Cigarette smoke has bacterium- and ATG16L1 allele-specific effects on monocyte xenophagy promoting survival of pathogenic E. coli in Crohn's Disease

Anouk Regeling¹, Haukeline H. Volders², Tjasso Blokzijl², Elise M.J. van der Logt¹, Mariska Geuken², Gerard Dijkstra¹* and Klaas Nico Faber¹*

¹University of Groningen, University Medical Center Groningen, Department of Gastroenterology and Hepatology, Groningen, The Netherlands, ²University of Groningen, University Medical Center Groningen, Department of Laboratory Medicine, Groningen, The Netherlands

*Both last authors contributed equally to this work

Submitted
CHAPTER 5

ABSTRACT

BACKGROUND & AIMS
Smoking is a prominent environmental factor and ATG16L1-T300A an important risk allele associated with Crohn's disease (CD). The ATG16L1-T300A allele is enriched in CD patients who smoke. Here, we studied separate and combined effects of ATG16L1-T300A and cigarette smoke on phagocytosis, autophagy and bacterial killing (collectively xenophagy) of commensal and pathogenic E. coli strains in mouse and human monocytes.

METHODS
Mouse macrophages (RAW264.7) and human peripheral blood monocytes of ATG16L1-genotyped healthy individuals were isolated using CD14+ magnetic beads and exposed to different concentrations cigarette smoke extract (CSE). FITC-labeled zymosan, E. coli or adherent invasive E. coli (AIEC) were used to analyze monocyte phagocytic activity. AIEC were co-cultured with human monocytes to determine intracellular survival of the pathogen. Autophagy was analyzed by LC3 lipidation and LC3 mRNA levels, by Western blotting and Q-PCR, respectively.

RESULTS
Cigarette smoke dose-dependently reduced phagocytosis in RAW264.7 cells, while increasing LC3 mRNA levels and lipidation. Human monocytes homozygous for the ATG16L1-T300A risk allele showed increased E. coli and AIEC phagocytosis and autophagic activity compared to ATG16L1-T300 homozygous monocytes. Cigarette smoke did not affect phagocytic activity in ATG16L1-T300 homozygous monocytes, but increased LC3 lipidation, leading to more effective killing of AIEC. Cigarette smoke selectively suppressed phagocytosis of E. coli in ATG16L1-T300A homozygous monocytes, but not that of AIECs and was associated with impaired killing of AIEC.

CONCLUSION
Cigarette smoke has a bacterium- and ATG16L1 allele-specific effect on the xenophagic activity of human monocytes promoting survival of pathogenic adherent invasive E. coli.
INTRODUCTION

Crohn’s disease (CD) is an autoimmune disorder of the gastrointestinal tract, characterized by transmural discontinuous mucosal ulcerations in the gastrointestinal tract predominantly in the ileocolonic region. The underlying cause of CD is unknown, however, recent genome wide association studies revealed over 160 genetic loci associated with this disease. The most prominent CD-associated genes act in anti-bacterial defense, including bacterial recognition (e.g. NOD2) and processing and elimination of bacteria through the autophagy pathway (e.g. ATG16L1, IRGM), implying a prominent role of the innate immune system in disease initiation and/or progression. Still, individually and collectively, these genetic factors appear to confer only limited risk to the disease, estimated to maximally 15%. Thus, other more dominant factors contribute to the development of CD, including those in the patients’ environment and/or lifestyle.

The most prominent environmental risk factor for CD is cigarette smoke. Epidemiological studies revealed that smoking influences both the etiology and the disease course of CD. Not only is the number of smokers higher among CD patients compared to control populations, smoking is also associated with an increased prevalence of ileal disease, need for steroids and immunosuppressive therapy and the risk of relapses. Moreover, the need for surgery is twice as high in smokers.

A functional polymorphism in the autophagy gene ATG16L1 has been identified as a CD risk allele. ATG16L1-T300A (rs2241880; G > A) is a common non-synonymous SNP where the G major allele enhances risk for CD and results in a threonine-to-alanine substitution at amino acid position 300 (T300A) in the ATG16L1 protein.

