Activity of LL-37, CRAMP and antimicrobial peptide-derived compounds E2, E6 and CP26 against *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) is a major worldwide health problem in part due to the lack of development of new treatments and the emergence of new strains such as multidrug-resistant (MDR) and extensively drug-resistant strains that are threatening and impairing the control of this disease. In this study, the efficacy of natural and synthetic cationic antimicrobial (host defence) peptides that have been shown often to possess broad-spectrum antimicrobial activity was tested. The natural antimicrobial peptides human LL-37 and mouse CRAMP as well as synthetic peptides E2, E6 and CP26 were tested for their activity against *Mycobacterium tuberculosis* both in in vitro and in vivo models. The peptides had moderate antimicrobial activities, with minimum inhibitory concentrations ranging from 2 µg/mL to 10 µg/mL. In a virulent model of *M. tuberculosis* lung infection, intratracheal therapeutic application of these peptides three times a week at doses of ca. 1 mg/kg led to significant 3–10-fold reductions in lung bacilli after 28–30 days of treatment. The treatments worked both against the drug-sensitive H37Rv strain and a MDR strain. These results indicate that antimicrobial peptides might constitute a novel therapy against TB.

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1. Introduction

Tuberculosis (TB) [1], caused by the bacterium *Mycobacterium tuberculosis*, remains one of the leading causes of disease and mortality due to an infectious agent. According to recent data from the World Health Organization (WHO), in 2010 there were 8.8 million active TB cases worldwide and nearly 1.5 million deaths. It has been estimated that one-third of the human population carries *M. tuberculosis* and 10% of these people will develop active disease at some time in their lives, creating an enormous reservoir [2].

Treatment of pulmonary TB has become increasingly challenging due in part to the required long duration of therapy and the advent of multiple drug resistance. One of the most important factors is the emergence of multidrug-resistant (MDR) bacilli that has been associated with inadequate use of antibiotics and poor adherence to recommended treatment regimens [3]. In recent years, new strains have emerged, termed extensively drug-resistant (XDR), that are also resistant to second-line antibiotics such as fluoroquinolones and either kanamycin, amikacin or capreomycin. These strains lead to poor treatment outcomes and a considerably increased rate of mortality [4]. Recent reports suggest the possible existence of cases of completely resistant TB in the Middle East, raising concerns regarding how to treat these TB cases effectively [5].

In the past 40 years, no broadly successful new TB drug has been developed. Therefore, there is a strong drive to develop new treatments for TB and/or to improve those currently in use. Important advances have been made and there are several clinical trials underway that have utilised fluoroquinolones in place of ethambutol, leading to preliminary indications of a significant reduction in the duration of therapy and encouraging the possibility of an improvement in patient survival [6].

Antimicrobial peptides (AMPs) are gene-encoded, amphipathic, cationic peptides that are produced by several species of mammals, birds, reptiles and amphibians. These peptides can inhibit microbial growth through a variety of often complex mechanisms, including membrane interactions that lead to permeabilisation of cells, inhibition of cell wall synthesis, and entry into cells leading to inhibition

of macromolecular synthesis [7–9]. In addition, these peptides, also termed host defence peptides, can profoundly and favourably modulate innate immunity, upregulating protective immunity such as increasing the production of chemokines to recruit immune cells whilst dampening potentially harmful inflammation [9,10]. The major groups of AMPs in humans are the defensins and a single cathelicidin, LL-37. It has been reported that alterations in the production of these molecules increase susceptibility to infectious diseases, including TB [11]. Conversely, upregulation of cathelicidin LL-37 through use of vitamin D supplementation has been considered to be a potential strategy to improve TB infection outcomes, although current data do not necessarily favour this possibility [12].

