

Cigarette smoke impairs cytokine responses and BCG containment in alveolar macrophages

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Methods

Peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected into sodium heparin BD Vacutainer tubes and diluted with an equal volume of Ca⁺ and Mg⁺ free phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated by density sedimentation using Ficoll-Hypaque (Sigma-Aldrich, Steinham, Germany) using standardised techniques. Briefly, diluted blood in PBS was layered onto Ficoll and centrifuged at 400g for 25 minutes at room temperature. The resultant interface was removed and washed twice with PBS and centrifuged at 300g for 10 minutes. After the final wash the PBMC were adjusted to 1x10⁶/ml in complete medium.

Preparation of monocyte derived macrophages

PBMCs (obtained by density sedimentation as described above) were seeded at a concentration of 1x10⁶/ml into either 24 well plates (Corning) or 96 well plates (Corning) for mycobacterial uptake studies or cytokine and mycobacterial stasis assays, respectively. The cells were incubated at 37°C with 5% CO₂ for 6 days to ensure that the monocytes had acquired a macrophage phenotype (MDM). The adherent macrophages were presumed to be at 1x10⁵/ml, as mononuclear cells comprise approximately 10% of monocytes. Non-adherent cells were removed by washing with warmed RPMI prior to any further experiments.

Bronchoscopy and lavage technique

Alveolar lavage cells were obtained by bronchoscopy as follows. Following the administration of local anaesthetic gel and spray to the nose and pharynx, a flexible video-bronchoscope was passed through a nostril to the level of the vocal cords. Further lignocaine was sprayed onto the cords. No fluid was aspirated prior to passing the cords to minimize the risk of contamination of the bronchoscope suction channel with nasopharyngeal organisms.

Following further administration of lignocaine to the lower airways, the tip of the bronchoscope was wedged into the right middle lobe bronchus. A 300ml lavage using sterile saline, in 60 ml aliquots with a dwell time of 10 seconds was performed with low suction (<20cm H₂O). The lavage fluid was aspirated into a sterile 500ml Schott bottle and a maximal return attempted (patient tolerance and physical return). The fluid was then transported on ice directly to the laboratory for processing.

Preparation of alveolar macrophages

The volume of the BAL fluid obtained was documented. The BAL was passed through 2-ply gauze to remove any mucus and particulate debris then transferred into sterile 50ml conical tubes. Following centrifuging at 300g for 10 min at room temperature, the resultant pellets were combined and

reconstituted with 50 ml PBS. The cells were washed and centrifuged at 300g twice more then resuspended in 2ml of RPMI containing 10% human AB serum and 0.1%Fungin™ (Invivogen, France) and 100u/ml penicillin (Sigma Aldrich). Cell count and viability was determined by counting on a haemocytometer with trypan blue exclusion dye (Sigma Aldrich). Appropriate cell concentrations were prepared for each of the various experiments performed.

Assessing macrophage viability

Several techniques were used to determine macrophage viability dependent on the specific experimental question. To determine viability of macrophages exposed to cigarette smoke, macrophages were stained with trypan blue exclusion dye and counted using a haemocytometer. For flow cytometry experiments, macrophage viability was determined by 7AAD viability dye (eBiosciences) staining. Immediately prior to acquisition of the cells 10µl of 7AAD was added to the cells. Once acquired the cells were analysed on a FACsCalibur flow cytometer using *Cell Quest* software to determine the proportion of viable cells. For fluorescent microscopy, 7AAD was added to the cell suspension (or culture medium of adherent cells) immediately before viewing under a fluorescent microscope.

Preparation of cigarette smoke extract

To examine the effect of tobacco smoke on immune function, using in-vitro cell cultures, a common approach is to co-culture the cells with a cigarette smoke condensate prepared by passing tobacco smoke through culture media. The condensate prepared is then diluted to prevent toxic cell death. The exact concentration varies between research groups but a 10% extract is common.[1-3] Su and colleagues have proposed that the commonly used CSE of 10% is equivalent to smoking more than one pack of cigarettes per day.[4] We based our approach on well-validated techniques as well as considerable optimisation work as presented below. [5, 6]

Cigarette smoke extract (CSE) was obtained from the combustion of commercially available medium tar (10 mg tar & 0.8 mg nicotine) Marlboro Red® (Phillip Morris, USA) cigarettes. A single carton containing 10 individually wrapped cigarette cartons was purchased and the individual boxes were stored in sealed plastic bags at -20°C until required for use.[7] Individual boxes were allowed to equilibrate to ambient room temperature and humidity over 48 hours prior to use in experiments.

