Enhancement of Osteogenic Induction by LL37 Modified with a Collagen-Binding Domain In Vitro and In Vivo

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Abstract
Bone defect diseases, particularly those induced by inflammation, pose a challenge for the design of ideal drug-loading scaffolds that could facilitate bone repair and eliminate inflammatory pathogens. Antimicrobial peptides LL37 is considered a promising alternative loading drug due to its broad-spectrum antimicrobial effect and various bio-functions, including osteogenic induction. In this study, we synthesized modified LL37 by adding a collagen-binding domain (CBD) to achieve specific binding to collagen and a slow release pattern. The modified peptide was indicated to exhibit similar biological activities as natural LL37 on rat BMSCs, including promotion of migration activity, anti-inflammatory activity and osteogenic induction in vitro. Ectopic bone formation experiments further confirmed the angiogenesis and osteoinduction activities in vivo. Collectively, the results indicate that LL37-CBD may be a potential loading drug for preventative and curative applications in the treatment of inflammation-induced bone diseases.

Keywords LL37 · Collagen-binding domain · Osteoinduction

Background
Inflammation-induced bone diseases such as periodontitis and rheumatoid arthritis are characterized by severe bone loss (Hardy and Cooper, 2009; Redlich and Smolen, 2012). Restoration of these inflammatory bone defects is a major clinical and economic concern. In addition to the exciting progress made in the development of bone substitutes, loading drugs that aim to enhance the bioactivity of substitutes or accelerate regeneration are attracting increasing interest. Antimicrobial peptides (AMPs) are a class of functional molecules of the innate immune system with various bioactivities. Several members of this class have been found, one of which, named LL37, has attracted increasing attention. It is the only human member of the cathelicidin family, formed from the last 37 amino acid residues of the C-terminus of human cationic antimicrobial peptide 18 (hCAP-18) (Mai et al. 2017). LL37 is widely expressed in several bodily fluids and tissues, such as the epithelium and skin (Gustafsson et al. 2010). Along with its potent and broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, LL37 also exhibits multiple bio-functions including lipopolysaccharide (LPS)-neutralizing effects, cell migration promotion, cytokine production, and wound healing (Mai et al. 2017). In recent years, LL37 has been found to accelerate bone formation by regulating angiogenesis, recruiting stem cells (Kittaka et al. 2013) and even promoting osteogenic differentiation of bone mesenchymal stem cells (BMSCs) (Yu et al. 2018). Moreover, it significantly inhibited osteoclastic bone resorption in an LPS-stimulated calvarial osteolysis model (Yu et al. 2018). These multiple biofunctions owned by LL37 indicate promising potential applications, including in bone tissue regeneration, especially when combined with infection.

The clinical translation of LL37 is limited by its susceptibility to protease degradation, potential toxicity, poor bioavailability and other limitations (Johansson et al. 1998; Ramos et al. 2011; Sieprawska-Lupa et al. 2004). Thus, many studies have focused on overcoming the above limitations, including via peptide modification or combination.
with suitable delivery systems. Typically, administration of poly (lactic-co-glycolic acid) (PLGA) nanoparticles loaded with LL37 significantly promotes wound healing compared to individual administration (Chereddy et al. 2014). In addition, LL37-nanopore titania exhibited enhanced antibacterial ability and bone formation (Shen et al. 2019). However, these vehicles displayed a comparatively poor binding pattern. Loading systems often exhibit an initial drug-releasing burst, which may lead to premature loss or unwanted side effects topically since the binding is mainly dependent on interstitial capture or electrostatic attraction (King and Krebsbach 2012; Ruszczak and Friess 2003; Wallace and Rosenblatt 2003).

