A small mycobacteriophage–derived peptide and its improved isomer restrict mycobacterial infection via dual mycobactericidal–immunoregulatory activities

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ABSTRACT

Mycobacteriophages express various peptides/proteins to infect Mycobacterium tuberculosis (M. tb). Particular attention has been paid to mycobacteriophage-derived endolysin proteins. We herein characterized a small mycobacteriophage-derived peptide designated AK15 with potent anti-M. tb activity. AK15 adopted cationic amphiphilic α-helical structure, and on the basis of this structure, we designed six isomers with increased hydrophobic moment by rearranging amino acid residues of the helix. We found that one of these isomers, AK15-6, exhibits enhanced anti-mycobacterial efficiency. Both AK15 and AK15–6 directly inhibited M. tb by trehalose 6, 6′-dimycolate (TDM)–binding and membrane–disruption. They both exhibited bactericidal activity, cell selectivity, and synergistic effects with rifampicin, and none induced drug resistance to M. tb. They efficiently attenuated mycobacterial load in the lung of M. tb–infected mice. We observed that lysine, arginine, tryptophan and an α–helix are key structural requirements for their direct anti-mycobacterial action. Of note, they also exhibited immunomodulatory effects, including
inhibition of proinflammatory response in TDM–stimulated or *M. tb*–infected murine bone marrow–derived macrophages (BMDMs) and *M. tb*–infected mice, and induction of only a modest level of cytokine (tumor necrosis factor α [TNF–α] and interleukin–6 [IL–6]) production in murine BMDMs and a T–cell cytokine (interferin–γ [IFN–γ] and TNF–α) response in murine lung and spleen. In summary, characterization of a small mycobacteriophage–derived peptide and its improved isomer revealed that both efficiently restrain *M. tb* infection via dual mycobactericidal–immunoregulatory activities. Our work provides clues for identifying small mycobacteriophage–derived anti–mycobacterial peptides and improving those that have cationic amphiphilic α–helices.

Tuberculosis (TB) is a serious airborne fatal disease caused by *Mycobacterium tuberculosis* (*M. tb*) infection (1). In 2016, TB was the ninth leading cause of death worldwide and the leading cause from a single infectious agent (2). There were an estimated 10.4 million new TB cases, an estimated 1.3 million TB deaths among HIV–negative people and an additional 374,000 deaths among HIV–positive people (2). Worst of all, there were rapid emergences of drug resistant TB. Among the new TB cases in 2016, about 600,000 TB cases were resistant to rifampicin (RR–TB), the most effective first–line drug, of which 490,000 had multidrug-resistant TB (MDR–TB) (2). So far, global control of tuberculosis is mainly achieved by the development of effective vaccines, improved diagnostics, novel and shortened therapy regimens (3). Although several first–line effective anti–*M. tb* drugs, including rifampin, isoniazid, pyrazinamide, and ethambutol have displayed critical roles in control of TB, the rapid emergence of drug–resistant *M. tb* strains make them invalid (4). Given the severe global TB burden, it is urgent to find alternate ways to control *M. tb* infection.

Mycobacteriophages, the viruses of mycobacteria, have developed unique proteins or peptides to interfere with metabolic processes or cell membranes of mycobacterial hosts (4,5). It is reasonable to search for potent anti–mycobacterial peptides or proteins from mycobacteriophages (4). In the past decades, thousands of mycobacteriophages have been isolated, and more than 1,400 mycobacteriophage genomes have been completely sequenced (http://phagesdb.org/). Based on the genomic information, particular attention has been paid to mycobacteriophage–encoded lytic endolysins, also called enzybiotics, which were considered as potential anti–mycobacterial agents against a number of MDR– and extensively drug–resistant TB (XDR–TB) (6–10). Aside from mycobacteriophage–encoded lytic endolysins, only one mycobacteriophage–derived anti–mycobacterial peptide, PK34, was identified from the genomic information of mycobacteriophage D29 (4). Due to the horizontal genetic exchange, mycobacteriophage genomes are highly genetically diverse (11,12), providing a staggering large number of genes coding for products without functional annotation. Among the numerous mycobacteriophage–derived bioactive agents, we focus on the identification of small mycobacteriophage–derived anti–mycobacterial peptides. In last few decades, antimicrobial peptides have been considered as potential alternates for the therapy of bacterial infection with multiple advantages, such as low immunogenicity, selective affinity to negatively charged cell membrane of bacteria, diverse mechanisms of action, without development of drug resistance and showing synergistic effects with fist–line antibiotics (13–15). These advantages also make them good therapeutic weapons to combat tuberculosis.

In the present study, a small but potent cationic amphiphilic helical peptide derived from mycobacteriophage, designated as AK15, was characterized from the genomic information of
mycobacteriophage Che12. Besides, AK15-6, an improved isomer of AK15, was isolated by rearrangement of the amino acid residues to increase the hydrophobic moment of the helix. AK15 and AK15–6 efficiently restrained *M. tb* infection in vitro and in vivo. Their mechanism of action, cell selectivity, structural requirements, synergistic effects with rifampicin and induced drug resistance were investigated. The present work indicates that we can identify small but potent anti–mycobacterial peptide from mycobacteriophages, and we can optimize cationic amphiphilic helical anti–mycobacterial peptide by rearrangement of amino acid residues. Collectively, this study provides novel peptide candidates and new clues to find and design small but potent peptide antibiotics for control of *M. tb* infection.

**Results**

**A small anti–mycobacterial peptide, AK15, was identified from mycobacteriophage**

In order to identify small as well as potent anti–mycobacterial peptide from mycobacteriophage, a series of peptide candidates derived from mycobacteriophage with 10–30 amino acid residues in length were synthesized for anti–mycobacterial activity assay by MIC determination (Table S1). Among them, a small peptide named AK15, possessed a potent anti–mycobacterial activity against *M. tuberculosis* H37Rv (ATCC 27294) with a MIC value of 37.5 μg/ml. AK15 even kept active to rifampicin–induced drug–resistant *M. tuberculosis* H37Rv, clinically isolated *M. tb* (drug–susceptible or –resistant strains) with MIC values ranging from 18.75 to 75 μg/ml, respectively (Table 1). AK15 is a small peptide with a molecular weight of 2071.55 Da (Table 2). AK15 is derived from a hypothetical protein of mycobacteriophage Che12 with unknown function, which is a small peptide family conserved in many mycobacteriophages, including mycobacteriophage Adzzy, L5, DarthPhader, Alma, Pioneer, and SkiPole. The identification of AK15 provided a small but potent anti–mycobacterial peptide derived from mycobacteriophage.

**AK15 adopted amphiphilic α–helical confirmation**

To understand the structural characterization of AK15, its secondary structure was firstly predicted using a computational framework PEP–FOLD3. As shown in Fig. 1A, AK15 was predicted to adopt α–helical conformation. To verify the accuracy of the predicted structure, circular dichroism (CD) spectroscopy was performed to detect its secondary structure in a membrane–mimetic environment (TFE/H2O, 9:1). In accordance with the predicted structure, the CD spectra of AK15 exhibited double minima at 208 nm and 222 nm, which was indicative of the presence of α–helical conformation in TFE/H2O solution (Fig. 1B). In order to estimate the amphipathicity of this helix, a helix–wheel diagram was plotted using HeliQuest. As shown in Fig. 1C, the hydrophobic amino acid residues (shown in yellow and gray) of AK15 are mainly concentrated on one side of the helix, and the hydrophilic amino acid residues (shown in blue and purple) of AK15 are mainly concentrated on the other side, which formed hydrophobic side and hydrophilic side of the helix, respectively. Collectively, the structural characterization indicated that AK15 is a cationic amphiphilic α–helical peptide.

