CYTOTOXIC EFFECTS OF DIFFERENT TOBACCO PRODUCT PREPARATIONS ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Objective of the Study

Assess cytotoxic effects of combustible and non-combustible tobacco product preparations with human peripheral blood mononuclear cells.

Background

- Several experimental systems, particularly cell culture models, are utilized to assess the short-term effects of tobacco exposure.
- Cellular responses to smoke exposure (or its constituents) include:
  - marked cytotoxicity
  - induction of inflammatory responses
- Since inflammatory responses are primarily driven by hemato-poietic cell types, we focused on human Peripheral Blood Mononuclear Cells (PBMCs).
- Previously we showed that combustible and non-combustible tobacco product preparations differ in cytotoxicity and we have extended those initial findings.

Study Materials

Combustible Tobacco Product Preparations:

- **TPM (Total Particulate matter, also referred to as CSC, cigarette smoke condensate) prepared by smoking 3R4F reference cigarettes (35ml-60sec-2sec, puff volume, frequency and duration, respectively) and dissolving the particulate phase in DMSO.**
  - **Vehicle control: DMSO**
- **Whole Smoke-Conditioned Medium (WS-CM) prepared by passing smoke from 3R4F cigarettes through RPMI 1640 medium without phenol red.**
  - **Vehicle control: culture medium**

Study Materials

Non-combustible Tobacco Product Preparations:

- **ST/CAS (Smokeless Tobacco in Complete Artificial Saliva) prepared by extracting 2S3 smokeless tobacco (reference moist snuff) in Complete Artificial Saliva (CAS) for 2h followed by filtration.**
  - **Vehicle control: Complete Artificial Saliva (CAS)**

- **Pure Nicotine (Sigma) was used as a reference.**

Analysis of Tobacco Product Preparations Used in the Study

		Tobacco specific nitrosamines (TSNA)				benzo[a]pyrene
	Nicotine [µg/mL]	NNN [pg/mL]	NAT [pg/mL]	NAB [pg/mL]	NNK [pg/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	1258 ± 113	999 ± 58	212 ± 34	998 ± 99	< 0.013
ST/CAS (10%, W/V)	1408	164	92.4	≤ 10.3 but > 3.10	39.8	BDL

BDL=Below the Detection Limit

We quantitated the content of nicotine, NNN, NAT, NAB,NNK and B [a]P in TPM, WS-CM and ST/CAS. We have used nicotine content (expressed in µg/mL) of the preparations as a measure of exposure. TSNA and B[a]P values are derived from one analysis.

Peripheral Blood Mononuclear Cells (PBMCs)

What are PBMCs?

- **PBMCs are any circulating blood cells having a round nucleus. They are critical components of immune system and inflammatory responses.**

- **PBMCs consist of different cell subtypes: Example…**
  - **Helper T lymphocytes (CD4), Cytotoxic T lymphocytes (CD8), B cells, Monocytes and NK cells.**

Peripheral Blood Mononuclear Cells (PBMCs)

In this study:

- We collected blood from healthy human subjects who were non-tobacco users.
- Blood was processed within 2h of collection.
- PBMCs were isolated from density gradient centrifugation method and were stored frozen in liquid nitrogen for further use.

Dr. Bobbette Jones (RJRT) coordinated the collection of blood through a CRO and managed the clinical phase of the study per Good Clinical Practices.

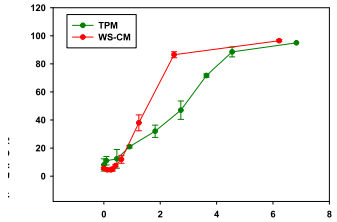
Assays Performed

Assay	Target cell marker	How does the assay work?
Cell death (7AAD)	Dead cells	7-Amino-Actinomycin D (7-AAD) is a nucleic acid dye that labels dead cells
Live/dead cell assay	Live cells & dead cells	Red stain binds metabolically active live cells & green stain binds dead cells
Apoptosis (Caspase-3)	Late apoptosis	Intracellular red stain that binds to active caspase-3
DNA damage (H2AX)	Double strand DNA breaks (DSBs)	Histone H2A in the nucleosome undergoes phosphorylation on ser-139 in DSBs (γH2AX)
Inflammation	IL-8	IL-8 ELISA

