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Evaluation of cytotoxicity of different tobacco product preparations ☆

Q1 Subhashini Arimilli [a,*], Brad E. Damratoski [a], Betsy Bombick [b], Michael F. Borgerding [b], G.L. Prasad [b]

[a] Department of Microbiology & Immunology, Wake Forest University School of Medicine, Wake Forest Biotech Place, Winston-Salem, NC 27157, United States
[b] R&D Department, R.J. Reynolds Tobacco Company, Winston-Salem, NC 27102, United States

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ABSTRACT

Acute exposure to cigarette smoke or its components triggers diverse cellular effects, including cytotoxicity. However, available data regarding the potential cytotoxic effects of smokeless tobacco (ST) extracts lack consensus. Here, we investigated the relative biological effects of 2S3 reference ST, and whether ST elicits differential cellular/molecular responses compared to combustible tobacco product preparations (TPPs) prepared from 3R4F cigarettes. Total particulate matter (TPM) and whole smoke conditioned medium (WS-CM) were employed as combustible TPPs, while the ST extract was used as non-combustible TPP. HL60, THP1 cells and human PBMCs were used to examine the effects of TPPs in short-term cell culture. Corresponding EC_{50} values, normalized for nicotine content of the TPPs, suggest that combustible TPPs induced higher cytotoxicity as follows: WS-CM $\geqslant$ TPM $\gg$ ST extract > nicotine. While all three TPPs induced detectable levels of DNA damage and IL8 secretion, the combustible TPPs were significantly more potent than the ST preparation. The major PBMC subsets showed differential cytotoxicity to combustible TPPs as follows: CD4 > CD8 > monocytes > NK cells. These findings suggest that, relative cytotoxic and other cell biological effects of TPPs are dose-dependent, and that ST extract is the least cytotoxic TPP tested in this study.

1. Introduction

Cigarette smoke is a complex chemical mixture containing more than 8000 different chemical compounds, of which about 69 are known or probable human carcinogens (Perfetti, 2011; Rodgman and Perfetti, 2008) The long-term effects of smoking have been thoroughly documented, particularly with respect to the development of cancer, COPD, respiratory infections and cardiovascular diseases (Sopori, 2002; Sopori and Kozak, 1998). Cigarette smoke is a dynamic and highly reactive mixture consisting of gas–vapor and particulate phases and is rich in free radicals (Church and Pryor, 1985; Pryor, 1997). One of the mechanisms through which cigarette smoke exerts its adverse effects is through the free radical and other oxidant-driven oxidative stress which triggers local and systemic inflammatory (US Department of Health and Human Services, 2010; van der Vaart et al., 2004). Thus, cigarette smoke exerts both direct effects at local areas of exposure (lung and oral cavity, for example) and systemic effects.

Smokeless (non-combustible) tobacco (ST) constitutes a large, diverse product category that is marketed globally. In this manuscript, we primarily focus on moist snuff available in the US. Fermented moist snuff is the form of moist snuff traditionally sold in the US. Fermented moist snuff, or dipping tobacco, is made primarily from dark air and dark fire-cured tobaccos with moisture content that is typically near 50% of the product weight. Snus is a moist snuff tobacco product prepared by the heat treatment, i.e., "pasteurization," of the tobacco (Lewis, 2008). Existing epidemiological data suggests that ST consumption is less harmful than smoking (Lewis, 2008; Stratton et al., 2001; Zeller et al., 2009). For example, the risk of lung cancer, COPD, CVD and oral cancer are significantly reduced in ST users relative to smokers (Gartner and Hall, 2008; Hatsukami et al., 2002; Lee and Hamling, 2009). The risk of some tobacco-related diseases, however, appears to be elevated in ST users, relative non-tobacco users (Henley et al., 2005).

The purpose of this study is to investigate the relative effects of exposure to smoke and ST in cultured cells. Several experimental systems, particularly cell culture models, are currently utilized to assess the biological effects of tobacco exposure (Andreoli et al., 2003). Researchers have employed a variety of preparations and individual smoke constituents to investigate the biological effects

Abbreviations: CAS, complete artificial saliva; CD, cluster of differentiation; COPD, chronic obstructive pulmonary disease; CVD, cardio vascular disease; DMSO, dimethyl sulfoxide; IL, interleukin; IRB, internal review board; NK cells, natural killer cells; PAHs, polycyclic aromatic hydrocarbons; PBMCs, peripheral blood mononuclear cells; ST, smokeless tobacco; TNF, tumor necrotic factor; TPM, total particulate matter; TSNAs, tobacco specific nitrosamines; TPPs, tobacco product preparations; WS-CM, whole smoke conditioned medium.

☆ Preliminary findings of the work were presented as a poster at the 64th Tobacco Science Research Conference (TSRC) held in Hilton Head, SC in 2010.

* Corresponding author. Address: Department of Microbiology & Immunology, Wake Forest Baptist Health, Room 2E-033, 575 Patterson Avenue, Wake Forest Biotech Place, Winston-Salem, NC 27101, United States. Fax: +1 336 716 9928.
E-mail address: sarimill@wfubmc.edu (S. Arimilli).

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of smoke exposure (Johnson et al., 2009). To assess the effects of smoke exposure the particulate fraction TPM (collected on Cambridge filter pads, also referred by some as cigarette smoke condensate) dissolved in DMSO has been extensively used. However, this fraction is devoid of the gas–vapor phase constituents such as acrolein and other reactive compounds (Johnson et al., 2009). Assessment of genotoxic and cytotoxic effects of exposure to whole smoke ideally is achieved by exposing cells directly to freshly generated smoke (Bombick et al., 1997). A commonly employed method to expose cells to whole smoke involves bubbling fresh smoke through aqueous media and using it as the whole smoke aqueous extract (Fukano et al., 2004; Phipps et al., 2010). This preparation is likely to consist of water-soluble constituents of cigarette smoke. Similarly, various preparations of ST in organic and aqueous media have been used (Rickert et al., 2009).

Well-recognized cellular responses to smoke or smoke-constituent exposure include marked cytotoxicity and induction of inflammatory responses, as noted by increased secretion of IL8 and other proinflammatory cytokines (Nordskog et al., 2005; Parsanejad et al., 2008). The chemokine IL8 mainly attracts neutrophils and lymphocytes and may result in neutrophilia (Goncalves et al., 2010; Kobayashi, 2008; Larsen et al., 1989; Tomita et al., 2003) and lymphocytosis (Jagels and Hugli, 1992; Terashima et al., 1998). In smokers, blood IL8 levels were significantly higher than those in non-smokers (Iho et al., 2003). IL8 seems to be a possible candidate chemokine playing a role in leukocytosis in smokers. There is limited information on the inflammatory responses and cytokine profiles resulting from exposure to ST (Seyedroudbari and Khan, 1998).

Here we investigate the cellular responses to treatment with cigarette smoke-derived and smokeless TPPs in short-term cell cultures. Since inflammatory responses are primarily driven by hematopoietic cell types, we utilized human peripheral blood mononuclear cells (PBMCs), HL60 and THP1 cells. We evaluated cytotoxicity, DNA damage and IL8 secretion to determine the relative effects of the TPPs. We also included nicotine as a test compound to assess the relative effects of the TPPs.