**Isomers of AK15 with increased hydrophobic moment were designed by rearrangement of amino acid residues**

The effects of hydrophobicity, charge, secondary structure and amphiphilicity on antimicrobial properties for antimicrobial peptides were extensively investigated on Gram–negative bacteria, Gram–positive bacteria and fungi (15–18). But the effects of these physicochemical parameters on anti–mycobacterial action remain unclear. Recently, several designed α–helical anti–mycobacterial peptides indicated that the cationic and
amphiphilic α-helix of these peptides are critical for their interaction with *M. tb* (15–17). Up to now, no investigation was conducted on the improvement of cationic α-helical anti-mycobacterial peptides by rearranging amino acid residues. In an attempt to obtain much more potent anti-mycobacterial peptides, we designed six isomers by rearranging the amino acid residues of the helix and plotted by HeliQuest. After helix–wheel diagram assay, six isomers with increased concentration of hydrophobic amino acid residues on one side and hydrophilic amino acid residues on the other side, and the other physicochemical parameters (molecular weight, net charge, hydrophobicity and α-helical confirmation) unaltered, were selected and synthesized for CD assay (Table 2, Fig. 1C). CD spectra of these six isomers displayed characteristic double minima at 208 nm and 222 nm, which confirmed that six isomers kept the same secondary conformation (α-helix) as AK15 after the rearrangement of amino acid residues (Fig. 1B). *In vitro* anti-mycobacterial activity assay against *M. tuberculosis* H37Rv showed that one of them, AK15–6, displayed enhanced anti-mycobacterial activity as reflected by a decreased MIC value (18.75 μg/ml) relative to AK15 (37.5 μg/ml Table 1&2).

**AK15 and its isomer AK15–6 showed bactericidal activity against *M. tb***

AK15–6 possessed the most effective anti-mycobacterial activity among the six isomers, and hence was selected for investigation of mechanism of action together with AK15. As described previously, a ≥3 log decrease in the initial inoculum was defined as bactericidal activity (19). As shown in Fig. 2, compared to the initial inoculum, a 3 log reduction in final CFU counts was observed after incubation of *M. tuberculosis* H37Rv with AK15 (75 μg/ml) for 7 days (Fig. 2A), and a 4 log reduction in final CFU counts was observed at 7 days after the incubation of AK15–6 (75 μg/ml) for 7 days (Fig. 2B). The results indicated that They both showed bactericidal activity against *M. tuberculosis* H37Rv at concentration of 75 μg/ml, and AK15–6 showed an enhanced bactericidal property against *M. tb* relative to AK15.

**AK15 and its isomer AK15–6 were directly bound to *M. tb***

It is well–known that the direct interactions between the positively charged peptides, and bacterial anionic phospholipids and acidic polymers drive initial association and accumulation of antimicrobial peptides onto the membrane, which constitutes the first step of the lytic process of cationic amphiphilic α-helical antimicrobial peptides (16,20). To address their potential anti-mycobacterial mechanism, the direct interactions between the peptides and *M. tb* were firstly investigated by incubation of *M. tuberculosis* H37Ra with FITC–labeled AK15 or AK15–6. As shown in Fig. 3, the incubation of PBS and scrambled AK15 (sAK15) with *M. tb* didn’t induce a significant increasing of fluorescence intensity. While the exposure of *M. tb* to FITC–AK15 (1 μg/ml) induced a significant increasing of fluorescence intensity, and the percentage of FITC–positive *M. tb* was increased by 25.3% relative to PBS-exposed *M. tb* (Fig. 3A). At the same concentration (1 μg/ml), the exposure of *M. tb* to AK15–6 induced a more remarkable increment of fluorescence intensity, and the fluorescence intensity was increased by 37.7% relative to PBS-exposed *M. tb* (Fig. 3B). Flow cytometry assay indicated that they both directly bound to *M. tb*, and AK15–6 showed an increased *M. tb*-binding ability relative to AK15.

**AK15 and its isomer AK15–6 showed apparent affinity to mycobacterial cord factor***

Mycobacterial cord factor, TDM, is the most abundant and toxic glycolipid produced on the mycobacterial cell surface (21). In order to understand the binding target of AK15 and AK15–6 to *M. tb*, surface plasmon resonance was performed to investigate whether the peptides can interact with TDM. After immobilizing AK15 or AK16-6 on the chips, TDM as analyte
was assayed at four concentrations (5, 10, 20, and 40 nM) in HBS-N buffer. As shown in Fig. 4, the binding of TDM to AK15 (Fig. 4A) and AK15-6 (Fig. 4B) was dose-dependent. The increment in resonance units (RU) by binding of TDM to immobilized-AK15 or AK15-6 demonstrated the direct interaction between TDM and AK15 or AK15-6. Kinetic analysis for AK15 or AK15-6 as ligand interacting with TDM as analyte indicated a rapid association of AK15 ($K_D = 3.19 \times 10^{-11}$ M) or AK15-6 ($K_D = 2.03 \times 10^{-11}$ M) with TDM to form AK15- or AK15-6-TDM complex and slow disassociation, and AK15-6 showed a higher affinity to TDM as compared to AK15.

**The pre-incubation of AK15 or AK15-6 with TDM blocked the anti-mycobacterial activity of the peptides**

To further investigate the interaction between the peptides and TDM, we assessed the anti-mycobacterial activity of the peptides followed by pre-incubation with TDM at 37 °C for 5 min. Compared to vehicle (PBS, DMSO)–treated *M. tuberculosis* H37Rv, the addition of 1, 10 or 100 μg/ml of TDM alone had no significant effect on *M. tb* replication, and the addition of 75 μg/ml of AK15 or AK15-6 alone significantly inhibited *M. tb* replication. While TDM blocked the anti-mycobacterial activity of AK15 or AK15-6 in a dose-dependent manner after the pre-incubation of 1, 10 and 100 μg/ml of TDM with 75 μg/ml of AK16 or AK15-6, respectively (Fig. 5). And 100 μg/ml of TDM completely blocked the anti-mycobacterial activity of 75 μg/ml of AK15 or AK15-6. The data further indicated that the peptides directly interacted with TDM.

**AK15 and its isomer AK15-6 increased the passive diffusion of the fluorescent dye into *M. tb***

Antimicrobial peptides are usually membrane–active agents which disrupt the cell membrane integrity and result in pore formation after their association and accumulation onto bacterial membrane (15). With direct binding of the peptides to *M. tb* and apparent affinity of the peptides to TDM, we next evaluated whether AK15 and AK15-6 could disrupt mycobacterial membrane by incubation of *M. tuberculosis* H37Ra with FITC–labeled dextran probe in the presence or absence of peptide. As shown in Fig. 6, incubation of *M. tuberculosis* H37Ra with PBS did not result in the uptake of FITC–dextran, while the exposure of *M. tb* to AK15 induced the uptake of fluorescence probe, and the exposure of *M. tb* to AK15-6 resulted in a greater uptake of the fluorescence probe than AK15 did. The data indicated that the peptides induced the passive diffusion of the fluorescent dye into *M. tb*.

**Increasing of fluorescence intensity after treatment of AK15–6 as compared to AK15 is indicative of an enhanced membrane damage and pore formation, allowing for greater uptake of the fluorescent probe. These results implied a membrane–disrupting mechanism of action of the peptides after direct binding to *M. tb***

To confirm the membrane–disrupting mechanism of action of the peptides, the surface morphology of *M. tuberculosis* H37Ra was visualized by SEM after exposure to peptides or vehicle (PBS). As shown in Fig. 7, PBS–exposed *M. tb* exhibited regular, smooth and intact surface, while AK15–exposed *M. tb* exhibited extensively rough and collapsed surface. In the same condition, AK15–6–exposed *M. tb* showed a greater change of surface morphology with rough and collapsed surface covered with irregular debris. The impaired surface morphology of AK15/AK15-6–exposed *M. tb* confirmed the membrane–disrupting mechanism of action of the peptides after direct binding to *M. tb*.

**AK15 and its isomer AK15-6 induced the leakage of intracellular ATP from *M. tb***

As described previously, mycobacterial membrane integrity disrupted by anti-mycobacterial peptides is accompanied by
leakage of intracellular contents including ATP (16). To further confirm the membrane–disrupting mechanism, we assessed the extracellular ATP levels after exposure of *M. tuberculosis* H37Ra to supra and sub–inhibitory concentrations of AK15 or AK15–6. Compared to PBS–exposed *M. tb*, AK15 or AK15–6 exposure significantly induced a concentration–dependent release of ATP from mycobacterial cells at concentrations above respective MIC value (Fig. 8). At a concentration of 75 μg/ml, AK15 and AK15–6 induced 68.4 and 105.7 nM extracellular ATP accumulation released from *M. tb*, respectively. The data further confirmed the membrane–disrupting mechanism of the peptides, and AK15–6 showed a stronger membrane–disrupting capacity than AK15 did.