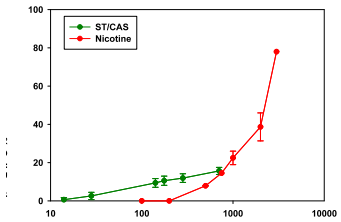
Effect of Different Tobacco Product Preparations on PBMC Cell Death

PBMCs were cultured in the presence of varying amounts of tobacco product preparations for 24h and cell death was measured. Data are presented as equi-nicotine units.

TPM & WS-CM



ST/CAS & Nicotine

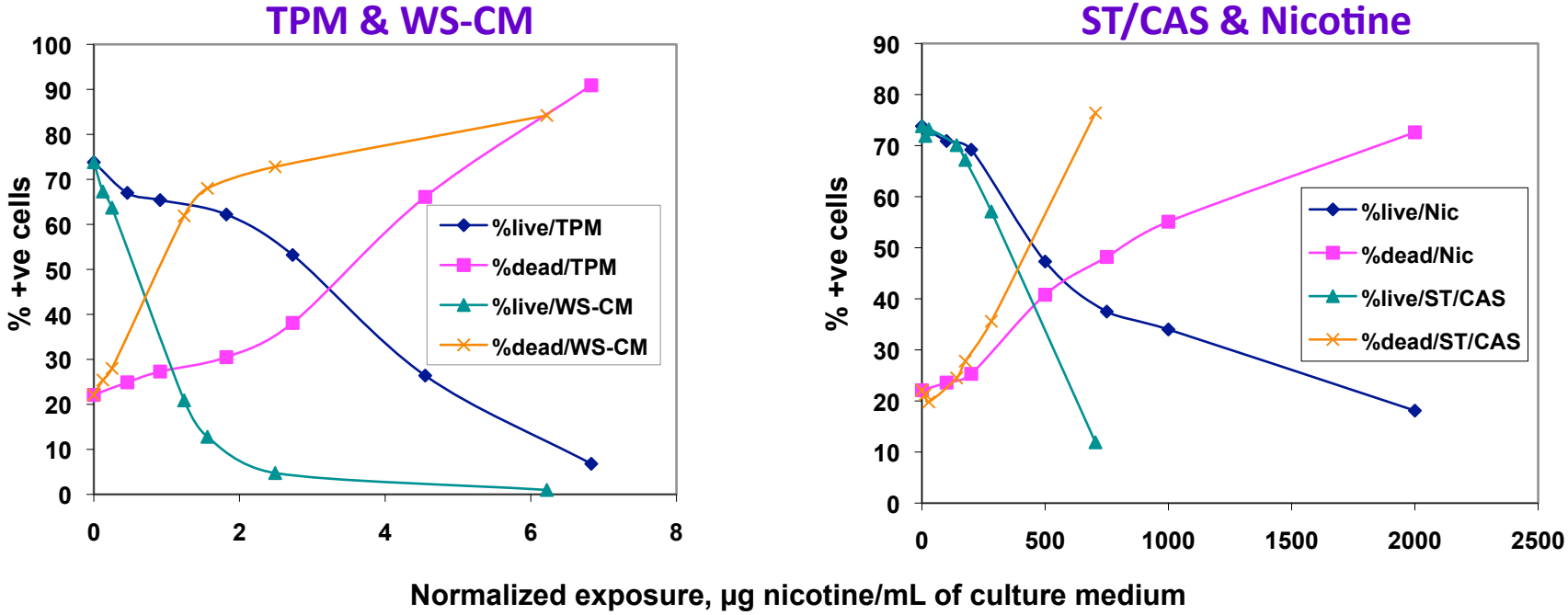


Normalized exposure, µg nicotine/mL of culture medium

TPM & WS-CM cause cell death in PBMCs at lower nicotine units than ST/CAS & pure nicotine.

