Smoking decreases the response of human lung macrophages to double-stranded RNA by reducing TLR3 expression

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Abstract

Background: Cigarette smoking is associated with increased frequency and duration of viral respiratory infections, but the underlying mechanisms are incompletely defined. We investigated whether smoking reduces expression by human lung macrophages (Mø) of receptors for viral nucleic acids and, if so, the effect on CXCL10 production.

Methods: We collected alveolar macrophages (AMø) by bronchoalveolar lavage of radiographically-normal lungs of subjects undergoing bronchoscopies for solitary nodules (n = 16) and of volunteers who were current or former smokers (n = 7) or never-smokers (n = 13). We measured expression of mRNA transcripts for viral nucleic acid receptors by real-time PCR in those AMø and in the human Mø cell line THP-1 following phorbol myristate acetate/vitamin D3 differentiation and exposure to cigarette smoke extract, and determined TLR3 protein expression using flow cytometry and immunohistochemistry. We also used flow cytometry to examine TLR3 expression in total lung Mø from subjects undergoing clinically-indicated lung resections (n = 25). Of these, seven had normal FEV1 and FEV1/FVC ratio (three former smokers, four current smokers); the remaining 18 subjects (14 former smokers; four current smokers) had COPD of GOLD stages I-IV. We measured AMø production of CXCL10 in response to stimulation with the dsRNA analogue poly(I:C) using Luminex assay.

Results: Relative to AMø of never-smokers, AMø of smokers demonstrated reduced protein expression of TLR3 and decreased mRNA for TLR3 but not TLR7, TLR8, TLR9, RIG-I, MDA-5 or PKR. Identical changes in TLR3 gene expression were induced in differentiated THP-1 cells exposed to cigarette smoke-extract in vitro for 4 hours. Among total lung Mø, the percentage of TLR3-positive cells correlated inversely with active smoking but not with COPD diagnosis, FEV1% predicted, sex, age or pack-years. Compared to AMø of never-smokers, poly(I:C)-stimulated production of CXCL10 was significantly reduced in AMø of smokers.

Conclusions: Active smoking, independent of COPD stage or smoking duration, reduces both the percent of human lung Mø expressing TLR3, and dsRNA-induced CXCL10 production, without altering other endosomal or cytoplasmic receptors for microbial nucleic acids. This effect provides one possible mechanism for increased frequency and duration of viral lower respiratory tract infections in smokers.

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Keywords: Lung, Cigarette smoking, Effects, Toll-like receptors, Macrophages, Alveolar
Background

Viral respiratory infections are major risk factors for exacerbations of chronic obstructive pulmonary disease (COPD) and asthma [1,2], contributing to enormous societal costs due to healthcare utilization and reduced productivity. The frequency and duration of viral respiratory infections is increased by cigarette smoking [3-6] through multiple mechanisms, including reduced mucociliary clearance, impaired production of epithelial defensins, and decreased neutrophil chemotaxis [reviewed in [7,8]]. Smoking also negatively impacts host defense by alveolar macrophages (AMø), a key phagocyte and source of inflammatory mediators. Acting via both nicotinic cholinergic receptors [9] and uncharacterized mechanisms [10], smoking decreases pro-inflammatory cytokine release by human AMø [11-13]. Cigarette smoke extract (CSE) also blunts the ability of AMø to be activated by IFN-γ produced by T cells and natural killer (NK) cells [14]. Whether smoking has additional effects predisposing to viral respiratory infections is unknown.

We hypothesized that cigarette smoke could also reduce the ability of AMø to detect and activate innate responses to viral respiratory viruses. Cells recognize viruses by sensing double-stranded (ds) RNA using TLR3, which is expressed on endosomes and in some cell types, on the cell surface [15,16]. The other endosomal microbe-associated molecular pattern (MAMP) receptors, TLR7, TLR8 and TLR9, are also important for recognizing single stranded RNA and bacterial DNA with unmethylated CpG dinucleotides, respectively. Alternatively, dsRNA can also be recognized via cytosolic sensors, such as double-stranded RNA-dependent protein kinase R (PKR), retinoic acid-inducible gene I (RIG-I), or melanoma differentiation-associated gene 5 (MDA-5) [17].

Experimentally, the response to dsRNA is measured using the synthetic analogue polyinosinic acid:cytidylic acid (poly(I:C)), which stimulates secretion of the chemokine CXCL10 (formerly IP-10) [18-20], in some cell types in an entirely TLR3-dependent fashion [21]. CXCL10 is important to recruit and activate neutrophils, lymphocytes and NK cells [22-24], which are crucial to limit viral replication. At concentrations 100-fold higher than needed for chemotactic activity, CXCL10 also has defensin-like antimicrobial activities [25] and can enhance Mø killing of intracellular Leishmania [26].

The purpose of this study was to determine the effect of smoking on endosomal and cytoplasmic receptors for nucleic acids. Our results demonstrate the susceptibility in human AMø of TLR3, but not of other receptors tested, to smoke-induced down-regulation.

Methods

Research subjects

Studies and consent procedures were performed in accordance with the Declaration of Helsinki at the VA Ann Arbor Healthcare System (all bronchoscopies and some clinically-indicated lung resections) and the University of Michigan Health System (some clinically-indicated lung resections, including all lung transplants and lung volume reduction surgeries) and were approved by their Institutional Review Boards (FWA 0000348 & FWA 00004969, respectively). All subjects understood the purpose of the study and gave written consent before any research procedures. All subjects underwent a complete history and physical examination by a Pulmonologist, spirometry, chest imaging, prospective collection of medication history, and complete blood count with differential, coagulation studies and chemistry panel.