**AK15 and its isomer AK15–6 showed cell selectivity to *M. tb***

With the *M. tb* (TDM)–binding and membrane–disrupting mechanisms of the peptides, we were interested to determine the relative specificity of AK15 and its isomer AK16–6 on mammalian cells, including their hemolytic activity against human and rabbit red blood cells, and cytotoxicity against murine BMDMs, RAW264.7 cells and Vero E6 cells. As shown in Table 3, AK15 and its isomer AK15–6 didn’t show any hemolytic activity and cytotoxicity against all tested mammalian cells at concentrations up to 200 μg/ml.

We next evaluated their inhibitory effects on other Gram–negative bacteria, Gram–positive bacteria and fungi by MIC assay. As show in Table S2, all the tested microorganisms were not sensitive to AK15 and its isomer AK15–6 at concentrations below 150 μg/ml. These results indicated that AK15 and its isomer AK15–6 showed cell selectivity to *M. tb*. As expected, AK15 and AK15–6 showed no significant cell–binding and membrane–permeating capacity to *E. coli* and *S. aureus* relative to *M. tb* (Fig. S1 & 2).

**Positively charged residues, aromatic residues and α–helix constitute the key structural requirements of the peptides**

As described previously, positively charged residues (arginine and lysine) are essential for the antimicrobial actions of many other antimicrobial peptides (22–24). In addition, aromatic residues (tryptophan and phenylalanine) are key residues for the antimicrobial activity, anti–inflammatory activity and cell selectivity of these peptides (16,23,25). In order to understand whether positively charged residues and aromatic residues are critical for the anti–mycobacterial activities of mycobacteriophage–derived AK15 and its isomer AK15–6, we substituted the positively charged residues and aromatic residues with alanine, respectively. As shown in Table 4, the substitution of arginine, lysine or tryptophan with alanine in AK15 and AK15–6 resulted in a significant decrease of anti–mycobacterial activity as reflected by MIC values increased by 2–8 folds, respectively. Besides, the co–substitution of arginine/lysine and tryptophan with alanine in AK15 and AK15–6 resulted in no detectable anti–mycobacterial activity at concentration up to 200 μg/ml. Additionally, a scrambled amino acid sequence of the helical AK15 (sAK15) also did not show any anti–mycobacterial activity at concentration up to 200 μg/ml (Table 4). The data indicated that lysine, arginine, aromatic residues and an α–helix are key structural requirements for the anti–mycobacterial activity of the peptides, and the positively charged residues and aromatic residues probably play synergistic roles in anti–mycobacterial activity of the peptides, which provides new insights to design small but potent α–helical anti–mycobacterial peptide enriched in positively charged residues (lysine, arginine) and aromatic residues (tryptophan).

**AK15 and its isomer AK15–6 showed none induced drug resistance to *M. tb***

Microbes are less likely to develop drug resistance against antimicrobial peptides. Hence, they were considered as promising candidates for peptide antibiotic development (24). We herein
investigated whether mycobacteriophage–derived peptides could induce drug resistance against *M. tuberculosis* H37Rv. To simulate drug resistance *in vitro*, mycobacteria were exposed to sub–therapeutic doses of rifampicin or peptides over 16 passages slightly modified as previous method (15). As shown in Table 5, the MIC value of rifampicin against *M. tuberculosis* H37Rv increased from 0.012 μg/ml to 1.5625 μg/ml (increased by 128 folds), which indicated that resistance of *M. tuberculosis* H37Rv against rifampicin did develop with sub–therapeutic treatment. In contrast, resistance of *M. tuberculosis* H37Rv against AK15 and AK15–6 did not readily develop with sub–therapeutic treatment of the peptides as shown by the consistent MIC values obtained over 16 passages. **AK15 and its isomer AK15–6 generated synergistic effects with rifampicin against *M. tb***

Although antimicrobial peptides exhibit such advantage without induced drug resistance, their clinical applications are still limited due to draw–backs such as poor stability *in vivo* (26). To overcome such shortcomings, the combinational usage of antimicrobial peptides and traditional antibiotics might be a promising way, which could produce synergistic effects between the peptides and antibiotics, and a greater antimicrobial effect (27,28). Given these, the interactive effects of mycobacteriophage–derived peptides with rifampicin (the most effective first line antibiotic in treatment of tuberculosis) were determined by chequerboard assay. As shown in Table 6, AK15 and its isomer AK15–6 exhibited synergistic effects with rifampicin with an FICI value of 0.5 in rifampicin–susceptible *M. tuberculosis* H37Rv. Further testing in rifampicin–resistant *M. tuberculosis* H37Rv (MIC=1.5625 μg/ml) produced similar results, whereas the AK15– or AK15–6–rifampicin combination generated synergistic effects against rifampicin–resistant *M. tuberculosis* H37Rv with an FICI of 0.50. Notably, peptide–rifampicin used in combination generated a lower minimum effective concentration for each agent. Synergistic effects of AK15/AK15–6 with rifampicin is likely attributed to the peptide–mediated permeation of rifampicin from outer membrane to cytoplasmic targets (15,16,24). As a result, the combinational usage of anti–mycobacterial peptide and antibiotic may slow the progression of drug–resistant *M. tb*, and even prevent the emergence of drug–resistant *M. tb*. **AK15 and its isomer AK15–6 exhibited thermal stability and low toxicity to mice**

In order to translate these promising findings to clinical trials, it is extremely important to evaluate the thermal stability, serum stability and acute toxicity of the peptides. As shown in Fig. S3A, after incubation at 37 °C for 96 h, the MIC values of the peptides against *M. tuberculosis* H37Rv just increased by 2–3 folds, which indicated that the peptides exhibited extremely thermal stability in aqueous solution.

We next detected the effect of human serum on the anti–mycobacterial activity of the peptides. As shown in Fig. S3B, the MIC values of the peptides against *M. tuberculosis* H37Rv gradually increased with intended incubation time. After incubation of the peptides with human serum for 4 h, the peptides exhibited no detectable anti–mycobacterial activity against *M. tuberculosis* H37Rv at concentration up to 200 μg/ml. Serum stability investigation indicated that AK15 and AK15–6 can be degraded by human serum proteins as many other AMPs.

We then evaluated the acute toxicity of the peptides to mice. The acute toxicity (24 h) of AK15 and AK15–6 was tested in female BALB/c mice by i.v. delivery. The highest amount of administered AK15/AK15–6 that does not kill tested mice is defined as the maximum tolerable dose. The maximum tolerable dose of AK15/AK15–6 by i.v. delivery was between 150 and 175 mg/kg, well above the doses (10 mg/kg) used in mouse models as mentioned below.
**AK15 and its isomer AK15–6 restrained TDM-stimulated inflammatory signaling activation and inflammatory cytokine production in murine macrophages**

As mentioned above, AK15 and its isomer AK15–6 directly inhibited *M. tb* replication by binding to *M. tb* (TDM) and disrupting mycobacterial membrane. To see if the peptides could affect TDM–induced inflammatory response, we evaluated their effects on inflammatory signaling activation and inflammatory cytokine production in murine BMDMs stimulated by TDM, which acts as the most immunostimulatory component derived from *M. tb*.

As illustrated in Fig. 9A, TDM stimulation markedly induced the activation (phosphorylation) of mitogen–activated protein kinases (ERK, JNK and p38), protein kinase B (Akt) and NF–κB (p65) signaling, and the addition of AK15 or AK15–6 (20 μg/ml) inhibited TDM–induced inflammatory signaling activation in murine BMDMs.