Cigarette smoking apparatus

A standardized cigarette-smoking device was constructed based on the apparatus used in several studies published by Freed and co-workers.[5, 6] A single filtered cigarette was connected via 5mm high flow PVC tubing (Gilson) to a peristaltic pump (Minipulse® evolution, Gilson). For each extract preparation, freshly sterilized tubing and connections were used.(Figure E1) A fixed smoking time of 5min 21 sec at a flow rate

of 125cc/min was used after demonstrating a reliability of <1mm variability in distance from the filter after the specified time (coefficient of variance 2.5%). (Figure E2, Table E1).

A sterile 50cc conical tube (Corning, Corning NY, USA) was inserted inline with the cigarette and the pump using a rubber stopper with two glass tubes as connection ports. For each extract preparation, new sterilised tubing and connections were used. The sterile 50ml conical flask was filled with 10ml warmed (37°C) RPMI and the rubber stopper and connections inserted into the tube under sterile conditions. The tube and connectors were then transferred to the fume hood for preparation of the extract.



Figure E1 Cigarette smoking machine

The cigarette smoking apparatus consists of the peristaltic pump, PVC tubing and 50ml conical flask containing culture medium. Smoke is extracted from the cigarette and bubbled through the culture medium before exhausting through the pump.

Optimisation of cigarette smoke extract preparation

All smoke extract preparation was performed in a fume hood. The hood cover was closed to the same height for all experiments and the extraction fan only switched on after completion of the smoking time. A fixed pump rate of 45 RPM with 5mm PVC tubing achieved a flow rate of 125cc/min equivalent to published methods.[5, 6] Several cigarette brands were tested to establish the time taken to smoke the cigarette to within 1 cm of the filter.

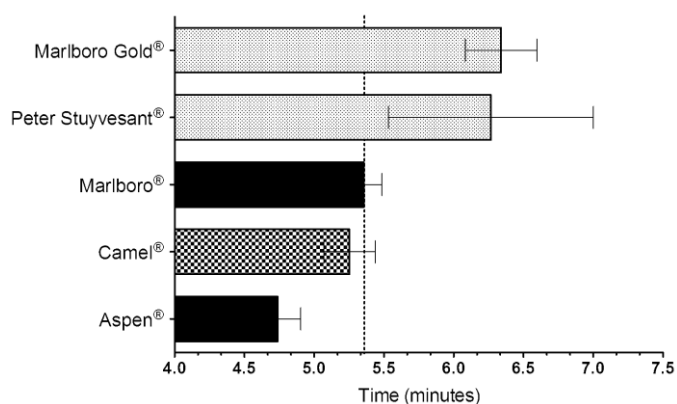


Figure E2 Smoking time for various cigarette brands

The time taken for 5 cigarettes of each brand to burn to 1 cm from the filter was recorded using a fixed pump rate. The individual bars represent the mean 'smoking time' with error bars depicting the standard deviation. The dotted vertical line represents the 5min 21 sec 'smoking time' for the brand of cigarette used in all experiments.

Using the Marlboro Red[®] cigarettes (Phillip Morris, USA) at a fixed flow rate of 125cc/min, a smoking time of 5 minutes and 21 seconds after an initial 5 second ignition period was established. (Table E1) This time reliably smoked the cigarette to 10mm (\pm 0.8mm) from the filter. Smoke remaining within the 50ml conical tube after completion of the smoking time, was allowed to dissolve in the medium by gentle shaking for 30 seconds.

Table E1 Variability of 'smoking time' between cigarette brands and products within brand products

Cigarette brand	Mean time to burn to 1cm of filter	Standard deviation	Coefficient of variance
Aspen[®]	4min 44 sec	10 sec	3.5%
Peter Stuyvesant[®]	6 min 16 sec	44 sec	11.7%
Marlboro Red[®]	5 min 21 sec	8 sec	2.5%
Marlboro Gold[®]	6 min 20 sec	15 sec	4.1%
Camel[®]	5 min 15 sec	11 sec	3.5%

The concentration of CSE produced by this method was defined (by convention) as a 100% solution. Dependent on the experimental protocol, appropriate dilutions were prepared to produce a final concentration ranging between 0.1% - 10% in cell culture medium. Cigarette smoke extract was produced freshly for each experimental intervention and was used within 20 minutes of preparation. Two aliquots of 100% cigarette smoke extract were immediately frozen and stored at -80°C for mass spectrometry determination of nicotine content.

