ORIGINAL ARTICLE

Cigarette smoke impairs cytokine responses and BCG containment in alveolar macrophages

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ABSTRACT

Background There is a strong epidemiological link between smoking and tuberculosis (TB), but the association is confounded by socioeconomic and other factors. A direct relationship between cigarette smoke and poor treatment-related outcomes in patients with TB is therefore questionable. We investigated whether constituents of tobacco smoke impair mycobacterial host immune responses in vitro.

Methodology Preparation of a cigarette smoke extract (CSE) from Marlboro Red cigarettes was standardised and reproducibility verified by mass spectroscopy. Macrophages were derived from peripheral blood monocytes (MDM) and alveolar macrophages from bronchoalveolar lavage fluid from healthy non-smoking volunteers. Mycobacterial uptake (flow cytometric detection of fluorescence using green fluorescent protein-labelled BCG), cytokine responses (ELISA) and mycobacterial containment (colony forming units) was evaluated in both macrophage populations with and without co-culture with CSE, nicotine and a nicotine receptor blocker.

Results Cigarette smoke failed to impair the uptake of mycobacteria by monocyte-derived or alveolar macrophages. CSE (vs no CSE) reduced the mean (SD) BCG-driven macrophage (MDM) interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and interleukin 10 (IL-10) responses by 56.4 (18.6)%, 67.0 (33.4)% and 77.7 (27.7)%, respectively ($p<0.001$). Nicotine alone impaired IL-10 and TNF-α production by 48.8 (37)% and 49 (50)%, respectively ($p<0.05$) through an α-7 nicotine receptor-independent mechanism. In 5-day cultures, CSE impaired mycobacterial (BCG) containment in both monocyte-derived and alveolar macrophages.

Conclusions Cigarette smoke attenuates effector cytokine responses and impairs mycobacterial containment within infected human macrophages derived from the peripheral blood and alveolar compartments, thus supporting the hypothesis that cigarette smoke subverts mycobacteria-related immunity.

Key messages

What is the key question?

- Is the epidemiological association of smoking and heightened risk (approximately double) of tuberculosis (TB) disease and death merely a confounder of socioeconomic or other factors or is it supported by biologically plausible mechanistic data?

What is the bottom line?

- An animal study has demonstrated higher *M tuberculosis* colony growth in mice exposed to cigarette smoke but substantial human data are lacking.

Why read on?

- This study provides novel human data demonstrating several biologically plausible mechanisms by which tobacco smokes subverts human immunity and possibly increases the risk for TB disease.

INTRODUCTION

There are an estimated 1.3 billion smokers worldwide.[1] Tobacco smoking is the most important preventable cause of death, with an estimated 8% of all adult deaths per year (5 million people) attributable to tobacco smoking. More than 80% of these deaths occur in the developing world.[1] One-third of the world's population is thought to be latently infected with *Mycobacterium tuberculosis* and an additional 8.8 million new cases are diagnosed with active tuberculosis (TB) each year.[2] Several modifiable factors including malnutrition, overcrowding, poverty and HIV co-infection are associated with susceptibility to and spread of active TB.[3] In recent years, smoking has been confirmed as another risk factor for TB. Three comprehensive independent systematic reviews and meta-analyses support this association.[4–6] Compared with non-smokers, smokers have almost twice the risk of TB infection and of progression from latent to active disease. Smokers are also almost twice as likely to die from active TB.[4–6] Based on these data, an estimated 15.8% of TB cases worldwide are probably attributable to tobacco smoking, higher than that attributable to HIV infection (~11%), alcohol abuse (~8%) and diabetes (~7%).[7]

In the light of these facts, implementation of smoking cessation strategies has been proposed as an important component of TB control programmes in tandem with addressing other modifiable factors such as overcrowding, poverty and alcohol abuse.[8] However, in poorly resourced regions, smoking cessation programmes will incur additional cost and place extra demands upon already overburdened clinic staff. It is therefore rightly questioned what priority smoking cessation should receive, particularly because the epidemiological association is weakened by confounding factors such as overcrowding, poverty and alcohol

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usage, and there is limited experimental data to support the biological plausibility for the association between smoking and TB.[9 10] In animal models, mice exposed to cigarette smoke for 14 weeks have a significantly higher mycobacterial burden in the lungs and spleen 30 days after challenge with aerosolised *M tuberculosis*.[9] In non-TB models, cigarette smoke has been demonstrated to impair phagocytic function (*Staphylococcus*, *Listeria*, *Candida*) and cytokine responses (lipopolysaccharide, *Legionella*).[11–15] There are no human data on the impact of cigarette smoke extract (CSE) on mycobacterial containment. We hypothesised that constituents of tobacco smoke may attenuate effector cytokine responses and mycobacterial containment in human alveolar and monocyte-derived macrophages (MDM).

METHODS

Participants and obtaining macrophages

Healthy HIV-negative non-smoking participants were recruited to provide venous blood samples and/or undergo bronchoscopy and bronchoalveolar lavage. Detailed methods used are provided in the online supplement.

MDM were prepared from peripheral blood mononuclear cells obtained by density sedimentation through Ficoll-Hypaque. The peripheral blood mononuclear cells were seeded at a concentration of 1×10^6/mL into 24-well plates and allowed to adhere for 6 days. Alveolar macrophages were obtained by bronchoscopy with low-pressure suction using a 300 mL sterile saline lavage. Non-adherent cells were removed at 4 h and appropriate cell concentrations were prepared for each of the experiments performed.

Macrophage infection with BCG

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) expressing green fluorescent protein (BCG-GFP) was used in all infection experiments. MDM were infected with BCG-GFP at a multiplicity of infection of 2:1 and for alveolar macrophages at 2.5:1. MDM were washed with warm phosphate-buffered saline after 18 h to remove non-ingested mycobacteria.

Preparation of CSE and nicotine

A standardised cigarette smoking device was constructed based on the apparatus used in the studies reported by Freed and coworkers (see online supplement). Reproducibility of the extract was assessed using an ABSciex 3200 Qtrap mass spectrometer connected to an Aglient 1200 Series high-performance liquid chromatography. For each experiment, fresh extract was used and added to cultures within 15 min of preparation.

Determination of mycobacterial uptake

Flow cytometric analysis was performed to determine the number of macrophages containing intracellular BCG-GFP. Immediately before acquisition of the cells, 10 µL 7-aminoactinomycin D (7AAD; eBiosciences) was added in order to establish cell viability. Once acquired, the cells were analysed on a FACsCalibur using Cell Quest software.

