulate the extracellular matrix
- Matrix alterations to Bruch's membrane are early events in AMD
- The transcriptome to smoke is complex with both impaired and compensatory responses

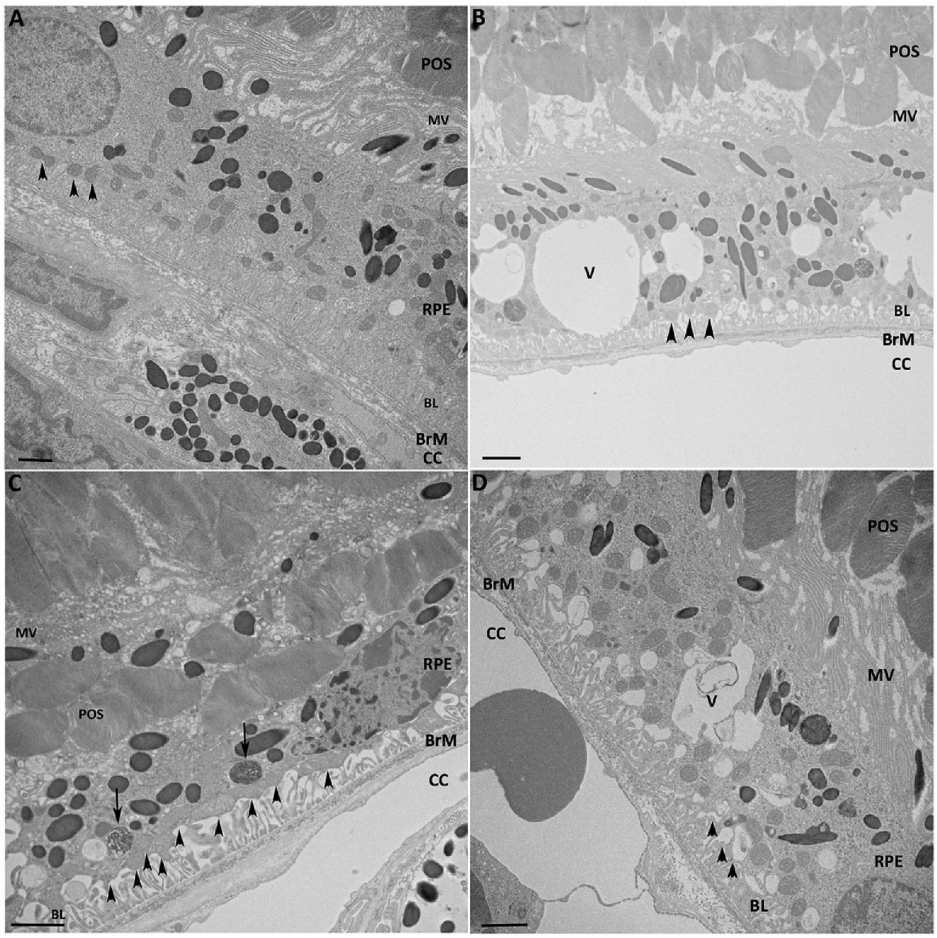

Figure 1.

Ultrastructural changes to the RPE after chronic smoking. **A.** Normal morphology and ultrastructure of the RPE from a C57BL6J mouse raised in air. Apical microvilli (MV) surround photoreceptor outer segments (POS). The basolateral (BL) RPE has normal infoldings (arrowheads). A variety of ultrastructural changes are seen in the RPE from C57BL6J mice exposed to smoke for 6 months. **B.** The microvilli are shortened, multiple vacuoles (V) are seen within the cell body, and the basal infoldings are shortened and widened (arrowheads). **C.** More severe mesenchymal shape to the RPE cell with similar changes to the microvilli and basal infoldings as in (B). Undigested POS (arrows) are seen in the basal region of the cell. **D.** Vacuoles with membranous debris are seen. BrM, Bruch's membrane, CC, choriocapillaris. Bar = 5 μm.