Bronchoalveolar lavage (BAL) cohort

We recruited 13 healthy never-smoker volunteers and 23 subjects with a smoking history who either were scheduled to undergo bronchoscopy in the evaluation of solitary pulmonary nodules (n = 16) or who volunteered (n = 7). Among the 23 subjects who had ever smoked, 18 were active smokers (<six months since quitting) and 5 were former smokers (>six months since quitting). Of those with any smoking history, subjects (n =10) with ≥10 pack years, a ratio of forced expiratory volume in 1 second to forced vital capacity (FEV1/FVC) >0.7, normal spirometry, and no clinical diagnosis of COPD represent control smokers. Subjects (n =13) with a smoking history, FEV1/FVC <0.7 and abnormal spirometry were considered to have COPD. All subjects were without evidence of lung infection, interstitial lung disease or collagen vascular disease.

Importantly, not all types of experiments were performed on cells from every subject in this cohort, and conversely, some subjects were used for more than one type of experiment. The characteristics of the BAL subjects used in each type of experiments are summarized in Tables in the Results sections; demographic and clinical data for this entire cohort are shown in Additional file 1: Table S1.

Lung tissue cohort

Lung tissue was collected from consented subjects undergoing clinically-indicated resections for pulmonary nodules, lung volume reduction surgery, or lung transplantation (n = 25). Using the same definitions as in the BAL cohort, this cohort comprised seven control smokers and 18 subjects with COPD. This cohort was used exclusively for flow cytometric analysis of total lung Mø. Characteristics of this cohort are summarized in the
Results section, and demographic and clinical data for individual subjects are shown in Additional file 2: Table S2.

**BAL procedure and cell preparation**

BAL was performed in the right middle lobe and lingula of the volunteers and in whichever of these sites was contralateral to the nodule in the clinically-indicated bronchoscopies, in which the research BAL was performed as early as feasible, and always before any biopsies or brushings. We instilled 100 ml normal saline per site, using a 30 ml syringe and gentle manual suction. BAL fluid was filtered through sterile gauze to remove mucus, and cells were washed thrice with PBS, with centrifugation between washes.

BAL cells (>95% AMø by Wright-Giemsa-stained cytospins) were either immediately processed for flow cytometry and immunohistochemistry or were cultured briefly to purify AMø by adherence. For this purpose, cells were resuspended in complete medium (RPMI 1640 containing 25 mM HEPES, 2 mM l-glutamine, 1 mM pyruvate, 100 U/ml penicillin/streptomycin, 10% heat-inactivated AB human serum (all from Invitrogen, Carlsbad, CA), and 55 μM 2-ME (Sigma Chemical, St. Louis, MO), and were plated at 2 x 10⁵ cells/well in sterile 24-well plates. Cells were incubated for 1.5 h in 5% CO₂ at 37°C, washed to remove nonadherent cells, then incubated in AIM-V serum-free medium (Invitrogen) under experimental conditions. For TLR3 stimulation, adherence-purified AMø were cultured in AIM-V alone or with poly(I:C) at 50 μg/ml for 24 h.

**Lung sample preparation**

Lung tissue was dissected free of any areas containing nodules, cancers or evidence of infection by a Pathologist before being released to the study. Lung sections weighing approximately 3 g were dispersed using a Waring blender with centrifugation between washes.

BAL cells (>95% AMø by Wright-Giemsa-stained cytospins) were either immediately processed for flow cytometry and immunohistochemistry or were cultured briefly to purify AMø by adherence. For this purpose, cells were resuspended in complete medium (RPMI 1640 containing 25 mM HEPES, 2 mM l-glutamine, 1 mM pyruvate, 100 U/ml penicillin/streptomycin, 10% heat-inactivated AB human serum (all from Invitrogen, Carlsbad, CA), and 55 μM 2-ME (Sigma Chemical, St. Louis, MO), and were plated at 2 x 10⁵ cells/well in sterile 24-well plates. Cells were incubated for 1.5 h in 5% CO₂ at 37°C, washed to remove nonadherent cells, then incubated in AIM-V serum-free medium (Invitrogen) under experimental conditions. For TLR3 stimulation, adherence-purified AMø were cultured in AIM-V alone or with poly(I:C) at 50 μg/ml for 24 h.

**Differentiation and treatment of THP-1 cells**

THP-1 cells obtained from ATCC (Manassas, VA) were grown according to the supplier’s guidelines. Cells were plated at 4 x 10⁵ cells/well in 24-well plates, and after adherence, were differentiated by exposure to 150 ng/ml phorbol myristate acetate (PMA) and 0.01 μM vitamin D3 for 48 hrs, and then were washed with fresh complete medium before exposure to various concentrations of cigarette smoke-extract (CSE) for 4 hr. Fresh CSE was prepared by bubbling the mainstream smoke of two University of Kentucky Tobacco Health Research Institute standardized cigarettes (lot 2R4F), using a Jaeger-Baumgartner Cigarette Smoking Machine (C.H. Technologies, New Jersey) driven by dry compressed air, into 20 ml RPMI 1640; the result was defined as 10% CSE. CSE was filter-sterilized using a 0.22 μm membrane and was used the same day.

