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Extracts from presumed “reduced harm” cigarettes induce equivalent or greater toxicity in antigen-presenting cells

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Abstract

The tobacco industry has promoted certain cigarette products with claims that their use may be less harmful to the smoker as they purportedly deliver lower amounts of toxic chemicals compared to conventional cigarettes. This study was designed to compare the relative antigen presenting cellular toxicity of Eclipse, a presumed reduced exposure product (PREP) cigarette, when compared with the reference research 3R4F cigarettes (Kentucky University). Utilizing a murine macrophage cell line, murine bone marrow derived dendritic cells (DCs) and human monocyte-derived DCs incubated with extracts generated from Eclipse and Kentucky reference 3R4F cigarettes, we determined the relative toxic effects of the different cigarette smoke extracts on cellular viability, oxidative stress, T-helper-1 (Th-1) polarizing cytokine production and general gene expression. Eclipse and 3R4F cigarette smoke extracts induced equivalent oxidatively-mediated cellular heme oxygenase-1 (HO-1) protein levels in macrophages and DCs. Cellular viability determination demonstrated greater induction of cell death by apoptosis and necrosis by Eclipse extracts in DCs. The production of the key Th-1 polarizing cytokine interleukin-12 (IL-12) by activated DCs or macrophages was suppressed to an equivalent or greater extent by Eclipse extracts. Microarray studies performed on bone marrow derived murine DCs incubated with Eclipse or 3R4F cigarette extracts showed identical genotoxic profiles. These studies imply that presumed reduced harm Eclipse cigarettes induce equivalent or greater antigen presenting cell dysfunction relative to 3R4F cigarettes and illustrate the importance of independent validation and

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testing of similar products claimed to be associated with reduced toxicity relative to other cigarettes.

Keywords

cigarette smoke extract; antigen presenting cell; gene array; Eclipse; dendritic cell

Introduction

Cigarette smoking is a leading cause of preventable mortality in the world (Rostron, 2012). In spite of the undeniable evidence of harm associated with cigarette smoking, the habit remains unacceptably prevalent in both developed and developing countries. In addition, current smoking cessation strategies are associated with a relatively low sustained abstinence rate, even though sustained smoking cessation has been demonstrated to reduce long-term cancer, vascular and respiratory complications (Kenfield et al., 2008). While in principle the most effective means to eliminate disease due to smoking is to eliminate all tobacco product use, this is not likely to happen in the foreseeable future, and other approaches are needed for control of the worldwide tobacco disease epidemic. In consideration of this, the principle of tobacco harm reduction has been proposed, with the intent of reducing the adverse health effects of smokers who are either unwilling or unable to abstain from smoking (Stratton et al., 2001; Warner and Martin, 2003). Tobacco harm reduction would potentially form a component of a comprehensive tobacco control program, and might be of benefit in reducing long-term morbidity and mortality in highly addicted smokers (Lawson, 2012; Le Houezec and Aubin, 2013; Stratton et al., 2001).

In 1999, the United States Food and Drug Administration commissioned a study by the Institute of Medicine with the specific goal of addressing the feasibility of tobacco harm reduction, a concept embracing approaches aimed at minimizing harm and decreasing total morbidity and mortality without completely eliminating tobacco or nicotine exposure (Stratton et al., 2001). The expert panel recommended that reduction of tobacco-induced harm through use of potential reduced exposure products is a feasible and justifiable public health policy, provided that relevant basic and clinical research is conducted with the intent of establishing the true potential for harm reduction (Stratton et al., 2001) (Stratton K, 2001). Eclipse^{RM} is a cigarette introduced by RJ Reynolds Tobacco that fits in the description of a potential reduced exposure product because unlike other cigarettes, Eclipse heats rather than burns tobacco (Slade et al., 2002). In addition to Eclipse, other cigarettes have also been marketed with the label of presumed reduced exposure products; however Eclipse is a commercial tobacco product that has been more extensively characterized (Brown et al., 1998; Fields et al., 2005; Slade et al., 2002). Prior studies, mostly funded by the makers of Eclipse, have shown that smoke condensate generated from Eclipse cigarettes cause less mutagenicity, cytotoxicity, and genotoxicity *in vitro* when compared with other cigarettes (Fields et al., 2005; Meckley et al., 2004). While intriguing, these studies may have been biased by certain methodological approaches.

