EMBO Molecular Medicine

Gut microbiota-CRAMP axis shapes intestinal barrier function and immune responses in dietary gluten-induced enteropathy

Zhengnan Ren, Li-Long Pan, Yiwen Huang, Yu Liu, Hongbing Chen, He Liu, Xing Tu, Yanyan Liu, Binbin Li, Xiaoliang Dong, Xiaohua Pan, Hanfei Li, Yu Fu, Birgitta Agerberth, Julien DIANA, and Jia Sun
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

16th Mar 2021

Dear Prof. Sun,

Thank you for the submission of your manuscript to EMBO Molecular Medicine, and please accept my apologies for the delay in getting back to you. We have received feedback from two of the three reviewers who agreed to evaluate your manuscript. Should referee #3 provide a report, we will send it to you, with the understanding that we will not ask for an additional revision. As you will see from the reports below, both referees find the study interesting and important. However, they also raise important criticism that I would like you to address in a major revision of the current manuscript.

Addressing the reviewers' concerns in full will be necessary for further considering the manuscript in our journal. Please note that EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. However, we realize that the current situation is exceptional on the account of the COVID-19/SARS-CoV-2 pandemic. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This manuscript by Ren and colleagues investigates the function of gut microbiota-CRAMP-mediated modulation of intestinal barrier function and immune responses during gluten-induced enteropathy. The authors find that mice with GIE have altered microbiota compositions, which contributes to the CRAMP degradation in intestinal epithelium. Most intriguingly, they report that exogenous CRAMP treatment markedly ameliorate damage of intestinal structure and immune responses. Overall, these findings are interesting but the following issues need to be addressed.

1. In Fig.1E, the expression of CRAMP was co-localized with DAPI instead of E-cadherin, whether CRAMP could be translocated into nucleus?
2. In Fig.3, despite the production of duodenal CRAMP in CRAMP/gluten group is more than that in CRAMP (prophy)/gluten group, no significant different occurs in their therapeutic effect. So the timing of CRAMP administration is more important for GIE treatment?
3. Could CRAMP treatment lead to the alteration of gut microbiota and how dose gut microbiota regulate the CRAMP production in intestinal epithelium? Metabolites or other mediators? Whether endogenic CRAMP from intestinal epithelium is required for inhibiting Pseudomonas aeruginosa, which protects intestinal epithelium and furthermore contributed to intestinal CRAMP production.
4. As shown in all flow cytometry data, the percentage of immune cells was presented, the authors should also provide the absolute number of these cells. Moreover, what are mechanisms by which CRAMP administration modulate macrophages, Tregs and DCs.
5. Can CRAMP be used in clinical practice? Please comment

Referee #2 (Remarks for Author):

The authors showed an interesting and complete study on the role of cathelicidin-related antimicrobial peptide (CRAMP) in gluten-induced enteropathy (GIE). The study demonstrated that CRAMP production was defective in GIE and CRAMP administration ameliorated GIE. The authors further provided evidence that GIE-associated gut dysbiosis contributed to defective intestinal CRAMP production and GIE development. Thus, gut microbiota-CRAMP axis represents a potential therapeutic strategy for human GIE (celiac disease). The experimental designs are logical and well-described. In general, the data are convincing and support their conclusion. However, a few concerns need to be addressed.

1. In Figure 1, epithelial CRAMP production was defective in mice with GIE (sensitized and maintained on a gluten diet), compared with control mice (sensitized but maintained on a gluten-free diet). What is the level of CRAMP in non-sensitized mice on a gluten vs gluten-free diet? This could clarify if the defect was due to T cell-mediated sensitization (or not).
2. The authors demonstrated that CRAMP inhibited the expression of IL-15 using ex vivo isolated epithelial cells from mouse duodenum. This is an important observation and could this be repeated in human epithelial cells?
3. In Figure 4, LL-37 reduced EGFR phosphorylation and MyD88 expression via MMP activity. The authors further discussed that MyD88 is known to be associated with zonulin. It will be more convincing if the authors can provide direct evidence that LL-37 regulates zonulin in human epithelial cells.
4. Was the exogenous CRAMP peptide pro-form or mature form? The authors should add more precise information on the CRAMP peptide used in this study.
5. Please specify whether the intestinal microbiota in Figure 6 and S5 was extracted from duodenum or feces.
6. The microbiota sequencing study showed mice with GIE had increased Pseudomonas aeruginosa and reduced Akkamansia muciniphila. P. aeruginosa was shown to modulate CRAMP. Do A. muciniphila play any role in regulating CRAMP expression, GIE, or modulation of P. aeruginosa? This should be discussed.

