An enhanced variant designed from insect defensin DLP4 against *Staphylococcus aureus* CVCC 546

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- Peptide design and characterization
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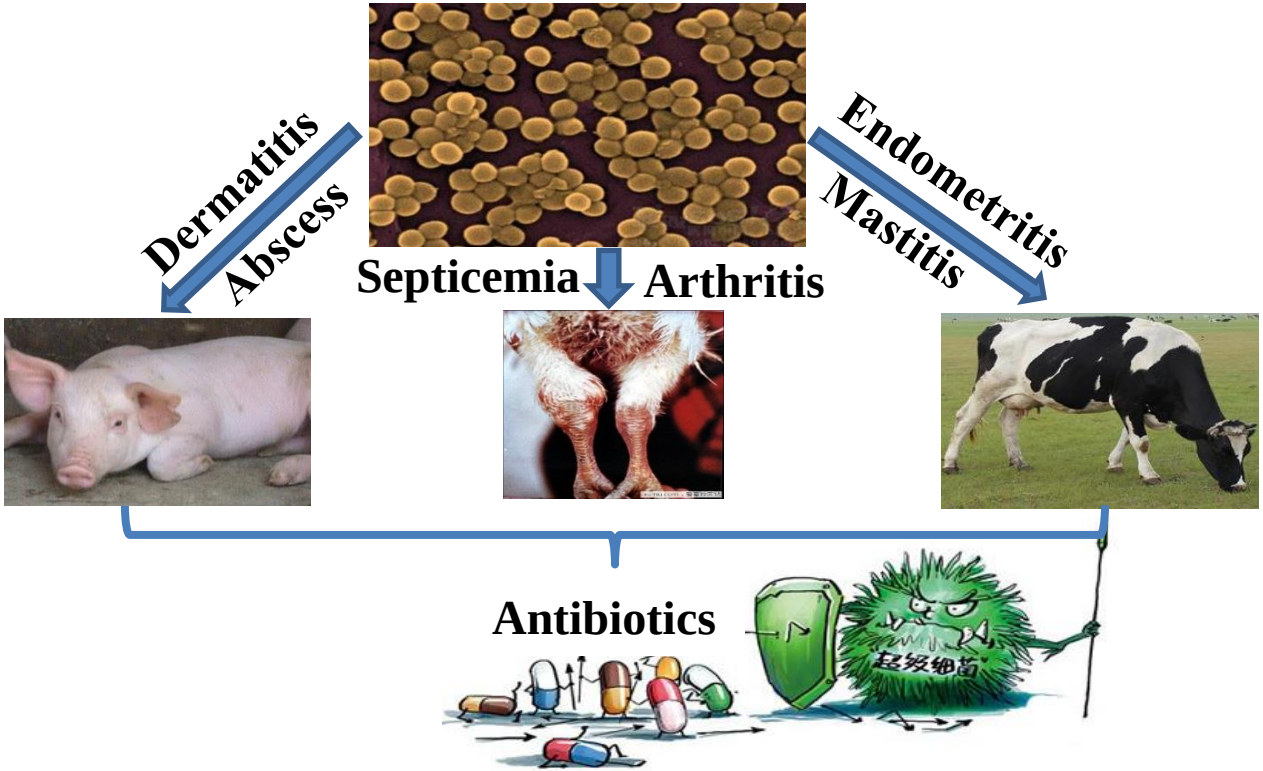
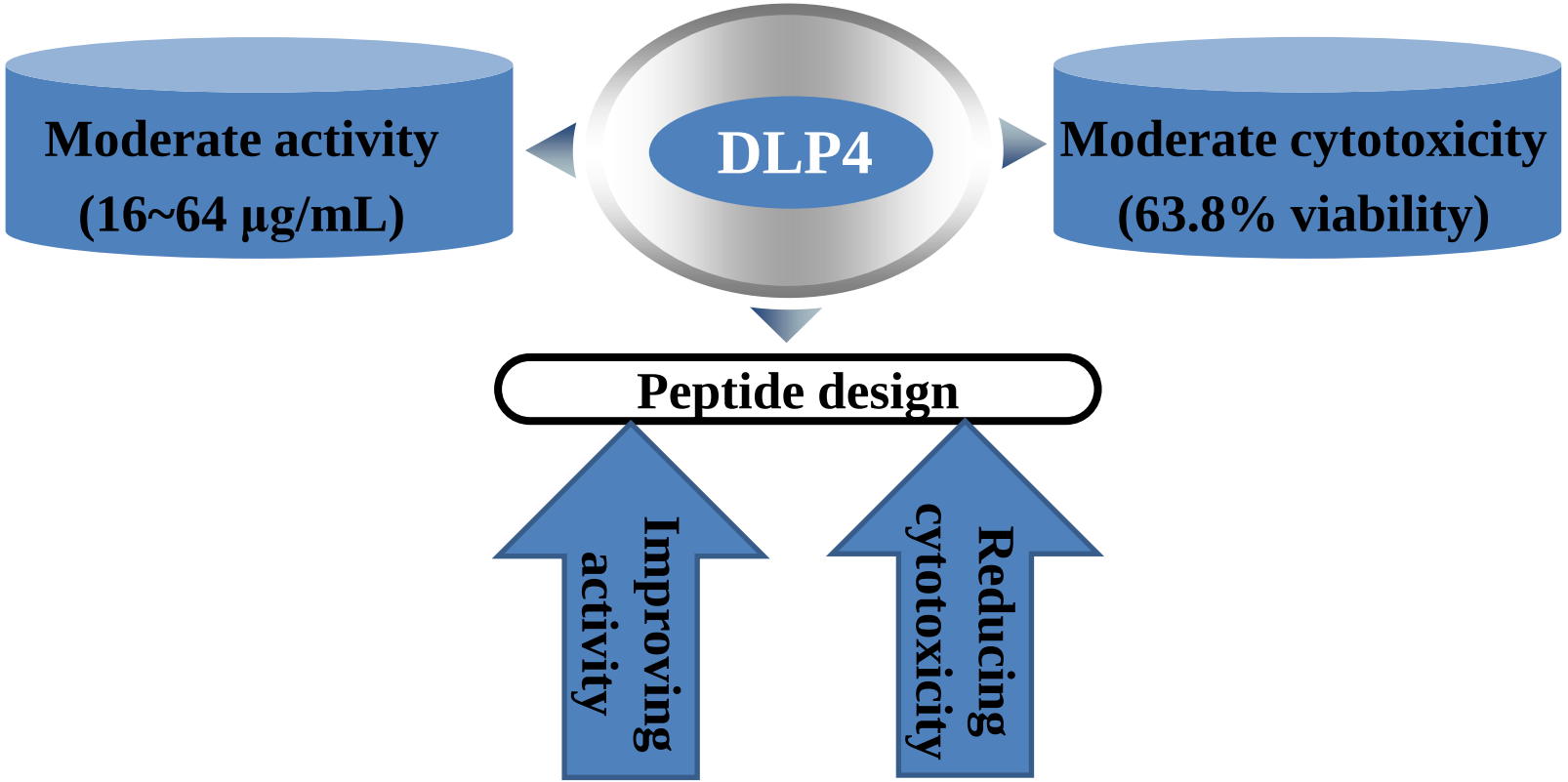
Background



World Health Organization

Methicillin and vancomycin-resistant bacteria is high-crisis (WHO, 2017).