As a result, TDM significantly induced inflammatory cytokine production in murine BMDMs, including IFN–γ, TNF–α, IL–1β, IL–6 and IL–10, while the peptides significantly attenuated these inflammatory cytokine production (Fig. 9B). As mentioned before, AK15–6 showed a higher binding affinity to TDM than AK15 did. AK15–6 herein also showed a higher inhibitory efficiency against TDM–induced inflammatory signaling activation and inflammatory cytokine production in murine BMDMs than AK15 did. It is more likely that the inhibitory effects of the peptides on TDM–stimulated inflammatory response in murine BMDMs are attributable to their binding to TDM and neutralizing the immunostimulatory activity of TDM. Similarly, the peptides also significantly attenuated the pro–inflammatory cytokine production in *M. tb*–infected murine BMDMs (Fig. S4).

**AK15 and its isomer AK15–6 efficiently protected mice from *M. tb* infection**

Although several anti–mycobacterial peptides were well investigated *in vitro*, most of them lacked anti–mycobacterial assay *in vivo*. Given the potent anti–mycobacterial and anti–inflammatory abilities of the peptides with low toxicity to mammalian cells and mice, we next evaluated the *in vivo* protective effects of AK15 and AK15–6 against *M. tuberculosis* H37Rv infection in mice. Compared to PBS–treated mice, AK15 or AK15–6 treatment (10 mg/kg) significantly reduced the mycobacterial loads, granulomatous responses (number and size) and inflammatory cytokine production (IFN–γ, TNF–α, IL–1β, IL–6 and IL–10) in lungs of *M. tb*–infected mice (Fig. 10). Besides, AK15 treatment (10 mg/kg) generated a better protective effect than PK34 did, and AK15–6 therapy (10 mg/kg) did show the best protective effect among them, which were consistent with the results observed *in vitro*. At a dose of 10 mg/kg, AK15–6 reduced about 79.0% mycobacterial load, 72.2% granuloma number, 67.5% granuloma size, 66.2% IFN–γ, 58.6% TNF–α, 55.1% IL–1β, 61.8% IL–6 and 64.1% IL–10 production in lungs as compared to PBS–treated mice, respectively (Fig. 10). The data indicated that the peptides efficiently restrained *M. tb* replication and inflammatory responses *in vivo*, but sustained certain levels of pro–inflammatory cytokine production *in vivo*.

**AK15 and its isomer AK15–6 enhanced modest basal immune responses in BMDMs and mice**

In addition to direct antimicrobial activities, many antimicrobial peptides also showed diverse immunomodulatory activities, including induction of proinflammatory cytokines. We next assessed whether the peptides affected the basal immune response of BMDMs and mice by detecting their effects on proinflammatory cytokine production without TDM–stimulation and *M. tb* infection.

As shown in Fig. S5, the incubation of BMDMs with AK15 or AK15–6 (20 μg/ml)
elicited a modest level of TNF–α production ($P<0.05$), as well as IL–6 production.

As described previously, the formation of an early granuloma is primarily regulated by IFN–γ and TNF–α released from the infected cells to restrain bacterial spread (29). Besides, the granuloma is mainly composed by CD4$^+$ and CD8$^+$ T cells (29), and IFN–γ–CD3$^+$ T cells have been considered as dominant immunological cells to clear $M. \text{tb}$ (30). Thus, we particularly investigated the effects of AK15 and AK15–6 on T–cell cytokine (IFN–γ and TNF–α) response in mice without $M. \text{tb}$ infection. As shown in Fig. 11&S6, the i.v. injection of AK15 or AK15–6 (10 mg/kg) elicited a modest IFN–γ and TNF–α response by CD4$^+$ and CD8$^+$ T cell in lung. For example, AK15 and AK15–6 (10 mg/kg) significantly elicited IFN–γ–CD4$^+$, TNF–α–CD4$^+$ and TNF–α–CD8$^+$ T cell response in lung at week 1 followed by i.v. injection of the peptides as compared to vehicle (PBS)–treated mice. Similarly, the i.v. injection of AK15 or AK15–6 (10 mg/kg) simultaneously elicited a modest IFN–γ and TNF–α responses by CD4$^+$ and CD8$^+$ T cells in spleen (Fig. S7). For instance, AK15 and AK15–6 (10 mg/kg) significantly elicited IFN–γ–CD4$^+$, IFN–γ–CD8$^+$ and TNF–α–CD8$^+$ T cell responses in spleen at week 1 followed by i.v. injection of the peptides as compared to vehicle (PBS)–treated mice. The data indicated that AK15 and AK15–6 could modestly elicit the expression of pro–inflammatory cytokines in BMDMs and mice.

Discussion

Due to the limitation of bacillus Calmette–Guérin vaccine and rapid emergence of drug–resistance, $M. \text{tb}$ is depriving millions of people of their life worldwide every year. We are obligated to look for alternative and/or supplemental ways to combat with $M. \text{tb}$ infection. The remarkable genetic diversity of mycobacteriophage may provide a wealthy platform of mycobacteriophage metaproteome to identify potent anti–mycobacterial peptides with potential for treatment of $M. \text{tb}$ infection. In the previous study, we identified a mycobacteriophage–derived anti–mycobacterial peptide PK34 with a MIC value of 50 μg/ml against $M. \text{tuberculosis}$ H37Rv (ATCC 27294), which proved that we can identify anti–tubercular peptides from mycobacteriophages (4). PK34 is composed of 34 amino acids with a molecular weight of 3957.55 Da. It is widely recognized that the large size that correspond to higher production cost for drug development limit the widespread use of peptide antibiotics (15). In present study, we focused on identification of mycobacteriophage–derived anti–mycobacterial peptide with small size. To our surprise, a small peptide, AK15, is composed of 15 amino acid residues with a lower molecular weight (2071.55 Da) as compared to PK34 (3957.55 Da). AK15 possessed a more potent anti–mycobacterial activity against of $M. \text{tuberculosis}$ H37Rv (ATCC 27294) with a MIC value of 37.5 μg/ml as compared to PK34 (50 μg/ml). AK15 even kept active to rifampicin–induced drug–resistant $M. \text{tb}$ infection. The identification of AK15 with potent anti–mycobacterial activities provided a smaller mycobacteriophage–derived peptide candidate for anti–mycobacterial drug development with lower production cost. Combined with PK34 identified from mycobacteriophage previously (4), there are a total of two anti–mycobacterial peptides derived from mycobacteriophages. Although the true roles of these peptides in mycobacteriophage lifestyle are entirely unknown, the membrane-disrupting capacity of these peptides imply that they possibly facilitate the injection of deoxyribonucleic acid of mycobacteriophage to mycobacterial host or release of mycobacteriophage from mycobacterial host during infection.

Secondary structural analysis indicated that AK15 adopted cationic amphiphilic α–
helical structure. Majority of AMPs adopt this arrangement and their antimicrobial activities are largely attributed to such cationic amphiphilic α–helical structure (22,31). The interactions between antimicrobial peptides and bacterial membrane are modulated by various physicochemical properties including peptide hydrophobicity, charge, secondary structure and amphiphilicity (32,33). The roles of these physicochemical parameters of peptides in their antimicrobial properties are well focused on Gram–positive bacteria, Gram–negative bacteria and fungi (22,31–34). More recently, anti–mycobacterial assay of several designed peptides revealed that positive charges, amphiphilicity and α–helix of such peptides are critical for their interactions with M. tb (15–17). However, there was no information about optimization of anti–mycobacterial peptide through rearranging amino acid residues. In this study, we tried to improve the anti–mycobacterial potency of AK15 by rearranging the amino acid residues of its amphilic helix to increase the hydrophobic moment, and kept molecular weight, net charge, hydrophobicity and α–helical confirmation unaltered. After rearrangement, AK15–6, one of the six isomers, exhibited an enhancement of anti–mycobacterial activity. Previous study indicated that hydrophobic moment plot methodology is not a generally reliable predictor of structure–function relationship of α–helical peptide (35). In accordance with that, the other five isomers of AK15 with increased hydrophobic moment did not show an enhanced anti–mycobacterial potency (Table 2). Besides, we also designed several isomers of AK15 with decreased hydrophobic moment, and not all of them showed a decreased anti–mycobacterial activity (Data not shown). As described previously, hydrophobic moment is a measure of the amphiphilicity of a helix (18). A more likely reason is that the increment of concentration of hydrophobic amino acid residues on one side and hydrophilic amino acid residues on the other side resulted in the increment of the hydrophobic moment (Fig.1, Table 2), which in turn increased the amphiphilicity and enhanced the interaction of AK15–6 with M. tb.