Cytokine assays

Cytokine concentrations were determined using commercially available ELISA kits and performed according to the manufacturers' instructions. Triplicates of each experimental condition were prepared and pooled whenever sufficient cells were available. To explore the hypothesis that nicotine can modulate tumour necrosis factor α (TNF-α) production, experiments were conducted using an α7 receptor blocker, α-bungarotoxin (Sigma Aldrich). Macrophages were pre-incubated with α-bungarotoxin-FITC (1.5 µg/mL) for 15 min prior to the addition of nicotine and infection with BCG.

Mycobacterial containment endpoints

To determine the capacity of human macrophages to contain mycobacterial infection, adherent macrophages were infected with BCG and then cultured for 5 days in the presence of 10% CSE. On days 1, 2, 3 and 5, intracellular colony forming units (CFUs) were determined.

Statistical methods

Statistical comparisons were made with the appropriate parametric (t test) and non-parametric tests (Mann–Whitney U test) and, where applicable, paired tests (paired t test or Wilcoxon matched pairs signed rank test). For data involving more than two categories, an analysis of variance (ANOVA) was used (one-way ANOVA or repeated measures ANOVA as appropriate). To correct for multiple comparisons the Tukey test was used. A p value of 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism (V.5.00, GraphPad Software, San Diego, California, USA, http://www.graphpad.com) and OpenEpi (V.2.3.1. http://www.OpenEpi.com, updated 19 September 2010).

RESULTS

CSE and toxicity

The CSE was prepared 26 times over a 9-month experimental period. The mean (SD) concentration of nicotine in the extract was 6.4 (2.6) µg/mL. Exposure of adherent MDM to concentrations of CSE >10% for 24 h showed a dose-dependent reduction in cell viability (see figure E4 in online supplement). Furthermore, at concentrations of ≥20%, spontaneous cell detachment increased (see figure E5 in online supplement). Experiments were conducted using 10% CSE. For uptake and cytokine experiments, CSE was added to the macrophage cultures immediately prior to infection.

Mycobacterial uptake

MDM viability was not affected by BCG infection after 18 h or by co-exposure to either nicotine or cigarette smoke. The mean (SD) percentage viability of uninfected unexposed MDM (determined by 7AAD staining) was 63.3 (5.5)%. Exposure to 10% CSE or 1 µg/mL nicotine did not affect viability (CSE: 67.7 (4.05)%, nicotine: 64.1 (3.8)%; p=0.59). After BCG infection, MDM viability (67.3 (5.8)%) was not significantly different from uninfected macrophage viability (exposed or unexposed) nor from that of infected/CSE-exposed macrophages (68.1 (2.7)%) or infected/nicotine-exposed macrophages (60.9 (9.7)%; p=0.63).

Significant variability in BCG uptake was seen between individuals at 4 h and 18 h. At 4 h BCG uptake was low, but significantly higher in unexposed MDM than in those exposed to 10% CSE (4.5 (2.6)% vs 3.2 (1.6)%; p=0.03). At 18 h, however, no significant difference in uptake was demonstrated between unexposed MDM and those exposed to 10% CSE (24.9 (6.9)% vs 21.3 (6.8)%; p=0.09, figure 1).

Alveolar macrophages exhibited a higher uptake of BCG at 18 h (42.6 (17.4)%), but this was not significantly different from alveolar macrophages exposed to 10% CSE (44.3 (16.1)%) or 1 µg/mL nicotine (35.9 (16.8)%); p=0.28, figure 1). Viability of alveolar macrophages was not affected by infection or co-exposure to 10% CSE or nicotine (see figures E6 and E7 in online supplement).

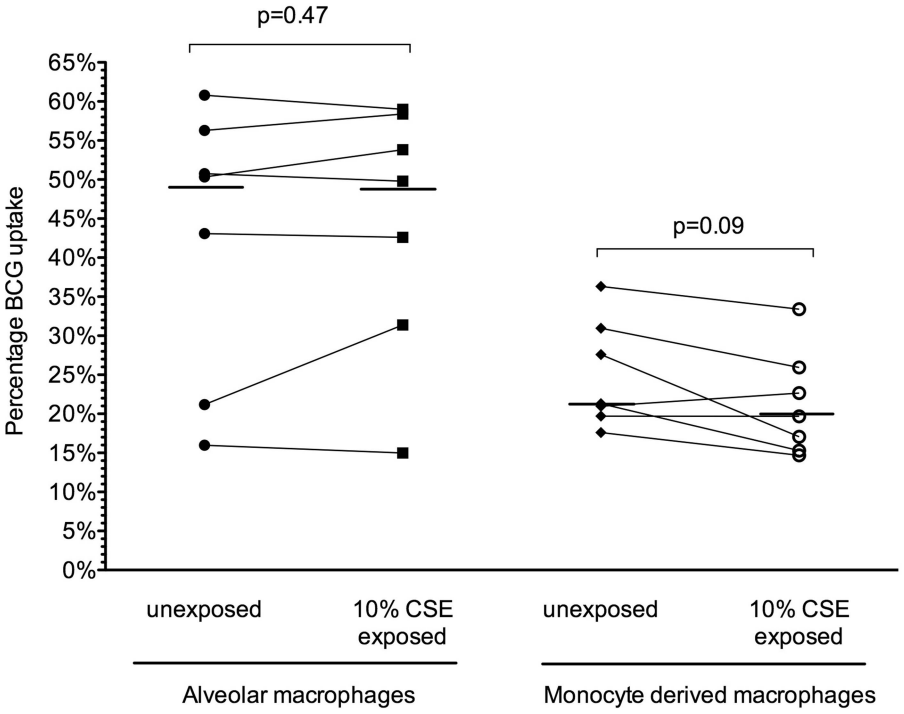

Figure 1 BCG-GFP uptake by alveolar and monocyte-derived macrophages after 18 h. Uptake (percentage of cells positive for GFP by flow cytometry) is depicted for subjects (n=7) following 18 h of infection. Uptake by macrophages with and without exposure to 10% cigarette smoke extract (CSE) from individual donors is depicted by joined lines. The horizontal line represents the median value for all individuals.

