Supplementary Materials for

Discovery of anti-inflammatory physiological peptides that promote tissue repair by reinforcing epithelial barrier formation

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Figs. S1 to S11

Other Supplementary Material for this manuscript includes the following:

Data file S1
Fig. S1 Mouse peritoneal tissues secrete humoral factors that induce tight junction assembly in A431 cells

A, Representative images of extracted cell–cell boundaries immunostained with anti-α-catenin antibodies. Bar, 20 µm. B, List of the fluorescence intensities of claudin-1 and the cell–cell boundary area in samples treated as in (A). C, Schema of the PCM treatment of A431 cells. D, Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with PCM prepared from control mice or LPS-treated mice, or control HBSS for 3 h. Bar, 20 µm. E, Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (D) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. F, Relative barrier permeability measured by paracellular tracer flux analysis using FITC-dextran (4 kDa) in A431 cells (n = 3 independent samples). G, Western blot analysis of A431 cells treated with PCM or control HBSS using anti-claudin-1 and anti-keratin 5 antibodies. Dried PCM was eluted in Laemli buffer and 50 µg of proteins were loaded for each lane. Similar results were obtained in two independent experiments. H, Western blot analysis of PCM prepared from control mice or LPS-stimulated mice administered anti-A1AT C-terminus antibodies. Similar results were obtained in two independent experiments. Dunnett’s test (E) and two-tailed t-test (F). *p < 0.05.
Fig. S2 PCM induces TJ assembly in various types of epithelial cells

A, Immunofluorescence of claudin-4 and α-catenin in MCF7 cells treated with PCM or HBSS for 3 h. Bar, 20 µm. B, Quantification of relative claudin-4 intensity at cell–cell boundaries of MCF7 cells shown in (A) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments.

C, Immunofluorescence of claudin-4 and α-catenin in HT29 cells treated with PCM or HBSS for 3 h. Bar, 10 µm. D, Quantification of relative claudin-4 intensity at cell–cell boundaries of HT29 cells shown in (C) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments.

E, Immunofluorescence of claudin-4 and α-catenin in EpH4 cells treated with PCM or HBSS for 3 h. Bar, 20 µm. F, Quantification of relative claudin-4 intensity at cell–cell boundaries of EpH4 cells shown in (E) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. two-tailed t-test (B, D, F). ***p < 0.001.
**Fig. S3 Claudin-assembling factors in PCM are heat-stable small molecules**

**A.** Schema of the experimental procedure for characterization of PCM. **B,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with PCM with or without heat treatment or control HBSS. Bar, 20 µm. **C,** Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (B) (n = 6 images from two independent samples). Similar results were obtained in two independent experiments. **D,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with PCM dialyzed with a molecular weight cut-off 3.5-kDa or 1-kDa membrane or control HBSS. Bar, 20 µm. Similar results were obtained in two independent experiments. Dunnett’s-test (C). ***p < 0.001.
Fig. S4 Purification of the claudin-assembling factors in PCM

A, Schema of the purification procedure for claudin-assembling factors in PCM. B, Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with HBSS, PCM, or eluted fractions of Q-sepharose and Con A-sepharose columns. Bar, 20 µm. C, Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (B) (n = 5 images). D, Silver staining of each purification step. Similar results were obtained in two independent experiments. E, List of peptides identified in the Con A-elute fraction and derived from extracellular proteins. F, Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with anti-A1AT or rabbit IgG antibody-immunoprecipitated PCM, or with HBSS or PCM for 3 h. Bar, 20 µm. G, Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (F) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. H, Elution profile of peptides separated by a C18 column for immunoprecipitants of PCM obtained with anti-A1AT antibodies (bottom). Two-step linear gradient programs were used (upper) (see Methods). Similar results were obtained in two independent experiments. I, Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with eluted fractions of the C18 column or HBSS. Bar, 20 µm. J, Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (I) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. K, Amino acid sequences of the 22-aa C-terminal peptides of A1AT, JIPm35, JIPm36, and JIPm40, identified in the active fractions (e3–5) of the C18 column. L, Elution profiles of synthetic 22-, 35-, 36-, and 40-aa C-terminal peptides of A1AT (100 µg) on the C18 column (bottoms). Dunnett’s-test (G, J). *p < 0.05, ***p < 0.001.
**Fig. S5 JIPs induces TJ assembly in various types of epithelial cells**

