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β-catenin promotes intracellular bacterial killing via suppression of *Pseudomonas aeruginosa*-triggered macrophage autophagy

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Abstract

Objective: To investigate β-catenin-mediated bacterial elimination during *Pseudomonas aeruginosa* infection of macrophage-like RAW264.7 cells.

Methods: Cell viability and catenin beta 1 (*CTNNB1*) expression in RAW264.7 cells following *P. aeruginosa* infection versus uninfected cells, were detected by cell counting kit-8 assay and β-catenin Western blots. RAW264.7 cells with *CTNNB1* overexpression were established with β-catenin lentivirus using flow cytometry and clonogenic limiting dilution assays. Bacterial killing was measured by plate counts; phagocytosis and nitric oxide (NO) were measured by flow cytometry; and reactive oxygen species (ROS) were measured using Griess reaction. Autophagy was determined by microtubule-associated protein 1 light chain 3 alpha-phosphatidylethanolamine conjugate (LC3-II) protein levels and formation of LC3 puncta, using Western blot and immunofluorescence staining.

Results: Following *P. aeruginosa* infection, RAW264.7 cell β-catenin levels were reduced in a time- and multiplicity of infection-dependent manner. *CTNNB1* overexpression was associated with increased *P. aeruginosa* elimination, but had no effect on RAW264.7 cell phagocytosis, ROS and NO. *CTNNB1* overexpression reduced LC3-II levels and formation of LC3 puncta, suggesting autophagy inhibition. Rapamycin/starvation-induced autophagy resulted in reduced bacterial killing following *P. aeruginosa* infection.

Conclusion: β-catenin may promote bacterial killing via suppression of *P. aeruginosa*-induced macrophage autophagy.

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Introduction

Pseudomonas aeruginosa is one of the most common pathogenic bacteria associated with various opportunistic infections, particularly in immunocompromised individuals.[1] Antibiotics are administered to treat bacterial infections, however, the frequent failure to clear multi-drug-resistant bacteria has shifted the focus of research to explore innate host defences.[2]

Macrophages are one of the most important primary immune response cells, and during pathogen challenge, macrophages engulf and eradicate invading pathogens through the recognition of pathogen-associated molecular patterns.[3] Oxygen-dependent bactericidal systems, such as reactive oxygen species (ROS)[4] and reactive nitrogen species,[5] as well as autophagy,[6–9] are reported to have the ability to fight against invading bacteria.

β-catenin, encoded by the catenin beta 1 (*CTNNB1*) gene, is a key molecule of the canonical Wnt pathway, and is reported to have the ability to modulate host defense.[10] β-catenin is thought to promote increased ROS[11] and nitric oxide (NO) levels,[12] both of which play a crucial role in *P. aeruginosa* elimination. Autophagy has been reported to promote degradation of β-catenin,[13] and activation of β-catenin signalling is shown to inhibit autophagy,[14] suggesting that there is crosstalk between β-catenin signalling and autophagy. Further investigation is required to clarify the underlying mechanism of β-catenin-related modulation of *P. aeruginosa* elimination.

The aim of the present study was to investigate the potential role of β-catenin in modulating bacterial clearance and autophagy, through *in vitro* infection of murine macrophage-like cells with *P. aeruginosa*.

Materials and methods

Preparation of Pseudomonas aeruginosa

P. aeruginosa cultures (Catalogue No. ATCC 19660; American Type Culture Collection, Manassas, VA, USA) were maintained in *Pseudomonas* isolation agar (BD Biosciences, San Jose, CA, USA). To prepare a bacterial inoculum, a loopful of culture from a peptone-tryptic soy broth (0.25% tryptic soy broth [Sigma Chemical Co., St. Louis, MO, USA] and 5% Bacto-Peptone [Difco Laboratories, Detroit, MI, USA]) was used to inoculate 25 ml of peptone-tryptic soy broth. Bacteria were cultured at 37°C on a rotary shaker for 18 h to an optical density (at 540 nm) of approximately 1.6, then centrifuged at 6 000 *g* for 10 min at 15°C, washed once with sterile saline (0.85% NaCl, pH 7.2), and resuspended in saline to a concentration of approximately 2.0×10^{10} CFU/ml using a standard curve relating viable counts to optical density.

Mammalian cell culture

Murine macrophage-like RAW264.7 cells (Catalogue No. ATCC TIB-71; American Type Culture Collection) and 293T cells (Catalogue No. ATCC CRL-3216; American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100U/ml streptomycin and 1% L-glutamine (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), at 37°C/5% CO_2.

Lentivirus preparation and transduction

β-catenin and control lentiviruses were produced by transient transfection in 293T cells.[15] Briefly, β-catenin lentivirus vector, encoding β-catenin and the fluorescent marker protein mCherry, was purchased from Addgene (Cambridge, MA, USA; Catalogue No. 24312). A control lentivirus was obtained by removing the β-catenin sequence of the β-catenin lentivirus vector using BamHI restriction enzyme (Catalogue No. R0136; New England Biolabs, Ipswich, MA, USA) followed by self-ligation.

