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Article

Antimicrobial peptide LL-37 forms complex with bacterial DNA to facilitate blood translocation of bacterial DNA and aggravate ulcerative colitis

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ABSTRACT

Bacterial DNA (bacDNA) is frequently found in serum of patient with ulcerative colitis (UC) and Crohn's disease, even blood bacterial culture is negative. How bacDNA evades immune elimination and is translocated into blood remain unclear. Here, we showed that bacDNA avoids elimination and disables bacteria-killing function of antimicrobial peptide LL-37 (Cramp in mice) by forming complex with LL-37, which is inducible after culture with bacteria or bacterial products. Elevated LL-37-bacDNA complex was found in plasma and lesions of patients with UC. LL-37-bacDNA promoted inflammation by inducing Th1, Th2 and Th17 differentiation and activating toll-like receptor-9 (TLR9). The complex also increased paracellular permeability, which possibly combines its inflammatory effects to promote local damage and bacDNA translocation into blood. Cramp-bacDNA aggravated mouse colitis severity while interference with the complex ameliorated the disease. The study identifies that inflammatogenic bacDNA utilizes LL-37 as a vehicle for blood translocation and to evade immune elimination. Additionally, bacteria may make a milieu by releasing bacDNA to utilize and resist host antimicrobial peptides as a 'trojan horse'.

1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a major public health problem. Especially, UC is a complex chronic inflammatory disorder with high direct economic burden and bad effects on quality of life [1]. However, the pathogenesis of IBD is not fully understood. In the intestinal milieu with abundant presence of microbes, and this enteric bacteria not only play a key role in intestinal homeostasis and function, but also in the onset and perpetuation of chronic intestinal inflammation [2]. On the one hand, innate tolerance to commensal microorganisms must be maintained to benefit from the colonization. On the other hand, local interactions between hosts and pathogenic organisms determine the outcome of an infection. The studies that animal models fail to develop disease under germ-free conditions [3,4], along with the fact that the distal ileum and colon, which have the highest bacterial concentrations are more susceptible to infection [5] further certify the resident enteric bacteria play a key role in the development of IBD. The host has evolved multifarious defensive or homeostatic mechanisms towards microbial infections or harmless colonization.

One of these mechanisms for host intestinal innate defense system is equipped with an array of antimicrobial peptides in order to maintain an initial barrier against the enteric bacteria. Defensins and cathelicidins are two major families of antimicrobial peptides for human [6]. These gene families differ significantly in expression in IBD. The human cathelicidin (known as LL-37, Cramp in mice), for example, is found increased in inflamed and non-inflamed mucosa in patients with UC but not in patients with CD [7]. In addition, bacterial infections at epithelial surfaces are associated with increased expression of LL-37 [8]. LL-37 has a broad range of bactericidal activities against gram-positive and gram-negative

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bacteria at substantially lower peptide concentrations [9,10]. LL-37-mediated lysis of bacteria results in release of bacterial DNA (bacDNA) with potential proinflammatory effects in the host. Indeed, it has been reported that bacDNA but not bacterial lipopolysaccharide (LPS) was detected in 51.7% of patients with UC and 42.4% of patients with CD, while no bacDNA is found in serum of healthy controls [5]. BacDNA contains multiple unmethylated CpG DNA repeats, which display strong inflammatogenic properties [11]. However, as an inflammatory stimulator, bacDNA might be degraded by DNase or engulfed by macrophages in the blood or colon tissues of UC, suggesting that there are factors could protect bacDNA from clearance.

Compelling evidences have been provided that LL-37 forms complexes with the released DNA or RNA from the dying cells in psoriasis, and such complexes are highly protected from DNase or RNase degradation [12,13]. Our previous study has indicated that the mitochondrial DNA (mtDNA) could also be protected by LL-37, and the formed LL-37-mtDNA complex plays key roles in the activation of leukocytes and promoting atherosclerosis [14]. Considering that the increased expression of LL-37 in patients with UC and the highly correlation of bacDNA with UC [15–17], we suppose that bacDNA forms complex with LL-37 to avoid immune clearance and aggravate UC. We now found that bacDNA evades immune elimination by forming complex with LL-37 and drives chronic inflammatory activation in UC.

2. Materials and methods

2.1. Ethics

This study was approved by the Institutional Review Board and the Animal Care and Use Committee of Kunming Institute of Zoology (SMKX-2016023) and the First Affiliated Hospital of Kunming Medical University (YAH2016-0006). Human specimens (Table S1 online) were collected with the informed consent of the patients prior to the study.

2.2. Antibodies

Monoclonal anti-LL-37 antibody (Santa Cruz, sc-21578), monoclonal antibody against LC3 (Cell Signaling Technology, 3868S), monoclonal anti-TLR9 antibody (Santa Cruz, sc-47723), and secondary antibodies including Cy3-labeled anti-rabbit IgG (H + L) antibody (KPL, 072-01-15-06), Cy3-labeled anti-mouse IgG (H + L) antibody (KPL, 072-01-18-06), and goat anti-rabbit IgG H&L antibody (Alexa Fluor 488) (abcam, ab150077) were purchased from corresponding manufacturer. Antibodies against LL-37-bacDNA and Cramp-bacDNA were prepared as described below.

2.3. Peptides synthesis

Peptides of LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPR TES), KR-37 (KRKSFDGFKELILKGRQEIKFKRDIFLVNLRETRPSV, LL-37's scrambled control peptide), Cramp (GLLRKGGEKIGEKLKKIG QKIKNFFQKLVPQPE), and GK-33 (GKLGIKERLGPGEEQKKLPVNFKFQ KIKQIGLK Cramp's scrambled control peptide) were synthesized by GL Biochem (Shanghai, China). Their purity was confirmed to be greater than 98% by reversed phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry analysis.

2.4. Antimicrobial tests

Escherichia coli (*E. coli*) (ATCC 25922), *Bacillus subtilis* and *Staphylococcus aureus* (ATCC 2592) were obtained from Kunming Medical University. Bacteria culture and antimicrobial tests were performed according to our previous report [18].

2.5. Preparation of bacteria DNA (bacDNA)

Bactria DNA isolation kit (TIANamp Bacteria DNA Kit, DP302) was used to isolate bacDNA from *E. coli*. DNA concentration and purity were determined by using a spectrophotometer and electrophoresis (1% agarose gel in Tris–acetate ethylenediaminetetraacetic acid (EDTA) (TAE) buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA, pH 8.5). Some of bacDNA was labeled with Alexa Fluor 647 using the ULYSIS Nucleic Acid Labeling Kits (Molecular Probes) according to the protocol provided by manufacturer.

2.6. Preparation of peptide-bacDNA complex

For generation of peptide-bacDNA complex, 20 μg of peptide (LL-37, KR-37, Cramp or GK-33) was mixed with 4 μg bacDNA in 20 μL of PBS buffer (1.5 mmol/L KH_2PO_4, 2.7 mmol/L $Na_2HPO_4 \cdot 7H_2O$, 0.15 mol/L NaCl, pH 7.4) for 15 min at room temperature according to our previously described method [14].

2.7. Nuclease protection assays

BacDNA (320 ng) or LL-37/KR-37-bacDNA complex (1920 ng) alone or after incubation with 5 μg/mL porcine DNase II (Sigma) at 37 °C in 0.1 mol/L sodium acetate buffer (pH 4.7) containing 20 mmol/L EDTA for 15 min, the products were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Residual bacDNA was also quantified by fluorimetry using PicoGreen (Invitrogen) [14].

2.8. Polyclonal antibodies production

According to the previous methods [14,19], rabbit polyclonal antibodies against LL-37-bacDNA and Cramp-bacDNA were produced. Briefly, rabbits (2 kg, male) were primed by subcutaneous injections with 600 μg peptide-bacDNA complex mixed with 1 mL complete Freund's adjuvant (Sigma–Aldrich Inc.) on day 0, followed by subcutaneous booster injections of the half dose of the antigen mixed with 500 μL incomplete Freund's adjuvant (Sigma–Aldrich Inc.) on day 14, 28, and 42, respectively. The titer and specificity of polyclonal antibody were measured by indirect enzyme-linked immunosorbent assay (ELISA) according to our previous methods [14].

2.9. BacDNA release from E. coli to form LL-37-bacDNA complex

E. coli was first cultured in Luria-Bertani (LB) broth at 37 °C to exponential-phase, after diluting to 4×10^5 colony-forming units (CFU)/mL, the bacteria were then stimulated with LL-37 at sub-minimal inhibitory concentration (0.1 μg/mL) for different time (5, 10, 20, 30, 45, 60, 120, 240, 480 or 960 min). After stimulation, the supernatant of *E. coli* was collected and the level of LL-37-bacDNA was determined by ELISA.