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Thank you very much for taking time to review this article and for your valuable comments.

1. In Fig.1E, the expression of CRAMP was co-localized with DAPI instead of E-cadherin, whether CRAMP could be translocated into nucleus?

Ans: Thank you. We and other research groups have reported that although CRAMP can form complexes with released DNA in diseases such as psoriasis and systemic lupus erythematosus (PMID: 17873860; 21389263), it is found to be present in the cytoplasmic compartment in many other conditions (PMID: 22927244; 28069814; 26253786; 31976546; 31420464). As rightly pointed out by you, localization of CRAMP was not well represented by earlier graphs due to the use of slice scanner. Time taken for drying of slides as required by the slice scanner may have caused fluorescence quenching and artifacts. We have repeated the immunofluorescent experiments on CRAMP localization using an ultra-high-resolution confocal microscope (LSM880, Carl Zeiss, Germany) to timely capture the staining. As shown in the revised Figure 1E, CRAMP was co-localized primarily with E-cadherin. The related information has also been updated in the revised expanded view files.

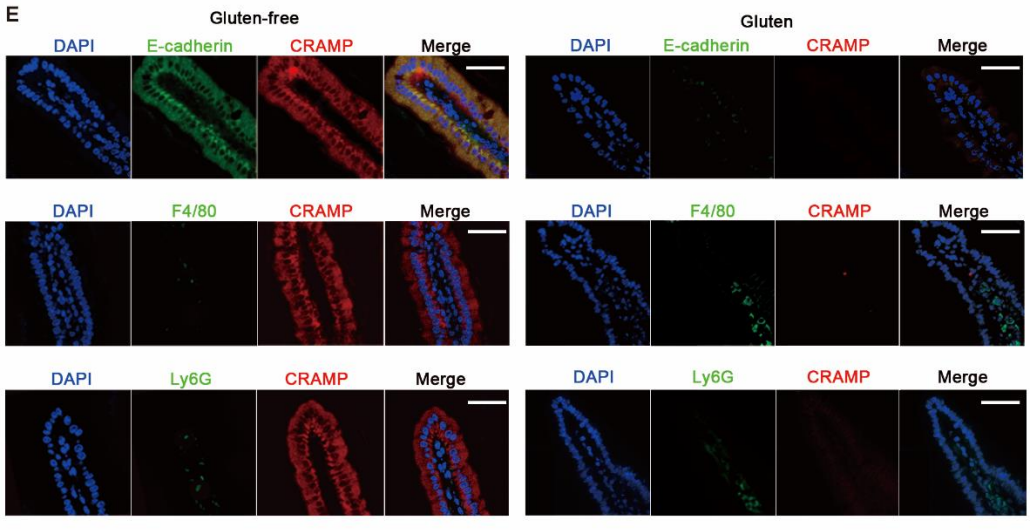

Figure 1E. Localization and expression of CRAMP (red), E-cadherin (green), F4/80 (green) and Ly6G (green) in duodenum by immunofluorescent staining. Representative photomicrographs of individual and merged staining were shown. Nuclei were stained with DAPI (blue). Scale bar: 50 μm.

2. In Fig.3, despite the production of duodenal CRAMP in CRAMP/gluten group is more than that in CRAMP (prophy)/gluten group, no significant different occurs in their therapeutic effect. So, the timing of CRAMP administration is more important for GIE treatment?

Ans: Thank you. In our study design, CRAMP/gluten group and CRAMP (prophy)/gluten group received 7 injections of CRAMP (0-6 weeks, once a week) after adoptive T cells transfer and 2 injections of CRAMP (-2 and -1 weeks, once a week) before adoptive T cells transfer, respectively (Figure 3A). Not surprisingly, CRAMP as determined at the end of treatment (6 weeks after adaptive gliadin-specific T cells transfer) was higher in CRAMP/gluten group than in CRAMP (prophy)/gluten group (Figure 3B). Comparing these two groups, we observed more pronounced effect of CRAMP given as in CRAMP/gluten group in the upregulation of duodenal tight junction proteins occludin and ZO-2 than in CRAMP (prophy)/gluten group (Figure 3E). For other markers, comparable effects were observed between the two treatment groups. We agree with you that the timing of CRAMP administration is important for GIE treatment.