2. Material and methods

2.1. Tobacco products

Total particulate matter (TPM) was prepared by smoking 3R4F reference cigarettes (University of Kentucky) using the standard ISO method (Johnson et al., 2009) (35-60-2, puff volume in mL, inter puff interval in sec and puff duration in sec, respectively) and dissolving the particulate phase in DMSO. Borgerding et al. have analyzed the composition of the reference 2S3 moist snuff (manuscript under revision). Per those analyses, the 2S3 moist snuff contains dark fire cured tobacco (25.3%), air cured tobacco (7.8%), burley stems (3.72%). sodium carbonate (0.72%), sodium chloride (7.32%) and moisture (54.81%). Smokeless tobacco extract was prepared by extracting 2S3 smokeless tobacco reference product (North Carolina State University Tobacco Services Analytical Laboratory) in complete artificial saliva (CAS) including active enzymes for 2 h followed by filtration (Chou and Hee, 1994). Whole smoke conditioned media (WS-CM) was prepared by passing smoke from 3R4F cigarettes through RPMI 1640 medium (Invitrogen, Grand Island, NY) without phenol red. Neat nicotine (Sigma–Aldrich, Milwaukee, WI) was used as a reference. Aliquots of frozen TPPs were analyzed for nicotine, tobacco specific nitrosamines (TSNAs) and polycyclic aromatic hydrocarbons (PAHs) contents at Labstat International (Kitchener, Ontario, Canada) (Table 1). A preliminary description of the ST extraction conditions were presented at the 64th TSRC conference held in Hilton Head, SC.

2.2. Cell lines and isolation of PBMCs

HL60 (human lymphoblast cell line), THP1 (human monocyte-macrophage cell line) were obtained from ATCC (Manassas VA, USA). HL60 and THP1 cell lines were maintained in RPMI 1640 media containing 10% Fetal Bovine Serum with 1% L-glutamine, 1% penicillin and streptomycin. Fresh blood was collected from healthy donors (who were non-consumers of tobacco products) at a local clinical Contract Research Organization (Piedmont Medical Group, Winston-Salem, NC) under IRB approval. PBMCs were isolated from fresh blood by standard density gradient centrifugation by using Isolymph (CTL Scientific Supply Corp., Deer Park, NY) (method described in a poster presented at 64th TSRC, Hilton Head, SC; Arimilli et al., in press). Isolated PBMCs were cryopreserved in the liquid nitrogen tanks for further use.

2.3. Cell Staining and flow cytometry

PBMCs, HL60, and THP1 cells were treated with varying concentrations of nicotine units of WS-CM, TPM, ST/CAS and pure nicotine as indicated. Dosing (nicotine units) was based on an equi-nicotine exposure paradigm. Unless indicated, all treatments were performed for 24 h. Cell culture media supernatants were collected for IL8 assessment and the cells were washed in phosphate-buffered saline, fixed with Cytofix & Cytoperm (BD Biosciences, San Jose, CA) and stained for caspase 3-PE and ser-139 phosphorylated H2AX (γ-H2AX-Alexa Fluor 647). Cell death was also assessed by staining with 7AAD (BD Biosciences, San Jose, CA) and using a Live cell/Dead cell vitality assay kit (Cell Signaling, Danvers, MA). PBMCs were further stained with CD4 or CD8-perCP, CD14-FITC and CD56-PE to determine which leukocyte subsets might be more susceptible to the *in vitro* cytotoxic effects of the tobacco products. The percent positive staining of the cells was measured using a flow cytometer (BD Biosciences, San Jose, CA)) and the flow data were analyzed using Cell Quest (BD Biosciences, San Jose, CA) and Flow Jo (Tree Star, Ashland, OR) software.

2.4. IL8 assay

IL8 was measured by ELISA kit from R&D Systems (Minneapolis, MN) according to the manufacturer's protocol.

2.5. Fluorescence microscopy

After intracellular staining of cells with Alexa Fluor 647-conjugated H2AX (BD Biosciences, San Jose, CA), the cell pellet was spotted on the microscopic slide, cover-slipped with a small drop of DAPI gold (Molecular Probes, Grand Island, NY) and analyzed with a Nikon Eclipse fluorescence microscope at 40X magnification. Images were captured using a Q Imaging digital camera and processed using Q Capture software.

2.6. Review of abbreviations

We have used a number of abbreviations in this manuscript and a brief summary of those is included in the following. We have prepared total particulate matter (TPM) and whole-smoke conditioned medium (WS-CM) from 3R4F cigarettes. Smokeless tobacco extracts of 2S3 moist snuff were prepared in complete artificial saliva (CAS) and they are referred as ST/CAS. Collectively, the combustible (TPM and WS-CM) and non-combustible (ST/CAS) tobacco product preparations are abbreviated as TPPs. Peripheral blood mononuclear cells (PBMCs) have been isolated from human blood. Selected leukocyte subsets, viz., cytotoxic T lymphocytes, helper T lymphocytes, monocytes and natural killer cells (NK cells), were labeled for respective cell surface markers known as cluster of

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Table 1
Chemical analysis of different TPPs.

	Nicotine (μg/mL)	NNN (ng/mL)	NAT (ng/mL)	NAB (ng/mL)	NNK (ng/mL)	B[a]P (ng/mL)
TPM	1716 ± 12	116 ± 2	182 ± 5	22.6 ± 3.3	116 ± 2	11.3 ± 0.1
WS-CM	12.44 ± 0.44	1.3 ± 0,1	0.99 ± 0.05	0.21 ± 0.03	0.99 ± 0.09	<0.013
ST/CAS	1408	164	92.4	⩽10.3 but >3.10	39.8	BDL

BDL- Below the Detection Limit. Tobacco product preparations were analyzed for key tobacco constituents. TPM was prepared in DMSO; ST/CAS was prepared as 10% solution in CAS; WS-CM was prepared in cell culture medium. The data shown here are derived from a batch of tobacco product preparations.

differentiation (CD) marker proteins with specific antibodies. The labeled samples were detected by flow cytometry.

3. Results

3.1. Tobacco product preparations (TPPs)

In this study, we sought to evaluate the effects of combustible and non-combustible TPPs, which are chemically very distinct. The TPM phase of cigarette smoke (Johnson et al., 2009), has been previously employed in numerous cell culture studies as a model for particulate phase smoke exposure. We also tested an additional combustion preparation by using whole smoke-conditioned medium (WS-CM). Exposure to the conditioned medium was measured in terms of the number of cigarettes smoked for preparation of the medium and/or the absorbance at A320 nm. Exposure to ST, on the other hand, may be measured in terms of the strength of the extract (weight of ST used/volume of the medium used for extraction; example, 10% extract) (Rickert et al., 2009). Thus, no common method exists for comparing of the effects of *in vitro* exposure to different TPPs.

Nicotine, TSNAs and PAHs in TPM (prepared in DMSO), WS-CM (generated in RPMI cell culture medium) and ST extract (prepared in CAS) were quantified. We used nicotine content (shown in Table 1), expressed in μg/mL of cell culture medium, as a common measure of exposure to the different TPPs.

3.2. Cytotoxic effects of TPPs on HL60 and THP1 cell lines, and human PBMCs

In the first set of experiments we measured the effect of TPPs on HL60 and THP1 cell lines, and freshly isolated PBMCs. A dose-dependent increase in cell death was detected in response to TPPs and nicotine in all the three cell types (Fig. 1). Treatment with TPM and WS-CM at low nicotine units (2–6 μg/mL) resulted in significant cell death (Fig. 1, left panels) compared to ST/CAS and nicotine (Fig. 1, right panels). Cell death in TPM and WS-CM exposed cells reached 100 percent at substantially low nicotine units (2–4 μg/mL and ~6 μg/mL, respectively). Human PBMCs appeared to be more sensitive to WS-CM treatment at lower range compared to exposure to TPM, whereas in HL60 and THP1 cell lines were more sensitive to TPM. Since the cytotoxic effects of WS-CM significantly diminished upon storage at 4 °C (preliminary data; not shown), freshly generated WS-CM was used in all of the experiments. Typically, cells were exposed to freshly generated WS-CM within 2 h of preparation.