Effect of Different Tobacco Product Preparations on PBMC Live cell/Dead cell Assay

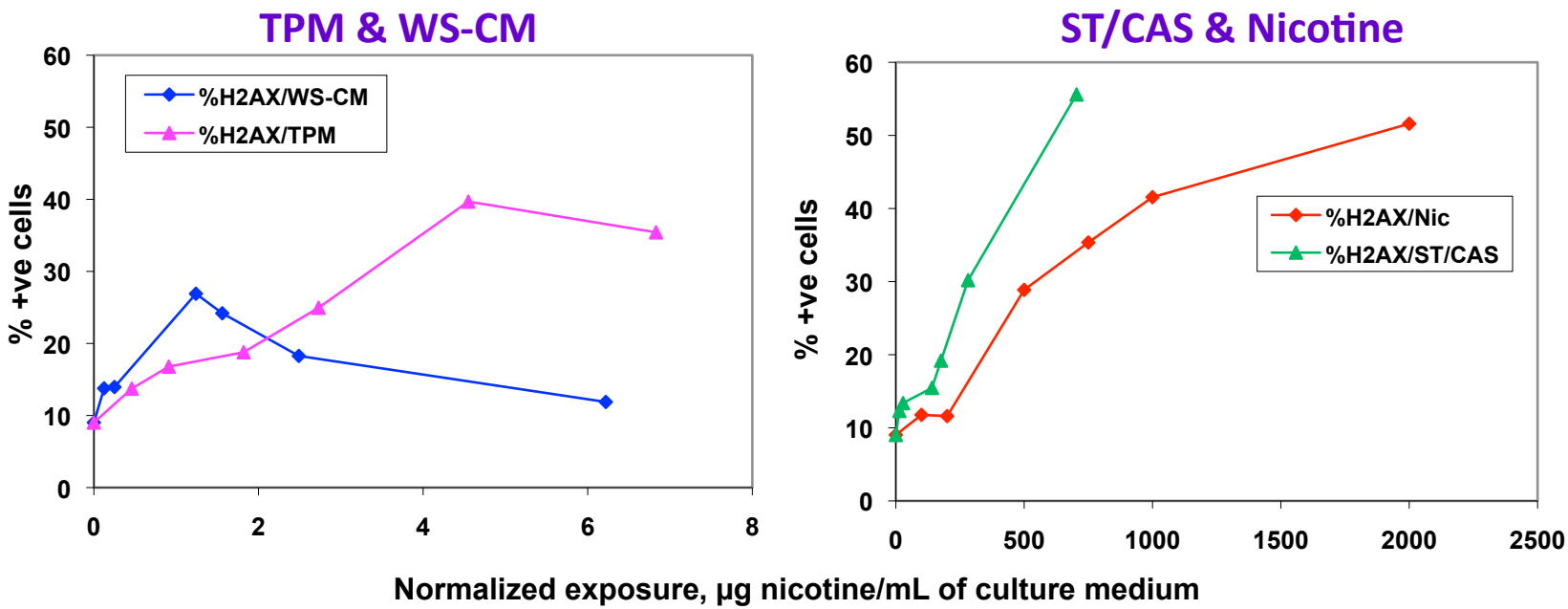
PBMCs were cultured with varying amounts of tobacco product preparations for 24h and cell death was measured by live cell and dead cell assay.



TPM & WS-CM are cytotoxic at much lower nicotine units than ST/CAS and nicotine in live cell/dead cell assay.