**Quantitative real-time PCR**

RNA was isolated using RiboPure kits (Applied Biosystems, Foster City, CA), TURBO DNA-free (Applied Biosystems) to remove genomic DNA, and Retroscript kits (Applied Biosystems) for reverse-transcription. We performed quantitative real-time PCR using the Stratagene Mx3000P (LaJolla, CA), with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous reference, and commercial primer-probe sets (Taqlman chemistry, Applied Biosystems). Transcript levels were reported relative to a calibrator of unstimulated THP cells, or AMø from never smokers, as appropriate, as calculated on the thermocycler.

**Flow cytometry**

BAL cells were resuspended in 100 μL staining buffer (1% FA Buffer (BD Bioscience, San Jose, CA), 1% FCS, 0.01% sodium azide) per tube. We used antibodies against the following antigens (clones in parentheses): CD45 (2D1) (eBioscience, San Diego, CA), TLR3 (40C1285.6), TLR7 (polyclonal), TLR9 (26C593.2) (Imgenex Corp., San Diego, CA), and TLR8 (303F1.14) (Dendritics, Lyon, France). To detect intracellular receptor expression, cells were treated with Fixation and Permeabilization Buffers (eBioscience). In all experiments, we used isotype-matched controls, analyzed cells on an LSR II flow cytometer (BD Bioscience), and collected a minimum of 10,000 CD45+ events per sample, using FACS Diva software with automatic compensation, and FlowJo analysis software (Tree Star, Inc., Ashland, OR). Details of instrument setup have been described recently [29].

**Immunohistochemistry**

Cytosperms were immersed in cold acetone followed by methanol plus hydrogen peroxide. The slides were stained with biotinylated anti-human TLR3 (Imgenex) or isotype control antibodies, followed by Universal ABC peroxidase complex and 3-aminop-9-ethylcarbazole peroxidase substrate. Slides were counter-stained with hematoxylin and photographed using an Olympus BX51 digital camera.

**CXCL10 analysis**

CXCL10 levels were determined using Biosource Multiplex Assays (Invitrogen) and a Luminex 200™ (Luminex Corporation, Austin, TX).
Statistical analyses
Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA) except as specified. We used the nonparametric unpaired Mann–Whitney test to determine differences between two groups (smokers versus never-smokers; healthy subjects versus COPD subjects) in mRNA and flow cytometry data; the nonparametric Kruskal–Wallis one-way ANOVA to compare multiple conditions on THP cells in vitro; and ANOVA with Bonferroni’s Multiple Comparison post-hoc testing to determine differences between smokers and never-smokers in stimulated cytokine secretion. We used Spearman non-parametric analysis to correlate TLR3 mRNA and protein expression with clinical variables and performed linear regression using SPSS (IBM Corp.; Armonk, NY). A two-tailed $p$ value of $< 0.05$ was considered significant.

Results

TLR3 expression is selectively decreased in AMø of smokers

To test whether cigarette smoking impacts expression by human AMø of TLRs implicated in defense against respiratory viruses, we first analyzed adherence-purified AMø obtained by BAL of 11 current or former smoking subjects and six never-smokers (Table 1) using quantitative real-time PCR. Results showed significantly decreased TLR3 mRNA transcripts in smokers compared with never-smokers ($p = 0.0015$; Mann–Whitney test) (Figure 1A), but no significant differences in transcripts for TLR7, TLR8 or TLR9, which are also found in the endosome (Figure 1A). We also found no significant differences between smokers and never-smokers in mRNA transcripts for the cytoplasmic dsRNA receptors RIG-I, MDA-5 and PKR (Additional file 3: Figure S1).

These findings were supported by two measures of AMø protein expression. Flow cytometry (Additional file 4: Figure S2) permitted identification of specific staining for TLR3 and TLR9 amongst AMø of never-smokers, although expression of TLR7 was very low (Additional file 4: Figure S2, middle panel) in both groups, and was not analyzed further. Comparing BAL samples from smokers ($n = 13$), and never-smokers ($n = 10$) (Table 2), we found a significantly decreased percentage ($p < 0.0001$, Mann–Whitney test) of AMø positive for intracellular TLR3 in AMø of smokers (Figure 1B). Note that these experiments contain some of the same subjects studied in Table 1, as well as other BAL subjects, as described in Methods; for additional details about individual subjects, see Additional file 1: Table S1). TLR3 was not detected on the surface of unpermeabilized AMø of either group (not shown). There were no differences between smokers ($n = 6$) and never-smokers ($n = 5$) in the percentage of AMø expressing intracellular TLR9 (Figure 1B) (or TLR7 and TLR8, data not shown). Similarly, immunohistochemical staining of BAL cytopsins (Additional file 1: Table S1) demonstrated greatly reduced TLR3 expression in the AMø from smokers, relative to AMø from the never-smokers (Figure 1C). These independent data indicate that reduction in TLR3 protein expression measured by flow cytometry did not result simply from greater autofluorescence in AMø of smokers.