One day before transfection, 10 cm dishes were seeded with 4×10^6 293T cells. For each dish, 10 μg lentivirus vectors (β-catenin or control) were mixed with 3 μg pCMV-VSV-G envelope plasmid (Catalogue No. 8454; Addgene) and 10 μg pCMV-dR8.2 packaging plasmid (Catalogue No. 8455; Addgene). The solution was adjusted to 450 μl with water and mixed with 50 μl 2.5 M $CaCl_2$. A precipitate was formed by drop-wise addition of 500 μl 2 × HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, and 50 mM HEPES, pH 7.05), and this was added directly to the 293T cells. The 293T cells were then incubated at 37°C/5% CO_2, the culture media were replaced at 12 h following transfection, and the cells were incubated for a further 36 h. At 36 h, the conditioned media were pooled from the 10 cm dishes, centrifuged at 4000 *g* for 5 min, and then centrifuged at 50 000 *g* for 140 min. The lentiviral pellet was resuspended in 400 μl 0.1% bovine serum albumin in 280–315 mOsm/kg phosphate buffered saline (PBS, pH 7.4; Invitrogen). An HIV p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Catalogue No. 0801002; Zeptometrix, Buffalo, NY, USA) was used to detect the viral titer, according to the manufacturer's instructions. For lentivirus transduction, RAW264.7 cells were infected with β-catenin lentivirus or control lentivirus at MOI values of 1 for 6 h at 37°C/5% CO_2. The cells were then cultured at 37°C/5% CO_2 for a further 24 h following transduction for fluorescence-activated cell sorting.

Establishment of RAW264.7 cells with stable CTNNB1 *expression*

RAW264.7 cells infected with lentivirus were harvested and resuspended in PBS with 1% FBS. Cell suspensions were analysed and sorted using a BD LSRFortessa™ cell analyser (BD Biosciences) according to the manufacturer's instructions. During flow cytometry analysis, cell debris and cell aggregates were gated out, and the top 5–10% of the brightest mCherry-expressing cells were sorted. Results were analysed using FlowJo software, version 7.6 (Stanford University, Stanford, CA, USA). A total of 1×10^5 sorted cells were plated onto 6 cm culture dishes for clonogenicity. Clonogenic limiting dilution assays were performed in 96-well plates at cell densities of 1–10 cells/well, with a minimum of 5 replicate wells per cell seeding density. Culture media was changed at days 4, 7 and 11 using a multichannel pipette. Single cell clones expressing mCherry were observed by fluorescence microscopy (Olympus IX53, Olympus, Japan) and subcultured at 37°C/5% CO_2 for further assays.

Western blot analyses

Total protein was extracted from the RAW264.7 cells using lysis buffer (1 mM phenylmethylsulphonyl fluoride, 1% protease inhibitor cocktail [Catalogue No. P8340; Sigma] and 1 mM dithiothreitol). Cytoplasmic and nuclear protein were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Catalogue No. 78833; ThermoFisher Scientific), according to the manufacturer's instructions. Lysate protein concentrations were measured using a Quick

Start™ Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. A total of 30 μg of protein per sample were separated by 10% sodium dodecyl sulphate-polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% milk for 2 h at room temperature, then incubated with: a 1:1 000 dilution of rabbit anti-β-catenin (Catalogue No. 8480; Cell Signalling Technology, Danvers, MA, USA); a 1:1 000 dilution of rabbit anti-microtubule-associated protein 1 light chain 3 alpha (LC3; Catalogue No. NB100-2331; Novus Biologicals, Littleton, CO, USA); a 1:2 000 dilution of rabbit anti-β-actin (Catalogue No. 4970; Cell Signalling Technology); or a 1:2 000 dilution of rabbit anti-Histone (Catalogue No. 3638; Cell Signalling Technology) primary antibody at 4°C overnight. Membranes were washed three times for 5 min each in Tris buffered saline-Tween (10 mM Tris, 154 mM NaCl [pH 7.5], and 0.1% Tween 20). The immune complexes were then incubated with a 1:5 000 dilution of IRDye® 800CW donkey anti-rabbit IgG secondary antibody (LI-COR, Lincoln, NE, USA) for 1 h at room temperature, then washed again as above. Immunodetection was performed using an Odyssey® CLx Infrared Imaging System (LI-COR) according to the manufacturer's instructions. β-catenin and LC3 levels were quantified by relative integrated density values after normalization to β-actin, and are presented as percentage of control values.

Cell viability assay

Cell viability was determined using a cell counting kit (CCK)-8 assay (Catalogue No. KGA317; Nanjing KeyGEN BioTECH, Jiangsu, China). Briefly, 1×10^4 cells were seeded into 96-well plates one day prior to *P. aeruginosa* infection. Cells were then infected with *P. aeruginosa*, followed by addition of 10 μl CCK-8 reagent into the plate. Uninfected cells were used as controls. Absorbance was measured at 450 nm using a Bio-Rad ELISA plate reader (Bio-Rad Laboratories).

Intracellular bacterial killing assay

Six-well plates were seeded with transduced β-catenin stable RAW264.7 cells or transduced control cells. Following infection with *P. aeruginosa*, cells were further cultured for 30 min in media with 300 μg/ml gentamicin to eradicate any extracellular bacteria. Cells were then washed with PBS and lysed in 1 ml of water containing 0.1% Triton X. The number of bacteria in the lysates was determined by counting colony forming units (CFUs) in *Pseudomonas* isolation agar (BD Biosciences). Intracellular bacterial load is presented as CFU per 10^6 cells ± SEM.

Phagocytosis assay

Transduced RAW264.7 cells were infected with *P. aeruginosa* that had been stained with FilmTracer™ Green Biofilm (Invitrogen) according to the manufacturer's instructions. Following 1 h incubation with stained *P. aeruginosa*, RAW264.7 cells were washed with PBS, followed by incubation with PBS containing 0.1% trypan blue, to erase extracellular fluorescence. Intracellular fluorescence, which indicated bacteria engulfed by RAW264.7 cells, was analysed using a Coulter EPICS XL-MCL™ flow cytometer and associated software (Beckman Coulter, Brea, CA, USA), according to the manufacturer's instructions.

