Supplementary Materials for

A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion

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Materials and Methods

Animals

All WT, knockout and transgenic mice were male 8-12 week old on the C57BL/6 background bred in house. Animals were anesthetized using isofluorane and killed by cervical dislocation prior to collection of samples. For *in vivo* experiments animals were anesthetized and 200 µl of test reagents were administered intrarectally using a blunt needle 10 min prior to cervical dislocation and sample collection. For some experiments DSS (Dextran Sodium Sulfate, TdB Consultancy, Uppsala, Sweden) was dissolved in animal drinking water to 3% (w/v) and animals were allowed to drink freely. The DSS water was given before the 12 h night period and the experiments were performed 12, 36 or 84 h later. mCherry labelled human MUC2 transgenic mice (RedMUC2^{98trTg}) were generated by pronuclear injection of an mCherry-MUC2-98tr containing BAC clone was generated by homologous recombination of BAC clone RP13-870H17 with an mCherry targeting cassette inserted between the MUC2 CysD1 and CysD2 domains. All animal experimental procedures were approved by the Swedish Laboratory Animal Ethical Committee in Gothenburg.

The following knock-out mice strains were utilized: Tlr2$^{-/-}$ generated by Carsten J. Kirschning, Technical University of Munich, Munich, Germany; Tlr4$^{-/-}$, MyD88$^{-/-}$, IL18$^{-/-}$, and Trif$^{-/-}$ generated by Shizuo Akira, Osaka University, Osaka, Japan; Nlrp3$^{-/-}$ generated by Jürg Tschopp, University of Lausanne, Lausanne, Switzerland; Nlrc4$^{-/-}$ and Caspase 11$^{-/-}$ generated by Vishva Dixit, Genentech, South San Francisco, CA; Nlrp6$^{-/-}$ generated by Gabriel Nuñez, University of Michigan, Ann Arbor, MI; Caspase 1/11$^{-/-}$ generated by Tara Seshadri, BASF Bioresearch, Worcester, MA; IL1αβ$^{-/-}$ generated by Yoichiro Iwakura, University of Tokyo, Tokyo, Japan; and Rag1$^{-/-}$ generated by Virginia E. Papaioannou, MIT, Cambridge, MA.

Reagents

All reagents were Ultrapure grade and purchased from Invivogen unless otherwise stated. Final concentrations used to treat explants are indicated in brackets: LPS from *E. coli* O111:B4 (200 µg/ml), *E. coli* diphosphoryl Lipid A (Sigma; 10 µg/ml), LTA from *S. aureus* (200 µg/ml), P3CSK4 (50 µg/ml), DNA from *E. coli* (200 µg/ml), flagellin from *B. subtilis* (50 µg/ml), MDP (200 µg/ml), iE-DAP (200 µg/ml) and Carbachol (Sigma; 1 mM). Experiments requiring fluorescently tagged reagents utilized: *E. coli* LPS-Alexa488 (ThermoFisher; 200 µg/ml), P3CSK4-Rhodamine (50 µg/ml), P3CSK4-FITC (EMC Microcollections; 50 µg/ml) and Dextran-Alexa488 (ThermoFisher; 0.5 mg/ml). Reagents were reconstituted in Krebs-mannitol buffer, H$_2$O (P3CSK4) or DMSO (Lipid A, Carbachol). LPS, Lipid A, P3CSK4 and LTA stocks were sonicated prior to use.

Intestinal explant mucus measurements

Intestinal tissues were prepared for *ex-vivo* mucus analysis as previously described (*13*). Briefly, intestinal tissues were collected by dissection and flushed with Krebs buffer to remove luminal content and unattached mucus. The muscle layer was removed by microdissection and tissue was mounted in a horizontal chamber system and maintained at 37°C with basolateral Krebs-glucose buffer perfusion and apical Krebs-mannitol buffer. Removal of the muscular layer improves viability and allowes serosal addition of compounds. The senGC cells and system was working in the same way with an intact muscular layer, Fig. S1A. To visualize the mucus formed on the apical side of the tissue explant, the mucus was overlaid with charcoal. The surface charcoal and epithelium was observed by a stereomicroscope and the difference making up the mucus thickness measured using a glass micropipette connected to a micrometer. Mucus thickness was measured every 15 min from t=0-60

min. Inhibitors were added to the apical or basolateral buffer at t=0 and mucus secretion was stimulated at t=30 (unless stated otherwise) and mucus growth rate before and after treatment was calculated. Transepithelial potential difference (PD) was recorded using electrodes linked to the basolateral and apical chambers via agar bridges. The PD remained constant during the experiments showing that ion channels were functoning normally.

Intestinal explant live imaging

Intestinal tissue explants were prepared and chamber mounted using the same method as described above. For mucus penetrability analysis the tissue was stained by adding Calcein violet (ThermoFisher; 1 µg/ml) to the basolateral Krebs-glucose buffer and the mucus was overlaid with 1 µm Fluosphere beads (ThermoFisher) as described previously (20). Tissue and beads were visualized by generating confocal z-stacks using an LSM 700 microscope equipped with a x20 water immersion objective (Zeiss). For live imaging of GC secretion MUC2-mCtg explants were used and Hoechst-34580 (Sigma; 1 µg/mL) was included in the apical Krebs-mannitol buffer. For live imaging of inflammasome activity tissues were treated with Z-YVAD-R110 Caspase1/11 fluorogenic peptide probe (ThermoFisher; 100 µM) for 30 min at RT. For live imaging of Ca^{2+} signaling in GCs, RedMUC2^{98trTg} explants were incubated with apical HEPES buffer (pH 7; 140 mM NaCl) containing Fluo4 dye (ThermoFisher; 5 µM) for 30 min at 4°C. The apical buffer was then replaced with fresh HEPES and the tissue incubated for 30 min at 37°C prior to imaging. Imaging of GC secretion and Ca^{2+} signaling was performed by acquiring continuous confocal z-stacks over a 1 h period.