In contrast, cytotoxic effects of ST/CAS and nicotine were observed at much higher levels of nicotine units. Detectable cell death was observed in cells treated with nicotine and ST/CAS only at several hundreds of micrograms of nicotine units. Consistent with published data (Yoshida et al., 1998), maximal cell death with nicotine treatment was observed at >3.0 mg/mL (Fig. 1, right panels). Although cell death was detected in ST/CAS-treated cells, maximal cell death was not achieved due to the interference by the CAS, which was the vehicle control for ST/CAS treatment. CAS at higher concentrations induced significant cell death, independent of ST. For example, addition of CAS to medium [final concentration 33% (1:2 dilutions) in culture medium] caused death in approximately 80–90 percent of cells. Cells exposed to ST/CAS up to 704 μg/mL nicotine units showed a maximum of 20 percent cell death. As a result, it was not possible to experimentally determine the concentration of ST/CAS at which maximal cell death is caused. Similar solvent effects were not apparent to TPM, as DMSO (as a vehicle control) did not cause noticeable cell death at the concentrations used. In terms of nicotine units, ST/CAS and nicotine were non-cytotoxic in all cell types tested at the levels the combustible TPPs were maximally cytotoxic.

We further tested the cytotoxic effects of TPPs in a different assay method, known as Live/Dead cell assay. The proportion of live cells decreased with a concomitant increase in the dead cells as the exposure to TPM and WS-CM increased (Fig. 2A and C). As observed with the 7AAD method (Fig. 1, left panel) both TPM and WS-CM were highly cytotoxic to both HL60 and THP1 cells. In contrast, a 20 percent decrease in live cell population was noted only at markedly higher concentrations (704 μg/mL nicotine units) of ST extract or nicotine (900 μg/mL) (Fig. 2B and D). As indicated above, a maximum amount of 704 μg/mL nicotine units of ST/CAS could be employed due to the cytotoxic effects of the solvent. At 2 mg/mL of nicotine, 60–70 percent of cell death was noted in HL60 and THP1 cells as indicated by a decline in live cell population and increase in the fraction of dead cells (Fig. 2B and D). Collectively, these studies showed that the combustible TPPs are far more cytotoxic compared to either ST or pure nicotine.

3.3. EC_{50} values of TPM, WS-CM, ST/CAS and nicotine

The EC_{50} is defined as the concentration at which 50 percent of the cells were no longer viable in a 24 h assay and the values are expressed as μg nicotine units/mL. EC_{50} values of different TPPs based on cell death as measured by 7AAD staining for PBMCs, HL60 and THP1 cell lines are presented in Table 2. The EC_{50} values of combustible preparations (TPM and WS-CM) were determined to be between 1.5–2.5 μg/mL nicotine units for all the cell lines tested. As discussed in the previous section, we were unable to calculate the EC_{50} value for ST/CAS using 20% ST/CAS due to the interfering cytotoxic effects of the solvent CAS. Hence, for the purposes of this study, we adapted 704 μg/mL nicotine units as apparent EC_{50} value, even though the true EC_{50} concentration is likely to be much higher. Nicotine EC_{50} values were highest, indicating the least cytotoxic effects relative to combustible TPPs, TPM and WS-CM in all the cell types. The EC_{50} values of nicotine ranged from 1420–1800 μg/mL, depending on the cell type. Thus, the observed cytotoxic effects of TPPs are not driven by nicotine itself.

3.4. Mechanisms of cell death induced by TPPs

To understand the cytotoxic effects caused by combustible and non-combustible TPPs, we investigated the underlying molecular mechanisms. Cigarette smoke has been reported to induce DNA damage and apoptosis. We evaluated DNA damage by measuring double-stranded DNA breaks by quantifying phosphorylated

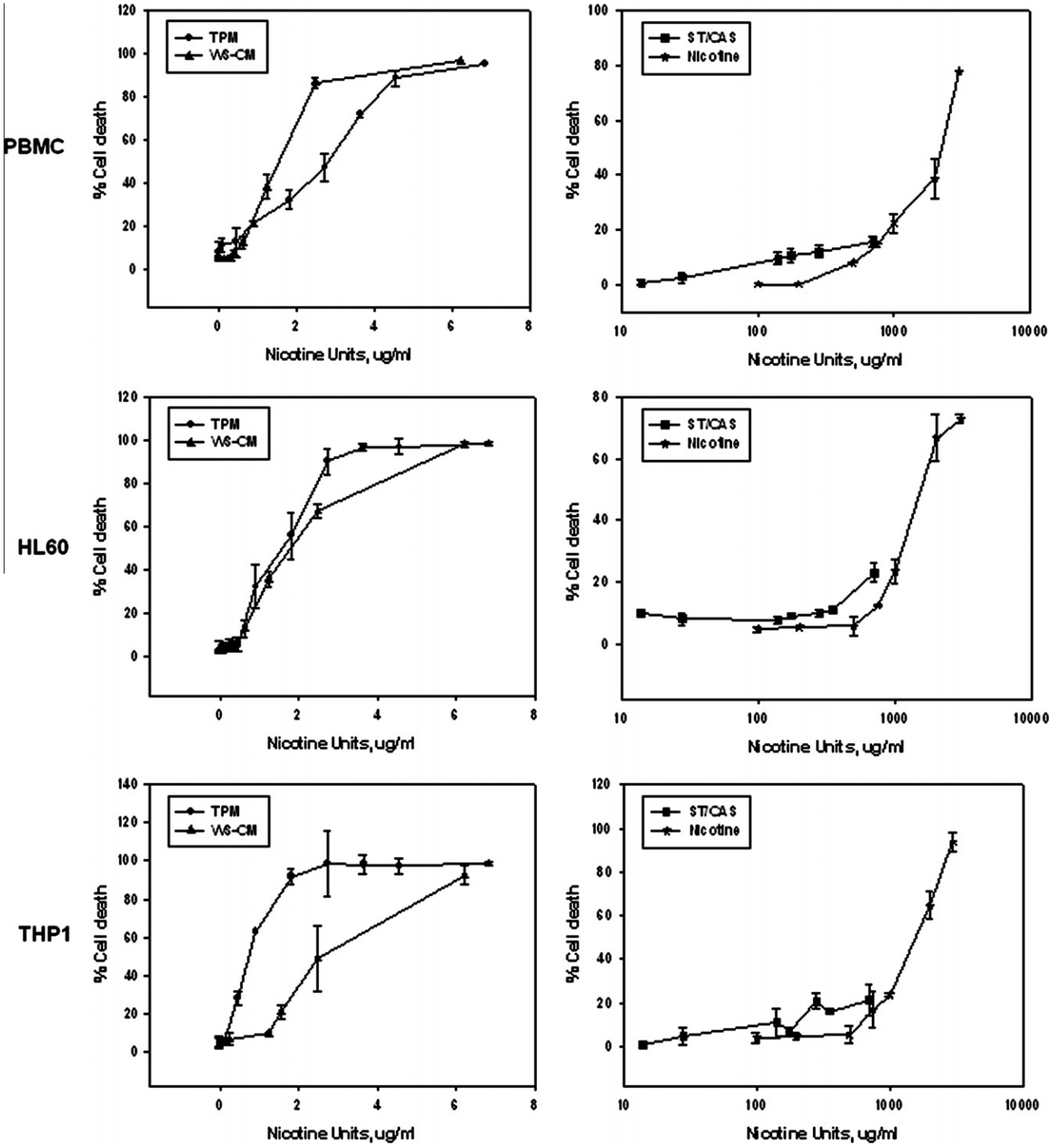

Fig. 1. Dose-dependent cell death of PBMCs, HL60 and THP1 cell lines exposed to different tobacco product preparations. PBMC, HL60 and THP1 cells were exposed to the indicated concentrations of TPM, WS-CM, ST/CAS and nicotine for 24 h. Cell death was measured by 7AAD staining. Cells were treated with TPM and WS-CM (left panels), and ST/CAS and nicotine (right panels). Note markedly different scaling for ST/CAS and nicotine. Each point represents the mean ± SEM of four experiments. We subtracted CAS induced cell death values from the ST/CAS values to plot the ST/CAS cell death curves.