Collagen is a component of the extracellular matrix (ECM) and one of the most extensively used bone scaffolds due to its excellent properties. Several previous studies have described the collagen tethering of biomolecules or antimicrobial drugs via various short sequences, named the collagen-binding domain (CBD), to achieve specific binding and slow release (Lozeau et al. 2017; Onaizi and Leong 2011; Visser et al. 2009). Visser et al. (Visser et al. 2009) produced a modified rhBMP-2 with an additional CBD and showed not only that rhBMP2–CBD had an increased affinity for collagen but also that it maintained its osteoinductive activity. Furthermore, CBD-mediated delivery of LL37 onto collagen scaffolds has been proposed (Lozeau et al. 2017). However, the potential applications of this system for bone tissue engineering were not investigated. Accordingly, we aimed to introduce LL37 modified with CBD (LL37-CBD) as a loading drug onto collagen scaffolds and explore its bone regenerating effect. It is expected that this composite substitute could exert a more durable and prominent promotion effect on bone regeneration.

Materials and Methods

LL37-CBD Synthesis

A conserved sequence derived from human vWF was selected (WREPSFCALS) (Takagi et al. 1992), and the modified antimicrobial peptide LL37-CBD was synthesized by Bioyargene Biotechnology Inc (Wuhan, China) using the solid-phase peptide synthesis (SPPS) technique. A FLAG™ domain (DYKDDDDK) linker sequence was included to preserve flexibility (Bagheri et al. 2009; Lozeau et al. 2015, 2017; Onaizi and Leong 2011). HPLC and MS were used to confirm the peptide purity and relative molecular mass, respectively.

CD Spectroscopy

CD spectra were employed to analyse the secondary structures of peptides or proteins. Spectra were obtained at 190–280 nm for 40 µM LL37 or LL37-CBD solutions in pure water or 50% (vol/vol) TFE, respectively, through a CD spectrometer (JASCO, Japan). The 50% TFE solution was used to simulate the hydrophobic environment of the cell membrane. Then, the levels of secondary structure components were analysed using DICHROWEB software.

Peptide Loading and Release

Type I collagen was extracted from rat tail tendons according to the method described in our previous study (Wang et al. 2020). In brief, the tendons were dissolved in acetic acid and centrifuged. The supernatant was dialyzed for 5 ~ 7 days in dipotassium phosphate solution. Then, the collected hydrogel was rinsed with deionized water, lyophilized to form a homogeneous paste, and transferred to a columniform mould with the same dimensions (8 mm diameter, 2 mm thickness). The morphology of the materials was examined using scanning electron microscopy (SEM, JSM6460, JEOL, Japan).

The solidified collagen scaffolds were immersed in peptides (LL37 or LL37-CBD) dissolved in sterile phosphate-buffered saline (PBS, pH 7.4) and rotated on a rotator (Heidolph Duomax 1030, Germany) for 2 h. Subsequently, the collagen scaffolds were rinsed twice with PBS, which was added to the corresponding immersed solution. An LL37 ELISA Kit (CUSABIO Biotech, Wuhan, China) was used to measure peptide concentrations before loading and after rinsing, and peptide binding was evaluated. Then, the scaffolds with either LL37-CBD or LL37 were immersed in release medium (sterile PBS supplemented with 1% (wt/v) bovine serum albumin) and rotated on a rotator. At specific time points, the release medium was collected and replaced with fresh medium. Peptide concentrations in the collected medium were measured using an ELISA Kit, which can be compared with the initial amount of peptide absorbed to determine cumulative release.

Isolation and Culture of BMSCs

BMSCs were prepared as previously described (Li et al. 2013). Briefly, under sterile conditions, both the tibia and femur bones of 3-week-old Sprague–Dawley rats were isolated, with careful removal of the attached muscles thereafter. Then, the marrow was flushed out by using Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DMEM/F-12, Gibco, CA, USA) containing 10% foetal bovine serum (FBS, Gibco, CA, USA), 2 mmol/L
l-glutamine and 100 U/ml penicillin/streptomycin. The cell suspension was seeded and then cultured at 37 °C with 5% CO₂. Nonadherent cells were removed by replacing the medium with fresh medium after 24 h. Adherent cells were passaged with TrypLE. In this study, cells at passages 3 ~ 5 were employed.