Inhibitors
Stock inhibitor solutions were prepared in DMSO and added to the apical buffer unless otherwise stated. Dynasore (Sigma; 100 µM), DPI (Sigma; 100 µM), Ac-YVAD-cmk Caspase1/11 inhibitory peptide (Sigma; 200 µM), basolateral atropine (Sigma; 50 µM), NAC (Sigma; 0.5 mM), Mito-TEMPO (Sigma; 0.5 mM), Pepinh-MYD MyD88 inhibitory peptide (Invivogen; 50 µM), carbenoxolone (Sigma; 75 µM), tetrodotoxin (Abcam; 100 µM), BAPTA-AM (ThermoFisher; 200 µM), EGTA (Sigma; 2 mM), Xestospongin-C (Tocris, 100 µM) and Ryanodine (100% ethanol solubilized; Tocris; 100 µM). All inhibitors were incubated with explant tissue for 30 min prior to different treatments, with the exception of carbenoxolone which was incubated for 1 h.

Estimation of stool and colonic mucus soluble endotoxin:
Stool samples were collected by dissection and transferred to tubes containing 1 ml PBS. For mucus samples unflushed colonic tissues containing no fecal material were opened and pinned flat to silicon dissection plates. Mucus and underlying mucosal tissue were collected using 2 mm biopsy punches (Miltex) and transferred to tubes containing 1 ml PBS. A 5 mm steel bead (Qiagen) was added to each tube and samples were homogenized for 1 min using a Fast-Prep 24 instrument (MP-Biomedicals). Homogenates were clarified by centrifugation at 5,000xg for 10 min and supernatants were collected. Supernatant endotoxin was quantified using LAL Chromogenic Endotoxin Quantitation Kits (ThermoFisher) with PBS used as the negative control and data was normalized to stool or mucus volume. All buffers, plastics and dissection equipment were sterile and pyrogen free. Pipette tips and sample tubes were maximum recovery grade (Axygen).

Estimation of stool and colonic mucus bacterial load

Stool and mucus were collected (as described in previous section) and transferred to 1 ml TE buffer supplemented with 0.5% SDS (w/v) and 200 µg/ml proteinase K (Qiagen) in Lysing Matrix E tubes (MP Biomedicals). Lysing tubes were incubated at 55˚C for 1 h after which DNA was isolated using mechanical disruption and phenol:chloroform:isoamylalcohol extraction as previously described *(28). Extracted DNA was precipitated using ethanol and 0.2 M NaCl. DNA was pelleted by centrifugat*ion at 20,000xg for 20 min and the pellet was washed with 70% ethanol. Finally DNA pellets were rehydrated in 100 µl TE buffer and stored at -20˚C until used. In order to allow quantification of relatively low copy number bacterial 16S DNA in mucus samples by qPCR, the ratio of 16S DNA to total DNA was increased by limited cycle number (LCN) PCRs amplifying the whole 16S gene (20). 50 µL LCN PCRs were prepared using HotStar Taq Plus PCR Mastermix (Qiagen), 0.2 µM universal forward primer 27F (AGAGTTTGATCMTGGCTCAG), 0.2 µM universal reverse primer 1492R (CGGTTACCTTGTTACGACTT) and 500 ng template DNA. Thermocycling conditions were: 1 cycle of 95˚C for 5 min; 16 cycles of 94˚C for 1min, 55˚C for 1 min, 72˚C for 1.5 min; 1 cycle of 72˚C for 10 min. 16S standards (quantified E. coli 16S DNA), contamination controls and no template controls were amplified at the same time as samples. Amplified samples, standards and controls were then analyzed by qPCR to determine the total number of 16S copies. Briefly, 20 µl qPCRs were prepared using 2 µl of LCN PCR amplifications as template, SsoFast EvaGreen qPCR Supermix (Bio-Rad) and 0.3 µM each of universal 16S primers 926F (AAACTCAAAKGAATTGACGG) and 1062R (CTCACRRCACGAGCTGAC). DNA extracted from stool (25 ng) was used in qPCRs directly without LCN-PCR enrichment. qPCRs were analyzed on a CFX96 instrument (Bio-Rad) and resulting Cq values were exported from CFX manager software (Bio-Rad) and calibration curves constructed from 16S standard data. Calibration curves were used to calculate the total original 16S copy number from stool and mucus sample DNA extractions and data was normalized to sample volume.

Explant and whole tissue histology

For explant histology tissues were submerged in 4% (w/v) neutral buffered formalin for 1 h. Fixed tissue was incubated in 30% (w/v) sucrose at 4°C for 15 h and then embedded in Optimal Cutting Temperature compound prior to cryosectioning. Sections were washed in PBS and stained with Hoechst-34580 (Sigma; 1µg/ml) to visualize nuclei. Stained tissue sections were cover-slipped with Prolong-Gold Antifade mounting medium (ThermoFisher) and visualized by confocal microscopy using an LSM 700 microscope with a x40 oil immersion objective (Zeiss). For whole tissue histology colonic tissue containing a stool pellet was submerged in Methacarn fixative for at least 24h (*1*). Fixed tissue was paraffin embedded and sectioned, followed by dewaxing and staining using a combination of Alcian Blue and Periodic acid-Schiff (AB/PAS) stains (*1*).

Explant whole mounts

Explant tissue was fixed in 4% neutral buffered formalin for 1 h and then permeabilized with 0.5% (v/v) Triton X-100 for 15 min and washed with PBS. Tissue was stained for 1 h with 10 µg/ml Hoechst-34580 (Sigma) to visualize nuclei and 1:500 Phalloidin-Alexa647 (ThermoFisher) to visualize actin. Stained tissue was washed with PBS, transferred to a microscope slide and cover-slipped with Prolong-Gold Antifade mounting medium (ThermoFisher). Whole mounts were visualized by generating confocal z-stacks using an LSM 700 microscope equipped with a x40 oil immersion objective (Zeiss).

