

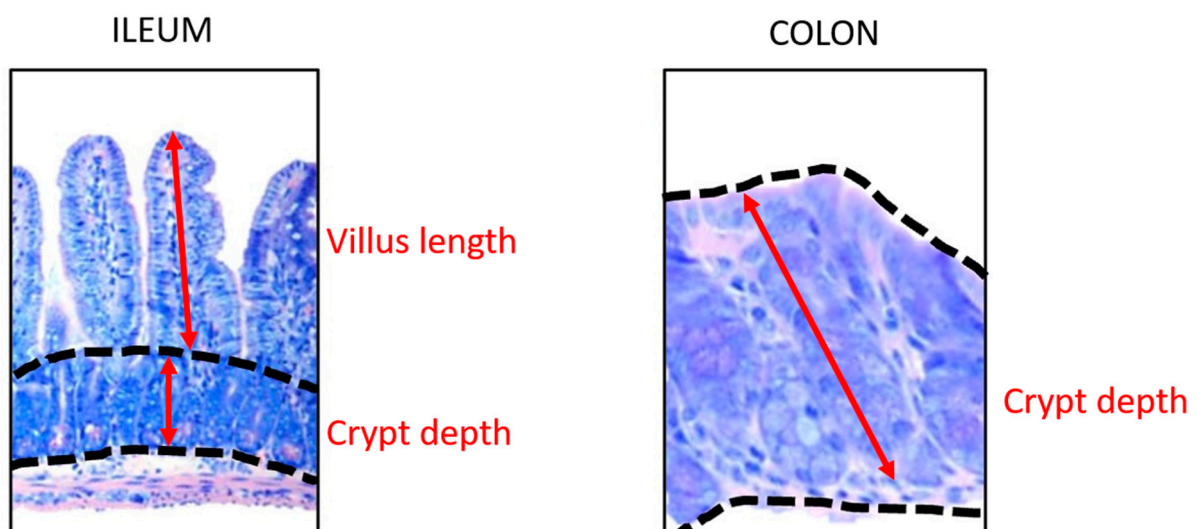
Supplementary methods

Chronic Exposure to Both Electronic and Conventional Cigarettes Alters Ileum and Colon Turnover, Immune Function, and Barrier Integrity in Mice.

Histological analysis

At necropsy, ileum and colon tissue were fixed in 4% formaldehyde overnight, processed, and embedded in paraffin wax by an automatic sample preparation system (LOGOS One, Milestone, Valbrembo, Italy). Serial histological sections of 4 μm thickness were cut, deparaffinised, rehydrated, and stained with May Grünwald Giemsa (MGG; Carlo Erba, Val de Reuil, France). Images were acquired with a DM5500 B microscope (Leica Microsystems, Wetzlar, Germany). Histomorphometric analyses were performed using Image J software (NIH). Epithelial area in the colon, villus height, and crypt depth in the proximal and distal small intestine were measured according to the scheme presented below. At least 100 well-oriented mucosa, villi, and crypts were measured in at least 5 individual mice from each group.

Scheme of histomorphometric measures



Quantitative RT-PCR

Small intestinal and colonic tissue samples were homogenised with ceramic beads using Precellys Lysing Equipment (Bertin Technologies, Montigny le Bretonneux, France). Total RNA was extracted with the NucleoSpin RNA kit (Macherey-Nagel, Hoerd, France). Transcript levels of genes were quantified with the StepOne™ Real-Time PCR system using a SYBR Green PCR master mix (Thermo Fisher Scientific, Illkirch, France). The primer sequences were designed using the National Library of Medicine Primer Designing Tool and are presented in Table 1 below. Melting curve analyses were performed for each sample and gene to confirm the specificity of the amplification. Three reference genes were tested for this study: b-Actin, Gapdh, and Polr2a. Polr2A was found to be the most stable and showed no variation between treatments, so was chosen as reference gene. Additional targets were quantified using the TaqMan Fast advanced Master Mix and Taqman assays (Thermo Fisher Scientific, Illkirch, France) and are presented in Table 2. Quantification of target gene expression was based on the comparative cycle threshold (Ct) value. The fold changes in the target genes were analysed by the $2^{-\Delta\Delta C_t}$ method.

