Video Article

Methods to Evaluate Cytotoxicity and Immunosuppression of Combustible Tobacco Product Preparations

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Abstract

Among other pathophysiological changes, chronic exposure to cigarette smoke causes inflammation and immune suppression, which have been linked to increased susceptibility of smokers to microbial infections and tumor incidence. *Ex vivo* suppression of receptor-mediated immune responses in human peripheral blood mononuclear cells (PBMCs) treated with smoke constituents is an attractive approach to study mechanisms and evaluate the likely long-term effects of exposure to tobacco products. Here, we optimized methods to perform *ex vivo* assays using PBMCs stimulated by bacterial lipopolysaccharide, a Toll-like receptor-4 ligand. The effects of whole smoke-conditioned medium (WS-CM), a combustible tobacco product preparation (TPP), and nicotine were investigated on cytokine secretion and target cell killing by PBMCs in the *ex vivo* assays. We show that secreted cytokines IFN-γ, TNF, IL-10, IL-6, and IL-8 and intracellular cytokines IFN-γ, TNF-α, and MIP-1α were suppressed in WS-CM-exposed PBMCs. The cytolytic function of effector PBMCs, as determined by a K562 target cell killing assay was also reduced by exposure to WS-CM; nicotine was minimally effective in these assays. In summary, we present a set of improved assays to evaluate the effects of TPPs in *ex vivo* assays, and these methods could be readily adapted for testing other products of interest.

Video Link

The video component of this article can be found at https://www.jove.com/video/52351/

Introduction

A substantial body of knowledge points to the adverse health effects of chronic cigarette smoking, including cardiovascular disease (CVD), chronic obstructive pulmonary disease (COPD) and cancer[1,2]. Chronic cigarette smoking has been known to cause inflammation and immune suppression, and these alterations are reported to contribute to increased risk of microbial infection and cancer in smokers[3]. *In vitro* and *ex vivo* techniques are useful in elucidating the molecular basis of the pathophysiological effects of cigarette smoke[4-9] (**Table 1**) and are recognized as important tools for guiding the emerging regulation of various tobacco products[10,11].

For example, we have demonstrated that combustible tobacco product preparations (TPPs) such as whole smoke-conditioned medium (WS-CM) and total particulate matter (TPM) are far more cytotoxic and damaging to DNA than non-combustible TPPs or nicotine[12,13]. Consistent with the published work, it was recently reported that combustible TPPs or nicotine[12,13]. Consistent with the published work, we recently reported that combustible TPPs caused marked immunosuppression. This was evidenced by suppression of Toll- like receptor (TLR)-ligands, stimulated cytokine secretion, and targeted cell (K562) killing by PBMCs in an *ex vivo* model[14]. Given the significance of inflammation in cigarette smoke-induced disease processes, further optimization of the assay conditions to evaluate the immune modulatory effects of cigarette smoke is presented in this report.

The *ex vivo* assays typically measured intracellular and secreted cytokines as well as the cytolytic function of cytotoxic T and NK cells in K562 cell killing assays[14]. The assays involved pre-incubation with WS-CM and nicotine and subsequent stimulation of PBMCs with TLR agonists over a period of 3 days; the final readouts are performed using enzyme-linked immunosorbent assays (ELISAs) and/or flow cytometry. We utilized bacterial lipopolysaccharide (LPS), which binds to TLR-4 receptors and stimulates PBMCs resulting in the production of intracellular cytokines and secretion of cytokines. In addition to optimization of the various assay steps for evaluating the immunomodulatory effects of TPPs, we also present methods for isolating PBMCs, cell death assays, and IL-8 quantification. These methods may be applied to address other research questions and further refined to evaluate tobacco products in the regulatory context.

Table 1. Published reports of *in vitro* and *ex vivo* methods used to study varilus pathophysiological effects of tobacco product preparations. CS, cigarette smoke medium; CSC, cigarette smoke condensate; CSE, cigarette smoke extract; ELISA, enzyme-linked immunosorbent assay; GADPH, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction; RT, real time quantitative polymerase chain reaction; TS, tobacco smoke.

Author (Year of study)	Laan *et al.* (2004)	Moodie *et al.* (2004)	Oltmanns *et al.* (2005)	Vayssier (1998)	Witherden *et al.* (2004)	Birrell *et al.* (2008)
Cells used	Human bronchial endothelium cells (BEAS-2B), human neutrophils	Human alveolar epithelial cells (A549)	Human airway smooth muscle cells (HASMC)	Human premonocytic U937 cells, human monocytes	Alveolar type II epithelial cells (ATII)	Human monocytic cell line (THP-1), human lung macrophages
TPP used	CSE	CSC	CSE	TS	CSE	CS
Method used	ELISA, qPCR, migration, electromobility shift	Immunohisto-chemistry, electrophoresis, Arrayscan kit, RT-PCR, ELISA	ELISA, RT-PCR, qPCR, electrophoresis	Gel-mobility shift	Light microscopy, electron microscopy, electrophoresis, ELISA	qPCR, ELISA, E-toxate kit (Sigma), p65 plate assay (TransAM), electrophoresis, various immunoassay kits
Measure	IL-8, GM-CF, AP-1, NF-κB, migration	Histone acetyltransferases, histone deacetylases, NF-κB, IL-8, p-I κB-α, GADPH	HO-1, GADPH, RANTES, IL-8, eotaxin	Heat shock/ stress proteins (HSP/Hsp70), HF transcription factor, NF-κB, TNF-α	Surfactant protein (SP-A, SP-C), IL-8, MCP-1, GRO-α, TNF-α, IL-1β, IFN-γ	IL-8, IL-1β, IL-6, TNF-α, MIP1-α, GRO-α, MAPK/JNK/ERK phosphorylation, cJUN:DNA binding, glutathione, p65:DNA binding
End result	CSE down-regulates cytokine production via suppression of AP-1 activation.	H_2O_2 and CSC enhance acetylation of histone proteins, decrease histone deacetylase activity, differentially regulate proinflammatory cytokine release.	Cigarette smoke may cause the release of IL-8 from HASMC, enhanced by TNF-α, 20% CSE less IL-8 release, Inhibition of eotaxin and RANTES by cigarette smoke.	TS activated HF transcription factor, which was associated with Hsp70 overexpression and inhibition of NFkB binding activity and TNF-α release.	Reduced ATII cell-derived chemokine levels compromise alveolar repair, contributing to cigarette smoke-induced alveolar damage and emphysema.	Data provide mechanistic explanation for why smokers have increased respiratory infections. Suppression of the innate response is accompanied by an increase in IL-8.

