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Regulation of gene expression by tobacco product preparations in cultured human dermal fibroblasts

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Abstract

Skin fibroblasts comprise the first barrier of defense against wounds, and tobacco products directly contact the oral cavity. Cultured human dermal fibroblasts were exposed to smokeless tobacco extract (STE), total particulate matter (TPM) from tobacco smoke, or nicotine at concentrations comparable to those found in these extracts for 1 h or 5 h. Differences were identified in pathway-specific genes between treatments and vehicle using qRT-PCR. At 1 h, *IL1α* was suppressed significantly by TPM and less significantly by STE. Neither *FOS* nor *JUN* was suppressed at 1 h by tobacco products. *IL8*, *TNFα*, *VCAM1*, and *NFκB1* were suppressed after 5 h with STE, whereas only *TNFα* and *NFκB1* were suppressed by TPM. At 1 h with TPM, secreted levels of IL10 and TNFα were increased. Potentially confounding effects of nicotine were exemplified by genes such as *ATF3* (5 h), which was increased by nicotine but suppressed by other components of STE. Within 2 h, TPM stimulated nitric oxide production, and both STE and TPM increased reactive oxygen species. The biological significance of these findings and utilization of the gene expression changes reported herein regarding effects of the tobacco product preparations on dermal fibroblasts will require additional research.

Keywords

Tobacco product preparations; Pro-inflammatory cytokines; Interleukin-8; Tumor necrosis factor alpha; Vascular cell adhesion molecule 1; Nuclear factor of kappa light polypeptide gene; enhancer in B-cells 1

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Introduction

Cigarette smoke exerts both systemic and direct effects at local areas of exposure including the oral cavity (Sopori, 2002; van der Vaart et al., 2004). Exposure to cigarette smoke or its constituent phases alters cytokine expression in various cell types, e.g. human aortic endothelial cells, THP-1 monocyte macrophages (Nordskog et al., 2005), and normal human bronchial epithelial cells (NHBEs) (Fields et al., 2005). The particulate phase of cigarette smoke, total particulate matter (TPM), also commonly known as cigarette smoke condensate (CSC), uniquely influences the expression of cytokines depending on cell type (Nordskog et al., 2005).

Acute exposure to cigarette smoke or its constituent phases can cause oxidative stress, inflammation, and cytotoxicity. Exposure to TPM induces: the cytochrome P450 family of genes in normal epidermal keratinocytes, oral dysplasia cells, and primary oral carcinoma cells (Nagaraj et al., 2006); the hemoxygenase gene in human alveolar epithelial cells (Fukano et al., 2006); cytokines including interleukin-8 (*IL8*) in primary human peripheral blood mononuclear cells, human lymphoblasts (HL60) (Arimilli et al., 2012), and NHBEs (Parsanejad et al., 2008); and caspases in THP-1 monocytes (Arimilli et al., 2012), oral squamous cell carcinoma cells, and normal human gingival epithelial cells (Gao et al., 2013). Gene expression/transcriptomic changes of cigarette smoking have been proposed as biomarkers of lung cancer, chronic obstructive pulmonary disease, and other smoking-related diseases (Gower et al., 2011).

Smokeless tobacco (ST) products comprise a large and diverse category of tobacco products consumed worldwide. The European and US studies indicate that ST consumption, relative to smoking, is associated with significantly reduced risk of serious diseases including lung cancer, oral cancer, and chronic obstructive pulmonary disease (Lee and Hamling, 2009). However, information on cellular responses following exposure to ST preparations is relatively limited, and the cellular effects appear to depend on how the ST extracts are prepared. Recent reports (Arimilli et al., 2012; Gao et al., 2013) show that combustible tobacco product preparations are far more cytotoxic than ST preparations. Information on the comparative effects of ST and combustible tobacco (e.g., TPM) preparations on gene expression is also limited and would be valuable in understanding biological and pathophysiological effects of different tobacco product categories.

TPM upregulates gene and protein expression of pro-inflammatory cytokines including *IL1 α* , *IL1 β* , *IL6*, and *IL8* in human fibroblast-like synoviocyte cells (Shizu et al., 2008) and initiates an inflammatory response in vocal fold fibroblasts (Branski et al., 2011). This study examined immediate early gene expression changes, secreted cytokine levels, and oxidative stress in actively growing normal adult human dermal fibroblasts (HDFa) in culture as a model for comparing the relative effects of smokeless tobacco extract (STE), TPM from reference cigarettes, and nicotine at non-cytotoxic levels.

Methods

Materials

Reagents and cell culture materials were purchased from the following sources: normal adult human dermal fibroblasts, fibroblast basal media (FBM), fibroblast supplemental growth factors (hydrocortisone hemisuccinate, HLL supplements [human serum albumin, linoleic acid, and lecithin], rh FGF β , rh EGF/TGF, β -1 supplement, rh insulin, and ascorbic acid), and 0.1% gelatin solution, American Type Culture Collection (ATCC; Manassas, VA); fetal bovine serum, Atlanta Biologicals, Inc. (Lawrenceville, GA); GlutaMAX I and TRIzol, Life Technologies (Grand Island, NY); Dulbecco's Phosphate Buffered Saline (DPBS), Lonza (Walkersville, MD); penicillin (100 units·mL⁻¹) and streptomycin (100 μ g·mL⁻¹) solution (Pen–Strep), phenol red, and dimethyl sulfoxide (DMSO), Sigma-Aldrich (St. Louis, MO); β -mercaptoethanol, Calbiochem, (La Jolla, CA); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Thermo Fisher Scientific (Waltham, MA); and DAF-FM diacetate, Life Technologies (Grand Island, NY). A 5 mM stock solution of DAF-FM diacetate was prepared by diluting DAF-FM diacetate with GMP Biotechnology Performance Certified anhydrous DMSO.

TPM, STE, and CAS were prepared by Labstat International, Kitchener, Ontario, Canada (Arimilli et al., 2012; Gao et al., 2013). CAS was prepared using a protocol based on the work of Chou and Hee (1994). Components in CAS were: mucin (1.35 g/l), potassium chloride (475 mg/l), sodium chloride (700 mg/l), calcium chloride dehydrate (185 mg/l), di-potassium hydrogen phosphate (420 mg/l), magnesium chloride hexahydrate (105 mg/l), urea (45 mg/l), D-(+)-glucose (100 mg/l), α -amylase (100,000 U/l), lysozyme (750 U/l), and acid phosphatase (4 U/l). STE was prepared by extracting 2.5 g of 2S3 smokeless tobacco reference product (North Carolina State University Tobacco Services Analytical Laboratory) in 25 ml CAS for 2 h followed by filtration to produce a 10% STE stock solution. CAS served as the solvent control for all experiments using STE. TPM was prepared by smoking 3R4F reference cigarettes (University of Kentucky), collecting the particulate phase on glass fiber, and dissolving the particulate in DMSO. For experiments using TPM, DMSO served as the solvent control. Chemical analyses for nicotine and tobacco specific nitrosamine contents were performed by Labstat.