Interestingly, the ATG16L1-T300A allele does not seem to affect basal, non-selective (or canonical) autophagy, a process principally designed to recycle cellular components during times of nutritional limitations, but rather affects pathogen processing after their internalization (e.g. xenophagy). ATG16L1 hypomorphic (ATG16L1-HM) mice exhibit an aberrant Paneth cell morphology and function similar to those found in CD patients carrying the ATG16L1-T300A (risk) allele. It is, however, unknown whether the Paneth cell abnormalities directly promote CD development. It is evident that ATG16L1-HM mice are more susceptible to dextran sodium sulfate (DSS)-induced colitis, in particular when pre-exposed to the murine norovirus (MNV), and that this requires the presence of normal gut microbiota. These findings provided the first experimental evidence for an interaction between genetic susceptibility and environmental factors that cause CD. The ATG16L1 genotype may be particularly relevant for environmental factors associated with CD, since it was shown that...
active cigarette smoking and homozygosity for the ATG16L1-T300A risk variant synergistically enhanced the risk for CD\textsuperscript{22}. These observations directed us to analyze molecular mechanisms that underlie the gene-smoking interactions in CD.

Functional impairment of the autophagy process by genetic mutations results in reduced and ineffective elimination of intracellular bacteria, including adherent-invasive \textit{E. coli} (AIEC). This pathogen colonizes ileal CD lesions and invades and replicates within host macrophages\textsuperscript{23}, thereby promoting production of pro-inflammatory cytokines\textsuperscript{24,25}. In this study, we developed an \textit{ex vivo} monocyte model to assess the interaction between cigarette smoke and the ATG16L1-T300A allele on CD-associated innate immune functions, including phagocytosis of \textit{E. coli} and CD-associated AIEC and (canonical) autophagy. We also evaluating the impact of this polymorphism on the intracellular survival of AIEC in the presence of cigarette smoke. The use of \textit{ex vivo} monocytes allows functional evaluation of this risk variant on innate immunity and the impact of environmental influences on CD. This may provide knowledge of how the genetic susceptibility is linked to the patient’s lifestyle, in this case smoking.

**MATERIALS AND METHODS**

**CELL CULTURE CONDITIONS AND BACTERIAL STRAINS**

Mouse macrophages (RAW264.7) were cultured in DMEM glutamax medium in T75 flasks (Greiner bio-one, Alphen aan den Rijn, The Netherlands) supplemented with 10\% v/v heat-inactivated fetal calf serum and 1\% gentamicine at 37°C in a humidified atmosphere of 5\% CO\textsubscript{2} until 50\% confluency was reached. Cells from exponentially growing cultures were used for all experiments.

CSE was prepared as described\textsuperscript{26} by passing smoke from 2 standard 3R4F research cigarettes (Tobacco research institute, Kentucky 3R4F, University of Kentucky, KY, USA) through 25 ml culture medium, which was defined as 100\% CSE. Freshly-prepared CSE was used within 30 min and diluted directly into culture medium for the indicated concentrations. Adherent invasive \textit{Escherichia coli} strain LF82 serotype O83:H1 (AIEC) and the non-invasive \textit{Escherichia coli} strain K-12 Top 10 (\textit{E. coli}) were grown in Luria-Bertani (LB) medium at 37°C.

**GENOTYPING FOR ATG16L1 POLYMORPHISMS**

Forty healthy volunteers were genotyped for ATG16L1 and the 3 main NOD2 variants (Table 1). DNA was extracted from whole blood as previously described\textsuperscript{27} and ATG16L1 genotyping was performed using the TaqMan single nucleotide
Table 1. ATG16L1 and NOD2 genotypes of healthy volunteers. Forty healthy volunteers were genotyped for ATG16L1 and the 3 functional NOD2 variants. Monocytes were isolated from the volunteers that were homozygous for the major allele (G=ATG16L1-T300A) or for the minor allele (A=ATG16L1-T300) of ATG16L1 (rs2241880) and homozygous wildtype for all 3 NOD2 variants.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>ATG16L1</th>
<th>NOD2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T300A</td>
<td>R702W</td>
</tr>
<tr>
<td>1</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>2</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>3</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>4</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>5</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>6</td>
<td>AG</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>7</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>8</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>9</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>10</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>11</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>12</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>13</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>14</td>
<td>AG</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>15</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>16</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>17</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>18</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>19</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>20</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>21</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>22</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>23</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>24</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>25</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>26</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>27</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>28</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>29</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>30</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>31</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>32</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>33</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>34</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>35</td>
<td>AA</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>36</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>37</td>
<td>AG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>38</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
<tr>
<td>39</td>
<td>AA</td>
<td>Wildtype</td>
</tr>
<tr>
<td>40</td>
<td>GG</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
polymorphism assay C_9095577_20 on ABI PRISM 7700 sequence detector (Applied Biosystems, CA, USA). All forty healthy volunteers were non-smoking at time of the experiments and only those who were homozygous for ATG16L1-T300 (protective variant), ATG16L1-T300A (risk variant) and wildtype for NOD2 were selected. All volunteers gave written informed consent.