Protocol

NOTE: Written Informed consent to do this study was obtained at a local clinical research unit under IRB approval, per Good Clinical Practices. Processing of blood, isolation of PBMCs and other cell culture experiments are performed under sterile conditions, using microbiologically sterile supplies and reagents.

1. WS-CM Preparation

1. Generate WS-CM as previously described[12].
    1. Prepare WS-CM by passing smoke from four 3R4F reference cigarettes through Roswell Park Memorial Institute (RPMI) 1640 medium without phenol red using the following smoking regimen: 35-60-2, puff volume in ml, puff interval in sec, and puff duration in sec, respectively. Each preparation generates a 20 ml sample.

2. Label tube(s) with date, time completed, and cigarette name and number. Store the 500 µl aliquots at -80 °C immediately after smoking is completed.
3. Analyze aliquots of frozen WS-CM to determine the levels of nicotine, tobacco specific nitrosamines, and polycyclic aromatic hydrocarbons as previously described[12].

2. Isolation of PBMCs

1. Collect fresh blood from healthy donors (who are non-consumers of tobacco products) Isolate PBMCs from fresh blood as described below under a separate approval from Wake Forest Baptist Health IRB[15].
    1. Prior to the arrival of the blood bag, have a 500 ml bottle, scissors, isolation buffer, and Dulbecco's phosphate buffered saline (DPBS) (RT) ready in a biosafety level 2 (BSL-2) cell culture hood. Isolation buffer must be protected from light.
    2. Hold the blood bag upside down and cut the tube just below where it has been clamped leaving at least 3 cm of tubing.

3. Remove the cap from the 500 ml bottle and hold the tube above the bottle opening. Pick up the blood bag and invert it to allow the blood to flow freely from the bag into the bottle until the bag is empty. Allow the blood to flow onto the inside wall of the bottle versus straight down as to avoid creating bubbles.
4. Pour isolation buffer into the bottle at a 1:5 ratio of isolation buffer to blood. Cap the bottle tightly and gently invert it, end-over-end, 10 times. Leave the bottle in the cell culture hood to incubate with lights off, for 1 hr at RT.
5. A light, straw-colored layer will build up above the blood. Remove this layer using a 25 ml serological pipette into 50 ml conical tubes. The collected amount may vary from 50 - 300 ml, depending on the subject who donated blood.
6. Centrifuge the tubes at 200 x g for 10 min at RT.
7. Aspirate the translucent supernatant, leaving the dark blood-colored pellet. The pellet will be loose but viscous.
8. Pipette 3 ml of isolation buffer into 15 ml conical tubes.
9. To the resuspend pellet, add 20 ml of DPBS for every 50 ml of straw-colored liquid collected in step 2.1.5. Vortex to mix thoroughly, and consolidate the resuspended liquid from multiple tubes. This liquid contains suspended blood cells.
10. With a transfer pipet, transfer 5 ml of the suspended blood cells onto 3 ml of isolation buffer in step 2.1.8. Tilt the 15 ml conical tube that contains the cell suspension slowly and gently to create two separate layers. Centrifuge the tubes at 400 x g for 40 min at RT with minimal acceleration and without brake.
11. Use a transfer pipet to remove the resulting cloudy middle layer (buffy coat) containing PBMCs into a 50 ml conical tube. Avoid drawing other clear layers below it. Transfer no more than 25 ml into each 50 ml conical tube.
12. Add 25 ml of cold running buffer (or more to fill the entire remaining volume of the 50 ml conical tube) to wash the cells. Centrifuge the cells at 400 x g for 10 min at 4 °C.
13. Resuspend the pellet with 10 ml running buffer. This contains PBMCs. Count the cells and use immediately or place on ice for freezing.

3. Freezing and Thawing the PBMCs

1. Centrifuge the PBMCs that were collected in step 2.1.13 for 10 min at 400 x g.
2. Fill the freezing container with isopropyl alcohol per the manufacturer's instructions. CAUTION: Isopropyl alcohol is flammable and acutely toxic.
3. Resuspend the pellet with RPMI 1640 medium (4 °C) containing 20% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). This is RPMI 1640 freezing medium. Resuspend the pellet with an amount of freezing medium that will result in a suspension having about 5 x 10^7 cells/ml. The number of cells available for freezing will vary.
4. Dispense 1 ml aliquots of cell suspension into 2 ml cryotubes and place the cryotubes in the freezing container. Place the freezing container with cryotubes in a freezer at -80 °C to store O/N and then remove the cryotubes and transfer to store in a cryogenic freezer between -150 °C to -190 °C.
5. Remove the cryotube from cryogenic storage and thaw it rapidly with gentle agitation in a water bath at 37 °C.
6. Immediately transfer the thawed PBMCs in the cryotube into a 15 ml conical tube with 10 ml RPMI 1640 complete medium (4 °C) containing 10% FBS, 1% Pen/Strep and 1% L-glutamine. The contents of the thawed cryotube should be transferred as soon as possible to obtain maximal cell viability[15].
7. Centrifuge the PBMCs at 400 x g for 10 min.
8. Resuspend the pellet with 5 ml RPMI 1640 complete medium and count the cells.
9. Measure cell viability by established methods such as trypan blue exclusion method. Generally cell viability with this method is about 90 - 95%. The PBMCs are now ready to use in experiments.

4. Cell Death Determination

NOTE: Dilutions listed here are for the purpose of this study. The dilutions can be changed accordingly.

1. Dilute WS-CM or nicotine in a 96-well plate using RPMI complete medium to a total volume of 100 µl/well, as indicated below.
2. Dilute WS-CM to the following concentrations: 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 µg/mL of equi-nicotine units (based on the nicotine content in WS-CM)[12].
3. Dilute nicotine in RPMI medium to the following concentrations: 100, 200, 500, 750, 1000, 2000, and 3000 µg/ml. CAUTION: Nicotine is acutely toxic and environmentally hazardous.
4. Add 100 µl of PBMCs suspended in RPMI complete medium to each well at a concentration of 1 x 10^6 cells/well. The total volume of cells plus WS-CM or nicotine will be 200 µl/well.
5. For the purpose of this study, prepare two sets of plates as above. Cover the plates and incubate one plate for 24 hr and one plate for 3 hr at 37 °C and 5% CO_2. Adjust time points as necessary.
6. Wash the cells at RT by centrifuging at 300 x g for 3 min, aspirating the supernatant, vortexing the bottom of the plate with plate covered and finally resuspending cells with 200 µl of ice cold running buffer, and repeat the washing step one more time.
7. Add 95 µl of running buffer followed by 5 µl of 7-aminoactinomycin D (7AAD) to each well for a total volume of 100 µl/well. Incubate the plate in the dark at RT for 15 min.
8. Add 100 µl of running buffer to cluster tubes. Transfer the entire volume of cell suspension from each well of the plate to the cluster tubes and acquire the samples on flow cytometer.
9. Determine the percentage of 7AAD-positive cells using flow cytometry analysis software.