Kits for assays were purchased from the following sources: Qiashredder columns, Qiagen RNeasy mini kits, RT² Profiler PCR Arrays for Human Transcription Factors and for Human Signal Transduction PathwayFinder, RT² First Strand Kits, RT² SYBR Green qPCR Mastermix, and RNase-Free DNase Sets, Qiagen Inc. (Valencia, CA); high capacity cDNA reverse transcription kits and TaqMan Expression Assays, Applied Biosystems (Foster City, CA); FastStart Universal Probe Master (Rox), Roche (Indianapolis, IN); Cytometric Bead Array (CBA) human inflammatory cytokine kits, BD Biosciences (San Jose, CA); and total reactive oxygen and nitrogen species (RO/NS) and superoxide detection kits, Enzo Life Sciences Inc. (Farmingdale, NY).

Cell culture and treatments

HDFa were maintained in serum-complete media comprised of FBM supplemented with 10% FBS, GlutaMAX I (2 mM), Pen–Strep (1:100 dilution), and phenol red (2 μ M) in a humidified 5% CO₂ incubator at 37 °C. HDFa were plated at a density of 4×10^5 cells in 60 mm tissue culture dishes in serum-complete media, such that cells were 50% to 80% confluent and actively proliferating at the time of each experiment. After 20 to 24 h, serum-complete media were removed, cells were rinsed twice with DPBS, and media were changed to serum-free defined media comprised of (1) FBM, GlutaMAX I, Pen–Strep, phenol red, and fibroblast supplemental growth factors (hydrocortisone hemisuccinate [1 μ g/ml], human serum albumin [500 μ g/ml], linoleic acid [0.6 μ M], lecithin [0.6 μ g/ml], rh FGF β [5 ng/ml], rh EGF/TGF [5 ng/ml], β -1 supplement [30 pg/ml], rh-insulin [5 μ g/ml], and ascorbic acid [50 μ g/ml]), or (2) FBM, GlutaMAX I, Pen–Strep, and phenol red. Appropriate test article or vehicle was added, and HDFa cells were exposed to these products at 37 °C and 5% CO₂ for 1 h or 5 h. At the designated time following treatment, cells were collected for RNA isolation, or media were collected for the determination of cytokine release.

For treatments, 1% STE was prepared by diluting 10% STE in CAS to yield a solution with 141.6 μ g/ml nicotine content, a concentration within the range of salivary levels (70 to 1560 μ g/ml) in ST users (Petro, 2003). Nicotine hydrogen tartrate salt (NIC) was dissolved in CAS to yield a solution with a nicotine concentration of 141.6 μ g/ml (NIC/CAS). Equivalent volumes of CAS, STE/CAS, and NIC/CAS were administered to cells. TPM was administered at a nicotine concentration of 4 μ g/ml, a concentration found to be non-cytotoxic for time periods $\geq$ 24 h (Gao et al., 2013) and within the range of salivary levels (0.9 to 4.6 μ g/ml) in smokers (Robson et al., 2010). NIC was dissolved in DMSO to yield a solution with a nicotine concentration of 4 μ g/ml (NIC/DMSO). Equivalent volumes of DMSO, TPM, and NIC/DMSO were administered to cells.

RNA isolation and cDNA preparation

The cell monolayer was rinsed twice with DPBS and dislodged with TRIzol containing 1% β -mercaptoethanol. Cells were transferred to Qiashredder columns and disrupted by sedimentation at 14,000 rpm for 2 min. Supernatant was collected and RNA was isolated using the Qiagen RNeasy mini kit. Purity and quantity of RNA were measured on the NanoDrop 2000 (Thermo Fisher Scientific Inc., Wilmington, DE). For RT² Profiler PCR Arrays, RNA isolation included an on-column DNase digestion using an RNase-Free DNase Set (Qiagen) and total RNA was converted into first strand cDNA while genomic DNA was also removed from the RNA using an RT² First Strand Kit comprised of genomic DNA elimination buffer, RT buffer, primer and external control mix, RT enzyme mix, and RNase-free H₂O. For follow-up experiments, total RNA was converted to single-stranded cDNA using a high capacity cDNA reverse transcription kit comprised of RT buffer, RT random primers, dNTP mix, reverse transcriptase, and RNase-free H₂O.

Gene expression arrays

For array detection of gene expression differences between groups, qRT-PCR analyses were performed using RT² Profiler Arrays and RT² SYBR Green qPCR Mastermix. Two sets of arrays were performed: (1) Human Signal Transduction PathwayFinder; and (2) Human

Transcription Factors. All data were normalized to the average of three housekeeping genes: 60s ribosomal protein L13a (*RPL13A*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and beta-actin (*ACTB*). Fold changes are reported relative to vehicle control. All data were analyzed using the RT² Profiler PCR Array Data Analysis Web Portal (Qiagen) for converting cycle time to fold change.

For follow-up experiments, qRT-PCR analyses were performed using TaqMan Expression Assays and FastStart Universal Probe Master (Rox). Target genes included: *FOS*, *IL1 α* , *JUN*, *IL8*, *TNF α* , *VCAM1*, *NF κ B1*, and *ATF3*. Three to five independent experiments with two technical replicates for each were performed. All data were normalized to the average of three housekeeping genes: *RPL13A*, *GAPDH*, and *ACTB*. Fold changes are reported relative to basal control (at time $t = 0$ h). Comparisons between treated and vehicle values were analyzed using REST 2009 (Relative Expression Software Tool V2.0.13, Qiagen, Inc., Valencia, CA). This software tool compares a sample group and control group, and tests the group differences for significance by applying a Pair Wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002). Based on the recommendations of Pfaffl et al. (2002), 2000 randomizations were performed for each analysis.

Cytometric Bead Array assays

Pro-inflammatory cytokine release in HDFa was determined by using Cytometric Bead Array (CBA) assays for human cytokine inflammatory responses. We measured cytokines by immunoadsorption to solid phase antibodies specific for IL1 β , IL6, IL8, IL10, IL12p70, and TNF α (BD Biosciences). Three to four independent experiments were performed for each treatment. All data were analyzed using FlowJo (Tree Star Inc., Ashland, OR) software. Statistical analysis (one-way ANOVA followed by Dunnett's Multiple Comparison Test) was performed using Prism 6.00 software (GraphPad Software, Inc., San Diego, CA).