Cell Proliferation Assay

To investigate the effects of LL37-CBD on the cellular proliferation of BMSCs, the CCK-8 assay was performed using the Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. First, different concentrations of LL37 or LL37-CBD were administered to the culture medium of the BMSCs (1 × 10⁵ cells/well) for 12, 24 and 48 h prior to adding the CCK-8 solution. Then, the optical density (OD) of the CCK-8 reagent was measured at 450 nm with a microplate reader (Infinite 200, Tecan, Switzerland). The group with BMSCs alone was used as a control group.

Migration Assay

Transwell plates (8 μm pore size; Costar, Corning, NY, USA) were used to perform the cell migration assay in accordance with the manufacturer’s recommendations. Briefly, 100 μl of a serum-free suspension (1 × 10⁵ cells/ml) of BMSCs was seeded into each upper well. Then, 6 μM LL37-CBD or LL37 or an equal amount of solvent was placed in the upper wells as well. Growth medium with 10% serum was added to the lower well. After incubation for 6 h at 37 °C, the lower cells were fixed with 4% paraformaldehyde for 15 min and incubated with 0.1% crystal violet to stain the migrated cells. Then, the cells in five random microscopy fields were counted using an inverted microscope.

LPS-Neutralizing Activity

BMSCs (5 × 10⁴ cells) were cultured in 12-well plates. LPS (Invitrogen, Carlsbad, CA, USA) from Porphyromonas gingivalis at 1 μg/ml with or without LL37-CBD or LL37 (1 μM and 5 μM) was added to each well, and the cells were then cultured at 37 °C. Untreated culture media were used as a control group. At specific time points (12 h, 24 h, 48 h), supernatants were collected and employed to measure the release of IL-1 and TNF-α by ELISA kits according to a protocol provided by the manufacturer (R&D Systems). The experiment was performed in triplicate.

Alizarin Red Staining

BMSCs at passage 3 (1 × 10⁵ cells/ml) were seeded in 6-well plates, and 6 μM LL37-CBD or LL37 was administered to the osteogenic medium, followed by changing the medium every 3 days. After 14 days of osteogenic induction, the cells were fixed with 4% paraformaldehyde for 15 min and incubated with alizarin red (Cyagen, USA) for 10 min. Excess stain was eliminated by three washes using PBS.

ALP Activity Determination

After osteogenic induction for 7 and 14 days, the BMSCs were treated with Triton for 30 min, and the supernatants were collected. ALP activity was tested according to the manufacturer’s protocol for the assay kit (Jiancheng, Nanjing, China). The product was detected by evaluating the absorbance at 520 nm using a microplate reader.

Real-Time PCR

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated by reverse transcription using 2 μg of total RNA and PrimeScriptTM RT Master Mix (TaKaRa, Kusatsu, Japan) followed by amplification with SYBR Premix Ex Taq II (TaKaRa, Kusatsu, Japan), which was carried out on a Light Cycler 480 (Roche, Basel, Switzerland). The specific oligonucleotide primers used are provided in Table 1. The cycling profile for the real-time PCR (45 cycles) was as follows: 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 60 s. The relative expression of genes was normalized to that of GAPDH. Three independent samples in each group were analysed, and three parallel repeats were analysed for each sample.

Table 1 Primers used for real-time polymerase chain reaction

| Gene | Forward primer sequence (5′–3′) | Reverse primer sequence (5′–3′) |
|------|--------------------------------|--------------------------------|
| ALP  | TATGGCTCACCTGCTTCACG | GCTGTCCATTTTGGGCTCTTG |
| Runx2| TTAGGGCGATTCCTCATCC | GTCAGAAGTTGGCAGTTGCAT |
| OCN  | GCCCTGACTGCTTCTGCCTCT | TCACCACCTTACTGCCCCCTCCT |
| GAPDH| GCAAGTTCAACGGCACAG | GCCAGTGAAGCTCAGCACAT |
Establishment of Ectopic Subcutaneous Models

Twenty-four male Sprague–Dawley rats (250 ~ 300 g in weight) were used. The rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (120 mg/kg). The hair around the area for incisions was shaved, and the operative regions were disinfected with povidone iodine. Subcutaneous implantation was performed through four longitudinal incisions near the midline of the dorsal skin (Fig. 1). Then, subcutaneous pockets were prepared with a blunt instrument and received implants from four different groups (Group 1, nonloaded collagen scaffold; Group 2, 0.4 nmol/mg LL37-loaded scaffold; Group 3, 0.4 nmol/mg LL37-CBD-loaded scaffold; Group 4, 0.9 nmol/mg LL37-CBD-loaded scaffold). The wounds were closed with absorbable sutures after implantation. Postoperative intraperitoneal injection of penicillin was performed to prevent infection (8 × 10^4 U/each). The animals were sacrificed after 8 weeks. The subcutaneous implant samples were separated and fixed in 4% paraformaldehyde for subsequent histological assays.