Ex-vivo ROS quantification in colonic epithelial cell suspensions

Epithelial cells were isolated from colonic tissues by disassociating them from the underlying lamina propria. Colons were first thoroughly flushed with Krebs buffer then were inverted using a blunt needle and syringe. One end of the tissue was ligated with nylon thread then the colon was inflated with Krebs buffer and the other end of the colon ligated in a similar manner. Inverted/inflated colons were transferred to 10 ml Hanks balanced salt solution with 5% (v/v) FCS, 5 mM EDTA and 1 mM DTT and incubated at 37°C in a shaking incubator for 30 min. Tissue was vortexed for 1 min to disassociate cells and the remaining intact tissue was removed. The cell suspension was passed through a 40 µm filter (BD Biosciences) to remove clumped cells and the filter was washed with 10 ml PBS. Cells were centrifuged at 100xg for 10 min and pellets resuspended in 2 ml of 140 mM pH 7 HEPES buffer. Cells were loaded with ROS sensitive DCFDA-AM dye (Sigma; 10 µM) and Mito-TEMPO (Sigma; 200 µM) to quench mitochondrial ROS production. Cell suspensions were divided into different treatment groups and different inhibitors added to the concentrations detailed previously. Cells were incubated for 1 h at 37°C then distributed to wells on black 96-well plates. Different bacterial molecules or vehicle buffer was added to wells and plates were incubated for 30 min at 37°C. DCFDA fluorescence was measured using a Victor plate reader (PerkinElmer) and data for bacterial molecule treated wells normalized to vehicle treated controls.

Ex-vivo FRAP

Colonic tissue explants were prepared and chamber mounted as described above. The apical Krebs-mannitol buffer was supplemented with Calcein Violet tissue stain (ThermoFisher; 1 µg/ml) and incubated in the dark at RT for 30 min. Explants were thoroughly washed 5 times with fresh Krebs-mannitol and incubated for 30 min at 37°C. The tissue surface was imaged and FRAP data acquired using an LSM 700 confocal microscope with a x20 water immersion lens and 405 nm laser with Zen acquisition software (Zeiss). For each FRAP measurement Calcien violet fluorescence was initially recorded over a 0.0256 mm^2 experimental area of tissue using 1% laser power for 1 min (pre-bleach). A 1,200 $µm^2$ sub-region of this area (the target) was then bleached using 15% laser power for 15 s, followed by recording of fluorescence over the experimental area using 1% laser power for 10 min (recovery). Fluorescence data for the target region was first normalized to a non-target region to control for photobleaching then baseline corrected to control for background fluorescence. Data was normalized to average pre-bleach data to allow relative comparison of FRAP experiments from different tissues. For experiments analyzing the effect of gap junction inhibition on FRAP, carbenoxolone (Sigma; 75 µM) was added to the apical Krebs-mannitol buffer during staining and incubation steps.

Ex-vivo bacterial crypt clearance

Citrobacter rodentium (strain ICC169) was grown to mid-log phase in BHI broth and 1 ml OD_{600} 0.1 culture was centrifuged at 5,000xg for 5 min. Cell pellets were resuspended in 1ml PBS containing Baclight Red cell stain (ThermoFisher; 0.5 µM) and incubated at 37°C for 30 min. Labelled bacteria were washed 4 times in PBS then resuspended in 100 µl Krebs-mannitol buffer. Chamber mounted colonic explants were prepared as described above with apical Krebs-mannitol containing Syto9 nucleic acid stain (ThermoFisher; 10 µM) and incubated for 30 min at RT. Tissue was then washed with fresh Krebs-mannitol buffer and the attached mucus was disrupted by vigorous aspiration with a micropipette. Labelled *C. rodentium* was then applied to the tissue and allowed to sediment for 5 min before the apical chamber was refilled with Krebs-mannitol buffer. Labelled tissue and bacteria were visualized before and after treatment with LPS by generating confocal z-stacks using an LSM 700 microscope with a x20 water immersion lens.