**MONOCYTE ISOLATION FROM HUMAN PERIPHERAL BLOOD**

Heparinized blood was obtained from healthy volunteers homozygous for ATG16L1-T300 or ATG16L1-T300A. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep™ gradients according to suppliers’ instructions (Axis-Shield PoC As, Norway). Monocytes were further purified using CD14 monoclonal antibodies conjugated to microbeads according to the manufacturer’s protocol (Miltenyi Biotec, Leiden, The Netherlands). The purity of monocytes was evaluated by fluorescent staining with CD14-FITC antibody (Miltenyi Biotec) and flowcytometry. Primary monocytes were cultured in RPMI-1640 glutamax medium supplemented with 10% v/v heat-inactivated fetal calf serum, penicillin (50 U/ml), streptomycin (50 µg/ml) and fungizone (5 µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂.

**ANALYSIS OF AUTOPHagy**

RAW264.7 cells or primary monocytes were exposed to different concentrations of CSE (0-30% v/v) for 16 hours. When indicated, chloroquine (CQ, 50 µM; Sigma, MO, USA) was added to the cell culture media 2 hours prior to cell harvesting to inhibit lysosomal degradation of LC3B and allow accumulation of LC3B-II. Rapamycin (200 nM; Sigma) was used as a positive control for autophagy induction. The LC3B-I/LC3B-II ratio was determined by quantifying protein band intensities on Western blots using an enhanced chemiluminescence SuperSignal West Dura detection system (Pierce, IL, USA).

**FITC-LABELING OF E. COLI**

Formaldehyde-fixed E. coli and AIEC were labeled with fluorescein isothiocyanate (FITC, #F7250, Sigma) by incubation of 1 mg/ml FITC solution in 0.1 M Na₂CO₃, pH 9.5 for 1 hour at room temperature (RT) with occasional mixing, followed by several wash steps with 0.1 M Na₂CO₃, pH 9.5. Bacterial concentrations were determined by optical density at 600 nm (OD₆₀₀), assuming that an OD₆₀₀ of 1 equals 1x10⁹ bacteria. For FITC-labeling of zymosan, 10 mg/ml of zymosan A (S. cerevisiae, #Z4250; Sigma) was
sonicated (50-60 kHz) to prevent aggregation, followed by the labeling procedure as for *E. coli*. Additionally, FITC-labeled zymosan was opsonized by incubating 1 mg/ml with 50 µl human serum for 45 min at 37°C during constant mixing (1400 rpm), followed by several centrifugation and wash steps with 0.1 M Na₂CO₃, pH 9.5. Zymosan concentration of 1 mg/ml equaled 5x10⁸ particles.

**Phagocytosis assays**

RAW264.7 or primary human monocytes were plated at 5x10⁵ cells per ml on coverslips in 12-well plates (Greiner bio-one). Subsequently, cells were incubated with different concentrations of CSE for 30 min prior to challenge with FITC-labeled *E. coli*, zymosan or AIEC at a 1:10 cell-particle ratio for 10 (*E. coli*) or 30 min (zymosan and AIEC). Subsequently, cells were washed with ice-cold phosphate-buffered saline (PBS), and fixed with 4% formaldehyde for 10 min at room temperature in the dark and mounted on glass slides using Vectashield mounting medium containing Dapi (Vector laboratories, CA, USA). Phagocytosis was evaluated microscopically by counting the number of monocytes containing FITC-labeled particles and the number of particles per monocyte. At least 300 cells were counted for each condition. Internalisation of zymosan, *E. coli* or AIEC was confirmed by confocal laser scanning microscopy.