It is apparent that cigarette smoke profoundly alters the function of antigen presenting cells in ways that predisposes to immune dysregulation and the development of tobacco induced diseases like chronic obstructive pulmonary disease (COPD), asthma, and lung cancer. Of central importance in the regulation of innate and adaptive host immunity are antigen-presenting cells like macrophages and dendritic cells (DC), which regulate immunity relevant to the pathogenesis of many smoking-induced diseases including cancer and lung diseases (Mellman and Steinman, 2001). The current study was designed to evaluate the relative toxicity of Eclipse cigarette extracts compared to reference cigarette extracts on antigen-presenting cell function. To determine whether Eclipse cigarettes induce greater, equivalent or less immune cell toxicity relative to reference cigarettes, *in vitro* studies were performed on macrophage and dendritic cell (DC) survival and viability, activation and gene expression.

Methods

Cells and general reagents

All reagents were purchased from Sigma unless otherwise specified. Human monocyte derived DCs were generated from blood samples obtained from non-smoking adults as previously described (Vassallo et al., 2005). RAW 264.7 murine macrophage cells were purchased from American Type Culture Collection. Murine bone marrow derived DCs were generated using recombinant murine GM-CSF (10ng/ml R&D systems) and recombinant murine IL-4 (10ng/ml R&D systems) as previously described (Inaba et al., 1992).

Generation of cigarette smoke extracts

The generation of soluble components from mainstream cigarette smoke (cigarette smoke extract or CSE) has been utilized by many investigators, including our group (Kroening et al., 2008; Vassallo et al., 2008; Vassallo et al., 2005), as a model to understand the effect of soluble cigarette smoke constituents on cells *in vitro*. Cigarette smoke extracts were generated as previously described (Blue and Janoff, 1978; Carnevali et al., 1998; Kroening et al., 2008; Vassallo et al., 2008; Vassallo et al., 2005). The smoking apparatus consisted of a 50-ml plastic syringe with a three-way stopcock, to which a cigarette and a sterile plastic tip were attached. One end of the plastic tip was attached to a three-way stopcock and one end was immersed in a 50-ml conical tube containing 30 ml of sterile PBS pre-warmed to 37°C for 30 min. All materials used were sterile and used only once. CSE was prepared by drawing 35 ml of cigarette smoke into the syringe and then bubbling the smoke into the PBS. The cigarette was smoked up to 1-cm butt length. One cigarette was smoked per 10 ml of PBS solution, yielding a 100% CSE solution. Following preparation, the solution was filtered through 0.2-µm filters. Because cigarette smoke contains many volatile components, the CSE generated was used within minutes of preparation and prepared fresh for every experiment. The cigarettes used as a reference were 3R4F University of Kentucky's Tobacco and Health Research Program cigarettes which represents the average low-“tar” American cigarette (University of Kentucky, Kentucky, USA). The 3R4F cigarettes were specifically designed to represent cigarettes marketed in typical segments of the American market and are used as a standard against which other cigarettes may be compared (Bodnar et al., 2012). The nicotine content in RPMI media with 1% Eclipse CSE was determined to be 453ng/ml

while the nicotine content with 1% 3R4F Kentucky cigarette CSE was 174ng/ml (nicotine content measured by HPLC-tandem mass spectrometry in our institutional clinical nicotine analysis lab (Moyer et al., 2002)). These values refer to the final nicotine concentrations measured in a 1: 100 dilution of the freshly prepared CSE solution (thus the 2% CSE in culture would have twice this nicotine content). Measurement of nicotine in CSE provides context regarding corresponding *in vivo* exposures to nicotine, which are reported to range from 10 to 100ng/ml depending on the number of cigarettes smoked and other variables (Moyer et al., 2002).

Determination of relative cellular toxicity of Eclipse CSE compared to 3R4F CSE

To determine the effect of CSE generated from Eclipse and 3R4F cigarettes on antigen presenting cell viability, freshly generated CSE was added to 1×10^6 /ml human monocyte-derived DCs (generated as described in ref (Vassallo et al., 2005)) suspended in RPMI and 10% fetal calf serum. Following a 24-hour period of incubation, DCs were washed with ice cold PBS, and determination of apoptotic and necrotic cells was undertaken using a commercially available Annexin V-PI kit as recently described (Vassallo et al., 2005).

Suppression of interleukin-12 (IL-12) production by Eclipse and 3R4F CSE

Prior studies have shown that CSE significantly suppresses the production of IL-12 by activated DCs. Measurement of IL-12 protein secreted in cell culture supernatants by either human monocyte-derived DCs or RAW macrophage cells was performed using ELISA as previously described (Vassallo et al., 2008; Vassallo et al., 2005). Human DCs and RAW macrophages were plated in 6-well plates (1×10^6 /ml cells in 3mL of RPMI supplemented with 10% fetal calf serum). DCs were activated with 100ng/ml of lipopolysaccharide (LPS) while RAW macrophages were activated with a combination of 100ng/ml LPS and 5ng/ml interferon-gamma. CSE generated from either Eclipse or 3R4F cigarettes were added to the cells in concentrations of 1%, 2% and 3%. One hour later LPS and interferon-gamma was added to activate the cells. Cells were then incubated for 24 hours and supernatants were collected, centrifuged at high speed to remove cells or debris, and IL-12p70 levels measured using commercially available murine and human IL-12 ELISA kits (eBioscience).