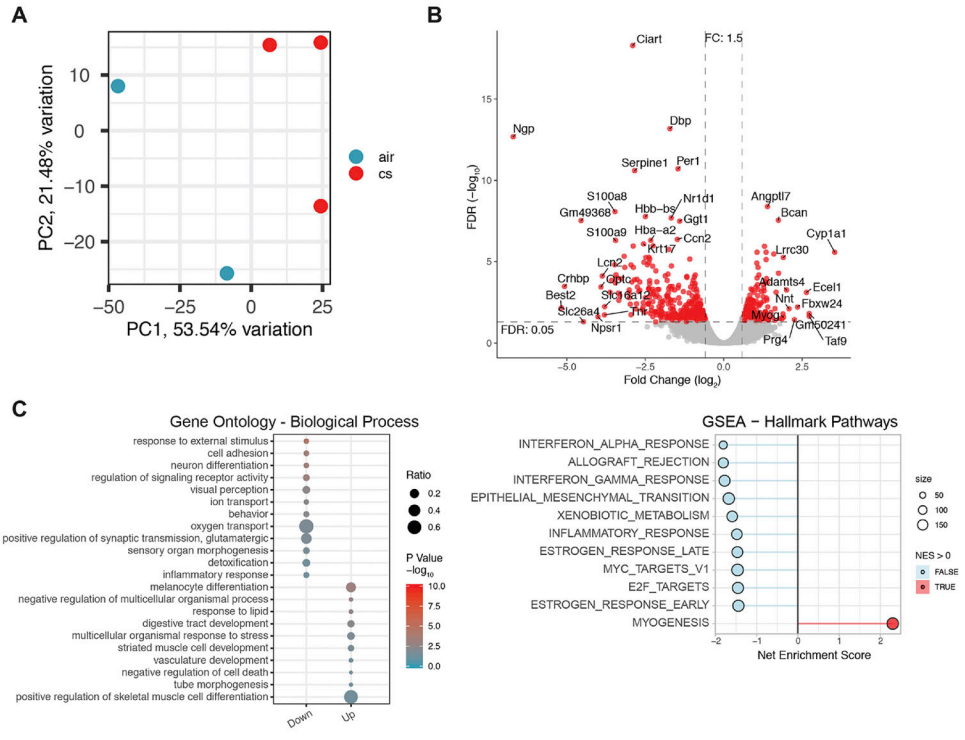

Figure 2.

Overview of differentially expressed genes. **A.** Principle Component Analysis for all expressed genes, totaling 15,559 genes. The samples clustered into two distinct groups of mice exposed to chronic cigarette smoking (CS) and air. **B.** Volcano plot of DEGs shows separation between air and CS treated mice. **C.** Gene Ontology enrichment of biological processes (BP) with chronic smoking exposure. Down-regulated genes from smoking are pooled in cluster1, and up-regulated genes in cluster2. The ratio of genes changed in each BP is reflected by the dot size. p-value is reflected by dot color, with red as the most confident change. **D.** Gene set enrichment analysis using the Hallmark Pathways. Pathways enriched in the down-regulated genes are indicated with a negative net enrichment score (light blue) and those enriched in the up-regulated genes have a positive enrichment score (red). Size indicates the number of genes in the pathway.