Effect of Different Tobacco Product Preparations on PBMC DNA Damage

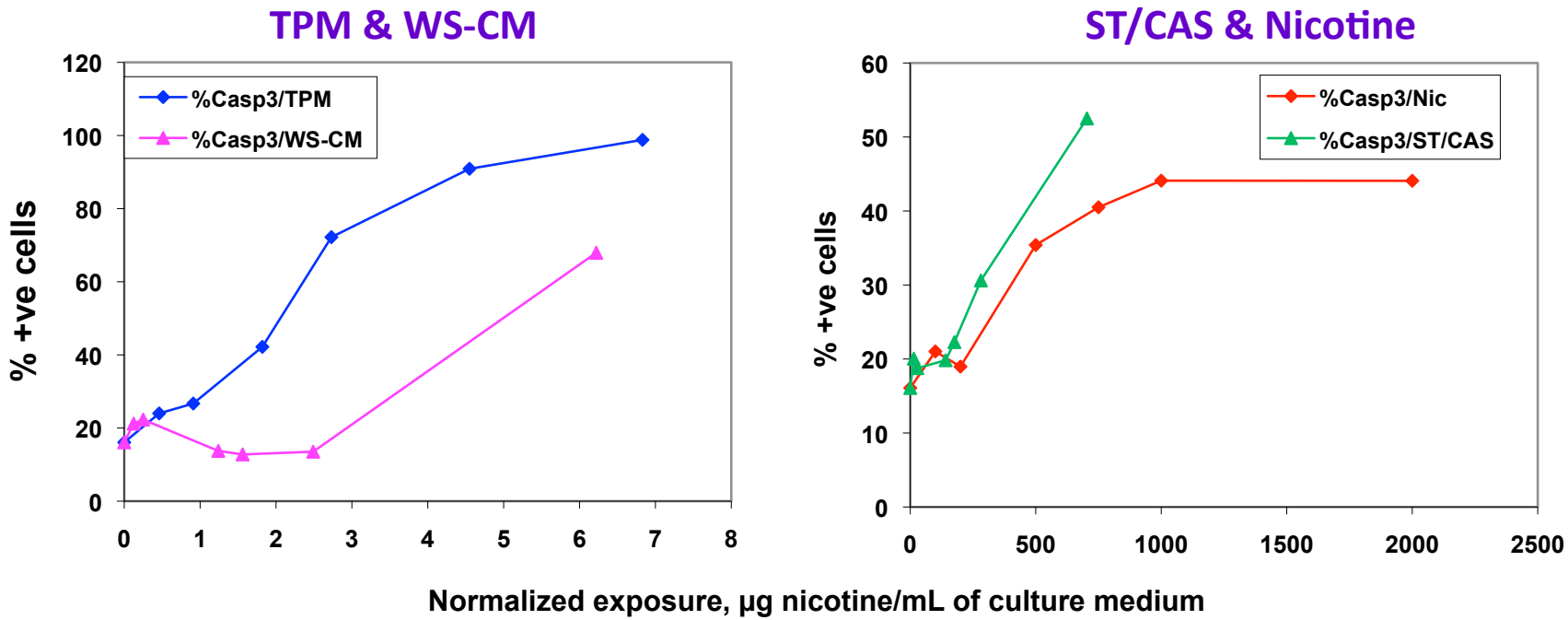
PBMCs were cultured in the presence of varying amounts of tobacco product preparations for 24h and double stranded breaks were measured by H2AX staining.



TPM & WS-CM induced DNA double stranded breaks at much lower nicotine units than ST/CAS and nicotine.

Effect of Different Tobacco Product Preparations on PBMC Apoptosis

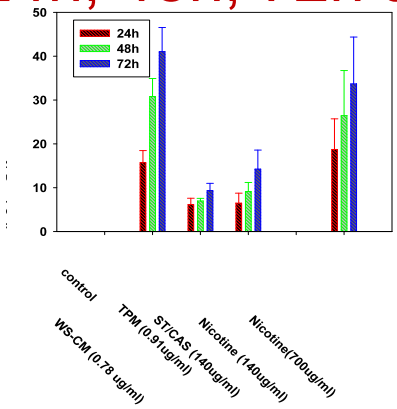
PBMCs were cultured in the presence of varying amounts of tobacco product preparations for 24h and apoptosis was measured by active caspase-3 staining.



TPM & WS-CM induced apoptosis at much lower nicotine units than ST/CAS and nicotine.