Determination of cellular heme-oxygenase 1 (HO-1) levels

Intracellular HO-1 protein levels were determined as a surrogate of cellular oxidative stress. HO-1 levels were determined following 6 hours of incubation of either human monocyte-derived DCs or macrophages with either 2% Eclipse CSE or 2% 3R4F CSE. Whole cell lysates were prepared on ice using RIPA buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris). Whole cell protein levels were determined using a Bradford assay (Pierce) and 50µg of total cellular protein separated on a polyacrylamide gel, transferred to nitrocellulose, and immunoblotting performed using a polyclonal anti-HO-1 antibody (Stressgen, 1:2000 dilution). Beta-actin levels were detected on blots using a commercially available polyclonal antibody (Sigma-Aldrich, 1:5000 dilution).

Determination of relative toxicity of Eclipse and 3R4F CSE on global gene expression by bone marrow derived murine DCs

Murine bone marrow DCs were generated from the long bones of C57B6 mice as described by Inaba et al (Inaba et al., 1992). Following culture in GM-CSF and IL-4 for 9 days, cells were washed, enumerated and 1×10^6 /ml cells were resuspended in RPMI with 10% fetal calf serum. A total of 5×10^6 cells were plated in 6-well plates and cultured with media only (negative control), or with media containing the following: LPS (100ng/ml; positive control), LPS (100ng/ml) + 2% 3R4F CSE, or LPS (100ng/ml) + 2% Eclipse CSE. The experiment was repeated 4 times using an identical approach, generating 16 total samples for analysis. After 4 hours of incubation in an incubator at 37°C, cells were collected and mRNA prepared as previously described. RNA quality was determined on all samples generated using an Agilent 2100 Bioanalyzer in the Mayo Clinic institutional genomics core facility: all samples showed an RNA Integrity Number > 7.5. Complimentary DNA (cDNA) products were generated from 10ug of starting RNA per each sample, using the GeneChipR IVT Express Kit (Affymetrix) according to manufacturer's instructions. cDNA products were fluorescently labeled and hybridized to the Affymetrix Mouse 430 2.0 arrays (Affymetrix) according to the manufacturer's instructions at the Mayo Clinic Genome Facility – Gene Expression Core (GEC).

Statistical Analysis

General statistical analysis: All data are shown as the means $\pm$ SEM, unless otherwise stated, and were tested for statistical significance using ANOVA with relevant posttests where appropriate. Statistical differences were considered to be significant if p was <0.05. Statistical analysis was performed using SigmaPlot12. Relative levels (see Fig. 4C) of phosphorylated cellular ERK protein is represented relative to cellular actin levels by densitometry using Image J software (<http://imagej.nih.gov/ij/>).

Statistical Analysis of Microarray Data: 30 total arrays were generated: 6 arrays in each group multiplied 5 times. The analysis initially focused on the expression of critical dendritic cell immune-regulatory genes; these included cytokine genes, chemokines and chemokine receptor genes, adhesion molecules, co-stimulatory genes, and cell cycle and apoptosis related genes (Complete Table of genes in this primary analysis is included in Supplemental Table 1). In addition to this primary analysis, two other complementary analyses were performed: (a) identification of genes for non-stimulated DCs that showed statistically different expression levels between Eclipse and research CSE stimulation, and (b) identification of genes for the LPS stimulated DC that showed statistically different expression levels between Eclipse and 3R4F CSE stimulation. Analyses were performed on the perfect match data using the log base 2 transformation. Data was assessed for quality utilizing Affymetrix quality control metrics as well as RNA degradation, pre and post normalization MvA plots and box and whisker plots. Robust multi-array analysis was used to quintile normalize and summarize the probes into probe-sets (Bolstad et al., 2003; Irizarry et al., 2003a; Irizarry et al., 2003b). Per probe-set differential expression was assessed using one-way ANOVA with contrast statements to test for between group differences. P-values and false discovery rate q-values were used to rank genes in order of significance. The R statistical package was used for the statistical analyses of microarray data.