Previous studies by our group have reported that during *M. tuberculosis* infection of lung epithelial cells, there was a high production of β-defensins-3 and -4, and both were associated with mycobacteria in the lung, suggesting their possible participation in clearance of *M. tuberculosis* [13,14]. Subsequently, it was reported that in murine TB models, BALB/c mice produced low quantities of murine β-defensins-3 and -4 during late progressive TB, and when both defensins were overproduced by intratracheal administration of isoleucine (a defensin inducer) these animals efficiently controlled infection both by drug-sensitive and drug-resistant bacilli [14,15]. In addition, it has been shown that the interaction of a 19-kDa lipopeptide of *M. tuberculosis* with Toll-like receptor-2 on the macrophage surface upregulated the expression of vitamin D receptor leading to the induction of cathelicidin LL-37, promoting the killing of intracellular *M. tuberculosis* [16,17].

Recently, methodologies have been developed to enable the enhanced design of AMPs (e.g. [18]). Rational substitution studies led to an enhanced 26-amino-acid β-helical peptide CP26 derived from a hybrid peptide comprising the amphipathic α-helical N-terminal region of cecropin A and the hydrophobic N-terminal α-helix of the bee venom peptide melittin [17]. Peptide array methods and substitution studies, starting from the smallest known broad-spectrum natural AMP bactenecin (also known as bovine dodecapeptide), led to peptides E2 (also known as Bac8c), an 8-amino-acid peptide, as well as E6 (also called Sub3), a 12-amino-acid peptide, both of which demonstrated enhanced activity against a range of pathogenic Gram-positive and Gram-negative bacteria and the yeast *Candida albicans* [18,19].

In this study, the antimicrobial activity of five natural and synthetic peptides against *M. tuberculosis* was evaluated in an in vitro setting.

2. Materials and methods

2.1. Peptide synthesis and design

Peptides were synthesised by the Peptide Synthesis Facility, Biomedical Research Centre at the University of British Columbia (Vancouver, Canada) using tertiary butyloxycarbonyl (tBOC) solid-phase synthesis. Peptides were purified by high-performance liquid chromatography to >95% purity and were confirmed by mass spectrometry.

The following peptides were utilised: mouse CRAMP (GLL-RKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ) [20]; human LL-37 [20,21] (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES); E2 (also termed Bac8c; RIWVIWRR-NH₂) [18,19]; E6 (also termed Sub3; RRWRIVVIRVRR-NH₂) [18,19]; and CP26 (KWKSFIKKLT-SAAKKVVTTAKPLISS) [22]. Briefly, these peptides were selected as either natural peptides with moderate antimicrobial activity (LL-37 and CRAMP) or as broad-spectrum synthetic peptides with moderate to good antimicrobial activity (E2, E6 and CP26).

2.2. Mycobacterium tuberculosis strain growth

The drug-sensitive *M. tuberculosis* strain H37Rv (ATCC) and a MDR strain (clinical isolate, resistant to first-line antibiotics) were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% glycerol, 10% oleic acid–albumin–dextrose–catalase (OADC enrichment media; Becton Dickinson, Franklin Lakes, NJ) and 0.02% Tween 80 at 37 °C. Mid-log phase cultures were used for all experiments.

2.3. Microdilution colorimetric reduction assay

Susceptibility testing utilising resazurin (Trek Diagnostic, Westlake, OH) as an indicator of residual bacterial viability was performed in Costar® 96-well flat-bottom plates (Corning Inc., Corning, NY) as described previously [23]. Briefly, all test wells contained 100 μL of OADC-supplemented Middlebrook 7H9 growth medium. Then, 100 μL of the diluted peptide at the highest concentration starting at 12.8 μg/mL was added to one well. The contents of the wells were mixed thoroughly and 100 μL was transferred into the next well; the process was then repeated, thus creating serial two-fold dilutions. In addition to the tested peptides, rifampicin (8.0 μg/mL) was used as a positive control, and medium without any compound was used as a negative control in each plate. Peptides were tested in the concentration range 0.4–12.8 μg/mL.