5. EC_{50} Determination

1. The EC_{50} values of WS-CM and nicotine are determined by 7AAD-positive staining of PBMCs.

2. The EC_{50} value is defined as the concentration at which 50% of the cells were no longer viable in a 24 hr assay, and the values are expressed as µg of equi-nicotine units/ml.
3. For the purposes of this study, the EC_{50} values were determined to be 1.56 µg/ml and 1,650 µg/ml for WS-CM and nicotine, respectively.

6. Secreted Cytokines

NOTE: Dilutions listed here are for the purpose of this study. The dilutions can be adjusted accordingly.

1. Dilute WS-CM in a 96-well plate using RPMI complete medium to a total volume of 100 µl/well at the concentration of 0.3, 1.56, 3, and 5 µg/ml of equi-nicotine units.
2. Dilute nicotine to the following concentrations: 100, 200, 500, 750, 1000, 2000, and 3000 µg/mL. CAUTION: Nicotine is acutely toxic and environmentally hazardous.
3. Add 100 µl of PBMCs suspended in RPMI complete medium to each well at a concentration of 1 x 10^6 cells/well. The total volume of cells plus WS-CM or nicotine will be 200 µl/well.
4. Cover the plate and incubate for 3 h at 37 °C and 5% CO_2.
5. Wash the cells at RT by centrifuging at 300 x g for 3 min, aspirating the supernatant, vortexing the bottom of the plate with plate covered and finally suspending cells with 200 µl of ice cold running buffer and repeating the washing step one more time.
6. Add 200 µl of RPMI complete medium, and repeat the washing step one more time.
7. Add 200 µl of 10 µg/ml LPS medium to each well.
8. Cover the plate and incubate for 4 hr, 24 hr, 48 hr, or 72 hr at 37 °C and 5% CO_2. Adjust incubation times as necessary.
9. Centrifuge the plate at 300 × g for 3 min.
10. Take 175 µl of supernatant from each well and store in a freezer at -80 °C to perform the assays in steps 7 and 8.

7. Cytometric Bead Array Assay

1. Thaw the cell supernatants prepared from **step 6.10** and use in the CBA assay. Perform the cytometric bead array (CBA) assay as per the manufacturer's instructions.

8. IL-8 ELISA

1. Thaw the cell supernatants from **step 6.10** and use in the IL-8 ELISA. Perform the ELISA assay as per the manufacturer's instructions.

9. Intracellular Staining and Flow Cytometry

NOTE: Dilutions listed here are for the purpose of this study. The dilutions can be adjusted accordingly.

1. Dilute WS-CM in a 96-well plate using RPMI complete medium to a total volume of 100 µl/well at the concentration of 0.3, 1.56, 3, and 5 µg/ml of equi-nicotine units.
2. In the same plate, dilute nicotine to the following concentrations: 2, 10, 50, 100, 500, 2000, and 4000 µg/ml. CAUTION: Nicotine is acutely toxic and environmentally hazardous.
3. Add 100 µl of PBMCs suspended in RPMI complete medium at a concentration of 1 × 10^6 cells/well. The total volume of cells plus WS-CM or nicotine will be 200 µl/well.
4. Cover the plate and incubate for 3 hr at 37 °C and 5% CO_2.
5. Wash the cells at RT by centrifuging at 300 × g for 3 min, aspirating the supernatant, vortexing the bottom of the plate with plate covered and finally resuspending cells with 200 µl of ice cold running buffer, and repeat the washing step one more time.
6. Add 200 µl of RPMI complete medium to the plate, and repeat the wash step one more time.
7. Prepare the working concentrations of 2 µl/ml GolgiPlug and 10 µg/ml LPS using RPMI complete medium and add 200 µl to each well.
8. Incubate the plate for 6 hr at 37 °C and 5% CO_2.
9. At the end of step 9.8, wash the cells with running buffer (4 °C) and spinning at 300 x g for 3 min at 4 °C.
10. Add 100 µl of Cytofix to each well and incubate for 20 min at 4 °C.
11. Wash the cells 3 times as described in step 9.9 with 1x Permwash (4 °C) at 300 x g for 3 min at 4 °C.
12. Add 45 µL of 1x Cytoperm to each well followed by 5 µl of each of one of the following antibodies to each well: TNF-α-Alexa Fluor 488, IFN-γ V500, MIP-1α PE. Incubate at 4 °C for 30 min.
13. Wash the cells two times as described in step 9.9 with 1x Permwash (4 °C) and one time with running buffer (4 °C) at 300 x g for 3 min at 4 °C.
14. Resuspend the cells with 200 µl of 2% paraformaldehyde (4 °C). Transfer the cells into 12 × 75 mm tubes, and analyze the samples on flow cytometer. CAUTION: Paraformaldehyde is corrosive, acutely toxic and a health hazard.

10. K562 Killing Assay

NOTE: K562 cells should be grown in culture at 37 °C and 5% CO_2 with RPMI complete medium until they reach 80% confluence before the assay.

1. Prepare a 5 mM carboxyfluorescein succinimidyl ester (CFSE) stock solution by adding 18 µl of DMSO to the vial.
2. Dilute WS-CM or nicotine in a 96-well plate using RPMI complete medium to a total volume of 100 µl/well to achieve the desired equi-nicotine units or nicotine concentrations for each well. CAUTION: Nicotine is acutely toxic and environmentally hazardous.