A large numbers of antimicrobial peptides showed many draw–backs, including high cytotoxicity against mammalian cells and broad spectrum against microbes, which limited their clinical application. In this study, AK15 and its isomer AK15–6 did not show any cytotoxicity and hemolytic activity toward the tested mammalian cells at concentration up to 200 μg/ml (Table 3). In addition, AK15 and its isomer AK15–6 did not show any antimicrobial activity against the tested Gram–positive bacteria, Gram–negative bacteria and fungi at concentration below 150 μg/ml (Table S2). The results indicated that mycobacteriophage–derived AK15 and its isomer AK15–6 showed cell selectivity to M. tb. Similar results were observed in mycobacteriophage–derived anti–mycobacterial peptide PK34, which also showed cell selectivity to M. tb (4). It is more likely that mycobacteriophages are viruses that specifically infect mycobacterial hosts, and these mycobacteriophage–derived anti–mycobacterial peptides may show specificity to target the highly complex cell wall, cell membrane or mycolic acids and peptidoglycan–arabinogalactan polymer on outer membrane of M. tb such as TDM (4,21).

Real time interaction of mycobacteriophage–derived AK15 or AK15–6 with TMD by surface plasmon resonance confirmed that they both showed apparent affinity to TDM, but TDM did not bind to sAK15, which demonstrated the binding specificity of TDM to the peptides (Fig. S8). In addition, the pre–incubation of TDM (100 μg/ml) with AK15 or AK15–6 (75 μg/ml) completely blocked the anti–mycobacterial activities of the peptides. The data indicated that the pre–incubation of TDM with the peptides led to the formation of TDM–AK15 or –AK15–6 complex and blocked the
binding of AK15 or AK15–6 to TDM on the outer membrane of *M. tb*, which in turn blocked the anti–mycobacterial activities of the peptides. The direct interaction of the peptides with TDM demonstrated that TDM comprised a critical binding target of the peptides, and the binding of AK15 or AK15–6 to TDM on outer membrane of *M. tb* constituted the first step of the anti–mycobacterial process. The apparent binding affinity of AK15 and AK15–6 to TDM at least partly interpreted the cell selectivity of the peptides to *M. tb*.

As mentioned above, it is well known that proinflammatory response is extremely crucial for clearing off *M. tb* infection. However, AK15 and its isomer AK15–6 exhibited anti–inflammatory response by inhibition of cytokine production in TDM–induced BMDMs and *M. tb*–infected mice, which seemed to be a contradiction with their anti–mycobacterial activity in *vivo*. It is most likely that the anti–inflammatory activity of the peptides may be attributed to a consequence of their anti–mycobacterial effect by direct binding to TDM and inactivation of *M. tb*. In other word, peptide treatment led to a reduction of mycobacterial load, which consequently led to a reduction of granuloma formation and proinflammatory cytokine production in the lung of *M. tb*–infected mice.

Recently, cytokines in tuberculosis have been considered as a two–edged sword in TB pathogenesis (29). *M. tb* can orchestrate a cytokine storm to activate and/or evade host immune responses during infection (29). Thus, the inhibition of a large amount of cytokine production in TDM–stimulated murine BMDMs and *M. tb*–infected mice by the peptides might be a good aspect for controlling *M. tb* infection as reflected by a better outcome after AK15/AK15–6 treatment. In addition to the inhibition of a large amount of cytokine production, AK15 and AK15–6 simultaneously exhibited a modest enhancement of the basal immune response by eliciting a certain level of proinflammatory cytokines. Considering the critical roles of proinflammatory cytokine in clearing off *M. tb* infection, the induction of certain level of regulatory cytokines by the peptides both in *vitro* and in *vivo* may thus complement their anti–mycobacterial activities. We thereby concluded that AK15 and its isomer AK15–6 exhibit immunomodulatory effects against *M. tb* infection by inhibiting cytokine storm and enhancing host basal immune response.

In order to compare mycobactriophage–derived peptides with other published anti–mycobacterial peptides, we selected four well–studied small anti–mycobacterial peptides for anti–mycobacterial assay in the same conditions as AK15 and AK15–6, and summarized many other small cationic anti–mycobacterial peptides reported in previous papers (41–43). As shown in Table S3, the selected four well–studied small anti–mycobacterial peptides possessed potent anti–*M. tb* activities against the tested mycobacterial strains with MIC values ranging from 11.2 to 120.4 µM, while AK15 and its isomer AK15–6 possessed MIC values ranging from 9.1 to 36.2 µM. The data indicated that AK15 and AK15–6 showed a comparable anti–*M. tb* activities with Pin2[14], Pin2[17] and IDR–HH2. Then, we summarized the anti–mycobacterial performance of many other small cationic anti–mycobacterial peptides from previous papers in Table S4. Performance comparison indicated that the well–studied small cationic anti–mycobacterial peptides showed different anti–mycobacterial efficiencies with MIC values ranging from 0.7 to 141 µM, and mycobactriophage–derived AK15 and its isomer AK15–6 exhibited potent anti–mycobacterial activities as most of the well–studied peptides with MIC values ranging from 4.53 to 36.2 µM (Table 1). In future, the anti–mycobacterial potency as well as stability of AK15 and AK15–6 might be further improved by many ways such as D–enantiomer residue
substitution, polyethylene glycol conjugation, hyaluronic acid nanogel conjugation, chitosan nanogel conjugation, and combinational usage with antibiotics, among many others.

In summary, we characterized a small cationic amphiphilic α–helical anti–mycobacterial peptide (AK15) derived from mycobacteriophage. Besides, an improved isomer named AK15–6 was designed by rearranging the amino acid residues of the helix. AK15 and AK15–6 efficiently restrained mycobacterial replication by binding to TDM and disrupting mycobacterial membrane. Lysine, arginine, tryptophan and an α–helix are key structural requirements for their anti–M. tb activities. They showed bactericidal activity, cell selectivity and synergistic effects with rifampicin, and none induced drug resistance to M. tb. In addition, they simultaneously exhibited immunomodulatory effects by inhibiting the production of a large amount of proinflammatory cytokines and enhancing the basal immune responses of host. Combined with the small size, AK15 and AK15–6 might be good candidates for anti–mycobacterial peptide antibiotic development. Our study provides novel insights to identify small but potent anti–mycobacterial peptide from mycobacteriophage, and highlights new clues to design and improve small and potent anti–mycobacterial peptide.

**Experimental procedures**

**Ethics approval**

BALB/c mice (20 ± 2 g, female) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd and housed in pathogen–free facility in accordance with the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China, 1998) and animal experiment procedures were approved by the Animal Care and Use Committee as well as the Ethical Committee of Soochow University (SYXK2017–0043). All surgery was performed under sodium pentobarbital anesthesia with minimum fear, anxiety and pain.

Collection of venous blood from healthy volunteers was approved by the Ethical Committee of Soochow University. All donors provided informed consent in written form.

**Mycobacteria, cells and peptides**

*M. tuberculosis* H37Rv (ATCC 27294), *M. tuberculosis* H37Ra and clinically isolated *M. tb* strains were provided by Affiliated Hospital of Zunyi Medical University or Kunming Medical University, and were cultured in a Middlebrook 7H9 medium or enumerated on 7H11 agar supplemented with 10% oleic acid–albumin–dextrose–catalase, 0.50% glycerol, and 0.05% Tween 80.

Murine bone marrow–derived macrophages (BMDMs) from BALB/c mice were collected (44) and cultured in RPMI–1640 containing L–glutamine, pyruvate, β–mercaptoethanol (all from Sigma, USA), antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, Gibco, USA), 10% fetal bovine serum and 20 ng/ml of murine recombinant M–CSF (Biolegend) for 7–8 days. Differentiated BMDMs were re–plated for experiment. Vero E6 cells were gifted by Dr. Chunsheng Dong and cultured in DMEM supplemented with antibiotics and 10% FBS. All cells were cultured in a humidified incubator under 5% CO₂ at 37°C.