Background


Moderate activity
(16~64 μg/mL)
DLP4
Moderate cytotoxicity
(63.8% viability)
Peptide design
Improving activity
Reducing cytotoxicity

Background

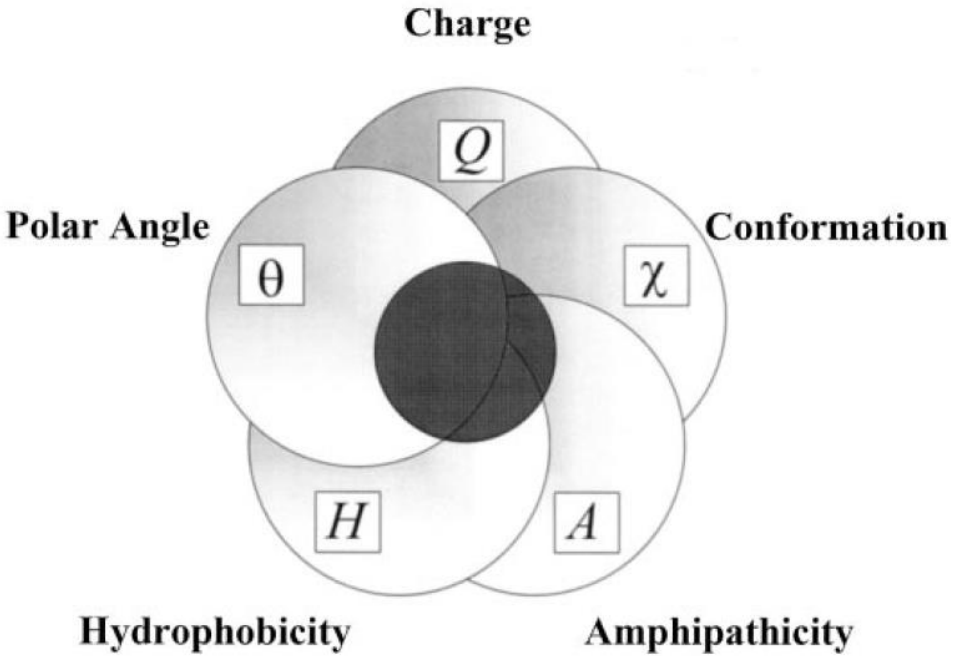


Figure 1. Interrelationship among structural determinants in antimicrobial peptides.

Yeaman MR, Yount NY. Pharmacol Rev. 2003, 55(1):27-55.

Peptide design and characterization

- Peptide design
- Expression & characterization

Peptide design

Table 1. Physicochemical parameters of DLP4 and ID13.

Peptide	Sequences	MW (Da)	Net charge	GRAVY
DLP4	ATCDLLSPFKVGHAACAAHCI ARGKRGGWCDKRAVCNCRK	4269.05	6	-0.13
ID13	ATCDLLSPFKVGHAACAAHCI ARGKRGGWCDGRAVCNCRK	4197.93	5	-0.042

➢ The Lys32-substituted DLP4 by Gly, designated as ID13, showed **decreased charge, increased hydrophobicity, and shorter amino acid side chain.** It comprises an N-terminal loop, a central amphipathic α-helix and a C-terminal antiparallel β-sheet.

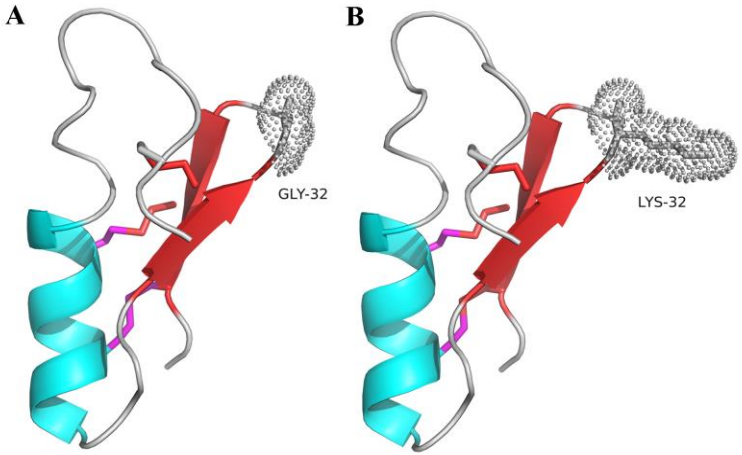


Figure 2. Molecular modeling of **(A)** ID13 and **(B)** DLP4.