3. Add 100 µl of PBMCs into RPMI complete medium at a concentration of 1.5 x 10^6 cells/well. The total volume of cells with WS-CM or nicotine will be 200 µl/well.
4. Cover the plate and incubate for 3 hr at 37 °C and 5% CO_2.
5. Wash the K562 cells by adding 10 ml of DPBS and centrifuge at 400 x g for 8 min at RT.
6. Resuspend the cells with 10 ml of DPBS and count the K562 cells.
7. Prepare CFSE working solution by adding 1 µl of CFSE stock solution to 1 ml DPBS.
8. Add 1 ml of the CFSE working solution to 1 ml of the K562 cell suspension containing 1 - 2 x 10^7 cells. Vortex and incubate precisely for 2 min at RT.
9. Immediately add 200 µl of FBS. Vortex and incubate precisely for 2 min at RT.
10. Add 10 ml of RPMI complete medium and centrifuge the tube at 400 x g for 8 min at RT.
11. Remove the RPMI supernatant and break the pellet and resuspend the cells with 10 ml of RPMI complete medium and count the CFSE-labeled K562 cells.
12. Wash the PBMCs by centrifuging the plate at 300 x g for 3 min at RT. Aspirate the supernatant by decanting the liquid. Replace the cover and vortex the bottom of the plate. Add 200 µl of RPMI complete medium and repeat washing step one more time.
13. Add CFSE-labeled K562 cells at a ratio of 1:15 (100,000 K562s:1.5 x 10^6 PBMCs) to each sample well of the PBMC plate and further incubate for 5 hr at 37 °C and 5% CO_2.
14. Wash the cell mix by centrifuging at 300 x g for 3 min at RT. Aspirate the supernatant by discarding the liquid. Place the cover and vortex the bottom of the plate.
15. Add 200 µl of running buffer and repeat the washing step described in step 10.14 one more time.
16. Add 95 µl of running buffer followed by 5 µl of 7AAD to each well for a total volume of 100 µl/well. Incubate the plate in the dark at RT for 15 min.
17. Add 100 µl of running buffer to each well and transfer the entire volume of cell suspension from each well of the plate to the cluster tubes and acquire the samples on flow cytometer.
18. Determine the percentage of 7AAD-positive and CFSE-positive cells using flow cytometry analysis software.

Representative Results

The results were presented as mean ± standard error of the mean (four donor samples). The student's t-test between treated and untreated control samples was performed using Excel software as well as t-test comparisons for all treatments with their corresponding controls. The statistical significance was indicated by: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

To measure the effect of exposure to WS-CM and nicotine, PBMCs were treated with different concentrations of WS-CM and nicotine for 3 h or 24 hr. Cell death was measured by the robust and reliable 7AAD staining method, as 7AAD intercalates into double-stranded nucleic acids and can penetrate cell membranes of dying or dead cells[16]. A dose-dependent increase in cell death was observed with WS-CM at 24 hr, with a near 80% cell death detected at 4 µg/ml of equi-nicotine units (**Figure 1A**). A comparable degree of cell death was noted only at 3,000 µg/ml nicotine (**Figure 1B**). Since we exposed PBMCs for 3 hr with WS-CM and nicotine to assess their effect of cytokine induction and ability to kill target cells, it was necessary to determine whether WS-CM causes significant cytotoxicity following 3 hr treatment. PBMCs treated with 5 µg/ml of equi-nicotine units of WS-CM did not experience significant toxicity (<5%; **Figure 1A**). Treatment with nicotine for 3 hr caused measurable (8%) cell death only at 2,000 µg/ml (**Figure 1B**). Thus, exposure to WS-CM or nicotine at the indicated doses and treatment periods did not cause significant cytotoxicity under the experimental conditions.

To measure the immunomodulatory effects, PBMCs were stimulated with LPS for different time periods, and the secreted cytokines were measured by the CBA assay. To optimize the LPS stimulation time, we exposed PBMCs to LPS stimulation for 4 hr, 24 hr, 48 hr, and 72 hr, and secreted cytokines were measured. For example, LPS stimulation for 24 hr yielded maximal secretion of the cytokines IL-6 and IL-8 (**Figure 2**), and extended time periods did not result in further increases (IL-6) or decreases (IL-8) in cytokine secretion. Similar results were obtained with other secreted cytokines such as IL-10, IL-1β and TNF-α (data not shown).

The pilot time-course experiments suggested that maximal production of cytokines in responses to TLR-4 stimulation by LPS occurs by 24 hr, and hence, secreted cytokines were measured at 24 hr in all subsequent experiments. Treatment with WS-CM and nicotine, followed by stimulation of TLR-4 receptor with LPS, resulted in a dose-dependent decrease of secreted cytokines (**Figure 3**). Treatment with WS-CM resulted in profound decreases of IFN-γ (**Figure 3A**), TNF (**Figure 3B**), IL-10 (**Figure 3C**) and IL-6 (**Figure 3D**) at low equi-nicotine units (1.56 µg/ml). Suppression of cytokines was also evident with nicotine. However, the suppression of cytokines with nicotine occurred at significantly higher doses, and the degree of suppression varied among individual cytokines. For example, IFN-γ appeared to be significantly suppressed with nicotine at 50 µg/ml, whereas IL-6 was suppressed at the highest concentration (4 mg/ml) tested (**Figurie 3D**). Next, we measured IL-8 levels in the same samples using an ELISA assay. IL-8 secretion was effectively suppressed in WS-CM-exposed PBMCs; while nicotine's suppressive effects were significant at 2 mg/ml (**Figure 4**).

The levels of intracellular cytokines were quantified in WS-CM- and nicotine-treated cells upon stimulation with LPS. Previously we stimulated PBMCs for 3 days and added Golgiplug during the last 6 hr of incubation to measure intracellular cytokines[14]. We now significantly reduced the incubation period by combining stimulation with LPS and Golgi plug to a total of 6 hr and measured intracellular cytokine-positive cells (**Figure 5**). **Figure 5** illustrates a dose-dependent percent reduction in IFN-γ-positive cells (**Figure 5A**), TNF-α-positive cells (**Figure 5B**) in both nicotine- and WS-CM- exposed PBMCs, whereas a dose-dependent percent reduction in MIP-1α-positive cells (**Figure 5C**) was observed in WS-CM-exposed PBMCs. In this assay we observed a decrease in the number of intracellular cytokine-positive cells after exposure to WS-CM at lower equi-nicotine units (1.56 µg/ml). Both secreted cytokine and intracellular cytokine assays showed similar results in terms of IFN-γ levels or IFN-γ-positive cells.