Peptides were purchased from Synpeptide Co.Ltd. (Shanghai, China). The crude peptides were purified, analyzed by RP–HPLC and MALDI–TOF MS to confirm that the purity was higher than 98%.

**Peptide candidate selection**

Up to now, more than 1400 mycobacteriophage genomes have been completely sequenced (http://phagesdb.org/), which provided a wealthy library to identify anti–mycobacterial peptides (4,45). By mining mycobacteriophage genomes in the U.S. National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/), we selected and synthesized a series of small...
Mycobacteriophage-derived anti-mycobacterial peptides

mycobacteriophage-encoded peptides or their derivatives for anti-mycobacterial assay in vitro. These peptide candidates exhibited a high degree of sequence conservation in different mycobacteriophages, and were composed of 10–30 amino acid residues and positively charged.

To improve the anti-mycobacterial potency of the interesting peptide candidate, isomers were designed by rearranging the amino acid residues to increase concentration of hydrophobic amino acid residues on one side, and hydrophilic amino acid residues on the other side by helix–wheel diagram assay. Isomers with increased hydrophobic moment, and unaltered amino acid composition, secondary confirmation, net charge, molecular weight, and hydrophobicity, were selected for anti-mycobacterial activity assay by MIC determination.

Minimum inhibitory concentration assay

Minimum inhibitory concentrations (MICs) of peptides against M. tuberculosis, Gram–positive, Gram–negative and fungi were determined according to previous methods (15,34). Briefly, a series of two–fold peptide dilutions were prepared in a 96–well plate (100 µl/well) and added to an equal volume of bacterial cells (approximately 10^5 CFU/ml, 100 µl/well) in each well. The plates were then incubated at 37°C and read after 7 days for M. tuberculosis and 18 h for Gram–negative bacteria, Gram–positive bacteria and fungi. The MIC was defined as the lowest peptide concentration at which no growth of microorganism was observed visually or measured at 600 nm.

Structural analysis

An online computational framework was performed to predict the secondary structure (http://bioserv.rpbs.univ–paris–diderot.fr/services/PEP–FOLD3) (46). Physical and chemical parameters of the peptides were analyzed using the software package provided by the Expert Protein Analysis System (ExPASy) proteomics server (http://www.expasy.org/tools/).

The helix–wheel plot was constructed by HeliQuest (http://heliquest.ipmc.cnrs.fr/) (31).

The secondary structural confirmation was verified by CD analysis. Peptides (0.2 mg/ml) were dissolved in membrane–mimetic environment (TFE/H2O, 9:1). CD spectra were collected on a Jasco–810 spectropolarimeter (Jasco, Tokyo, Japan) with a 1 mm path–length cell (25 °C, 0.2 nm interval from 190 to 260 nm). For each spectrum, the data from 3 scans were averaged and smoothed by Jasco–810 software. CD spectra were expressed as the mean residue ellipticity (θ) in deg·cm^2·dmol^−1.

Mycobacterial killing kinetic assay

Mycobacterial killing kinetic assay was performed according to previous report (47). Tubes containing 7H9 broth (10 ml) with peptide at concentrations of 0.5×MIC, 1×MIC and 2×MIC were inoculated with M. tuberculosis H37Rv (approximately 10^7 CFU/ml) and incubated at 37°C for 4, 7, 14 and 21 days, respectively. At each time point, aliquots were removed and diluted in a series of Difco Middlebrook 7H9 broth for the determination of viable counts. Diluted samples were plated onto Difco Middlebrook 7H11 agar plates and total mycobacterial counts were determined after incubation at 37°C for 4 weeks.

Hemolysis and cytotoxicity

Hemolytic activity was evaluated by incubating a series of two–fold diluted peptides with human and rabbit red blood cells at 37°C for 30 min. Hemoglobin concentration was measured by monitoring the absorbance at 540 nm. The positive control of 1% Triton X–100 (v/v) was determined as 100% hemolysis, and 0.9% saline was used as negative control (34).

Cytotoxicity was determined by incubating a series of two–fold diluted peptides with RAW264.7 cells, murine peritoneal macrophages and Vero E6 cells in 96 well plates (2×10^4 cells/well) (34). After incubation with peptides or vehicle (PBS) at 37°C for 24 h, CCK–8 solution (10 µl/well) was added. After incubation for an
additional 2–4 h, absorbance at 450 nm was measured.

**Flow cytometry analysis**

*M. tuberculosis* H37Ra, *E. coli* and *S. aureus* cells were washed twice with PBS and exposed to FITC–labeled AK15 or AK15–6 (1 μg/ml) for 5 min at 37 °C. PBS and FITC–labeled sAK15 served as control, respectively. After exposure, bacterial cells were washed three times with PBS, assayed on a FACScalibur flow cytometer, and analyzed by Cell Quest software (BD Immunocytometry).

**Surface plasmon resonance**

The binding interaction of peptides with mycobacterial cord factor (TDM) was measured using a Biacore 3000 instrument (Biacore, Piscataway) at 25 °C according to the previous method (4). Peptides were immobilized as ligand on a CM5 sensor chip using amine–coupling kit (GE Healthcare, USA) according to the manufacturer’s instruction. In brief, around 1000 RU immobilized peptide was obtained. Different dilutions of TDM (5, 10, 20, 40 nM) in running buffer (HBS–N buffer, GE Healthcare) were injected as analytes for 3 minutes at a flow rate of 20 μl/minute for binding analysis. The surface of the CM5 sensor chip was regenerated with 40 mM NaOH. The values of binding affinity of TDM for the peptides were calculated by BIAevaluation 3.0 software (Biacore) selecting 1:1 Langmuir binding model for the kinetic calculation.

**Effect of TDM on the anti–mycobacterial activity of the peptides**

Peptides (750 μg/ml), TDM (10, 100 and 1000 μg/ml), and peptide (750 μg/ml) mixed with 10, 100 or 1000 μg/ml of TDM were incubated at 37°C for 5 min with 150 rpm shaking. Then peptide, TDM, peptide–TDM mixture, or a same volume of vehicle (PBS, DMSO) were inoculated with *M. tuberculosis* H37Rv (approximately 10^7 CFU/ml), respectively. The final concentrations of TDM were 1, 10 and 100 μg/ml, respectively. After incubation at 37°C for 7 days, the viable mycobacteria were counted as mentioned above.

**Confocal microscopy**

*M. tuberculosis* H37Ra, *E. coli* and *S. aureus* suspension (400 μl, 5×10^5 CFU/ml) was seeded into an 8 well–cover slip chamber. Then an equal volume of PBS solution containing peptide (75 μg/ml) and FITC–dextran (150 kDa, 250 μg/ml, Sigma) were added. After incubation for 1 h at 37°C with constant shaking at 200 rpm, the bacterial cells were washed three times with PBS to remove the free FITC–dextran. The images were acquired using Nikon A1 confocal microscope.

**ATP bioluminescence assay**

Extracellular ATP levels after incubation of *M. tuberculosis* H37Ra with peptides were detected as described previously (16). Briefly, *M. tuberculosis* H37Ra was washed and re–suspended in 10 mM phosphate buffer (OD_600=0.4). A series of peptide dilutions were prepared with the same buffer, and added to an equal volume of *M. tb* suspension (300 μl). After incubation at 37°C for 2 h, samples were centrifuged at 5000 g for 5 min. The ATP levels in the supernatant (extracellular ATP concentrations) were determined using ATP assay kit (Beyotime, Shanghai, China) according to the manufacturer’s instruction.

**Scanning electron microscopy**

*M. tuberculosis* H37Ra cells (approximately 2×10^7 CFU/ml) were incubated with peptides (187.5 μg/ml) or an equal volume of vehicle (PBS) at 37 °C for 4 h. After centrifugation at 1000 g for 10 min, mycobacterial cells were prepared for SEM analysis as described previously (15). The surface morphology was observed with a Hitachi S–4800 SEM following the manufacturer’s instruction.

**Drug resistance stimulation**

Drug resistance was induced in *M. tuberculosis* H37Rv by repeated treatment with
rifampicin, peptide or the same volume of vehicle (DMSO, PBS) for 16 passages and checked via MIC measurement. For each passage, *M. tuberculosis* H37Rv was exposed to sub–MIC concentration of rifampicin or peptide (1/8 of MIC at that particular passage) until growing to the log phase, and the MICs of peptide and rifampicin against *M. tuberculosis* H37Rv were determined as mentioned above (15).