**RNA isolations and quantitative RT-PCR**

Total RNA was extracted from human monocytes with trizol (Sigma), reverse transcribed and analyzed for gene expression using real time Q-PCR (ABI PRISM 7700 sequence detector; Applied Biosystems, NY, USA) as described²⁸. Sequences for LC3B primers were 5’-AGA GTA GAA GAT GTC CGA CTT ATT CGA-3’ (sense), 5’-CTG CTT CTC ACC CTT GTA TCG TT-3’ (antisense) and 5’ FAM-TCC AAC CAA AAT CCC GGT GAT AAT A-TAMRA 3’ (probe). Primer sequences for the housekeeping gene β-actin were 5’-CGG CTA CCA CAT CCA AGG A-3’ (sense), 5’-CCA ATT ACA GGG CCT CGA AA-3’ (antisense) and 5’ FAM-CGC GCA AAT TAC CCA CTC CCG A-TAMRA 3’ (probe). Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (CT value) were normalized to the endogenous control (β-actin) and correlate inversely with initial mRNA levels.

**Total protein extraction and Western blotting**

Total protein lysates of cells were prepared using NP40 lysis buffer (25 mmol/l HEPES, 2 mmol/l EDTA, 150 mmol/l Kac, 0.1% NP40, 10 mmol/l NaFl, 1 mmol/l PMSF, 1 mmol/l
DTT, aprotinin, leupeptide and prostatine (each 1 µg/ml), pH 7.2). After four cycles of freezing (liquid nitrogen) and thawing (37°C), total lysates were centrifuged (10 min, 12,500xg, RT) to remove cell debris and the supernatant was stored at -80°C until further use. Protein concentrations were determined using the BioRad Dc protein assay (BioRad, CA, USA). Absorbance was measured at 750 nm on an EL800 universal microplate reader (Bio-Tek instruments, VT, USA). For Western blot analysis, 20 µg of total protein was fractionated on 12% SDS polyacrylamide gels and transferred onto nitrocellulose membrane (GE Healthcare Europe, Diegem, Belgium). After blocking with 0.5% skim milk in 0.1% PBS-Tween-20 for 1 hour at room temperature (RT), membranes were probed with mouse monoclonal α-LC3B antibody (1:500; Cell Signaling, Danvers, USA) or with monoclonal α-GAPDH (1:50.000; Calbiochem, CA, USA) in 0.5% BSA, 0.1% PBS-Tween-20 overnight at 4°C. Membranes were incubated with horseradish peroxidase conjugated secondary antibodies in 0.1% PBS-Tween-20 for 1 hour at RT and target proteins were visualized using an enhanced chemiluminescence SuperSignal West Dura detection system (Pierce). GAPDH was used as loading control.

**Bacterial survival assay**

Bacterial survival/killing was measured using the gentamicin protection assay as previously described. Briefly, cells were infected at a multiplicity of infection (MOI) of 100 bacteria per monocyte. After 30 min of incubation at 37°C with 5% CO₂, infected monocytes were washed twice with PBS and fresh culture medium containing 50 µg/ml of gentamycin was added to kill extracellular bacteria. After incubation of 1 hour, infected cells were washed twice with PBS and lysed by 1% Triton X-100 (Sigma) for additional 5 min at room temperature. The Triton X-100 cell lysate was plated onto LB agar and incubated overnight at 37°C to determine the number of colony forming units (CFUs) recovered from the lysed monocytes. The number of surviving bacteria was expressed as the mean percentage of the number of bacteria recovered after 30 min post-infection, defined as 100%.

**Metabolic activity**

Metabolic activity of cells was determined using WST-1 reagent following the instructions of the manufacturer (Roche, CA, USA). Cells exposed to 5 mmol/l H₂O₂ were used as a control for complete suppression of metabolic activity.
AN INTERACTION BETWEEN SMOKING AND ATG16L1-T300A

STATISTICAL ANALYSIS
Data are expressed as means ± SD or SEM of 3 independent experiments performed in duplicate. Statistical significance was calculated using Student’s t-test, or one way analysis of variance (ANOVA) in combination with a Bonferroni correction for multiple comparisons. A P value of <0.05 was considered statistically significant.