Determination of nicotine concentration

The University of Cape Town Division of Pharmacology analytical & research laboratory performed the mass spectrometry for the determination of nicotine concentrations. Briefly, aliquots of cigarette smoke extract were analysed using a ABSciex 3200 Qtrap mass spectrometer connected to an Aglient 1200 Series HPLC (High Performance Liquid Chromatography). A series of nicotine standards (Sigma) and quality controls were prepared in HPLC grade water (Merck, Germany). Standards, controls and unknowns were diluted 1000 fold, in 50% Acetonitrile: 0.1% Formic acid (Merck, Germany), with Reserpine (Sigma) as an internal standard. 5 μ l of the samples were injected onto a Phenomenex Luna Hilic column (50 x 2mm x 3 micron), using Acetonitrile and 0.1% formic acid as mobile phase.

Reproducibility of the cigarette smoke extract

5 Marlboro Red[®] cigarettes (10 mg tar and 0.8 mg nicotine) were sequentially smoked using the standardized protocol. Individual nicotine concentrations were calculated for each of the five prepared extracts. The mean (SD) concentration of nicotine obtained from the 5 sequentially smoked cigarettes was

12.5(3.4) $\mu\text{g/ml}$ and the coefficient of variance was 28.17%. Nicotine concentrations are known to vary within brands (Marlboro Gold[®] vs. Marlboro Red[®]) and within brand products (Marlboro Red[®] bought in Kenya vs. Marlboro Red[®] bought in America).[7]

The nicotine concentrations of one low tar and two medium tar brands were compared. Camel[®] (medium tar: 10 mg tar, 0.8 mg nicotine), Marlboro Red[®] (medium tar: 10 mg tar, 0.8 mg nicotine) and Marlboro Gold[®] (low tar: 0.8 mg tar, 0.5 mg nicotine) were compared. Nicotine concentrations in CSE differed across all three brands. CSE prepared from Camel[®] cigarettes had the highest mean (SD) concentration of nicotine: 13.9(2.4) $\mu\text{g/ml}$ compared to Marlboro Red[®] 9.1(1.9) $\mu\text{g/ml}$ and Marlboro Gold[®] 4.9(0.9) $\mu\text{g/ml}$; $p < 0.001$. (Figure E3) The coefficient of variance was similar across all three brands: 17.6%, 18,3% and 21.1% respectively.



Figure E3 Nicotine concentrations in cigarette smoke extract from different tobacco brands

Each bar represents the mean nicotine concentration in prepared cigarette smoke extract from two medium and one low tar brand. Five cigarettes were smoked for each brand with error bars representing the SEM. Each brand produced statistically different nicotine concentrations ($P < 0.001$; ANOVA).

Cigarette smoke extract reproducibility over time

Two aliquots of all prepared cigarette smoke extract were immediately frozen and stored at -80°C for later batched nicotine determination. Using nicotine standards ranging from 30–200 $\mu\text{g/ml}$ the coefficient of variance of the mass spectrometer determined standard concentrations was 14.9%.

Reproducibility of the cigarette extract was best when multiple extracts were produced on the same day (set up phase): mean (SD) nicotine concentration of 12.5(3.4) $\mu\text{g/ml}$ coefficient of variance (CV%) 28.2%. During the first 6-month optimisation phase (total of 16 separate CSE prepared), the mean (SD) concentration of nicotine was 10.7(5.2) $\mu\text{g/ml}$ (CV%= 48.4%). For the 9 month experimental phase (total of 26 prepared extracts) the mean (SD) nicotine concentration was 6.4(2.6) $\mu\text{g/ml}$ ($p = 0.006$ compared to optimisation phase); CV%=40.3%. The level of reproducibility is similar to that published by Vassalo et al

who had a coefficient of variance of 32% in nicotine concentration using a similar technique of liquid chromatography-tandem mass spectrometry. [8]

Infection of macrophages

Mycobacterium Bovis Bacillus Calmette Guérin expressing green fluorescent protein (BCG-gfp); provided by Prof. B Ryffel (Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, South Africa) was used in all infection experiments. Stock was grown in enriched Middlebrook 7H9 broth supplemented with Hygromycin B (50ug/ml) for selection of recombinant mycobacteria. MDM were infected with BCG-gfp at a multiplicity of infection (MOI) of 2:1 and for alveolar macrophages at an MOI of 2.5:1. These MOI were chosen based on preliminary optimization experiments demonstrating that lower MOI resulted in poor BCG uptake. MDM were washed with warm PBS after 18hours to remove non-ingested mycobacteria.