Results

Eclipse CSE induces equivalent cellular toxicity *in vitro* as 3R4F CSE

To determine the relative toxicity of extracts prepared from reference 3R4F cigarettes and Eclipse cigarettes, freshly prepared CSE was added to the culture media of human monocyte-derived DCs for a 24-hour period. Following this, determination of apoptotic and necrotic cells was undertaken using flow cytometric determination of Annexin V and PI staining. For this experiment, viability was arbitrarily defined as 90% of cells without Annexin V or PI positivity. CSE concentrations 2% did not diminish DC viability: >90% viability was observed with both 3R4F CSE and Eclipse CSE (Figure 1). Incubation of DCs with CSE concentrations 4% was associated with increased cellular apoptosis and necrosis (Figure 1). When compared with 3R4F CSE, Eclipse CSE caused similar *in vitro* cell death by either apoptosis or necrosis (Figure 1). As a result, subsequent experiments were conducted with CSE concentrations of 3% or less to minimize cell death or non-specific cellular toxicity as confounders of the analyzed specific antigen presenting cell responses.

Eclipse CSE induces equivalent or greater suppression of IL-12 production from activated antigen presenting cells

In prior studies, we showed that CSE significantly suppresses the production of IL-12 from activated DCs (Vassallo et al., 2005). Since IL-12 is a critical Th-1 polarizing cytokine involved in anti-tumor responses, we sought to determine whether Eclipse CSE would induce equivalent, less or greater suppression of IL-12 generation by activated DCs and macrophages. When DCs incubated with Eclipse CSE or 3R4F CSE were concurrently stimulated with 100ng/ml of LPS, the production of IL-12 was significantly reduced compared with DCs incubated with LPS alone (Figure 2). Eclipse CSE was even more potent than 3R4F in IL-12 suppression at all concentrations tested (Figure 2A: Anova, $p < 0.01$ for 1, 2 and 3% Eclipse CSE compared with 3R4F CSE). In a parallel experiment, we determined the IL-12 suppressive effect of Eclipse CSE on macrophage IL-12 production using the murine macrophage cell line RAW 264.7 stimulated with 100ng/ml LPS and 5ng/ml IFN γ . Eclipse CSE suppressed the production of IL-12 from activated macrophages; the suppressive effect was equivalent to that observed with 3R4F CSE (Figure 2B: Anova, $p > 0.05$ for all comparisons between Eclipse and 3R4F CSE samples).

Eclipse CSE induces more cellular oxidative stress than 3R4F CSE

In prior studies we demonstrated that CSE-induced oxidative stress is an important mechanism by which CSE modulates DC functions, including secretion of IL-12 (Kroening et al., 2008; Vassallo et al., 2005). To determine the cellular oxidative stress induced by Eclipse vs 3R4F CSE, levels of HO-1 protein were determined by immunoblotting (HO-1 is a 32 kD inducible isoform of the heme-oxygenase system that plays a critical cytoprotective role during cellular oxidative stress (Lin et al., 2007; Vile et al., 1994)). Both CSE preparations induced substantial augmentation of DC (Figure 3A) HO-1 protein levels. Quantitative analysis by densitometry shows that augmentation of cellular HO-1 by Eclipse CSE is higher than that observed with 3R4F CSE (Figure 3B; Anova, $p = 0.002$ for Eclipse vs 3R4F samples). The pre-incubation of DCs with the anti-oxidant n-acetyl-cysteine completely abrogated the induction of HO-1 by either CSE preparations, thereby confirming

that CSE-induced oxidative stress is a primary mechanism for the observed augmentation of cellular HO-1 levels (Figure 3A and B). Similar findings were observed in murine macrophage levels following incubation with either 3R4F or Eclipse CSE (Figure 3C and D). As observed with DCs, both 3R4F and Eclipse CSE stimulated macrophage HO-1 protein levels through oxidative stress (abrogated by NAC; Figure 3C). Densitometric analysis of the immunoblot shows enhanced induction of HO-1 protein by Eclipse CSE when compared with 3R4F stimulated macrophages (Figure 3D; Anova $p=0.001$ for Eclipse vs 3R4F samples).