RESULTS
PHAGOCYTIC CAPACITY OF MOUSE MACROPHAGES IS DOSE-DEPENDENTLY REDUCED BY CIGARETTE SMOKE
Pathogen clearance can be subdivided into 2 consecutive steps; phagocytotic uptake and subsequent autophagic degradation of the microbe(s) (= xenophagy). We first analyzed whether cigarette smoke extract (CSE) affects microbial uptake by mouse RAW264.7 macrophages. As shown in Figure 1A, CSE dose-dependently reduced (-70% at 30% CSE) the number of RAW264.7 cells that contained FITC-labeled *E. coli*. Importantly, CSE did not induce cell death at these conditions. The metabolic activity actually increased significantly (+60% at 30% CSE) compared to the untreated cells (Figure 1E), similar as we observed before for the human cell lines DLD-1 (intestinal epithelial cells) and Jurkat (T-lymphocytes) (Chapter 3; Regeling *et al.*, submitted). The average numbers of internalized particles per macrophage was slightly decreased and only significant at 30% CSE (Figure 1B). Internalization of FITC-labeled zymosan particles by RAW264.7 cells was similarly reduced by CSE (Figure 1C and D), indicating a generalized impairment of phagocytosis and not a selective effect towards specific pathogens. Moreover, it shows that truly the uptake of particles is impaired and that the decreased number of particles is not a result of enhanced intracellular clearance of phagocytosed microbes.

CIGARETTE SMOKE EXPOSURE ENHANCED LC3B-I AND -II LEVELS DOSE-DEPENDENTLY IN MOUSE MACROPHAGES
Next, we analyzed the effect of CSE on autophagy in RAW264.7 cells. CSE dose-dependently enhanced the level of lipidated LC3B (LC3B-II; Figure 2A), the biochemical marker for canonical autophagy. LC3B-II levels were even further enhanced when the RAW264.7 cells were treated with chloroquine (Figure 2B) that prevents its lysosomal breakdown, indicating that the autophagic flux is enhanced in CSE-treated RAW264.7 cells. Remarkably, also transcript levels of LC3B were significantly enhanced by CSE (Figure 2C). Increased levels of LC3B were already detectable at concentrations of 10% CSE at 2 hours and accumulated quickly to a significant 4-fold increase within
Figure 1. CSE dose-dependently decreased phagocytosis of FITC-labeled *E. coli* and zymosan in mouse RAW264.7 cells. RAW264.7 cells were cultured in the presence of different concentrations of CSE (0-30%). The phagocytic activity and average numbers of engulfed particles was measured by quantifying incorporation of FITC-labeled *E. coli* (A,B) or FITC-labeled zymosan particles (C,D) using fluorescence microscopy. A and C show the total number of particles per 100 monocytes, B and D show the number of particles per monocyte. Data represent means ± SEM. (A,B) n=5, *P<0.01 vs. 0% CSE. (B,D) n=5, *P<0.01 vs. 0% CSE. (E) The metabolic activity of CSE-exposed RAW264.7 cells was quantified using the WST-1 assay and was significant enhancement at CSE concentrations of 10-30%. 5 mmol/l H<sub>2</sub>O<sub>2</sub> was used as control for full suppression of metabolic activity.
AN INTERACTION BETWEEN SMOKING AND ATG16L1-T300A

Figure 2. CSE dose-dependently enhanced LC3-II levels in RAW264.7 cells. RAW264.7 cells were cultured in the presence of different concentrations of CSE (0-30%). Autophagic activity was monitored by (A) the conversion of LC3-I to LC3-II using Western blotting. Rapamycin was used as positive control for autophagy induction and (B) LC3-II lipidation was evaluated by Western blotting in the presence of 10% CSE with and without chloroquine to suppress lysosomal degradation of LC3-II. (C) mRNA levels of LC3 using Q-PCR. Data represent means ± SD of at least three independent experiments. *P<0.0001 vs. 0% CSE. #P<0.0001 vs. H2O2. GAPDH was used as a loading control.

10 hours after exposure to 30% CSE (Figure 2C). This suggests that smoke-exposed macrophages not only rely on basal levels of LC3B to promote autophagy, but also enhance their autophagic capacity by transcriptional adaptations.