Monocyte-derived macrophages were infected with BCG-gfp at a multiplicity of infection (MOI) of 2:1 and for alveolar macrophages at an MOI of 2.5:1. These MOI were chosen based on preliminary optimisation experiments demonstrating that lower MOI resulted in poor BCG uptake after 18 hours. For infection of adherent macrophages, aliquots were thawed and mycobacterial clumps disrupted by passing the bacteria through a 27g insulin syringe several times. After 18 hours the macrophages were washed with warm PBS to remove any bacteria that did not gain access into the cells.

Mycobacterial containment experiments

Macrophages were infected as described above for 18 hours following infection non-ingested organisms were removed by washing adherent cells three times with warm RPMI. Fresh culture medium supplemented with 10% FBS was added to all experimental wells. CSE was added to triplicate wells to a final concentration of 10%. No further CSE was added on subsequent days. On days 1,2,3,5 supernatants were removed and adherent cells lysed to release intracellular bacteria. The CFU count was determined by growth on 7H10 solid media. The number of viable organisms at each time point thus reflected the ability of the macrophages to contain / restrict intracellular mycobacterial growth. At each time point visual inspection of control and CSE exposed wells was performed to identify excess cell loss in either condition.

Determination of mycobacterial uptake by flow cytometry

Flow cytometric analysis was performed to determine the number of macrophages containing intracellular BCG-GFP. Cold PBS with 20mM EDTA (Sigma-Aldrich) was added to all wells for 10 minutes to facilitate detachment of adherent macrophages. Macrophages were washed in PBS containing 1% human serum and 0.1% sodium azide (Sigma-Aldrich) and resuspended in the same buffer (FACs buffer). Immediately

prior to acquisition of the cells, 10µl of 7AAD (eBiosciences) was added in order to establish cell viability. Once acquired, the cells were analysed on a FACsCalibur using *Cell Quest* software. Monocytes and macrophages were identified according to their specific size and granularity characteristics (forward scatter against side scatter) in a primary gating strategy and further gated on FL1 and FL3. The BCG-GFP, (FL1) was plotted against the 7AAD (FL3) emission to determine mycobacterial uptake and macrophage viability.

Cytokine assays

IFN- γ concentration was determined using the QuantiFERON[®]-TB Gold (In-tube) whole blood IFN-gamma ELISA kit (Cellestis, Carnegie Victoria, Australia). Briefly, supernatants were thawed and brought to room temperature with the specified kit reagents. Replicate kit standards were prepared ranging from 0-4IU/ml (160pg/ml). After a 30 minute incubation with enzyme substrate the reaction was stopped and the optical density (OD) values were obtained within 5 minutes with a 450nm filter and a 620nm reference filter. OD values were manually inputted into the QuantiFERON[®]-TB Gold IT Analysis Software (Cellestis). Automated software quality control confirmed validity of the data and generated IFN- γ concentrations for each well.

TNF- α and IL-10 concentration was determined using the Human TNF- α and IL-10 Ready-SET-Go! ELISA Kit (eBiosciences, #88-7347, #88-7906) Briefly, supernatants were thawed and brought to room temperature with the specified kit reagents. Standards were prepared to generate standard curve ranges of 4 – 500 pg/ml (TNF- α) and 2– 300 pg/ml (IL-10). 100µl of supernatant was combined with 100µl of conjugate and incubated overnight at 4°C. OD values were manually entered into an Excel spread sheet and a standard curve was generated using the serial standard dilutions. Cytokine concentrations were then calculated by reading the OD of the test samples off the standard curve.