Cytokine production

IFN-γ production by MDM was measured at 4 h and 18 h after infection. At 4 h the mean (SD) IFN-γ production was minimal (0.05 (0.01) IU/mL) and was not affected by co-exposure to 10% CSE (0.04 (0.18) IU/mL; p=0.43). At 18 h, IFN-γ production by unstimulated MDM as well as those exposed to 10% CSE remained negligible (0.06 (0.03) IU/mL and 0.06 (0.03) IU/mL, respectively; p=1.0). Following BCG infection, a significant increase in mean (SD) IFN-γ was detected (0.28 (0.18) IU/mL). However, macrophages co-exposed to 10% CSE during infection demonstrated significantly less IFN-γ production (0.10 (0.04) IU/mL; p=0.001, figure 2). Production of IFN-γ was confirmed by intracellular staining for IFN-γ and by demonstrating upregulation of IFN-γ mRNA transcription (see figures E8 and E9 and table E2 in online supplement). Due to high basal cytokine production by alveolar macrophages in the 24 h after lavage, cytokine data for alveolar macrophages were not interpretable (data not shown).

TNF-α production was measured at 18 h after infection with BCG. Similarly to IFN-γ, negligible amounts of TNF-α were secreted by unstimulated and CSE-exposed macrophages (mean (SD) 0.73 (1.8) pg/mL and 1.7 (5.3) pg/mL, respectively; p=0.3). Following infection, TNF-α production increased significantly (137.5 (111.7) pg/mL) but was significantly reduced after co-exposure to 10% CSE (21.63 (45.97) pg/mL; p<0.001 vs control, figure 3).

Interleukin 10 was measured at 18 h after infection with and without 10% CSE exposure. The results were similar to those for TNF-α and IFN-γ production. Unstimulated and CSE-exposed macrophages produced little or no measurable IL-10 (0.65 (1.65) pg/mL and 0.0 (0.0) pg/mL, respectively). Following infection, IL-10 production was 27.68 (22.5) pg/mL and was significantly reduced by co-exposure to CSE during infection (4.64 (6.75) pg/mL; p<0.001, figure 3).

Compared with the maximal cytokine production of unexposed MDMs, 10% CSE reduced mean (SD) BCG-driven IFN-γ, TNF-α and IL-10 production by 56.4 (18.6)%, 67.0 (33.4)% and 77.7 (27.7)%, respectively. Alveolar macrophage cytokine production and response to CSE or nicotine exposure was not interpretable as basal cytokine production was high when tested 24 h after lavage (data not shown).

Cytokine response to nicotine exposure

Exposure of MDM to nicotine (1 μg mL) alone (as opposed to whole smoke extract) resulted in significantly reduced BCG-driven IL-10 production (40.1 (60.3) pg/mL vs 11.3 (16.4) pg/mL; p=0.03). TNF-α production was similarly reduced but did not reach statistical significance (181.6 (186.1) pg/mL vs 78.7 (52.5) pg/mL; p=0.08, figure 3). The calculated mean (SD) reduction in cytokine production in response to nicotine exposure was 48.8 (37)% for IL-10 and 49 (50)% for TNF-α. Blocking of the nicotine α7 receptor with α-bungarotoxin did not restore TNF-α production by cells exposed to CSE or nicotine (figure 4).

Mycobacterial containment

Over a 5-day period, BCG-infected MDM and alveolar macrophages exposed to 10% CSE showed higher CFU counts not accounted for by adherent cell numbers, as the latter did not change (figure 5). At each time point, prior to cell lysis to perform the CFU count, control and CSE-containing wells were inspected under an inverted microscope. No difference in the number and integrity of adherent macrophages could be identified.

DISCUSSION

Our studies of the effect of CSE on the responses of human MDM and alveolar macrophages to mycobacterial infection (BCG) demonstrate that, while the uptake of mycobacteria remained unaffected, the production of key cytokines in the immune response to TB infection (ie, TNF-α, IFN-γ and IL-10) was significantly reduced by exposure to CSE. Furthermore, nicotine alone similarly impaired both IL-10 and TNF-α production, suggesting that it too contributes to this effect. Since the effect of smoke that does not contain nicotine was not examined, it is not clear whether other components of cigarette