H2AX histones, which rapidly bind to double-stranded DNA breaks. The phosphorylation of H2AX histone has been used as a marker for double-stranded DNA breaks in general (Albino et al., 2004; Rothkamm and Lobrich, 2003) and in particular to cigarette smoke-induced DNA lesions (Albino et al., 2006).

THP1 cells were treated with TPM, WS-CM, ST/CAS and nicotine at EC_{50} concentrations (nicotine units) as indicated for 24 h. γ-H2AX staining was visualized by fluorescent microscopy (Fig. 3, middle panel). Nuclei were identified with DAPI stain (Fig. 3 left panel). The merged images indicate that the γ-H2AX staining was localized to nuclear DNA (Fig. 3, right panel). As evident from the staining of γ-H2AX, WS-CM at 2.5 μg/mL nicotine units induced significant DNA damage, although TPM also caused detectable DNA damage. In contrast, ST/CAS at 704 μg/mL nicotine units showed less double stranded DNA damage. Treatment with nicotine at 1800 μg/mL (11 mM) resulted in double-stranded DNA damage. Next, we measured the double-stranded DNA damage by flow cytometric quantification of γ-H2AX staining in THP1

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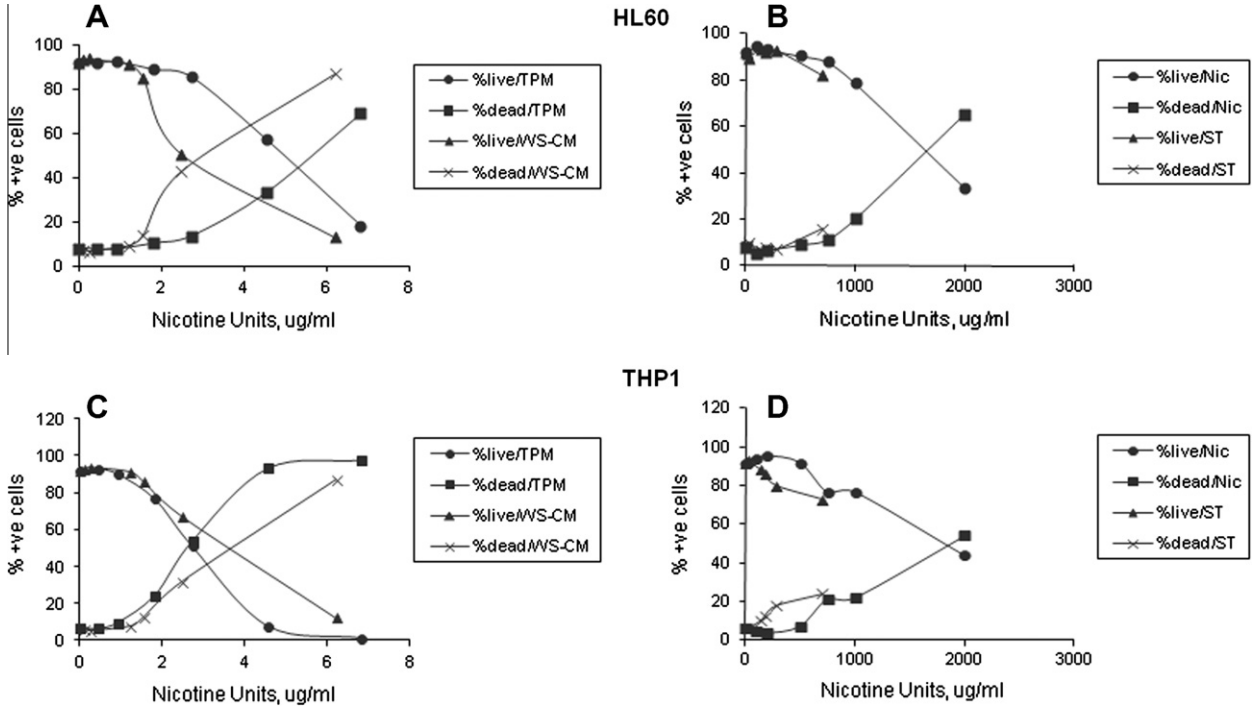

Fig. 2. Cytotoxic effects of TPM, WS-CM, ST/CAS and nicotine on HL60 and THP1 cells. HL60 cells (panels A and B) and THP1 cells (panels C and D) were exposed to the indicated equi-nicotine concentrations of TPM, WS-CM, ST/CAS, and nicotine for 24 h and measured the cell death by Live/Dead cell assay. Shown is one example of three independent experiments.

Table 2
Summary of EC_{50} values of different TPPs for PBMCs, HL60 and THP1 cell lines.

	PBMC	HL60	THP1
TPM	2.67 ± 0.15	1.55 ± 0.31	1.48 ± 0.07
WS-CM	1.55 ± 0.07	1.62 ± 0.07	2.5 ± 0.5
ST/CAS	>700	>700	>700
Nicotine	1650 ± 70 (10 mM)	1420 ± 52 (8 mM)	1800 ± 52 (11 mM)

The EC_{50} values are expressed as μg equi-nicotine units/mL. EC_{50} is the concentration at which 50 percent of the cells are no longer viable in a 24 h assay measured by 7AAD staining. Each value represents the mean ± SEM of four experiments. It should be noted that with ST/CAS, an EC_{50} value could not be determined. Therefore, a maximum dose tested using a 20% ST/CAS solution is provided.

cells (Fig. 4A and B). Although both WS-CM and TPM caused detectable DNA damage at low levels (4–7 μg/mL of nicotine units), the WS-CM is more potent relative to TPM. ST/CAS appears to be ineffective in causing DNA damage at lower concentrations tested in this study. Nicotine at low (1–7 μg/mL) or medium (~700 μg/mL) levels of exposure, did not cause significant double-stranded DNA damage. However, nicotine at the highest concentration (2000 μg/mL) caused significant double-stranded DNA breaks (Fig. 4B). These data suggest that the combustible TPPs, WS-CM and TPM induce double-stranded DNA damage at significantly lower nicotine units than ST or nicotine.

Next, we investigated whether the cell death caused by TPPs might occur through caspase 3-mediated pathways. THP1 cells were stained with antibodies against activated caspase 3 and quantified by flow cytometry. As evident from Fig. 4, panels C and D, the participation of caspase 3 in cell death pathways varied depending on the treatment. For example, WS-CM treatment appeared to activate caspase 3 well above the concentrations required to induce cell death, suggesting a potential for non-apoptotic cell death. TPM, on the other hand, profoundly activated caspase 3 in a dose-dependent manner, indicating a significant participation of caspase 3-mediated apoptotic cell death (Fig. 4C). ST/CAS which caused marginal cell death appears to activate caspase 3 proportionately at the doses tested (Fig. 4D). Similarly, nicotine at cytotoxic levels caused readily detectable activation of caspase 3, suggesting a role for caspase 3 and apoptosis in the cytotoxicity of nicotine. Thus, TPPs appear to cause cell death through multiple mechanisms, involving both caspase 3 dependent and independent pathways.