Bacterial DNA extraction and Illumina MiSeq sequencing

Genomic DNA was extracted from colon luminal content using the DNA Stool kit (Macherey Nagel, Hoerd, France). The quantity and purity of DNA (expressed as the ratio of absorbance at 260 and 280 nm) were assessed using a NanoDrop® spectrophotometer (Ozyme, Saint-Cyr-l'école, France). The sequencing library was generated by amplifying the V3-V4 regions of the bacterial 16S-rRNA gene using 16S rRNA amplicon generation for MiSeq with the primers Bact-0341 (CCTACGGGNGGCWGCAG) and Bact-0785 (GACTACHVGGGTATCTAATCC). Individual samples were barcoded, pooled to construct

the sequencing library, and sequenced using an Illumina MiSeq system (Illumina, Evry, France) to generate paired-end 2x300 bp reads.

Table 1.

Gene	Primer sequences
mTbx21-F	CCAAAGGATTCCGGGAGAA
mTbx21-R	CTCGTATCAACAGATGCGTACATG
mTnf-F	CCA-CCA-CGC-TCT-TCT-GTC-TA
mTnf-R	GAG-GCC-ATT-TGG-GAA-CTT-CT
mlfng-F	ACTGGCAAAAGGATGGTGAC
mlfng-R	GCTGATGGCCTGATTGTCTT
mGata3-F	GCAGAACCGGCCCTTAT
mGata3-R	ACAGTTCGCGCAGGATGTC
mlI4-F	CATGCACGGAGATGGATGTG
mlI4-R	TGGTGTCTTCGTTGCTGTGAG
mlI13-F	CCA-ATT-GCA-ATG-CCA-TCT-ACA
mlI13-R	GGG-CCT-TGC-GGT-TAC-AGA
mlI25-F	GTACCAGGCTGTTGCATTCTTG
mlI25-R	CTGACGGTGTGGGTTCCC
mlI9-F	TGT-GTG-CTG-CAT-GAC-CAA-CAA
mlI9-R	GGC-AAT-GCT-GCT-GAT-TCT-CCT
mRorc-F	CTTCCTCAGCGCCCTGTGT
mRorc-R	TGAGAACCAGGGCCGTGTAG
mFoxp3-F	CAACATGGACTACTTCAAGTACCACAATA
mFoxp3-R	GATGGCCCATCGGATAAGG
mlI10-F	CCC-TTT-GCT-ATG-GTG-TCC-TT
mlI10-R	TGG-TTT-CTC-TTC-CCA-AGA-CC
mTgfb1-F	CCC GAA GCG GAC TAC TAT GCT
mTgfb1-R	GTT TTC TCA TAG ATG GCG TTG TTG
mlI12a-F	CGAGACTCTGCGCCAGAAAC
mlI12a-R	GCAGGATGCAGAGCTTCATTT
mlI1a-F	GCTTGAGTCGGCAAAGAAATC
mlI1a-R	GAGAGAGATGGTCAATGGCAGA
mlI1b-F	AGCTCTCCACCTCAATGGAC
mlI1b-R	AGGCCACAGGTATTTTGTGCG
mlI18-F	CACTTCTCCCCTGTGGTGTG
mlI18-R	GCTGTGCCAGTCTGCAGTT
mlI6-F	TACACATGTTCTCTGGGAAATCGT
mlI6-R	AAGTGCATCATCGTTGTTCATACA
mRetnla-F	CCCTGCTGGGATGACTGCTA
mRetnla-R	CTCCACTCTGGATCTCCCAAGA
mVegfa-F	AAC-GAT-GAA-GCC-CTG-GAG-TG
mVegfa-R	GCT-GGC-TTT-GGT-GAG-GTT-TG

mGzmb-F	CAGCAAGTCATCCCTATGGTAAA
mGzmb-R	TCTAGTCCTCTTGGCCTTACTC
mCcl2-MCP1 F	AGGTCCCTGTCATGCTTCTG
mCcl2-MCP1 R	TCTGGACCCATTCTTCTTG
mCcl3-MIP1a F	AGATTCCACGCCAATTCATC
mCcl3-MIP1a R	CTCAAGCCCCTGCTCTACAC
mCcl5-Rantes-F	GTGCTCCAATCTTGCAATCGT
mCcl5-Rantes-R	ACACACTTGGCGGTTCTTC
mCcl20-F	CGA-CTG-TTG-CCT-CTC-GTA-CA
mCcl20-R	AGG-AGG-TTC-ACA-GCC-CTT-TT
mCxcl1-F	GGCGCCTATCGCCAATG
mCxcl1-R	CTGGATGTTCTTGAGGTGAATCC
mCyp1a1-F	CCA-CCT-GCT-GAG-GCT-AAA-CAG
mCyp1a1-R	TGC-CCC-CCA-CAT-GCA
mCyp2a4-F	TCG-AGG-AGC-GCA-TCC-AA
mCyp2a4-R	AAT-GAA-AGC-ACC-GTT-CGT-CTT-C
<i>mPolr2a-F</i>	CCCACAACCAGCTATCCTCAA
<i>mPolr2a-R</i>	GGTGCTGTGGGTACGGATACA

Table 2.