ROS and NO production

Reactive oxygen species were measured in RAW264.7 cells using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen), as a sensitive probe that is

deacetylated and oxidized by cells to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Flow cytometry was used to detect DCF fluorescence, which indicated ROS production in the RAW264.7 cells. To determine NO production, the culture supernatant was pooled from replicate plates following *P. aeruginosa* infection. NO production was determined by detecting the content of nitrite in supernatants using Griess reagent (Sigma).

Immunofluorescence assay for LC3

β-catenin-expressing and control transduced RAW264.7 cells were seeded onto sterile glass cover slips and infected with *P. aeruginosa*, then fixed with 4% paraformaldehyde. Cells were incubated with a 1:200 dilution of rabbit anti-LC3 primary antibody (Novus Biologicals) overnight at 4°C, then washed three times in PBS (pH 7.4), before adding a 1:500 dilution of Alexa Fluor 488-conjugated anti-rabbit IgG (Green; Catalogue No. 4412; Cell Signalling Technology) secondary antibody for 1 h at room temperature. Stained cells were observed using an Olympus IX53 fluorescence microscope (Olympus Company, Tokyo, Japan), and autophagy induction was quantified by calculating the mean number of LC3 puncta/cell out of 100 cells per sample.

Experimental conditions

RAW264.7 cells were infected with *P. aeruginosa* at multiplicity of infection (MOI) values of 1 or 5, for 6, 12, 24, 36, and 48 h at 37°C/5% CO_2 and β-catenin levels were assessed by Western blot. Nuclear and cytosolic protein fractions were assessed by Western blot in cells that were infected with *P. aeruginosa* at an MOI value of 1 for 6, 12, 24, 36, and 48 h. RAW264.7 cells were also infected with *P. aeruginosa* at MOI values of 0.1, 1, 5, 10, 20 and 50 for 6 h and 12 h. β-catenin levels were assessed by Western blot and cell viability was evaluated. Uninfected cells were used as controls.

Transduced RAW264.7 cells (overexpressing *CTNNB1* or controls) were infected with *P. aeruginosa* at the following MOI values and durations of infection: MOI of 10 for 1 h or 2 h at 37°C/5% CO_2 for intracellular bacterial killing assay; MOI of 5 for 1 h at 37°C/5% CO_2 for phagocytosis flow cytometry analysis; and MOI of 1 for 12 h or 24 h at 37°C/5% CO_2 for analysis of NO.

To further explore the underlying mechanism of β-catenin in bacterial killing, autophagy was determined in transduced RAW264.7 cells (overexpressing *CTNNB1* or controls) that were infected with *P. aeruginosa* at the following MOI values and durations of infection: MOI of 1 for 0, 1, 2, 4, and 8 h at 37°C/5% CO_2 for measuring LC3-I and LC3-II protein levels by Western blot; and MOI of 1 for 0, 4 and 8 h at 37°C/5% CO_2 for immunofluorescence LC3 assays for LC3 puncta formation. Rapamycin (Catalogue No. 37094; Sigma) and starvation (amino acid-free EBSS medium; Catalogue No. 24010043; GIBCO, Carlsbad, CA, USA) were used to induce autophagy in RAW264.7 cells (overexpressing *CTNNB1* or controls). Cells were treated with either 100 nM rapamycin or 0.1% v/v DMSO (Sigma) control for 1 h and then infected with *P. aeruginosa* for the bacterial killing assay. Autophagy was assessed by Western blot analysis of LC3-I and LC3-II levels, and the effect of rapamycin and starvation on bacterial killing was determined following infection with *P. aeruginosa* at an MOI value of 10 for 1 h and 2 h.

Statistical analyses

All experiments were performed in triplicate and repeated three times, and data are presented as mean ± SD or mean ± SEM from the three independent experiments. Statistical analyses were conducted using Prism software, version 5.0 (GraphPad

Software, La Jolla, CA, USA). Parametric data were analysed using an unpaired, two-tailed Student's *t*-test, and a P value < 0.05 was considered to indicate statistical significance.

Results

Reduced RAW264.7 cell β-catenin levels following P. aeruginosa *infection*

Investigation of RAW264.7 cell *CTNNB1* expression during *P. aeruginosa* infection with MOI values of 1 or 5 for 6–48 h, showed that β-catenin levels were significantly increased at early time-points (12 h for MOI of 1, Figure 1(a) and 1(d); and 6 and 12 h for MOI of 5, Figure 1(b) and 1(e); all $P < 0.001$), and decreased at 36 and 48 h (MOI of 1, Figure 1(a) and 1(d); and MOI of 5, Figure 1(b) and 1(e); all $P < 0.001$) versus uninfected controls. Cytosolic and nuclear protein fractions were also assessed for β-catenin levels in cells following *P. aeruginosa* infection versus uninfected cells. Nuclear β-catenin levels were shown to be increased at