ATG16L1-T300A ENHANCES E. COLI PHAGOCYTOSIS AND AUTOPHAGY IN HUMAN PRIMARY MONOCYTES

In order to analyze a potential involvement of the ATG16L1 risk allele on monocyte function, we next analyzed the phagocytotic and autophagic activity of primary human monocytes homozygous for the ATG16L1-T300 (protective) or the ATG16L1-T300A (risk) allele. ATG16L1-T300A homozygous monocytes showed a 2.5-fold
enhanced uptake of FITC-labeled *E. coli* compared to *ATG16L1-T300* homozygous monocytes (Figure 3A). Since *ATG16L1* is required for autophagy and may directly affect clearance of *E. coli* in monocytes, we analyzed whether *ATG16L1-T300* homozygous monocytes may show enhanced autophagy. Figure 3B shows that LC3-II levels are increased in monocytes homozygous for the *ATG16L1-T300A* (risk) allele compared to control monocytes (Figure 3B), both in the absence and presence of chloroquine. Chloroquine treatment hardly changed the LC3-II/I ratio in *ATG16L1-T300* homozygous monocytes, revealing a >10-fold predominance of LC3-I, indicating low activity of autophagy. In contrast, chloroquine enhanced the LC3-II/I ratio from 0.46 to 1.16 in *ATG16L1-T300A* homozygous monocytes, indicating that *ATG16L1-T300A* homozygous monocytes exhibit increased canonical autophagy.

**Cigarette Smoke-mediated Alteration on Innate Immune Functions in ATG16L1-T300A Monocytes**

In order to analyze whether cigarette smoke alters innate immune functions of primary human monocytes homozygous for either one of the *ATG16L1* alleles, we next analyzed the phagocytotic and autophagic activity of primary human monocytes homozygous for the *ATG16L1-T300* (protective) or the *ATG16L1-T300A* (risk) allele. Primary monocytes are more sensitive to CSE compared to RAW264.7 cells and significant effects on phagocytosis of FITC-*E. coli* were already detected at 1% CSE. Concentrations >5% induce toxicity in these cells (data not shown). Remarkably, CSE...
AN INTERACTION BETWEEN SMOKING AND ATG16L1-T300A

**Figure 4.** ATG16L1 allele-specific effects of cigarette smoke on monocyte innate immune functions. Human peripheral blood monocytes homozygous for the ATG16L1-T300 (A,C) or the ATG16L1-T300A (B,D) variant were cultured in the presence of different concentrations of CSE (0-5%) and the phagocytic incorporation of FITC-labeled *E. coli* was quantified using fluorescence microscopy (A,B) and the metabolic activity by WST-1 assays (C,D). 5 mmol/l H₂O₂ was used as control for full suppression of metabolic activity. (E) LC3-II lipidation in human peripheral blood monocytes carrying ATG16L1-T300 (protective) or ATG16L1-T300A (risk) variant was evaluated by Western blotting after exposure to various concentrations of CSE (1-5%) in the presence of chloroquine. The LC3II/I ratio reflects the level of autophagic flux. (A) n=4 ± SEM, *P<0.001 vs. 0% CSE. (B) n=5 ± SEM, *P<0.001 vs. 0% CSE. (C,D) n=3 ± SEM, *P<0.05 vs. 0% CSE. (E) Representative of n=3.

did not reduce the phagocytic activity of ATG16L1-T300 homozygous monocytes (Figure 4A), which show low basal levels of phagocytosis (Figure 3A). In sharp contrast, CSE strongly reduced the phagocytic activity of ATG16L1-T300A homozygous monocytes already at 1% CSE (Figure 4B) to levels comparable to ATG16L1-T300 homozygous monocytes. Since LC3II levels are increased in monocytes homozygous for the ATG16L1-T300A (risk) allele compared to control monocytes (Figure 3B), we analyzed if cigarette smoke may affect autophagy similarly as phagocytosis (e.g. reduction). Remarkably, CSE enhanced LC3II levels and the LC3II/I ratio increased with increasing CSE concentrations, peaking already at CSE concentrations of 1%. Similar to this, LC3 levels were also enhanced in ATG16L1-T300 control monocytes, although not as strongly as in ATG16L1-T300A homozygous monocytes (Figure 4E). Together, we conclude that the enhanced phagocytic activity of monocytes expressing the CD-associated susceptibility variant ATG16L1-T300A is strongly suppressed by cigarette smoke. Simultaneously, the increased canonical autophagy
in these monocytes is further enhanced by CSE, without a decrease in cell viability (Figure 4C and D).