**A,** Western blot analysis of synthetic peptides JIP$_{m35}$ and JIP$_{m35\text{-mut1}}$ (17.5 µg each) with anti-A1AT C-terminus antibodies. Similar results were obtained in two independent experiments. **B,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with the indicated peptides at 20 µM or control HBSS for 3 h. See Fig. 1H. Bar, 20 µm. **C,** Immunofluorescence of ZO-1 and occludin in A431 cells treated with JIP$_{m35}$ at 20 µM or control HBSS for 3 h. Bar, 20 µm. Similar results were obtained in two independent experiments. **D,** Immunofluorescence of claudin-1 and E-cadherin in A431 cells treated with or without JIP$_{m35}$ (55 µM) in DMEM for 3 h. Bar, 10 µm. **E,** TER measurements of A431 cells treated with or without JIP$_{m35}$ in DMEM for 3 h (55 µM, n = 3 independent samples). **F,** Immunofluorescence of claudin-4 and E-cadherin in MCF7 cells treated with or without JIP$_{m35}$ (65 µM) in E-MEM for 3 h. Bar, 10 µm. **G,** TER measurements of MCF7 cells treated with or without JIP$_{m35}$ in E-MEM for 3 h (65 µM, n = 3 independent samples). **H,** Immunofluorescence of claudin-4 and E-cadherin in HT29 cells treated with or without JIP$_{m35}$ (65 µM) in McCoy’s 5A medium for 3 h. Bar, 10 µm. **I,** TER measurements of HT29 cells treated with or without JIP$_{m35}$ in McCoy’s 5A medium for 3 h (65 µM, n = 3 independent samples). two-tailed t-test (E, G, I). n.s., not significant. *p < 0.05, **p < 0.01.
**Fig. S6** JIP$_m$35 and JIP$_m$42/CAAP48 do not inhibit the protease activity of trypsin, elastase, and chymotrypsin

A, Measurement of the protease activity of trypsin using an Amplite Universal Fluorimetric Protease Activity Assay Kit. The increase in fluorescence intensity was directly proportional to protease activity as protease catalyzed hydrolysis yielded fluorescent dye-labelled short peptides (n = 3 independent samples). Measurement of protease activity of B, human neutrophil elastase (HNE), C, porcine pancreas elastase (PPE), and D, chymotrypsin using an Amplite Universal Fluorimetric Protease Activity Assay Kit (n = 3 independent samples). E, TNFα release from LPS-stimulated mouse neutrophils with or without JIP at 18h (n = 3 independent experiments). F, CXCL1 release from LPS-stimulated mouse neutrophils with or without JIP at 18h (n = 3 independent experiments). Tukey’s test (A-D), Dunnett’s test (E, F). n.s., not significant. **p < 0.01, ***p < 0.001.
**Fig. S7 Assessment of the neutralizing activity of anti-JIPs antibodies and involvement of MMP in TJ assembly**

**A,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with 20 µM JIPm35 or control HBSS for 3 h in the presence of anti-JIPs antibodies or control preimmune serum. Bar, 20 µm. **B,** Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (A) (HBSS, JIPm35, and JIPm35 + anti-JIPs Ab; n=5 images from two independent samples, JIPm35 + preimmune; n=6 images from two independent samples). Similar results were obtained in two independent experiments. **C,** Immunofluorescence of JIPm35, ZO-1, and occludin in A431 cells treated with 20 µM JIPm35 or control HBSS for 3 h in the presence or absence of anti-JIPs antibodies or preimmune serum. Bar, 20 µm. Similar results were obtained in two independent experiments. **D,** TER measurements of EpH4 cells treated with or without JIPm35 (70 µM) for 3 h in the presence or absence of anti-JIPs antibodies or preimmune serum (n=4 independent samples). **E,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with the products of Fig. 2G or control HBSS for 3 h. Bar, 20 µm. **F,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with CCM prepared from control, DSS-treated mice, or from DSS-recovery mice in the presence or absence of GM6001, or control HBSS for 3 h. See Fig. 2H. Bar, 20 µm. Tukey’s test (B, D). *p < 0.05, ***p < 0.001.
**Fig. S8 JIPs localize at cell–cell junctions and induce cortical assembly of ZO-1**