As a functional measure, the ability of WS-CM- and nicotine-treated PBMCs to kill target K562 cells was determined. **Figure 6A** depicts representative flow cytometric raw data of 7AAD-positive staining of target (CFSE-labeled K562) cell killing by control, WS-CM-exposed, and nicotine-treated PBMCs. Numbers in the box represent percent 7AAD-positive CFSE-labeled K562 cells. Exposure to 1.56 µg/ml WS-CM significantly reduced the killing ability of the effector cells in PBMCs compared with control and the lower doses. Nicotine treatment at low and

high doses did not interfere with the cell killing. **Figure 6B** shows a dose-dependent decrease in percent K562 cell killing from multiple donors in a single experiment.

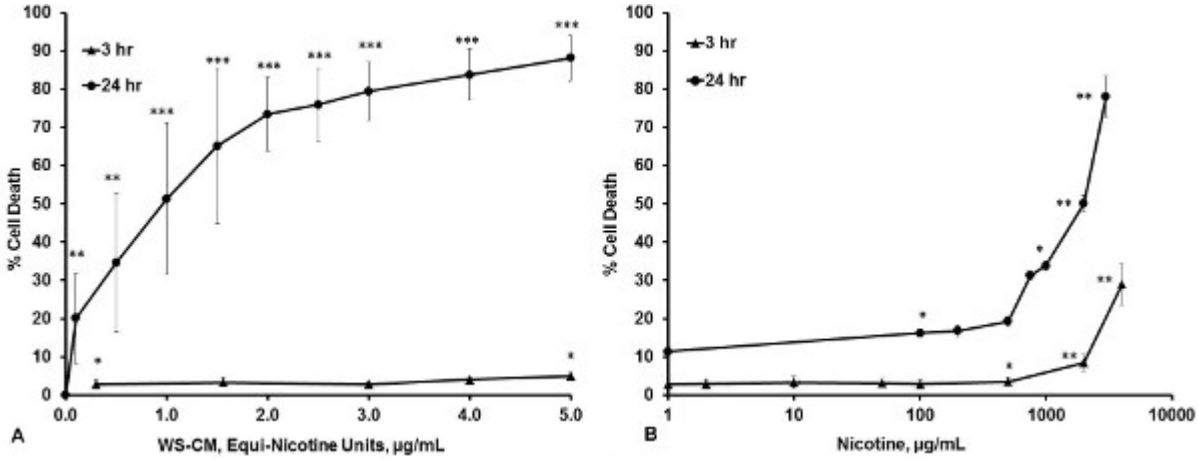

Figure 1. Cell death of PBMCs after exposure to increasing concentrations of equi-nicotine units of WS-CM (µg/ml) and nicotine (µg/ml). PBMCs were treated with WS-CM for 3 hr and 24 hr (**A**) and with nicotine for 3 hr and 24 hr (**B**). Cell death was measured by 7AAD staining. Each point represents the mean ± SD error bars of four donors from a representative experiment. The statistical significance is indicated by: *P < 0.05; **P < 0.005, ***P < 0.0005.

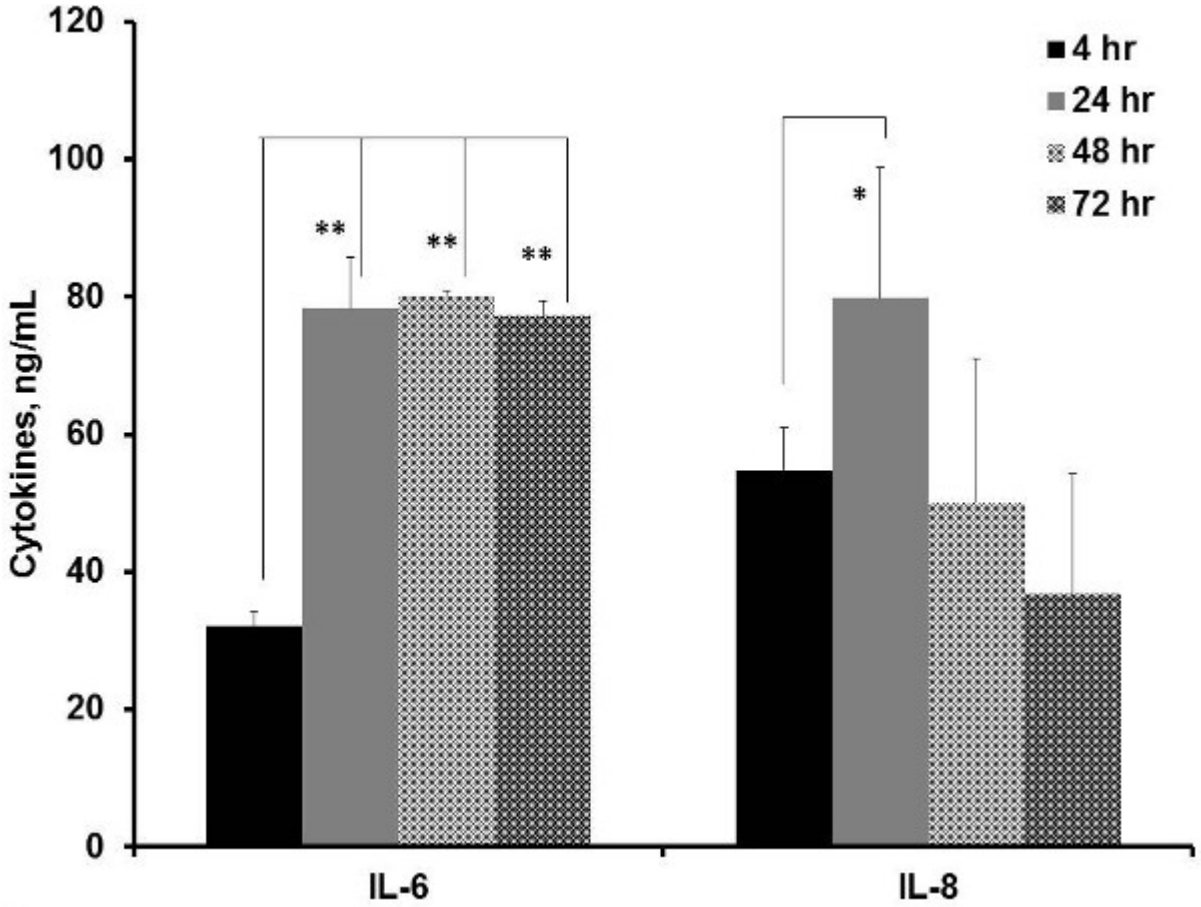

Figure 2. Secretion of IL-6 and IL-8 by PBMCs stimulated with LPS at different time points. PBMCs were stimulated with LPS for 4 hr, 24 hr, 48 hr, and 72 hr. Levels of IL-6 and IL-8 cytokines in the cell culture supernatants were determined using Human Inflammatory Cytokine Kit (CBA kit) and flow cytometry. These data represent the mean ± SD error bars from one of the two independent experiments using PBMCs from three different donors. The statistical significance is indicated by: *P < 0.05; **P < 0.005.