2.10. PBMC, pDC and PMN isolation and stimulation

Whole blood (EDTA-anticoagulated) from healthy donors was obtained from Kunming Blood Center of Yunnan Province. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences AB SE-751 84 Uppsala, Sweden) according to the protocol provided by the manufacturer. Plasmacytoid dendritic cells (pDCs) were isolated from PBMCs using the Blood Dendritic Cell Isolation Kit II, human (Miltenyi Biotec, Germany). Polymorphonuclear

granulocytes (PMNs, or neutrophils) were isolated by using polymorphprep™ (Axis-Shield) according to the separation procedure provided by the manufacture. Purified PBMCs, pDCs and PMN were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Carlsbad, CA, U.S.A.) supplemented with 5% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 5% carbon dioxide (CO_2).

For stimulation, pDCs or PMNs were seeded into 96-well plates at 1×10^5 cells per well in 200 μL RPMI 1640, the cells were then stimulated by the following samples: 0.9% salt water (control), KR-37 (10 μg/mL), LL-37 (10 μg/mL), bacDNA (2 μg/mL), KR-37-bacDNA complex (12 μg/mL) or LL-37-bacDNA complex (12 μg/mL). After 16 h incubation, supernatants of pDCs and PMN were collected. Level of interferon (IFN)-α, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, TNF-α, monocyte chemotactic protein (MCP)-1 and IFN-γ in the supernatants were measured by using the corresponding ELISA kits (RayBiotech, Inc or Dakewe, Beijing, China), respectively.

2.11. Effects of E. coli infection on Cramp-bacDNA formation

Male C57BL/6 J mice (6–8 weeks) were purchased from the Beijing HFK Bio-technology Co. Ltd (Beijing, China). Mice were intravenously (i.v.) injected with *E. coli* (5×10^7 CFU/mouse). After the challenge by *E. coli* for different time, mouse plasma was collected to detect amounts of Cramp and Cramp-bacDNA by ELISA.

2.12. The effects of Cramp-bacDNA on cytokines secretion in vivo

Male C57BL/6 J mice (6–8 weeks) were i.v. administered with Cramp-bacDNA (500 μg/kg) by caudal vein. After administration for different time (0.5, 1, 2, 4 or 6 h), the plasma was collected to measure the amounts of cytokines by ELISA or flow cytometry using the BD CBA Flex Set system according to the protocol provided by the manufacturer.

2.13. Western blot and ELISA analysis

Human colon homogenates from healthy volunteers and patients with UC were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%), and then transferred to polyvinylidene difluoride (PVDF) membranes. A mouse monoclonal anti-LL-37 antibody was used in the immunoreactivity. The concentration of LL-37-bacDNA or Cramp-bacDNA complex in human plasma or mouse plasma was determined by ELISA using anti-LL-37-bacDNA or anti-Cramp-bacDNA complex antibody.

2.14. Confocal microscopy

For immunostaining, cells or tissue slices were first fixed in 4% paraformaldehyde of PBS buffer at 4 °C for 30 min, and further permeabilized with 0.01% Triton X-100 in PBS for 15 min before being treated with 10% BSA for 1 h at 25 °C. LL-37, LL-37-bacDNA, LC3, or TLR9 was visualized by using the corresponding antibody. 4,6-Diamidino-2-phenylindole (DAPI, 10 ng/mL, Roche Diagnostics) was used to stain DNA. After washing with PBS for three times (5 min per time) to remove excess primary antibody, the slices were incubated with fluorescently-labeled secondary antibody at 37 °C for 1 h. The slices were imaged with Olympus FluoView 1000 confocal microscope (Olympus, Melville, NY, U.S.A.) according to the protocol provided by the manufacturer.

2.15. Effects of Cramp or Cramp-bacDNA on intestinal inflammation induced by dextran sulfate sodium

Intestinal inflammation of Male C57BL/6J mice (6–8 weeks) was first induced by 3% dextran sulfate sodium (DSS, 36–50 kDa; MP Biomedicals) in the drinking water ad libitum according to the previous method [20]. After treatment with DSS for 5 days, some mice were intrarectally administrated by Cramp (100 μg/mouse), GK-33 (100 μg/mouse), bacDNA (20 μg/mouse), Cramp-bacDNA (120 μg/mouse) or GK-33-bacDNA (120 μg/mouse), some mice were treated with an anti-Cramp-bacDNA antibody (50 (Ab1) or 100 (Ab2) μg/mouse) or an isotype control antibody (50 (IgG1) or 100 (IgG 2) μg/mouse) 1 time per day for 4 consecutive days. The mice were then sacrificed to collect plasma and colonic tissues for cytokines measurement or hematoxylin-eosin (H&E) staining. Disease Activity Index (DAI) including body weight loss, stool consistency and faecal blood using the haemoccult test were recorded every day. DAI was calculated according to previous reports [21,22].

2.16. Effects of LL-37-bacDNA on $CD4^+$ cell differentiation

PBMCs (1×10^7 cells/mL) were seeded into 12-well microtiter plates, and incubated with test sample (PBS control, LL-37 (10 μg/mL), bacDNA (2 μg/mL) or LL-37-bacDNA (12 μg/mL)) for 24 h in RPMI-1640 at 37 °C in a humidified atmosphere containing 5% CO_2. After another 5 h incubation with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL)/Ionomycin (1 μg/mL), the Th cell subtypes [23] were analyzed by flow cytometry using fluorescent-labeled antibodies (BD Biosciences) including HU CD4 PerCP-Cy5.5 (RPA-T4), HU IL-17A PE MAB (N49-653), HU IFN-GMA FITC MAB (B27), HU IL-4 APC MAB (MP4-25D2), HU FOXP3 ALEXA 488 MAB (259D/C7), HU CD25 PE MAB (M-A251) or APC Mouse Anti-Human CD4 (RPA-T4). Data were acquired on LSR Fortessa (BD, Biosciences) and analysed using FlowJo. In addition, the plasma concentration of IL-6, TNF-α, IFN-γ and IL-4 was analyzed by Cytometric Bead Arrays FlexSet (BD Biosciences). All the analysis was according to manufacturer's instructions.

2.17. Macrophages differentiation and stimulation

Human monocytic THP-1 cells were induced to differentiate to macrophages (by PMA (100 ng/mL) for 72 h) in RPMI-1640 (Gibco, Carlsbad, CA, U.S.A.) supplemented with 5% fetal calf serum, 100 U/mL penicillin and 100 μg/ml streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2 [24]. For imaging, macrophages were seeded into a 24-well culture plate (10^5 cells per well) containing 15 mm diameter round glass coverslips. For stimulation, the macrophages were incubated with LL-37 (2 μg/mL or 10 μg/mL), bacDNA (0.4 μg/mL or 2 μg/mL) or LL-37-bacDNA (2.4 or 12 μg/mL) for 2 h to assess formation of TLR9-positive or LC3-positive structures by confocal microscopy, or incubated with 0.9% salt water (control), bacDNA (2 μg/mL) or LL-37-bacDNA (12 μg/mL) in the presence or absence of ODN-TTAGGG (2 μmol/L) for 18 h to measure IFN-α production, or incubated with LL-37-bacDNA complex (12 μg/ml) for 2 h to determine autophagic vacuole by transmission electron microscopy (TEM). Macrophages were stimulated with bacDNA (2 μg/ml, Alexa 647-labeled) alone or complexed with LL-37 (10 μg/mL) for 4 h to determined the co-localization of DNA and LC3 by confocal microscopy.

2.18. Transmission electron microscopy

After incubation with LL-37-bacDNA (12 μg/mL) for 2 h, the macrophages were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide at 4 °C for 2 h. The fixed cells were dehydrated with increasing concentrations of ethanol from 70 to 100%, infiltrated

with Epon-Araldite resin for 1 h, embedded in pure resin, and then cured at 40 °C for 24 h, followed by 60 °C for 48 h. Ultrathin sections (70–80 nm) were obtained using an ultramicrotome (RMC MT6000-XL) and stained with uranyl acetate and lead citrate. The sections were observed under a TEM (JEOL, JEM-1011, Japan) according to the manufacturer's instruction [14,25].

2.19. Paracellular permeability assay

Paracellular permeability assay was performed according to a previous study [26]. The human colon cancer HCT-8 cells were first seeded on 24-well culture plate containing 15 mm diameter round glass coverslips, the cells were then cultured for 9–14 d in RPMI-1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco) and 2% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. Test sample (saline, LL-37 (40 μg/mL), bacDNA (8 μg/mL) or LL-37-bacDNA complex (48 μg/mL)) was then added and incubated for another 6 h. After the incubation, the cells in coverslips were fixed in 4% paraformaldehyde for 10 min and used for immunofluorescence staining to detect occludin expression. Images of immunofluorescence were acquired by Olympus FluoView 1000 confocal microscope (Olympus, Melville, NY, U.S.A.). Total proteins in the membrane fractions of HCT-8 cells were extracted using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. Occludin in the membrane fractions was analyzed by Western blot.