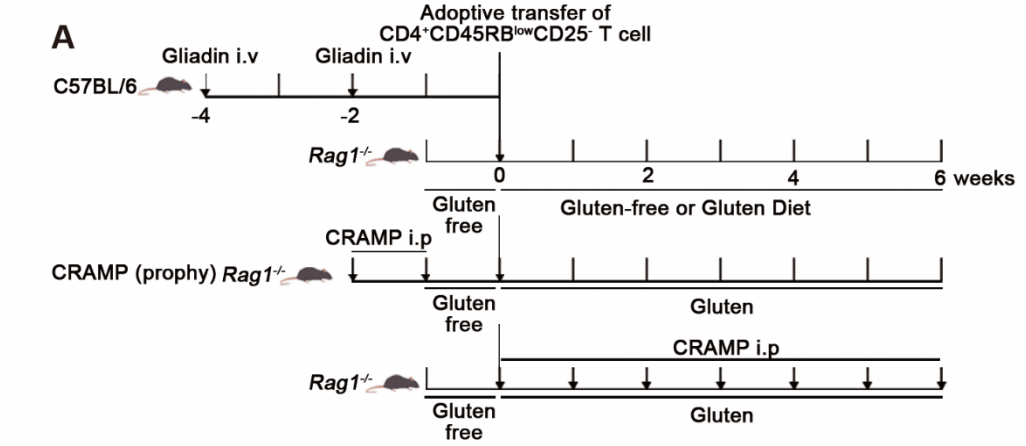

Figure 3A. Animal protocol

3. Could CRAMP treatment lead to the alteration of gut microbiota and how dose gut microbiota regulates the CRAMP production in intestinal epithelium? Metabolites or other mediators? Whether endogenic CRAMP from intestinal epithelium is required for inhibiting Pseudomonas aeruginosa, which protects intestinal epithelium and furthermore contributed to intestinal CRAMP production.

Ans: CRAMP, an endogenous antimicrobial peptide, has been shown to have an important role in the maintenance of gut microbiota homeostasis (PMID: 29440355; 33292444; 15814717; 31679249). CRAMP treatment could lead to the alteration of gut microbiota. For example, CRAMP administration attenuated enterohemorrhagic *Escherichia coli*-induced microbiota disruption (PMID: 28062699) and inhibited colitis-associated microbiota increase (PMID: 22507188).

How microbiota regulates intestinal CRAMP production is still not clearly demonstrated. However, indirect *in vitro* evidence has shown that gut pathogens may produce proteases to degrade and inactivate cathelicidin, such as *Pseudomonas aeruginosa*-stimulated LasB, *Proteus mirabilis*-stimulated ZapA and *Streptococcus pyogenes*-stimulated SpeB

(PMID: 19756242; 12366839). Here we demonstrated that feeding mice with two *P. aeruginosa* strains caused reduced duodenal production of CRAMP (Figure 6I) and the degradation of CRAMP in GIE could be attributable to the production of the protease LasB by *P. aeruginosa* (Figure 6E).

An important role of endogenous CRAMP against *P. aeruginosa* has been demonstrated in other disease contexts, such as lung infection and keratitis (PMID: 22634613; 17898271). Our ongoing unpublished data have suggested that CRAMP deficient mice had increased *P. aeruginosa*, and thus a role for endogenous CRAMP in inhibiting *P. aeruginosa*. Although CRAMP and *P. aeruginosa* are likely mutually regulating, we would prefer to keep the focus of this manuscript on the role of gut microbiota-CRAMP axis on GIE. Effect of CRAMP deficiency or exogenous CRAMP treatment on *P. aeruginosa* during GIE will be thoroughly reported as a follow-up study.

4. As shown in all flow cytometry data, the percentage of immune cells was presented, the authors should also provide the absolute number of these cells. Moreover, what are mechanisms by which CRAMP administration modulate macrophages, Tregs and DCs.

Ans: Thank you. We used the dot plot to present flow cytometry data. The total cell number in one FACS figure was constantly 10,000 (10^4), and the absolute cell number was percentage x 10^4.

Our data (Figure 5C and 5D) suggested that CRAMP promoted the modulatory phenotypes of aldehyde dehydrogenase $(ALDH)^+$ macrophages and DCs. ALDH is a key enzyme for retinoic acid production, which has an important role in inducing Treg. Also, our data suggested that CRAMP promoted conversion of cDCs to the modulatory phenotype ($CD103^+$ DCs) (Figure 5C), which is important for retinoic acid-dependent Treg generation (PMID: 17620362; 20068222). Earlier studies have suggested additional mechanisms of cathelicidin by regulating the TLR4 ligand LPS activity (PMID: 21441450) or by regulating the translocation of NF-κB subunit (PMID: 16456005) to modulate the recruitment and phenotypes of macrophages.