Histological Assays

After embedding the samples in paraffin, 5-μm-thick serial sections were prepared. HE staining was performed according to the instructions. Briefly, cell nuclei were stained with Mayer’s haematoxylin, and cell plasma and extracellular matrix were stained with eosin.

For immunohistochemical staining, osteogenic markers (Runx2 and OCN) were detected. Selected slides were dewaxed and rehydrated, and endogenous peroxidase activity was eliminated. Non-specific proteins were blocked with 3% BSA for 30 min. Then, the slides were incubated with Runx2 antibodies (Affinity) and OCN antibodies (Servicebio) overnight at 4 °C. Secondary antibody (HRP-labelled) incubation was performed after removing the primary antibodies. The antibody complexes were visualized by diaminobenzidine (DAB) chromogenic agent, and the images were captured using a slice scanner (Aperio AT2, Leica). The quantification procedure was conducted by Aperio ImageScope software.

Statistical Analysis

All data were analysed using the SPSS software package and are expressed as the mean ± standard deviation (SD) with three replications. The level of significance was determined by one-way analysis of variance (one-way ANOVA) followed by a Bonferroni post hoc test for multiple comparisons. A p value < 0.05 was considered statistically significant.
Results

Synthesis of LL37-CBD and Preparation of the Collagen Scaffold

The modified peptides were functionalized at their C-terminus with the CBD selected from human von Willebrand factor (vWF) and synthesized at 95.22% purity, as confirmed by high-performance liquid chromatography (HPLC). Mass spectrometry (MS) revealed that the relative molecular mass of LL37-CBD was 6665.64, which was largely consistent with the calculated estimate. A pure collagen scaffold was prepared as a round disk with an 8 mm diameter and 2 mm thickness (Fig. 2).

Secondary Structures of LL37 Peptides with Altered CBD Sequences

The amphiphilic secondary structures were investigated via circular dichroism (CD) spectroscopy, as shown in Fig. 3. In pure water, the spectra of LL37-CBD and LL37 nearly overlapped, indicating similar secondary structures in aqueous solution for these two peptides. In 50% trifluoroethanol (TFE) solution, both spectra exhibited relatively steep peaks compared to the spectra in water at 208 and 222 nm (red arrow), indicating that more significant alpha helices would be formed in this membrane-like environment. The levels of specific secondary structures (helix, beta strand, turn, random) were identified based on CD spectral data. In aqueous solution, there were slight differences between LL37 and LL37-CBD in the secondary structures content. The levels of helices were significantly increased in 50% TFE. Similar levels could be calculated for the helix and turn structures, while differences could be seen in the levels of beta strand and random structures in 50% TFE between LL37 and LL37-CBD.

Modification with the CBD Increases the Amount of LL37 Adsorbed and Improves Retention on Collagen

The collagen-binding abilities of LL37-CBD and unmodified LL37 were evaluated by ELISA. With the CBD modification at its terminus, we hypothesized that modified LL37 would be more specifically adsorbed on collagen and cause slower release over time. As shown in Fig. 4a, after 2 h of incubation, a significantly higher amount of LL37-CBD had been adsorbed on the collagen than of unmodified LL37. Subsequently, we measured the cumulative release curves for both peptides (Fig. 4b). In general, the total cumulative release of LL37-CBD and LL37 was 10.8% and 38.4%, respectively, after 2 weeks. Interestingly, the released amounts in the initial 12 h were the most significant for both groups. Except for this initial rapid release profile, the overall release curve was almost horizontal for LL37-CBD group. Additionally, greater amounts of LL37 were released between each time interval than of LL37-CBD ($p < 0.05$).