Paracellular permeability was also assessed by measuring transepithelial electric resistance (TER) according to a previously described method [27]. HCT-8 cells were seeded in the 24-well Transwell™ chambers (polycarbonate membrane, filter pore size: 0.4 mm; filter area: 0.33 cm^2; Costar, USA) and grown for 9–14 d in the same culture conditions. TER was measured using an epithelial voltohm meter (Millicell® ERS-2, Millipore) according to the manufacture's instruction. When TER value was up to 700 Ohm·cm^2, the test sample (saline, LL-37 (40 μg/mL), bacDNA (8 μg/mL) or LL-37-bacDNA complex (48 μg/mL)) was added and the TER value was recorded at different time point (0, 6, 12 or 24 h).

2.20. Statistical analyses

Data obtained from independent experiments were presented as mean ± SD. All statistical analyses were two-tailed, with 95% confidence intervals (CI), and conducted by using GraphPad prism 4. Results were subjected to an unpaired t test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. LL-37 or E. coli infection induces formation of LL-37/Cramp-bacDNA complex

Previous studies have proved that *E. coli* plays key roles in the pathogenesis of UC, and among the identified microorganisms by DNA sequencing from the serum of patients with IBD, *E. coli* was detected as the highest percentage [5,28]. Thus, we used bacDNA originated from *E. coli* to prepare LL-37/Cramp-bacDNA complex and anti-LL-37/Cramp-bacDNA complex rabbit polyclonal antibody (Fig. S1 online). As a human antimicrobial peptide, LL-37 contains strong microbicidal ability against bacteria. At sub-minimal inhibitory concentration (MIC) (0.1 μg/mL), LL-37 was incubated with *E. coli* to test whether LL-37 can form complex with bacDNA released from the bacterium. After treatment for 4 h by LL-37, LL-37-bacDNA complex was detected in the *E. coli* culture medium (Fig. 1a). The highest concentration of LL-37-bacDNA complex was up to 700 ng/mL after 16 h treatment with LL-37 (Fig. 1a). This result implies that LL-37-bacDNA complex can be formed after interaction between LL-37 and bacteria.

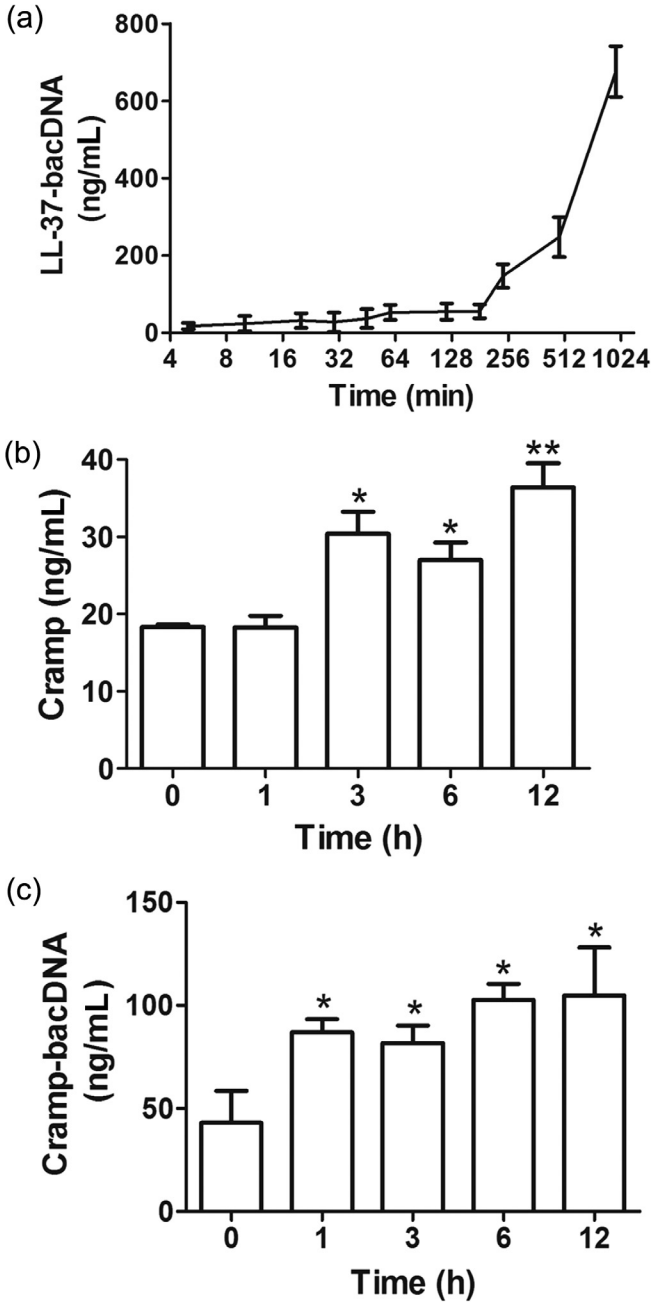

Fig. 1. LL-37 or *E. coli* infection induces formation of LL-37/Cramp-bacDNA complex. (a) The formation of LL-37-bacDNA complex in the supernatant of *E. coli* was detected by using ELISA after stimulation by LL-37 for different time. After injection with *E. coli*, levels of Cramp (b) and Cramp-bacDNA (c) in mouse plasma were determined by ELISA. Bars represent mean ± SD of at least three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Many reports have indicated that LL-37 or Cramp production can be induced by bacteria infection [29–31]. We investigated whether *E. coli* infection would promote Cramp-bacDNA complex production *in vivo*. After stimulation by intravenously injecting *E. coli* (100 μL, 5×10^7/mL) into mice tail for 1, 3, 6 and 12 h, the concentration of Cramp in mouse plasma was increased by ~1, 67, 50 and 110% (Fig. 1b), while that for Cramp-bacDNA complex, which was detected by using the anti-Cramp-bacDNA antibody (Fig. S1b online), was increased by ~93, 114, 140 and 149%

(Fig. 1c), respectively. We conclude that upon microbial infection, LL-37/Cramp was up-regulated to kill bacteria, however, the peptide-mediated lysis of bacteria thus results in bacDNA release and further promoting the formation of LL-37/Cramp-bacDNA complex *in vivo*.

3.2. Elevated levels of LL-37-bacDNA complex are found in plasma and lesions of patients with UC

To investigate whether LL-37-bacDNA complex is associated with human UC, the level and distribution of LL-37-bacDNA in plasma and lesions of patients with UC were analyzed. Western blot analysis indicated that LL-37 was highly expressed in colon of patients with UC in comparison with the healthy controls (Fig. 2a, b), which is in accordance with previous study [7]. The concentration of LL-37-bacDNA in plasma of UC patients (153.9 ± 7.547 ng/mL) was significant greater than that in healthy individuals (129.5 ± 4.313 ng/mL) ($n = 20$, $P = 0.0097$) (Fig. 2c). To determine whether bacDNA forms a complex with LL-37 in the lesion of patient's specimens, cellular localization of LL-37 and LL-37-bacDNA complex in the lesion was examined. Compared with normal tissue, UC lesions exhibited ~5 and ~15 fold increase of LL-37 (Fig. 2d, e) and LL-37-bacDNA complex (Fig. 2d, f), respectively. Collectively, these data indicated that LL-37-bacDNA is associated with human UC.

3.3. LL-37-bacDNA complex is resistant to DNase II degradation and disables LL-37's antimicrobial function

The bacDNA and LL-37-bacDNA complex in the presence or absence of DNase II were subjected to agarose gel electrophoresis. As showed in Figure S2a online, the bacDNA alone was degraded by DNase II while the complex was retained in the loading wells, indicating that LL-37-bacDNA complex is resistant to DNase II digestion. In addition, the complex of control peptide KR-37 with bacDNA was partly degraded after incubation with DNase II, suggesting that LL-37 is a specific protector of bacDNA. The protective ability of LL-37 on bacDNA was further quantified by a fluorometric assay. After 60 min treatment with DNase II, more than 70% of bacDNA alone or in complex with KR-37 was degraded, while only about 20% of bacDNA in LL-37-bacDNA complex was degraded (Fig. S2b online). In addition, it was found that LL-37's antimicrobial functions were inhibited after binding with bacDNA (Fig. S3 online), suggesting that by forming complex with LL-37, bacDNA disables LL-37's bacteria-killing effect.

