Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Il18 conditional knockout mice with floxed *Il18* alleles or floxed *Il18r* alleles were generated using standard homologous gene recombination techniques. The targeting vectors were electroporated into JM8 ES cells (C57/Bl6 genetic background), the screening was performed by long range PCR and the Neo selection cassette was deleted in vivo by crossing with FRT transgenic mice. Floxed mice were then crossed with Flk-1-cre transgenic mice (Motoike et al., 2003) to generate tissue-specific conditional deletion of the IL-18 or IL-18R genes in endothelial and hematopoietic cells, or with Villin-cre mice (Madison et al., 2002) to delete in intestinal epithelial cells. The different alleles (WT and floxed) were identified by PCR on genomic DNA using the following primers:

IL-18 Floxed Allele:

Forward: CTCAGTGGTCATTTCCTCTCTCTGA

Reverse: CAGACCAACCCTTGATCTAGAGC

IL-18R Floxed Allele:

Forward: ATGGAGATTCCATGGTGCTG

Reverse: TCATGTGAAGCACTGCCTTT

Cre Transgene:

Fwd: TTCCCGCAGAACCTGAAGATGTTCG

Rev: GCCAGATTACGTATATCCTGGCAGC

IL-18$^{-/-}$ mice have previously been reported (Takeda et al., 1998). IL-18bp deletion was generated using Cas9/CrispR technology directly into C57BL/6 mice as described (Wang et

al., 2013). Briefly, guide RNAs were designed to target both the 5' UTR and 3' UTR of the IL-18BP gene to generate a 2.5kb deletion of the entire protein coding region of *IL18bp*. Targeted DNA sequence for guide RNAs:

5' UTR: TTGGTTCTGGCTCCATTTCA

3' UTR: ATCTACCAGAGGTGGAAGCA

Primers used to confirm WT allele and KO allele are:

WT Fwd: TTTCAAGGCTCCTTTGCACT

WT Rev: ACTGCTGGAGACCAGGAAGA

KO Fwd: GAAGTTGCTCCTTCCTGTGG

KO Rev: TGGTCAGAGCTGAGTGGATG

All experiments were performed using littermate control, cohoused mice, as indicated in the figure legends. For cohousing experiments, age- and gender-matched *Il18r*$^{fl/fl}$, *Il18r*$^{\Delta/EC}$ and *Il18*$^{-/-}$ mice were co-housed at 1:1:1 ratio for 8 weeks. All animal experimentation was performed in compliance with Yale Institutional Animal Care and Use Committee protocols.

Histology

Colons were fixed in Bouin's medium and embedded in paraffin. Blocks were serially sectioned along the cephalocaudal axis of the gut to the level of the lumen; the next 5 mm-thick section was stained with hematoxylin and eosin. For goblet cell and mucus layer preservation ex vivo, immediately after excision, colons were submerged in Ethanol–Carnoy's fixative at 4°C for 1-2 hours. Fixed colon tissues were embedded in paraffin, cut into 5 μm sections and stained with Alcian blue/PAS.

Immunofluorescence

For in-situ immunofluorescence, distal colon was dissected, fixed in 4% paraformaldehyde for 4 h at 4 °C, followed by incubation in 10% Sucrose/PBS for 1 h, 20% Sucrose/PBS for 1 h and 30% Sucrose/PBS overnight at 4 °C. Tissue samples were frozen in OCT on dry ice and kept in -80 °C until sectioning. Sections 8-10 µm thick were prepared with a cryostat (Leica). Colon sections were permeabilized with Perm/Wash buffer (BD Biosciences) for 10 min or with 1% Triton X-100 (Sigma) in PBS for 20 min and blocked with Protein Block (Dako) for 7 min. Primary antibodies included rabbit anti-MUC2 (Santa Cruz H-300; 1:200), rat anti-ZO-1-eFluor570 (eBioscience 41-9776; 1:200) and mouse anti-β-catenin-Alexa488 (Cell Signaling 2849; 1:200). Fucose was stained with UEA-1-FITC (Sigma; 2.5 µg/ml). Primary and secondary antibodies were incubated in Perm/Wash buffer for 1 h. After washing with Perm/Wash buffer, sections were mounted with ProLong Gold (Invitrogen), covered and sealed with nail polish. Confocal imaging was done with a Nikon-Ti microscope combined with UltraVox spinning disk (PerkinElmer) and data was analyzed using the Volocity software (PerkinElmer). Widefield imaging was done with Leica DMI6000B inverted microscope and data was analyzed using the LAS-AF software.

Isolation of Intestinal Epithelial cells

Epithelial cells were obtained from freshly isolated colons and flushed from both ends with sterile PBS. Colons were then opened longitudinally and thoroughly washed in PBS. The colon was then incubated in 10 ml PBS containing 30 mM EDTA and 1.5 mM DTT on ice for 20 min. The colon was then removed and briefly washed in PBS and incubated in 10 ml PBS containing 30 mM EDTA at 37 °C at 200 RPM for 10 min. The cells were then subjected to 30 sec vigorous shaking and the colon tissue removed. The cells were then centrifuged at 1000 g for 5 min at 4 °C, washed in PBS containing 10% FBS and spun for a further 5 min at 4 °C at 1000 g. Cell pellets constituted isolated intestinal epithelial cell fractions.

Gene Expression Analysis

RNA was extracted using TRIzol reagent (Invitrogen) or the RNeasy Plus mini kit (QIAGEN), reverse transcribed (SuperScript III, Invitrogen) and the expression of specific genes was analyzed by real time PCR on an ABI 7500 machine using TaqMan Gene Expression Assays (Life Technologies). After RNA extraction from animal colons exposed to DSS, the Dynabeads mRNA purification kit (Life Technologies) was used to remove contaminating DSS prior to cDNA synthesis.

16S rRNA Gene Sequencing, Bacterial Genome Sequencing, and Statistical Analyses

Aliquots of frozen faecal pellets were processed for DNA isolation as described previously (Palm et al., 2014). 16S rRNA sequencing of the V4 region and bacterial genome sequencing were performed on an Illumina miSeq using barcoded primers. Microbial diversity and statistical analyses were performed by Lefse (Segata et al., 2011).

Colon Explants

Colon explants (0.5 cm) were obtained from isolated colons following flushing of the lumen with PBS. Explants were weighted and placed in 24 well plates in 500 µl α-MEM (Gibco) containing 10% FBS and 1x Penicillin-Streptomycin (Gibco) for 24 h at 37 °C. Supernatants were clarified by centrifugation at 12,000 g in 4 °C for 10 min and stored at -80 °C until use in ELISA.

ELISA

IL-18 ELISA was conducted using the Mouse Platinum ELISA Kit (eBioscience) as per the manufacturer instructions.

Statistical Analysis

Statistical significance was calculated by using a two-tailed Student's t-test with GraphPad Prism Software Version 6.03 (GraphPad Software). Statistical significance is presented as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Data are represented as mean ± SEM as indicated in the text.

SUPPLEMENTAL REFERENCES

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