Nitric oxide and total reactive oxygen species/oxidative assays

Preparation of cells for nitric oxide and oxidative stress/superoxide assays—

HDFa were plated at a density of 5000 cells per well in 96-well black wall, clear bottom plates in serum-complete media, such that cells were approximately 70% confluent and actively proliferating at the time of each experiment. Wells that contained media only (no cells) were designated as reagent controls. After 20 to 24 h, serum-complete media were removed, cells were rinsed twice with DPBS, and media were changed to serum-free assay media comprised of FBM, HEPES (10 mM), GlutaMAX I (2 mM), and Pen-Strep.

Nitric oxide assays—DAF-FM diacetate solution (5 mM) was diluted in HEPES-containing serum-free assay media to yield a 5 μ M working solution to be used for the detection of nitric oxide. Detection solution was added to wells containing cells and wells to be used as reagent controls, and the plate was covered and allowed to incubate for 1 h at 37 °C. A single test article (CAS, STE, NIC/CAS, DMSO, TPM, or NIC/DMSO) was then added to duplicate wells containing cells and duplicate reagent controls. The plate remained uncovered and was immediately read in the FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments Inc., Winooski, VT) at 37 °C using a bottom reading and a fluorescein filter set (Excitation = 485/20, Emission = 528/20). Fluorescence was read and recorded in one-

min increments up to 40 min and in 5-min increments from 45 min to 60 min using Gen5 2.00 software (Bio-Tek). Fluorescence was calculated by subtracting average readings for duplicate reagent blanks (wells with no cells) from average readings for duplicate wells containing cells. Data from three individual experiments were presented. The difference between fluorescence at 10 min and 35 min for each treatment group was calculated and reported as accumulated fluorophores (arbitrary units) per min. Statistical analysis (one-tailed unpaired t-test) was performed using Prism 6.00 software (GraphPad).

Oxidative stress/superoxide assays—An RO/NS detection kit (Enzo) was used to determine effects of tobacco products on oxidative stress and superoxide in HDFa. Reagents in this kit detect either a wide range of reactive species including hydrogen peroxide, peroxynitrite, hydroxyl radicals, nitric oxide, and peroxy radicals, or fluorescence specific for superoxide. After the media change, the plate was allowed to incubate at 37 °C for 30 or 90 min. Media were removed and detection solution was added to each well. Following a 30-min incubation at 37 °C, duplicate wells containing cells and duplicate reagent controls were treated with a single test article (CAS, STE, NIC/CAS, DMSO, TPM, or NIC/DMSO) and allowed to incubate at 37 °C for 30 min. The plate was uncovered and immediately read in the FLx800 Microplate Fluorescence Reader (Bio-Tek) at 37 °C using a bottom reading. A fluorescein filter set (Excitation = 485/20, Emission = 528/20) was used to detect oxidative species while a rhodamine filter set (Excitation = 540/25, Emission = 620/40) was used to detect superoxide. A fluorescence endpoint for each well was read and recorded using Gen5 2.00 software (Bio-Tek). Relative fluorescence units were calculated by subtracting average readings for duplicate reagent blanks (wells with no cells) from average readings for duplicate wells containing cells. Data from three individual experiments were presented. Statistical analysis (one-tailed unpaired t-test) was performed using Prism 6.00 software (GraphPad).

Graphical data—Data graphs were created using GraphPad Prism 6.00 for Windows (GraphPad).

Results

Genes of interest

Fig. 1 presents the results of a gene expression array analysis as a correlation between STE and complete artificial saliva (CAS) vehicle exposure, and between TPM and DMSO vehicle exposure for 1 h and 5 h for the expression of genes targeting signal transduction pathway markers and transcription factors. Complete lists of genes screened in these arrays can be found at the manufacturer's website (<http://www.sabiosciences.com/ArrayList.php>). Based on the deviation between STE versus CAS vehicle, and reports relating groups of genes in cell signaling, genes were selected for follow-up as potential biomarkers for the effects of STE. Particular attention was paid to determining differences between the effects of STE versus TPM or nicotine.

Suppression of gene expression by STE and TPM

A change of media to defined media containing supplemental growth factors and CAS vehicle significantly increased the expression of *IL8*, *TNF α* , *VCAM1*, and *NF κ B1* after 5 h (Fig. 2A). This effect was blunted or not observed in cells treated with defined media containing the DMSO vehicle, reflecting a difference from the effect of components of the CAS vehicle (Malpass et al., 2013). STE significantly decreased the *IL8* expression (relative to vehicle control), while TPM significantly increased the response after 5 h in culture, and these effects were not due to nicotine. Both STE and TPM significantly decreased the *TNF α* expression, and these responses were not due to nicotine. STE significantly decreased the *VCAM1* expression after 5 h in culture, while TPM elicited no response. STE and TPM both reduced *NF κ B1* expression at a level that did not reach statistical significance (STE: $P = 0.073$). When considered in combination, STE was able to robustly reduce expression of this tetrad of genes compared with CAS vehicle, and these responses were not the result of nicotine exposure at the concentrations found in this extract. The pattern of suppression of this tetrad of genes was distinctive for STE, as it was not mimicked by exposure to TPM. These four proteins have functional interactions with each other (Ashkenazi, 2002; Gao and Issekutz, 1996; Larsen et al., 1989).

We also examined the effects of nicotine since it is a component of tobacco products and could regulate cellular functions (Arredondo et al., 2003; Fan et al., 2011). An example of a nicotine responsive gene identified during these studies was *ATF3*. The concentration of nicotine found in this STE (141.6 μ g/ml) increased *ATF3* expression relative to vehicle (Fig. 2B). Interestingly, the effect of STE on *ATF3* expression was not significantly different from vehicle. This observation suggests that components of the STE were able to attenuate the response to nicotine within this extract. Unlike the higher concentration of nicotine (141.6 μ g/ml) found in STE, the lower concentration of nicotine (4 μ g/ml) found in the TPM significantly decreased *ATF3* expression relative to vehicle. TPM did not attenuate this effect.

The level of *IL1 α* mRNA was not altered significantly by a change in media containing supplemental growth factors after 1 h (Fig. 3A). TPM significantly decreased *IL1 α* mRNA levels, and this was not due to nicotine. STE elicited a similar response, but this did not reach statistical significance ($P = 0.055$).