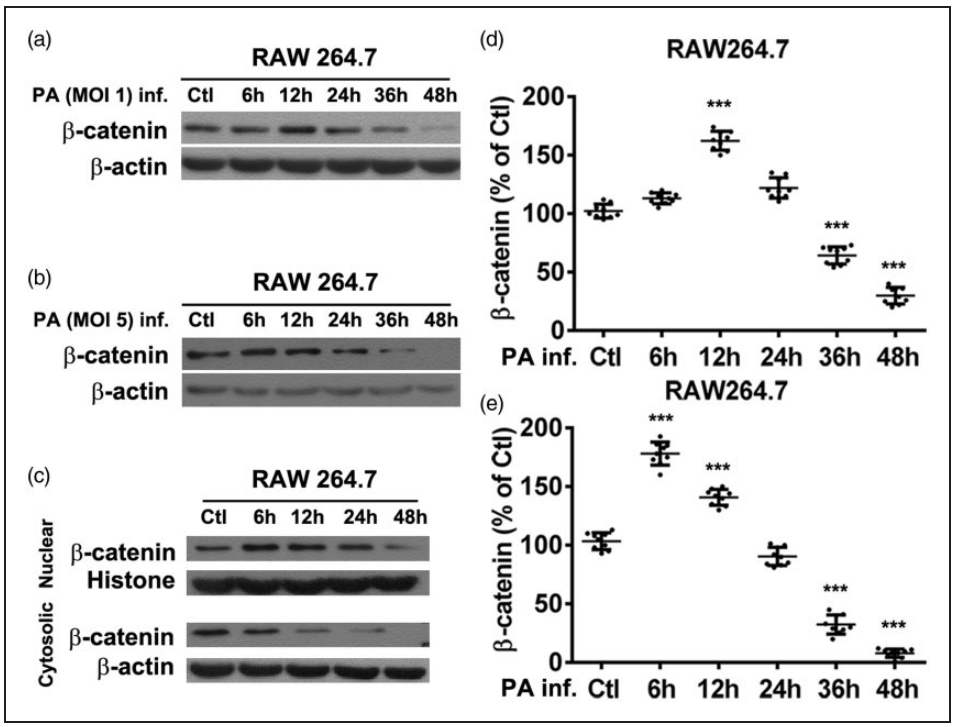

Figure 1. Representative Western blots of β-catenin levels from murine macrophage-like RAW264.7 cells infected with *Pseudomonas aeruginosa* at multiplicity of infection (MOI) values of (a) 1 and (b) 5, for 6–48 h; (c) Representative Western blots of nuclear and cytosolic β-catenin levels following RAW264.7 cell infection with *P. aeruginosa* at a MOI of 1 for 6–48 h, showing increased nuclear β-catenin levels at 6, 12 and 24 h and decreased levels at 48 h versus controls, and decreased cytosolic β-catenin levels at all time-points versus controls; Whole cell β-catenin relative integrated density values normalized to β-actin, presented as percentage of control values for (d) *P. aeruginosa* MOI 1 and (e) *P. aeruginosa* MOI 5 showing β-catenin levels increased at early time-points then reduced in a time-dependent manner following *P. aeruginosa* infection. Data presented as mean ± SD of three individual experiments; ***$P < 0.001$ versus control (unpaired, two-tailed Student's *t*-test); PA, *P. aeruginosa*; inf., infection duration; Ctl, control.

early time points (6, 12 and 24 h, Figure 1(c); all $P < 0.001$; data not shown) and decreased at 48 h following *P. aeruginosa* infection versus controls (Figure 1(c); $P < 0.001$, data not shown), while cytosolic β-catenin levels were reduced following infection at all time-points (Figure 1(c); all $P < 0.001$; data not shown).

In RAW264.7 cells infected with *P. aeruginosa* at MOI values of 0.1–50 for 6 h (Figure 2(a) and 2(c)) and 12 h (Figure 2(b) and 2(d)), β-catenin levels were significantly increased versus controls at lower MOI values (MOI of 1 and 5 at 6 h, and MOI of 1, 5 and 10 at 12 h; all $P < 0.001$) and were significantly reduced versus controls at MOI values of 20 and 50 at 6 h and 12 h infection duration (all $P < 0.001$). *P. aeruginosa* infection of RAW264.7 cells at MOI values of 0.1–50 for 6 h (Figure 2(e)) or 12 h (Figure 2(f)) did not affect cell viability.