Expression & characterization

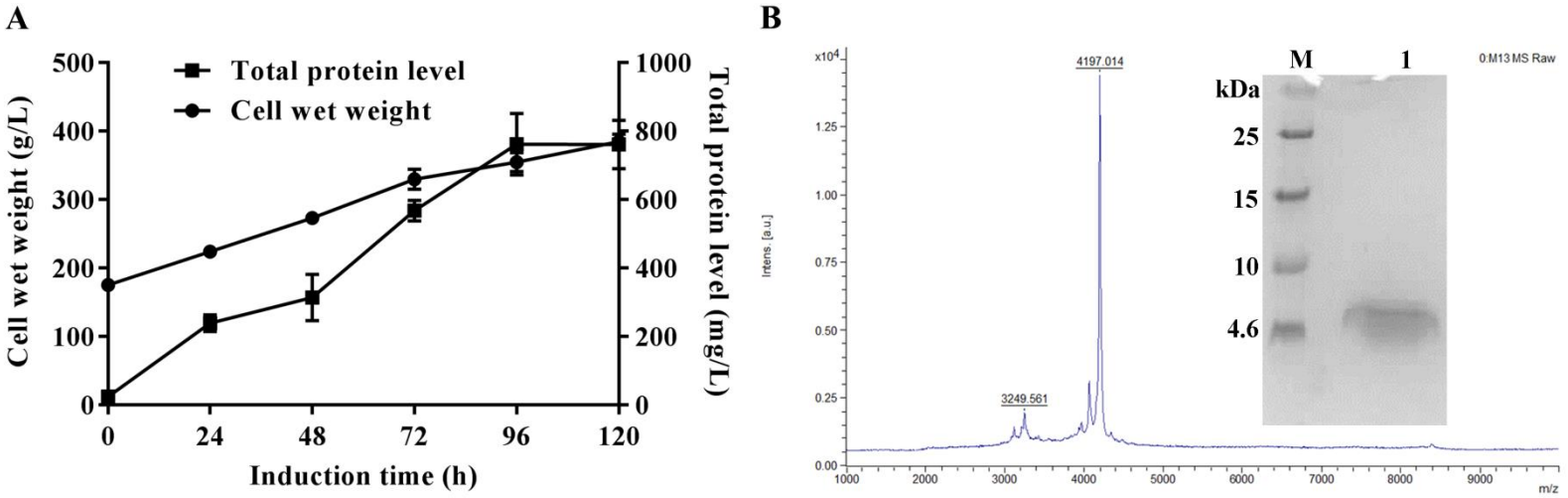


Figure 3. (A) Expression, **(B)** purification and characterization of ID13.

- **Total protein level:** 761 mg/L; **Cell wet weigh:** 385 g/L; **MW:** 4197.01Da.

Bioavailability of peptide ID13

- Antimicrobial activity
- Stability of peptide ID13
- Hemolytic activity and cytotoxicity
- Time-kill assay
- Postantibiotic effect (PAE)

Antimicrobial activity

- **G^+ bacteria:**

ID13: 4~8 μg/mL

DLP4: 16~64 μg/mL

Van: 0.25~1 μg/mL

- **G^- bacteria:**

ID13 : >128 μg/mL

DLP4: >128 μg/mL

Van: >64 μg/mL

Table 2. MICs of the designed peptides against pathogenic strains.

Species and strains	MIC					
	ID13		DLP4		Vancomycin	
	μM	μg/mL	μM	μg/mL	μM	μg/mL
Gram-positive bacteria						
Staphylococcus aureus CVCC 546	0.95	8	3.75	16	0.67	1
S. epidermidis ATCC 12228	1.91	8	1.87	64	0.67	1
Streptococcus pneumoniae CVCC 2350	0.95	4	1.87	32	0.34	0.5
S. suis CVCC 3928	0.95	4	3.75	16	0.17	0.25
Gram-negative bacteria						
Escherichia coli ATCC 25922	>30.50	>128	>29.98	>128	86.15	128
E. coli K88	>30.50	>128	>29.98	>128	43.08	64
Salmonella pullorum CVCC 533	>30.50	>128	>29.98	>128	86.15	128
S. Enteritidis CVCC 3377	>30.50	>128	>29.98	>128	86.15	128

Stability of peptide ID13

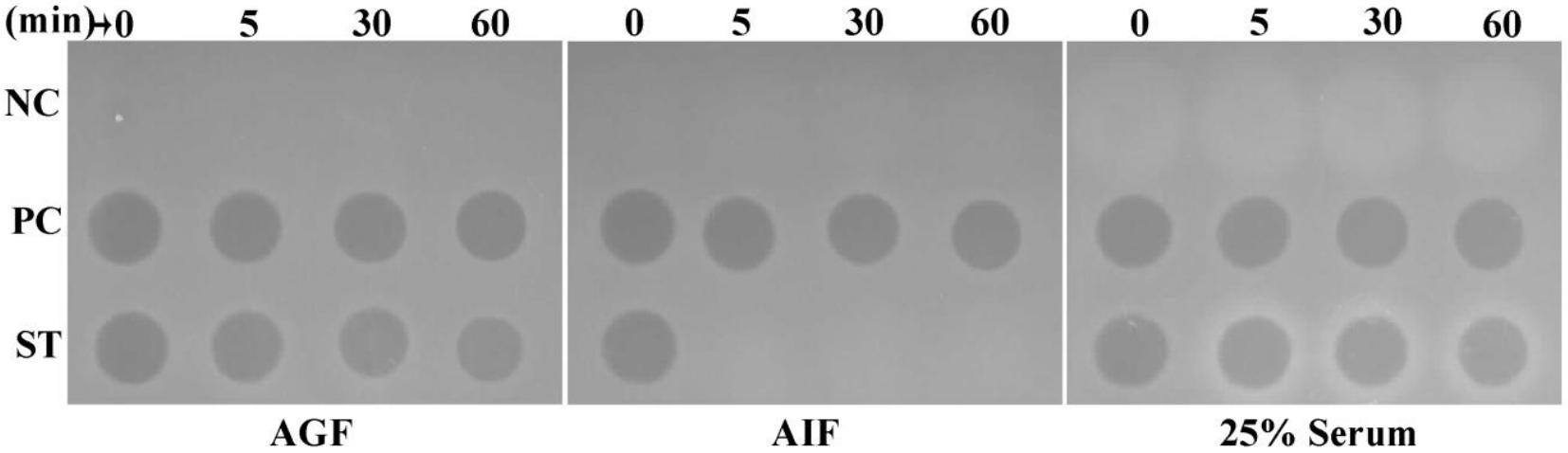


Figure 4. The stability of peptide ID13 in artificial gastric fluid **(AGF)**, artificial intestinal fluid **(AIF)** and serum. NC: negative control; PC: positive control; ST: stability tested of peptide ID13 prepared in AGF, AIF, or 25% serum.

- **Stability:** AGF: 90.79%; AIF: proteolysis in <5 min; 25% Serum: 99.54%.