Statistical and image analysis

All statistical analyses were 1- or 2-way ANOVA with post-hoc testing and were performed using Prism 6 software (Graphpad). FRAP time-series were produced using Zen software (Zeiss) and fluorescence data extracted directly for analysis. To quantify GC secretion and bacterial clearance from crypts, confocal z-stack time-series were produced using Zen then exported to Imaris software (Bitplane). For secretion, individual GC thecas were mapped to isosurfaces using the mCherry-MUC2 signal and isosurface volumes were tracked over time. To analyze bacterial clearance from crypts, individual bacteria were mapped using Baclight Red signal and crypt opening locations were mapped manually. 3D spatial coordinate data was extracted for each and the distance of individual bacteria from crypt openings was calculated.
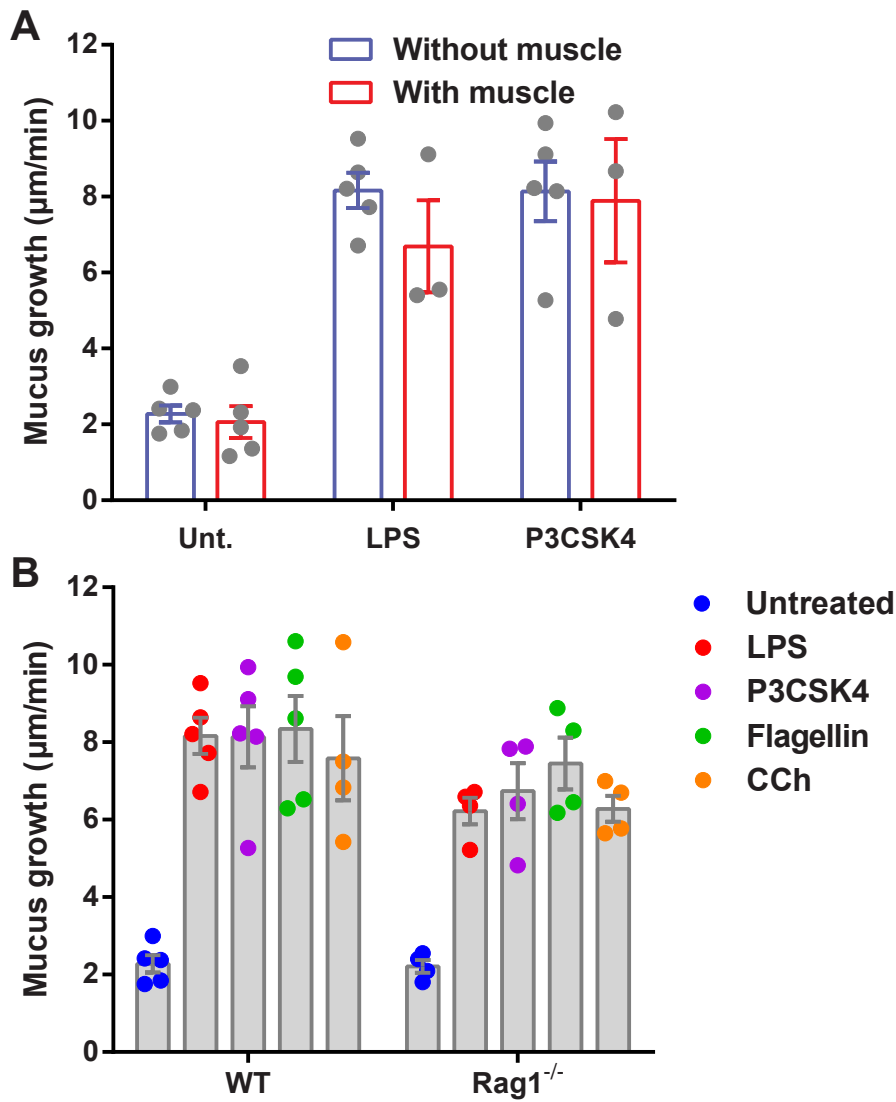
Figure S1. Muc2 secretion in response to TLR-ligands is preserved in intact tissue and tissue lacking mature lymphocytes. (**A**) Quantification of mucus growth in untreated or LPS or P3CSK4 treated explants from intact tissue where the muscle layer had not been removed. Explant data