Plates were incubated at 37 °C for 5 days. On Day 5, 20 μL of 0.01% resazurin solution and 12 μL of sterile 10% Tween 80 solution were added to several control wells containing *M. tuberculosis* but no antibacterial agent and plates were incubated again for 24 h under the same conditions. If the *M. tuberculosis* viability controls tested positive for resazurin reduction, resazurin was added to all wells. The minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration that prevented the reduction of resazurin and therefore a colour change from blue to pink. Previous studies by our group suggest that some AMPs may induce dormancy or a bacteriostatic state in *M. tuberculosis* [24]. To examine this, 10 μL from the lowest concentration that did not reduce resazurin was serially diluted and seeded onto 7H10 agar plates supplemented with Middlebrook OADC enrichment media and incubated for ≥21 days at 37 °C to observe whether *M. tuberculosis* re-growth occurred.

2.4. Experimental model of progressive pulmonary tuberculosis in BALB/c mice

The experimental model of progressive pulmonary TB has been described in detail elsewhere [25]. Briefly, male BALB/c mice aged 6–8 weeks were anaesthetised in a gas chamber using 0.1 mL per mouse of sevoflurane, and each mouse was infected by endotracheal instillation with 2.5×10^5 live bacilli. Mice were maintained in the vertical position until they underwent spontaneous recovery. Infected mice were maintained in groups of five in cages fitted with micro-isolators. Animal work was performed in accordance with Mexican national regulations on Animal Care and Experimentation (NOM 062-ZOO-1999).

2.5. Treatment of infected mice with peptides

After 60 days of infection, animals were arbitrarily allocated into four groups. Peptide treatment started 60 days after infection, when advanced progressive disease was well established. In the first experiments conducted to determine the in vitro MIC of each peptide, it was determined that doses at or near to 3.2 μg/mL for all peptides were able to kill *M. tuberculosis*. Thus, a dose of 32 μg in 100 μL of saline solution (ca. 1 mg/kg) was used for the therapeutic experiments. Three independent experiments were performed. All

groups of animals received the corresponding dose three times a week for up to 4 weeks by intratracheal instillation, since preliminary studies indicated no efficacy via the intraperitoneal delivery route. Six animals in each group were sacrificed at 7, 14 and 28 days after starting treatment. The efficiency of each peptide treatment was determined by quantifying the lung bacillary loads by assessing CFUs and the extent of tissue damage by histopathology.

2.6. Determination of CFUs in infected lungs

Lungs were homogenised with a Polytron® homogeniser (Kinematica, Lucerne, Switzerland) in sterile tubes containing 1 mL of 0.05% Tween 80 in phosphate-buffered saline (PBS). Five dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Laboratories) enriched with OADC-enriched medium (Becton Dickinson, Sparks, MD). The number of colonies was counted after 21 days of incubation.

2.7. Preparation of lung tissue for histology

The lungs from each of three different animals per time point and group were perfused intratracheally with ethyl alcohol (J.T. Baker, Mexico City, Mexico). Lungs were then dehydrated and embedded in paraffin (Oxford Labware, St Louis, MO), sectioned and stained with haematoxylin and eosin. The percentage of the lung surface affected by pneumonia was determined using an automated image analyser (Axiovert M200; Carl Zeiss, Oberkochen, Germany).

2.8. Ultrastructural analysis of treated Mycobacterium tuberculosis

Determination of the ultrastructural damage to *M. tuberculosis* caused by treatment with the different AMPs was evaluated using transmission electron microscopy. Briefly, bacilli were cultured in Middlebrook 7H9 broth (Difco Laboratories) supplemented with Middlebrook OADC enrichment media (BBL; BD, Franklin Lakes, NJ) until logarithmic phase was achieved. Viable bacilli (1×10^7) were placed in the wells of 96-well plates and were exposed to the corresponding AMP for 18 h at the MICs determined previously using the resazurin assay. Subsequently, fixation was performed with 4% paraformaldehyde in PBS and the fixed bacilli suspension was treated with 0.05 mM NH_4Cl in PBS to block free aldehyde groups. The bacterial suspension was then centrifuged to form a pellet that was later dehydrated with graded ethyl alcohol solutions and embedded in LR White hydrosoluble resin (London Resin Company, London, UK). Thin sections of 70–90 nm width were placed on nickel grids and were contrasted with uranium salts (Electron Microscopy Sciences, Fort Washington, PA) and were examined with a Zeiss M-10 electron microscope (Carl Zeiss).