Hemolytic activity and cytotoxicity

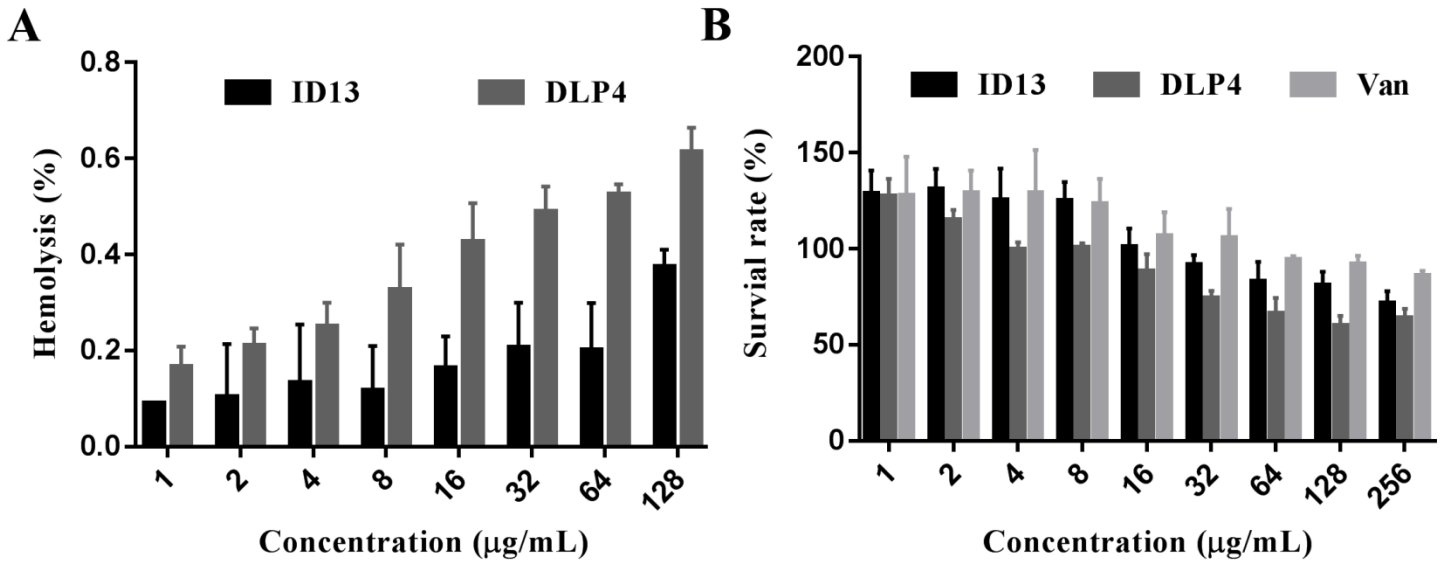


Figure 5. (A) Hemolysis of the ID13 against mouse erythrocytes. **(B)** Cytotoxicity of ID13 against RAW 264.7.

- **Hemolytic activity:** ID13: 0.38%; **cytotoxicity:** ID13: 80.91% viability.

Time-kill assay and PAE

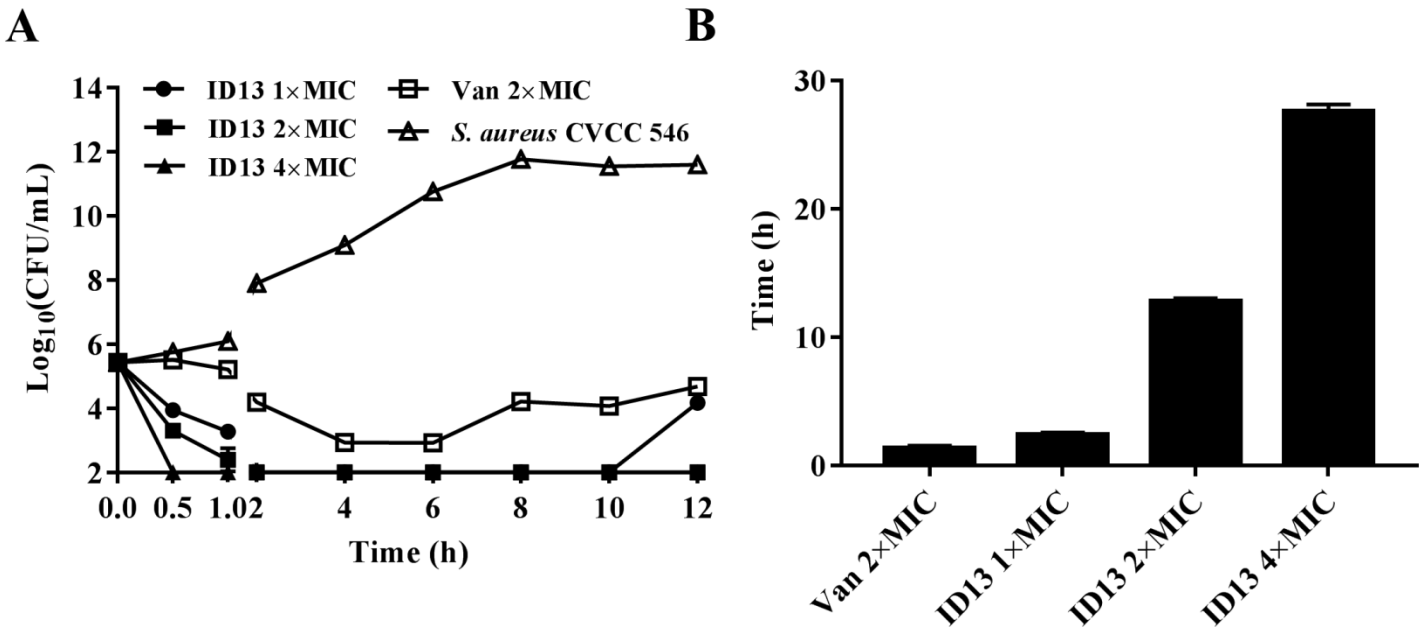


Figure 6. (A) Time-kill assay, and **(B)** PAEs of ID13 and vancomycin (Van) against *S. aureus* CVCC 546.

- ID13 killed over 99.99% of pathogens within 1 h, produced a PAE of 12.78 h.

Efficacy of peptide ID13 in vitro

- Membrane permeabilization & DNA binding
- Morphologic alterations
- Intracellular bactericidal activity
- LTA neutralization
- ID13-antibiotics synergism

Membrane permeabilization & DNA binding

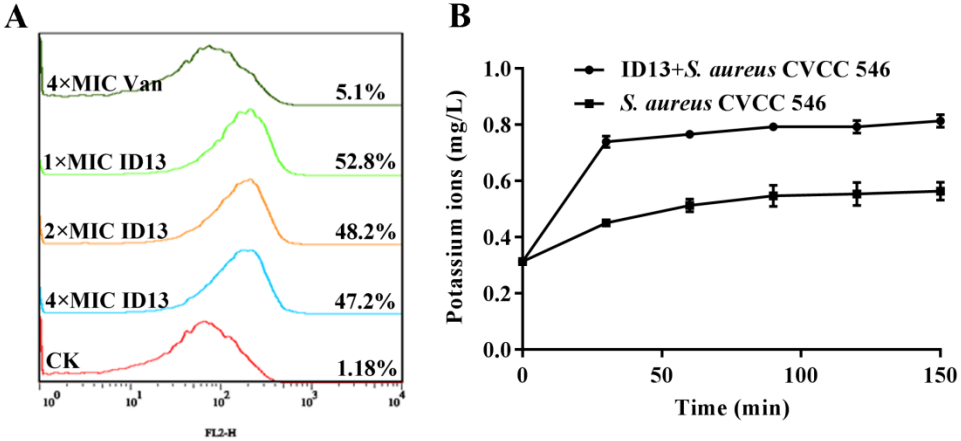


Figure 7. (A) Flow cytometric analysis **(B)** and the release of K^+ of *S. aureus* CVCC 546.