In univariate analyses including both smokers and never-smokers, TLR3 RNA transcripts correlated inversely with both FEV$_1$ % predicted and subject age (Figure 2A-B). Considering only those with a history of smoking, there was no correlation with pack-years (Figure 2C). However, in a linear regression, none of the variables (smoker versus never-smoker, age, sex, FEV$_1$ % predicted) reached statistical significance for mRNA transcripts. AMø positivity for TLR3 protein did not correlate in univariate analyses with FEV$_1$ % predicted (Figure 2D) or pack-years of smoking exposure (Figure 2F), but did correlate with subject age (Figure 2E). In a linear regression, smoking status (smoker vs. never-smoker) was strongly associated with TLR3+ AMø ($p = 0.035$), but sex and FEV$_1$ % predicted remained insignificant. When we analyzed smokers with COPD versus smokers without COPD, no significant differences were seen in TLR3 mRNA transcripts (COPD, 0.23 ± 0.16 vs. no COPD, 0.49 ± 0.49, mean ± SEM dRNA; $p = 0.52$, unpaired $t$ test) or flow cytometric results (COPD, 12.2 ± 12.2 vs. non-COPD, 10.4 ± 5.2; mean ± SEM % TLR3-positive AMø, $p = 0.87$, unpaired $t$ test). Despite the use of inhaled corticosteroids (ICS) by ∼50% of the smoking subjects, the decrease in the fraction of TLR3+ AMø of smokers was not a result of steroid usage ($p = 0.79$, determined by $t$-test comparing TLR3 mRNA transcript expression between ICS users and non-users; $p = 0.92$ when comparing TLR3 protein measurements by flow cytometry).

Table 1 Characteristics of BAL subjects used for RNA studies

| Group       | Smokers | Never-smokers | p value |
|-------------|---------|---------------|---------|
| Subjects, n | 11      | 6             |         |
| Sex ratio, M/F | 11/0 | 2/4            | 0.003   |
| Age, years (SD) | 65.5 (7.2) | 37.7 (12)  | 0.002   |
| Smoking, pack-years (SD) | 65.5 (23.1) | 0 (0)       | 0.0008  |
| Smoking status (Former/Current) | 2/9 | 0/0          | 0.003   |
| FEV$_1$, % pred. (SD) | 57 (22.1) | 101 (8.7)  | 0.002   |
| FEV$_1$/FVC% (SD) | 58.5 (15.6) | 86.8 (4.9) | 0.0007  |
| ICS use (yes/no) | 7/4 | 0/6          | 0.02    |

ICS Inhaled Corticosteroid Use, M Male, F Female. Data are represented as mean (SD), ratio of current smokers to former smokers, or fraction of ICS users (yes/no). All FEV$_1$ values are pre-bronchodilator. $p$ values calculated using Mann Whitney test.
Thus, these secondary analyses supported a significant effect of smoking on AM\(\text{\textbeta}\) expression of TLR3. However, due to the distribution of our subjects, we were unable to exclude the possibility that our findings resulted from the preponderance of women in the never-smoker group and of men among the smokers in this cohort, or from the significant difference in ages between the volunteer and clinical subjects.

Reduced TLR3 expression in lung M\(\text{\textbeta}\) correlates with smoking status but not COPD stage

In apparent contradiction to our findings, a recent paper primarily using immunohistochemistry in lung tissue sections reported that the percentage of TLR3-positive lung M\(\text{\textbeta}\) were significantly increased in smokers compared with never-smokers [30]. To address this discrepancy and the questions about the possible effects of age and sex in
our BAL subjects, we studied a different cohort of subjects (n = 25) undergoing clinically-indicated lung resection procedures (Table 3). This cohort also had the advantage of having more nearly balanced ratios of male and female subjects, as well as a wide range of spirometry values. We used mechanical disaggregation of tumor-free lung parenchyma to produce a single cell suspension of high viability containing both AMø and interstitial lung Mø, which we identified as autofluorescent, CD45+, high side scatter cells.

Flow cytometric analysis permitted objective quantification of the percentage of individual lung Møs positive for intracellular TLR3, relative to simultaneously analyzed isotype-control monoclonal antibodies (Figure 3A, B). We found the percentage of TLR3-positive lung Møs from current smokers was significantly reduced (p = 0.006) compared to the percentage of TLR3-positive lung Møs from former smokers (Figure 3C). By contrast, considering these same individuals, there were no significant differences in percentages of lung Mø expressing TLR3 between those with normal pulmonary function and those with COPD (Figure 3D). Nor was there a correlation between the percentage of TLR3-positive lung Mø by flow cytometry and either FEV1 % predicted or duration of smoking in pack-years (Additional file 5: Figures S3A & B). In a linear regression, smoking status (current vs. former) was significantly associated with TLR3 positivity (p = 0.0001). In a linear regression, smoking status (current vs. former) was significantly associated with TLR3 positivity (p = 0.0001).

**Table 2** Characteristics of BAL subjects used for flow cytometry studies

| Group                        | Smokers | Never-smokers | p value   |
|------------------------------|---------|---------------|-----------|
| Subjects, n                  | 13      | 10            |           |
| Sex ratio, M/F               | 11/2    | 1/9           | 0.0006    |
| Age, years (SD)              | 60.3 (8.9) | 42.2 (13.7) | 0.002     |
| Smoking, pack-years (SD)     | 47.4 (33.7) | 0 (0)       | 0.0001    |
| Smoking status (Former/Current) | 3/10   | 0/0           | 0.0001    |
| FEV1, % pred. (SD)           | 83.5 (23.6) | 100.7 (10.5) | 0.077     |
| FEV1/FVC% (SD)               | 72.5 (14.3) | 84.2 (5.7) | 0.0249    |
| ICS use (yes/no)             | 4/9     | 0/10          | N.S.      |

ICS Inhaled Corticosteroid Use; M Male, F Female. Data are represented as mean (SD), ratio of current smokers to former smokers, or fraction of ICS users (yes/no). All FEV1 values are pre-bronchodilator. p values calculated using Mann Whitney test.