3.4. LL-37-bacDNA complex escapes from autophagic recognition

As an inflammatory stimulator, free bacDNAs released from bacteria might be degraded by DNase or engulfed by macrophages. As reported above, by forming complex with LL-37, bacDNA was resistant to DNase II (Fig. S2 online). We therefore investigated whether LL-37-bacDNA complex could be eliminated by macrophages. The invading pathogens could be eliminated by autophagy, which is an evolutionarily conserved and regulated degradation system [32]. We found that the numbers of microtubule-associated protein 1 light chain 3 (LC3)-positive deposits representing autophagosomes [33], were increased in macrophages after 2 h-treatment with LL-37, bacDNA or LL-37-bacDNA complex (Fig. 3a, b). LL-37-bacDNA complex induced more LC3 aggregates than LL-37 or bacDNA (Fig. 3a, b). Moreover, electron microscopy also confirmed autophagic activation with formation of autophagosome-like vacuoles induced by LL-37-bacDNA (Fig. 3c). Immunoblot analysis indicated the same tendency (Fig. 3d, e).

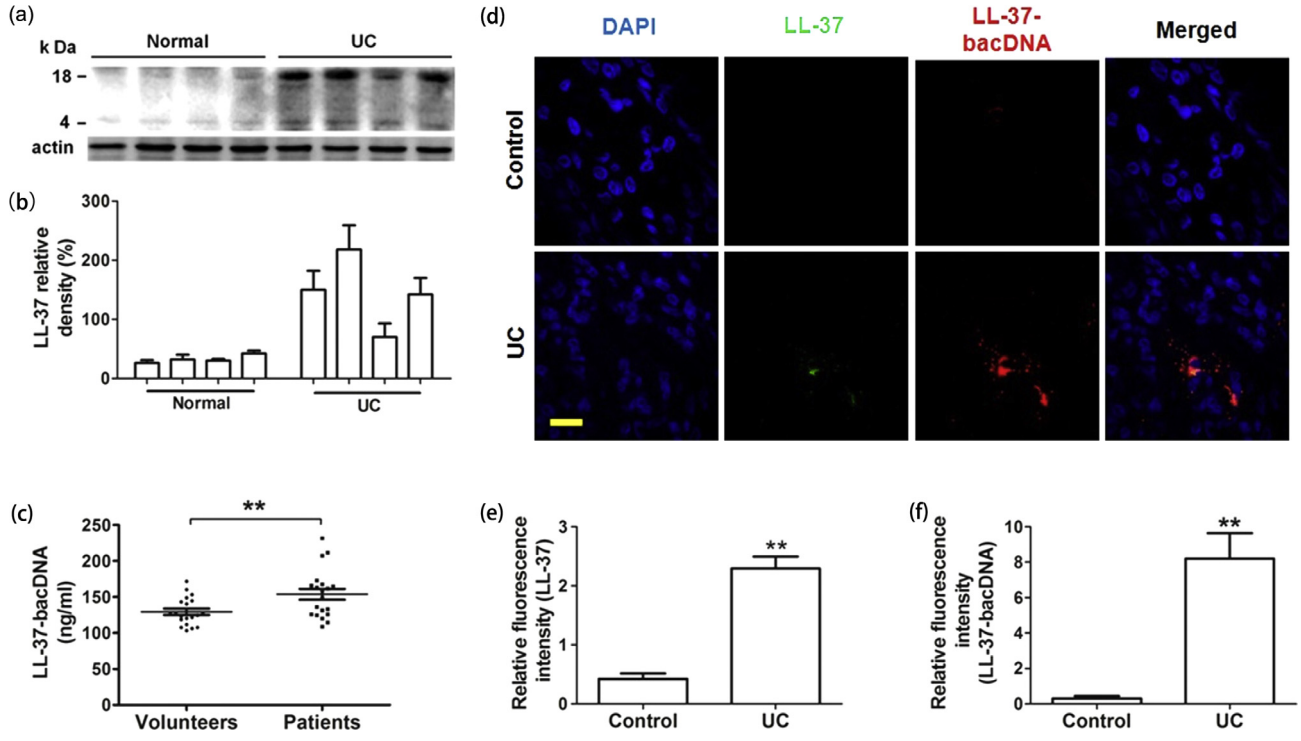

Fig. 2. Elevated LL-37-bacDNA complex is found in colon and plasma of patients with UC. (a) Western blot analysis and quantitative results (b) of protein extracts of colon from volunteers (Normal) and patients with UC (UC) using anti-LL-37 and anti-β-actin (loading control) antibody, respectively. (c) Plasma concentrations of LL-37-bacDNA in plasma from patients with UC ($n = 20$) and volunteers ($n = 20$) were determined by ELISA using anti-LL-37-bacDNA antibody. (d-f) Confocal microscopy analysis of LL-37 and LL-37-bacDNA in colon samples. Colon samples of patients with UC and healthy people were labeled with either anti-LL-37 (green) or anti-LL-37-bacDNA complex antibody (red) to detect the presence of LL-37 and LL-37-bacDNA. The cell nuclei were labeled by DAPI. Representative image (d) and quantitative results of LL-37 (e) and LL-37-bacDNA (f) were shown. Scale bar = 20 μm. Bars represent mean ± SD of at least three experiments. $^{**}P < 0.01$.