ID13 could penetrate the membrane of *S. aureus* CVCC 546, inducing an increase in potassium ion leakage.

ID13 bound to *S. aureus* CVCC 546 genome DNA and led to the change of DNA conformation.

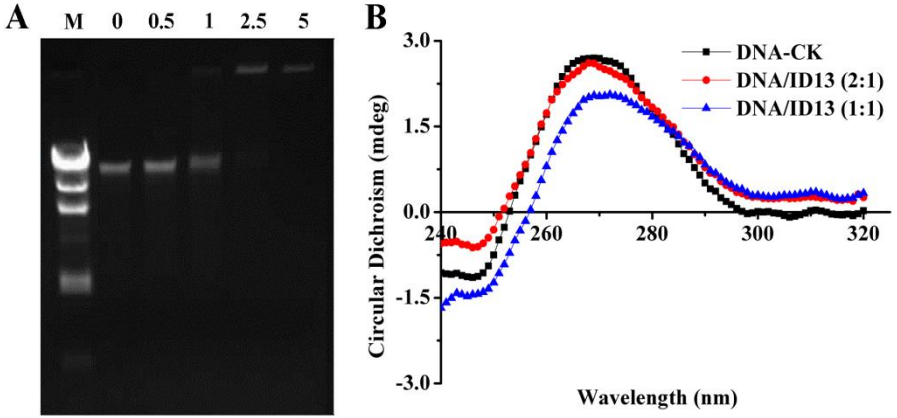


Figure 8. (A) Gel retardation analysis and **(B)** CD spectra of the binding of ID13 to *S. aureus* genomic DNA.

Morphologic alterations

Control: Intact surfaces.
ID13: cell membrane perforation and deformation.

Control: complete microscopic surface and a dense internal structure.
ID13: destruction of cell wall, release of intracellular contents, the formation of mesosome-like structure, and rupture of the cells.

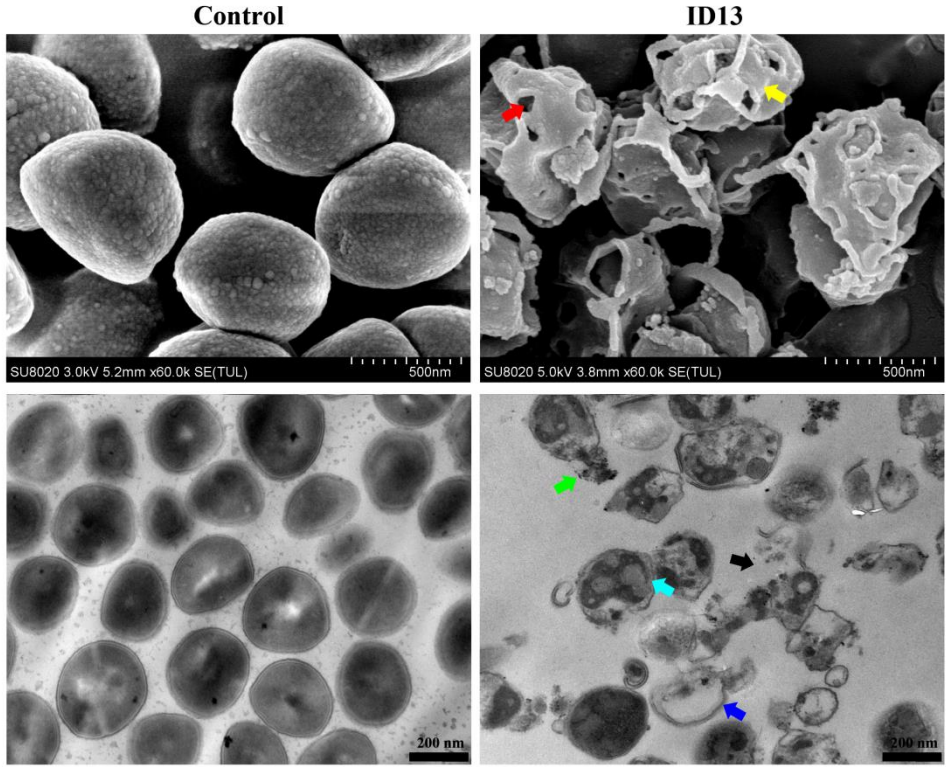


Figure 11. (A) SEM and **(B)** TEM micrographs of *S. aureus* CVCC 546.

Intracellular bactericidal activity

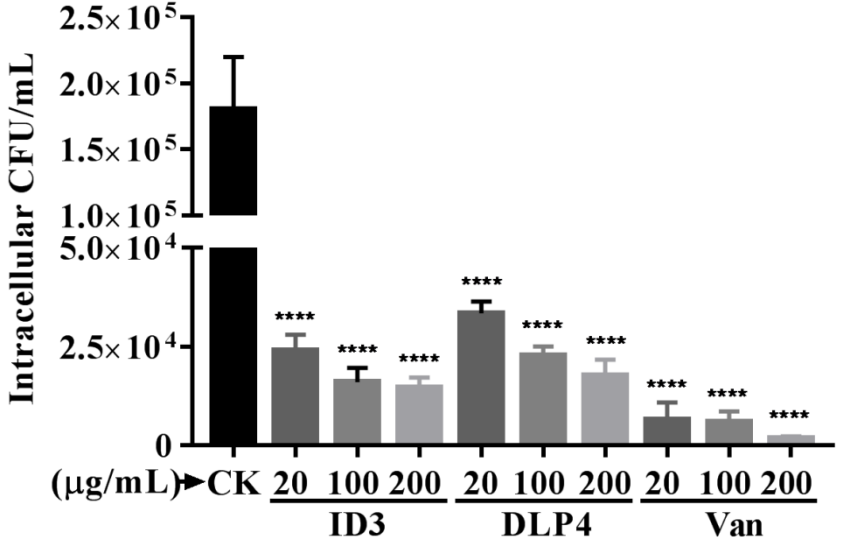


Figure 9. (A) bactericidal activity against intracellular *S. aureus* CVCC 546.