Fibroblast exposure to perturbations, including growth factors (Gilley et al., 2009), mechanical forces (Kook et al., 2009), and oxidative stress from cigarette smoke extracts (Bagloli et al., 2008), results in changes in the expression of the immediate early gene c-*FOS* or c-*JUN*. At 1 h following the change of media to defined media containing supplemental growth factors and vehicle, there was a 25- to 35-fold increase in the expression of *FOS*, and 1- to 2-fold increase of *JUN* over basal (Fig. 3B). STE failed to alter the increase in *FOS* expression. TPM significantly augmented *FOS* expression, and this was not due to nicotine. Phosphorylation of JUN proteins controls the expression of cytokines, such as granulocyte colony-stimulating factor, IL6, and *TNF α* , through transcriptional and posttranscriptional pathways (Wagner, 2010). In our study, neither STE nor TPM altered the expression of *JUN* after 1 h exposure.

Cytokine release

TPM significantly increased the release of IL10 and TNF α after 1 h in comparison to DMSO vehicle (Table 1). TPM did not alter the release of these cytokines after 5 h and did not produce an effect on the release of IL1 β , IL6, IL8, or IL12p70 after 1 h or 5 h. Nicotine (4 μ g/ml and 141.6 μ g/ml) and STE did not alter the release of IL1 β , IL6, IL8, IL10, IL12p70, or TNF α after 1 or 5 h.

Nitric oxide accumulation

A study by Arnold et al. (1977) suggested that nitric oxide generated from cigarette smoke could activate guanylate cyclase and increase tissue levels of cyclic guanosine 3',5'-monophosphate (cyclic GMP). Our findings indicating that STE and TPM alter the expression of certain pro-inflammatory cytokines led us to hypothesize that STE or TPM generates nitric oxide, which in turn, could induce changes in gene expression. We found that TPM significantly increased the rate of nitric oxide production between 10 and 35 min relative to vehicle (DMSO) (Fig. 4) while nicotine (4 μ g/ml) produced no effect. STE and nicotine (141.6 μ g/ml) did not alter the generation of nitric oxide relative to vehicle (CAS).

Oxidative stress and superoxide generation

Oxidative stress and inflammatory cytokine production can be regulated in a complex fashion. RO/NS can act as mediators in the regulation of the redox balance, which is relevant to our study since certain transcription factors such as NF κ B are responsive to oxidants (Han et al., 2009). We found that both STE and TPM increased the generation of oxygen species relative to vehicle (CAS and DMSO, respectively) and these effects were not due to nicotine (Fig. 5). STE did not increase the generation of superoxide to a statistically significant extent, whereas nicotine (141.6 μ g/ml) alone marginally but significantly increased levels of superoxide (Fig. 6). TPM and nicotine (4 μ g/ml) did not alter the generation of superoxide relative to vehicle (DMSO).

Discussion

The main purpose of this study is to comparatively evaluate the effects of short-term exposure to STE and TPM on gene expression in a standardized active fibroblast cell culture model. When sub-confluent, cultured fibroblasts proliferate and tend to arrange in a monolayer of elongated spindle-shaped cells. As fibroblasts reach confluence, they undergo contact inhibition, a directional inhibition of movement and proliferation that occurs when one fibroblast contacts another (Abercrombie, 1970). It has been shown that gene expression in cells may differ between proliferating versus confluent cultures (Kuppers et al., 2010). Sorrell and Caplan (2004) suggest that studies using monolayers of fibroblasts more closely reflect the status of these cells in an "early wound repair situation". We sought biomarkers of tobacco product exposure using cells that had not become contact inhibited in order to more closely mimic the behavior of active dermal fibroblasts in tissue.

Proliferation and differentiation of skin fibroblasts are regulated by various growth factors that are expected to be found in serum (Takehara, 2000). The use of defined serum-free media containing supplemental growth factors known to enhance fibroblast survival

standardizes the media formulation. This offers the advantages that conditions of the experiments can be more easily replicated and the media are selective for the particular cell type (Freshney, 2010). In our study, we followed a protocol in which culture media were replaced at the initiation of the assay with a defined serum-free media containing growth factors. For the determination of gene expression changes, cells were exposed to vehicle, tobacco products, or nicotine at the time of this media change. Basal control levels were determined in cells isolated at time $t = 0$ h from media without supplemental growth factors.

The concentration of STE administered to cells was based on previous studies in which 1% STE was added to cultured cells (Bernzweig et al., 1998; Payne et al., 1994) and reports which indicate that salivary nicotine concentrations in ST users range from 70 to 1560 $\mu\text{g/ml}$ (Petro, 2003; Petro et al., 2002). The low concentration of TPM used in our study was based on previous reports which indicate that nicotine concentrations in unstimulated saliva of smokers range from 0.9 to 4.6 $\mu\text{g/ml}$ (Robson et al., 2010), and data demonstrating that higher concentrations of TPM may be cytotoxic (Gao et al., 2013).

STE used for in vitro testing is typically extracted from ground tobacco with water, cell culture media, buffer, or artificial saliva. Cigarette smoke is collected for analysis by various methods including accumulation on a filter pad, in a cold trap, or as whole smoke. CSC is prepared by collecting smoke fractions as a condensate, typically in a cold trap, but sometimes in impaction traps, and by electrostatic precipitation (Johnson et al., 2009). TPM, sometimes referred to as CSC in the literature (Johnson et al., 2009), is collected from the mainstream smoke from cigarettes onto Cambridge filter pads and extracted with DMSO (Bombick et al., 1998; McKarns et al., 2000). In our study, vehicles were representative of the extraction methods with STE in artificial saliva and TPM in DMSO. Nicotine was used at the same concentration as the test article and was delivered in the appropriate vehicle.

Our investigation identified a pattern of immediate early gene expression changes in fibroblast cultures treated with STE, i.e. decreased expression of *TNF α* , *IL8*, and *VCAM1*. Although a decline in *TNF α* is observed with TPM, the response is different for *IL8* which was increased by TPM; the expression of *VCAM1*, however, was not influenced by TPM. *NF κ B1* was reduced in cultures treated with either STE or TPM, but did not reach a level of statistical significance. *IL1 α* expression was suppressed significantly by TPM and was decreased by STE.

The results of this study demonstrate that STE modulates select early inflammatory responses in our model system (Fig. 7). Fibroblasts can secrete IL8 both constitutively and in response to pro-inflammatory stimuli. Elevated levels of IL8 in in vitro studies negatively affect the morphology and function of fibroblasts (Iocono et al., 2000). It has been observed that α -amylase is responsible for a CAS-induced increase in genes for certain pro-inflammatory cytokines including *IL8* and *VCAM1* in HDFa (Malpass et al., 2013). Our data indicate that STE inhibits or reverses this stimulation of *IL8* expression, suggesting that STE may reverse some of the effects of salivary pro-inflammatory mediators. Our data also suggest that STE may have less negative impact on the morphology and function of fibroblasts than TPM which increased *IL8* expression.