2.9. Statistical analysis

Data were analysed by parametric two-way analysis of variance (ANOVA) with Tukey's post-test or a non-parametric Kruskal–Wallis multiple comparisons test with Dunn's post-test. GraphPad 5.02 software (GraphPad Inc., La Jolla, CA) was used to perform the analysis. For all analyses, a *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Antimicrobial activity of CRAMP, LL-37, E2, E6 and CP26 in vitro

For pre-clinical testing of antimycobacterial drugs, the most versatile and efficient technique utilises resazurin for determining residual *M. tuberculosis* viability [23].
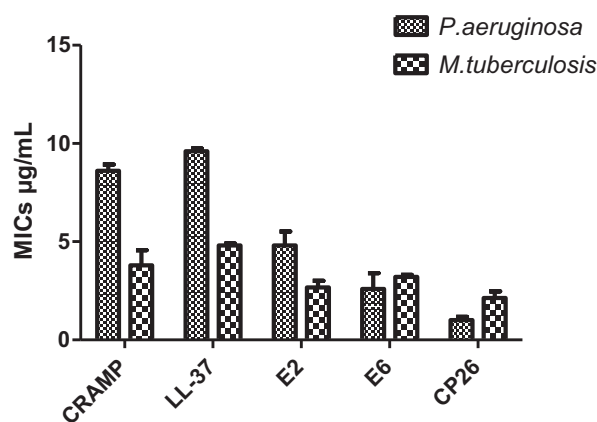

Fig. 1. The effect of antimicrobial peptides (AMPs) human LL-37, mouse CRAMP, E2, E6 and CP26 on the growth of *Mycobacterium tuberculosis*. *Mycobacterium tuberculosis* strain H37Rv was incubated with increasing concentrations of the indicated AMP to determine the minimum inhibitory concentration (MIC). Data are expressed as the mean ± standard deviation of three independent experiments, each performed in duplicate.

This assay was performed here to evaluate the capacity of selected AMPs to inhibit the growth of *M. tuberculosis* strains. Fig. 1 shows that all peptides had strong antimicrobial activity against *M. tuberculosis*, with CP26 being the most efficient (MIC = 2.1 ± 0.33 µg/mL), followed by E2 and E6 (MICs = 2.6 ± 0.34 µg/mL and 3.2 ± 0.10 µg/mL, respectively). Interestingly, these three optimised synthetic peptides all showed higher activity than the natural human (LL-37) and mice (CRAMP) cathelicidins, with 1.5–2-fold lower MICs. The fast-growing bacteria *Pseudomonas aeruginosa* was included as a control under the same conditions, showing similar results as those obtained for *M. tuberculosis*.

3.2. Ultrastructural changes in Mycobacterium tuberculosis in response to antimicrobial peptides

To examine the cytotoxic effect of these AMPs against *M. tuberculosis*, the ultrastructure of bacilli after treatment with these peptides was examined. In previous studies, it was demonstrated that antimicrobial cationic peptides bind to negatively charged molecules of the membrane and cell wall components such as lipoarabinomannan in *M. tuberculosis* and lead to membrane disruption [26]. To investigate the effects of semisynthetic peptides E2, E6, LL-37, CRAMP and CP26 on *M. tuberculosis*, bacilli incubated with these peptides were studied using electron microscopy.