A, Immunofluorescence of JIP\textsubscript{m35} in A431 cells treated with 20 µM JIP\textsubscript{m35} or control HBSS for 3 h. Bar, 20 µm. Similar results were obtained in two independent experiments. B, Immunofluorescence of rabbit IgG and ZO-1 in A431 cells transfected with anti-JIPs antibodies or preimmune (rabbit whole serum, respectively). Transfected cells were treated with 20 µM JIP\textsubscript{m35} or control HBSS for 3 h. Bar, 20 µm. Similar results were obtained in two independent experiments. C, Immunofluorescence images of rabbit IgG and ZO-1 in A431 cells microinjected with anti-JIPs antibodies or preimmune (rabbit whole serum, respectively). Transfected cells were treated with 20 µM JIP\textsubscript{m35} or control HBSS for 3 h. Bar, 20 µm. Similar results were obtained in two independent experiments. D, Input peptides and IgG, which were used in the liposome co-sedimentation assay (Fig. 3B), were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Similar results were obtained in two independent experiments. E, Immunofluorescence of ZO-1 in L cells treated with PCM or control HBSS for 3 h. Bar, 10 µm. F, Quantification of the ZO-1 intensity in L cells in (E) (n = 3 images from two independent samples). Similar results were obtained in two independent experiments. G, Immunofluorescence of ZO-1 in HeLa cells treated with PCM or control HBSS for 3 h. Bar, 20 µm. H, Quantification of the ZO-1 intensity in HeLa cells in (G) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. I, Phase separation assay of ZO1-GFP at 2.6 µM and 6.5 µM with and without 5 µM JIP\textsubscript{m35}. The amount of condensed ZO1-GFP was not significantly altered by JIP\textsubscript{m35}. Bar, 10 µm. Similar results were obtained in two independent experiments. J, Recruitment of ZO1-GFP by lipid-coated beads with and without 5 µM JIP\textsubscript{m35}. Membranes containing 10 mol% PI(4,5)P\textsubscript{2} were used as a positive control. JIP\textsubscript{m35} coated membrane did not recruit ZO1-GFP indicating no direct interaction. Bar, 5 µm. Similar results were obtained in two independent experiments. two-tailed \( t \)-test (F, H). *\( p < 0.05 \), **\( p < 0.001 \).
Fig. S9 Effects of activating toxins and inhibitors of G alpha protein on TJ assembly

**A,** List of activating toxins and inhibitors of G alpha proteins. **B,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with Cholera toxin (5 µg/ml) and Pasteurella multocida (5 µg/ml) or control HBSS for 3 h. Bar, 20 µm. **C,** Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 shown in (B) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. CT; Cholera toxin, Pm; Pasteurella multocida. **D,** Immunofluorescence of claudin-1 and α-catenin in A431 cells treated with JIP₃₅ (20 µM) or control HBSS for 3 h in the presence or absence of NF449 (10 µM), Pertussis toxin (5 µg/ml), or YM-254890 (1 µM). Bar, 20 µm. **E,** Quantification of relative claudin-1 intensity at cell–cell boundaries of A431 cells shown in (D) (n = 5 images from two independent samples). Similar results were obtained in two independent experiments. Dunnett’s-test (C, E). n.s., not significant. ***p < 0.001.
Fig. S10 G12/13 mediates JIPs-induced TJ assembly

A, Western blot analysis of A431 cells introduced with G12 and/or G13 siRNA by anti-G12, anti-G13, and anti-GAPDH antibodies. Asterisk indicates a non-specific band. Similar results were obtained in two independent experiments. B, Immunofluorescence of claudin-1, ZO-1, and F-actin in A431 cells transfected with G12 and/or G13 siRNA. Bar, 20 µm. Similar results were obtained in two independent experiments. C, Western blot analysis of EpH4 cells transfected with G12 and/or G13 siRNAs with anti-G12, anti-G13, and anti-GAPDH antibodies. Similar results were obtained in two independent experiments. D, Immunofluorescence of claudin-3, ZO-1 and F-actin in EpH4 cells transfected with G12 and/or G13 siRNAs for 5 days followed by incubation with or without 70 µM JIP m35 for 3 h. Bar, 20 µm. Similar results were obtained in two independent experiments. E, Coomassie brilliant blue staining of GST-Gα protein used for the GTPase assay in Fig. 3G. Similar results were obtained in two independent experiments.
**Fig. S11 JIPs are effective on the DSS-induced colitis mice**

**A**, Low magnification, wide range H&E staining of colon sections at day 10 in Fig. 2B. Colons of 1.5 cm from the anus are shown. Bar, 100 µm. Similar results were obtained in two independent experiments. **B**, Low magnification, wide range H&E staining of colon sections at day 10 in Fig. 4C. Colons of 1.5 cm distance from the anus are shown. Bar, 100 µm. Similar results were obtained in two independent experiments. **C**, Stools of mice treated as in Fig. 4A (n = 3 independent mice). Bar, 1 cm. **D**, Immunofluorescence of Gr-1 in colon sections from mice treated as in Fig. 4A. Bar, 20 µm.