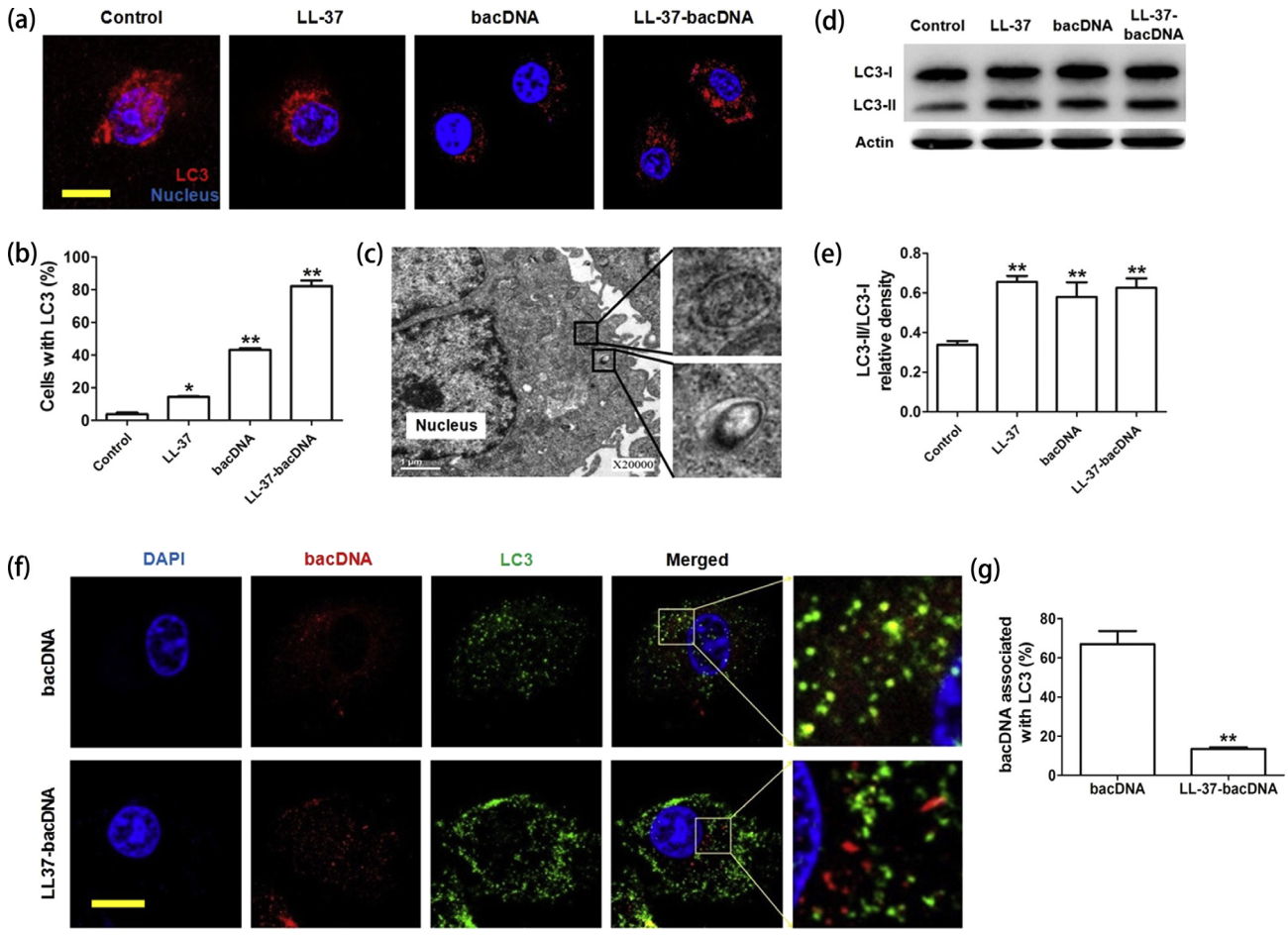

Fig. 3. LL-37-bacDNA evades autophagic recognition. (a, b) Formation of autophagosome in macrophages after induced by test sample. Macrophages were incubated with test sample for 2 h to assess formation of autophagosome using LC3 antibody (red). Cell nuclei were stained by DAPI (blue). Representative image (a) and quantitative result (b) were shown. (c) TEM images showed autophagic vacuoles in macrophages treated by LL-37-bacDNA complex for 2 h. (d, e) Immunoblot analysis showed increasing amounts of endogenous LC3-I and LC3-II in macrophages after stimulation with test samples for 2 h. β-actin was probed as the loading control. Representative image (d) and quantitative result (e) were shown. (f, g) After stimulation for 4 h with bacDNA (Alexa Fluor 647-labeled) alone or complexed with LL-37, co-localization of DNA and LC3 in macrophages were visualized by confocal microscopy. Confocal images of representative cells (f) and quantitative result (g) were shown. Scale bar in (a) and (f) represents 20 μm. Bars represent mean ± SD of at least three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Confocal fluorescence study was then performed to examine possible co-localization of Alexa Fluor 647-labeled bacDNA alone or premixed with LL-37 with LC3. As showed in Fig. 3f, g, most bacDNA-positive deposits (red) were associated with LC3 (green) after 4 h-treatment in macrophages, suggesting that most bacDNA was engulfed by autophagosome. However, after pretreatment with LL-37, there was only a little co-localization between bacDNA and LC3 (Fig. 3f, g), suggesting that the LL-37-bacDNA complex evades autophagic recognition.

3.5. LL-37/Cramp-bacDNA complex promotes the secretion of inflammatory cytokines in vitro and in vivo

LL-37-bacDNA complex has been found to escape elimination as mentioned above, which promoted us to investigate the effects of the complex on inflammation. We found that only LL-37-bacDNA complex, but neither LL-37 or bacDNA alone nor KR-37-bacDNA complex was able to induce secretion of IFN-α and TNF-α in pDCs (Fig. 4a, b). In addition, the LL-37-bacDNA complex showed much stronger ability to induce pDCs activation (Fig. S4 online).

Neutrophils and their inflammatory mediators play key roles in the pathogenesis of UC [34]. The amounts of inflammatory cytokines in neutrophils including IL-6, IL-8, TNF-α, MCP-1 and IFN-γ were also measured after treatment with LL-37, KR-37, bacDNA, KR-37-bacDNA or LL-37-bacDNA. LL-37-bacDNA significantly induced IL-6, IL-8, TNF-α and MCP-1 secretion in neutrophils, and the increased amounts of these four cytokines were much greater than those of LL-37 or bacDNA alone or KR-37-bacDNA (Fig. 4c-f). LL-37-bacDNA complex showed no obvious effect on the secretion of IFN-γ in neutrophils (Fig. S5 online).

Since both bacDNA and relevant cytokines can be detected in the serum of patients with UC [5], we suspected that the high levels of cytokines in serum may be related to LL-37-bacDNA complex-induced inflammation. Thus, we measured several inflammatory cytokines release in mouse serum after intravenous injection of Cramp-bacDNA complex. Upon the complex injection, TNF-α was up-regulated ~31 and 189 folds at 30 and 60 min post-injection, respectively (Fig. 4g). IL-6 (Fig. 4h), IFN-γ (Fig. 4j) and IL-17A (Fig. 4k) was increased by ~140, 8 and 22 folds at 2 h post-injection, respectively. IL-1β reached ~120 folds increase after 4 h administration (Fig. 4i). No significant effects of Cramp-bacDNA on the secretion of another two cytokines including IL-4 and IL-10 (Fig. S6 online) were found. Together, these data indicated that LL-37/Cramp-bacDNA complex has a marked ability to activate pDC and neutrophils and promotes the secretion of inflammatory cytokines.

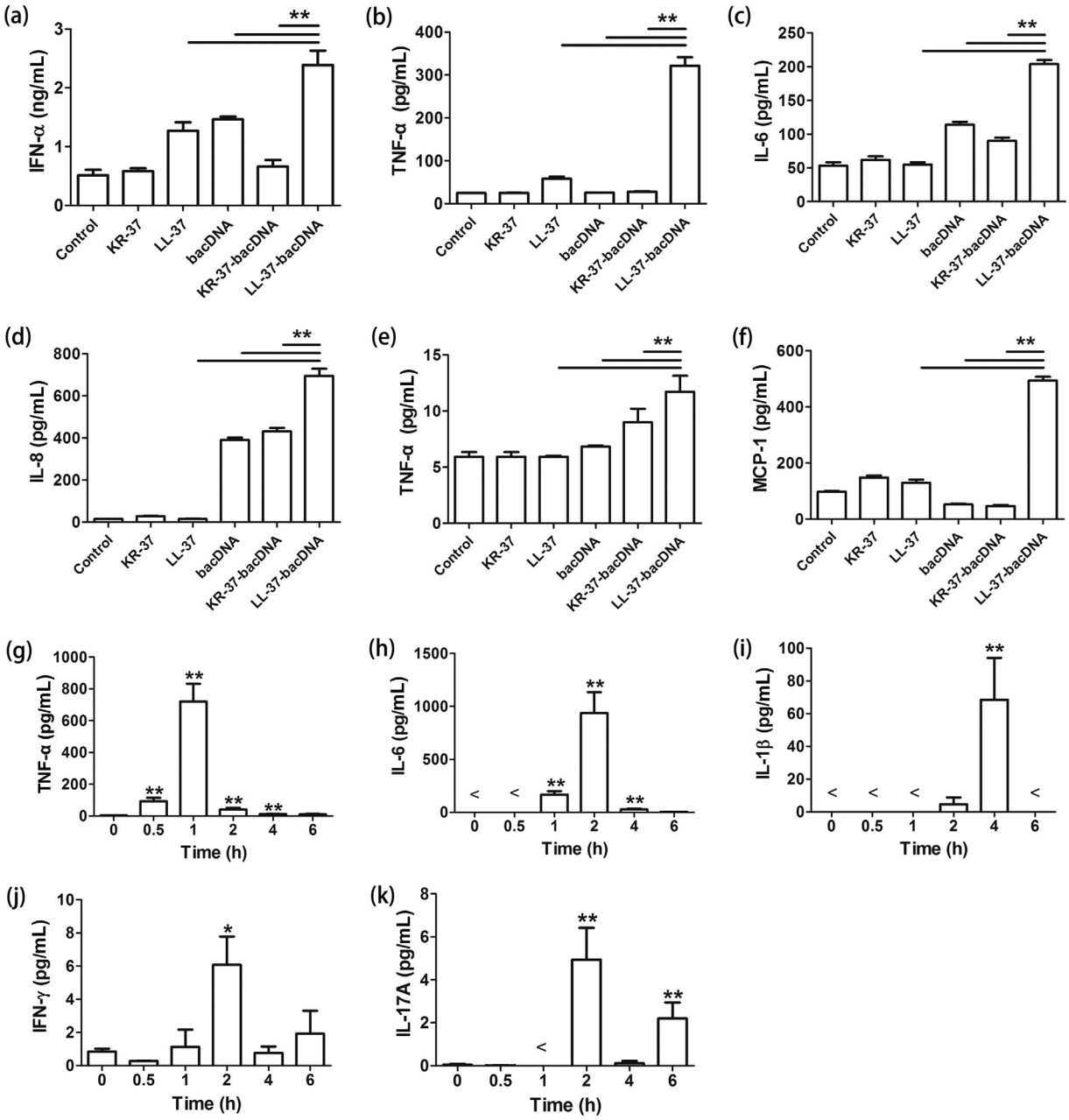

Fig. 4. LL-37/Cramp-bacDNA promotes secretion of inflammatory factors *in vitro* and *in vivo*. (a-f) LL-37/Cramp-bacDNA promoted the secretion of inflammatory factors in pDCs and neutrophils. After stimulation by test sample for 16 h, levels of IFN-α (a) and TNF-α (b) in the supernatant of pDC, and IL-6 (c), IL-8 (d), TNF-α (e) and MCP-1 (f) in the supernatant of neutrophils were determined by ELISA. (g-k) Cramp-bacDNA intravenous injection promoted secretion of inflammatory factors in mice. After injection of Cramp-bacDNA (500 μg/kg) via the mouse tail vein, levels of TNF-α (g), IL-6 (h), IL-1β (i), IFN-γ (j) and IL-17A (k) in the plasma of the mice were determined at different time point. Bars represent mean ± SD of at least three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.6. LL-37-bacDNA complex triggers TLR9 activation