Control untreated bacilli showed a well-defined, homogeneous and slightly electron-lucent cell wall, whilst the cytoplasm was generally electron dense with some medium-sized vacuoles (Fig. 2). Incubation with peptide E2 produced substantial abnormalities in the cell wall, including thinning, budding and thickening of the wall as well as condensation of the cytoplasm producing an electron-lucent area under the wall that was more prominent at one pole (Fig. 2). Incubation with peptide CP26 led to an almost complete disappearance of the cell wall, with only a thin superficial rim of electron-dense material evident around the bacteria, whilst the cytoplasm exhibited large vacuoles or was condensed leading to a shrinking of the whole bacillus (Fig. 2). Incubation with peptide E6 also induced significant abnormalities in the cell wall, including extreme thinning of the wall which alternated with thickened areas and regions of vesicular budding that could be visualised as an irregular surface coexisting with extreme cytoplasmic condensation leading to a large
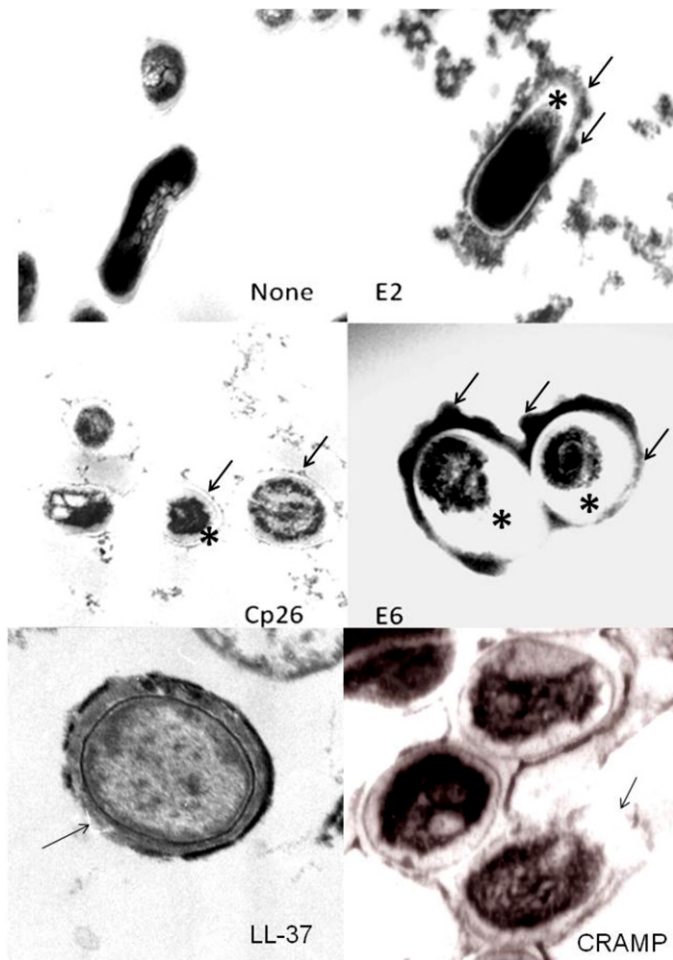
Fig. 2. Representative micrographs of conventional electron microscopy and immunoelectron microscopy of *Mycobacterium tuberculosis* strain H37Rv treated with antimicrobial peptides. In contrast to the untreated control bacteria, bacilli incubated with a minimum inhibitory concentration of peptides E2, CP26, E6, LL-37 or CRAMP showed cell wall thinning, budding or disruption (arrows), and cytoplasmic condensation producing a broad electron-lucent halo between the wall and the condensed cytoplasm (*).

Fig. 3. Effect of antimicrobial peptide (AMP) treatment on (A) pulmonary bacilli burden and (B) tissue damage (pneumonia) during advanced experimental tuberculosis. (A) Mice were infected with the drug-sensitive H37Rv *Mycobacterium tuberculosis* strain and after 60 days were treated three times per week with 32 μg of the indicated AMP in 100 μL of saline solution. All AMPs decreased the lung bacillary loads in comparison with non-treated mice. (B) Percentage of lung surface affected by pneumonia determined by automated morphometry. Results are expressed as the mean ± standard deviation. $P < 0.05$ was considered statistically significant. Asterisks show the kinetic points where there was statistical difference in CFUs between the control group and the rest of the groups. None of the AMPs induced a statistically significant decrease or increase in the pneumonic area compared with the control group.

electron-lucent space between the cell wall and the cytoplasm (Fig. 2). E6 induced the most striking subcellular abnormalities, however the general theme of cell wall destruction/modification and cytoplasmic condensation was evident for all peptides, including LL-37 and CRAMP, which induced a homogeneous increase of the electron-lucent cell wall surrounded by a thin electron-dense rim (Fig. 2).