Fig. 2 Prepared collagen scaffold. The pure collagen scaffold was prepared as a round disk (a). SEM images of the morphology of the collagen scaffolds (b). Scale bar = 100 μm

Fig. 3 Circular dichroism (CD) analysis of LL37 and LL37-CBD in pure water and 50% TFE. Representative CD spectra for 40 μM LL37 and LL37-CBD (①: LL37 in 50% TFE, ②: LL37-CBD in 50% TFE, ③: LL37-CBD in water, ④: LL37 in water) (a). Based on the CD spectrum results, the levels of secondary structures, including helix, beta strand, turn and random structures, were calculated (b) (Color figure online)
LL37-CBD Exhibits Less Cytotoxicity than and Similar Migration Activity as LL37

We presumed that modification with CBD may not cause excessive changes in its biological activity. Thus, we first used the CCK-8 assay to evaluate the influence of LL37-CBD on cell viability (Fig. 5). As expected, LL37-CBD exhibited similar proliferation on BMSCs as LL37 at concentrations below 10 μM. Interestingly, at a concentration of 14 μM, LL37-CBD displayed less cytotoxicity than LL37 (p < 0.05). Additionally, compared to the control group, a significantly greater number of cells in the 6 μM LL37-CBD treatment group migrated into the lower well of the Transwell plate (Fig. 6a–c). Quantitatively, there was a significant difference between the control group and the other two groups (p < 0.05) (Fig. 6d).

Inhibition of LPS-Induced Cytokine Expression

BMSCs were stimulated with LPS (1 μg/ml) with or without the peptides (1 or 5 μM), and the changes in cytokines were determined by ELISA kits. Compared with the control group, quantitative results showed that 1 μM LL37-CBD or LL37 partially inhibited cytokine expression induced by LPS, while 5 μM LL37 caused a marked decrease in TNF-α and IL-1 expression, which almost reached the control level (Fig. 7). The results were correlated with the potency of the peptides, even that of the peptide modified with CBD to inhibit the binding of LPS to BMSCs.

LL37-CBD Shows a Similar Ability as LL37 to Promote Osteogenic Differentiation of BMSCs In Vitro

LL37 enhanced the osteogenic differentiation of BMSCs in our previous research. As shown in Fig. 8, the LL37-CBD and LL37 treatment groups showed stronger and more extensive alizarin red staining of calcium nodules than the control group. Osteogenic differentiation was further confirmed by the determination of alkaline phosphatase (ALP) and osteogenesis-related mRNA expression assayed by RT-PCR. ALP expression was detected at 7 and 14 days of induction, exhibiting enhanced expression levels compared with the controls at both time points (Fig. 9). The relative expression of bone formation-related genes was detected by RT-PCR. ALP, OCN and Runx2 were increased in the LL37-CBD and LL37 groups at both 3 and 7 days of induction (Fig. 10).

Histological Analysis

In the 4-week groups, the implants exhibited preservation of a certain fraction of the raw materials, while in the 8-week groups, the samples were almost completely degraded and absorbed. The infiltration of inflammatory cells was evident. The effect of LL37 on angiogenesis could be verified since the experimental groups formed more new blood vessels than the control group. Among the experimental groups, the groups supplemented with LL37-CBD appeared to promote more evident angiogenesis (Fig. 11).

Furthermore, osteoinduction was identified by immunohistochemical staining. As a significant marker of osteogenic differentiation, the expression of OCN and Runx2 was detected. Although there was no obvious sign of new bone matrix formation, the results showed that positive staining for OCN and Runx2 was visible in the LL37 groups and most obvious in the LL37-CBD groups. There was virtually no obvious positive staining in the control groups at 4 weeks. Additionally, the differences were further validated via quantitative analyses, which showed increasing expression in the LL37-CBD groups, while no significant difference existed between the 0.4 and 0.9 nmol/mg LL37-CBD groups (Fig. 12).
Discussion