3.5. Cytotoxic effects of TPM, WS-CM, ST/CAS and nicotine on different leukocyte subsets

To better understand the effects of different TPPs on human PBMCs, we sought to determine whether the TPPs exert differential cytotoxicity against PBMC subtypes. The PBMCs were treated with TPPs at the concentrations used in the cytotoxicity studies, described above. The subtypes were marked by cell type-specific markers, as indicated: helper T cells (CD4), cytotoxic T cells (CD8), monocytes (CD14) and NK cells (CD56). The leukocytes were co-stained with 7AAD, and the double positive cells were measured by flow cytometry (Figs. 5 and 6A) to determine which leukocyte subset was susceptible to TPPs. WS-CM was more toxic than the TPM in all the cell types tested. Helper T cells (CD4) appeared to be more sensitive to TPM and WS-CM than cytotoxic T cells (CD8) with respect to cell death (Figs. 5 and 6A); NK cells appeared to be least sensitive to the cytotoxic effects of TPPs under the conditions tested. Consistent with the data obtained with unfractionated PBMCs, ST/CAS was the least toxic tobacco preparation. Combined exposure to TPM and WS-CM resulted in a further increase in cell death of all cell types relative to either treatment alone.

Assessment of DNA damage by flow cytometric quantification of γ-H2AX staining revealed that CD8 cells are more vulnerable to DNA damage by TPPs than CD4 cells (Fig. 6B). TPM, WS-CM (either separately or together) induced more DNA damage in monocytes. The data presented suggest that treatment with the combustible TPPs is more cytotoxic and causes a higher level of DNA damage (Fig. 6B) to the leukocyte subsets.

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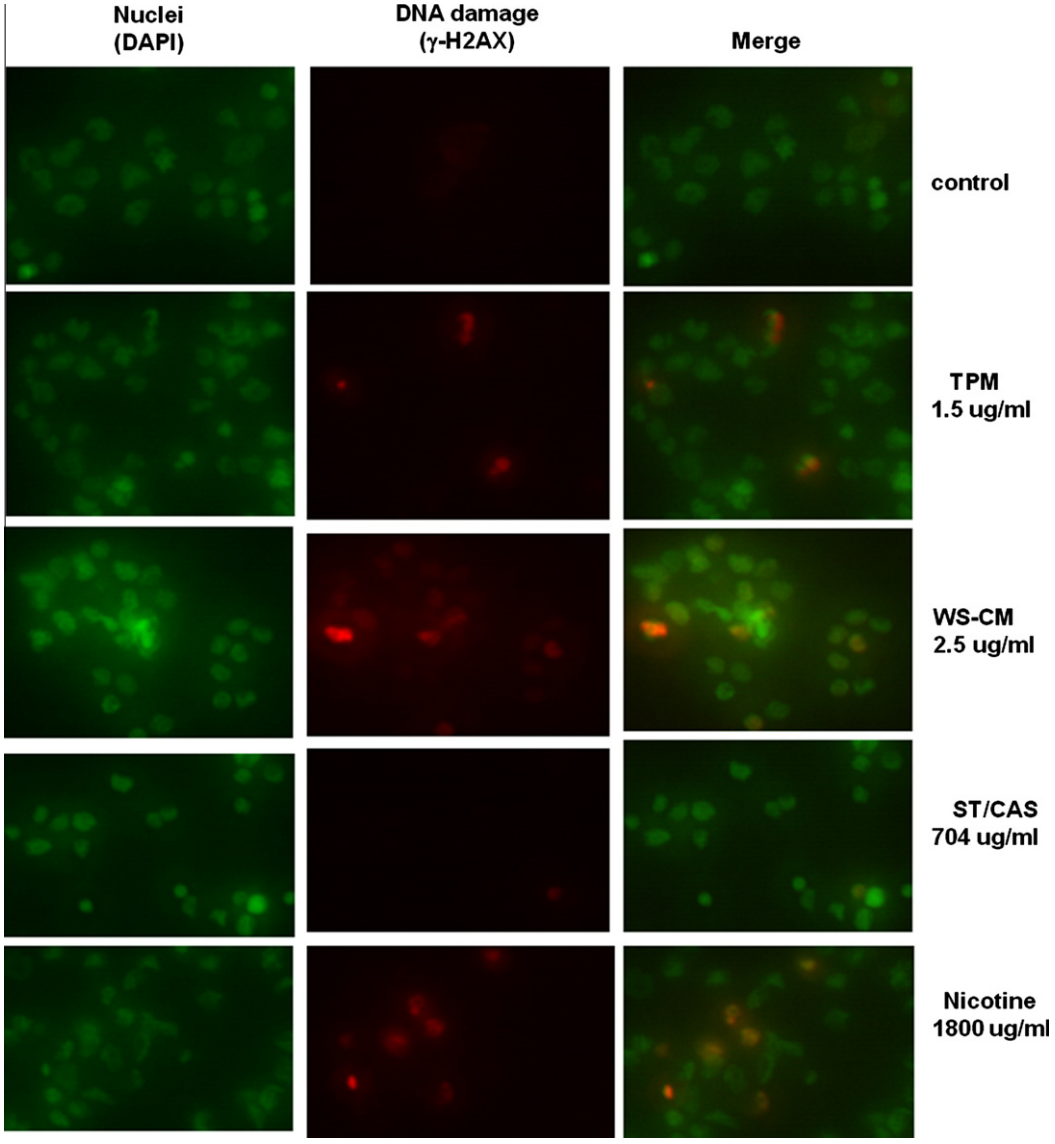

Fig. 3. Fluorescence microscopy of THP1 cells exposed to different tobacco product preparations at EC_{50} concentrations. THP1 cells were exposed to TPM,WS-CM, ST/CAS and nicotine at EC_{50} concentrations for 24 h and double-stranded DNA breaks were measured by staining with γ-H2AX (middle panel), DAPI nuclear stain (left panel) and merged images (right panel).

3.6. IL8 secretion of PBMCs, HL60 and THP1 cell lines by TPM, WS-CM, ST/CAS and nicotine treatment

Exposure to cigarette smoke has been shown to cause inflammatory responses in chronic smokers, and many *in vitro* studies have shown that treatment with TPM (cigarette smoke condensate) results in induction of several cytokine genes (Swenson et al., 2011) and secretion of cytokines (Nordskog et al., 2005; Xu et al., 2011). We investigated whether the combustible and smokeless TPPs differ in their ability to induce cytokine secretion. In the initial experiments, secretion of several cytokines was evaluated in response to treatment with the TPPs and nicotine (data not shown). The panel of cytokines at the screening stage included IL1b, IL6, IL8, IL10, IL12 and TNF-α. These pilot studies revealed that exposure to TPPs resulted in secretion of IL8 by PBMCs, HL60 and THP1 cells. Subsequent experiments were performed to evaluate dose-dependent effects of the reagents on IL8 secretion.

TPM and WS-CM treatments resulted in dose-dependent increases in secretion of IL8 in PBMCs and HL60, which decreased at higher concentrations, presumably due to exposure-related cell death (Fig. 7, left panel). The THP1 cell line showed a moderate secretion of IL8 with lower concentrations of WS-CM but not with TPM. Whereas nicotine and ST/CAS induced dose-dependent IL8 secretion, similar to the other data presented in this manuscript, the cytokine induction occurred at much higher nicotine units of exposure compared to the combustible TPPs. Nicotine did not induce detectable IL8 in either PBMCs or THP1 cells at the concentrations used (Fig. 7, right panel). However, nicotine treatment resulted in dose-dependent secretion of IL8 in HL60 cells. In terms of IL8 secretion, it appears that THP1 cells are less responsive to the TPPs relative to other cells used in this study. In summary, these data show cell type-specific effects of the TPPs and nicotine and that the combustible TPPs are generally more potent inducers of IL8 secretion, than ST/CAS and nicotine at equi-nicotine units.

