ONLINE SUPPLEMENT

Obesity accelerates *Helicobacter felis*-induced gastric carcinogenesis by enhancing immature myeloid cell trafficking and T_H17 response

MATERIALS AND METHODS

Mice

Male C57BL/6J mice were infected with *Helicobacter felis* (strain ATCC 49179) or sterile broth as previously described.[1] Briefly, mice received 3 doses of *H. felis* (10^{10} colony-forming units/mL) via oral gavage every other day. Mice were fed ad libitum either a standard control diet (CD, 13% of calories from fat, PicoLab Rodent Diet 20) or a high fat diet (HFD, 45% of calories from fat, Research Diets, Inc. D12451). Diets have roughly comparable caloric density (CD 4.07 kcal/g, HFD 4.73 kcal/g). Body mass was measured weekly from 1 to 68 weeks. Percentage body fat was measured by dual-energy x-ray absorptiometry (DEXA, PIXImus) after anesthetizing with 20% ketamine + 10% xylazine in PBS.

Histopathology and immunohistochemistry

After euthanasia at 10 and 15 months post-infection (PI), stomachs were removed, cut along greater curvature and photographed. Inflammation and dysplasia was identified upon gross view of the stomach as white lesions within the normally red epithelium of the gastric corpus. Percentage of affected corpus was estimated by tracing the affected corpus area and total corpus area in Adobe Photoshop on digital gross pictures. Stomachs then were fixed in 10% neutral buffered formalin, processed using standard techniques, embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin (H&E). H&E slides were graded for pathological parameters by a board-certified veterinary pathologist, according to previously defined criteria.[2] Perigonadal (visceral) and subcutaneous adipose tissue was fixed in 10% formalin, stained with H&E, and adipocyte size was quantified using Image Pro. Immunohistochemistry (IHC) was performed on 4 µm sections with avidin-biotin-peroxidase complex kits (Vector Laboratories) and counterstained with Mayer's hematoxylin. The antibodies used were mouse anti-Stat3 (Cell Signaling Technology) anti-F4/80 (eBioscience), anti-CD4 (Abcam), anti-IL-17A (R&D), and anti-Ki67 (Abcam).

Protein Quantification

To quantify serum proteins, mice were fasted overnight and blood was collected. Serum Interleukin (IL)-6, leptin, resistin, plasminogen activator inhibitor-1 (PAI-1), IL-17A, granulocyte macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL4, chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, and CXCL5 were measured by milliplex (Millipore). Serum CCL7 protein was measured by enzyme-linked immunosorbent assay (ELISA, Peprotech). For gastric protein measurement, gastric tissue was snap frozen, homogenized in tris-buffered RIPA (Boston BioProducts), and total protein was quantified (BioRad). Samples were analyzed using an IL-17A ELISA (eBioscience).

Quantitative RT-PCR

RNA was extracted from tissues using TRIzol reagent (Invitrogen) and cDNA was synthesized using Superscript III cDNA Amplification System (Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions were performed using a QuantiTect SYBR green PCR kit (QIAGEN) and primers (supplementary table 1) on an ABI Prism 7300 (Applied Biosystems). MicroRNAs were quantified using the Taqman MicroRNA Assay (Applied Biosystems). Samples were analyzed in triplicate and normalized against GAPDH as an internal control. Relative changes in gene expression were calculated using the ΔΔCt method.

Flow cytometry

Samples for flow cytometry were prepared as follows. Fresh adipose tissue was weighed, minced in 10 mL PBS + BSA + EDTA (Sigma) and centrifuged. Floating tissue was isolated and digested with agitation in a solution of DMEM + BSA + Liberase TM (Roche) + DNase I (Sigma) (3 mL/gram adipose tissue). The cell suspension was passed through a 250-μm nylon mesh and centrifuged to obtain the adipose stromal vascular section (SVF). Stomach tissue was minced, digested with agitation, first in HBSS + EDTA + DTT and then in RPMI 1640 + pronase + collagenase (Sigma). Cell suspension was centrifuged to obtain gastric leukocytes. Hind leg femur and tibia bones were flushed with PBS + 2% FBS and centrifuged to obtain bone marrow cells. Spleen tissue was crushed in PBS + 2% FBS and centrifuged to obtain splenocytes. The adipose SVF, gastric leukocytes, splenocytes, and blood were incubated in BD Pharm Lyse (BD Pharmingen), washed, and distributed into V-bottomed well plates (10^6 cells, Falcon). Plated cells were incubated with the following antibodies for 30 minutes at room temperature: anti- Gr-1-PerCP, Ly6G-APC-Cy7 (BioLegend), CD11b-APC, F4/80-FITC, Ly6C-PE (eBioscience) CD3ε-PerCP, CD4-FITC or CD4-APC, and/or CD8α-PEcy7 (BD Biosciences). The cells were then washed, reconstituted in PBS + 2% FBS + DAPI and analyzed using a BD LSRII. Cells in gastric tissue normalized to 10,000 total cells; cells in blood and spleen normalized to total cells.