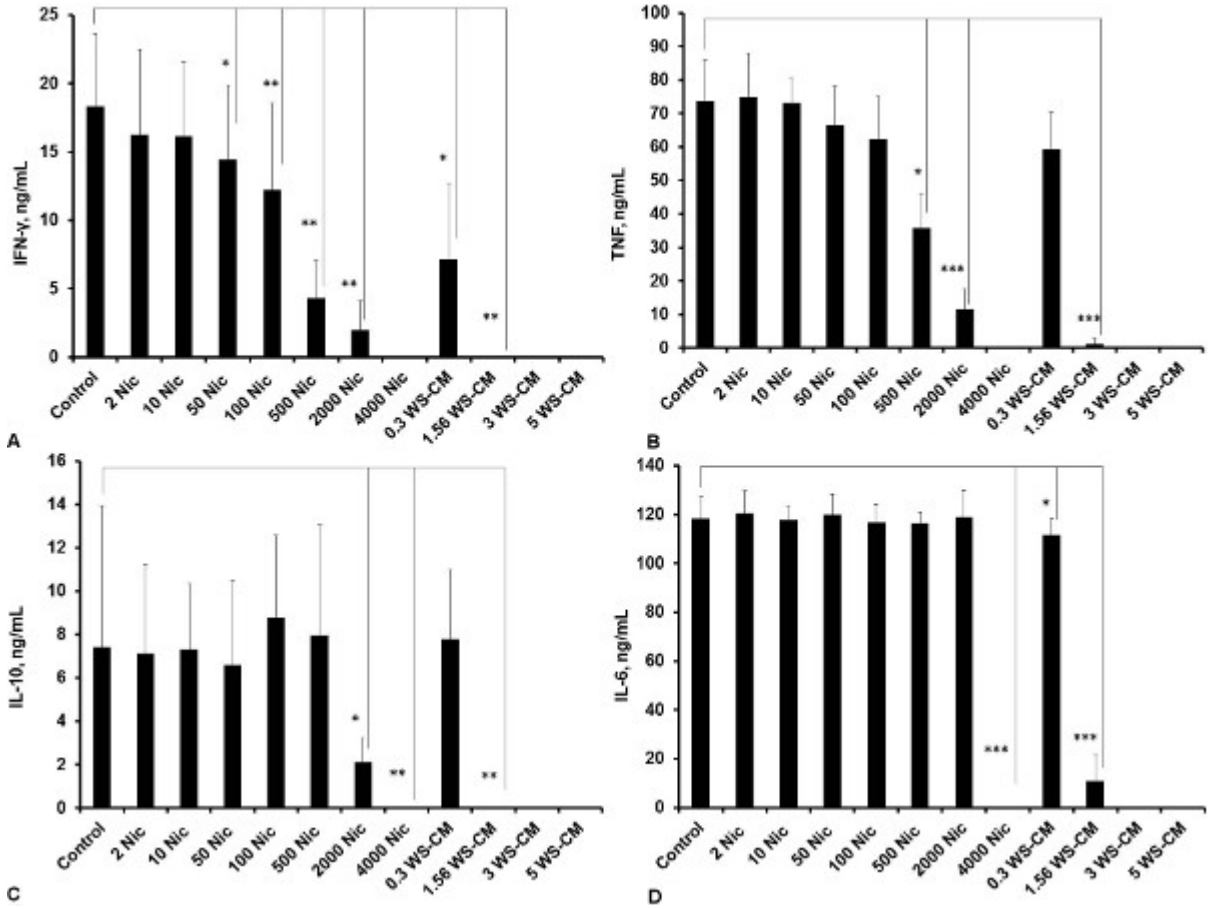

Figure 3. Reduction of cytokine secretion by PBMCs treated with increasing concentrations of equi-nicotine units of WS-CM (µg/ml) and nicotine (µg/ml) following LPS stimulation. PBMCs were exposed to different concentrations of WS-CM and nicotine for 3 hr and stimulated with LPS for 24 hr. Levels of IFN-γ (**A**), TNF (**B**), IL-10 (**C**), and IL-6 (**D**) cytokines in the cell culture supernatants were determined using a Th1/Th2 CBA assay and flow cytometry. This data represents the mean ± SD error bars from one of the three independent experiments using PBMCs from four different donors. The statistical significance is indicated by: *P < 0.05; **P < 0.005; ***P < 0.0005. Higher concentrations of WS-CM were also statistically significant with ***P < 0.0005.