where the muscle layer had been removed is reproduced from Fig. 1A. (**B**) Quantification of mucus growth in untreated or LPS, P3CSK4, flagellin or CCh treated colonic explants from WT or *Rag1*^{-/-} mice. WT data is reproduced from Fig. 3A.
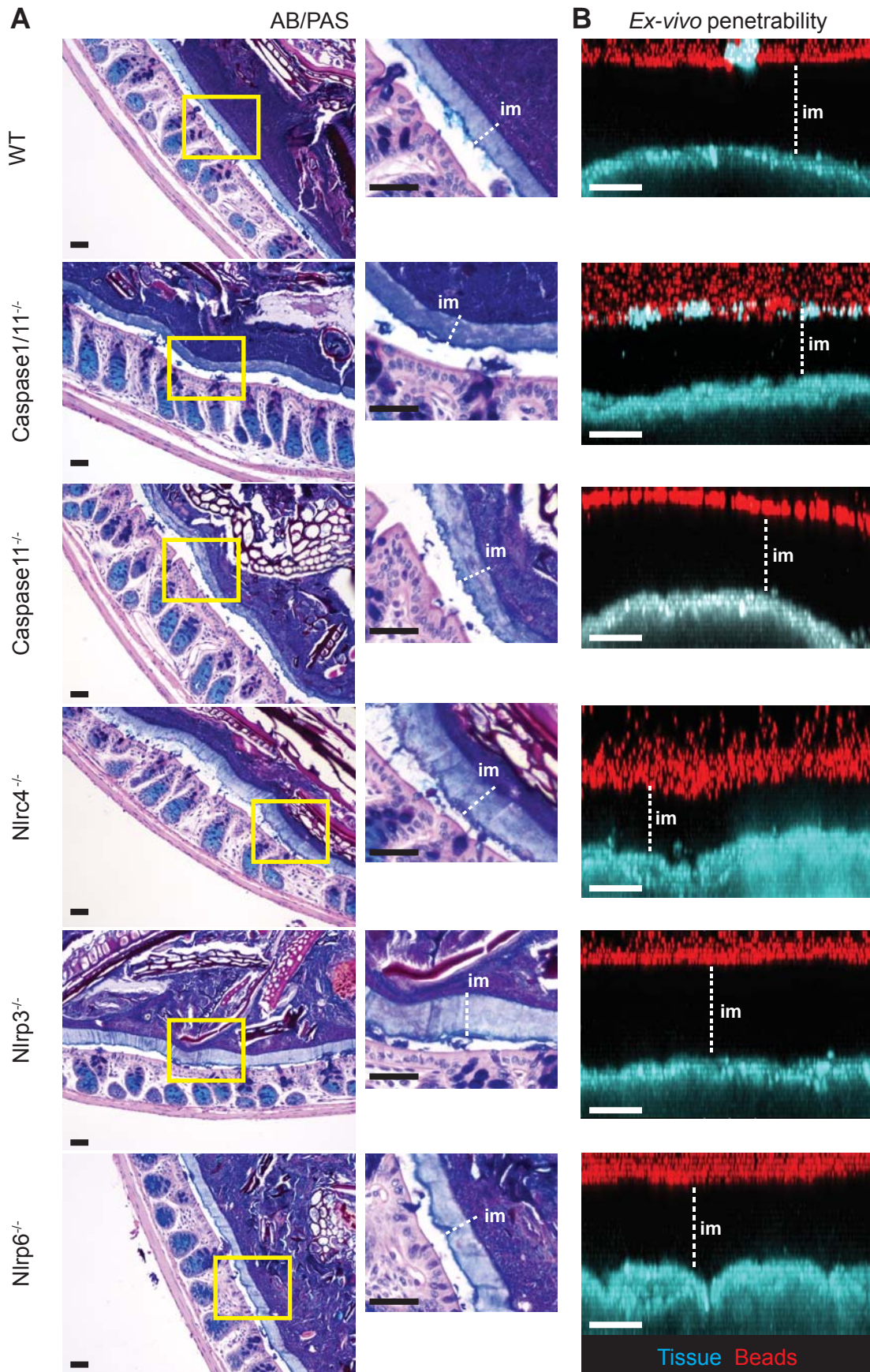
Figure S2. Inflammasome deficiency does not alter the colonic mucus phenotype. (**A**) Methacarn-fixed distal colon sections from WT and inflammasome KO mice were stained with AB/PAS to visualize the colonic mucus. Magnified region in yellow box (right panel), inner mucus layer (im; white dashed line). (**B**) Confocal z-stacks of colonic explants overlaid with 1 μm beads. Tissue (blue), beads (red), inner mucus layer (im; white dashed line). Explants shown in (B) correspond to genotypes shown in adjacent images in (A). Scale bars 50 μm.
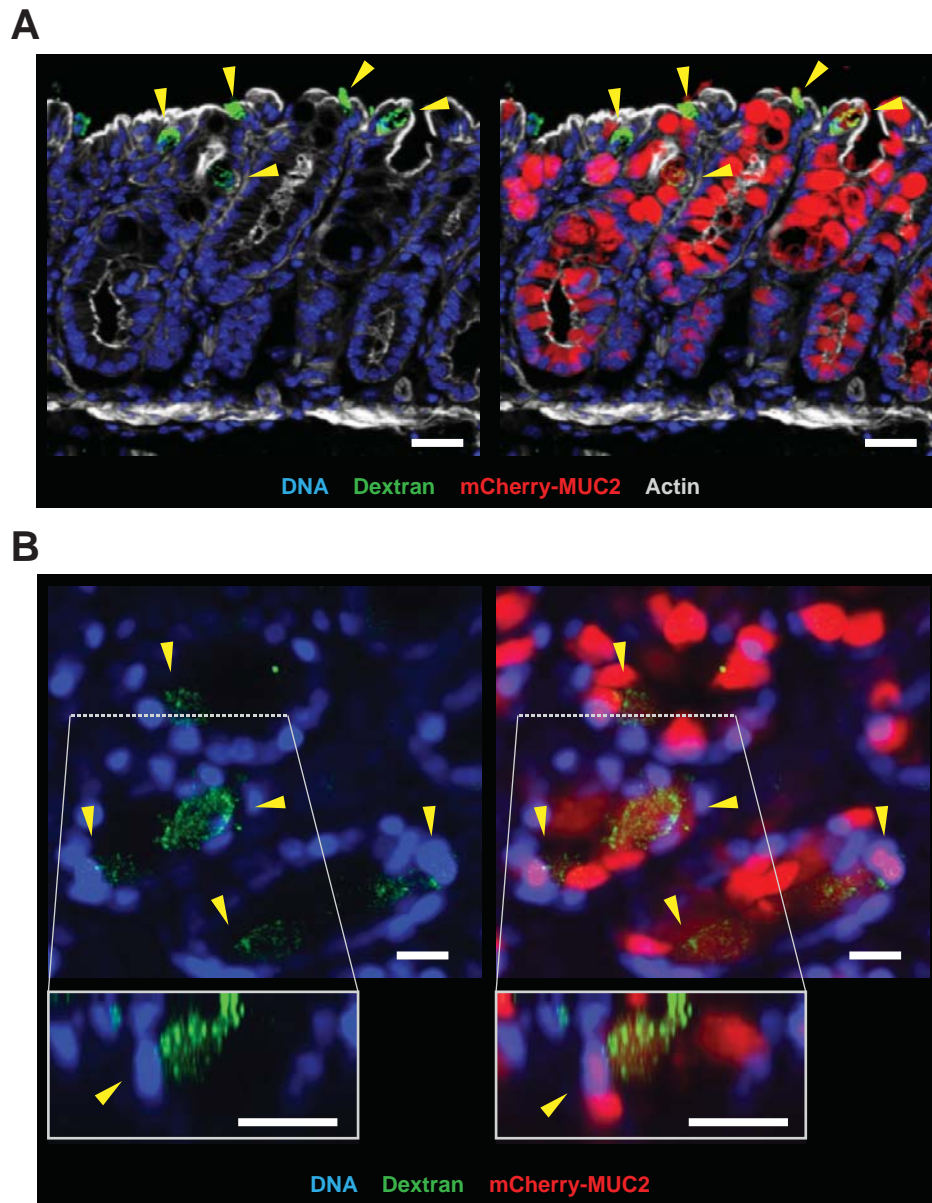
Figure S3. Endocytotic colonic goblet cells are restricted to the upper crypt and can be identified *in vivo*. (**A**) Confocal micrographs of cryosections of RedMUC2[98trTg] tissue treated with fluorescent dextran *ex vivo*. (**B**) Confocal micrographs of live of RedMUC2[98trTg] tissue obtained after 10 