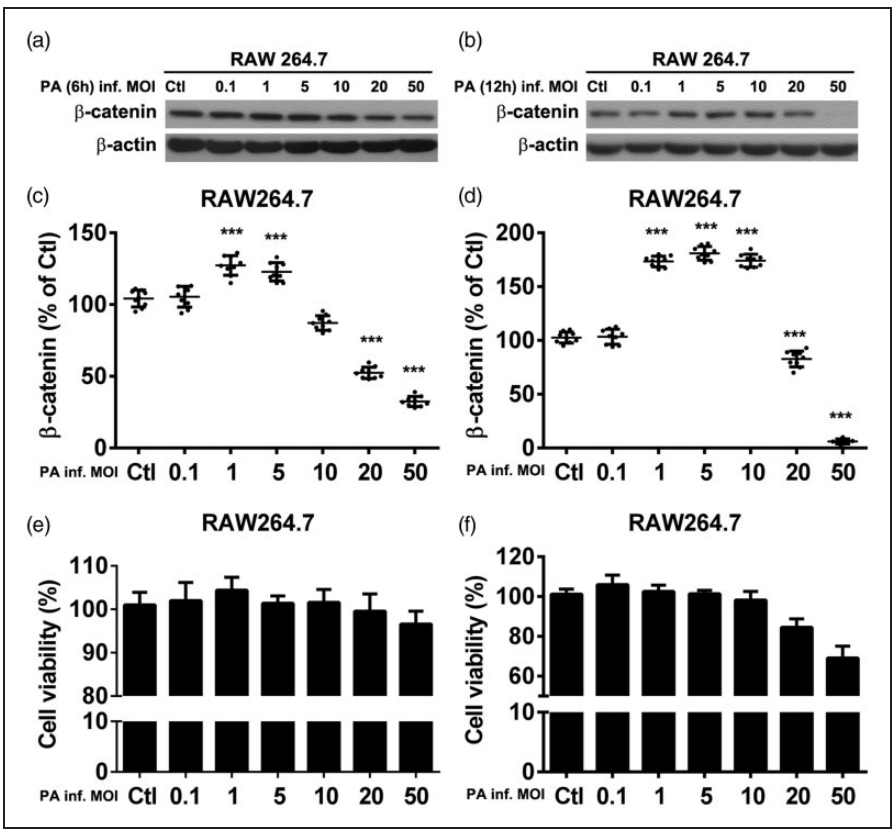

Figure 2. Representative Western blots of β-catenin levels from murine macrophage-like RAW264.7 cells infected with *Pseudomonas aeruginosa* at multiplicity of infection (MOI) values of 0.1–50 for (a) 6 h or (b) 12 h; Whole cell β-catenin relative integrated density values normalized to β-actin, presented as percentage of control values for (c) cells infected for 6 h and (d) cells infected for 12 h (β-catenin levels reduced with increasing *P. aeruginosa* multiplicity of infection values); Cell viability of RAW264.7 cells infected with *P. aeruginosa* at MOI values of 0.1–50 for (e) 6 h or (f) 12 h, evaluated by cell counting kit-8 assay. Data presented as mean ± SD of three individual experiments; ***$P < 0.001$ versus control (unpaired, two-tailed Student's *t*-test); PA, *P. aeruginosa*; inf., infection; Ctl, control.

Creation of a RAW264.7 cell line with stable CTNNB1 *expression*

To investigate the role of β-catenin in *P. aeruginosa* infection, RAW264.7 cells were infected with β-catenin lentivirus or control lentivirus. Flow cytometry showed that 7.3% and 11.6% mCherry expressing cells were sorted in β-catenin lentivirus and control lentivirus transduced cells, respectively (Figure 3(a)). Sorted cells were assessed for mCherry (red fluorescence) expression using fluorescence microscopy (Figure 3(d)), and clonogenic limiting dilution assays isolated single cell control and stable *CTNNB1*-expressing clones (Figure 3(e)). The presence of β-catenin was detected by Western blot (Figure 3(b) and (c)), and was shown to be significantly increased in the β-catenin transduced monoclone versus the control monoclone ($P < 0.001$; Figure 3(c)).

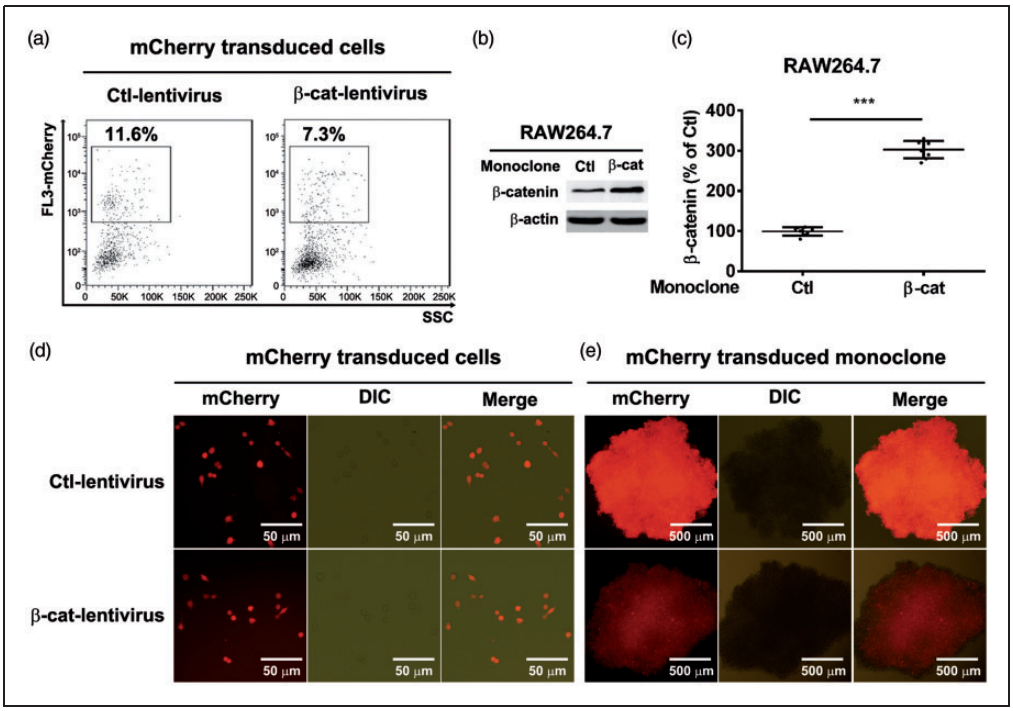

Figure 3. Construction of murine macrophage-like RAW264.7 cells with stable catenin beta 1 expression: (a) Flow cytometry scatter plot showing RAW264.7 cells transduced with control lentivirus or β-catenin lentivirus, sorted according to mCherry marker expression; (b) Representative Western blot showing β-catenin levels in control and β-catenin-transduced monoclones; (c) β-catenin relative integrated density values normalized to β-actin, presented as percentage of control transduced cells (***$P < 0.001$ versus controls); (d) Representative fluorescence micrographs showing mCherry expressing control lentivirus or β-catenin lentivirus cells sorted by flow cytometry; and (e) Representative fluorescence micrographs showing mCherry expressing control lentivirus or β-catenin lentivirus monoclones. Data presented as mean ± SD of the mean for triplicate experiments; Ctl, control; β-cat, β-catenin; FL3, fluorescence 3; SSC, side scatter; DIC, differential interference contrast.