As expected, the CBDs allowed specific binding onto I-type collagen, in accordance with other similar studies (Lozeau et al. 2017; Visser et al. 2009). Despite the desirable slow-release pattern, as shown, the release amount in the initial 12 h was 64.3% and 32.8% of the total 2-week cumulative release for the LL37-CBD and LL37 groups, respectively. This may be explained by the fact that most of the initial release was of extra peptides detained on the outer surface via interstitial capture. The differences between the two groups were significant, which may indicate the application potential for loading drugs for implanted scaffolds. Instead of premature and excessive release, the loading peptides modified with CBDs could be released gradually into the surrounding extracellular matrix along with the gradual degradation of collagen.

It is crucial that the addition of the CBD domain does not interfere with the original biofunction. A great number of studies on CBD-mediated collagen attachment have included growth factors (Lin et al. 2006; Takagi et al. 1992; Zhao et al. 2007), bone morphogenetic protein (BMP) (Lu et al. 2012; Visser et al. 2009) and streptavidin (Sistiabudi and Ivanisevic 2008), most of which confirmed that the modified molecule could retain its major activities even with CBD linking. For some macromolecules, modification with CBD may cause unexpected changes or even loss of their original biological activity. The reasons were speculated to be that auxiliary modification may interfere with the characteristic spatial configuration of macromolecules such as epidermal growth factor (EGF) (Hall et al. 2000; Nishi et al. 1998). From this perspective, the choice of small-molecule compounds is another option. In our study, we found that LL37-CBD exhibited similar proliferative and chemotactic activity towards BMSCs as unmodified LL37 at low concentrations. LL37-CBD displayed less cytotoxicity at a peptide concentration of 14 μM. The most widely accepted mechanism for the cytotoxicity or antimicrobial action of AMPs is permeabilization followed by membrane disruption (Brogden 2005; Xhindoli et al. 2015). This insertion into the bilayers depends on the random C-terminus (Dannehl et al. 2013). From the CD results, in the 50% TEF solution, modification with the CBD sequence mainly decreased the random structure, which may account for the lower cytotoxicity at high concentrations of LL37-CBD. On the other hand, many studies have tried to identify the essential region of LL37 that is responsible for the bioactivity or antimicrobial effect. Interestingly, it was found that the N-terminal fragment is more likely to be involved in immunoregulation, chemotaxis, peptide oligomerization and proteolytic resistance (Murakami et al. 2004; Oren et al. 1999). Therefore, in this study, CBD was added to the C-terminal tail; thus, the chemotactic effect and proliferative effect were not altered significantly.

Several studies have found that LL37 shows a strong binding affinity and neutralizing capacity for LPS through electrostatic and hydrophobic interactions (Cowland et al. 1995; Larrick et al. 1995; Turner et al. 1998), which is physiologically relevant since LPS composes the external structure of the membrane for most Gram-negative bacteria. Additionally, common inflammatory mediators, such as TNF-α, IL-1β and RANKL, can be over-secreted under
induction by LPS (Tang et al. 2011), leading to most inflammatory conditions that may advance to inflammatory bone diseases or septic shock. It is the concave hydrophobic surface of LL-37, consisting of phenylalanine located at positions 5, 6, 17 and 27, that directly recognizes and binds to LPS (Oren et al. 1999; Porcelli et al. 2008). Moreover, as shown by other research (Rosenfeld et al. 2006), the neutralizing interaction between LL37 and LPS is not enough; it
is also necessary to dissociate the LPS aggregates, as well as compete with them for the CD14 receptor in the cell. In addition, it was speculated that LL37 may exert its neutralization effect by regulating the NF-κB pathway induced by activation of LPS-binding Toll-like receptors (Mookherjee et al. 2006). Therefore, it is remarkable that modification with the CBD does not affect the bioactivity of LL37 or even the ligand-receptor binding capacity. LL37-CBD maintained a similar neutralization capacity against LPS, and at a concentration of 5 μM, these peptides almost completely inhibited the secretion of TNF-α and IL-1β induced by 1 μg/ml LPS, suggesting the potential application of LL37-CBD in inflammation-related bone repair.