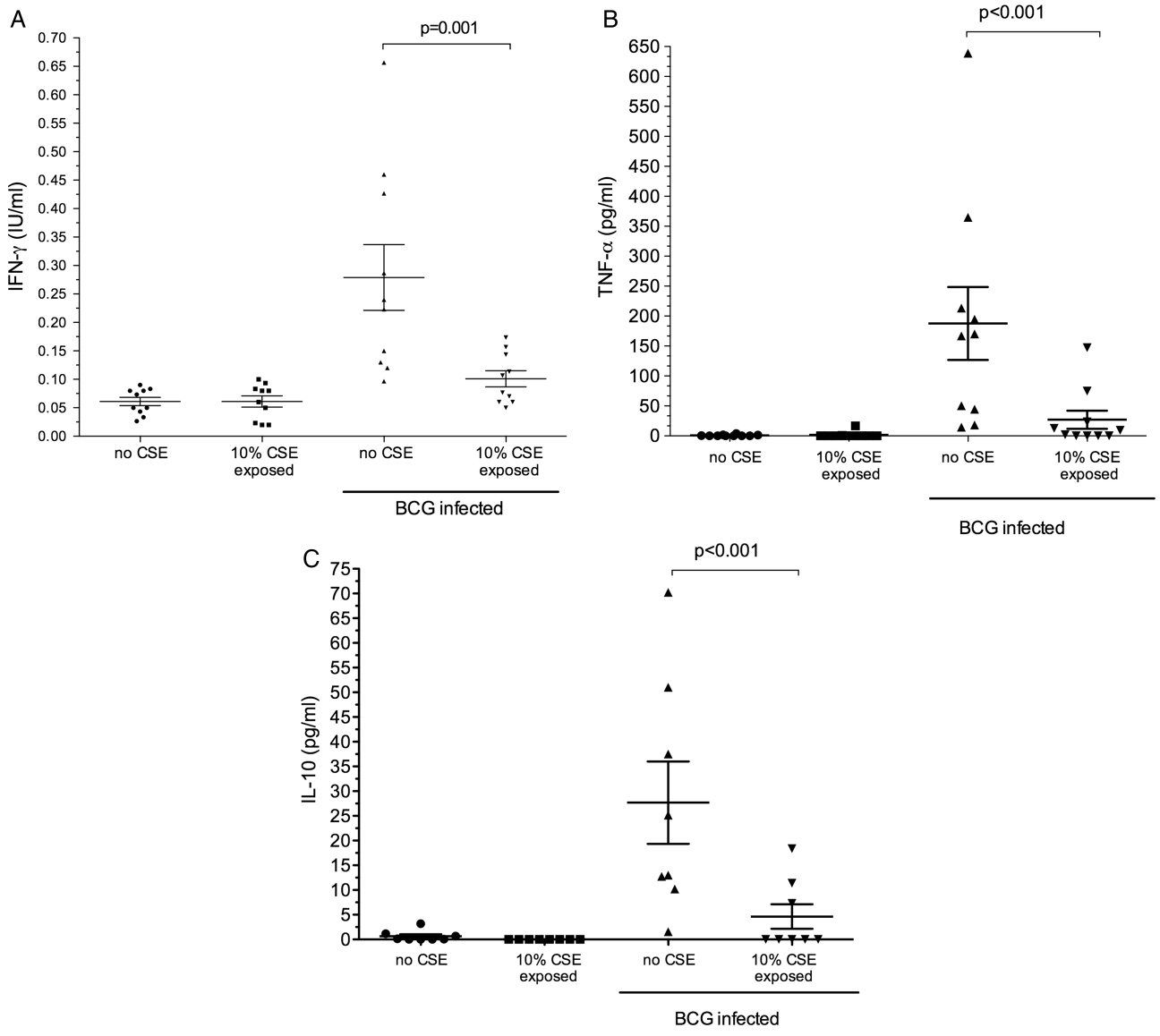

Figure 2 Cytokine production at 18 h by monocyte-derived macrophages with and without exposure to cigarette smoke extract (CSE). Each plot represents cytokine production by monocyte-derived macrophages from individual donors. The mean and (SEM) is represented by the horizontal line and error bars. Cells were exposed to BCG for 18 h (BCG-infected); (A) interferon γ (IFN-γ; n=10); (B) tumour necrosis factor α (TNF-α; n=10); (C) interleukin 10 (IL-10; n=8).

smoke have a similar effect. We have also demonstrated that both alveolar macrophages and MDM exposed to CSE had significantly higher intracellular bacillary loads after 5 days in culture. The mechanism whereby this occurs and the contribution of other cell types (such as regulatory T cells[16]) and the observed changes in cellular cytokine release in response to CSE exposure remain unclear and require further study. Collectively, these data provide biological plausibility and support a direct link between smoking and TB, an association which is also confounded by socioeconomic and other factors. To our knowledge, this is the first report of the deleterious impact of CSE on the ability of human macrophages to contain mycobacterial growth. The data strongly support the need for controlled trials about the impact of smoking cessation on TB outcomes.

Intracellular control of mycobacterial replication is a critical step in limiting disease progression.[17] Both the virulence of the infecting organisms and the bacterial load affect the ability of macrophages to contain infection.[18 19] Shang and colleagues demonstrated that smoke-exposed mice had a significantly higher bacterial burden in the lungs and spleen 30 days after infection.[9] Additionally, greater numbers of foci of inflammatory cells but reduced influx of CD4 and CD8 effector and memory T cells were present, suggesting impairment in both innate and adaptive responses. However, murine data may not reflect the situation in the human host. To our knowledge, the impact of CSE on the mycobacterial burden in human cells has hitherto not been investigated. In our 5-day human macrophage model, intracellular CFU counts were significantly higher in macrophages exposed to tobacco smoke. We further interrogated several mechanisms that may underpin these observations.

We first investigated the impact of CSE on mycobacterial uptake, about which there are limited data. Using a myelomonocytic cell line (THP-1), Shang and colleagues infected differentiated THP-1 cells with H37Rv during co–exposure to CSE or nicotine. The number of ingested mycobacteria was only measured at 1 h post infection, at which time no difference in uptake was noted.[9] Our data, which evaluated several time points, are consistent with these findings and also support the findings of