Results

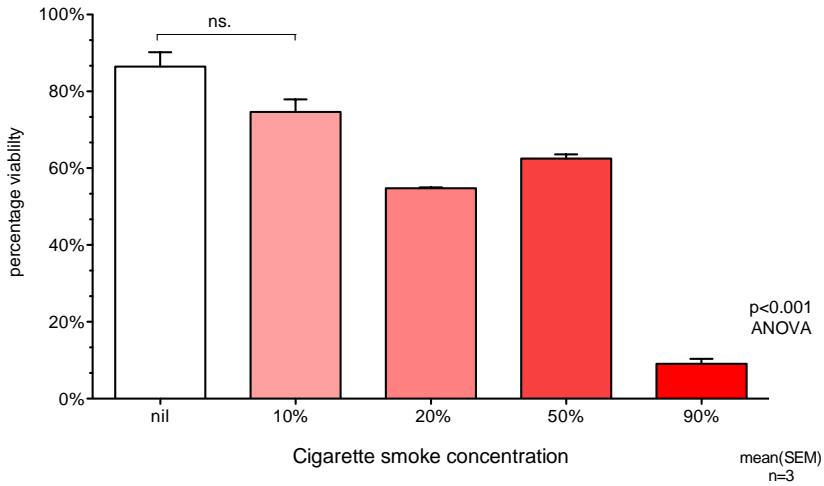


Figure E4 Viability of Macrophages exposed to increasing doses of cigarette smoke extract

Each bar represents the percentage of viable cells (negative staining for 7AAD) harvested after 24hour exposure to cigarette smoke exposure in increasing concentrations in full culture medium.

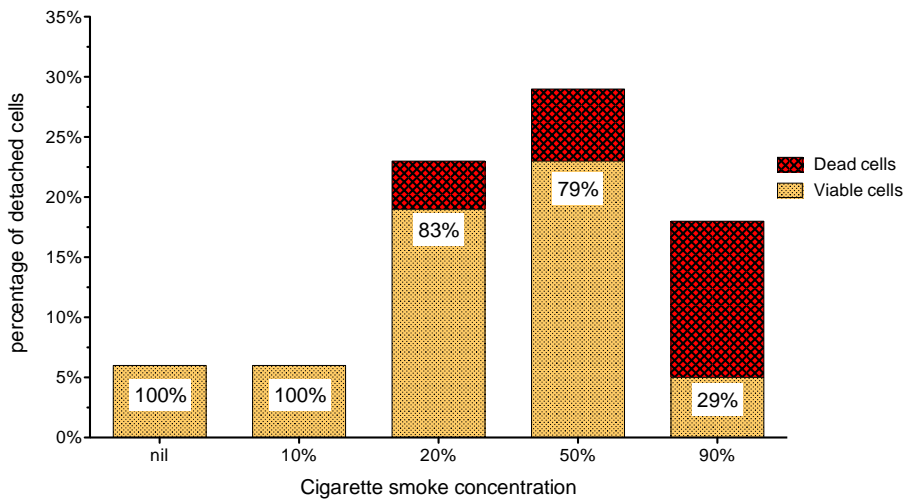


Figure E5 The effect of cigarette smoke on macrophage detachment and viability.

Each bar represents the percentage of cells recovered in the supernatant after 24 hours. The yellow shaded fraction and number indicates the proportion of the recovered cells that were viable, the red fraction the proportion that were non viable (positive for trypan blue).

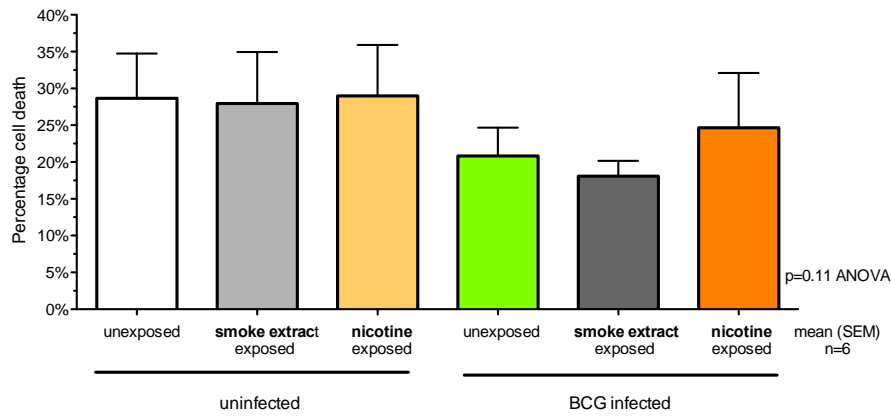


Figure E6 Viability of alveolar macrophages following BCG infection

Alveolar macrophage viability was determined by 7AAD staining following 18 hour infection and exposure to either tobacco smoke extract or nicotine. Percentage cell death depicted on the y-axis represents the percentage staining positive with 7AAD.