Overall, these observations indicated that the cell wall and membrane are important targets of these peptides, whilst the observation of a condensed cytoplasm is consistent with osmotic activity and perhaps also DNA binding. Overall, these observations mirrored those for other peptides in Gram-positive bacteria [22].

3.3. Effect of intratracheal administration of LL-37, CRAMP, E2, E6 and CP26 during late progressive tuberculosis produced by the drug-sensitive strain H37Rv

The mean of the highest MIC for all peptides against *M. tuberculosis* in vitro was 3.2 μg/mL, thus we decided to use a dose of 32 μg in 100 μL of saline solution (ca. 1 mg/kg), which was administered intratracheally three times a week. Treatment was started 60 days post infection, when advanced active TB was well established. In comparison with control mice in which bacilli numbers
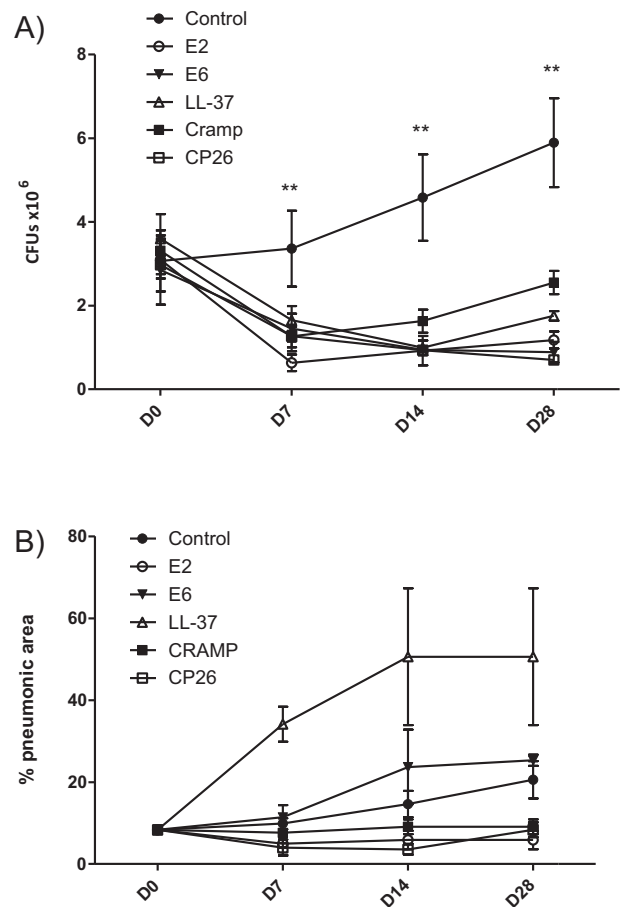
increased progressively over the 28 days with a net doubling over this time, animals treated with each of the tested peptides showed a significant reduction in bacilli loads during the entire treatment (Fig. 3A). For the mouse and human cathelicidin peptides CRAMP and LL-37, there was a similar initial decrease in bacterial load after 7 days but the bacteria appeared to grow thereafter, albeit at a slower rate than the untreated control, consistent with the weaker antimicrobial activity of these peptides. For the three synthetic peptides, the bacilli did not re-grow, and for E6 and CP26 there was an apparently increasing therapeutic effect between 7 days and 28 days.