**NO SIGNIFICANT CIGARETTE SMOKE-MEDIATED REDUCTION IN PHAGOCYTOSIS OF AIEC**

Crohn’s disease is characterized by the accumulation of adherent invasive *E. coli* (AIEC) that are able to survive and replicate in monocytes and escape xenophagic degradation in autophagy deficient cells triggering excessive TNF-α production. We next analyzed whether the *ATG16L1* allele and cigarette smoke affect AIEC phagocytosis similarly as non-adherent *E. coli* used in the preceding experiments. *ATG16L1-T300A* homozygous monocytes showed enhanced phagocytosis of FITC-labeled AIEC compared to *ATG16L1-T300* homozygous monocytes (Figure 5A), similar as observed for non-adherent *E. coli*. However, CSE did not significantly change AIEC phagocytosis in *ATG16L1-T300* (protective) (B) or *ATG161-T300A* homozygous monocytes (risk) (C). n=3±SEM, *P<0.05.

**CIGARETTE SMOKE AFFECTS AIEC SURVIVAL IN ATG16L1-T300A MONOCYTES**

In a final experiment, we analyzed survival of internalized AIEC after exposure to *ATG16L1-T300* or *ATG16L1-T300A* homozygous monocytes in the absence or presence of 2% CSE. CSE exposure strongly reduced the survival of AIEC in *ATG16L1-T300* homozygous monocytes by approximately 70% (Figure 6), most likely due to a strong induction of autophagy in the presence of the *ATG16L1* protective allele. In contrast, no reduction in AIEC survival was observed *ATG16L1-T300A* homozygous monocytes.
AN INTERACTION BETWEEN SMOKING AND ATG16L1-T300A

Figure 6. Cigarette smoke reduces AIEC survival in ATG16L1-T300 monocytes, but not in ATG16L1-T300A monocytes. Survival of internalized AIEC was analyzed in ATG16L1-T300 (protective) or ATG16L1-T300A (risk) homozygous monocytes exposed to 0% CSE (set to 100) and 2% CSE (shown as percentage of survival in control (0% CSE) monocytes). Colony forming units (CFUs) of AIEC were counted 20 hours after plating the monocyte-AIEC TX-100 lysate onto LB agar. n=4±SEM, *P<0.05.

monocytes exposed to CSE, suggesting an impairment in CSE-enhanced xenophagy of AIEC in ATG16L1-T300A homozygous monocytes.

DISCUSSION

The link between cigarette smoke exposure and susceptibility to CD has been recognized for many years, but the mechanisms underlying this association are poorly understood. In this report, we demonstrate that cigarette smoke exposure directly affects CD-associated innate immune processes. We show that cigarette smoke enhances canonical autophagy in mouse macrophages (RAW264.7), while interfering with phagocytotic uptake of E. coli and zymosan (= yeast) particles. Moreover, we show that the ATG16L1-T300A allele enhances phagocytosis and canonical autophagy in primary human monocytes. Cigarette smoke suppressed phagocytosis of non-adherent E. coli in ATG16L1-T300A homozygous monocytes, but not that of CD-associated AIEC promoting survival of pathogenic AIEC. Taken together, our data reveal a molecular mechanism how cigarette smoke may induce and maintain intestinal inflammation, in particular in patients with an impairment in autophagy.

Pathogen uptake (phagocytosis) and subsequent bacterial destruction (xenophagy) lies at the heart of the innate immune response. To determine if cigarette smoke affects these innate immune processes, we performed experiments, using a macrophage cell line, RAW264.7, and observed a smoke-dependent reduction in phagocytosis of E. coli or zymosan. Since zymosan is non-degradable by phagocytes, this indicates a non-selective effect of cigarette smoke on pathogen uptake, rather
than enhanced intracellular bacterial clearance. Further, RAW264.7 cells showed CSE-mediated induction of endogenous LC3B-II to similar levels of rapamycin-treated cells, a potent inducer of autophagy. Cigarette smoke also enhanced unlipidated LC3B-I levels, which is the result of cigarette smoke-induced transcription of the LC3 gene. CSE/CQ-treated RAW264.7 cells showed an additional increase of LC3B-II by cigarette smoke, indicating that these cells exhibit an enhanced autophagic flux and that the initially observed increase was not due to an impaired degradation of LC3B-II nor associated with an increase in cell death. These findings are consistent with previous reports, showing cigarette smoke-induced autophagy both in vitro and in vivo in intestinal cells, alveolar macrophages and human umbilical vein endothelial cells (HUVECs)29-33, supporting a generalized role of cigarette smoke in modulating autophagy in various cell types throughout the body.