Time Dependent Cell Death of PBMC with Different Tobacco Product Preparations

PBMCs were treated at indicated concentrations for 24h, 48h, 72h and cell death was measured.



A lower concentration of WS-CM causes greater time-dependent cell death in PBMCs than TPM, ST/CAS or nicotine.

Cytotoxicity of Different Tobacco Product Preparations - EC_{50} Values

The EC_{50} is determined to be the concentration at which 50% of the cells are no longer viable in a 24h cell death assay and the values are expressed as µg/ml equi-nicotine units.

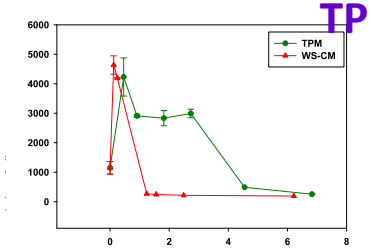
Treatment	EC_{50} (µg/ml)
TPM	2.67±0.15
WS-CM	1.55 ±0.07
ST/CAS	>700*
Nicotine	1650 ±70

EC_{50} values in terms of nicotine units suggests differential toxicity of tobacco product preparations as follows: WS-CM >TPM > ST/CAS, nicotine. * The highest concentration of ST/CAS tested in this study.

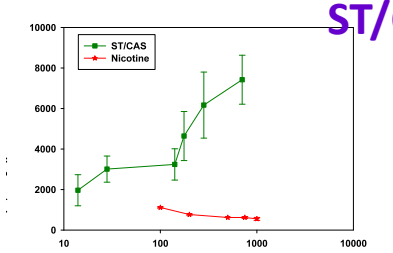
IL-8 Levels in PBMCs Treated with Tobacco Product Preparations

IL-8 is a chemokine and one of the major mediators of the inflammatory response. It serves as a chemical signal that attracts neutrophils at the site of inflammation. A well recognized cellular response to exposure to smoke or its constituents is IL-8 secretion.