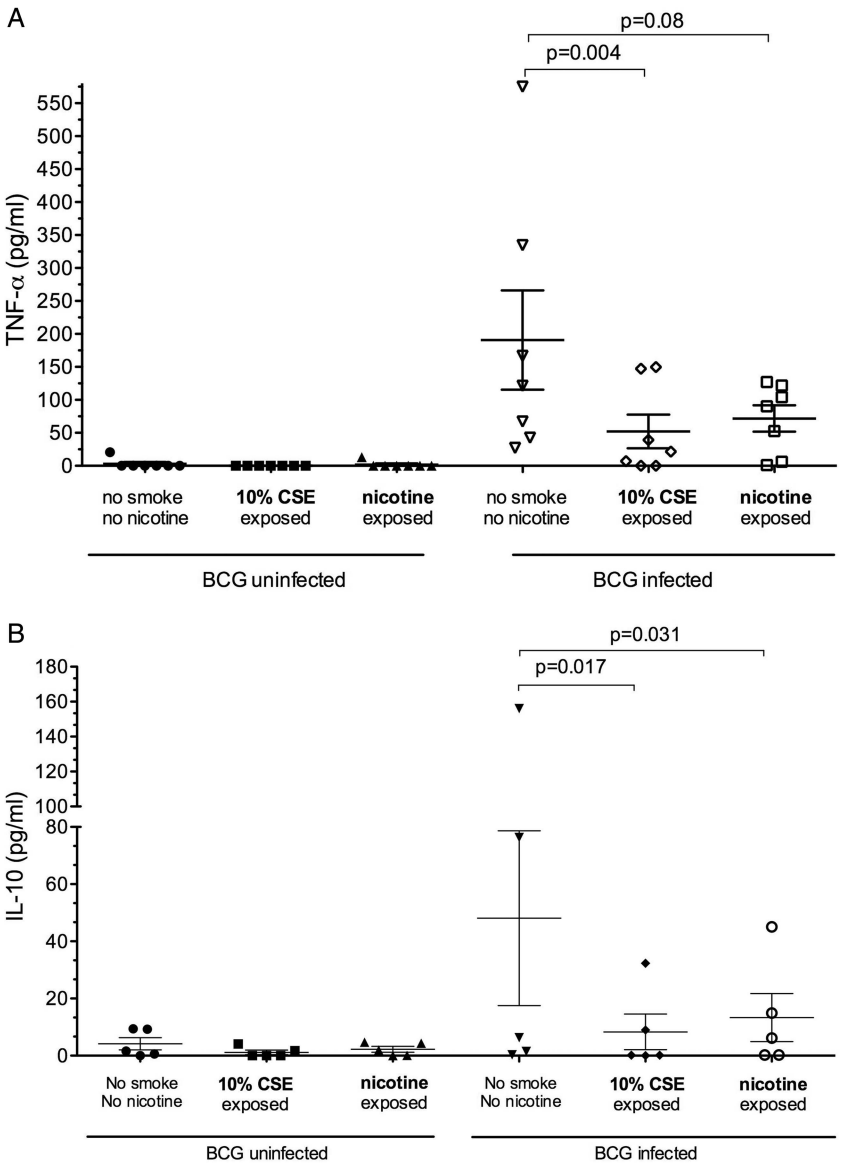

Figure 3 Effect of nicotine on cytokine production by monocyte-derived macrophages. Each scatter plot represents cytokine production by monocyte-derived macrophages unexposed or exposed to cigarette smoke extract (CSE) or nicotine, and uninfected (left hand panel) or following an 18 h BCG infection (right; BCG-infected). The horizontal bars represent mean (SEM) values. (A) Tumour necrosis factor α (TNF-α) responses (n=7). (B) Interleukin 10 (IL-10) responses (n=5).

Aldo *et al*[10] where no impairment of BCG-specific phagocytosis by alveolar macrophages was detected. These reports contrast with the effect of tobacco smoke on phagocytosis of organisms other than mycobacteria. Cigarette smoke has been shown to impair macrophage phagocytosis of several organisms including *Listeria*,[11] *Haemophilus*,[20] *Staphylococcus*,[13] *Streptococcus*[21] and *Candida*.[12] The reason for such organism-specific differences remains unclear. Interestingly, Berenson *et al*,[20] in a study using *Haemophilus*, showed reduced phagocytosis by alveolar macrophages but not MDM from smokers, suggesting functional differences between cells from different compartments. By contrast, we observed no such differences using BCG.

Next we evaluated cytokine responses in human cells after co-exposure to mycobacteria and either tobacco smoke or nicotine. Although there are data for non-mycobacterial stimuli (lipopolysaccharide (LPS), *Escherichia coli*), there are no data for mycobacterial-specific responses. CSE broadly attenuated cytokine production (IL-10, TNF-α and IFN-γ) from BCG-infected macrophages. This is consistent with the findings of studies in which stimuli other than mycobacteria were used. Ouyang demonstrated reduced TNF-α, IL-β, and IFN-γ in phytohaemagglutinin-stimulated human peripheral blood mononuclear cells following CSE exposure. Similarly, Wewers and Hagiwara, using alveolar lavage cells, showed a reduction in TNF-α (following LPS stimulation) and IFN-γ (following phorbol myristate acetate stimulation), respectively.[14 15] There are also data about the effect of nicotine on cytokine responses in models other than TB. IL-10 production was impaired in MDM from healthy non-smokers exposed to nicotine patches[22] but, in a murine alveolar macrophage cell line infected with *Legionella*, in contrast to other cytokines, IL-10 was unaffected.[23] In our study there was a consistent reduction in IL-10 production but a non-significant reduction in TNF-α production after nicotine exposure. The mechanism underlying these observations remains unclear, but data from non-TB models indicate that smoking may impair signalling through TLR2/4[24] and several intracellular pathways (NFKB, PI3 K and MAPK).[25 26] To further interrogate the mechanism underlying our findings, we blocked the α7 receptor. Davies *et al*,[27] based on the work of Wang *et al*,[28] hypothesised that exposure to cigarette smoke may impair TNF-α production through the action of nicotine on the α7 receptor. However, unlike these findings

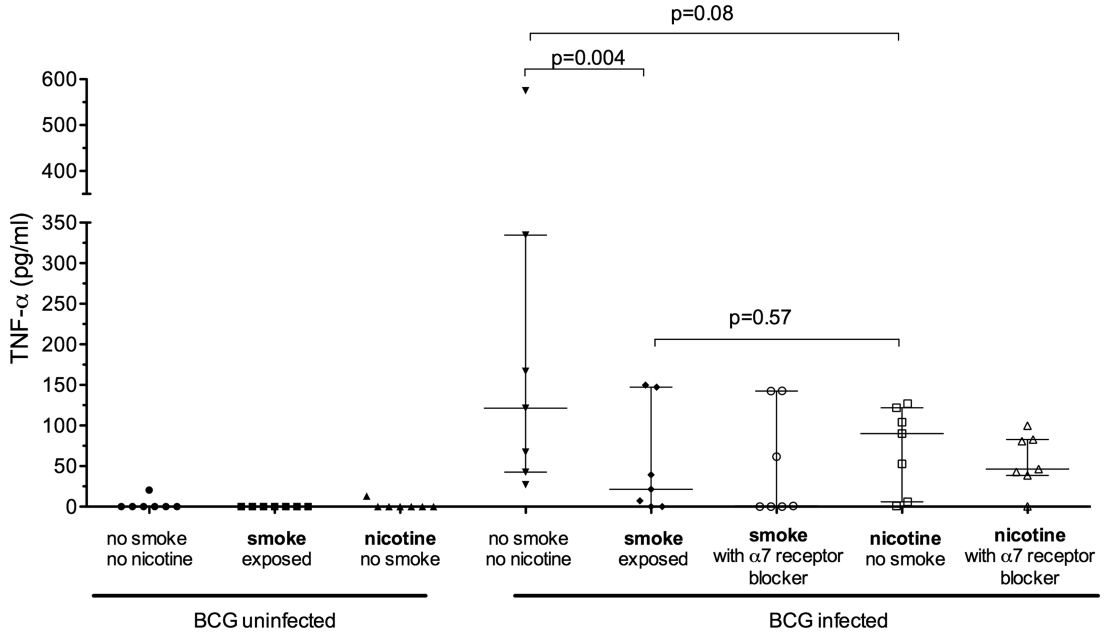

Figure 4 Effect of nicotine on tumour necrosis factor α (TNF-α) production by monocyte-derived macrophages. Each scatter plot represent the production of TNF-α by monocyte-derived macrophages either unexposed or exposed to 10% cigarette smoke extract (CSE) or 1 μg/mL nicotine (n=7). BCG-infected macrophages (18 h) were exposed during infection to CSE/nicotine and a α7 nicotine receptor blocker (α-bungarotoxin). The horizontal and error bars represent median and IQR values.