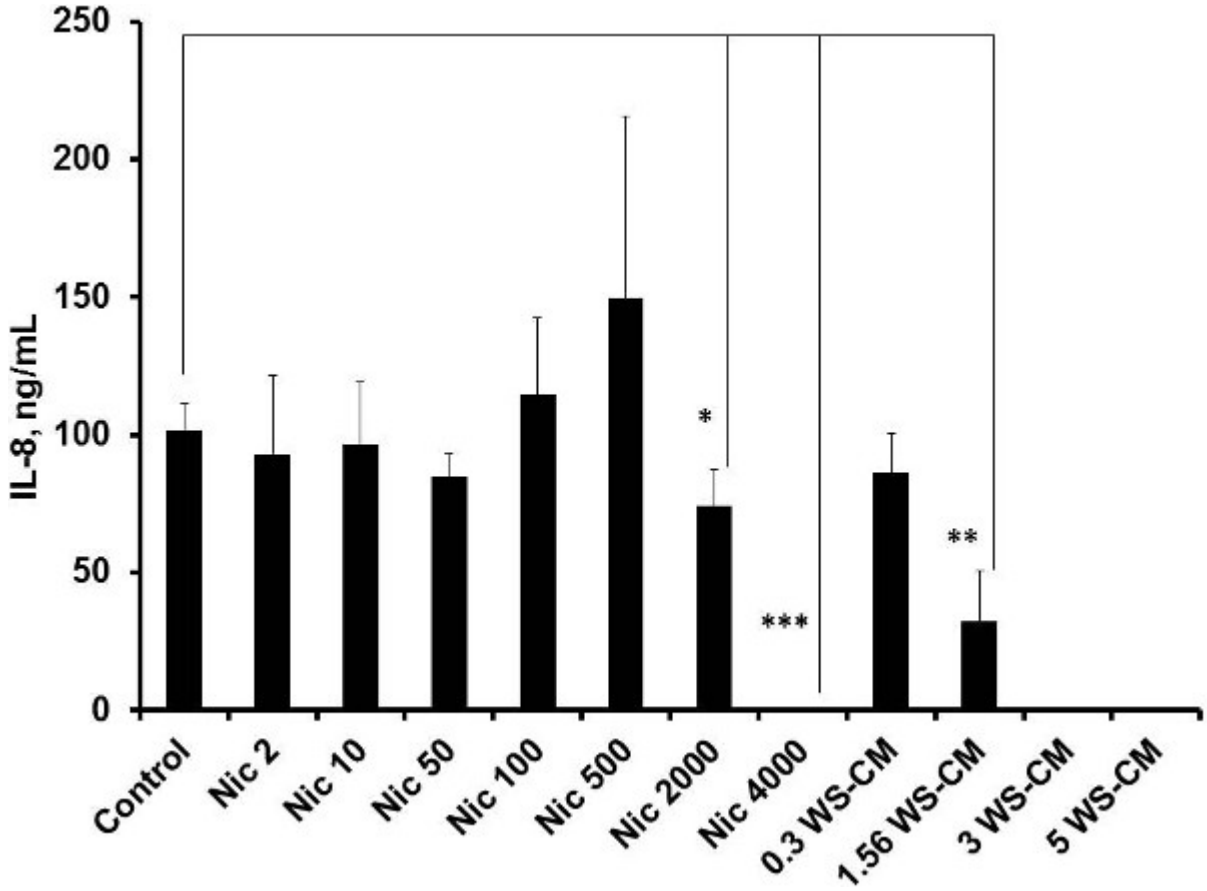

Figure 4. Attenuation of IL-8 secretion by PBMCs treated with increasing concentrations of equi-nicotine units of WS-CM (µg/ml) and nicotine (µg/ml) following LPS stimulation. PBMCs were exposed to WS-CM and nicotine, stimulated with LPS for 24 hr, and secreted IL-8 was measured by ELISA. These data represent the mean ± SD error bars from one of the three independent experiments using PBMCs from four different donors. The statistical significance is indicated by: *P < 0.05; **P < 0.005; ***P < 0.0005. Higher concentrations of WS-CM were also statistically significant with ***P < 0.0005.

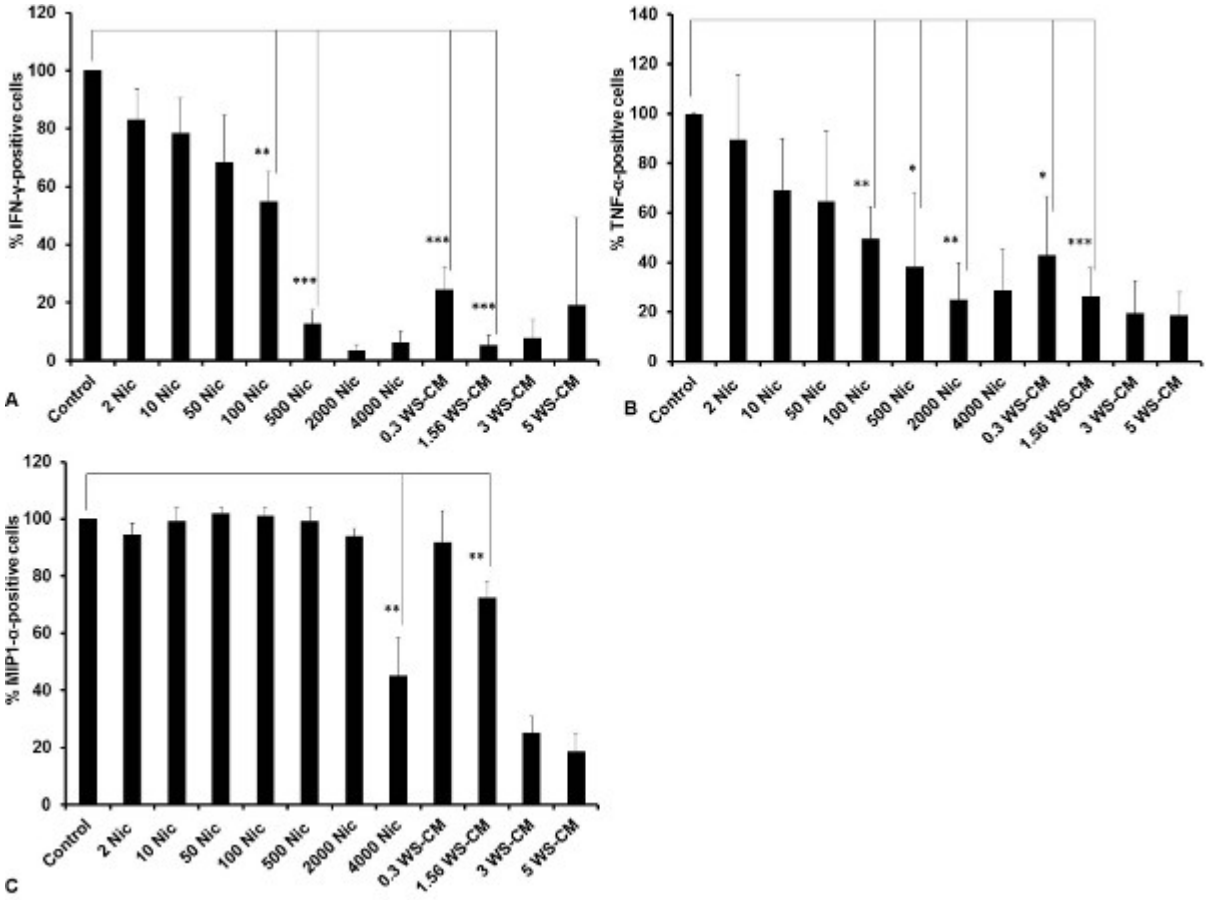

Figure 5. Reduction of intracellular cytokine-positive cells in PBMCs treated with increasing concentrations of equi-nicotine units of WS-CM (µg/ml) and nicotine (µg/ml). PBMCs were exposed to varying concentrations of WS-CM and nicotine for 3 hr and stimulated with LPS and Golgiplug for 6 hr. The vehicle control for WS-CM and nicotine was RPMI complete medium. Intracellular IFN-γ-positive (**A**), TNF-α-positive (**B**), and MIP-1α-positive (**C**) cells were quantified by flow cytometry. These data represent the mean ± SD error bars from one of the four independent experiments using PBMCs from four different donors. The statistical significance is indicated by: *P < 0.05; **P < 0.005; ***P < 0.0005. Higher concentrations of WS-CM were also statistically significant with ***P < 0.0005.