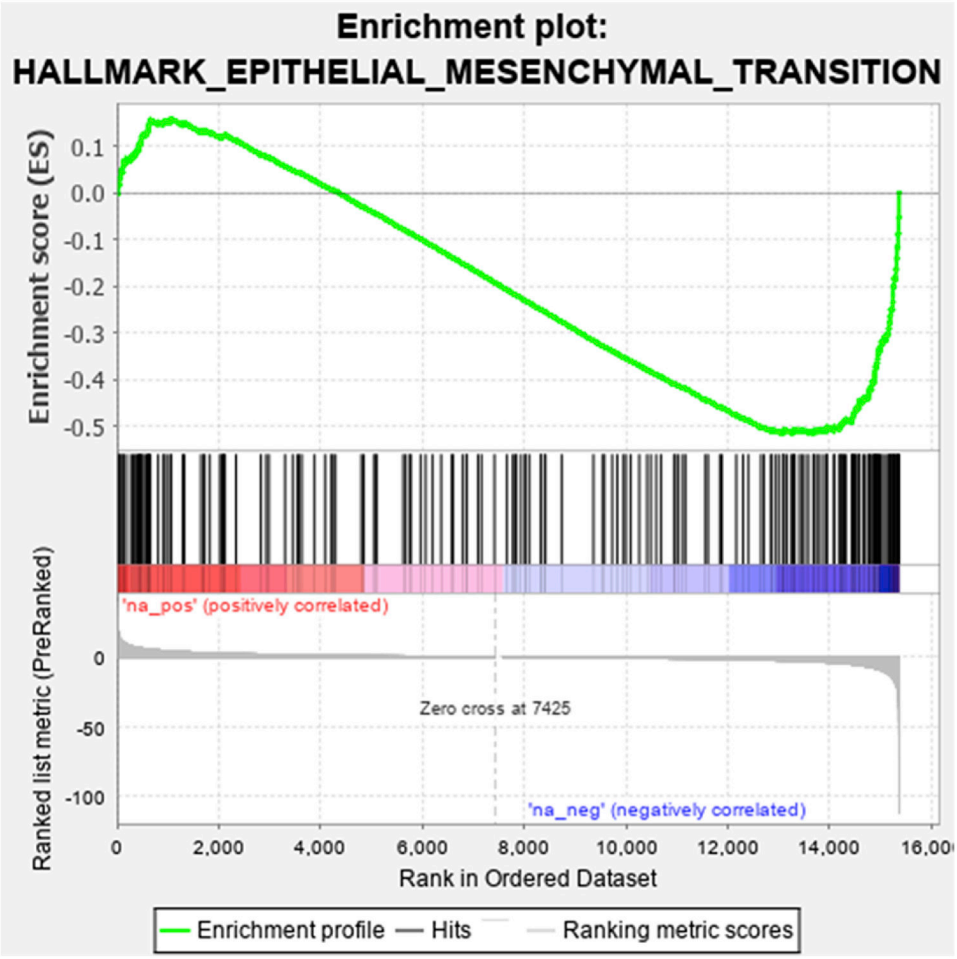

Figure 3.

Enrichment plot of differentially expressed genes (DEGs) induced by chronic smoking in the RPE that are related to Epithelial mesenchymal transition. DEGs were ranked from positive to negative fold change and compared to the Broad Institute's GSEA EMT 184 gene set. The enrichment score is plotted, which shows that the majority of DEGs were downregulated.

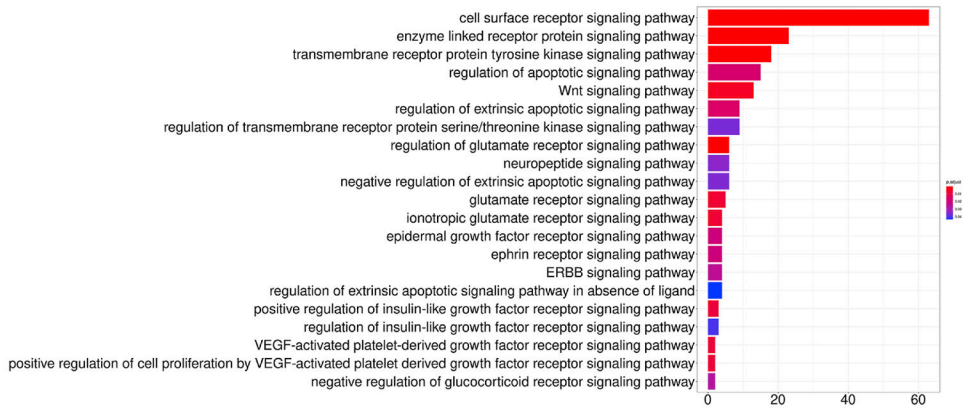

Figure 4.

Signal transduction pathways involved in the major processes were identified by GO analysis and then String analysis, enrichment analysis by selecting the keyword “pathway” to identify the involved genes that were altered by chronic smoking. The signaling pathways with the strongest likelihood of involvement are indicated by the p values. The number of genes that were differentially expressed is also shown.