TLR9 plays key roles in recognizing unmethylated microbial DNA, leading to secretion of type I IFNs [14,35]. We demonstrated that LL-37-bacDNA complex triggers pDC and neutrophils activation, resulting in secretion of inflammatory cytokines (Fig. 4). Whether LL-37-bacDNA complex triggers inflammation is dependent on TLR9 pathway is unknown. Here, we found that the number of TLR9-positive vesicles was increased after treatment with LL-37, bacDNA and LL-37-bacDNA complex in a dose-dependent manner, with LL-37-bacDNA showing the strongest ability to stimulate formation of TLR9 aggregates (Fig. 5a, b). Furthermore, IFN-α secretion induced by LL-37-bacDNA complex was inhibited by the oligonucleotide (ODN)-TTAGGG (Fig. 5c), which selectively blocks TLR9 pathways. These data indicated that LL-37-bacDNA complex triggers cytokine secretion is dependent on TLR9.

3.7. LL-37-bacDNA complex increases paracellular permeability

Occludin, the tight junction protein, plays a key role in maintaining intestinal impermeability [36]. As illustrated in Fig. 5d, LL-37-bacDNA complex (48 μg/mL) induced significant reduction and discontinuity of occludin in the cell membrane of human colon cancer cells (HCT-8), indicating tight junction destruction and paracellular permeability. The decreased TER value after treatment by LL-37-bacDNA complex (Fig. 5e) further indicated that the complex induces the paracellular permeability in HCT-8 cells. In addition, western blot analysis also indicated that LL-37-bacDNA

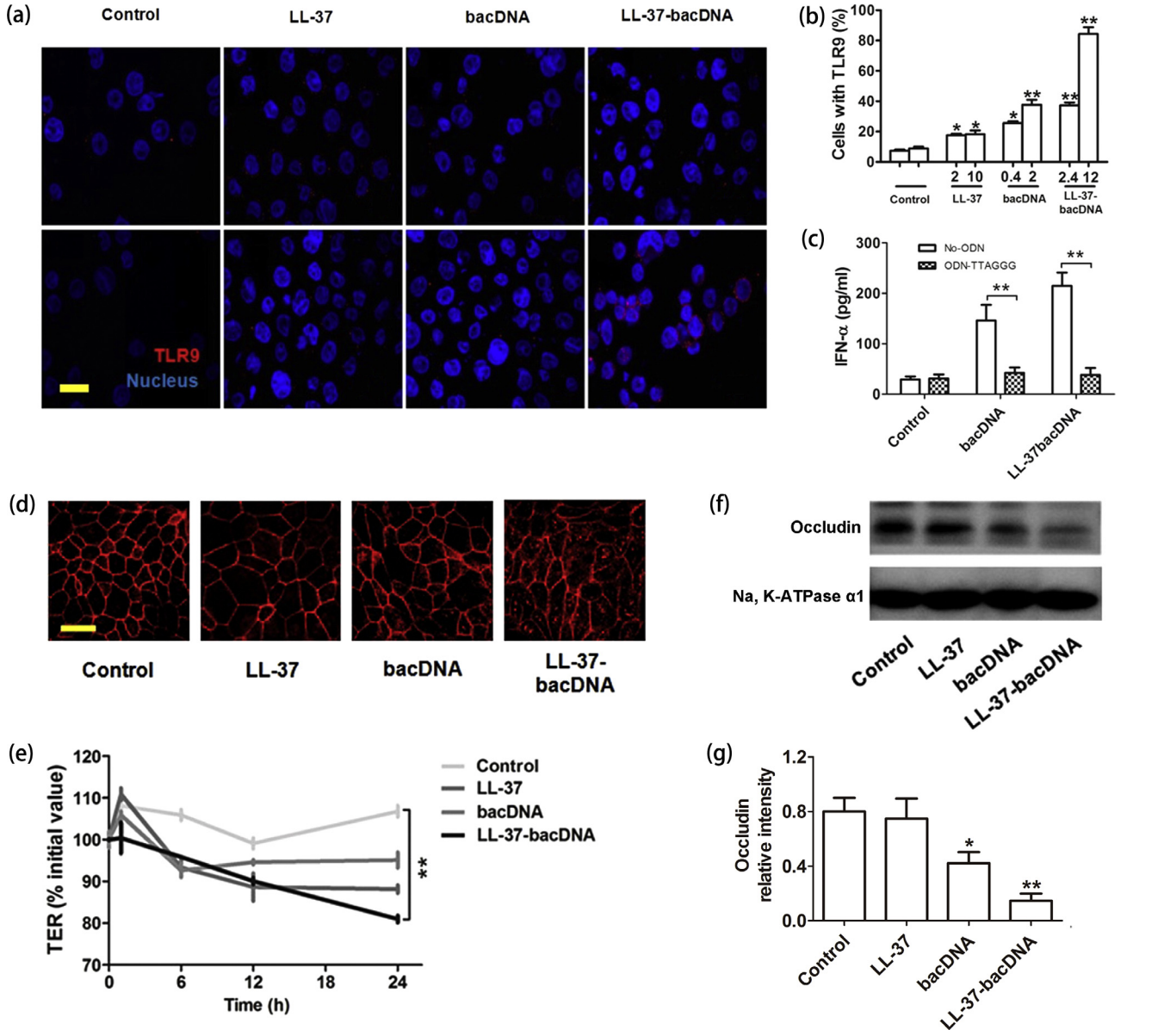

Fig. 5. LL-37-bacDNA induces TLR9 activation and paracellular permeability. (a, b) Expression analysis of TLR9 in macrophages after induction by test sample by confocal microscopy. Macrophages were incubated with LL-37 (2 or 10 μg/mL), bacDNA (0.4 μg/mL or 2 μg/mL) or LL-37-bacDNA (2.4 μg/mL or 12 μg/mL) for 2 h to assess formation of TLR9-positive structures using anti-TLR9 antibody (red). Cell nuclei were stained by DAPI (blue). Representative image (a) and quantitative result (b) were shown. (c) IFN-α produced by macrophages after stimulation with 0.9% salt water (control), bacDNA (2 μg/mL) or LL-37-bacDNA (12 μg/mL) in the presence or absence of ODN-TTAGGG (2 μmol/L). (d) Immunofluorescence imaging showed that LL-37-bacDNA (48 μg/mL) induces reduction and discontinuity of occludin (red) in the cell membrane. (e) Effects of test samples (saline, LL-37 (40 μg/mL), bacDNA (8 μg/mL), or LL-37-bacDNA (48 μg/mL)) on TER value in HCT-8 cells. (f, g) Occludin expression analysis by western blot in membrane fractions of HCT-8 cells. Na, K-ATPase α1 was probed as loading control. Representative image (f) and quantitative result (g) were shown. (a, d) Scale bar represents 20 μm. Bars represent mean ± SD of at least three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

complex treatment decreased membrane occludin by comparison with control in HCT-8 cells (Fig. 5f, g). These data indicated that LL-37-bacDNA complex induces paracellular permeability by decreasing membrane occludin.

3.8. LL-37-bacDNA complex promotes CD4$^+$ T cell differentiation

IBD is characterized by activation of circulating CD4$^+$ T cells [37], while the cause of activation remains unknown. We demonstrated that LL-37-bacDNA induced an expansion of CD4$^+$ T cell differentiation. As illustrated in Fig. S7a, b, compared with ~6.5% CD4$^+$IFN-γ$^+$ cells (Th1) [23] in PBS-treated control, the percentage of these cells expanded by ~13% after treatment with LL-37-bacDNA complex (12 μg/mL). Through the percentage of Th1 cells was seems to increase after treated by LL-37 or bacDNA alone, however, there was no statistical significance (Fig. S7b online). Furthermore, the percentage of CD4$^+$IL-4$^+$ cells (Th2) (Fig. S7c, d online) and CD4$^+$IL-17A$^+$ cells (Th17) [23] (Fig. S7e, f online) was significantly greater in LL-37-bacDNA complex-treated group than that in controls. In contrast, LL-37-bacDNA complex had no effect on T regulatory (Treg) cells differentiation (Fig. S7g, h online). These data indicated that LL-37-bacDNA complex promotes Th1, Th2 and Th17 differentiation but fails to induce Treg cells.