Consistent with these findings, after 4 weeks of treatment with E2, CRAMP or CP26, histological examination revealed that the lung area affected by pneumonia tended to be smaller than in control mice but not significantly. In contrast, mice treated with LL-37 showed a modest but non-significant increase in lung area affected by pneumonia, whilst those treated with E6 showed a slight or no increase in the pneumonic area (Fig. 3B).
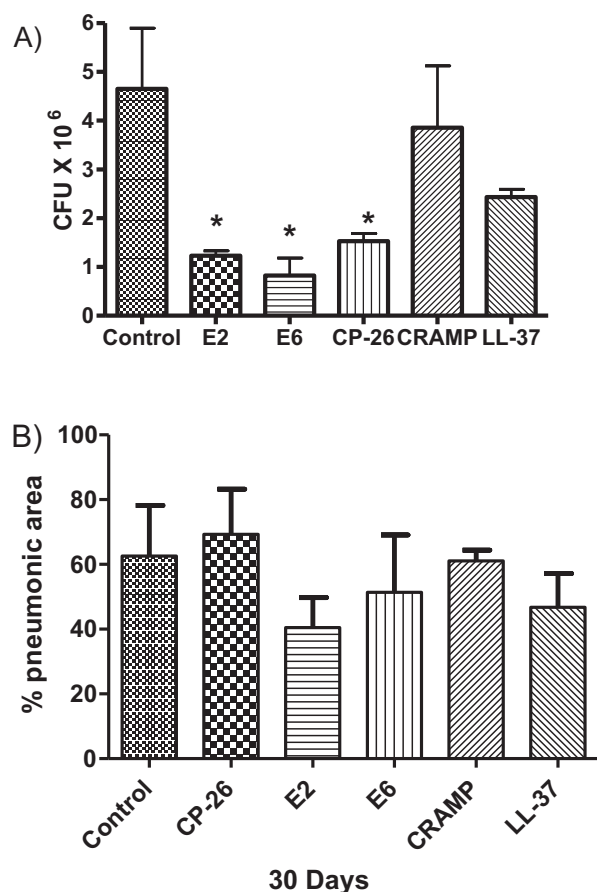
Fig. 4. Effect of antimicrobial peptides (AMPs) in the treatment of mice infected with a multidrug-resistant (MDR) strain of *Mycobacterium tuberculosis*. (A) Animals were infected with MDR strain and after 60 days were treated three times per week with 32 μg of the indicated AMP in 100 μL of saline solution. In comparison with the non-treated control animals, all of the semisynthetic peptides induced a significant decrease in the lung bacillary loads, whilst cathelicidins both from human and mouse induced a non-significant reduction of bacilli burdens. (B) In contrast, similar lung consolidation determined by automated morphometry was seen between treated and control groups. Results are expressed as the mean ± standard deviation. *P < 0.05 was considered statistically significant.

3.4. Effect of intratracheal administration of LL-37, CRAMP, E2, E6 and CP26 during late progressive tuberculosis produced by a multidrug-resistant strain

Owing to the emergence of MDR strains worldwide as well as the results observed in mice treated with the different peptides during infection with the drug-sensitive H37Rv strain, we studied whether this therapy had the ability to induce similar beneficial effects during late active disease in mice infected with a clinical isolate resistant to all first-line antibiotics. In comparison with control animals, MDR-infected mice treated with all three synthetic peptides demonstrated a significant 2.5–4.5-fold reduction in CFU counts (Fig. 4A). In contrast, whilst there was a trend towards activity for the natural peptides LL-37 and CRAMP, this was not significant. Although treatment with the different peptides led to a slight decrease in pneumonic area, these differences were again not statistically significant when compared with mice that received only saline solution (Fig. 4B).

4. Discussion

In the past decade, an increasing number of publications have suggested AMPs as molecules with great potential for the treatment of TB [11,27–29]. The present study demonstrates that several different AMPs showed a notable antimicrobial effect against the drug-sensitive *M. tuberculosis* strain H37Rv, in some cases even more than observed for a *P. aeruginosa* clinical isolate. Since previous studies by our group showed that β-defensin-2 and -3 might be involved in the maintenance of latency using a murine model [24], we wanted to assess whether treatment with these peptides would led to a decrease in metabolic activity in *M. tuberculosis*, making the mycobacterium unable to reduce resazurin. However, bacteria could not be re-grown from the wells containing the lowest inhibitory concentrations in the MIC assays, indicating that these peptides were in fact bactericidal rather than merely inducing a reduction of the metabolic activity in *M. tuberculosis*.