4. Discussion

We evaluated the cytotoxic effects of different TPPs on cells derived from hematopoietic lineage. A number of studies have assessed the effects of acute exposure to combustible TPPs. However, few have examined the effect of exposure to ST. The key findings of

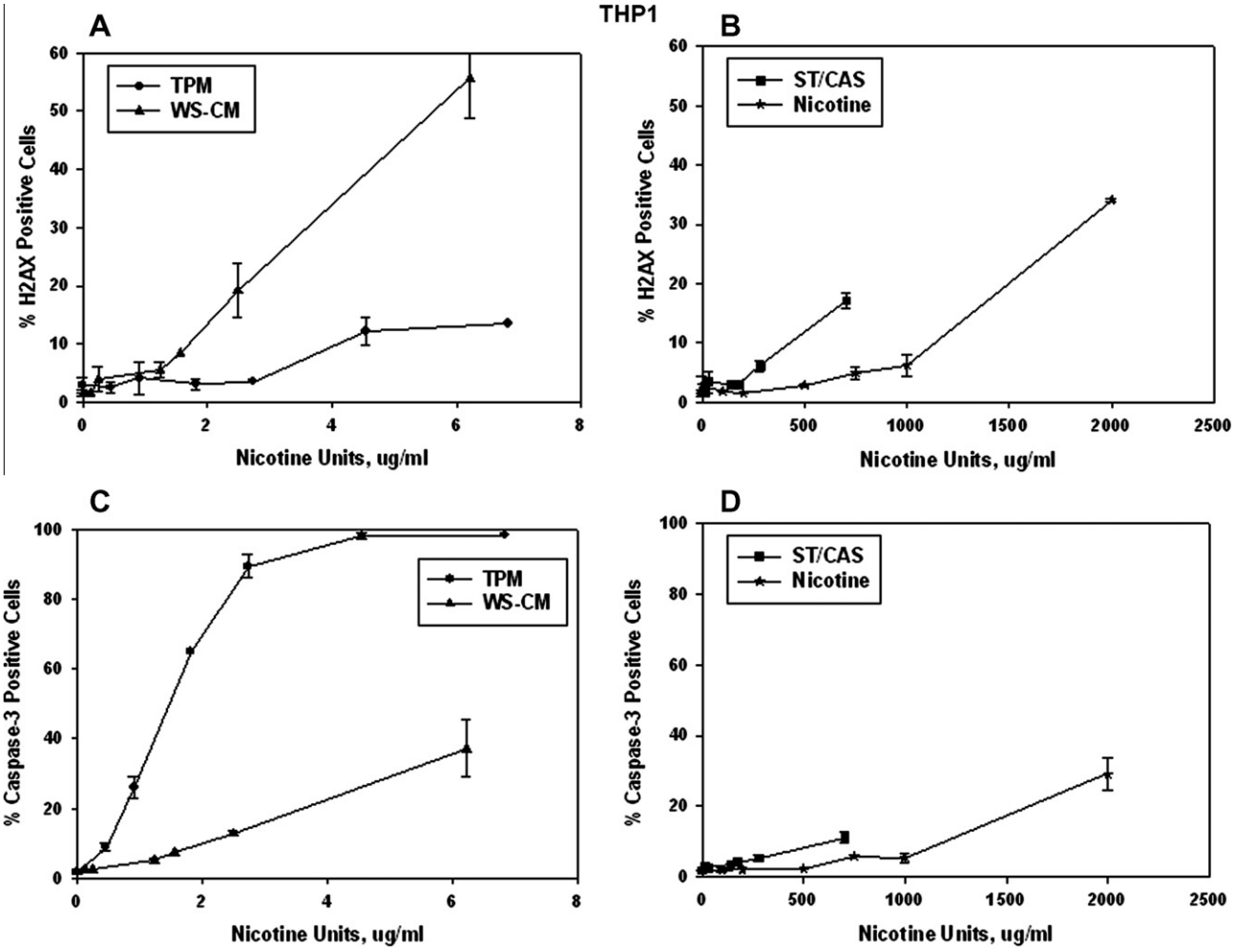

Fig. 4. Measuring DNA damage and caspase 3 activities of THP1 cells by different tobacco product preparations. Double-stranded DNA breaks were measured by staining with γ-H2AX (panels A and B) antibody and apoptosis was measured by labeling for activated caspase 3 (panels C and D) by flow cytometry. Each point represents the mean ± SEM of three independent experiments.

the study are: (1) the combustible TPPs, such as the WS-CM and TPM, were far more cytotoxic relative to the ST on an equi-nicotine basis in multiple cell culture models tested; (2) exposure to different TPPs induces graded cellular responses such as DNA damage and inflammation, and (3) the leukocyte subsets exhibit differential susceptibility to TPPs.

The results of the *in vitro* studies presented in this manuscript are consistent with the known relative risks associated with tobacco consumption and the relative risks associated with combustible and non-combustible tobacco products (Zeller et al., 2009). There is greater risk from the use of combustible tobacco products than from non-combustible tobacco products, such as smokeless tobacco products (Hatsukami et al., 2002; Zeller et al., 2009). Collectively the data from the *in vitro* studies presented herein clearly demonstrate cytotoxicity, DNA damage and inflammation (as measured by IL8 secretion) are observed at very low equi-nicotine units of TPM and WS-CM relative to ST and nicotine, with nicotine being the least toxic.

Data presented herein generally support the previously published findings that cigarette smoke exposure (TPM and WS-CM) induces inflammatory responses, DNA damage, apoptosis and marked cytotoxicity (Johnson et al., 2009). There is relatively limited information on the cytotoxic effects of exposure to smokeless TPPs, and few studies assessed the relative effects of exposure to combustible and non-combustible TPPs. Given the heterogeneous nature of the various tobacco product preparations, a direct comparison of the effects of the respective preparations is difficult.

One approach used to compare the relative effects of various TPPs exposures is to use the nicotine content of the preparations. An advantage of determining the chemical constituents of various TPPs also enables to compare the data obtained using different batches of the preparations and helps us understand the biological consequences of exposure. Since these preparations contain many chemicals, additional chemical analyses will be required. It is important to note that the observed biological effects in this study are attributable to the sample preparations used for exposure rather than to any given chemical constituent. Although this approach compares the relative effects of chemically different combustible and ST preparations, it is useful to broadly gauge differences in the biological effects, and has been used in the past to compare the relative mutagenic activities of the TPPs (Rickert et al., 2009, 2007).

Our finding that ST causes minimal cytotoxicity differs from some of the recently published work (Joyce et al., 2010; Lombard et al., 2010; Mitchell et al., 2010). Mitchell et al. (2010) reported extreme cytotoxicity to the exposure of 100 mg/mL (1%) ST and reported cell death after 3 h of exposure. The lack of consensus on the cytotoxic effects of ST may be attributed to the experimental conditions and methods used in the preparation of the ST. In the past, investigators have used ST extracts prepared by different