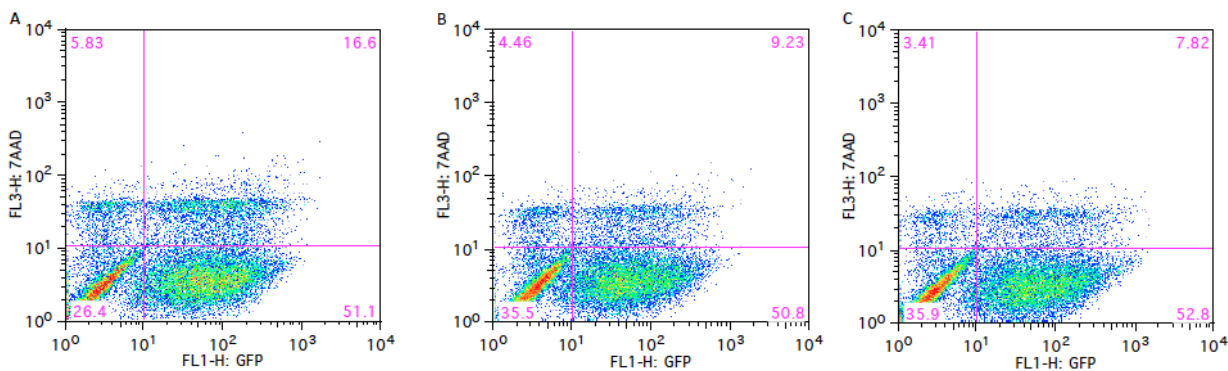


Figure E7 Flow cytometry dot-plots of alveolar macrophages BCG-gfp uptake and viability.

The representative flow cytometry dot-plots depict BCG-GFP uptake on the x-axis and 7AAD viability staining on the y-axis. **Panel A:** BCG-GFP infection without exposure. **Panel B:** Infection with co-exposure to 10% CSE. **Panel C:** Infection with co-exposure to 1 µg/ml nicotine. The bottom right quadrant of all panels represents alive and BCG-GFP infected macrophages.

Monocyte derived macrophage Interferon gamma production

Human and murine macrophages have been shown to produce IFN- γ , although T cells predominantly secrete it in response to infection.[9-11] Therefore to confirm that the macrophages in this MDM model were producing IFN- γ , further experiments were conducted. Three additional subjects were recruited and following the same experimental methods as previously described, harvested cells were prepared for flow cytometric analysis.

Cells were stained with surface markers CD3, CD4, CD33, CD14 and for intracellular IFN- γ . PBMCs: Monocytes, DCs and Macrophages were gated according to their characteristic FSC/SSC profile. The gated cells were negative for CD3 (dump gate for lymphocytes and NK T-cells). The gating strategy is depicted below: Briefly, CD3+ cells were gated to identify T- lymphocytes and NK T-cells and this gate was excluded from further analysis. Cells negative for the myeloid marker CD33 were also excluded. The macrophage population was subsequently identified by positive staining with anti CD14, a LPS receptor found on monocytes and macrophages (Figure E8). Using this gating strategy 40% of the cells were CD33 positive and 70% of CD33+ cells were CD14 positive. In the CD33+CD14+ population, 60% were positive for IFN- γ . (Figure E8) In the excluded CD3+ve population (not shown) 0.2% were IFN- γ positive.