The effects of acute smoke-exposure in vitro and the direct nature of the smoking effect was shown in vitro using differentiated cells of the human Mø cell line THP-1 by treatment with PMA plus vitamin D3. In four independent experiments, we found that CSE at concentrations of 1.25% and 2.5% significantly decreased expression of TLR3 transcripts (Figure 4A), but had no effect on transcripts of TLR7, TLR8 or TLR9 (Figures 4B-D). CSE at these concentrations had no effect on viability as assessed by trypan blue exclusion (not shown). CSE also had no significant effect on mRNA transcripts for RIG-I, MDA-5 or PKR at concentrations up to 2.5% CSE (Figure 4E-G). Thus, the effect of smoke exposure on TLR3 mRNA levels can be induced within as little as four hours in THP-1 cells differentiated to a mature Mø phenotype. These findings indicate that smoking reduces Mø TLR3 expression directly, and not secondarily due to an effect on another lung cell type or on the lung microbiome.

**AMø of smokers show reduced CXCL10 production in response to poly(I:C) stimulation in vitro**

Finally, we investigated whether decreased TLR3 expression on the AMø of smokers would affect CXCL10 production following poly(I:C) stimulation. These experiments were performed using AMø from subjects in the BAL cohort (Additional file 2: Table S2). CXCL10 concentrations were close to the level of detection in unstimulated cells from both smokers (n = 5) and never-smokers (n = 4) (Table 4) and did not differ significantly (Figure 5). Following poly(I:C) stimulation, AMø from never-smokers showed a 1000-fold increase in CXCL10 production, and differed significantly from the response of AMø from smokers, which showed only a 10-fold increase from unstimulated levels (Figure 5). These results illustrate a functional consequence of the difference in TLR3 expression between the two groups.

**Discussion**

We demonstrate that smoking reduces expression by human lung Mø of TLR3, a receptor for microbial dsRNA and endogenous danger signals. Relative to AMø of never-smokers, AMø of current smokers showed reduced TLR3 mRNA and protein, and lower secretion of CXCL10 in response to the viral dsRNA analogue poly(I:C). Smoking did not significantly reduce AMø expression of other receptors for viral nucleic acids in the cytoplasm (RIG-I, MDA-5 or PKR) or endosome (TLR7, TLR8 or TLR9). In a separate cohort, TLR3 protein expression by total lung Mø was also reduced in active smokers, relative to former smokers. TLR3 protein expressed as the percentage of positive lung Mø did not correlate significantly in either cohort with a diagnosis of COPD, FEV1% predicted or total history of smoking expressed as pack-years. The direct nature of the smoking effect was shown in vitro using differentiated cells of the human Mø line THP-1. These findings identify a
new mechanism by which smoking impairs anti-viral lung host defenses.

Our findings address the intersection of two highly prevalent clinical issues, host defense against respiratory viruses and smoking. Respiratory viruses are implicated, alone or as mixed infections, in most COPD exacerbations, including those of greatest severity [31,32]. Viral respiratory infections are also the leading cause of asthma exacerbations in adults and children [33,34], including those requiring hospitalization [35]. Although only some smokers develop COPD, those who continue to smoke sustain more frequent respiratory infections and faster lung function decline [36]. Smoking is also a key contributor to morbidity in asthma [37]. Asthmatic smokers experience worse symptom control, greater risk of hospitalization, and increased resistance to the therapeutic effects of inhaled corticosteroids [38-41]. Finally, even in individuals with normal spirometry, smoking is an independent risk factor for increased number and severity of respiratory infections [3]. Hence, these results

Figure 2 AMø TLR3 expression does not correlate with lung function or total duration of smoking exposure. AMø were harvested, processed and analyzed as described in the legend to Figure 1. A-C. TLR3 mRNA transcripts by quantitative real-time PCR (smokers, n = 11; never-smokers, n = 6, except in panel C, which includes only current or former smokers). D-F. Flow cytometric analysis of TLR3 protein expression (smokers, n = 13; never-smokers, n = 10, except in panel F which includes only current or former smokers). A. FEV1 % predicted; B. participant age (years); C. F. smoking history (pack-years); note the difference in the vertical scale of panel F. Blue circles, smokers; red crosses, never-smokers. p values by Spearman correlation.
provide another rationale to urge all smokers to quit smoking absolutely, regardless of pulmonary function or specific diagnosis.