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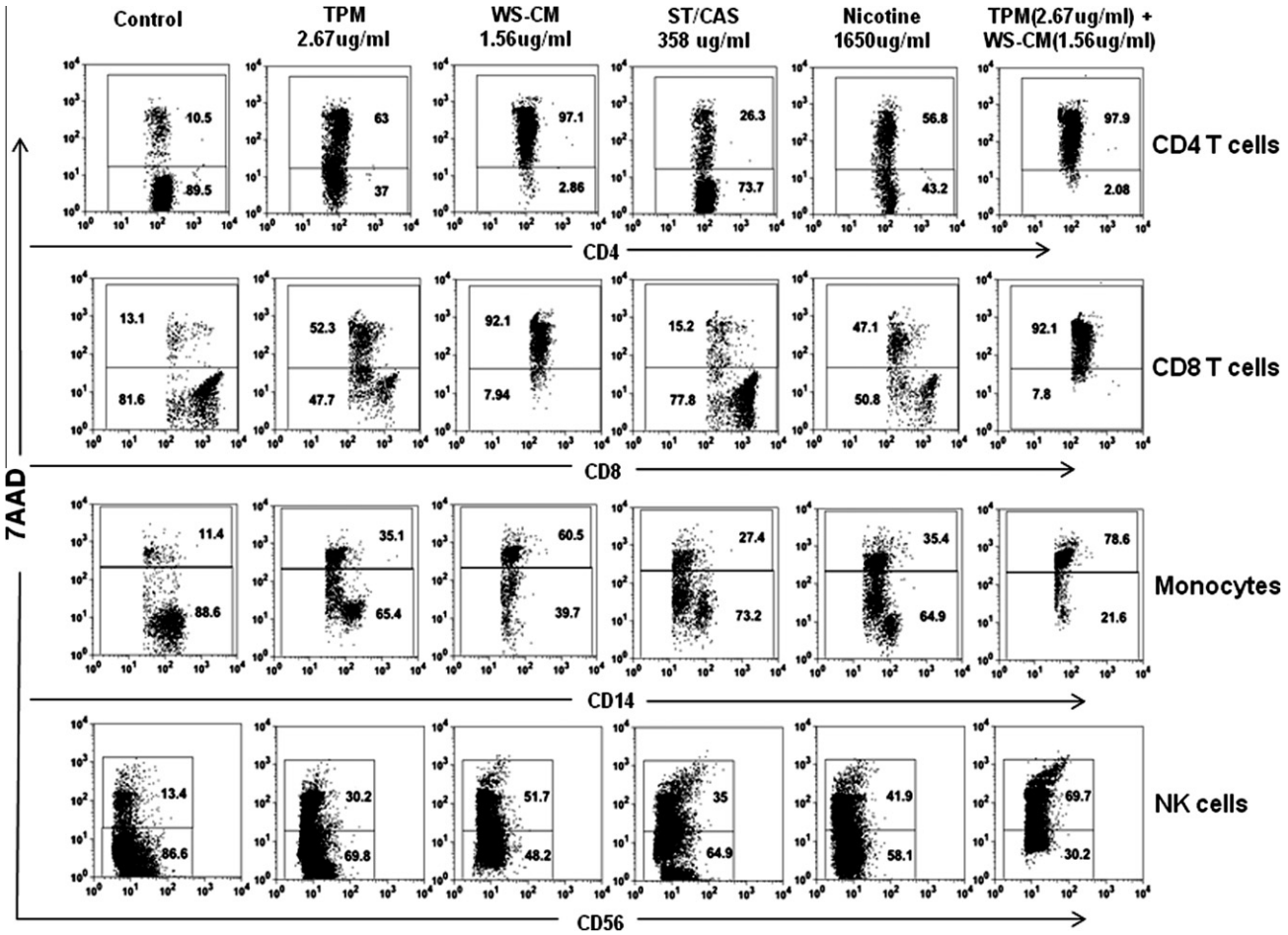

Fig. 5. Effect of TPM, WS-CM, ST/CAS and nicotine on leukocyte subsets. PBMCs were exposed to EC_{50} concentrations of TPM, WS-CM, ST/CAS and nicotine for 24 h and the cell death was measured by flow cytometry using 7AAD stain. Cells were labeled with antibodies specific to Helper T cells (CD4), cytotoxic T cells (CD8), monocytes (CD14) and NK cells (CD56). Data are representative of four independent experiments using four different donor PBMCs.

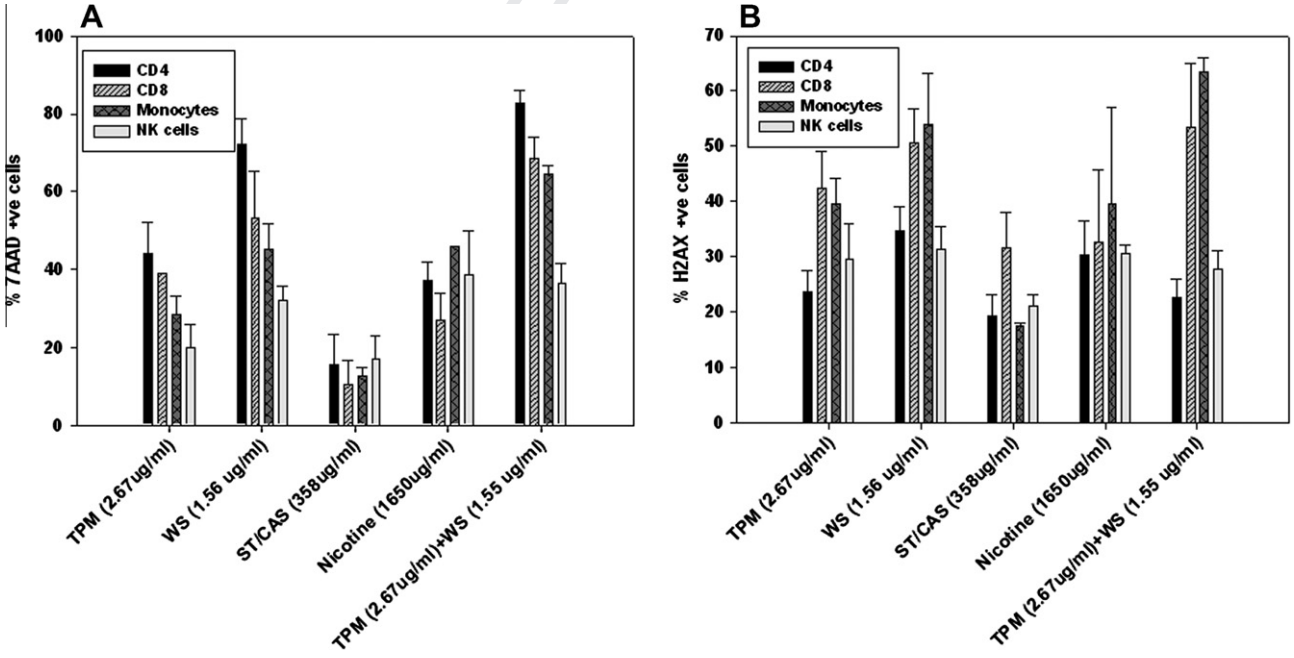

Fig. 6. Cytotoxic effects of TPM, WS-CM, ST/CAS and nicotine on different leukocyte subsets. PBMCs were exposed to EC_{50} concentrations of TPM and WS-CM for 24 h; the concentrations of ST/CAS and nicotine used were indicated. Cell death was measured by 7AAD (panel A) and DNA damage was assessed by γ-H2AX staining (panel B). Cells were also labeled to detect CD4, CD8, monocyte and NK cells by cell surface staining with appropriate antibodies. Data represents the mean ± SEM of four independent experiments from four different donor PBMCs.