obtained in a mouse model, using an antisense oligonucleotide specific for α7 receptors, we were not able to demonstrate a nicotine α7 receptor-dependent TNF-α response to mycobacterial infection in human cells. Further research is now required to define the mechanisms whereby CSE and nicotine subvert mycobactericidal responses. Such studies should also target whether CSE impacts T-helper cell profiles including regulatory T cells, which may also subvert mycobacterial containment[16] and are upregulated in smokers without COPD.[29]

Our study has several limitations. The model, as a proof of concept, using alveolar and MDMs, BCG-GFP and ex vivo CSE exposure, does not fully represent the lung environment during virulent *M tuberculosis* infection. BCG-GFP was available at our institution, allowed work to be safely performed outside a BSL3 facility and could be used for all experiments (uptake, cytokine stimulation and containment). It cannot be assumed that the responses to BCG, a non-virulent mycobacterium, that we observed, would be similar for virulent strains of mycobacteria. However, attenuation and subversion of containment of organisms would be expected to be even more pronounced with virulent mycobacteria, and our work provides a theory for how this might occur. Alveolar macrophage cytokine production may have been more informative if assessed after 24 h of culture; however, we chose this point to be consistent across experiments. We did not examine other cytokines such as IL-4, IL-17 that are important in the immune response to TB. We did not, in the first instance, evaluate cells from the blood or lungs of smokers. Smoking alters the white cell count and CD4/CD8 ratios in smokers,[30] and the macrophages from smokers' lungs are structurally different and contain carbon and tar[31] resulting in considerable autofluorescence, making the interpretation of flow cytometric results problematic.[32 33] Given these technical challenges which were borne out by our preliminary experiments, we elected to use cells from non-smokers in the first instance. We did not assess total fluorescence or the uptake of 'beads' as a control in our experiments. Other investigators have performed these experiments[10]; the assessment of phagocytosis by non-receptor mediated uptake was not central to our hypothesis. We did not interrogate specific subfractions of CSE except nicotine and, for reasons of safety, feasibility and complexity, we did not determine whether our findings are applicable to clinical strains of *M tuberculosis*. Furthermore, whether the concentrations of particulates and soluble constituents of CSE and the duration of exposure in our experiments adequately represent those that occur in the lungs of smokers is difficult to estimate. A variety of exposures have been used in previous studies.[13 34 35] Su and colleagues have proposed that the commonly used CSE of 10% is equivalent to smoking more than one pack of cigarettes per day.[35] Nevertheless, the biological significance of the differences in mycobacterial containment seen in our experiments (CSE vs no CSE) remains unclear.

In summary, we have demonstrated that, while not impairing uptake of mycobacteria, exposure to CSE of human macrophages derived both from blood monocytes and the lung attenuates cytokine production and impairs their ability to limit mycobacterial growth. The signalling pathways affected by CSE exposure are yet to be defined, as are the intracellular mechanisms by which mycobacterial growth is facilitated. These data suggest that the suppression of cytokine responses such as IFN-γ and TNF-α in the context of CSE is biologically meaningful and also provides direct evidence for the subversive effect of CSE in mycobacterial infection.

Contributors Conception and design: RNVZ-S, KD, PLS, EDB. Laboratory experiments: RNVZ-S, AB, RM, PLS, AE. Analysis and interpretation: RNVZ-S, PLS, AB, KD, PS, EDB. Drafting the manuscript and important intellectual content: RNVZ-S, PLS, EDB, KD, PS.

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Competing interests None.