Gene	Taqman assay ID
CCL4/MIP-1 beta	Mm00443111 m1
CCL11/Eotaxin	Mm00441238 m1
CCL19/MIP-3 beta	Mm00839966 g1
CD40 Ligand/TNFSF5	Mm00441911 m1
CXCL2/GRO beta/MIP-2/CINC-3	Mm00436450 m1
CXCL10/IP-10/CRG-2	Mm00445235 m1
EGF	Mm00438696 m1
FGF basic/FGF2/bFGF	Mm01285715 m1
Flt-3 Ligand/FLT3L	Mm00442801 m1
G-CSF	Mm00438334 m1
GM-CSF	Mm01290062 m1
IFN-alpha 2/IFNA2	Mm00833961 s1
IFN-beta	Mm00439552 s1
HMOX1	Mm00516005 m1
Cyp4f18	Mm07298284 m1
Cyp2a5	Mm00487248 g1
Txnrd1	Mm00443675 m1
Mt2	Mm00809556 s1
Cyp1a1	Mm00487218 m1
Aldh3a1	Mm00839312 m1
Ahrr	Mm00477443 m1
Nqo1	Mm01253561 m1

Cxcr1	Mm00731329 s1
Gpx2	Mm01286848 gH
Gsto1	Mm00599866 m1
IL-1ra/IL-1F3	Mm00434237 m1
IL-2	Mm00434256 m1
IL-3	Mm00439631 m1
IL-7	Mm01295803 m1
IL-15	Mm00434210 m1
IL-17/IL-17A	Mm00439618 m1
IL-33	Mm00505403 m1
Lymphotoxin-alpha/TNF-beta	Mm00440228 gH
PD-L1/B7-H1	Mm03048248 m1
TGF-alpha	Mm00446232 m1
TRAIL/TNFSF10	Mm01283606 m1
IL-22	Mm01226722 g1
CXCL3	Mm01701838 m1
CXCL5	Mm00436451 g1
CXCL11	Mm00444662 m1
CXCL16	Mm00469712 m1
CCL22	Mm00436439 m1
PDGF-A	Mm01205760 m1
PDGF-B	Mm00440677 m1
CCL28	Mm00445039 m1
CX3CL1	Mm00436454 m1
<i>RPLP0</i>	Mm00725448 s1
<i>PPIA</i>	Mm02342430 g1

Analysis of sequencing data

Bioinformatic analyses were performed using the QIIME2 pipeline (ver. 2020.2) [91]. The Divisive Amplicon Denoising Algorithm 2 (DADA-2) plug-in in QIIME2 was used to filter, dereplicate, identify chimeric sequences, and merge reads to obtain the set of amplicon sequence variants (ASVs) for each sample [92]. Then the representative sequences were picked for each ASV. The classify-sklearn plug-in in QIIME2, with the SILVA database (ver. 132), was applied to assign a taxonomic annotation to each representative ASV sequence. In the next step, ASVs identified as eukaryotic contamination (3 ASVs; 12 reads) and external contamination, identified with the decontam package (3 ASVs; 3119 reads), were filtered out

[93]. Diversity metrics (α and β) were obtained with the QIIME2 core-metrics-phylogenetic plug-in, with p-sampling depth parameter fixed to 13781 reads which corresponded to the total frequency that each sample should be rarefied to prior to computing diversity metrics. This sampling depth allowed retention of >61% of reads and the discarding of only one sample. Tests for differential relative abundance were performed with corncob at the order, family, and genus levels [94]. Spearman's correlation were performed using Prism 8 software (GraphPad Prism software v8).

References

91. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857, doi:10.1038/s41587-019-0209-9.
92. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nat. Methods* **2016**, *13*, 581–583, doi:10.1038/nmeth.3869.
93. Davis, N.M.; Proctor, D.M.; Holmes, S.P.; Relman, D.A.; Callahan, B.J. Simple Statistical Identification and Removal of Contaminant Sequences in Marker-Gene and Metagenomics Data. *Microbiome* **2018**, *6*, 226, doi:10.1186/s40168-018-0605-2.
94. Martin, B.D.; Witten, D.; Willis, A.D. Modeling Microbial Abundances and Dysbiosis with Beta-Binomial Regression. *Ann. Appl. Stat.* **2020**, *14*, 94–115, doi:10.1214/19-aos1283.