**Chequerboard assay**

Synergistic interactions between peptide and rifampicin were detected by chequerboard assay as described previously (15,48). Briefly, a series of two–fold rifampicin or peptide dilutions were prepared, and equal volume of rifampicin (50 μl/well) and peptide (50 μl/well) were mixed in a 96–well plate. Then *M. tuberculosis* H37Rv (approximately 10^6 CFU/ml, 100 μl/well) was added into each well. After incubation at 37 °C for 7 days, microbial growth was observed visually or recorded spectrophotometrically at 600 nm.

**Effects of the peptides on TDM–stimulated inflammatory signaling activation and cytokine production**

BMDMs (1×10^6/well) were cultured in RPMI–1640 containing 2% FBS in a 6–well culture plate. TDM (5 μg/ml; Sigma, USA), and peptide (20 μg/ml) were added and incubated for 1 h. After incubation, BMDMs were harvested and lysed with RIPA lysis buffer (Beyotime, China). Total protein (40 μg) was separated by SDS–PAGE, and transferred to a polyvinylidene difluoride membrane. The inflammatory signals were detected as described previously (4).

To detect the effect of the peptides on TDM–stimulated inflammatory cytokine production in macrophages, BMDMs (2.5×10^5/well) were plated into a 24–well culture plate in RPMI–1640 containing 2% FBS. TDM (5 μg/ml; Sigma, USA) and peptide (20 μg/ml) was added and incubated for 24 h. Cytokine levels in the supernatant were measured using mouse cytokine ELISA kits (eBioscience, USA) according to the kit instruction.

**Thermal stability and serum stability**

Peptide solution was prepared in sterile deionized water (2 mg/ml), and incubated at 37 °C for 0, 6, 12, 24, 48, 72 and 96 h. Thermal stability of the peptide was evaluated by detection the MIC value of the peptide against *M. tuberculosis* H37Rv after incubation of the peptide at 37 °C for 0–96 h (22).

To evaluate serum stability of the peptide, peptide solution was prepared in sterile deionized water (10 mg/ml), and peptide solution was mixed with human serum at a volume ratio of 1:4 to reach a final concentration of 2 mg/ml. The mixture was incubated at 37 °C for 0–6 h. Serum stability of the peptide was evaluated by determination of the MIC value of the peptide against *M. tuberculosis* H37Rv after incubation of the peptide with human serum for different times (22).

**In vivo anti–mycobacterial assay**

The in vivo protective effects of the peptides were determined in a mouse model of pulmonary tuberculosis using PK34 as a positive control. BALB/c mice (20 ± 2 g, n=6) were infected by intravenous injection of 2×10^6 CFU of *M. tuberculosis* H37Rv suspension in 7H9 medium. Mice were administered by intravenous injection of peptides (10 mg/kg, dissolved in PBS) once a day from day 1 to day 7 after *M. tb* infection. PBS treatment served as a negative control. Sham mice received the same volume of vehicle (PBS and 7H9 medium). Mice were sacrificed by cervical dislocation at 4 weeks post infection. The left lungs were taken, and homogenized in PBS (1 mg lung per ml PBS) on ice. Series of diluted homogenates (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} ) were plated onto Difco Middlebrook 7H11 agar plates, and CFUs were counted after culture at 37°C for 4 weeks. For cytokine measurement, homogenates of left lung were centrifugated at 12,000 g for 15 min. The supernatants were harvested for cytokine determination using ELISA kits (eBioscience,
USA) according to the manufacturer’s instruction. The right lungs were collected and fixed in 10% formalin solution for 24 h. After dehydration by an increasing concentration of alcohol, tissues were embedded in paraffin and sectioned into a thickness of 5 μm section using a histocut (Leica, Germany). Sections were stained with hematoxylin and eosin (H&E), and observed by light microscopy (Nikon Eclipse TE2000–S, Japan). Granulomatous response was calculated from the photographs using PhotoShop (Adobe Photoshop Element 2.0, Adobe Systems). The granuloma number of each mouse in the whole lung section was counted. The average granuloma size of each mouse was determined by randomly selecting 10 granulomas in 3 microscopic fields (36).

**Effects of the peptides on the basal immune response of BMDMs and mice**

BMDMs (2.5×10^5/well, 24–well culture plate) were incubated with peptide (20 μg/ml) in RPMI–1640 medium (2% FBS). After incubation for 24 h, cytokine levels in the supernatant were measured as mentioned above.

To investigate the effects of the peptides on the basal immune response of mice, BALB/c mice (20 ± 2 g, n=6) were intravenously injected with peptide (10 mg/kg, dissolved in PBS) once a day at week 1. Control mice received the same volume of vehicle (PBS), *M. bovis* BCG (10^6 CFU/mouse) or TP–5 (10 mg/kg, thymopentin, RKDVY, a clinically used immunomodulatory peptide). Mice were sacrificed at week 1, 2, 3 and 4, respectively. Pulmonary lymphocytes were isolated according to previous method (30). Briefly, lungs were cut and added with collagenase IV (1 mg/ml, Sigma) and DNase I (5 U/ml, Sigma). After digestion for 1.5 h at 37 °C, the digested lungs were mashed through a 70 μm Falcon strainer. Cells were centrifugated and resuspended in RPMI 1640 medium (2% FBS).

Pulmonary lymphocytes or splenocytes were stimulated with PMA (10 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) for 5 h in the presence of brefeldin A (10 μg/ml, BD Pharmingen). All antibody stains were performed on ice and centrifugation was carried out at 4°C. About 1 × 10^6 cells were first stained with surface markers of FITC–CD3, APC–Cy7–CD4, PerCP–CD8 (BD Pharmingen) for 30 min, then fixed and permeabilized by Cyto Fix/Perm (BD Pharmingen) for 30 min. After intracellular staining with FITC–IFN–γ and APC–TNF–α antibody (BD Biosciences), cells were assayed by flow cytometry as mentioned above.

**Statistical analysis**

Statistical analyses between two groups were done by Student’s t-tests using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). The threshold for significance was 0.05 or better. All experiments were repeated at least three times, and data were presented as mean ± SEM.

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Tables and Table Captions

Table 1. Anti-mycobacterial activity of AK15 in vitro

| M. tb | MIC (µg/ml) |
|-------|-------------|
| M. tuberculosis H37Rv | 37.5 (18.1 µM) |
| M. tuberculosis H37Rv (rifampicin–resistant) | 37.5 (18.1 µM) |
| M. tuberculosis H37Ra | 37.5 (18.1 µM) |
| M. tuberculosis WXY (CI) | 18.75 (9.1 µM) |
| M. tuberculosis CAS3 (CI) | 75 (36.2 µM) |
| M. tuberculosis FYX (CI, rifampicin–resistant) | 37.5 (18.1 µM) |

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates. CI: clinically isolated strain. The MIC values of rifampicin against rifampicin–induced drug–resistant M. tuberculosis H37Rv and clinically isolated drug–resistant M. tuberculosis FYX are 1.5625 and > 32 µg/ml, respectively.

Table 2. Amino acid sequence and physiochemical parameters of AK15 and its isomers, and their MICs against M. tuberculosis H37Rv in vitro

| Peptide | Amino acid sequence | MW (Da) | Net charge | Hydrophobicity | µH | MIC (µg/ml) |
|---------|---------------------|---------|------------|----------------|----|-------------|
| AK15    | AKKKLSRWWLRWWVK     | 2071.55 | +6         | 0.527          | 0.511 | 37.5 37.5 |
| AK15-1  | AKKKLVRWWLRWWSK     | 2071.55 | +6         | 0.527          | 0.613 | 37.5 37.5 |
| AK15-2  | ASKKLVRWWLRWWKK     | 2071.55 | +6         | 0.527          | 0.722 | 37.5 37.5 |
| AK15-3  | ALKKLSRWWKRWWVK     | 2071.55 | +6         | 0.527          | 0.732 | 37.5 37.5 |
| AK15-4  | AVKKSRLWLRWWKK      | 2071.55 | +6         | 0.527          | 0.766 | 37.5 37.5 |
| AK15-5  | ALKLLVRWWKRWWSK     | 2071.55 | +6         | 0.527          | 0.830 | 37.5 37.5 |
| AK15-6  | AKKLLRWWSRWWKK      | 2071.55 | +6         | 0.527          | 0.845 | 18.75 18.75 |

The hydrophobic moment (µH) was determined by HeliQuest (http://heliquest.ipmc.cnrs.fr/).