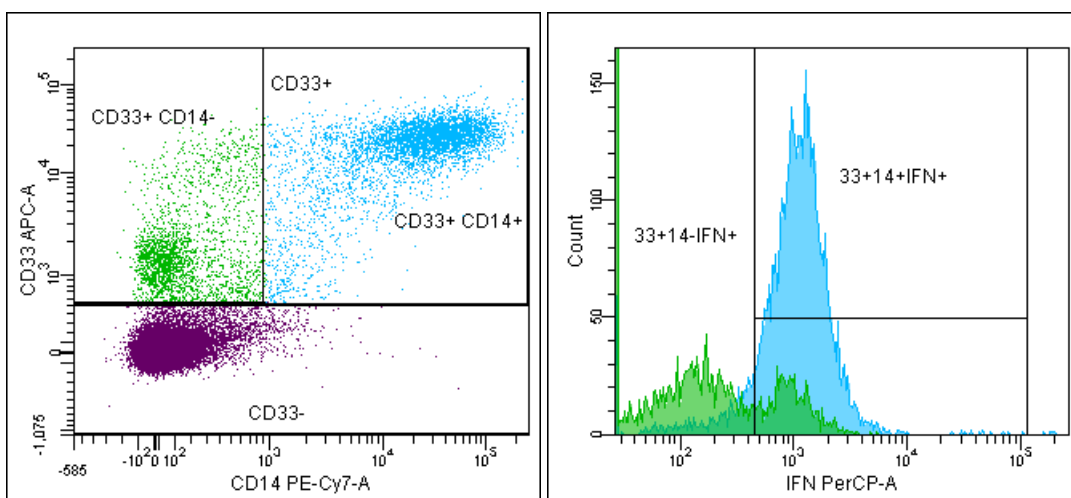


Figure E8 Flow cytometry gating strategy for the definition of cell types producing IFN- γ in response to BCG infection.

To determine the source of IFN- γ production, further experiments were conducted in order to isolate the CD14 positive (+) cells and to determine the production of IFN- γ in these cells using PCR. CD14 positive (+) cells were isolated as described. To confirm the purity of the CD14 positive (+) fraction prior to PCR, dual staining of anti CD3-/CD33+ was shown to be greater than 99% (duplicate experiments) in the CD14 fraction. (Table E2) A small percentage of CD33+ cells remained in the non-CD14 fraction, which was in keeping with the previous experiments demonstrating the presence of CD33 positive but CD14 negative cells.

Table E2 Purity of CD14 magnetic bead extracted cell fraction in unexposed and BCG infected conditions

		% CD3 -ve	% CD33 +ve	CD3 ⁻ CD33 ⁺ purity
Unexposed cells	CD14 fraction	99.8 %	99.4 %	99.2 %
	Non CD14 fraction	67%	42.5%	29%
BCG-infected	CD14 fraction	100%	99.6%	99.6 %
	Non CD14 fraction	88.4%	77.8%	68.4%

PCR for IFN- γ was performed on the CD14 +ve cell fraction as described above. Minimal IFN- γ mRNA copies, mean (SD), were present in the control (unexposed and uninfected) macrophages: 94.5(106.8) mRNA copies per 10⁶ copies of HuPO. Following BCG infection IFN- γ mRNA expression increased significantly to a mean (SD) 1016(16.97) copies per 10⁶ copies of HuPO; $p=0.003$. In BCG infected and CSE exposed macrophages the level of IFN- γ expression, mean (SD), was numerically lower: 320 (390) but did not reach statistical significance ($p=0.06$). These data, although only from two individuals, demonstrate the production of IFN- γ mRNA by the CD14 positive cells shown in the prior experiments to be IFN- γ positive by intracellular staining. The trend towards a reduced IFN- γ by co-exposure to cigarette smoke supports the ELISA findings.

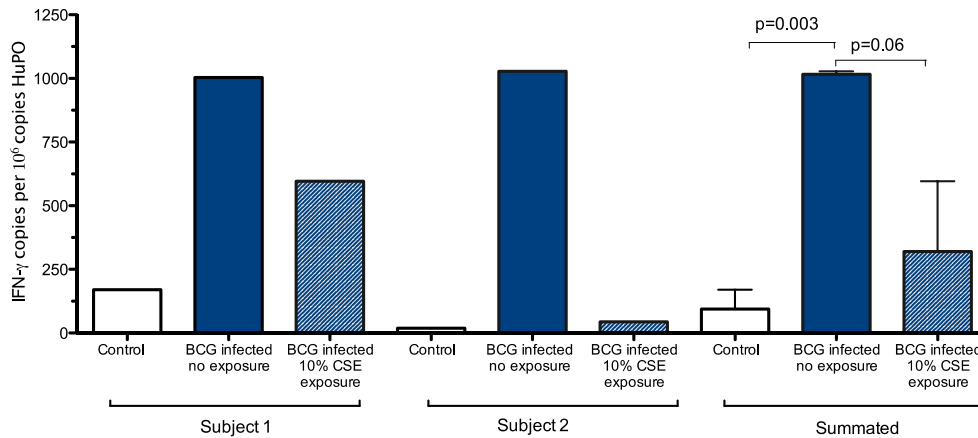


Figure E9 Interferon gamma (IFN- γ) mRNA transcription following BCG infection in CD14 positive cells.

IFN- γ mRNA was transcribed from CD14 cells (macrophages) purified by magnetic bead separation. Following conversion of RNA to cDNA, real time PCR was performed and quantitative amounts of IFN- γ mRNA calculated relative to the house keeping gene HPO.

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