Overexpression of CTNNB1 *enhanced intracellular bacterial killing in* P. aeruginosa*-infected RAW264.7 cells with no effect on phagocytosis, ROS and NO*

Plate count assays of bacterial killing following *P. aeruginosa* infection in RAW264.7 cells overexpressing *CTNNB1* versus transduced controls, showed that increased β-catenin reduced the bacterial burden at 1 h ($P < 0.001$, Figure 4(a)) and 2 h ($P < 0.01$, Figure 4(a)) following *P. aeruginosa* infection. Flow cytometry or Griess assay measurements showed that stable increased β-catenin levels had no effect on phagocytosis (Figure 4(b)), NO (Figure 4(c)) and ROS (Figure 4(d)).

Overexpression of CTNNB1 *enhanced bacterial killing through inhibiting* P. aeruginosa*-induced autophagy*

The underlying mechanism of β-catenin in bacterial killing was further assessed by measuring autophagy in RAW264.7 cells overexpressing *CTNNB1* versus transduced controls following *P. aeruginosa* infection. Autophagy was determined by Western blot analysis of LC3-I and LC3-II protein levels, and by immunofluorescence staining of endogenous LC3 distribution. *CTNNB1* overexpression (increased β-catenin) was associated with reduced LC3-II levels at 1, 2, 4 and 8 h following *P. aeruginosa* infection ($P < 0.001$ at 1, 4 and 8 h; $P < 0.05$ at 2 h; Figure 5(a) and (b)). Increased β-catenin was also shown to reduce the formation of LC3 puncta following *P. aeruginosa* infection ($P < 0.001$ at 4 and 8 h; Figure 5(c) and (d)).

Rapamycin and starvation were effective in inducing autophagy in RAW264.7 cells overexpressing *CTNNB1* and transduced controls, shown by Western blot analysis of LC3-1 and LC3-II levels ($P < 0.001$, rapamycin versus DMSO [Figure 6(a) and (c)]; and $P < 0.001$, starvation versus normal controls [Figure 6(b) and (d)]). The increased bacterial killing effect associated with *CTNNB1* overexpression was partly reversed by rapamycin ($P < 0.01$, DMSO versus rapamycin in β-catenin clones at 1 h; $P < 0.05$, DMSO versus rapamycin in β-catenin clones at 2 h; Figure 6(e)) and starvation ($P < 0.01$, normal treatment versus starvation in β-catenin clones at 1 h; $P < 0.001$, normal treatment versus starvation in β-catenin clones at 2 h; Figure 6(f)) following *P. aeruginosa* infection.

Discussion

As a multifunctional molecule, β-catenin is reported to be involved in diverse physiological and pathological processes,[16] however, the role of β-catenin in regulating bacterial elimination remains unclear. The present study demonstrated that β-catenin promoted *P. aeruginosa* clearance, possibly via suppressing *P. aeruginosa*-induced macrophage autophagy.

Variations in β-catenin levels have been shown to differ according to pathogen type during infection, for example, levels are reduced during *Salmonella*[17] and *M. tuberculosis*[18] infection, and are increased during *Shigella dysenteriae*[19] and *Helicobacter pylori*[20] infection. The present study indicated that β-catenin levels were downregulated in an infection duration- and multiplicity of infection-dependent manner following *P. aeruginosa* infection. Nuclear β-catenin levels were increased at 6, 12, and 24 h following infection, but cytosolic β-catenin levels decreased at all time-points, suggesting that β-catenin translocated to the nucleus following *P. aeruginosa* infection.

The present results indicating β-catenin degradation suggested that β-catenin may play a role in *P. aeruginosa* infection. Thus, a cell line that overexpressed *CTNNB1* was constructed for further investigation. A previous study by the present authors indicated that β-catenin promoted *P. aeruginosa*