- ID13 killed >99% intracellular *S. aureus* CVCC 546 in a dose-response manner.

LTA neutralization

➢**Proinflammatory cytokines:**

TNF-α ↓, IL-1β ↓, and IL-6 ↓

➢**Anti-Inflammatory cytokine:**

IL-10 ↑.

In a dose-dependent manner.

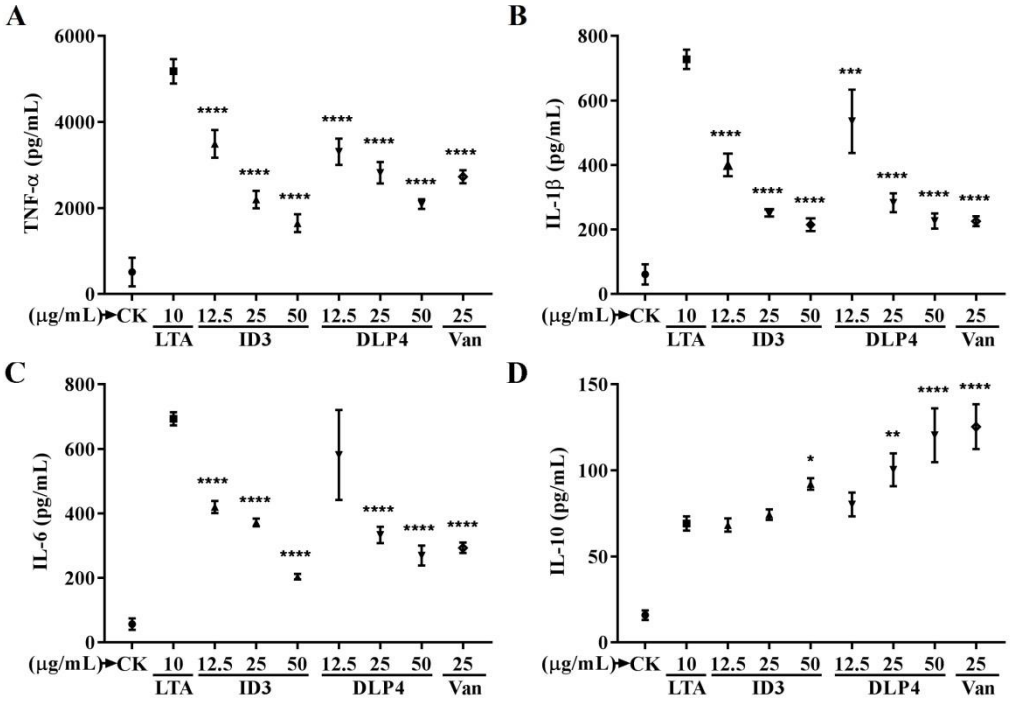


Figure 10. Regulation of LTA-induced inflammation cytokines.

ID13-antibiotics synergism

- Enhanced MIC_c of peptide ID13 and antibiotics.
- Synergism of peptide ID13 with antibiotics.

FICI≤0.5, 0.5<FICI≤1, 1<FICI≤4 and FICI >4 refers to synergy, additivity, indifference, and antagonism respectively.

Table 3. Combined application of peptide ID13 with antibiotics against *S. aureus* CVCC 546.

Combination[a]	Single	MIC_p/MIC_a	MIC_c	FICI
ID13+Van	ID13	8	1	0.25
	Van	0.5	0.0625	
ID13+Amp	ID13	8	2	0.375
	Amp	0.0625	0.0078	
ID13+Rif	ID13	8	1	0.1875
	Rif	0.03125	0.002	
ID13+Cip	ID13	8	1	0.25
	Cip	0.125	0.0156	

[a] Vancomycin: Van, ampicillin: Amp, rifampin: Rif, and ciprofloxacin: Cip.

Efficacy of peptide ID13 in vivo

- Mouse thigh infection model
- Mouse endometritis model

Mouse thigh infection model

- Causing a 1.8 log10 reduction of CFU counts in thighs;
- Decreasing TNF-α, IL-6, and IL-10 levels in serum;
- Superior to those of vancomycin.

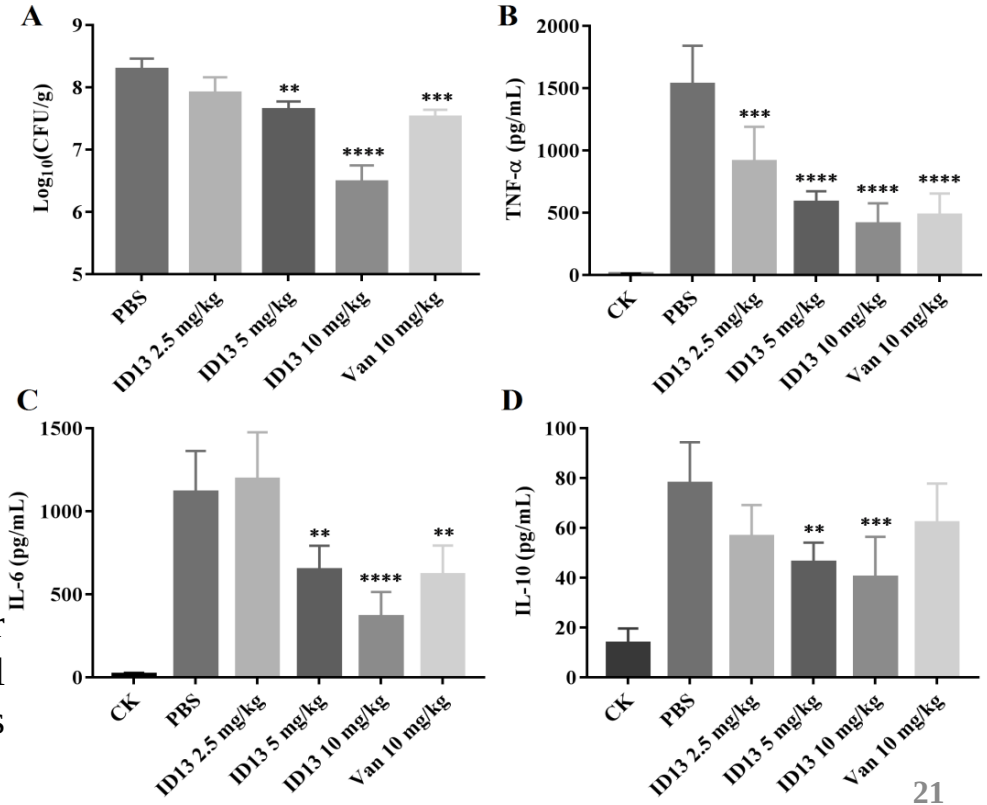


Figure 12. (A) Single i.p. treatment with ID13 or vancomycin (Van) in mouse thigh infection model (n=6). Effects of ID13 on inflammatory cytokines levels of **(B)** TNF-α, **(C)** IL-6 and **(D)** IL-10.