AMPs have complex multimodal mechanisms of action that have been proposed to involve several targets, including cell membrane-associated and intracellular targets [8]. These mechanisms have in common the initial interaction of positively charged peptides with the negatively charged cytoplasmic membrane and the insertion of peptides in the membrane owing to their amphipathic nature, leading to either membrane perturbation or translocation to cytoplasmic targets. The electron microscopy study, which showed disruption, thinning and budding of the bacterial cell wall after incubation of bacilli with the different peptides, suggests that their interaction with the membrane and/or cell wall might be an important mechanism to produce bacterial damage. This damage could in turn relate to triggering of autolytic mechanisms or interference with cell wall biosynthesis, both of which have been reported to be mechanisms by which peptides can act [8,9]. In addition, abnormalities such as bacterial cytoplasmic shrinkage are consistent with the peptide being taken up by cells [8]. Overall, we can conclude that peptides utilise complex mechanisms to produce *M. tuberculosis* damage, as observed for several peptides in other bacteria [22]. These peptides did not cause lysis of red blood cells at very high concentrations [18,19,22].

Treatment with the different peptides was initiated after 8 weeks of infection, when active disease was occurring, mimicking a common clinical situation in developing countries. Intratracheal instillation of the different peptides led to decreased lung bacillary loads. The activity of the peptides did not seem to be strongly related to their origin in that human LL-37, mouse CRAMP, bovine-derived E2 and E6 and insect-derived CP26 all had rather similar initial activities, with the natural peptides allowing slight re-growth of the bacillus. This indicates that neither the specific sequence not the origin of the peptides determined their activity, but rather their antimicrobial properties. Although none of the results were statistically significant, for three peptides (mouse CRAMP, E2 and CP26) slightly decreased pneumonia was observed, whilst LL-37 led to a modest but insignificant increase in pneumonia and E6 showed a slight or no effect. The modest suppression of pneumonia by mouse CRAMP, E2 and CP26 cannot be just due to the antimicrobial activity, which was similar for both the natural peptides and all three synthetic peptides. Indeed, it might suggest that there is another property of peptides that contributes to the suppression of pneumonia, such as an immunomodulatory, anti-inflammatory activity [9,10] that might differ among the peptides.

Cationic peptides like these have a variety of relevant properties, including suppression of inflammation, enhancement of cellular recruitment and a wound healing function. Thus, whilst the peptides were selected for their antimicrobial activities, other properties may assist in the control of *M. tuberculosis* infection. Intriguingly, the enhanced AMPs demonstrated apparently superior activities to the natural peptides, whilst LL-37 showed an increased (but not significantly) pneumonic area, perhaps due to the fact that it tends to be more cytotoxic. To evaluate the potential role of immune modulation, we are currently investigating peptides with enhanced immunomodulatory activities [30] to see

whether these are more successful at reducing the pneumonic area.

With regard to the MDR strain, the current results showed that intratracheal administration of peptides E2, E6 and CP26 in mice infected with this strain during the advanced phase of infection could significantly reduce lung bacillary loads. However, the reduction of pneumonia did not demonstrate significant differences when compared with control mice. Thus, similar to the H37Rv-infected mice, these results indicate that these peptides have an effective antimicrobial effect against MDR infection without affecting pneumonia.

In conclusion, these results show that repeated intrapulmonary administration of AMPs permits an efficient method of suppressing the growth of bacilli when they are administered during the late progressive disease induced by drug-sensitive or drug-resistant virulent mycobacteria. Although this treatment was not completely curative, these results suggest that, in conjunction with other more conventional treatments, inhalation therapy with AMPs would be a feasible treatment option in developing countries where there is an urgent need for new treatment options.

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