TPM & WS-CM



ST/CAS & Nicotine



Normalized exposure, µg nicotine/mL of culture medium

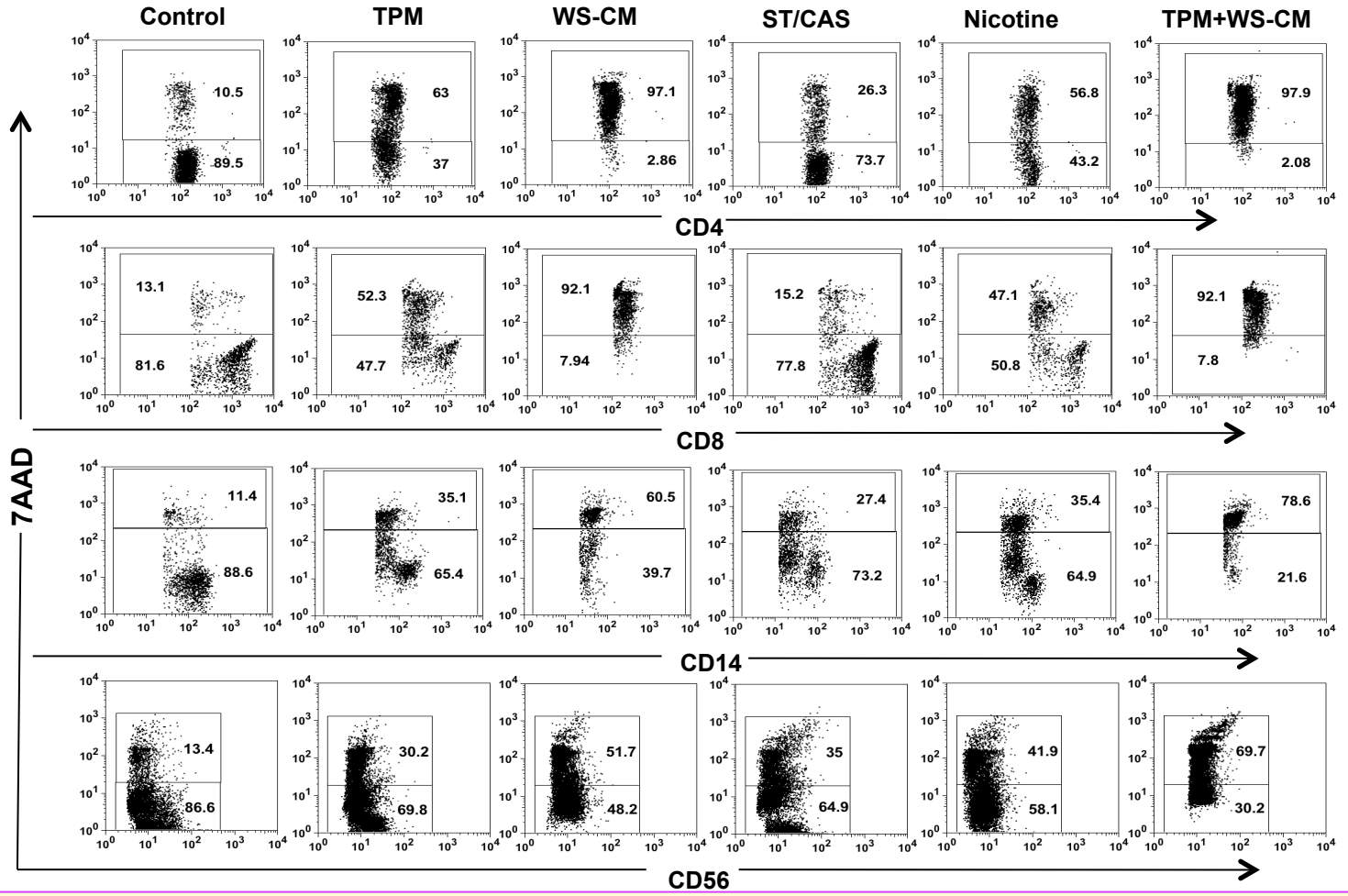
PBMCs secrete IL-8 with TPM, WS-CM & ST/CAS treatments

Cytotoxic Effects of Tobacco Product Preparations on Different Leukocyte Subsets of PBMCs