T cell differentiation assay

Naive T cells were sorted by fluorescence-activated cell sorting (FACS, FACSAria, BD) from the spleen and lymph nodes, based on a $CD4^+CD44^{low}CD62L^+$ profile using anti- CD4-PerCP-Cy5.5 (eBioscience), CD44-PE-Cy7, and CD62L-APC (BioLegend) antibodies. Sorted cells were placed in a 48-well plate pre-coated with anti-CD3ε (2 μg/mL) antibody ($2x10^6$ cells/well) and incubated for 3 days in RPMI 1640 + 10% FBS (Invitrogen) with 1% penicillin/streptomycin, 5 μM β-mercaptoethanol (Sigma), anti- CD28 (2 μg/mL), IFNγ (5 μg/mL) and IL-4 (5 μg/mL) (BioLegend). Recombinant proteins TGF-β, IL-6 and leptin were added separately and in combination for the 3-day duration as well. Cells were re-stimulated with PMA (50 ng/mL, Sigma) + ionomycin (1 μg/mL, Sigma) + Golgistop (BD Biosciences) in RPMI for 5 hours. After harvesting, cells were incubated with antibodies to surface antigens (specified in flow cytometry section above), fixed, permeabilized (BD Biosciences) and stained with anti- IL-17A-PE, IFNγ-FITC (eBioscience) and GM-CSF-FITC (BioLegend) antibodies. Cells analyzed using a BD LSRII.

BrdU Injection

Mice were injected twice with 5 mg/mL bromodeoxyuridine (BrdU) in saline (10 µL/g body mass), 1.5 hours apart, and sacrificed 18 hours later. Adipose tissue SVF was isolated as described above and stained with anti-BrdU (BD Pharmingen) and antibodies to surface antigens (specified in flow cytometry section above) following manufacturers' instructions. Cells were stimulated for 5 hours with PMA (50 ng/mL, Sigma) + ionomycin (1 µg/mL, Sigma) + Golgistop (BD Biosciences), fixed, permeabilized and stained with anti- IL-17A-PE, IFNγ-FITC and/or GM-CSF-FITC antibodies (BD Biosciences).

Chemokine infusion, migration, and stimulation assays

Chemokine infusion studies were preformed by tail vein injection of recombinant murine CXCL- 1, 2, 5, CCL2, or CCL7 (Peprotech) diluted in PBS. Injection of PBS alone was used as the control. Blood and bone marrow IMC populations were analyzed by flow cytometry. For migration assays, $CD11b^{+}Gr1^{+}$ cells were FACS sorted (FACSAria, BD) from bone marrow of uninfected C57BL/6J mice on chow diet. Sorted cells were placed in the top chamber of a 5-µm transwell plate ($2x10^{5}$ cells/well, Costar), with the chemokine of interest in the bottom chamber. After 4 hours, migrated cells were stained with F4/80, Ly6C, and Ly6G. Countbright Absolute Counting Beads (Invitrogen) were added for quantification and analysis. For CCL7 stimulation assays, adipose SVF cells were isolated, cultured in low glucose DMEM + 10% FBS (Invitrogen) + 1% penicillin/streptomycin (Sigma) and stimulated with recombinant murine IL-17A and/or GM-CSF (Peprotech). After 72 hours, supernatants were collected and CCL7 protein was quantified by ELISA (Peprotech).

Statistical Analysis

Statistical significance was evaluated using the student *t* test and expressed as mean ± standard deviation (SE), unless otherwise stated. Pathology scores were characterized by the Kruskal-Wallace test[3] followed by a comparison of H. felis HFD with the other 3 conditions using the nonparametric multiple comparison method of Konietscke et al.[4-9] *p*-values<0.05 were regarded as statistically significant. Study mice evaluated at 15 months PI, with n=4-10 per group, unless otherwise specified.

REFERENCES

1 Shibata W, Ariyama H, Westphalen CB, *et al*. Stromal cell-derived factor-1 overexpression induces gastric dysplasia through expansion of stromal myofibroblasts and epithelial progenitors. *Gut* 2013;**62**:192–200.