min *in vivo* intrarectal administration of fluorescent dextran. x/y-axis view (upper panels), x/z-axis view (lower panels); endocytotic GCs (yellow arrows), DNA (blue), dextran (green), mCherry-MUC2 (red), actin (grey). Scale bars 20 μm.
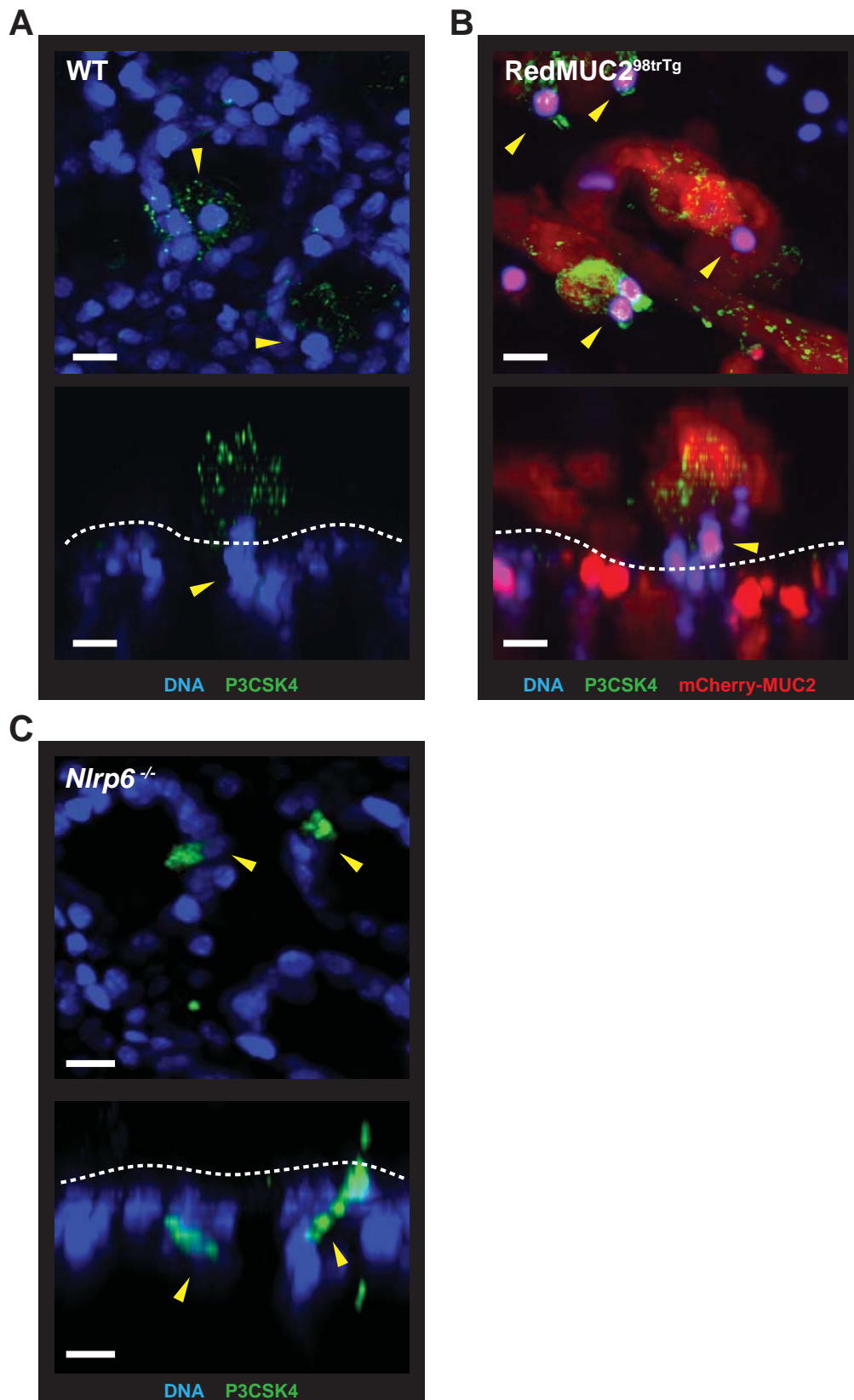
Figure S4. Nlrp6 dependent epithelial expulsion of senGCs after P3CSK4 endocytosis can be observed *in vivo*. Confocal micrographs of live WT (**A**), RedMUC2[98trTg] (**B**) and *Nlrp6^{-/-}* (**C**) tissue obtained after 10 min *in vivo* intrarectal administration of fluorescent P3CSK4. Endocytotic GCs (yellow arrows), DNA (blue), P3CSK4 (green), mCherry-MUC2 (red), tissue surface (dashed white line). Scale bars 20 μm.
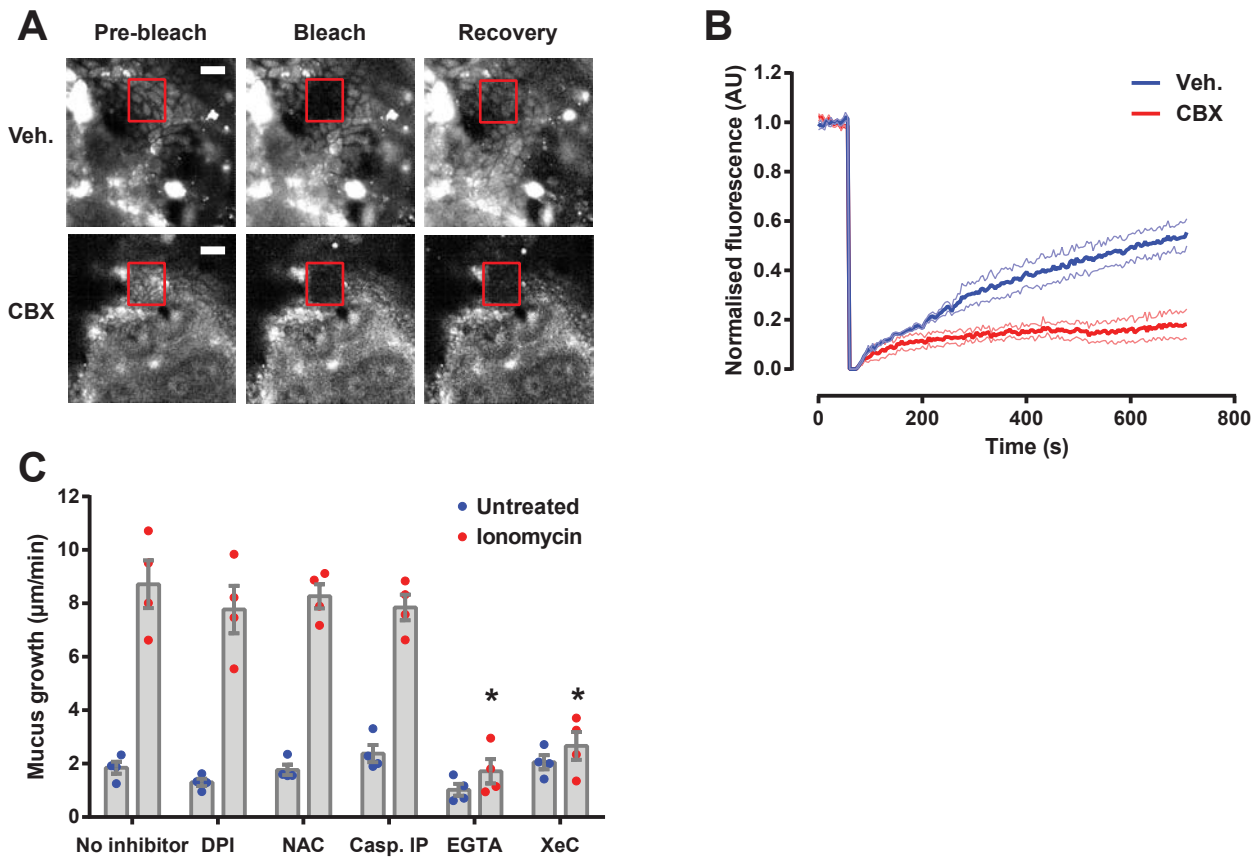
Figure S5. TLR-ligand induced Muc2 secretion requires functional gap junctions and Ca²⁺ signaling. (**A**) (**A**) Confocal micrographs from FRAP experiments in vehicle or CBX treated tissue: calcein violet (white), bleached area (red box). (**B**) Quantification of calcien violet fluorescence from FRAP experiment as shown in (H). (**C**) Quantification of mucus growth rate in untreated or ionomycin treated colonic explants with or without inhibitors. Error bars are SEM of 4-5 animals; significance calculated by Dunnett's multiple comparison of no inhibitor vs. inhibitor data (* $p<0.05$).