3.9. Cramp-bacDNA complex aggravates while its antibody alleviates mouse UC

To further study the role of Cramp-bacDNA complex in the development of UC, the C57BL/6J mice were provided with 3% dextran sodium sulfate (DSS) to induce UC according to previous

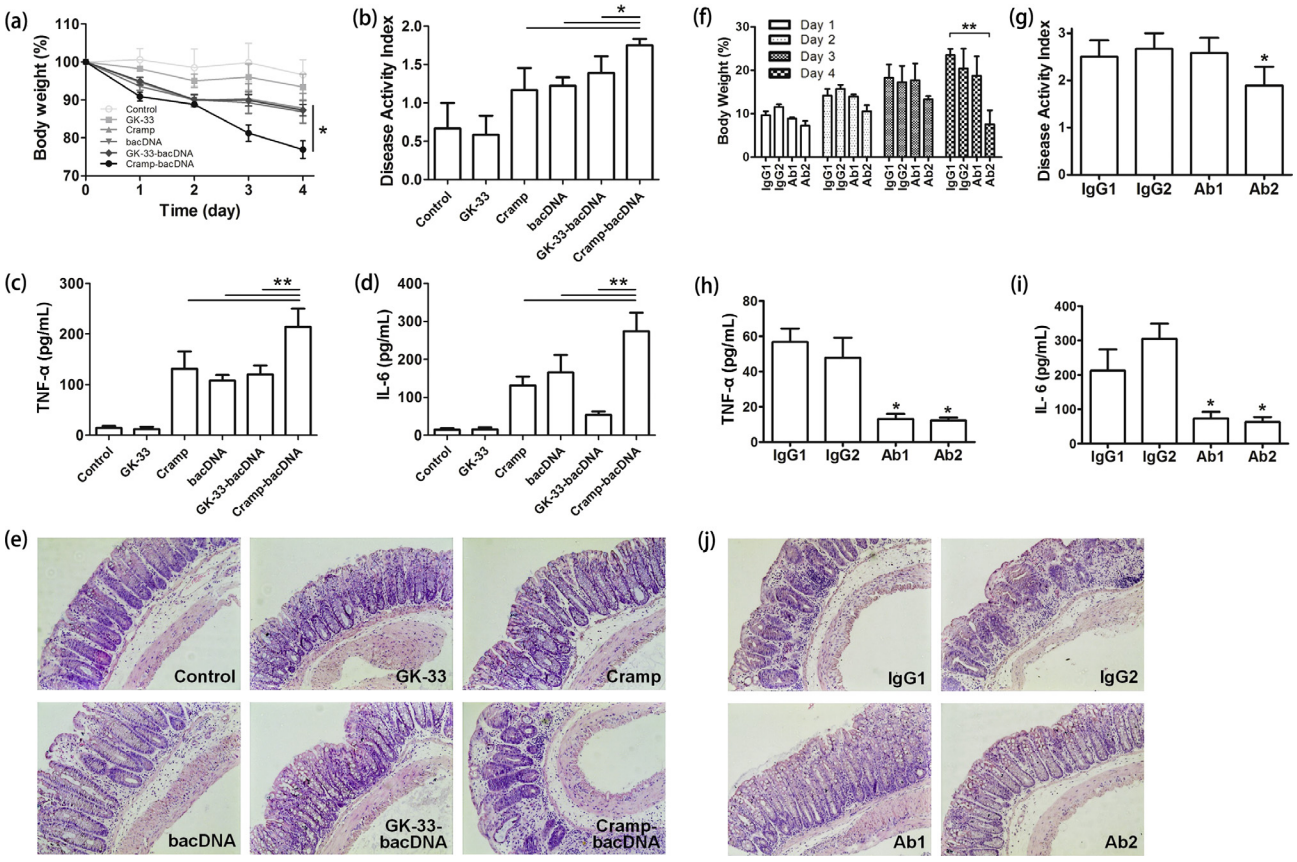

Fig. 6. Cramp-bacDNA complex aggravates mouse UC while its antibody alleviates it. (a-e) Cramp-bacDNA complex aggravated mouse UC. Mice were subjected to 3% DSS to induce UC for 5 days, and saline (Control), GK-33 (100 μg/mouse), Cramp (100 μg/mouse), bacDNA (20 μg/mouse), GK-33-bacDNA (120 μg/mouse) or Cramp-bacDNA complex (120 μg/mouse) were intracolonically injected into mice 1 time per day for 4 days to assess severity of colitis. Body weight change (a), DAI (b), the plasma levels of TNF-α (c) and IL-6 (d) were measured, the histopathological pictures of colon were also shown (e). (f-j) Anti-Cramp-bacDNA antibody alleviated mouse UC. Mice were provided with 3% DSS to induce UC for 5 days, the mice were then treated with intrarectal administration of anti-Cramp-bacDNA antibody (50 (Ab1) or 100 (Ab2) μg/mouse) or an isotype control antibody (50 (IgG_1) or 100 (IgG_2) μg/mouse). Body weight change (f), DAI (g), the levels of IL-6 (h) and TNF-α (i) were recorded, and colon histopathological pictures were shown (j). (e & j) Original magnification, 40×. Bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

report [38], the complex or controls were then intracolonically injected into mice after 5 days inducement by DSS for 4 consecutive days, the severity of colitis was assessed. The results showed that Cramp, bacDNA and Cramp-bacDNA complex aggravated UC symptoms including weight reduction (Fig. 6a) and DAI increment (Fig. 6b). Especially, Cramp-bacDNA complex showed the strongest ability to aggravate mouse UC with larger decrease in body weight and greater increase in DAI. The severity of colitis in different group was associated with an increase in plasma concentrations of TNF-α (Fig. 6c) and IL-6 (Fig. 6d). The amounts of IFN-γ (Fig. S8a online) and IL-4 (Fig. S8b online) in the plasma of different groups' mice showed no significant differences. Further histopathological analysis of the colon also confirmed that Cramp-bacDNA complex aggravates the disease severity. As illustrated in Fig. 6e, Cramp-bacDNA complex administration induced more severe symptoms of epithelial destruction, shortening or partial loss of crypt in the colon and accumulation of inflammatory cells by comparison with Cramp, bacDNA or GK-33-bacDNA complex-treated group.

Considering that LL-37/Cramp-bacDNA complex is highly associated with UC and Cramp-bacDNA directly aggravated UC in mice, we therefore asked whether the antibody against LL-37/Cramp-bacDNA complex exerts anti-UC effect. To test this hypothesis, after a 5-day inducement with 3% DSS, the mice were subjected to anti-Cramp-bacDNA antibody (Ab) or isotype control antibody (IgG) treatment. After a 4-day treatment, as expected, the UC symptoms including weight reduction and DAI were largely reversed (Fig. 6f, g). Consistent with the ameliorated disease severity, the concentration of TNF-α (Fig. 6h) and IL-6 (Fig. 6i) was found significantly decreased in anti-Cramp-bacDNA antibody-treated mice compared with control IgG-injected mice. No differences in amounts of IFN-γ (Fig. S9a online) and IL-4 (Fig. S9b online) were detected between anti-Cramp-bacDNA antibody treated and control IgG-injected mice. The histological changes including reduced number of inflammatory cells, signs of regeneration of crypts and restoration of colonic mucosa further indicated the improvement of colonic pathology by anti-Cramp-bacDNA antibody treatment (Fig. 6j). Together, these data indicated that Cramp-bacDNA complex aggravates UC development, and that interference with the complex may harbor valuable opportunities for UC treatment.

4. Discussion and conclusion

The persistent presence of bacDNA in blood and lymph nodes of IBD patients with negative blood bacterial cultures is a common event [5]. As an important inflammogenic factor with strong ability to induce inflammation, persistent bacDNA stimulation would cause chronic inflammatory disorder in hosts. Indeed, inflammatory cytokines have been found associated with bacDNA in serum of patients with IBD [5]. Thus, the key question is how bacDNA avoids elimination. Here, we provided the first evidence that the released bacterial nucleic acids upon LL-37-mediated lysis of bacteria were further protected by LL-37, resulting in delayed degradation (Fig. S2 online) or evasive elimination (Fig. 3f), further

leading to continuous activation of inflammatory responses (Fig. 4 and Fig. 5a-c).