2 Rogers AB, Houghton J. Helicobacter-based mouse models of digestive system carcinogenesis. *Methods Mol Biol* 2009;**511**:267-295.

3 Kruskal WH, Wallace WA. Use of ranks in one-criterion analysis of variance. *J Amer Stat Assoc* 1952;**47**:583-621.

4 Konietschke F, Brunner E, Hothon LA. Nonparametric relative contrast effects. Asymptotic Theory and Small Sample Approximations: University of Hannover 2008.

5 Munzel U, Hothon LA. A unified approach to simultaneous ranks tests procedures in the unbalanced one-way layout. *Biometric J* 2001;**43**:553-569.

6 Konietschke F, Hothorn LA, Brunner E. Rank-based multiple test procedures and simultaneous confidence intervals. *Electron J Stat* 2012;**6**:738-759.

7 Konietschke F. nparcomp, Version 2.0, 2012.

8 Ihaka R, Gentleman R. R: A language for data analysis and graphics. *J Comp Graph Stat* 1996;**5**:299-314.

9 R Development Core Team. R: A Language and Environment for Statistical Computing. 2.15.2 ed, 2012.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

(A) Representative DEXA scans of uninfected CD and HFD. (B) Percent body fat in study groups. (C) mRNA quantification of IL-6 and IL-11 in gastric corpus by qRT-PCR. n=4-6 mice per group, 12 months PI. **p*<0.05, $^{\#}$*p*<0.01, §p<0.001.

Supplementary Figure 2

(A) Representative plots, gating scheme and nomenclature for flow cytometry. Cells isolated from spleen, blood, and gastric tissue, stained with anti- CD45, CD11b, Gr1, F4/80, Ly6C, and Ly6G antibodies. Plots are pre-gated for live, single cells. (B) Statistical analyses for various IMC populations in blood, spleen, gastric tissue. Cells in gastric tissue normalized to 10^3 total cells; cells in blood normalized to total cells; cells in spleen normalized to total cells. (C) Transwell migration assay using FACS sorted $CD11b^+Gr1^+$ IMCs from uninfected/CD whole bone marrow. Migrated cells stained with anti-F4/80, anti-Ly6C, and anti-Ly6G antibodies, quantified with counting beads by flow cytometry. n=3-4 wells/condition, one representative shown of three independent experiments, *p*-values versus uninfected/CD. **p*<0.05, $^{\#}$*p*<0.01, §p<0.001.

Supplementary Figure 3

(A) IL-17A protein quantification in gastric corpus by ELISA. (B) Representative images of IL-17A IHC in stomach. Scale bar = 100 μm. (C) mRNA quantification of IL-17F, IL-21, IL-22, IL-1β in gastric corpus by qRT-PCR. **p*<0.05, $^{\#}$*p*<0.01, §p<0.001.

Supplementary Figure 4

(A) TNF-α and IL-1β mRNA quantification from whole visceral adipose tissue by qRT-PCR. (B) H&E-stained visceral adipose, representative images. Scale bar = 100 μm. Nuclei density calculated from total nuclei normalized to total adipocytes in each high power field. Average adipocyte size, calculated from H&E sections. At least 3 representative fields per mouse quantified. (C) Quantification of CCL7 mRNA from whole corpus gastric tissue by qRT-PCR. (D) Serum GM-CSF protein quantification by milliplex. **p*<0.05, $^{\#}$*p*<0.01, §p<0.001.

Supplementary Table 1

Primer sequences used in qRT-PCR analysis.