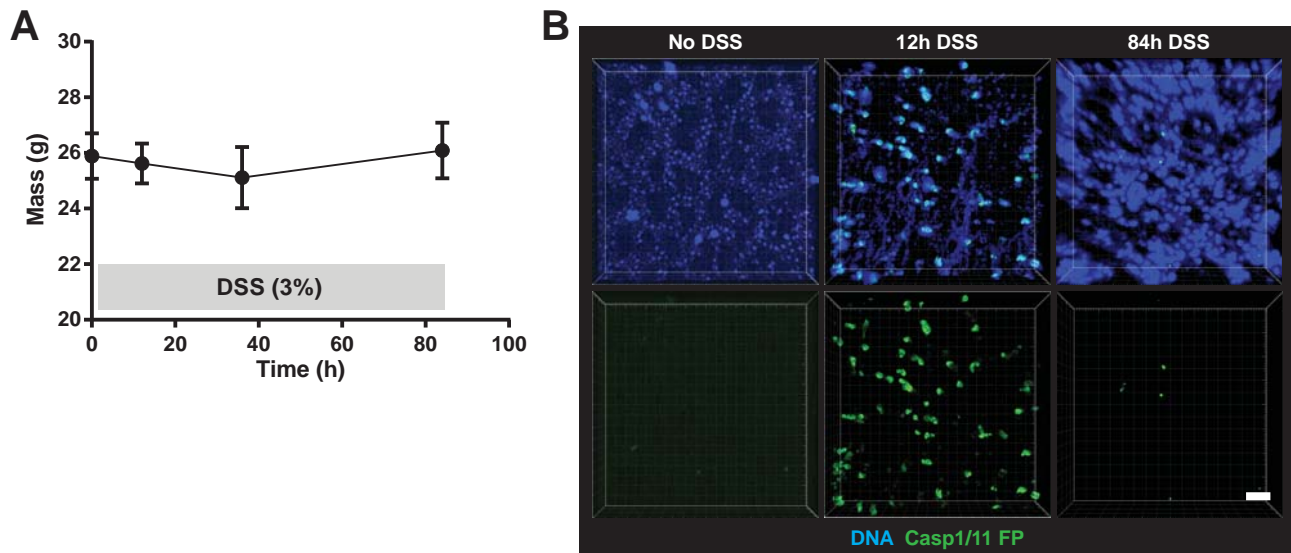
Figure S6. Short-term DSS treatment does not result in colitis, but does induce epithelial inflammasome activity. Mice were exposed to 3% DSS in drinking water; samples were collected after 0 (no DSS), 12, 36 and 84 h of DSS exposure. (**A**) Mass of mice from 0-84 h DSS exposure. (**B**) Confocal images of tissue treated with Casp1/11 FP probe after exposure to DSS. The images were used to generate the results presented in Fig. 7D. DNA (blue, upper panels), Casp1/11 FP (green, upper/lower panels). Scale bars 20 μm.
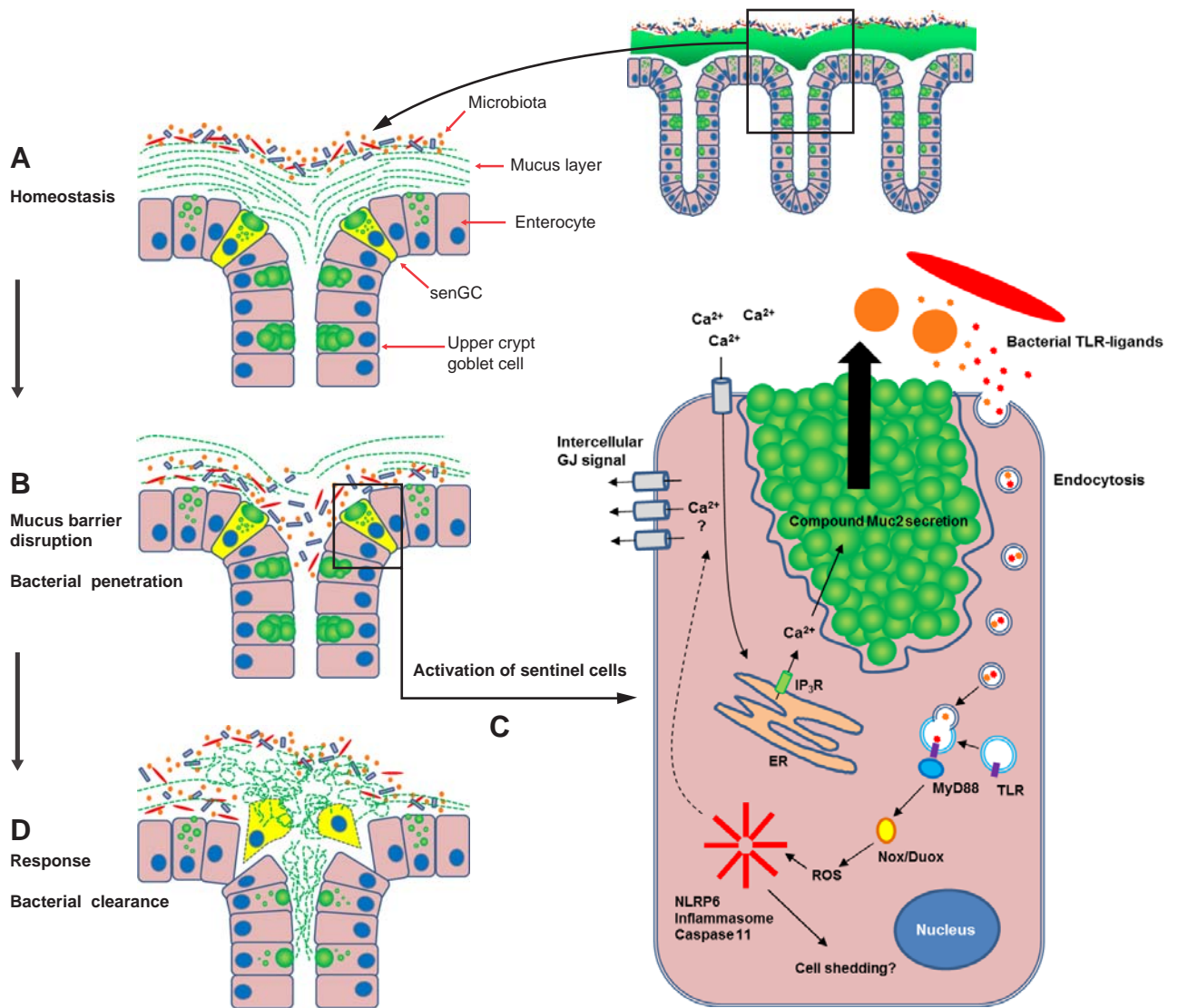
Figure S7. Sentinel GC activation model after disruption of the colonic mucus barrier. (**A**) Homeostatic conditions. (**B**) Disruption of the colonic mucus barrier and bacterial penetration to the crypt openings. (**C**) Intracellular pathways leading to senGC activation and Muc2 secretion. (**D**) Clearance of bacteria from the crypt opening by senGC mediated Muc2 secretion from upper crypt GCs.