Autophagy plays an essential role during infections by elimination of intracellular infective pathogens34-36. Genome wide association studies have indicated a prominent role for ATG16L1 in the etiology of CD. Functional studies have shown that ATG16L1 is not required for canonical autophagy, but is required for effective killing of internalized bacteria. These studies mostly used (RNAi-)silencing of ATG16L1 in a variety of epithelial- and monocyte cell lines to study ATG16L1 functionality18,20,21,25,37-39. However, such studies may not be representative for the functional effect of the ATG16L1-T300A risk variant that is associated with CD. Using primary monocytes from healthy volunteers either homozygous for the ATG16L1-T300 or ATG16L1-T300A allele we show that the ATG16L1-T300A allele enhances canonical autophagy (e.g. LC3-II levels and turnover) as well as the phagocytotic uptake of E. coli and CD-associated AIEC that colonize ileal lesions of CD patients23,40. AIEC are distinguishable from the non-pathogenic strain of E. coli in that they are able to adhere to and invade in intestinal epithelial cells37, to survive and replicate within macrophages and to induce high levels of pro-inflammatory cytokines24,25,41. The combination of an increased pathogenic uptake and the inability to kill intracellular AIEC results in a pro-inflammatory condition that may initiate and/or aggravate CD. At present, there is only one study that reported a potential relationship between the ATG16L1 gene and cigarette smoke in CD patients and reported a synergistic risk effect in CD22. Cigarette smoke enhanced canonical autophagy independent of the ATG16L1 genotype, taken into account that basal levels were much higher in the ATG16L1-T300A monocytes. However, cigarette smoke only suppressed uptake of commensal E. coli in ATG16L1-T300A homozygous monocytes, indicating that the ATG16L1-T300A polymorphism sensitizes monocytes to cigarette smoke
when the innate immune system is stressed. This is consistent with other studies showing cigarette smoke-dependent suppression of phagocytosis in alveolar macrophages\textsuperscript{29,30,33,42,43}. However, we observed an \textit{E. coli} strain-specific effect of cigarette smoke in \textit{ATG16L1-T300A} homozygous monocytes, as the uptake of AIEC was not reduced by cigarette smoke. CS-exposed \textit{ATG16L1-T300} monocytes showed significantly enhanced bactericidal activity towards AIEC, most likely because of the CSE-mediated induction of xenophagy. In contrast, CSE did not enhance bacterial killing of AIEC in \textit{ATG16L1-T300A} monocytes, which is fully in line with earlier reports that showed that the \textit{ATG16L1-T300A} risk variant leads to an impairment in autophagical processing of internalized bacteria\textsuperscript{18,37,44-46}.

Thus, these data offer an explanation for the synergistic effect of the \textit{ATG16L1-T300A} allele and cigarette smoking in CD, as the \textit{ATG16L1-T300A} allele enhances uptake of pathogenic bacteria, but are not sensitive to the smoke-induced autophagic processing of these pro-inflammatory microbes. This knowledge may guide counseling and therapy in CD patients, in particular those carrying risk alleles that affect autophagy.

ACKNOWLEDGEMENTS

\textit{E. coli} strain LF82 was a kind gift from A. Darfeuille-Michaud (Université Clermont I, Pathogénie Bactérienne Intestinale, Clermont-Ferrand, France).

REFERENCES


22. Fowler, EV, Doecke, J, Simms, LA, Zhao, ZZ, Webb, PM, Hayward, NK and Radford-Smith, GL. ATG16L1 T300A shows strong associations with disease subgroups in a large Australian IBD population: further support for significant disease heterogeneity. *Am J Gastroenterol* 2008;103:2519-2526.