Gene Expression Analysis of DCs incubated with Eclipse and 3R4F CSE

The primary analysis performed compared gene expression of a predetermined set of genes relevant to regulation of immunity (complete set of 271 genes compared between different groups is outlined in Supplementary Table 1). For the *a priori* 271 selected genes, the expression of Eclipse relative to 3R4F stimulated DCs was nearly identical, with none of the genes demonstrating statistically significant difference in expression when compared between Eclipse and 3R4F exposed DCs (Supplementary Table 1). This analysis is further pictorially depicted in Figure 4, which demonstrates substantial changes in gene expression between the LPS stimulated and control (unstimulated) DCs, but virtually identical heat map patterns between Eclipse and 3R4F DC gene expression. The demonstration of clear differences in gene expression between the LPS stimulated and control unstimulated DCs (as expected) is an internal control showing the ability of our experimental system to detect differences in gene expression under different conditions. The lack of substantive differences in gene expression induced by Eclipse and 3R4F CSE thus is likely due to relative equivalence in cellular effects on gene expression. In a secondary analysis, we determined which genes in the Eclipse and 3R4F groups differed at a predetermined statistical significance level of $p < 0.005$. Forty two genes were identified as differentially expressed between Eclipse and 3R4F (Table 1 and Figure 5). While the expression of these 42 genes in the Eclipse and 3R4F groups reached the predetermined statistically significant level, the overall difference in expression was minimal and the fold difference in expression was <2.0 for all these genes (Table 1). Of note, none of the genes from our *a priori* list of genes relevant to regulation of immunity obtained this level of significance. Furthermore, the q -values for all these probe sets was > 0.99 suggesting that although statistically significant, these differences may have occurred due to the large number of probe-sets tested in this analysis. Analysis of the 42 probes using DAVID (Database for Annotation, Visualization and Integrated Discovery) – an open access bioinformatics tool (Dennis et al., 2003) – revealed that only one regulatory pathway (the mitogen activated protein kinase or MAPK), contained more than one probe (1454119_AT and 1450177_AT). This suggests a potentially differential effect of Eclipse CSE on DC MAPK gene expression relative to 3R4F CSE; however, the relative difference between Eclipse and 3R4F was <1.1 .

Discussion

Cigarette smoking is a major cause of morbidity and mortality worldwide (Rostron, 2012; Xu et al., 2007; Yuan et al., 1996). Unfortunately, while the health risks posed by cigarette smoking are widely recognized, it remains an unacceptably prevalent habit with more than

one billion persons estimated to be active smokers globally. In addition to being a direct burden to those who practice the habit, cigarette smoking can also cause illness in bystanders through second-hand smoking (Beyer et al., 2009; Munteanu and Mihaltan, 2014). The development of “potential reduced exposure tobacco products” or PREPs has been suggested as a potentially useful component of a tobacco-disease control strategy, meant to complement smoking cessation strategies as a means to reduce the global tobacco disease burden (Stratton et al., 2001; Warner and Martin, 2003). The goal of the current study was to compare the relative antigen presenting cell toxicity of extracts generated from proposed reduced harm Eclipse cigarettes compared to extracts generated from a “standard” reference cigarette. Our *in vitro* studies using a relatively acute model of exposure, show that extracts produced from Eclipse cigarettes do not induce any less toxicity in antigen presenting cells when compared with extracts from reference cigarettes.

The Institute of Medicine defined PREPs as potentially harm-reducing with the provision that its use may lower total tobacco-related mortality and morbidity even though use of the product would continue to expose the user to certain tobacco-related toxins (Stratton et al., 2001; Stratton K, 2001). The Institute of Medicine report also noted that basic research is critically needed to objectively and independently evaluate the capacity of PREPs for harm reduction (Stratton et al., 2001; Stratton K, 2001). In the past two decades, tobacco companies have promoted a number of PREP cigarettes and products such as Omni, Accord and Eclipse. Eclipse was chosen for this study only as a representative PREP, and with the intent of characterizing relative cellular toxicity of this selected product that was originally marketed with claims of reduced toxicity, a contention based on the premise that it primarily heats rather than burns tobacco (Slade et al., 2002). In other published studies, Eclipse smoke condensate (prepared using a different methodology than the one used in the current study) was shown to induce less mutagenicity and cytotoxicity when compared with smoke condensate from the 1R4F Kentucky reference cigarette, which was representative of typical low ‘tar’ cigarettes sold in the US in the early 2000's (Fields et al., 2005). Exposure of human bronchial epithelial cells to extracts from Eclipse cigarettes was also shown to cause significantly less induction of genes associated with cellular stress and inflammation, when compared with epithelial cells challenged with condensate generated from Kentucky 1R4F cigarettes (Fields et al., 2005). That study used a significantly different method in the preparation of CSE (Fields et al., 2005). The CSE used in that study was prepared by collecting cigarette total particulate matter onto a Cambridge filter pad and extracted with dimethylsulfoxide (Fields et al., 2005). That extraction procedure is very different from the one used in this study, as it eliminates volatile agents that may be of high relevance biologically, and potentially may be crucial mediators of cellular functions relevant to the pathogenesis of diseases that afflict smokers. The different approaches used in the preparation of extracts may partially explain why the current study failed to show any reduced cellular toxicity when extracts from Eclipse cigarettes were compared with extracts from 3R4F cigarettes. Similarly, another study showed that DNA adducts in lung, heart and skin tissues of animals treated with condensate prepared from 1R4F cigarettes were higher than those observed with animals exposed to condensate generated from Eclipse cigarettes (Brown et al., 1998). That study was also performed with condensate prepared using a

technique that potentially eliminates a number of transient but potent cigarette smoke chemicals relevant to tobacco-induced diseases.