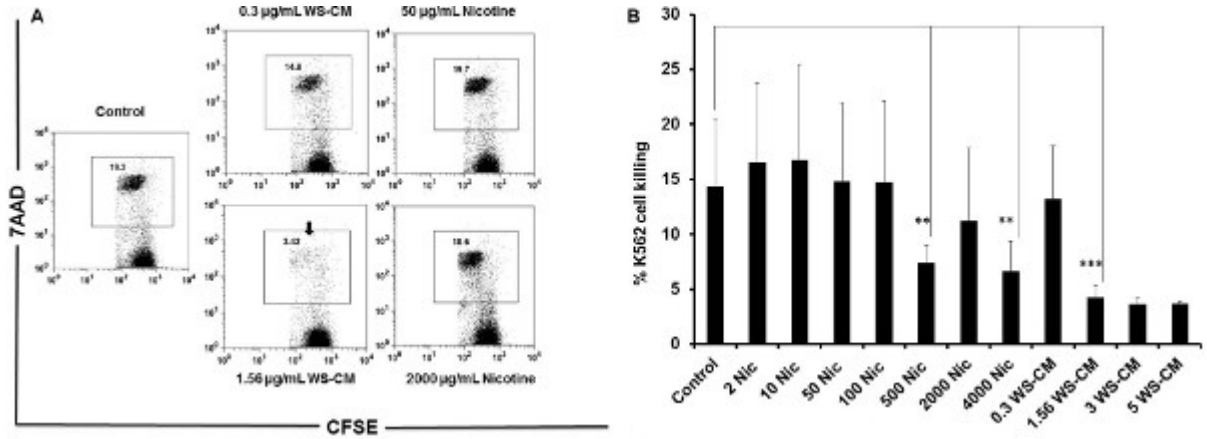

Figure 6. Reduction of PBMCs target killing ability with exposure to increasing concentrations of equi-nicotine units of WS-CM (µg/ml) and nicotine (µg/ml). PBMCs were treated with indicated concentrations of WS-CM and nicotine for 3 hr, and CFSE-labeled K562 cells were then added as target cells and incubated for additional 5 hr. Cells were stained with 7AAD and flow cytometry was used to gauge killing ability. **Figure 6A** shows the representative flow cytometry results with percent killing shown in the gated boxes. Arrow indicates reduced percent killing in 1.56 µg/ml exposed WS-CM. **Figure 6B** shows representative mean ± SD error bars from four independent experiments using PBMCs from four different donors. The statistical significance is indicated by: **P < 0.005; ***P < 0.0005. Higher concentrations of WS-CM were also statistically significant with ***P < 0.0005.

Discussion

We and others have previously demonstrated that treatment of PBMCs with TPPs suppresses several responses, including expression and secretion of cytokines and functional measures such as target cell killing[14]. The experimental methods described in the previous work require longer incubation periods and were modest in magnitude[14]. Given the potential applications of this attractive *ex vivo* model for basic and applied research, we investigated whether any of the assay parameters in these multi-step biological assays could be optimized. Here, we present simplified methods to measure TLR-mediated immune responses using a TPP-treated *ex vivo* PBMC model.

Isolation of viable PBMCs is a key requirement for these *ex vivo assays.* Given the individual variability and physiological status in potential donors, it is ideal to obtain PBMCs that are responsive. For the purpose of this study, we isolated PBMCs from generally healthy adult subjects using a previously published method[15]. Further, in this study to minimize variability among donors, we excluded those with allergies, infections, or who were taking any prescriptions or over the counter drugs such as aspirin. Additionally, isolation of PBMCs from freshly collected blood is desirable, as it ensures optimal viability and functionality of the cells in subsequent experiments.

Previous methods utilized stimulation with TLR agonists for 67–72 h to measure intracellular cytokines and secreted cytokines[14]. A time-course of cytokine secretion in TLR-stimulated cells (under control conditions with no WS-CM or nicotine) suggested that maximal secretion occurred at 24 hr. Furthermore, we combined the incubation with TLR agonists and Golgiplug and reduced the time of incubation to a total of 6 h for measuring intracellular cytokines. While this required a separate assay to measure cytokine-positive cells, the data are more robust compared with the previous method[14]. Consistent with the previous results, WS-CM strongly suppressed the induction of intracellular and secreted cytokines. Thus, these modifications led to substantially reduced assay times and yielded very similar results as those described in literature. While we have utilized flow cytometry and ELISA methods to evaluate cytokine levels, other techniques such as real time quantitative polymerase chain reaction (RT-qPCR) may also be utilized as a complementary technique.

Historically, target cell killing by effector PBMCs utilized radiolabeled methods (*e.g.*, ^{51}Cr-release assay). The method described in this report eliminates the use of radioactivity and employs loading cells with a fluorescent dye whose presence could be monitored by flow cytometry. Another advantage of the described method herein is that it is relatively rapid and may be adapted to high-throughput assays. Since the incubation period of target and effector cells is 5 hr, this assay may be completed in 8 hr. This in contrasts with other published methods, which are more involved as they require isolation of subtypes of PBMCs and activation/stimulation over a period of several days, or analysis of NK cell and K562 cell conjugates to monitor cytotoxicity[17,18]. Another advantage of the current method is that it uses cryopreserved PBMCs directly as effector cells, which affords greater flexibility.

In summary, we described several assays to determine the effect of TLR-mediated immune response in combustible TPP-treated PBMCs, which can be readily applied for testing different compounds. The use of cryopreserved components allows for significant flexibility, and together with established techniques such as flow cytometry, CBA assays, and ELISAs, robust and consistent results could be obtained rapidly across different laboratories.

Disclosures

The authors declare that there are no conflicts of interest.

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