Experimental Design

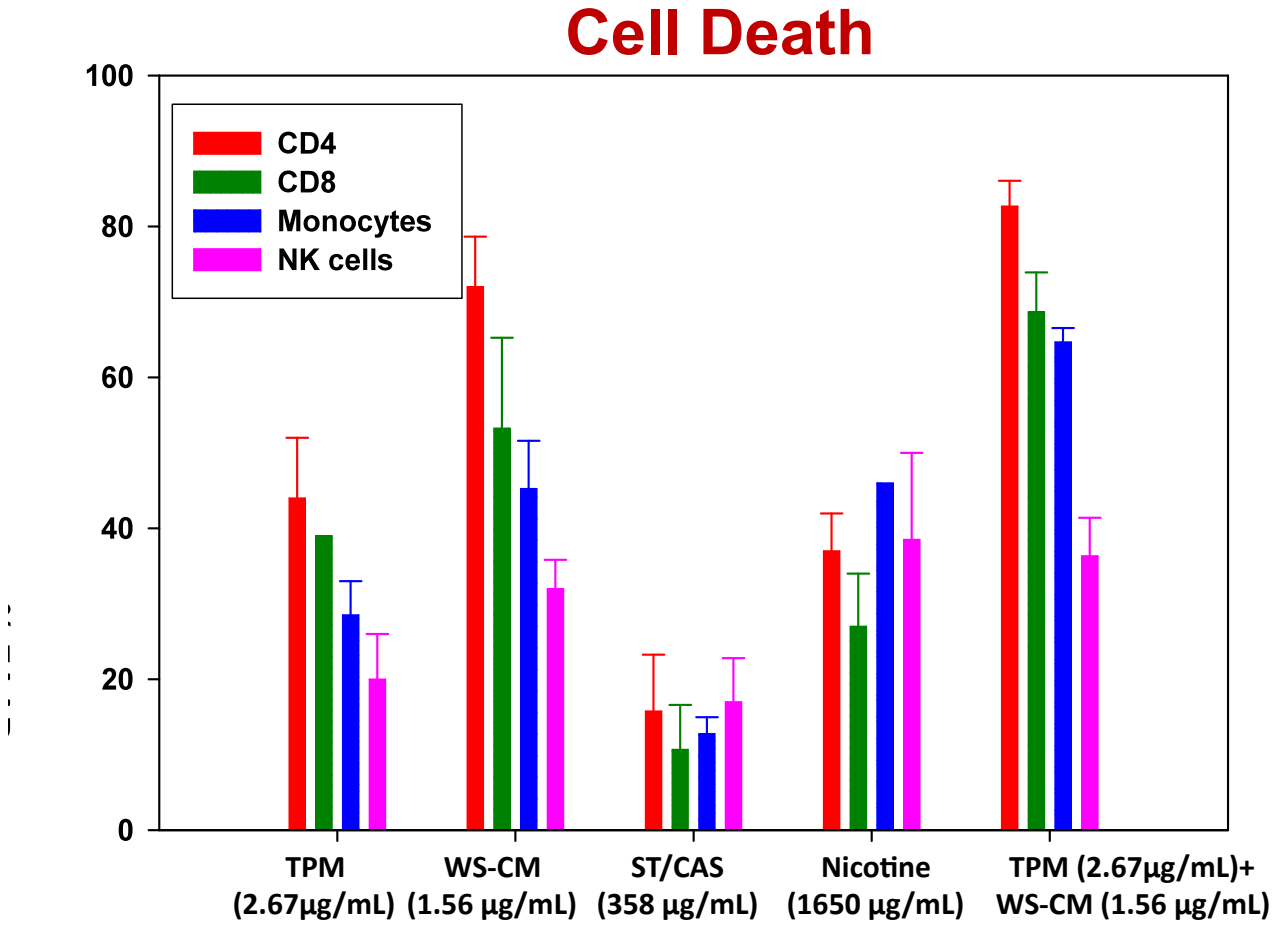
- PBMCs exposed
  - EC_{50} values
    - TPM (2.67 µg/mL)
    - WS-CM (1.56 µg/mL)
    - Nicotine (1650 µg/mL)
  - ST/CAS (358 µg/mL)
  - TPM+WS-CM (2.67 µg/mL)+ (1.56 µg/mL)
- For 24h and stained for
  - Helper T cells (CD4)
  - Cytotoxic T cells (CD8)
  - Monocytes (CD14)
  - NK cells (CD56)
- Measured cell death by 7AAD staining on the flow cytometer

Cytotoxic Effects of CSC, WS-CM, ST/CAS and Nicotine in Different Leukocyte Subsets of PBMCs



WS-CM > TPM > ST/CAS treatments induce cell death in all the leukocyte subsets. Leukocyte subsets exhibited differential cytotoxicity as follows: CD4 > CD8 > monocytes > NK cells.