Cigarette smoke constituents profoundly alter immune functions required for control of tumor development and spread (Barton et al., 1988; Ginns et al., 1985; Lu et al., 2007; Mian et al., 2008; Savage et al., 1991; Sopori, 2002; Tollerud et al., 1989; Vassallo et al., 2005). We and others have demonstrated that cigarette smoke exposure *in vivo* and CSE exposure *in vitro* profoundly suppress the function of DCs (Kroening et al., 2008; Nouri-Shirazi and Guinet, 2003; Nouri-Shirazi et al., 2007; Vassallo et al., 2008; Vassallo et al., 2005). Recent studies – including our own – have demonstrated that extracts prepared from mainstream cigarette smoke induce DCs oxidative stress (Kroening et al., 2008; McMaster et al., 2008), and potentially inhibit the production of interleukin-12p70 (IL-12p70), a pivotal cytokine in the orchestration of immunity to cancer and certain infections (Kroening et al., 2008; Lu et al., 2007; Vassallo et al., 2005). It is likely that the effect of smoking on antigen presenting cell function, and particularly DCs, is an important mechanism by which tobacco products induce cancer and predispose to infection (Lu et al., 2007). Among antigen presenting cells, DCs have a pre-eminent role in anti-tumor immunity (Lotze and Papamichail, 2004; Mellman and Steinman, 2001; Steinman and Banchereau, 2007). DCs produce T cell and NK cell priming cytokines like IL-12, engulf, process, and present self and foreign antigen, and possess considerable versatility in the induction of adaptive immunity relevant in host responses to tumors and infections (Lotze and Papamichail, 2004; Mellman and Steinman, 2001; Steinman and Banchereau, 2007). Due to the central role of DCs in the regulation of immunity, and the available evidence that smoking and CSE profoundly suppress DC function relevant for cancer elimination (Kroening et al., 2008; Lu et al., 2007; Vassallo et al., 2005), a primary focus of this study was to determine the relative *in vitro* toxicity of CSE from PREP cigarettes and standard cigarettes on DC functions.

The current study demonstrated no reduction in IL-12 production or oxidative stress induction by Eclipse CSE relative to reference CSE. Global gene expression analysis of inactivated as well as matured (LPS-activated) DCs showed nearly identical gene expression profiles in the presence of either Eclipse or 3R4F CSE. Through the measurement of cellular HO-1 protein levels, Eclipse extracts induced greater oxidative stress in both macrophages and DCs, relative to extracts from reference cigarettes. In the current study we demonstrate equivalence in gene expression effects and enhanced oxidative stress in cellular HO-1 analysis, and therefore refute the contention that Eclipse extracts have reduced toxicity. However, our study does not provide insight into the primary constituents in either Eclipse or 3R4F cigarette smoke responsible for suppression of IL-12 production, induction of oxidative stress or other specific DC functions. Nicotine has been implicated as a major cigarette smoke constituent involved in suppression of IL-12p70 production from murine DCs, but the concentrations of nicotine used in studies demonstrating this were appreciably greater than the ones that occur *in vivo* (Nouri-Shirazi and Guinet, 2003; Nouri-Shirazi et al., 2007). We have also observed that nicotine concentrations in the range of 50ng/ml or higher (typical serum concentrations of an active heavy smoker are in the range of 20-100ng/ml (Moyer et al., 2002)) induces significant suppression of IL-12p70 from activated DCs: however, concentrations <10ng/ml do not suppress IL-12p70 (published in reference

(Kroening et al., 2008)). Cigarette smoke and CSE are also a major source of oxidative stress, which is induced by a variety of chemicals, many of which exist in a transient state (Kroening et al., 2008; Yang et al., 2006). Among the many chemicals with the capacity of inducing oxidative stress are peroxynitrites (Yamaguchi et al., 2002), reactive oxygen species, inorganic and organic free radicals and hydrogen peroxide (Yan et al., 2005), acrolein (Risner and Martin, 1994), and other carbon and oxygen-based radicals. Rather than nicotinic stimulation, oxidative stress appears to be a more important mechanism by which smoking suppresses the generation of IL-12p70 from activated DCs (Kroening et al., 2008)). In the current study, we demonstrate no reduction in oxidative stress induction by Eclipse extracts, either in cellular oxidative stress response proteins (HO-1) or global analysis of oxidative response genes. On the contrary, increased HO-1 levels were observed in antigen presenting cells incubated with Eclipse extracts, indicating enhanced rather than suppressed oxidative potential.