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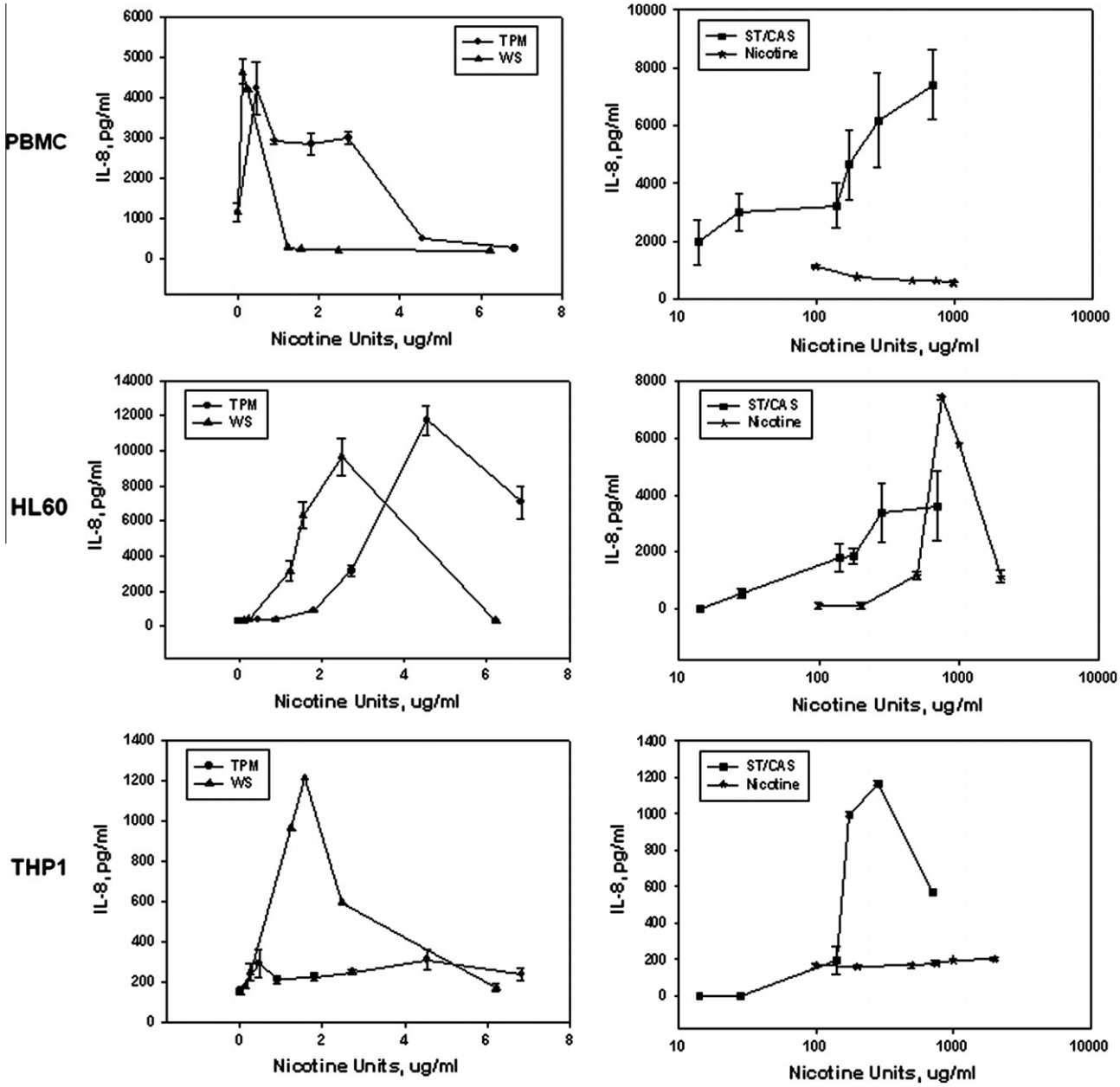

Fig. 7. Induction of IL8 secretion by tobacco product preparations: PBMC (from four donors), HL60 and THP1 cells were exposed to different TPPs at the indicated concentrations of TPM, WS-CM (left panel), and ST/CAS and nicotine (right panel) for 24 h. Culture supernatants were collected and IL8 cytokine levels were measured by Elisa. Data are representative of three independent experiments with ± SEM.

methods when treating cells (Mitchell et al., 2010; Petro, 2003; Petro et al., 1999). Although the reported nicotine concentrations (1.17 mg/mL) of those preparations are comparable to that used in the current study (in the range of 1.4 mg/mL; Table 1), the cytotoxic effects of ST extract are relatively modest and dose-dependent. A explanation for the lack of consensus is that a higher concentration of ST is achieved with a direct extraction method (Joyce et al., 2010; Lombard et al., 2010; Mitchell et al., 2010). It is not clear whether direct extraction into the medium of higher concentrations of ST would compromise the ability of the medium to support cell growth.

Exposure to cigarette smoke has been shown to induce necrotic or apoptotic cell death (Hellermann et al., 2002; Liu et al., 2005; Wickenden et al., 2003; Yang and Liu, 2004). We have assessed caspase 3 activation as a measure of apoptotic cell death. TPM induces marked and dose-dependent activation of caspase 3 in THP1 cells; WS-CM, on the other hand, does not. Both nicotine and ST/CAS also induce activation of caspase 3. Thus, it is likely that the mechanisms of cytotoxicity induced by the two phases of cigarette smoke could be different, and multiple cell death pathways could potentially contribute to cigarette smoke-induced *in vitro* cytotoxicity.

Exposure to cigarette smoke causes double-stranded DNA breaks, which can be measured by γ-H2AX levels (Albino et al., 2004, 2006). Our finding that ST exposure causes dose-dependent DNA damage is consistent with a previous report (Coppe et al., 2008). Nicotine, at higher concentrations (>8 mM), has been shown to induce internucleosomal DNA cleavage in HL60 and ML-1 leukemia cell lines (Yoshida et al., 1998). Consistent with these findings, the levels of γ-H2AX were clearly elevated after treatment with all TPPs and nicotine although the exposure required detecting DNA

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damage differed significantly among the reagents tested herein (ranging from a few micrograms/ml of equi-nicotine units in the combustible TPPS to 2000 μg/ml of nicotine) (Figs. 3 and 4A and B).

Exposure to the combustible TPPs has been suggested to cause oxidative stress and damage, which in turn leads to DNA damage (Albino et al., 2006). Additional work is necessary to elucidate whether the DNA damage caused by ST exposure at the doses tested involves similar mechanisms. The relatively low level of DNA damage caused by ST may be a contributing factor in the observed lower cytotoxicity from exposure to these products. Based on our (Fig. 4B) and published (Yoshida et al., 1998) data which show that nicotine–induced DNA damage occurs at high mM doses, it appears that nicotine may cause DNA damage and cell death through non-specific mechanisms.

One of the consequences of cigarette smoke exposure is the elicitation of inflammatory responses, as evidenced by secretion of IL8. IL8 is a critical signaling protein for recruitment of neutrophils and can be secreted by several cell types (Goncalves et al., 2010; Kobayashi, 2008; Larsen et al., 1989; Tomita et al., 2003). Expression and secretion of IL8 from acute exposure to the combustible tobacco preparation has been demonstrated (Fields et al., 2005; van Leeuwen et al., 2005; Yang et al., 2006).

There is limited information on the *in vitro* effects of tobacco products on PBMCs. To our knowledge, this is the first study to assess the effects of tobacco exposure on cytotoxicity and DNA damage to PBMC subsets. PBMC subsets exhibited differential cytotoxicity when exposed to TPM, WS-CM or TPM/WS-CM combination in the following order: CD4 > CD8 > monocytes > NK cells (Figs. 5 and 6A). It also appears that WS-CM is more potent in causing DNA damage and cell death compared to TPM. In contrast, the treatment with nicotine and ST/CAS was less cytotoxic and did not exhibit cell type selectivity. Double strand DNA breaks, as evaluated by γ-H2AX levels, suggest that CD8 and monocytes appear to exhibit higher γ-H2AX, compared to CD4 cells (Fig. 6B). Understanding the mechanisms of differential susceptibility of leukocytes to different TPPs and the consequent biological effects require additional research.

In summary, we have shown that exposure to combustible and non-combustible tobacco product preparations results in quantitatively different cellular responses, particularly cytotoxicity, DNA damage and inflammation. The combustible preparations TPM and WS-CM consistently perturbed cell physiology at much lower equi-nicotine units relative to ST and nicotine. Further work is necessary to define the molecular mechanisms of ST-induced biological effects. The research presented herein is an important step in that direction and understanding the effects of exposure to different tobacco products.

Conflict of interests

The authors declare that there are no conflicts of interest.

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