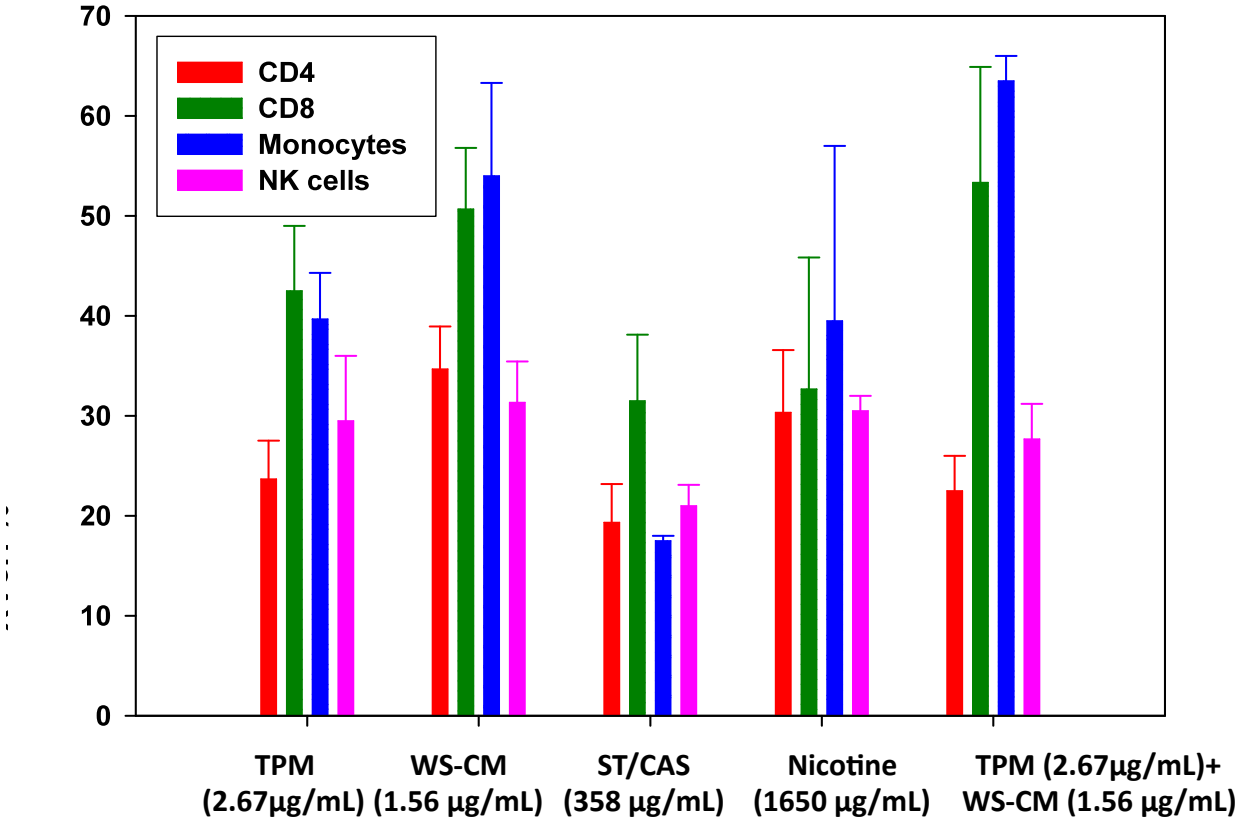
Cytotoxic Effects of TPM, WS-CM, ST/CAS and Nicotine in Different Leukocyte Subsets of PBMCs



WS-CM > TPM > ST/CAS treatments induce cell death in all the leukocyte subsets. Leukocyte subsets exhibited differential cytotoxicity: CD4 > CD8 > monocytes > NK cells.

Double Stranded Breaks with TPM, WS-CM, ST/CAS and Nicotine in Different Leukocyte Subsets of PBMCs

DNA Double Stranded Breaks



WS-CM > TPM > ST/CAS treatments induce double stranded breaks in all the leukocyte subsets. DNA double stranded breaks occur at greater frequencies in CD8 cells and monocytes than in CD4 and NK cells.
There is an additive effect of TPM+WS-CM.

Summary and Conclusions

- **The cytotoxic effects of tobacco product preparations on PBMCs vary.**
  - **As determined in this study, combustible tobacco product preparations are far more cytotoxic than the non-combustible tobacco product preparations: WS-CM>TPM>ST/CAS, nicotine.**

- **Exposure to tobacco product preparations elicited inflammatory responses and double stranded DNA damage in PBMCs.**
  - **Combustible tobacco product preparations were markedly more potent than the non-combustible tobacco product preparations.**
  - **Leukocyte subsets exhibited cell-type specific responses.**

- **These findings may be useful in understanding the biological effects of exposure to different categories of tobacco products.**

This work was funded by R.J. Reynolds Tobacco Company, and performed at Wake Forest Baptist Health under a collaborative research agreement.