The primary purpose of the current study is not argue a position in favor or against the use of reduced harm cigarettes as a means for controlling tobacco related diseases at the population level. The primary goal of the currently study was to generate data that independently tests claims made by tobacco manufacturers regarding the potential toxicities of novel products with the potential of reduced harm exposure. The use of an *in vitro* strategy as proposed herein may be considered a significant limitation. Further validation of *in vitro* findings by well-designed *in vivo* studies are indeed important to study toxicity profiles that assess the potential risks associated with whole animal exposure to the tobacco product. However, *in vitro* studies have the advantage of relative simplicity in design and reproducibility, rendering such assays useful bioassays for toxicity screening. Taken together, our findings clearly indicate that extracts generated from Eclipse cigarettes are not associated with any reduction in cellular toxicity on key immune cells involved in antigen presentation, and provide a starting point for the development of *in vitro* and other strategies by which to test further the potential immune toxicity of tobacco products with proposed reduced toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Human monocyte derived DCs (mDC) incubated with media (control), or varying concentrations of either 3R4F CSE (R-CSE) or Eclipse CSE (Ec-CSE): viability following 24 hours of incubation was determined by FACS using Annexin V (x-axis; apoptotic cells) and PI (y-axis; necrotic cells). The numbers shown in the box plots refer to the percentage of necrotic cells (upper left hand quadrant), apoptotic and necrotic cells (upper right hand quadrant), apoptotic cells (lower right hand quadrant), and viable cells (lower left-hand quadrant).

Figure 2.

A shows IL12p70 levels in supernatants of human monocyte derived DCs incubated with media (iDC or immature DC) and LPS-activated (100ng/ml; or mature DCs), in the presence of increasing concentrations (0-3%) of either 3R4F-CSE (R-CSE) or Eclipse-CSE. The suppression of IL-12p70 noted with Eclipse-CSE was significantly greater than with 3R4F-CSE (Anova, $p < 0.01$ for 1, 2 and 3% Eclipse CSE compared with 3R4F CSE). **B**) ELISA showing 12p70 levels from murine macrophages incubated with media (control) or a combination of LPS and IFN γ , in the presence of 0-3% of either 3R4F-CSE (R-CSE) or Eclipse-CSE. Comparison of the 2 groups (3R4F and Eclipse-CSE) by Anova shows no statistically significant difference ($p > 0.05$).

Figure 3.

A: Immunoblot of human DCs lysates generated from DCs incubated for 6 hours with either 2% Eclipse or 2% 3R4F CSE. **B)** Quantitative analysis of immunoblot findings in A) by densitometry (Anova, * $p=0.002$). **C)** Immunoblot of murine macrophage lysates following 6 hours of incubation with either 2% 3R4F or 2% Eclipse CSE. **D)** Densitometric analysis of the immunoblot findings in C) shows significantly higher induction of HO-1 protein by Eclipse CSE when compared with 3R4F stimulated macrophages (Anova, * $p=0.001$).

Figure 4.

Heat Map of the $\log_2$ Gene Expression for 271 probe-sets that were selected *a priori* to have genes that were significantly different than control (unstimulated cells). Gene expression is represented as darker blue for the probe-sets with lower expression to a bright yellow for the probe-sets with higher expression. This figure illustrates that in a set of genes defined *a priori*, demonstrating a contrast between the 3R4F and Eclipse and the control group, indicating that our model is adequately able to identify differential expression.

Figure 5. Heat map of the $\log_2$ Gene Expression for the 42 probe-sets that had $p < 0.005$ when comparing the 3R4F to the Eclipse cigarettes in our murine DC genomic experiment. Gene expression is represented as darker blue for the probe-sets with lower expression to a bright yellow for the probe-sets with higher expression.

Table 1

Table showing the 42 probe-sets representing genes that reached a statistically different expression level ($p < 0.005$) when comparing gene expression as fold-change of Eclipse CSE DCs relative to 3R4F challenged DCs, for all probe sets measured on the array. Despite these probe-sets having the smallest p-values when comparing the Eclipse and the 3R4F samples, the relative fold-changes are minor; with q-value's > 0.99 these significant differences are likely due to chance.