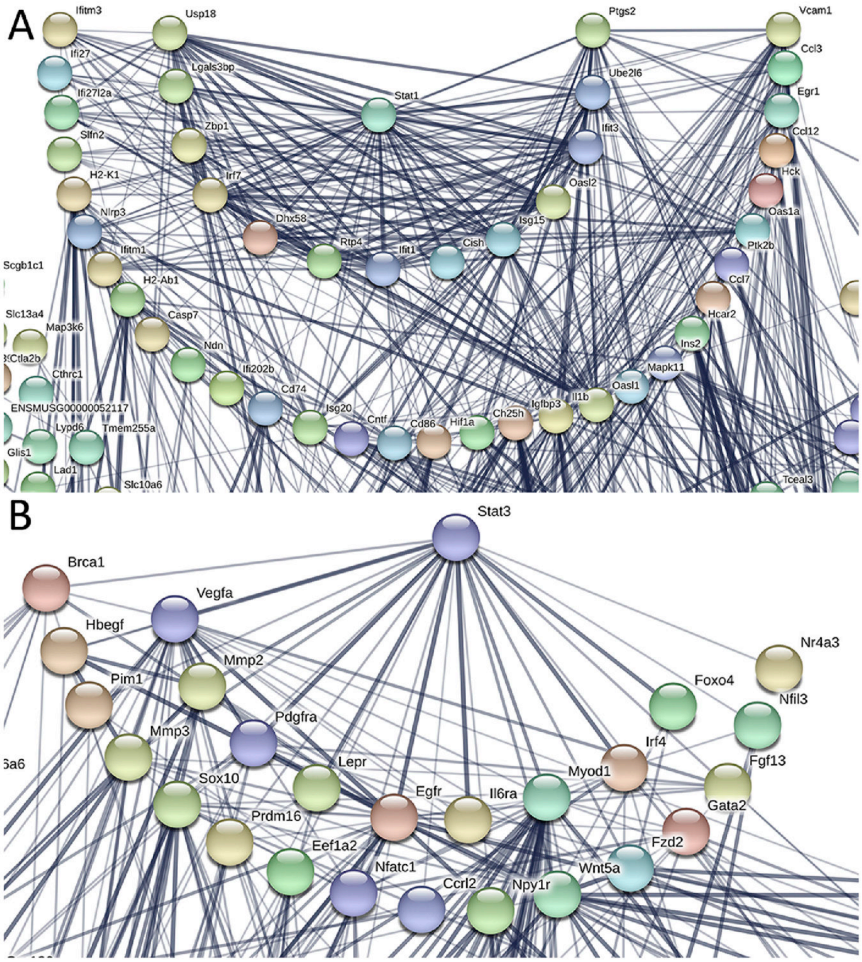

Figure 5.

String analysis of STAT1 and STAT3 signaling after chronic smoking in the RPE/choroid. The network was built based on high confidence evidence from both experimental protein-protein interaction and curated databases. The thickness of the lines that represent interactions is proportional to the STRING combined probability score. **A.** 44 genes are connected to Stat1 with chronic smoking. **B.** 25 genes are connected to Stat3 with chronic smoking.

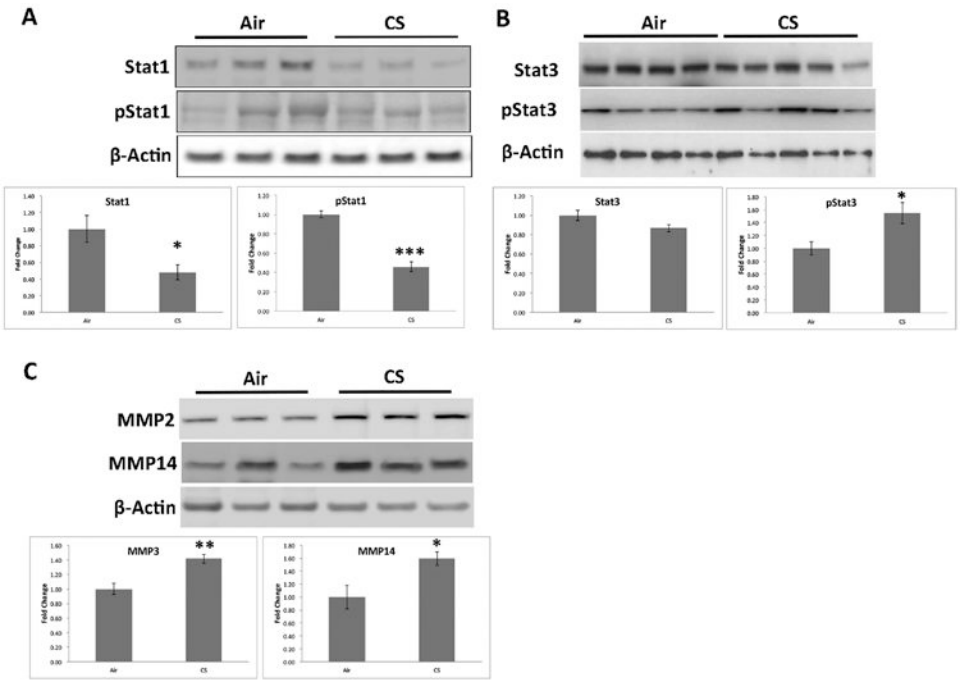

Figure 6.