5. Can CRAMP be used in clinical practice? Please comment

Ans: As far as we know, the effect and efficacy of the human ortholog of CRAMP, LL-37 have been evaluated or are under evaluation in clinical trials. The beneficial effect of LL-37 in promoting wound healing has been confirmed in venous leg ulcers (Akademiska Hospital, Sweden; PMID: 25041740). A phase II clinical trial on the therapeutic effect of LL-37 against melanoma was completed in December, 2020 (University of Texas MD Anderson Cancer Center, USA; https://clinicaltrials.gov/ct2/show/NCT02225366?term=LL-37&draw=2&rank=2). The effect of LL-37 on bacteria colonization, inflammation response and healing rate of diabetic foot ulcers is currently under evaluation in a phase II clinical trial in Fakultas Kedokteran Universitas (Indonesia; https://clinicaltrials.gov/ct2/show/NCT04098562?term=LL-37&draw=2&rank=4).

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Thank you very much for taking time to review this article and for your valuable comments.

1. In Figure 1, epithelial CRAMP production was defective in mice with GIE (sensitized and maintained on a gluten diet), compared with control mice (sensitized but maintained on a gluten-free diet). What is the level of CRAMP in non-sensitized mice on a gluten vs gluten-free diet? This could clarify if the defect was due to T cell-mediated sensitization (or not).

Ans: Thank you for this suggestion. We have performed additional experiments to detect and compare CRAMP levels in unsensitized *Rag1*$^{-/-}$/gluten-free and unsensitized *Rag1*$^{-/-}$/gluten mice (Figure EV1A). There was no significant difference between the two groups, suggesting that the defect in CRAMP production was dependent on sensitization by gliadin-specific T cells.

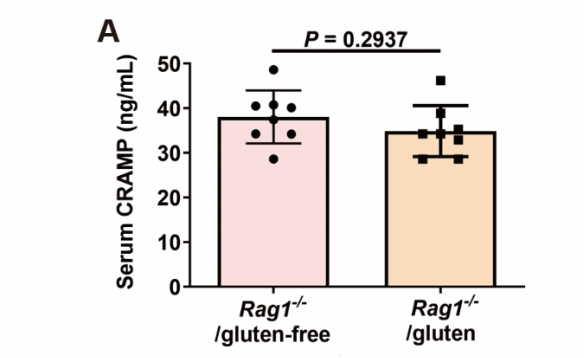

Figure EV1A. Serum CRAMP determination by ELISA. *Rag1*$^{-/-}$/gluten-free: unsensitized *Rag1*$^{-/-}$mice fed with gluten-free diet. *Rag1*$^{-/-}$/gluten: unsensitized *Rag1*$^{-/-}$ mice fed with gluten-containing diet. Data were representative and were the mean ± SD of three independent experiments with eight mice per group in each experiment. *P* values were calculated using two-tailed *t*-test.

2. The authors demonstrated that CRAMP inhibited the expression of IL-15 using ex vivo isolated epithelial cells from mouse duodenum. This is an important observation and could this be repeated in human epithelial cells?

Ans: Thank you. We have performed the experiment to analyze IL-15 expression in human epithelial cells and the new data have been added in new Figure EV3B. It was observed that LL-37 (the human ortholog of CRAMP) inhibited *IL15* expression in human

epithelial cells.

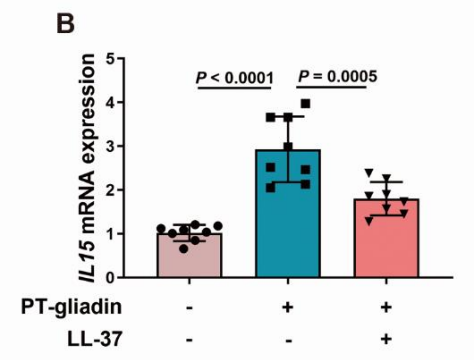

Figure EV3B. The mRNA levels of *IL15 in vitro* were measured by RT-qPCR. Data were representative and were the mean ± SD of three independent experiments. *P* values were calculated using ANOVA test with correction for multiple comparisons.

3. In Figure 4, LL-37 reduced EGFR phosphorylation and MyD88 expression via MMP activity. The authors further discussed that MyD88 is known to be associated with zonulin. It will be more convincing if the authors can provide direct evidence that LL-37 regulates zonulin in human epithelial cells.