TNF α mediates inflammation by activating leukocytes, enhancing adherence of neutrophils and monocytes to endothelium, promoting migration of inflammatory cells into the intercellular matrix, stimulating fibroblast proliferation, and triggering local production of other proinflammatory cytokines (Tracey and Cerami, 1994). TNF α binds to TNF α receptor 1 (TNFR1), triggering signaling cascades that activate NF κ B transcription factors (Chen and Goeddel, 2002), which suggests that STE and TPM may block or diminish the activation of NF κ B by inhibiting the expression of TNF α . Also, because the primary signaling function of TNF α at the cellular level is to induce transcription of proinflammatory cytokines including IL1, IL6, and IL8 and leukocyte adhesion molecules (Ashkenazi, 2002), STE-induced reductions in TNF α may lead to the observed decrease in expression of pro-inflammatory cytokines.

In normal cultured human skin, either IL1 α or TNF α can induce mRNA for IL8 in dermal fibroblasts, whereas IL1 α , but not TNF α , can induce mRNA for IL8 in keratinocytes (Larsen et al., 1989). Our finding of reduced expression of IL1 α and TNF α in HDFa exposed to tobacco products may have indirectly blunted the expression of IL8 as a result of the decreased expression of IL1 α and TNF α . The effects of STE and TPM on the release of IL8 and TNF α into the culture media do not mimic the effects of these tobacco products on gene expression for IL8 and TNF α . Perhaps the released protein levels did not change because protein synthesis and release are dependent upon additional factors beyond mRNA expression and stability. These other factors might include ribosomal regulation, appropriate folding and processing of the protein, and regulation of release. Furthermore, the net level of protein represents an equilibrium based upon rates of degradation as well as synthesis.

In dermal fibroblasts, VCAM1 mRNA is expressed constitutively in only trace amounts, but is rapidly up-regulated by IL1 α and TNF α at the mRNA, protein, and functional levels (Gao and Issekutz, 1996). Our results indicate that the STE-mediated decrease in VCAM1 expression may be due, at least partly, to reduced IL1 α and TNF α expression.

The pattern of decreased IL8, TNF α , and VCAM1 expression is not the result of exposure to nicotine at the same concentration and in the same vehicle as STE. Unlike the other genes we investigated, ATF3 appears to represent a gene that is altered by the concentration of nicotine present in STE. Our data demonstrate that nicotine at high doses (141.6 μ g/ml) induced an increase in ATF3 expression detectable after 5 h of exposure. This suggests that nicotine may alter the regulation of other genes and cellular processes, e.g. proliferation and differentiation (Albuquerque et al., 2009; Fujii et al., 2008). ATF3 may directly or indirectly regulate dimerization partners for ATF3 (AP-1 and CREB/ATF family proteins) and additional downstream targets (Allan et al., 2001). The AP-1 transcription factor composed mainly of Jun, Fos, and ATF protein dimers, mediates the regulation of gene expression in response to many physiological and pathological stimuli, e.g. cytokines, growth factors, stress signals, bacterial and viral infections, and oncogenic stimuli, and mediates the regulation of processes including proliferation, differentiation, apoptosis, and transformation (Hess et al., 2004). Stimulation of tissue culture cells with serum and whole organisms with physiological stresses greatly increases the level of ATF3 mRNA within 2 h (Liang et al., 1996). Our results indicate that nicotine appears to exert different effects on ATF3, depending on the dose, and further work is necessary to delineate those effects.

To investigate the mechanism by which STE and TPM alter the expression of pro-inflammatory cytokines, we tested the possibility that changes in gene expression might be due to nitric oxide or RO/NS/superoxide generation. Daghigh et al. (2002) reported that human gingival fibroblasts exhibit increased expression of inducible nitric oxide synthase (iNOS) and modulate nitric oxide synthesis in response to pro-inflammatory cytokines such as TNF α , IL1 β , and interferon gamma (IFN γ). We propose that our observation of an early increased release of TNF α produced by TPM is associated with the early increased production of nitric oxide observed in HDFa treated with TPM. Unlike TPM, STE does not alter the early production of nitric oxide in our model system. This suggests that changes in gene expression induced by STE may be due to mechanisms not involving nitric oxide.

We observed an increase in RO/NS in HDFa produced by STE and TPM, and these effects were not due to nicotine. The early increase in RO/NS in the TPM-treated HDFa may be related to the increased release of IL10 and TNF α into the culture media observed after 5 h. However, the lack of superoxide changes in response to STE treatment suggests that the changes in gene expression induced by STE are due to a mechanism other than superoxide generation. We note that our results using a model system of dermal fibroblasts alone in culture are likely to differ from other model systems in which fibroblasts co-exist with other cell types such as keratinocytes or in vivo situations in which fibroblasts are subjected to different environments.

We have utilized a cell culture model that allowed us to distinguish between gene expression changes elicited from serum and exposure to STE and TPM (test articles). Skin is the first source of immunological defense in response to a wound or abrasions, and fibroblasts enter this damaged skin in response to the factors that are found in the defined media containing fibroblast basal media and fibroblast supplemental growth factors obtained from ATCC. Smokeless tobacco products will come into contact with skin from oropharyngeal tissue, as well as face and hands. We selected a line of human skin fibroblasts easily obtained from ATCC which is a widely used resource. Cultured HDFa were grown within a limited number of passages. Non-confluent HDFa were used so that the cells could respond to environmental cues in the culture media, mimicking conditions in which fibroblasts would be activated to migrate into a wound for surface coverage and repair. Culture conditions were standardized with serum-free culture media being defined to include the basal fibroblast media and supplemental growth factors recommended for use in these cultures (American Type Culture Collection, 2013). These steps minimized variability in cell proliferation and matrix protein formation which would be likely to mediate cell surface alteration and immunoregulation (Takehara, 2000). Our results indicate that a limited number of genes associated with transcription and signal transduction regulation are altered as a result of supplemental growth factors in the culture media. Therefore, it was necessary to identify those changes in gene expression due to defined media containing growth factors that could also be influenced by the tobacco product preparations. Here, we have quantitated short term (1 h) and intermediate (5 h) changes in mRNA levels by comparing the response to the tobacco product preparations with the response to the defined media containing the appropriate dilution of the vehicle. Thus, it is important to note that the responses elicited by the tobacco product preparations are dynamic and further studies are necessary to

understand the biological significance of these gene expression changes detected through this pathway-specific profiling approach.