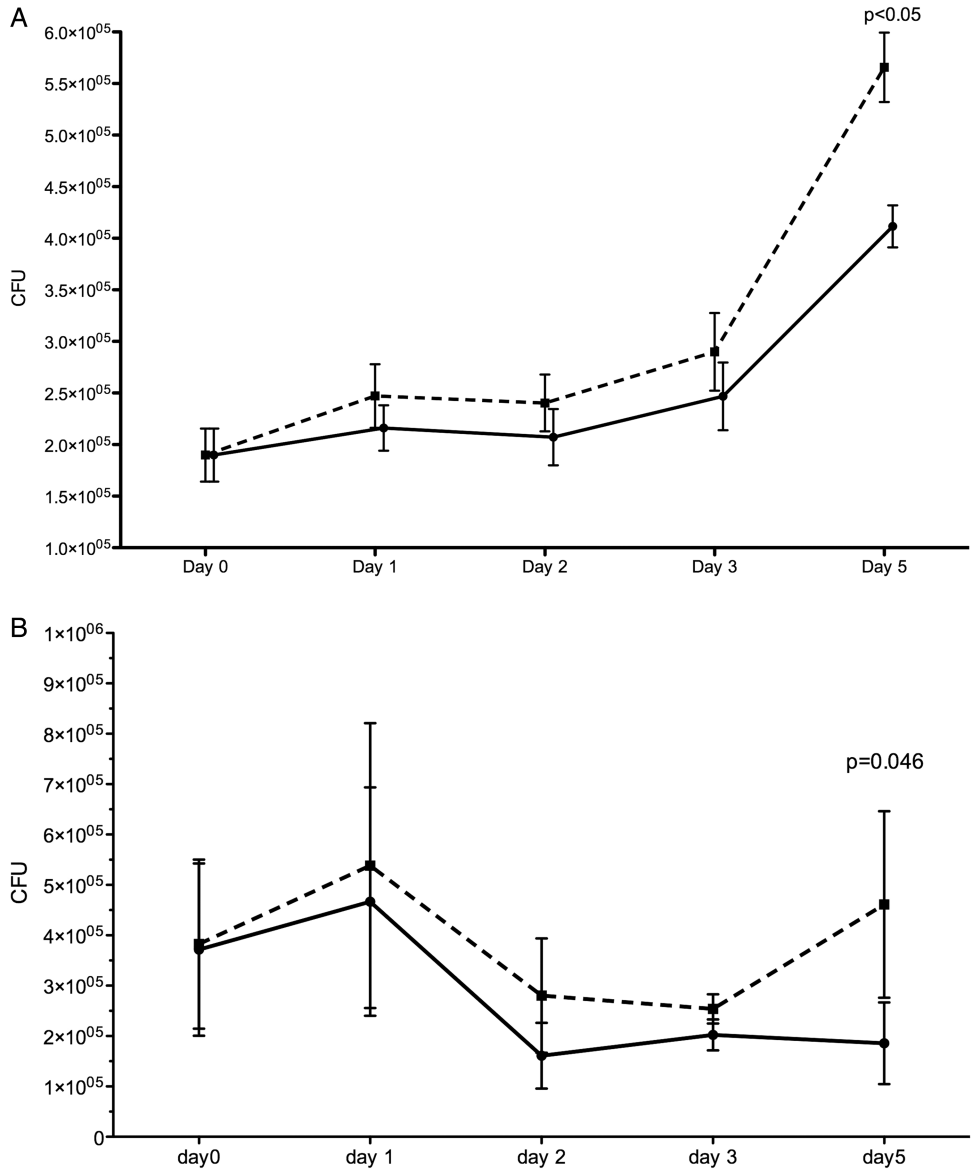

Figure 5 Serial BCG colony counts over 5 days in (A) monocyte-derived macrophages and (B) alveolar macrophages. Monocyte-derived macrophages and alveolar macrophages infected with BCG were cultured for 5 days after the addition of 10% cigarette smoke extract (CSE) on day 0 (post infection). The solid line represents unexposed macrophages and the dashed line represents CSE-exposed macrophages. Each day represents the time point (post infection) when macrophages were lysed and organism load derived by counting the number of colony forming units (CFU) on solid media. Monocyte-derived macrophages, n=9 (day 1–3) and n=5 (day 5); alveolar macrophages, n=5 (day 1–4) and n=4 (day 5).

Ethics approval The University of Cape Town Research Ethics Committee granted approval for this study.

Patient consent Written informed consent was obtained from all participants prior to enrolment in the study.

Provenance and peer review Not commissioned; externally peer reviewed.

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Online supplement

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 $1x10^6$/ml in complete medium.

Preparation of monocyte derived macrophages

PBMCs (obtained by density sedimentation as described above) were seeded at a concentration of $1x10^6$/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% CO_2 for 6 days to ensure that the monocytes had acquired a macrophage phenotype (MDM). The adherent macrophages were presumed to be at $1x10^5$/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 H_20). The lavage fluid was aspirated into a sterile500ml 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^{0}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 where 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.

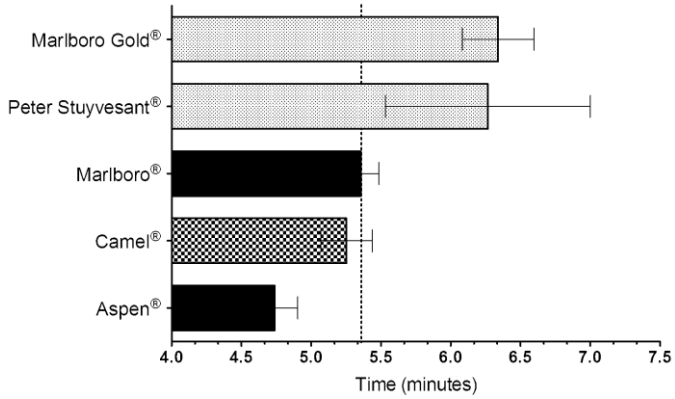

Figure E2 Smoking time for various cigarette brands

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 (± 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 µl 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)μg/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) μg/ml compared to Marlboro Red® 9.1(1.9) μg/ml and Marlboro Gold® 4.9(0.9) μg/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 μg/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) μg/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) μg/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) μg/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 7ADD (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

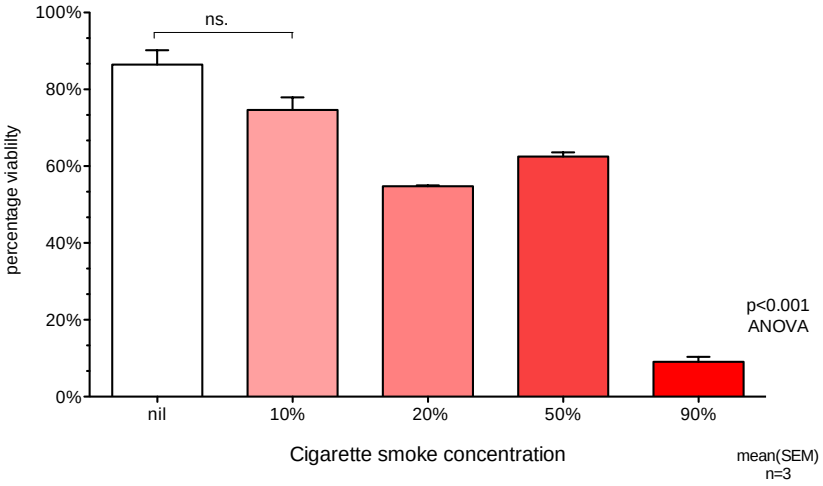

Figure E4 Viability of Macrophages exposed to increasing doses of cigarette smoke extract

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.

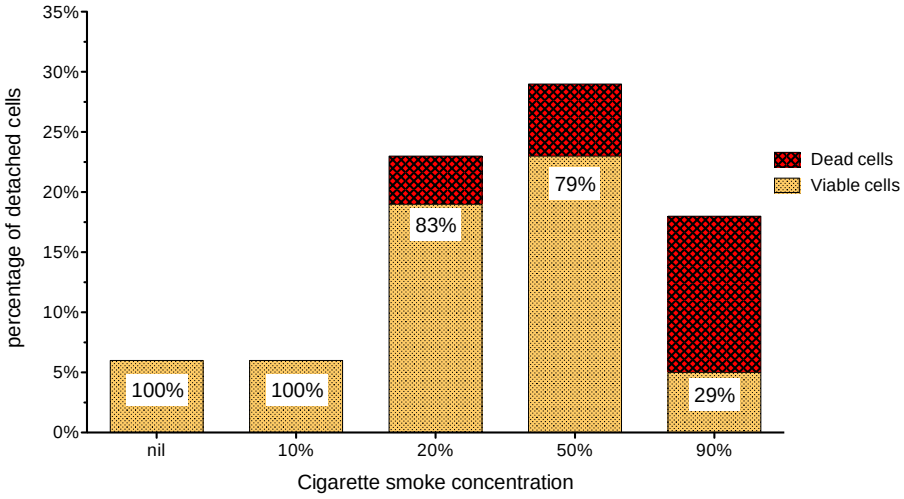

Figure E5 The effect of cigarette smoke on macrophage detachment and viability.

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).