Mouse endometritis model

Table 4. Changes of different white blood cells in mouse between negative and blank control (n=5)

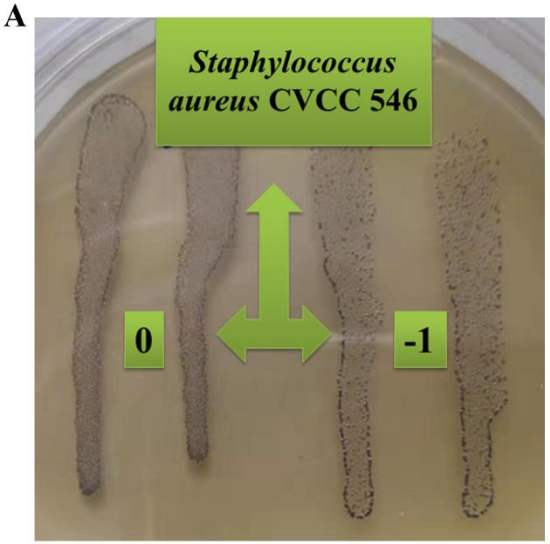
Group	WBC[a] (10^9/L)	NEU[b] (10^9/L)	LYM[c] (10^9/L)	NEUT%	LYM%
BC[d]	5.14±1.22	2.24±0.34	2.03±0.40	47.80±5.13	39.73±1.75
NC[e]	14.56±3.00**	9.65±2.00***	3.25±0.83*	66.24±1.51***	22.24±2.33****

[a] WBC: white blood cells; [b] NEU: neutrophils; [c] LYM: lymphocytes; [d] BC: blank control; e NC: negative control, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

➢ The number of white blood cells, neutrophils, and lymphocytes of the negative control was significantly higher than those of the blank control, which means that there may be bacterial infection in mouse endometritis model.

Mouse endometritis model

- **Drop plate method** was used for *S. aureus* CVCC 546 isolation and identification.
- **16S rRNA gene sequencing** further conformed the pathogen.



B

TTTTGGTACACCTTCGACGGCTAGCTCCTAAAAGGTTACTCCACCGGCTTCG
GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGA
ACGTATTCACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATG
TAGTCGAGTTGCAGACTACAATCCGAACTGAGAACAACTTTATGGGATTTG
CTTGACCTCGCGGTTTCGCTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTA
GCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGG
TTTGTCACCGGCAGTCAACTTAGAGTGCCCAACTTAATGATGGCAACTAAG
CTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG
CTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGAAGGCT
CTATCTCTAGAGTTGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTT
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CATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTC
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TAACGCTTGCCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGG
CTTCTGATTAGGTCCGTCAGATGTGCCAGTACTTACCATATGTCTTCCTAATA
CAGAGTTTACGATCGAAGACTTCTCATCAGCGCGTGCTCGCAGGTTTCGCC
TTGCGAGATCCTATGGTGCTCCGAAGATTGGACGGGCCATTCAGGGGGCAA

Figure 13. Pathogens isolation and identification. **(A)** Homogenate of uteri and 10 times dilutions were dropped on Baird-Parker egg yolk agar for *S. aureus* CVCC 546. **(B)** 16S rRNA gene sequence of *S. aureus* CVCC 546.

Mouse endometritis model

- **BC:** intact structures.
- **NC:** glands atrophy and disappearance, the infiltration of inflammatory cells, fibrous tissue hyperplasia, and the shedding of epithelial cells.
- **TG:** pathological conditions were improved with the ID13 and vancomycin administration.

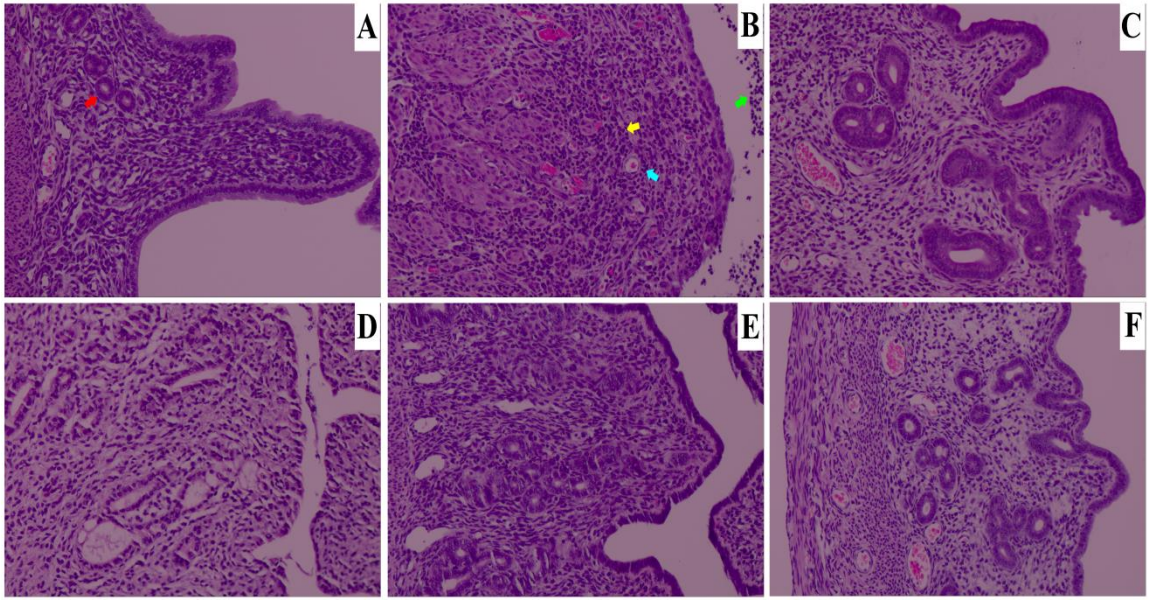

Figure 14. Histopathological analysis of uteri by H&E staining (×200). Histopathology of uteri of the **(A)** blank (**BC**) and **(B)** negative control (**NC**), **(C-F)** and groups treated **(TG)** by vancomycin (10 mg/kg), and ID13 (5, 10, or 15 mg/kg, respectively).