Overall, our results indicate that STE and TPM alter the expression of some critical immediate early genes involved in the inflammatory response in adult human dermal fibroblasts. The response to these two stimuli can be distinguished in this cell culture system based on the divergent expression of a select set of genes. These findings suggest that changes in the expression of certain pro-inflammatory cytokines and related genes in HDFa can be used in the investigation of cellular responses to the exposure of different tobacco products.

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References

- Abercrombie M. Contact inhibition in tissue culture. *In Vitro*. 1970; 6:128–142. [PubMed: 4943054]
- Albuquerque EX, Pereira EF, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiol. Rev.* 2009; 89:73–120. [PubMed: 19126755]
- Allan AL, Albanese C, Pestell RG, LaMarre J. Activating transcription factor 3 induces DNA synthesis and expression of cyclin D1 in hepatocytes. *J. Biol. Chem.* 2001; 276:27272–27280. [PubMed: 11375399]
- American Type Culture Collection. [Accessed April 3, 2013] Dermal fibroblasts; normal, human, adult (ATCC® PCS-201-012TM); product sheet PCS-201-012[1].pdf [online]. 2013. <http://www.atcc.org/Products/All/PCS-201-012#10C5F80529A547D6AA8B08636E2E2F44>
- Arimilli S, Damratoski BE, Bombick B, Borgerding MF, Prasad GL. Evaluation of cytotoxicity of different tobacco product preparations. *Regul. Toxicol. Pharmacol.* 2012; 64:350–360. [PubMed: 22996032]
- Arnold WP, Aldred R, Murad F. Cigarette smoke activates guanylate cyclase and increases guanosine 3',5'-monophosphate in tissues. *Science (New York, N.Y.)*. 1977; 198:934–936.
- Arredondo J, Hall LL, Ndoye A, Nguyen VT, Chernyavsky AI, Bercovich D, Orr-Urtreger A, Beaudet AL, Grando SA. Central role of fibroblast alpha3 nicotinic acetylcholine receptor in mediating cutaneous effects of nicotine. *Lab. Invest.* 2003; 83:207–225. [PubMed: 12594236]
- Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat. Rev. Cancer.* 2002; 2:420–430. [PubMed: 12189384]
- Baglolle CJ, Sime PJ, Phipps RP. Cigarette smoke-induced expression of heme oxygenase-1 in human lung fibroblasts is regulated by intracellular glutathione. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2008; 295:L624–L636. [PubMed: 18689604]
- Bernzweig E, Payne JB, Reinhardt RA, Dyer JK, Patil KD. Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. *J. Clin. Periodontol.* 1998; 25:246–252. [PubMed: 9543195]
- Bombick BR, Murli H, Avalos JT, Bombick DW, Morgan WT, Putnam KP, Doolittle DJ. Chemical and biological studies of a new cigarette that primarily heats tobacco. Part 2. In vitro toxicology of mainstream smoke condensate. *Food Chem. Toxicol.* 1998; 36:183–190. [PubMed: 9609391]

- Branski RC, Zhou H, Kraus DH, Sivasankar M. The effects of cigarette smoke condensate on vocal fold transepithelial resistance and inflammatory signaling in vocal fold fibroblasts. *Laryngoscope*. 2011; 121:601–605. [PubMed: 21298639]
- Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science (New York, N.Y.)*. 2002; 296:1634–1635.
- Chou CC, Hee SSQ. Bioassay-driven analysis of chewing tobacco extracts. *Environ. Toxicol. Chem*. 1994; 13:1177–1186.
- Daghigh F, Borghaei RC, Thornton RD, Bee JH. Human gingival fibroblasts produce nitric oxide in response to proinflammatory cytokines. *J. Periodontol*. 2002; 73:392–400. [PubMed: 11990440]
- Fan YY, Yu TS, Wang T, Liu WW, Zhao R, Zhang ST, Ma WX, Zheng JL, Guan DW. Nicotinic acetylcholine receptor alpha7 subunit is time-dependently expressed in distinct cell types during skin wound healing in mice. *Histochem. Cell Biol*. 2011; 135:375–387. [PubMed: 21437621]
- Fields WR, Leonard RM, Odom PS, Nordskog BK, Ogden MW, Doolittle DJ. Gene expression in normal human bronchial epithelial (NHBE) cells following in vitro exposure to cigarette smoke condensate. *Toxicol. Sci*. 2005; 86:84–91. [PubMed: 15858226]
- Freshney, RI. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. 6th ed. John Wiley & Sons, Inc.; Hoboken, New Jersey: 2010.
- Fujii T, Takada-Takatori Y, Kawashima K. Basic and clinical aspects of nonneuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. *J. Pharmacol. Sci*. 2008; 106:186–192. [PubMed: 18285654]
- Fukano Y, Oishi M, Chibana F, Numazawa S, Yoshida T. Analysis of the expression of heme oxygenase-1 gene in human alveolar epithelial cells exposed to cigarette smoke condensate. *J. Toxicol. Sci*. 2006; 31:99–109. [PubMed: 16772700]
- Gao JX, Issekutz AC. Expression of VCAM-1 and VLA-4 dependent T-lymphocyte adhesion to dermal fibroblasts stimulated with proinflammatory cytokines. *Immunology*. 1996; 89:375–383. [PubMed: 8958050]
- Gao H, Prasad GL, Zacharias W. Differential cell-specific cytotoxic responses of oral cavity cells to tobacco preparations. *Toxicol. In Vitro*. 2013; 27:282–291. [PubMed: 22960471]
- Gilley R, March HN, Cook SJ. ERK1/2, but not ERK5, is necessary and sufficient for phosphorylation and activation of c-Fos. *Cell. Signal*. 2009; 21:969–977. [PubMed: 19249353]
- Gower AC, Steiling K, Brothers JF II, Lenburg ME, Spira A. Transcriptomic studies of the airway field of injury associated with smoking-related lung disease. *Proc. Am. Thorac. Soc*. 2011; 8:173–179. [PubMed: 21543797]
- Han D, Ybanez MD, Ahmadi S, Yeh K, Kaplowitz N. Redox regulation of tumor necrosis factor signaling. *Antioxid. Redox Signal*. 2009; 11:2245–2263. [PubMed: 19361274]
- Hess J, Angel P, Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. *J. Cell Sci*. 2004; 117:5965–5973. [PubMed: 15564374]
- Iocono JA, Collieran KR, Remick DG, Gillespie BW, Ehrlich HP, Garner WL. Interleukin-8 levels and activity in delayed-healing human thermal wounds. *Wound Repair Regen*. 2000; 8:216–225. [PubMed: 10886812]
- Johnson MD, Schilz J, Djordjevic MV, Rice JR, Shields PG. Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco. *Cancer Epidemiol. Biomarkers Prev*. 2009; 18:3263–3304. [PubMed: 19959677]
- Kook SH, Hwang JM, Park JS, Kim EM, Heo JS, Jeon YM, Lee JC. Mechanical force induces type I collagen expression in human periodontal ligament fibroblasts through activation of ERK/JNK and AP-1. *J. Cell. Biochem*. 2009; 106:1060–1067. [PubMed: 19206162]
- Kuppers M, Ittrich C, Faust D, Dietrich C. The transcriptional programme of contact-inhibition. *J. Cell. Biochem*. 2010; 110:1234–1243. [PubMed: 20564218]
- Larsen CG, Anderson AO, Oppenheim JJ, Matsushima K. Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor. *Immunology*. 1989; 68:31–36. [PubMed: 2478449]
- Lee PN, Hamling J. Systematic review of the relation between smokeless tobacco and cancer in Europe and North America. *BMC Med*. 2009; 7:36. [PubMed: 19638245]