Gene	Primer sequence	Gene	Primer sequence
GAPDH	5'-GACATCAAGAAGGTGGTGAAGCAG-3'	CD4	5'-TTCGCCTTCGCAGTTTGATCGT-3'
	5'-ATACCAGGAAATGAGCTTGACAAA-3'		5'-CGGACTGAAGGTCACTTTGAACAC-3'
Mcl1	5'-GTGCCTTTGTGGCCAAACACTT-3'	CD2	5'-AGAAGGACCTGGACGTGAGGATT-3'
	5'-AGAACTCCACAAACCCATCCCA-3'		5'-TCAGGTTGGTCCACTGGTAACTCA-3'
Bcl-XL	5'-AGAGCCTTGGATCCAGGAGAAC-3'	NK1.1	5'-TGTCCTGACCCTGATTGGGATGA-3'
	5'-ACTGAAGAGTGAGCCCAGCAGA-3'		5'-CTCGGTGTAAAAGCCAGTCTTGTG-3'
c-Myc	5'-CCCACCACCAGCAGCGACTC-3'	CD20	5'-TGAGGGAATCAAAGGCTTTGGG-3'
	5'-CAGTGGGCTGTGCGGAGGTT-3'		5'-GCTGCCAGGAGTGATCCTGAAATA-3'
Survivin	5'-CTACCGCATCGCCACCTTCAA-3'	CD8a	5'-GCCAGTCCTTCAGAAAGTGAACTC-3'
	5'-ATCGGGTTGTCATCGGGTTCC-3'		5'-ATATCACAGGCGAAGTCCAATCCG-3'
GM-CSF	5'-GCCTGTCACGTTGAATGAAGAGGT-3'	F4/80	5'-CTTTGGCTATGGGCTTCCAGTC-3'
	5'-AGTAGCTGGCTGTCATGTTCAAGG-3'		5'-GCAAGGAGGACAGAGTTTATCGTG-3'
IL-17A	5'-CGCAAAAGTGAGCTCCAGAAG-3'	IL-1β	5'-CAAGCAACGACAAAATACCTGTG-3'
	5'-TTTCCCTCCGCATTGACACA-3'		5'-AGACAAACCGTTTTTCCATCTTCT-3'
IL-11	5'-CCGACTGGAACGGCTACTCC-3'	TNFα	5'-TGGCCCAGACCCTCACACTCAG -3'
	5'-CACGGCCCAGTCCAAGGTCA-3'		5'-ACCCATCGGCTGGCACCACT -3'
IL-4	5'-GTGCCAAACGTCCTCACAGC-3'	CCL5	5'-CTCACTGCAGCCGCCCTCTG-3'
	5'-CTGCAGCTCCATGAGAACACTAGA-3'		5'-TGGCGGTTCCTTCGAGTGACA-3'
IFNγ	5'-AGCAACAGCAAGGCGAAAAAG-3'	CXCL14	5'-CAAGATCCGCTACAGCGACGTG-3'
	5'-CGCTTCCTGAGGCTGGATTC-3'		5'-AGCTTAGGGTGCAGGCAGTGCT-3'
CCL20	5'-GAACTGGGTGAAAAGGGCTGTGAA-3'	CCL4	5'-CCTTCTGTGCTCCAGGGTTCTC-3'
	5'-TGTGCAGTGATGTGCAGGTGAA-3'		5'-CTGTCTGCCTCTTTTGGTCAGG-3'
Arg1	5'-GTGACTCCCTGCATATCTGCCAAA-3'	CCL8	5'-CTGCTCATAGCTGTCCCTGTCA -3'
	5'-AAGGTCTCTTCCATCACCTTGCCA-3'		5'-TTCCATGGGGCACTGGATATTG -3'
iNOS	5'-TGGCTCGGGATGTGGCTACC-3'	CCL3	5'-TCTGCAACCAAGTCTTCTCAGC-3'
	5'-TTGCCCCATAGGAAAAGACTGC-3'		5'-CTGCCGGTTTCTCTTAGTCAGG-3'
CXCL1	5'-GGCCCCACTGCACCCAAACC -3'	CCL2	5'-GCAGGTCCCTGTCATGCTTCTG-3'
	5'-TGTTGTCAGAAGCCAGCGTTCACC -3'		5'-GCAGGTCCCTGTCATGCTTCTG-3'
CXCL2	5'-CCACTCTCAAGGGCGGTCAAAA-3'	CCL7	5'-CAGCTCTCTCACTCTCTTTCTCCA -3'
	5'-TTCCCGGGTGCTGTTTGTTTTT-3'		5'-CTGTAGCTCTTGAGATTCCTCTTGG -3'
CXCL5	5'-GCAGGTCCACAGTGCCCTAC-3'	IL-17F	5'-CAAACCAGGGCATTTCTGTCC-3'
	5'-TGCGAGTGCATTCCGCTTAG-3'		5'-CTTCCTGACCCTGGGCATTGAT-3'
Saa3	5'-AGCGATGCCAGAGAGGCTGTTC-3'	IL-21	5'-ACGCTCACGAATGCAGGAGTACAT-3'
	5'-AGCAGGTCGGAAGTGGTTGG-3'		5'-AGCTGGGCTCTTGTTGAGTTGAGA-3'
IL-6	5'-CCGGAGAGGAGACTTCACAGAG-3'	IL-22	5'-GAGGTGGTGCCTTTCCTGACC-3'
	5'-CTGCAAGTGCATCATCGTTGTT-3'		5'-CAGTTCCCCAATCGCCTTGAT-3'