Probe Set ID	Fold Change*	Gene Title	Gene Symbol	Chromosomal Location	Entrez Gene
1457606_x_a	0.880	0.0002 1.0000 expressed sequence AU015228	AU015228	chr2 F1 2	99169
1447786_at	0.857	0.0002 1.0000 cytohesin 1	Cytl1	chr11 E2 11	19157
1442896_at	1.114	0.0009 1.0000 Heat shock protein 12A	Hspa12a	chr19 D2-D3	73442
1445228_at	1.116	0.0009 1.0000 Sprouty protein with EVH-1 domain 1, related sequence	Spred1	chr2 E5	114715
1439072_at	1.078	0.0010 1.0000 solute carrier family 1 (glial high affinity glutamate transporter), member 3	Slc1a3	chr15 A2 15 6.7 cM	20512
1445635_at	0.888	0.0010 1.0000 ---	---	---	---
1459275_at	1.108	0.0010 1.0000 ring finger protein 17	Rnf17	chr14 C3 14	30054
1425950_at	1.142	0.0013 1.0000 solute carrier family 17, member 9	Slc17a9	chr2 H4 2	228993
1421822_at	1.079	0.0016 1.0000 protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	Ppp2cb	chr8 A4 8 20.0 cM	19053
1431919_at	1.112	0.0018 1.0000 rotatin	Rtnn	chr18 E4 18	246102
1430232_at	0.907	0.0018 1.0000 RIKEN cDNA 4933407E14 gene	4933407E14Rik	chr15 15	71017
1420144_x_a	1.134	0.0019 1.0000 ring finger and CCH-type zinc finger domains 2	Rc3h2	chr2 B 2	319817
1454347_at	1.114	0.0019 1.0000 RIKEN cDNA 4930540E01 gene	4930540E01Rik	chr9 9	75174
1427721_at	1.130	0.0020 1.0000 ---	---	---	---
1437971_at	0.888	0.0021 1.0000 superkiller viralicidic activity 2-like 2 (<i>S. cerevisiae</i>)	Skiv2 2	chr13 D2.2 13	72198
1430882_at	1.062	0.0023 1.0000 ---	---	---	---
1444388_at	1.083	0.0023 1.0000 ---	---	---	---
1434121_at	0.900	0.0026 1.0000 leucine-rich repeat LGI family, member 4	Lgi4	chr7 B1 7 15.2 cM	243914
1433210_at	1.076	0.0028 1.0000 repetin	Rptn	chr3 3 F1-F2	20129
1430285_at	0.933	0.0029 1.0000 Ppp1r14c pseudogene	Gml14057	chr2 F1 2	1E+08
1427230_at	1.123	0.0029 1.0000 RIKEN cDNA B930041F14 gene	B930041F14Rik	chr4 E2 4	230991
1430728_at	1.138	0.0030 1.0000 keratin associated protein 5-5	Krtap5-5	chr7 F5 7	114666
1428536_at	1.103	0.0031 1.0000 potassium voltage-gated channel, subfamily G, member 4	Kcng4	chr8 E1 8	66733
1443040_x_a	0.895	0.0031 1.0000 Linker for activation of T cells family, member 2	Lat2	chr5 G2	56743

Probe Set ID	Fold Change*	Gene Title	Gene Symbol	Chromosomal Location	Entrez Gene
1447357_at	0.925	---	---	---	---
1446445_at	1.142	---	---	---	---
1438231_at	0.906	forkhead box P2	Foxp2	chr6(6 A2)	114142
1428986_at	1.073	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	Slc17a7	chr7 B4 7	72961
1432551_at	0.913	RIKEN cDNA 1700031F10 gene	1700031F10Rik	chr6 D1 6	73317
1446367_at	0.927	---	---	---	---
1439557_s_a	0.914	LIM domain binding 2	Ldb2	chr5 B3 5	16826
1415794_a_a	0.903	spindlin 1	Spin1	chr13 A5 13 31.0 cM	20729
1458556_at	1.090	---	---	---	---
1433558_at	1.114	disabled homolog 2 (Drosophila) interacting protein	Dab2ip	chr2 B 2	69601
1457295_at	0.925	trinucleotide repeat containing 6a	Tnrc6a	chr7 F3 7	233833
1450177_at	1.064	nerve growth factor receptor (TNFR superfamily, member 16)	Ngfr	chr11 D 11 55.6 cM	18053
1459339_at	1.073	---	---	---	---
1442690_at	1.118	transmembrane protein 143	Tmem143	chr7 B4 7	70209
1443926_at	1.076	---	---	---	---
1444777_at	0.883	Retinoic acid induced 14	Rai14	chr15 A2	75646
1454119_at	1.082	MAP kinase-interacting serine/threonine kinase 1	Mknk1	chr4 D1 4	17346
1437672_at	0.925	insulin receptor substrate 3	Ins3	chr5 G2 5	16369

* Fold-change: Eclipse CSE relative to 3R4F challenged DCs