- Liang G, Wolfgang CD, Chen BP, Chen TH, Hai T. ATF3 gene. Genomic organization, promoter, and regulation. *J. Biol. Chem.* 1996; 271:1695–1701. [PubMed: 8576171]
- Malpass GE, Arimilli S, Prasad GL, Howlett AC. Complete artificial saliva alters expression of proinflammatory cytokines in human dermal fibroblasts. *Toxicol. Sci.* 2013; 134:18–25. [PubMed: 23629517]
- McKarns SC, Bombick DW, Morton MJ, Doolittle DJ. Gap junction intercellular communication and cytotoxicity in normal human cells after exposure to smoke condensates from cigarettes that burn or primarily heat tobacco. *Toxicol. In Vitro.* 2000; 14:41–51. [PubMed: 10699360]
- Nagaraj NS, Beckers S, Mensah JK, Waigel S, Vigneswaran N, Zacharias W. Cigarette smoke condensate induces cytochromes P450 and aldo-keto reductases in oral cancer cells. *Toxicol. Lett.* 2006; 165:182–194. [PubMed: 16713138]
- Nordskog BK, Fields WR, Hellmann GM. Kinetic analysis of cytokine response to cigarette smoke condensate by human endothelial and monocytic cells. *Toxicology.* 2005; 212:87–97. [PubMed: 15885868]
- Parsanejad R, Fields WR, Steichen TJ, Bombick BR, Doolittle DJ. Distinct regulatory profiles of interleukins and chemokines in response to cigarette smoke condensate in normal human bronchial epithelial (NHBE) cells. *J. Interferon Cytokine Res.* 2008; 28:703–712. [PubMed: 18937544]
- Payne JB, Johnson GK, Reinhardt RA, Maze CR, Dyer JK, Patil KD. Smokeless tobacco effects on monocyte secretion of PGE2 and IL-1 beta. *J. Periodontol.* 1994; 65:937–941. [PubMed: 7823275]
- Petro TM. Modulation of IL-12 p35 and p40 promoter activity by smokeless tobacco extract is associated with an effect upon activation of NF-kappaB but not IRF transcription factors. *Int. Immunopharmacol.* 2003; 3:735–745. [PubMed: 12757742]
- Petro TM, Anderson LL, Gowler JS, Liu XJ, Schwartzbach SD. Smokeless tobacco extract decreases IL-12 production from LPS-stimulated but increases IL-12 from IFN-gamma-stimulated macrophages. *Int. Immunopharmacol.* 2002; 2:345–355. [PubMed: 11811937]
- Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 2002; 30:e36. [PubMed: 11972351]
- Robson NB, A.J. Wolff K. Salivary nicotine and cotinine concentrations in unstimulated and stimulated saliva. *Afr. J. Pharm. Pharmacol.* 2010; 4:61–65.
- Shizu M, Itoh Y, Sunahara R, Chujo S, Hayashi H, Ide Y, Takii T, Koshiko M, Chung SW, Hayakawa K, Miyazawa K, Hirose K, Onozaki K. Cigarette smoke condensate upregulates the gene and protein expression of proinflammatory cytokines in human fibroblast-like synoviocyte line. *J. Interferon Cytokine Res.* 2008; 28:509–521. [PubMed: 18729741]
- Sopori M. Effects of cigarette smoke on the immune system. *Nat. Rev. Immunol.* 2002; 2:372–377. [PubMed: 12033743]
- Sorrell JM, Caplan AI. Fibroblast heterogeneity: more than skin deep. *J. Cell Sci.* 2004; 117:667–675. [PubMed: 14754903]
- Takehara K. Growth regulation of skin fibroblasts. *J. Dermatol. Sci.* 2000; 24(Suppl. 1):S70–S77. [PubMed: 11137399]
- Tracey KJ, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.* 1994; 45:491–503. [PubMed: 8198398]
- van der Vaart H, Postma DS, Timens W, ten Hacken NH. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax.* 2004; 59:713–721. [PubMed: 15282395]
- Wagner EF. Bone development and inflammatory disease is regulated by AP-1 (Fos/Jun). *Ann. Rheum. Dis.* 2010; 69(Suppl. 1):i86–i88. [PubMed: 19995753]

Fig. 1.

Correlations between gene expression levels in response to STE versus CAS vehicle and to TPM versus DMSO vehicle at 1 h and 5 h in HDFa, measured using qRT-PCR for genes involved in (A) signal transduction and (B) transcription factors. Genes deviating from the regression by greater than 1.4-fold (lines on either side of the regression line) are listed as either being overexpressed or underexpressed by STE compared with CAS vehicle or by TPM compared with DMSO vehicle. Data are normalized to three reference genes as described in the Methods section. STE = smokeless tobacco extract; CAS = complete artificial saliva; TPM = total particulate matter; DMSO = dimethyl sulfoxide; Ct = cycle time.

Fig. 2.

Effect of STE or TPM at 5 h on *IL8*, *TNF α* , *VCAM1*, *NF κ B1*, and *ATF3* gene expression in HDFa. (A) Changes in gene expression due to STE or TPM, but not nicotine. (B) Changes in *ATF3* gene expression due to nicotine. Data indicate fold change relative to basal, measured using qRT-PCR, $n = 5$ for *IL8* and *TNF α* , $n = 3$ for *VCAM1*, *NF κ B1*, and *ATF3*. Error bars indicate SEM. Asterisks indicate significant difference from cells exposed to vehicle (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). VEH1 = defined media containing complete artificial saliva; STE = smokeless tobacco extract; NIC = nicotine; VEH2 = defined media containing dimethyl sulfoxide; TPM = total particulate matter.