Optimal defense against respiratory viruses requires coordination of multiple elements of innate and adaptive immunity [42], especially prompt production by epithelial cells of types I (alpha/beta) and III (lambda) IFNs [43-45]. Appropriate contributions by AMø are likely also important [42]. In response to viruses, AMø produce measurable amounts of type I IFNs [46] and of the type III IFN lambda 1 (IL-29) [47]. Human monocyte-derived Mø (MDMø) produce mRNA for type III IFNs in response to

Table 3 Characteristics of surgical lung tissue subjects

| Group                             | Smokers with COPD | Smokers without COPD | p value  |
|-----------------------------------|-------------------|----------------------|----------|
| Subjects, n                       | 18                | 7                    |          |
| Sex ratio, M/F                    | 10/8              | 5/2                  | 0.49     |
| Age, years (SD)                   | 61.8 (9.4)        | 54.4 (9.1)           | 0.74     |
| Smoking, pack-years (SD)          | 60.3 (41.4)       | 44.5 (23.9)          | 0.47     |
| Smoking status (Former/Current)   | 4/14              | 3/4                  | 0.11     |
| FEV1, % pred. (SD)                | 41.8 (27.7)       | 95.4 (11.2)          | 0.0008   |
| FEV1/FVC% (SD)                    | 42.5 (18.6)       | 76.0 (5.2)           | 0.0002   |
| ICS use (yes/no)                  | 12/6              | 1/6                  | 0.02     |

ICS Inhaled Corticosteroid Use, M Male, F Female. Data are represented as mean (SD), ratio of current smokers to former smokers, or fraction of ICS users (yes/no). All FEV1 values are pre-bronchodilator. p values calculated using Mann Whitney test.

Figure 3 In current smokers, the frequency of lung Mø expressing TLR3 protein is reduced relative to lung Mø of former-smokers. To isolate total lung Mø, areas of human lung parenchyma, resected for clinical indications and remote from any evidence of nodules or infection, were processed by mechanical disaggregation without the use of enzymes. Lung Mø were permeabilized, stained for TLR3 expression and analyzed by flow cytometry. A, B. Representative histograms of intracellular TLR3 expression; A, former smoker; B, current smoker. C. Percentage of lung Mø expressing TLR3 relative to smoking status; x’s represent former smokers (n = 17); circles represent current smokers (n = 8). D. Percentage of TLR3-positive by lung Mø relative to COPD status; as in panel C, x’s represent former smokers (n = 17); circles represent current smokers (n = 8); *, p < 0.05; NS, non-significant. The Mann–Whitney test was used to calculate p values.
stimulation via TLR3 or TLR4 [48] or to viruses [47,49]. A specific anti-viral role of AMø has been confirmed experimentally by cellular depletion in two animal models [50,51], but in a murine model of respiratory syncytial virus pneumonia, AMø depletion reduced early antiviral responses, increased viral load, but had no effect on late outcomes [52]. Hence, the significance of the current findings may vary with the particular viral pathogen. Additional studies will be needed to determine whether the effect we found on TLR3 expression translates into defective responses by the AMø of smokers to intact respiratory viruses.

These results expand the known effects of cigarette smoking on AMø function, which are clearly complex. Two studies using Affymetrix Human Genome U133 Plus 2.0 GeneChips found AMø from human smokers to have distinctive, globally altered gene expression profiles relative to non-smokers [53,54]. Interestingly, TLR3 is not mentioned as a differentially expressed gene in either study. Disparity from our results likely relates to the difference in methodology; importantly, we showed very reduced TLR3 protein expression. The more pronounced reduction we found in TLR3 protein relative to mRNA implies the possibility of post-transcriptional control.

Figure 4 Exposure to CSE specifically and rapidly reduces TLR3 gene expression in a differentiated human Mø cell line. THP-1 cells differentiated using PMA & vitamin D3 for 48 hours were cultured in complete medium alone or with various concentrations of CSE for an additional 4 hr, then analyzed by quantitative real-time RT-PCR. Data are mean ± SEM of four independent experiments, expressed as dRn (relative to differentiated THP-1 cells cultures in complete medium alone). A, TLR3; B, TLR7; C, TLR8; D, TLR9; E, RIG-I; F, MDA-5; G, PKR. Kruskal-Wallis one-way ANOVA was used to compare groups. *, p < 0.05, compared to cells that did not receive CSE exposure.
smoking-induced downregulation, an important issue for future studies.

AMα of human smokers are often described as being “activated” [55–57], but both they and AMα from COPD patients show impaired bacterial uptake and killing [58,59]. Production of TNF-α and IL-6 by AMα of smokers (and COPD patients, which often mixed current and former smokers) equaled or exceeded that of non-smokers, but was strikingly less inhibitable by steroids [58,59]. Production of TNF-α and IL-6 by AMα of smokers in response to poly(I:C) stimulation is impaired. Figure 5 Production of CXCL10 by AMα of smokers in response to poly(I:C) stimulation is impaired. AMα from never-smokers (n = 4) (white and red columns) and smokers (n = 5) (light and dark blue columns) were cultured for 24 h in either complete medium alone or with poly(I:C). Supernatants were collected and CXCL10 protein was measured by Luminex assay; results are expressed as the fold-increase in CXCL10 over the unstimulated control condition; note the logarithmic scale of the vertical axis. Data are mean ± SEM. One-way ANOVA with Bonferroni’s Multiple Comparison post-hoc testing was used to compare differences among groups, *, p < 0.01.