Ans: Thank you for this suggestion. We have performed additional experiments on the expression of zonulin by Western blot. As shown in the new Figure 4B, zonulin was downregulated by LL-37 in human epithelial cells.

4. Was the exogenous CRAMP peptide pro-form or mature form? The authors should add more precise information on the CRAMP peptide used in this study.

Ans: We used the mature form of CRAMP for exogenous treatment. We have added this description in ‘Materials & Methods’ in the ‘revised manuscript’.

5. Please specify whether the intestinal microbiota in Figure 6 and S5 was extracted from duodenum or feces.

Ans: The intestinal microbiota was extracted from duodenal content. We have specified this information in ‘expanded view materials&methods’ in ‘revised expanded view files’.

6. The microbiota sequencing study showed mice with GIE had increased Pseudomonas aeruginosa and reduced Akkamansia muciniphila. P. aeruginosa was shown to modulate CRAMP. Do A. muciniphila play any role in regulating CRAMP expression, GIE, or modulation of P. aeruginosa? This should be discussed.

Ans: Thank you for this comment. Indeed, there have been many studies on the protective effect of *A. muciniphila* on the intestinal barrier function (PMID: 23671105; 29472701; 31632373). However, the effect of *A. muciniphila* on CRAMP, GIE and *P. aeruginosa* is still unknown. In fact, ongoing work of our group suggested that the protective mechanisms of live *A. muciniphila* and pasteurized *A. muciniphila* on GIE were different. To keep a focused goal of this work, we will report the data as a follow-up study.

12th May 2021

Dear Prof. Sun,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Tables: Please rename Expanded View Files to "Appendix" with a table of content and rename tables to "Appendix Table S1 and S2". Also, correct their callouts in the text accordingly.
2) In the main manuscript file, please do the following:
- Correct/answer the track changes suggested by our data editors by working from the attached/uploaded document.
- Remove font colour.
- Specify author contributions for He Liu and Hanfei Li i.e., HeLi. and HaLi.
- Data availability section should include information only about datasets deposited in public repositories. Please use the following format to report the accession number of your data:

The datasets produced in this study are available in the following databases:
[data type]: [full name of the resource] [accession number/identifier] ([doi or URL or identifiers.org/DATABASE:ACCESSION])

Please check "Author Guidelines" for more information.
https://www.embopress.org/page/journal/17574684/authorguide#availabilityofpublishedmaterial
- Please merge "Funding" section with "Acknowledgements".
3) Source data: Please upload one file per figure.
4) Synopsis: Please check your synopsis text and image, revise them if necessary and submit their final versions with your revised manuscript. Please be aware that in the proof stage minor corrections only are allowed (e.g., typos).
- Synopsis image: Please consider revising the background colours (no colours is also an option) used in the graphical abstract and resize the image to 550 px-wide x (250-400)-px high.
5) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...
6) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.
7) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

In this revised the manuscript,the authors addressed all my concerns.

Referee #2 (Remarks for Author):

The authors have addressed all my concerns and the quality of the revised manuscript has been greatly improved. I think this manuscript fits well within EMBO Molecular Medicine as the authors focus primarily on studies that provide functional novel insights of translational significance in an appropriate in vivo and in vitro model, which are also conceptually novel and of broad interest. Therefore, I think this manuscript could be considered for publication as a regular paper in EMBO Molecular Medicine.

The authors performed the requested editorial changes.

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jia Sun, Julien Diana
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2021-14059