Reciprocal STAT1 and STAT3 signaling in the RPE/choroid after chronic smoking. **A.** Western blots of STAT1 and pSTAT1 from the RPE/choroid of air and smoke exposed mice. STAT1 and p-STAT1 were plotted as fold change of smoke to air treated mice. **B.** Western blots of STAT3 and pSTAT3 from the RPE/choroid of air and smoke exposed mice. STAT3 and p-STAT3 were plotted as fold change of smoke to air treated mice. **C.** Western blots of MMP3 and MMP14 from the RPE/choroid of air and smoke exposed mice. MMP3 and MMP14 were plotted as fold change of smoke to air treated mice. Data were normalized to β-actin. *p<0.05. **p<0.01, ***p<0.001.

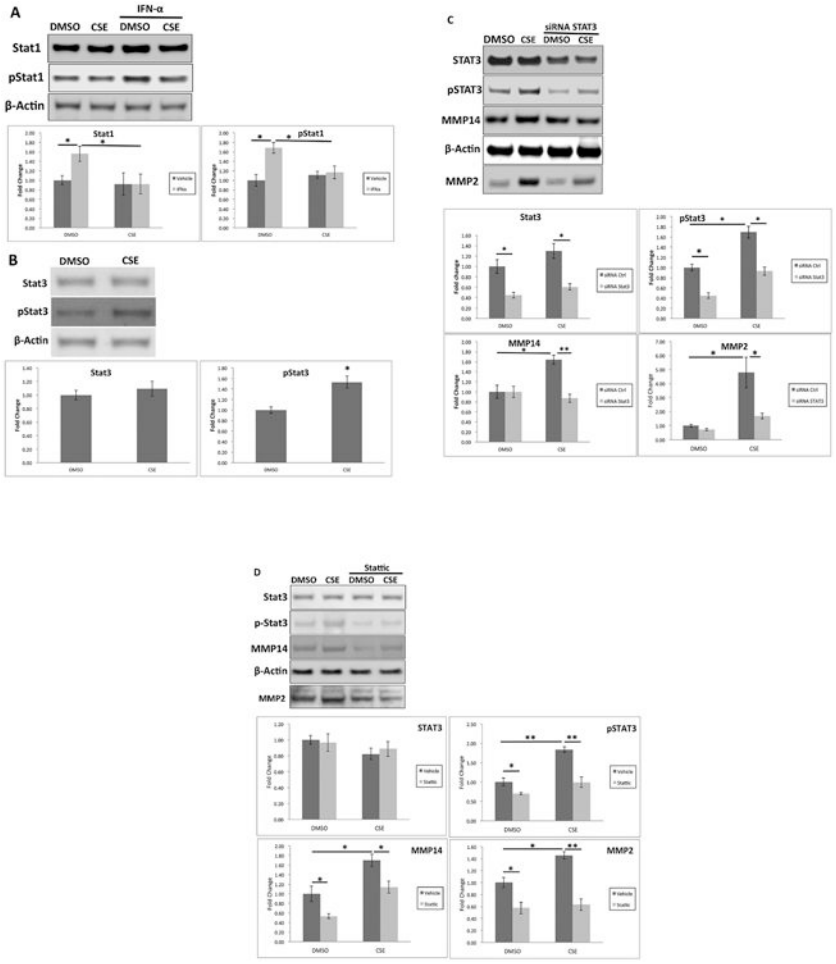

Figure 7.

STAT1 is decreased by cigarette smoke extract (CSE). **A.** RPE cells were treated with DMSO or 125 μg/ml CSE in the presence of 100 ng/ml IFN- α for 6 hrs. Western blots of STAT1 and pSTAT1 and their abundances were plotted as fold change. **B.** Cells were treated with DMSO or CSE for 24 hrs. Western blots of STAT3 and pSTAT3, and their abundances were plotted as fold change. *p<0.05. STAT3 silencing abrogates CSE-induced MMP14 production and MMP2 secretion. **C.** ARPE-19 cells were transfected with STAT3 siRNA and treated with 125 μg/ml CSE for 24 h. Western blot shows total STAT3, p-STAT3, and MMP14, and their abundances were plotted as fold change relative to DMSO-treated control siRNA treated cells. Data were normalized to β-actin. **D.** RPE cells were transfected with STAT3 siRNA, and treated with 125 μg/ml CSE for 24 h. Western blot shows total STAT3, p-STAT3, and MMP14 from cell lysates and MMP2 from the supernatant, and their abundances were plotted as fold change relative to DMSO-treated control siRNA treated cells. Data were normalized to β-actin. *p<0.05; **p<0.01.