Table 4 Characteristics of BAL subjects used for in vitro stimulation studies

| Group                  | Smokers | Never-smokers | p value |
|------------------------|---------|----------------|---------|
| Subjects, n            | 5       | 4              |         |
| Sex ratio, M/F         | 5/0     | 2/2            | 0.13    |
| Age, years (SD)        | 60 (11) | 39.5 (12.1)    | 0.06    |
| Smoking, pack-years (SD)| 47.4 (35.2) | 0 (0)      | 0.02    |
| Smoking status (Former/Current) | 0/5 | 0/0 | 0.007 |
| FEV1, % pred. (SD)     | 66.6 (30.6) | 106.5 (12)     | 0.06    |
| FEV1/FVC% (SD)         | 61.8 (19) | 81.0 (7.5)     | 0.10    |
| ICS use (yes/no)       | 3/2     | 0/4            | 0.10    |

ICS Inhaled Corticosteroid Use, M Male, F Female. Data are represented as mean (SD); ratio of current smokers to former smokers, or fraction of ICS users (yes/no). All FEV1 values are pre-bronchodilator. p values calculated using Mann Whitney test.
mRNA expression by MDMø of TLR8 and MDA5 and that MDMø of smokers had reduced mRNA expression of RIG-I and MDA-5, relative to MDMø of never-smokers [75]. That study does not mention TLR3 expression, which presumably was not altered in either case. Differences from our results likely reflect disparity both in cell type studied and possibly in methodology, as those results were obtained by Affimmetrix assay.

The current findings are interesting in regard to two recent murine studies. Gaschler and colleagues found attenuated in vitro secretion of TNF-α, IL-6 and CCL5 in response to poly(I:C) by AMø from smoke-exposed mice, but did not find decreased TLR3 expression [76]. That disparity from our results may reflect a species difference. Kang and colleagues found that cigarette smoke selectively augmented airway and alveolar inflammation induced by viral MAMPs and influenza virus, as measured by induction of type I IFN, IFN-γ, IL-18 and IL-12/IL-23 p40 and PKR activation [77]. Further analysis using knockout mice indicated a role for both acute TLR3-dependent and chronic TLR3-independent pathways, as well as a pathway dependent on mitochondrial antiviral signaling protein (MAVS), IL-18Rα, IFN-γ and PKR [77]. That important study, however, did not measure TLR3 expression by AMø, and its findings likely reflect the interaction of multiple cell types in vivo.

Considerable evidence indicates the importance of CXCL10 in antiviral host defense but also in development or prevention of certain lung diseases [78,79]. CXCL10 is one of three chemokines, along with CXCL9 and CXCL11, that are highly induced by IFN-γ, as well as by types I and III IFNs [49,80]. Of note, however, CXCL10 production is also induced directly by infection of human AMø by rhinovirus, of murine AMø by RSV and of human MDMø by influenza or HIV [19,20,81-83], making this cytokine a suitable endpoint to test the functional importance of the observed TLR3 downregulation. A key action of CXCL10 is inflammatory cell recruitment, acting via CXCR3, which is found on Mø, CD8+ T cells, activated CD4+ T cells (especially Th1 cells), NK cells and plasmacytoid dendritic cells [84]. CXCR3 is essential for recruitment of antigen-specific CD4+ T cells to the lungs in a murine model of parainfluenza pneumonia [85]. In a model of Coxsackievirus infection, CXCL10 was shown to limit viral replication via NK cell recruitment [24].

Markedly reduced induction of CXCL10 in AMø from smokers is of interest in the context of other recent human studies. CXCL10 is one of several M1-associated genes down-regulated in AMø of healthy smokers and even more so, in smokers with COPD, relative to healthy never-smokers [54]. Although significantly increased CXCL10 concentrations have been found in the sputum of COPD patients, when compared with nonsmokers but not with smokers without obstruction [86], the origin of that chemokine is unclear, as it is also produced by epithelial cells that significantly outnumber Mø in the airways. Both by showing the role of reduced TLR3 expression and by studying AMø, we extend a study of gene expression by MDMø, which found reduced expression of CXCL10 at baseline in smokers relative to never-smokers, and also that CSE reduced IFN-γ-induced CXCL10 production by MDMø of never-smokers [75].

Our study has several limitations. One is the absence of never-smokers from our surgical cohort, who were recruited before clinically-indicated resections. Another is the predominance in our BAL cohort of female never-smokers (11 of 13 subjects) versus a predominance of male current smokers with preserved lung function (eight of nine subjects) and COPD patients (11 of 11 subjects). Concerns that differences in sex or age could confound our BAL results should be reduced by the congruent effect of smoking in our surgical cohort, which comprised 40% female subjects and which showed no significant effect of age on TLR3 expression by lung Mø. Moreover, at equivalent levels of tobacco exposure, women appear to be at greater risk of lung function impairment [reviewed in [87]], the opposite of what would be expected if the current finding were a male-specific effect. Although we cannot formally exclude the possibility that differences in subject age explain the reduced expression of CXCL10 in response to TLR3, we consider that possibility unlikely, as serum levels of CXCL10 increase with age [88,89]. Other limitations are the absence of DLCO measurements, which precludes comparison of our results with those of Koarai and colleagues [30] on this point, and that because post-bronchodilator FEV1 values were not available on all subjects, we have presented and analyzed exclusively pre-bronchodilator FEV1 values.