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size estimation was not conducted in our study. Instead, we included in all our experimental groups subjected to statistical analysis at n > =6 , which met the requirement of the minimum 'n' for statistical analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least six independent mice per group from three independent experiements. All experiments were independently repeated as indicated in the Figure Legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In our study, potential outliers were tested using with Grubbs's test, and no subjects qualified for removal. No data were excluded from the analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All amimals were randomly allocated into experimental and control groups.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were allocated into experimental groups randomly.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The group allocation and analyses of H&E and immunofluorescence staining were made using blinded analysis.The samples were blinded by randomly numbering to hide group information. The qualification anaylses of staining images were carried out using ImageJ software.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal studies were designed to generate groups of equal size, using blinded analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Difference among two groups was determined using two-tailed t-test. Difference among three or more groups was determined analysis of variance (ANOVA) with Tukey's multiple comparison.
Is there an estimate of variation within each group of data?	Mean ± standard deviation or median ± interquartile range were calculated for each group as a measure of variation and have been described in the Figure Legends.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
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http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Western Blot: CRAMP, Innovagen AB (Lund, Sweden), PA-CRPL-100, Rabbit,pAb, 1:1,000; claudin-1, Abcam (Cambridge, UK), ab180158, Rabbit,pAb, 1:5,000; occludin, Abcam, ab216327, Rabbit,pAb, 1:1,000; zonulin, Abcam, ab131236, Rabbit,pAb, 1:10,000; TRAF6, Abcam, ab40675, Rabbit,pAb, 1:5,000; p65, Abcam, ab32536, Rabbit,pAb, 1:50,000; p-Akt, Cell Signaling Technology (MA, USA), 4060S, Rabbit,pAb, 1:1,000; Akt, Cell Signaling Technology, 4685S, Rabbit,pAb, 1:1,000; MyD88, Cell Signaling Technology, 4283S, Rabbit,pAb, 1:1,000; p-p65, Cell Signaling Technology,3033S, Rabbit,pAb, 1:1,000; ZO-1, ThermoFisher Scientific (MA, USA), 40-2200, Rabbit,pAb, 1:500; ZO-2, ThermoFisher Scientific,71-1400, Rabbit,pAb, 1:200; β-actin, Abclonal Technology (Wuhan, China), AC026, Rabbit,pAb, 1:200,000. Flow Cytometry: Alexa Fluor® 700 anti-mouse CD45, BioLegend (CA, USA), 103128; Brilliant Violet® 711 anti-mouse CD11c, BioLegend, 117349; Brilliant Violet® 605 anti-mouse CD11b, BioLegend, 101257; APC anti-mouse CD103, BioLegend, 121413; Brilliant Violet® 421 anti-mouse CX3CR1, BioLegend, 149023; Brilliant Violet® 421 anti-mouse CD3e, BioLegend, 100341; PE anti-mouse CD314 (NKG2D), BioLegend, 130207; APC anti-T-bet, BioLegend, 644813; Alexa Fluor® 488 anti-mouse FOXP3, BioLegend, 126405; PE anti-mouse ROR GAMMA (T) (B2D), eBioscience (MA, USA), 12-6981-80; PE-Cy™7 anti-mouse CD4, BD biosciences (MD, USA), 552775. Immunofluorescence: CRAMP,Proteintech (Hubei, China), 12009-1-AP, Rabbit,pAb, 1:50; E-cadherin, BD Biosciences (MD, USA), 610181, mouse,pAb, 1:100; F4/80, Abcam, ab6640, Rat,mAb, 1:100; Ly6G, Abcam, ab25377, Rat,mAb, 1:100; Anti-rabbit AlexaFluor® 555, ThermoFisher Scientific, A27039; anti-mouse AlexaFluor® 488, ThermoFisher Scientific, A11001; anti-rat AlexaFluor® 488,ThermoFisher Scientific, A21208.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Caco-2 (human colonic epithelial cells) [ATCC®HTB-37™].Authentication was guaranteed by the vendor (ATCC).All cell lines were regularly tested negative for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57BL/6J (male, 6 weeks, GemPharmatech Co., Ltd, Jiangsu, China); Rag1-/- (C57BL/6J background, male, 8 weeks, GemPharmatech Co., Ltd, Jiangsu, China); CRAMP deficient Cnlp-/- mice (C57BL/6J background, male, 8 weeks, The Jackson Laboratory, CA, USA); Cnlp-/-Rag1-/- mice (C57BL/6J background, male, 8 weeks, generated by backcrossing of Cnlp-/- mice with Rag1-/- mice for more than 10 generations.). All mice were maintained at the Animal Housing Unit of Jiangnan University (Jiangsu, China) under a controlled temperature (23 -25°C) and a 12 h light-12 h dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experimental protocols were approved by the Animal Ethics Committee of Jiangnan University [JN. No20181230c0500815(303) and JN. No20170614-20190225 (77)] and were performed in accordance with the guidelines.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Animal studies are reported in compliance with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This study was approved by the ethics committee of the Second Affiliated Hospital of Nanchang University [no. (2010) 041].
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All subjects provided informed consent before participation and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Mouse gut microbiota sequencing and assembly: Sequence Read Archive PRJNA686187 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA686187). The main data supporting the findings of this study are available within the article and its Expanded View Figures. Extra data are available from the corresponding author upon request.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	The source data have been uploaded.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A