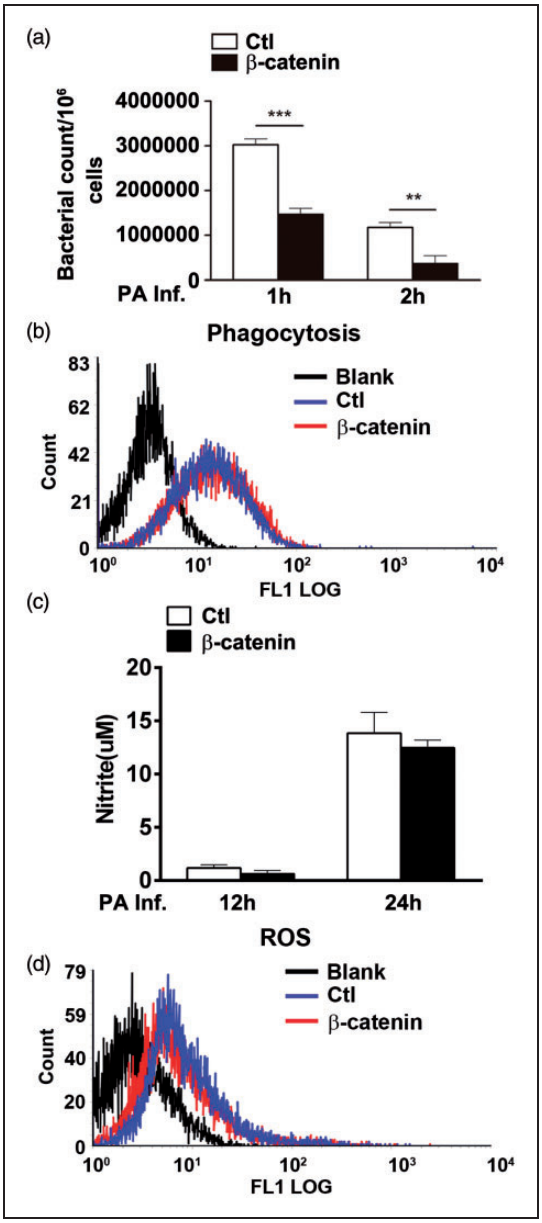

Figure 4. RAW264.7 cells transduced with β-catenin (encoded by catenin beta 1 [*CTNNB1*] gene) or controls were infected with *Pseudomonas aeruginosa*: (a) Plate counts showed that bacterial killing was increased in cells overexpressing *CTNNB1* versus controls at 1 h (***$P < 0.001$) and 2 h (**$P < 0.01$); (b) Flow cytometry analysis showing no difference in phagocytosis between cells overexpressing *CTNNB1* versus controls at 1 h following *P. aeruginosa* infection; (c) Stable end product nitrite measurements showing no difference in nitric oxide levels between cells overexpressing *CTNNB1* versus controls at 12 h and 24 h following *P. aeruginosa* infection; (d) Flow cytometry analysis showing no difference in reactive oxygen species (ROS) production between cells overexpressing *CTNNB1* versus controls at 6 h following *P. aeruginosa* infection. Data presented as mean ± SEM for triplicate experiments. PA, *P. aeruginosa*; inf., infection duration; Ctl, control; FL1, fluorescence 1.

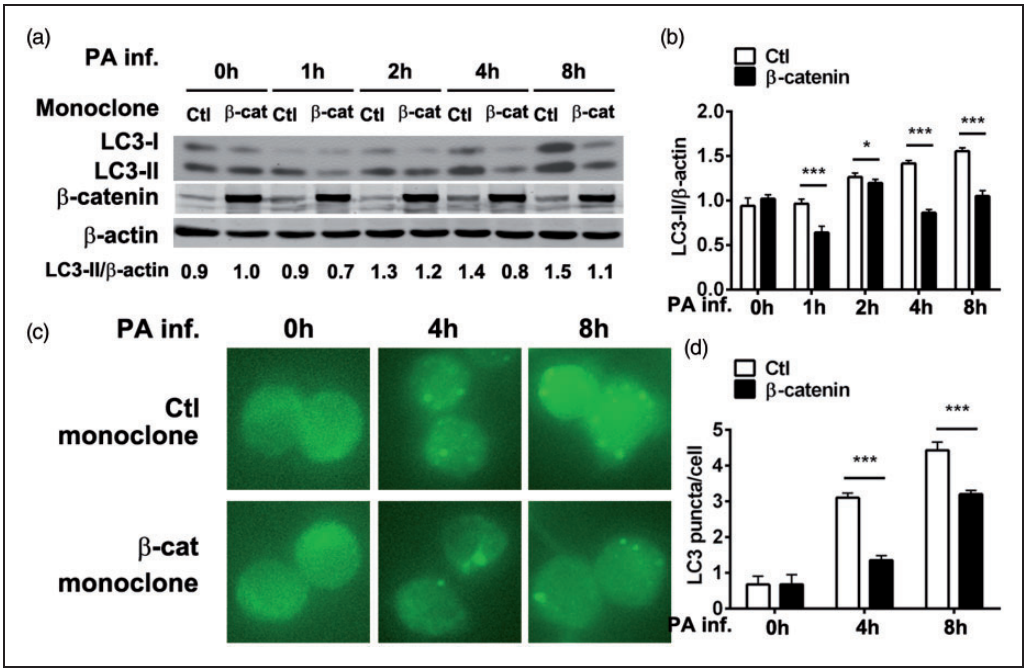

Figure 5. RAW264.7 cells transduced with β-catenin (encoded by catenin beta 1 [*CTNNB1*] gene) or controls were infected with *Pseudomonas aeruginosa* at a multiplicity of infection value of 1 and showed that β-catenin suppressed *P. aeruginosa*-triggered macrophage autophagy: (a) Representative Western blots showing microtubule-associated protein 1 light chain 3 alpha (LC3)-I, LC3-II and β-catenin protein levels with or without *P. aeruginosa* infection for 1, 2, 4 or 8 h (LC3-II:β-actin ratio shown underneath); (b) LC3 relative integrated density values normalized to β-actin, presented as ratio of control transduced cells showing lower LC3 levels in β-catenin versus control cells (*$P < 0.05$ at 2 h; ***$P < 0.001$ at 1 h, 4 h and 8 h); (c) Representative fluorescence micrograph showing endogenous LC3 stained cells following *P. aeruginosa* infection for 4 or 8 h; and (d) LC3 puncta quantified as the mean number of puncta/cell out of 100 cells per sample showing that stable overproduction of β-catenin reduced LC3 puncta formation (***$P < 0.001$ at 4 h and 8 h). Data presented as mean ± SEM of the mean for triplicate experiments. PA inf., *P. aeruginosa* infection duration; Ctl, control; β-cat, β-catenin.

clearance in bacteria infected cornea and macrophages, however, the mechanism of β-catenin in bacterial killing remained unclear.[10] The present study demonstrated that upregulated β-catenin levels promoted bacterial killing in *P. aeruginosa*-infected macrophages. β-catenin has been reported to produce ROS[9] and NO,[12] which are main components of oxygen-dependent bactericidal systems, however, β-catenin had no influence on ROS and NO production in the present study.