Up-regulation of LL-37 in inflamed and non-inflamed mucosa of patients with UC and the role of it in the pathogenesis of UC [7,8] suggest it may provide a protective effect from pathogenic organisms infection in epithelial surfaces of colon [39,40]. Indeed, both antimicrobial [9,10] and anti-inflammatory [41] functions have been assigned to LL-37. However, in the context of inflammatory response, LL-37 may also provide proinflammatory signals by inducing secretion of type I IFNs, resulting in induction of autoimmune and inflammatory diseases [12–14]. The presence of LL-37 in UC, which is specially characterized by continuous chronic inflammatory activation, may contribute to UC pathogenesis. Expectedly, by forming complex with bacDNA, LL-37-bacDNA complex induced pDC activation and secretion of IFN-α (Fig. 4a) and TNF-α (Fig. 4b). LL-37-bacDNA complex also involved in neutrophils activation and secretion of IL-6, IL-8, TNF-α and MCP-1 (Fig. 4c-f). Accordingly, the released cytokines and chemokines may further induce an amplification of inflammatory cascades by triggering pDCs and neutrophils activation (Fig. 4g-k) and eventually aggravate UC. High mount of LL-37-bacDNA complex was found in the plasma and lesions of patients with UC (Fig. 2) and the role of it (Cramp-bacDNA) was further demonstrated to promote UC in mice (Fig. 6). Thus, LL-37 may play a dual role in pathogenesis of UC. On the one hand, LL-37's protection against colitis may be related to the antimicrobial effect [42], stabilization of proinflammatory effects of the TLR9 ligand in the colon [39] and suppressing apoptosis [40], which may be primary responses of LL-37. On the other hand, the interaction of LL-37 with bacDNA may promote loss of elimination to bacDNA and aberrant immune responses, which may be secondary responses of LL-37. In addition, the antimicrobial effect of LL-37 was disabled after forming complex with bacDNA (Fig. S3 online).

The increased amount of LL-37-bacDNA complex in patients with UC may due to LL-37-mediated lysis of bacteria, resulting in releasing bacDNA and further forming complex with LL-37. Indeed, after incubation with LL-37 for 4 h, the LL-37-bacDNA complex could be detected in the *E. coli* culture medium (Fig. 1a). Previous study has demonstrated that *E. coli* genomic DNA induced LL-37 expression in vivo [39]. Here we found *E. coli* also induced high amounts of Cramp and Cramp-bacDNA complex in the plasma of mice (Fig. 1b, c), suggesting the formation of LL-37/Cramp-bacDNA complex results from the interaction of the peptide and bacteria in vivo. Furthermore, as a member of cathelicidin family of antimicrobial peptides, LL-37 in human and Cramp in mice show similar function in most cases [14,43], the present study also demonstrated that they play similar role in the pathogenesis of UC.

Local damage to gastrointestinal tract in IBD may result in T-cell activation and systemic inflammation. IBD has been characterized by activation of circulating $CD4^+$ T cells [37], which play key roles in immune-regulation and immune-activation. $CD4^+$ T cells are classified as 4 subtypes: IFN-γ-secreting Th1, which is involved in cellular immunity; IL-4-secreting Th2, which induces antibody production and plays a role in humoral immunity; IL-17 producing Th17, which plays a role in maintaining mucosal barriers and contributing to pathogen clearance at mucosal surfaces [23,44]. In the present study, we found that LL-37-bacDNA complex promotes Th1, Th2 and Th17 differentiation (Fig. S7a-f online) but fails to induce Treg cells (Fig. S7g, h online), indicating the role of the complex in regulation of inflammatory cell differentiation and inflammatory responses. This may partly explain the abnormal activation of $CD4^+$ T cells in patients with UC.

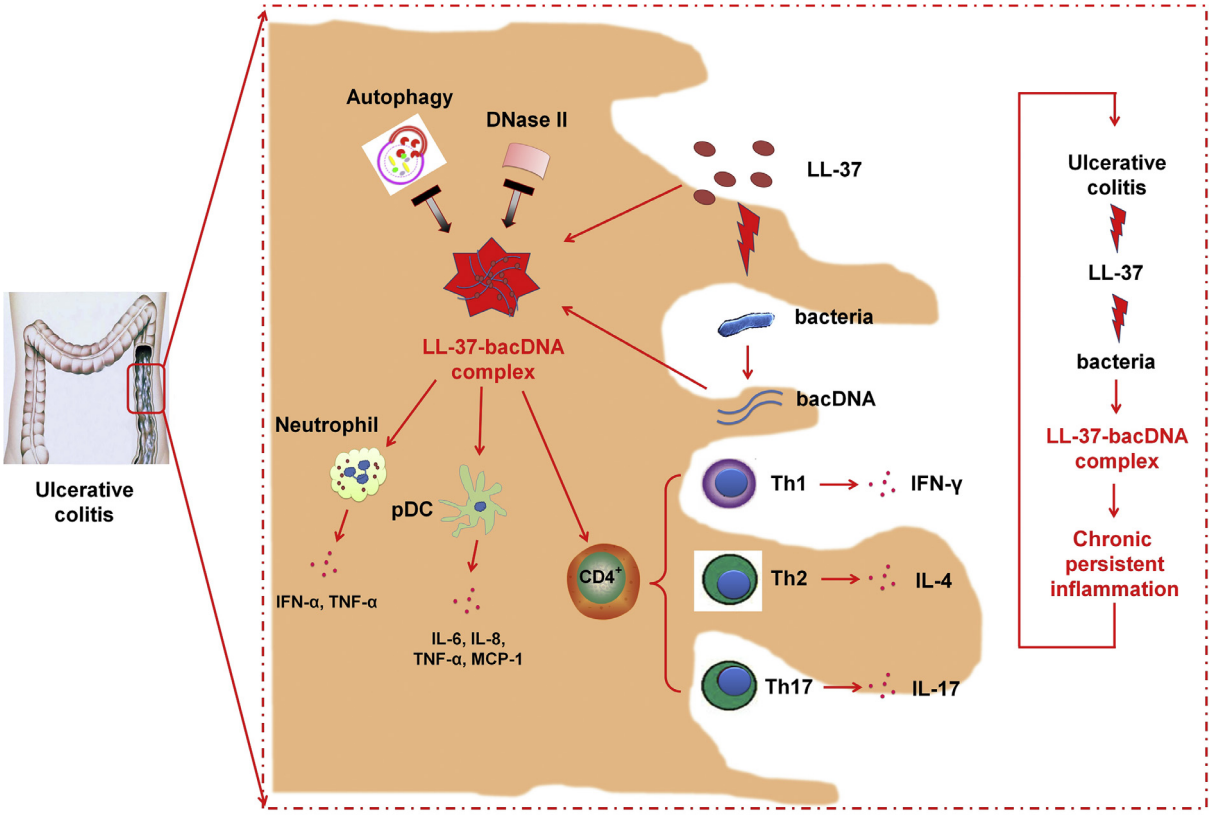

Fig. 7. Schematic diagram of LL-37-bacDNA's promotion on UC. BacDNA escapes immune elimination by DNase II and autophagy and induces chronic inflammation by forming complex with human antimicrobial peptide LL-37, which is up-regulated upon bacterial infection. LL-37-bacDNA, results from LL-37's bacteria-killing effect and subsequent bacterial DNA release. LL-37-bacDNA complex promotes the secretion of inflammatory cytokines and induces $CD4^+$ T cell differentiation.

IBD patients display increased paracellular permeability with tight junctions abnormalities documented in several studies [45,46]. A decreased expression and redistribution of tight junctions' constituents, such as occludins, claudins, and junctional adhesion molecules (JAM), have all been documented in IBD [47–50]. The underlying cause of IBD involves the disruption of mucosal barrier function is not clear. As illustrated in Fig. 5d-g, LL-37-bacDNA was found to increase paracellular permeability by down-regulating the expression of occludin, implying that LL-37-bacDNA damages mucosal integrity. Conceivably, the disruption of mucosal barrier function due to increased paracellular permeability facilitates blood translocation of bacteria and/or bacDNA. In addition, combined with inflammatory effects of LL-37-bacDNA, increased paracellular permeability induced by the complex probably aggravates local damage and UC as illustrated in Figure 6.

Taken together, the present study indicated that by forming complex with LL-37, bacDNA is resistant against DNase II and autophagy degradation, leading to the persistent presence of bacDNA in blood of patients with UC and indelible immune activation and cytokines generation and eventually aggravates UC. By down-regulating occludin to increase paracellular permeability and to disrupt mucosal barrier, LL-37-bacDNA facilitates blood translocation of bacDNA. These data provide new perspectives on the mechanism of bacDNA persistent presence in blood and chronic inflammation that accompanies UC, potentially providing a promising new therapeutic target (Fig. 7). In addition, after bacterium lysis induced by LL-37, bacDNA is released to disable microbicidal functions of LL-37 and may make a milieu for microbe survival as a 'trojan horse'.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scib.2018.09.014.

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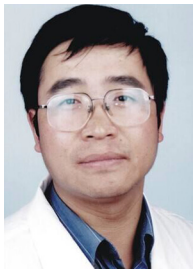

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