**Conclusions**

Active smoking reduces expression of TLR3 by human lung Mø, assayed both as AMø harvested by BAL and as a mixture of AMø and interstitial lung Mø from surgical tissue. This effect occurs via both down-regulation of mRNA transcripts, which can be induced in vitro within four hours, and from a more pronounced effect on TLR3 protein expression, which appears to be at least partially reversible on smoking cessation. Reduction in lung Mø TLR3 expression may be one mechanism contributing to the increased incidence of viral respiratory infections in smokers and to viral induction of acute exacerbations of COPD. Defining the effect of smoking on the phenotype of human AMø and other lung cell types is a crucial step in the translation of basic science into therapies.
Endnotes

Subjects were recruited via observational studies registered with ClinicalTrials.gov as NCT00281190, NCT00281203 and NCT00281229. These data were presented in part at the International Scientific Conference of the American Thoracic Society, May 17, 2010 in New Orleans, LA, and have been published in abstract form in Am J Respir Crit Care Med 2010; 181: A3875. Supported by R01 HL082480 (ILC, FJM), R01 HL056309 (JLC) and U01 HL098961 (JMB, JLC) from the USPHS; a Career Development Award (CMF) and a Merit Review Award (CMF) and a Research Enhancement Award Program (SWC, JMB, JLC) from the Biomedical Laboratory Research & Development Service, Department of Veterans Affairs. These investigations were also supported in part by the Tissue Procurement Core of the University of Michigan Comprehensive Cancer Center, Grant P30 CA69525, and the Lung Tissue Research Consortium (Clinical Centers), Grant N01 HR046162.

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Additional files

Additional file 1: Table S1. Summary of all BAL subjects demographics, smoking histories, spirometry & current smoking status. ICS, inhaled corticosteroid use; FC, flow cytometry; IHC, immunohistochemistry. Summarized data are shown in bold and are represented as mean (SD), ratio of current smokers to former smokers, or fraction of ICS users (yes/no). All FEV1 values are pre-bronchodilator.

Additional file 2: Table S2. Summary of all lung tissue subject demographics, smoking histories, spirometry & current smoking status. ICS, inhaled corticosteroid use. M, Male; F, Female. Summarized data are shown in bold and are represented as mean (SD), ratio of current smokers to former smokers, or fraction of ICS users (yes/no). All FEV1 values are pre-bronchodilator.

Additional file 3: Figure S1. A),TMA of current smokers show no reduction in mRNA expression of cytoplasmic dsRNA receptors, relative to A),TMA of never-smokers. RNA from A),TMA was isolated, depleated of contaminating genomic DNA, reverse-transcribed and analyzed by quantitative real-time RT-PCR using Taqman chemistry and specific primer-probe sets, normalized to GAPDH transcripts. Data are expressed on the horizontal axis as mean ± SEM for relative quantity (dRn), calculated in comparison to a single never-smoker who was arbitrarily designated the reference sample. Never-smokers (n = 6), red bars; smokers (n = 11), blue bars. The Mann-Whitney test was used to calculate statistical significance.

Additional file 4: Figure S2. Representative flow cytometry results. A),TMA were permeabilized, stained for expression of TLR3 (left-hand panels), TLR 7 (middle panel) and or TLR9 (right-hand panels), and analyzed by flow cytometry, gating on A),TMA (CD45+, high side scatter cells). A,B; specific staining (red line), isotype control staining (blue line). A, never-smoker, B, smoker.

Additional file 5: Figure S3. Lack of correlation of spirometry or total smoking history with lung A),TMA TLR3 expression in surgical cohort. Total lung Ma were harvested from excess lung tissue removed surgically for clinical indications as described in the legend to Figure 3. Lung Ma were permeabilized, stained for TLR3 expression and analyzed by flow cytometry, gating on CD45+, high side-scatter cells. Data are shown as the percentage of TLR3-positive lung Ma on the vertical axis versus A).

Abbreviations

AMø: Alveolar macrophage(s); BAL: Bronchoalveolar lavage; COPD: Chronic obstructive pulmonary disease; CSE: Cigarette smoke extract; Dr: Double-stranded; FEV1: Forced expiratory volume in 1 second; FVC: Forced vital capacity; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Ma: Macrophage(s); MDA-S: Melanoma differentiation-associated gene 5; MDMø: Monocyte-derived macrophage(s); NK: Natural killer; PKR: Double-stranded RNA-dependent protein kinase; PMCA: Phorbol myristate acetate; poly(C): Polyinosinic acid:cytidylic acid; RIG-I: Retinoic acid-inducible gene I; TLR3: Toll-like receptor 3.

Competing interests

JCT: Designed and performed experiments, analyzed data, produced graphs and tables, wrote the initial draft of manuscript, reviewed and approved final manuscript; CMF: designed and performed experiments; analyzed data and produced graphs and tables, wrote the final draft of the revised manuscript, reviewed and approved final manuscript; JPB designed and performed experiments, analyzed data and produced graphs and tables, reviewed and approved final manuscript; JS: performed and analyzed flow cytometry experiments, reviewed and approved final manuscript; TMA: performed and photographed immunocytochemistry experiments, participated in performance of other experiments, reviewed and approved final manuscript; ALM: designed and performed experiments; reviewed and approved final manuscript; FJM: secured research funding, participated in study design, analyzed data, participated in manuscript generation, reviewed and approved final manuscript; JMB: secured research funding, participated in study design, analyzed data, participated in manuscript generation, reviewed and approved final manuscript; JLC: secured research funding, oversaw study design, performed all subject-related activities including bronchoscopies, analyzed data, generated the final manuscript, and takes responsibility for the scientific integrity of the overall project.

Authors’ contributions

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