Fig. 3.

Effect of STE or TPM at 1 h on *IL1 α* , *FOS*, and *JUN* gene expression in HDFa. (A) Gene expression not altered by the change of media to defined media. (B) Gene expression altered by the change to defined media. Data indicate fold change relative to basal, measured using qRT-PCR, $n = 5$. Error bars indicate SEM. Asterisks indicate significant difference from cells exposed to vehicle ($*P < 0.05$). VEH1 = defined media containing complete artificial saliva; STE = smokeless tobacco extract; NIC = nicotine; VEH2 = defined media containing dimethyl sulfoxide; TPM = total particulate matter.

Fig. 4.

Effect of STE, TPM, or NIC on the generation of nitric oxide in HDFa. Data represent the rate of accumulation between 10 min and 35 min of treatment (mean $\pm$ SEM, $n = 3$).

Asterisk indicates significant difference from cells exposed to vehicle ($*P < 0.05$). VEH1 = vehicle 1 (complete artificial saliva); STE = smokeless tobacco extract; NIC = nicotine; VEH2 = vehicle 2 (dimethyl sulfoxide); TPM = total particulate matter.

Fig. 5. Effect of STE, TPM, or NIC on the generation of reactive oxygen/nitrogen species in HDFa. Data represent the fluorescence endpoint in cells after 90 min of treatment (mean $\pm$ SEM, $n = 3$). Asterisks indicate significant difference from cells exposed to vehicle ($*P < 0.05$). RFU = relative fluorescence units; VEH1 = vehicle 1 (complete artificial saliva); STE = smokeless tobacco extract; NIC = nicotine; VEH2 = vehicle 2 (dimethyl sulfoxide); TPM = total particulate matter.

Fig. 6.

Effect of STE, TPM, or NIC on the generation of superoxide in HDFa. Data represent the accumulation of fluorescence endpoint in cells after 90 min of treatment (mean $\pm$ SEM, $n = 3$). Asterisks indicate significant difference from cells exposed to vehicle ($*P < 0.05$). RFU = relative fluorescence units; VEH1 = vehicle 1 (complete artificial saliva); STE = smokeless tobacco extract; NIC = nicotine; VEH2 = vehicle 2 (dimethyl sulfoxide); TPM = total particulate matter.

Fig. 7. Summary of the expression of biomarkers of fibroblast function influenced by tobacco products. STE = smokeless tobacco extract; TPM = total particulate matter.

Table 1

Effect of STE, TPM, or NIC at 1 h and 5 h on HDFa release of cytokines IL1 β , IL6, IL8, IL10, IL12p70, and TNF α . Cytokines in culture media were measured using Cytometric Bead Array (CBA) assays.

	<i>n</i>	VEH1	STE	NIC 141.6 μ g/ml
1 h				
IL1 β	4	13.6 $\pm$ 1.1	13.9 $\pm$ 1.0	14.4 $\pm$ 1.1
IL6	4	9.0 $\pm$ 0.7	9.1 $\pm$ 0.8	9.8 $\pm$ 0.9
IL8	4	18.3 $\pm$ 1.8	17.9 $\pm$ 2.0	19.4 $\pm$ 2.1
IL10	4	5.7 $\pm$ 0.4	5.7 $\pm$ 0.4	6.2 $\pm$ 0.2
IL12p70	4	14.5 $\pm$ 4.8	14.6 $\pm$ 4.8	15.3 $\pm$ 4.3
TNF α	4	7.3 $\pm$ 0.3	7.6 $\pm$ 0.3	8.0 $\pm$ 0.4
5 h				
IL1 β	4	16.0 $\pm$ 1.2	16.3 $\pm$ 1.6	14.9 $\pm$ 0.7
IL6	4	26.8 $\pm$ 7.4	23.0 $\pm$ 3.6	33.3 $\pm$ 12.5
IL8	4	308.3 $\pm$ 124.4	119.1 $\pm$ 29.1	238.1 $\pm$ 116.1
IL10	4	6.4 $\pm$ 0.3	7.0 $\pm$ 0.2	6.3 $\pm$ 0.5
IL12p70	4	15.2 $\pm$ 4.5	16.0 $\pm$ 3.8	14.8 $\pm$ 4.7
TNF α	4	8.2 $\pm$ 0.5	8.7 $\pm$ 0.8	7.8 $\pm$ 0.5
	<i>n</i>	VEH2	TPM	NIC 4 μ g/ml
1 h				
IL1 β	3	13.4 $\pm$ 1.3	16.7 $\pm$ 0.9	13.5 $\pm$ 0.9
IL6	3	8.7 $\pm$ 0.6	10.8 $\pm$ 0.7	8.7 $\pm$ 0.7
IL8	3	20.4 $\pm$ 3.5	23.7 $\pm$ 3.8	17.0 $\pm$ 1.8
IL10	3	5.9 $\pm$ 0.6	8.9 $\pm$ 0.8*	5.9 $\pm$ 0.5
IL12p70	3	16.9 $\pm$ 7.5	23.9 $\pm$ 9.6	17.1 $\pm$ 7.7
TNF α	3	7.2 $\pm$ 0.2	10.8 $\pm$ 0.3***	7.5 $\pm$ 0.1
5 h				
IL1 β	3	13.9 $\pm$ 1.2	15.8 $\pm$ 1.1	13.6 $\pm$ 1.0
IL6	3	10.7 $\pm$ 1.8	12.3 $\pm$ 1.5	9.8 $\pm$ 1.7
IL8	3	43.8 $\pm$ 18.1	52.0 $\pm$ 19.2	41.2 $\pm$ 18.0
IL10	3	6.1 $\pm$ 0.4	7.5 $\pm$ 0.6	5.6 $\pm$ 0.4
IL12p70	3	16.0 $\pm$ 5.8	20.2 $\pm$ 8.3	15.2 $\pm$ 5.8
TNF α	3	7.6 $\pm$ 0.6	9.1 $\pm$ 0.4	7.0 $\pm$ 0.5

Data are the concentration of cytokines released (pg/ml) presented as mean $\pm$ SEM, *n* = 3–4.

Asterisks indicate significant difference from cells exposed to vehicle

VEH1 = vehicle 1 (complete artificial saliva); STE = smokeless tobacco extract; NIC = nicotine; VEH2 = vehicle 2 (dimethyl sulfoxide); TPM = total panicle matter.

* *P* < 0.05;

*** *P* < 0.001