The P2X7 receptor (P2X7-R) is a ligand-gated plasma membrane ion channel (Burnstock 2006). It is generally agreed that ATP is the only physiological agonist. Over the last several years, increasing evidence has suggested that ATP might not be the only agonist, especially at inflammatory sites (Di Virgilio et al. 2018). P2X7-R can be activated by various agonists, including LL37 (Adinolfi et al. 2005; Elsner et al. 2004; Tomasinsig et al. 2008), via poorly understood mechanisms of action. It has been confirmed that the osteoblastic differentiation of BMSCs is mainly mediated by P2X7-R (Ke et al. 2003; Li et al. 2015; Noronha-Matos et al. 2014; Sun et al. 2013). This was demonstrated by several classic experiments, which used different kinds of receptor agonists of P2X7-R to achieve osteoblastic differentiation (Li et al. 2015; Sun et al. 2013). Based on these studies, our previous research (Yu et al. 2018) also found that LL37 can promote osteoblastic differentiation via the activation of P2X7-R. The exact binding mechanism between LL37 and P2X7-R remains unknown. It was assumed that the functional interaction between them involves transmembrane segment-mediated binding, which, interestingly, occurred irrespective of their chirality or helix sense (Tomasinsig et al. 2008). As shown in our results, LL37-CBD displayed similar osteogenic activity in vitro, indicating that CBD does not interfere with the ligand-receptor binding capacity and osteogenic activity of LL37.

The in vivo osteoinductivity of LL37 has not been previously proven. With the positive results achieved from the in vitro experiments, an ectopic bone formation experiment was employed to further verify the osteoinductivity of LL37. It stands to reason that the initial vascularization formed within the implant can provide indispensable nutrients and maintain tissue viability. LL37 was found to assist in vascularization by recruiting vascular endothelial precursor cells to the damaged area and promoting cell proliferation. Furthermore, LL37 can directly induce angiogenesis mediated by formyl peptide receptor-like 1 expressed on endothelial
cells (Koczulla et al. 2003). The Haematoxylin and eosin (HE) staining results showed that only the implants with LL37-CBD displayed significant signs of angiogenesis. Similarly, immunohistochemical staining showed that both the high and low dosages of LL37-CBD caused a significant increase in OCN and Runx2 expression-positive areas, which was consistent with the preceding results. The angiogenic and osteogenic effects were impaired in

Fig. 10 mRNA expression by quantitative real-time RT-PCR at 3 days and 7 days. Treatment with LL37-CBD or LL37 caused an increase in the expression of ALP, OCN and Runx2 compared with the control group (*p<0.05)

Fig. 11 HE staining of the implants from different groups at 4 weeks and 8 weeks. A significant amount of bone matrix was unobservable in all groups. The implants with LL37-CBD displayed a more significant vascular scattered distribution (green arrows) than those with LL37 or the control groups. Scale bar = 50 µm (Color figure online)
the unmodified LL37 groups due to premature loss of the peptides. Retention of peptides onto collagen guarantees a sustained pattern, allowing peptides to be gradually released along with the degradation of scaffolds while simultaneously reducing degradation caused by protease from circulation or tissue fluid. These results illustrate that LL37-CBD can maintain good bioactivities in vivo, which is crucial in bone tissue engineering. A significant amount of newly formed bone matrix was unobservable, which might result from the relatively fast degradation of the collagen scaffolds.

**Conclusion**

In this work, we demonstrated that addition of the CBD achieved more durable retention of collagen and did not modify the biological activities of native LL37, including osteogenic induction, in vitro. Furthermore, ectopic bone formation experiments confirmed the osteoinduction performance of the LL37-CBD-loaded collagen scaffold. The versatile biological properties of LL37-CBD suggest the applicability of modified LL37 for bone tissue engineering, especially for inflammatory bone defects.

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**Data Availability** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no competing interests in this section.

**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The
in vivo experimental protocols were reviewed and approved by the Ethics Committee of Sun Yat-Sen University, China (Approval No. SYSU-IACUC-2019-000327).

Research Involving Human and Animal Participants This article does not contain any studies with human participants performed by any of the authors.

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