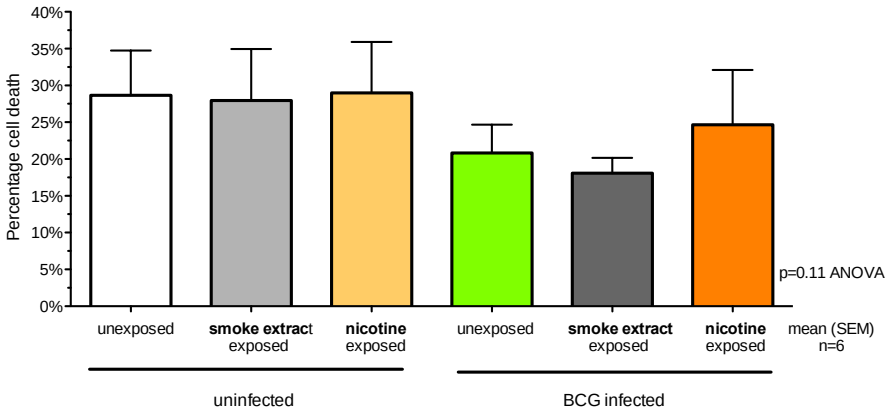

Figure E6 Viability of alveolar macrophages following BCG infection

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.

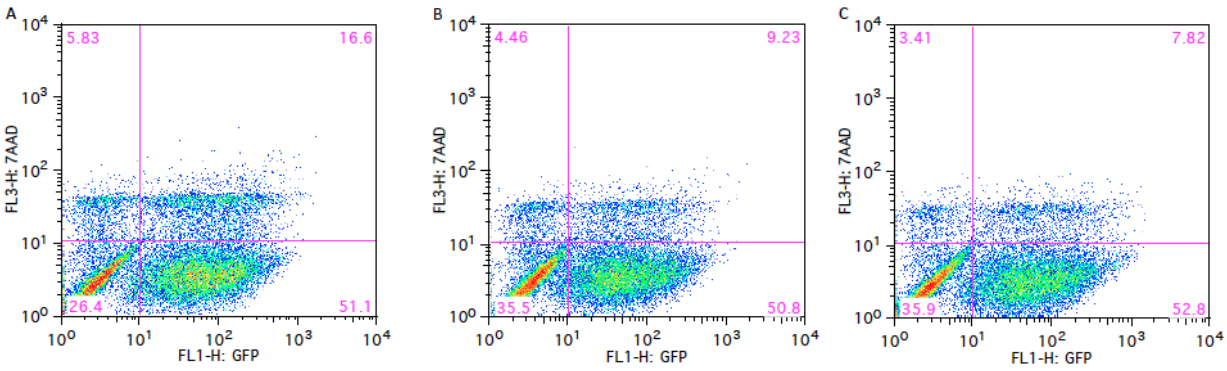

Figure E7 Flow cytometry dot-plots of alveolar macrophages BCG-gfp uptake and viability.

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.

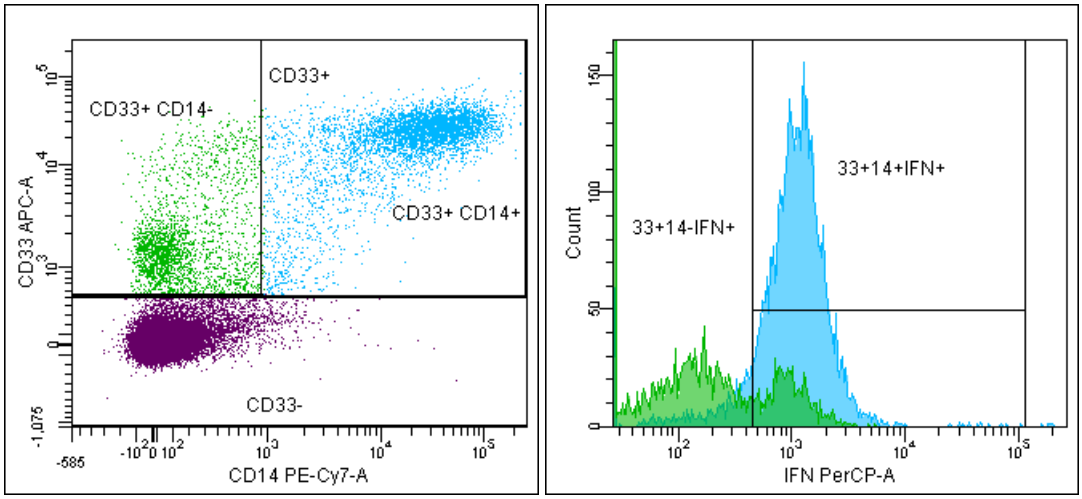

Figure E8 Flow cytometry gating strategy for the definition of cell types producing IFN-γ in response to BCG infection.

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 10^6 copies of HuPO. Following BCG infection IFN-γ mRNA expression increased significantly to a mean (SD) 1016(16.97) copies per 10^6 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.

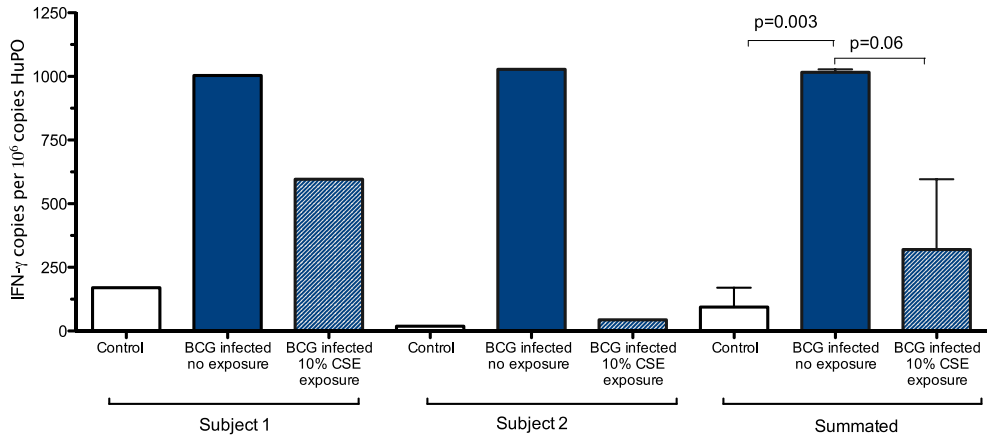

Figure E9 Interferon gamma (IFN-γ) mRNA transcription following BCG infection in CD14 positive cells.
IFN-γ mRNA was transcribed from CD14 cells (macrophages) purified by magnetic bead separation. Following conversion of RNA to cDNA, real time PCR was performed and quantitative amounts of IFN-γ mRNA calculated relative to the house keeping gene HPO.

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