Mouse endometritis model

- A significant increase was observed in TNF-α, IL-1β, IL-6, and IL-10 production induced by *S. aureus* CVCC 546.
- In contrast, the expression of these cytokines was remarkably decreased with administration of ID13 and vancomycin.

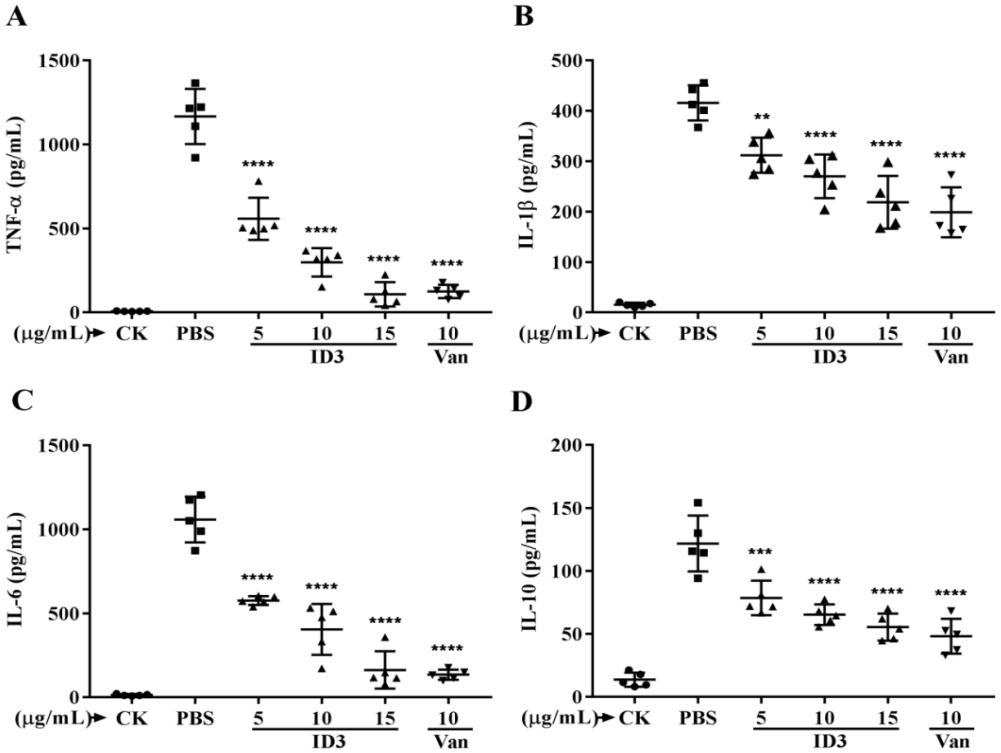

Figure 15. Changes of inflammatory cytokines **(A)** TNF-α, **(B)** IL-1β, **(C)** IL-6, and **(D)** IL-10 in serum from mouse.

Mouse endometritis model

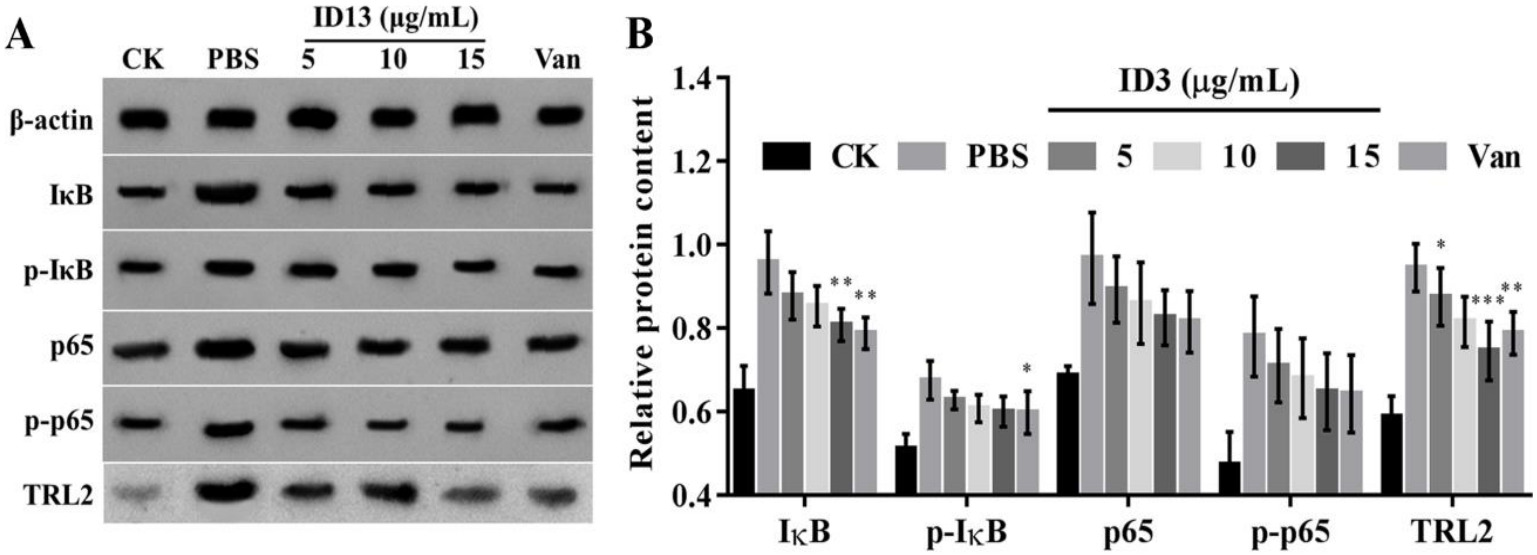

Figure 16. Effects of peptide ID13 on TLR2/NF-κB signaling.

- ID13 attenuated the expression of TLR2 and the phosphorylated NF-κB and IκB.

Conclusion

a. **To increase antimicrobial activity and decrease cytotoxicity of DLP4, the Lys32Gly mutation was generated;**
b. **ID13 showed higher activity, low cytotoxicity, and stability in artificial gastric fluid and serum;**
c. **ID13 showed preferable bactericidal activity and a long PAE;**
d. **ID13 penetrated and destroyed cell membrane, causing cell contents release;**
e. **ID13 bound to bacterial genomic DNA and destroyed its structure;**
f. **ID13 killed intracellular *S. aureus*, attenuated LTA-induced inflammation;**
g. **ID13 could be used in combination with traditional antibiotics;**
h. **ID13 had high efficacy in mouse model of thigh infection and endometritis.**

**** The above results are unpublished yet.****

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I Key Team Members

- **Jianhua Wang, PI (supervisor)**
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Thank you for your attention!

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