CAPTIONS FOR MOVIES

Movie S1. Vehicle treated RedMUC2^{98trTg} colonic explant
x/z-axis view of explant imaged by confocal microscopy; vehicle treatment occurred at t=2 min; mCherry-MUC2 (red). This movie is the negative control for Movie S2. Scale bar 40 µm.

Movie S2. LPS treated RedMUC2^{98trTg} colonic explant
x/z-axis view of explant imaged by confocal microscopy; LPS (0.2 mg/ml) treatment occurred at t=2 min; mCherry-MUC2 (red). Scale bar 40 µm.

Movie S3. LPS treated RedMUC2^{98trTg} colonic crypt top view
x/y-axis view of explant crypt imaged by confocal microscopy; LPS (0.2 mg/ml) treatment occurred at t=30 sec; mCherry-MUC2 (red), DNA (blue). Scale bar 10 µm.

Movie S4. LPS treated RedMUC2^{98trTg} colonic crypt side view
x/z-axis view of explant crypt shown in Movie S3; mCherry-MUC2 (red), DNA (blue). Scale bar 10 µm.

Movie S5. LPS treated RedMUC2^{98trTg} mCherry-MUC2 isosurface top view
x/y-axis view of mCherry-MUC2 mapped isosurfaces from explant crypt shown in Movie S3; LPS (0.2 mg/mL) treatment occurred at t=30 s; senGC (green), responsive GCs (orange) non-responsive GCs (blue), DNA (white). Scale bar 10 µm.

Movie S6. LPS treated RedMUC2^{98trTg} mCherry-MUC2 isosurface side view
x/z-axis view of mCherry-MUC2 mapped isosurfaces from explant crypt shown in Movie S5; senGC (green), responsive GCs (orange) non-responsive GCs (blue), DNA (white). Scale bar 10 µm.

Movie S7. LPS treated RedMUC2^{98trTg} colonic crypt loaded with Fluo4 Ca^{2+} indicator
x/y-axis view of explant crypt imaged by confocal microscopy; LPS (0.2 mg/ml) treatment occurred at t=30 s; mCherry-MUC2 (red), Fluo4 (green), DNA (blue). Scale bar 10 µm.