Autophagy plays a protective role in host defence against diverse intracellular pathogens,[6–9] and *P. aeruginosa* has been shown to induce autophagy in macrophages.[21,22] The role of autophagy in *P. aeruginosa* infection, however, remains unclear. One study demonstrated that autophagy increased bacterial killing during *P. aeruginosa* infection,[23] while another[24] found that impairment of autophagy did not influence intestinal accumulation of *P. aeruginosa*. Certainly, some researchers feel there is

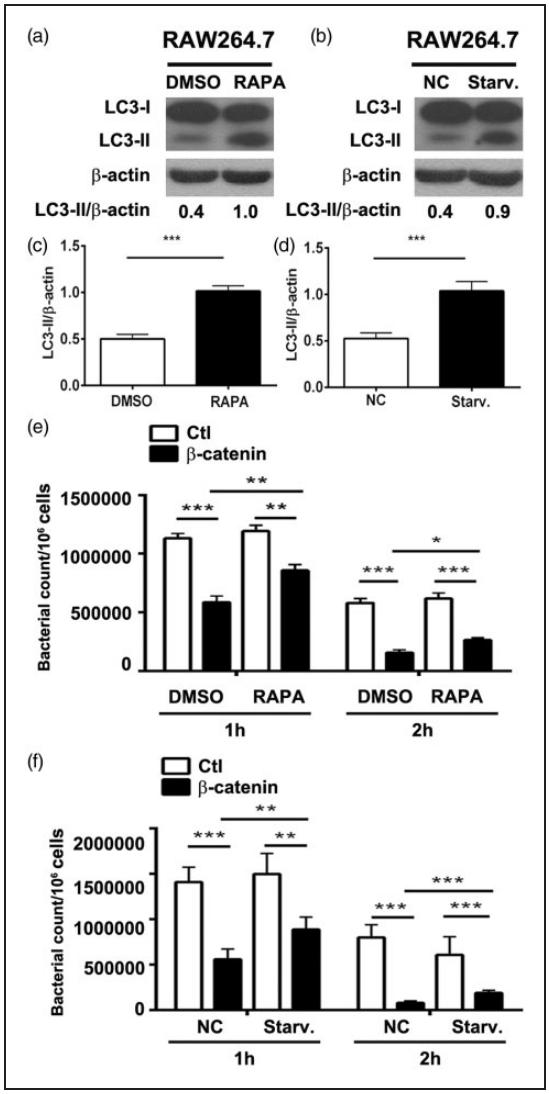

Figure 6. RAW264.7 cells transduced with β-catenin (encoded by catenin beta 1 [*CTNNB1*] gene) or control cells were infected with *Pseudomonas aeruginosa* at a multiplicity of infection value of 10 for 1 and 2 h: Microtubule-associated protein 1 light chain 3 alpha (LC3) protein levels were analysed by Western blot to confirm autophagy induction by (a and c) rapamycin and (b and d) starvation, with LC3-II:β-actin ratios shown (both $^{***}P < 0.001$ versus controls); (e) Plate counts showed significantly increased bacterial killing associated with increased β-catenin levels versus control cells in DMSO and rapamycin treated group at 1 h ($^{***}P < 0.001$ and $^{**}P < 0.01$, respectively) and 2 h (both $^{***}P < 0.001$), with a significantly reduced increase in bacterial killing following treatment with rapamycin ($^{**}P < 0.01$, DMSO versus rapamycin in β-catenin clones at 1 h; $^{*}P < 0.05$, DMSO versus rapamycin in β-catenin clones at 2 h); and (f) Plate counts showed significantly increased bacterial killing associated with increased β-catenin levels versus control cells ($^{***}P < 0.001$ and $^{**}P < 0.01$, at 1 h in normal treatment and starvation treatment group, respectively; $^{***}P < 0.001$, at 2 h), with a significantly reduced increase in bacterial killing following starvation ($^{**}P < 0.01$, normal treatment versus starvation in β-catenin clones at 1 h; $^{***}P < 0.001$, normal treatment versus starvation in β-catenin clones at 2 h); Data presented as mean ± SEM for triplicate experiments. Ctl, control; β-cat, β-catenin; RAPA, rapamycin; NC, normal control; Starv., starvation using amino acid-free EBSS medium.

strong evidence to show that autophagy plays a negative role in *P. aeruginosa* elimination.[22,25] In the present study, the increased bacterial killing effect associated with *CTNNB1* overexpression was partly reversed by rapamycin and starvation, suggesting that β-catenin promoted bacterial killing via autophagy suppression, which is consistent with the latter viewpoint.

In conclusion, the results of the present study suggest that β-catenin promotes bacterial elimination, in part through suppression of *P. aeruginosa*-induced macrophage autophagy. These results may highlight a potential therapeutic strategy for treating *P. aeruginosa* infection.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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