Table 3. Hemolysis and cytotoxicity of AK15 and its isomer AK15–6 against mammalian cells

| Peptide | Hemolytic activity | Cytotoxicity |
|---------|--------------------|--------------|
|         | Human RBC<sup>a</sup> | Rabbit RBC<sup>a</sup> | Murine BMDMs | RAW264.7 | Vero E6 cells |
| AK15    | ND                  | ND           | ND       | ND       | ND      |
| AK15–6  | ND                  | ND           | ND       | ND       | ND      |

<sup>a</sup>RBC, red blood cells. ND, no detectable hemolytic activity or cytotoxicity of the peptides against tested mammalian cells at concentration up to 200 µg/ml.
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Table 4. Key structural requirement assay for AK15 and its isomer AK15–6

| Peptide          | Amino acids sequence       | MIC (µg/ml) | H37Rv | WXY  | CAS3 |
|------------------|----------------------------|-------------|-------|------|------|
| AK15             | AKKKLLSRWRLRWVK            | 37.5        | 18.75 | 75   |      |
| AK15(R→A)        | AKKKLSAWLAWWVK             | 75          | 75    | 150  |      |
| AK15(K→A)        | AAAAAALSRWRLRWVAA          | 150         | 75    | 150  |      |
| AK15 (W→A)       | AKKKLSRAALRAAVK            | 150         | 150   | 150  |      |
| AK15 (R→A, W→A)  | AKKKLSAAALAAAVK            | >200        | >200  | >200 |      |
| AK15 (K→A, W→A)  | AAAAAALSAALRAAVA           | >200        | >200  | >200 |      |
| AK15–6           | AVKLLLWRWSRWKK             | 18.75       | 9.38  | 18.75|      |
| AK15–6 (R→A)     | AVKKLLAWWSAWWKK            | 75          | 37.5  | 37.5 |      |
| AK15–6 (K→A)     | AVAALLRWWSRWAA             | 75          | 75    | 75   |      |
| AK15–6 (W→A)     | AVKKLLRAASRAAKK            | 150         | 75    | 150  |      |
| AK15–6 (R→A, W→A)| AVKKLLAAASAAAKK           | >200        | >200  | >200 |      |
| AK15–6 (K→A, W→A)| AVAALLRAASRAAAA           | >200        | >200  | >200 |      |
| sAK15 (scrambled AK15) | WSKWKRKAIVWRLWK       | >200        | >200  | >200 |      |

aThese concentrations represent mean values of three independent experiments performed in duplicates.

Table 5. Drug resistance test of the peptides and rifampicin against M. tuberculosis H37Rv

| Stimulation drug | Tested drug | MIC(1) (µg/ml)a | MIC (16) (µg/ml)a |
|------------------|-------------|-----------------|------------------|
| PBS              | AK15        | 37.5            | 37.5             |
| AK15             | AK15        | 37.5            | 37.5             |
| PBS              | AK15–6      | 18.75           | 18.75            |
| AK15–6           | AK15–6      | 18.75           | 18.75            |
| DMSO             | Rifampicin  | 0.012           | 0.012            |
| Rifampicin       | Rifampicin  | 0.012           | 1.5625           |

aThese concentrations represent mean values of three independent experiments performed in duplicates. Peptide was dissolved in PBS, and rifampicin was dissolved in DMSO.

Table 6. Chequerboard assay of rifampicin and AK15 or AK15–6 against M. tuberculosis H37Rv

| M. tuberculosis | Drug combination | MIC (µg/ml) | FIC | FICIa |
|-----------------|------------------|-------------|-----|-------|
|                 |                  | Alone | Combination |       |
| H37Rv (RIF–susceptible) | Rifampicin | 0.012 | 0.003 | 0.25 | 0.5  |
|                  | AK15             | 37.5  | 4.69    | 0.25 | 0.5  |
|                  | Rifampicin       | 0.012 | 0.003 | 0.25 | 0.5  |
|                  | AK15–6           | 18.75 | 2.34  | 0.25 | 0.5  |
| H37Rv (RIF–resistant) | Rifampicin | 1.5625 | 0.3906 | 0.25 | 0.5  |
|                  | AK15             | 37.5  | 9.38   | 0.25 | 0.5  |
|                  | Rifampicin       | 1.5625 | 0.3906 | 0.25 | 0.5  |
|                  | AK15–6           | 18.75 | 4.69  | 0.25 | 0.5  |

aThe fractional inhibitory concentration index (FICI) was calculated for each combination using this equation: FICI= FICA+FICB, where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB= MIC of drug B
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in combination/MIC of drug B alone. FICI of $\leq 0.5$ was interpreted as synergy, $0.5 < \text{FICI} \leq 1.0$ as additive, $1.0 < \text{FICI} \leq 4.0$ as indifferent, and FICI $> 4.0$ as antagonism. These concentrations represent mean values of three independent experiments performed in duplicates.

**Figures and figure Legends**

**Figure 1. AK15 and its isomers adopted amphiphilic $\alpha$–helical structure.** (A) Secondary structure of AK15 predicted by computational framework PEP–FOLD3. (B) Circular dichroism spectra of AK15 and its isomers (0.2 mg/ml) in TFE/H$_2$O (9:1) environment. (C) AK15 and its isomers adopted amphiphilic helical structures constructed by HeliQuest. The hydrophobic residues are shown in yellow and gray, positively charged hydrophilic residues are shown in blue, non–charged hydrophilic residues are shown in purple.

**Figure 2. AK15 and its isomer AK15–6 showed bactericidal property against* M. tuberculosis* H37Rv.** (A) Killing curves of AK15 at different concentrations (0.5×, 1× and 2×MIC) after incubation for 4, 7, 14 and 21 days. (B) Comparison of killing curves between AK15 and its isomer AK15–6 against
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*M. tuberculosis* H37Rv at concentration of 75 μg/ml.

**Figure 3.** AK15 (A) and its isomer AK15–6 (B) were directly bound to *M. tuberculosis* H37Ra cells. *M. tuberculosis* H37Ra were washed twice with PBS and exposed to FITC–labeled AK15 or AK15–6 (1 μg/ml) at 37 °C. PBS and FITC–labeled sAK15 (scrambled AK15) were used as control, respectively. After incubation for 5 min, *M. tuberculosis* H37Ra cells were washed twice with PBS, assayed on a FACS calibur flow cytometer and analyzed by Cell Quest software (BD Immunocytometry). *P < 0.05, **P < 0.01.

**Figure 4.** AK15 (A) and its isomer AK15–6 (B) showed apparent affinity to mycobacterial cord factor (TDM). Interaction kinetics of peptides and TDM were determined by surface plasma resonance. Peptide was immobilized on a CM5 sensor chip as ligand, and TDM was diluted in a series of concentrations in HBS–N buffer. Response (resonance units, RU) are recorded for indicated
Figure 5. TDM blocked the anti-mycobacterial activities of AK15 (A) and its isomer AK15–6 (B). Peptides (750 μg/ml), TDM (10, 100 and 1000 μg/ml), and peptide (750 μg/ml) mixed with 10, 100 or 1000 μg/ml of TDM were incubated at 37°C and shook at 150 rpm for 5 min. Then peptide, TDM, peptide–TDM mixture, or a same volume of vehicle (PBS, DMSO) were incubated with M. tuberculosis H37Rv (approximately 10^7 CFU/ml), respectively. The final concentrations of TDM were 1, 10 and 100 μg/ml, and the final concentrations of peptides was 75 μg/ml. After incubation at 37°C for 7 days, the viable mycobacteria were counted. *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant.
Figure 6. AK15 and its isomer AK15–6 increased the passive diffusion of the fluorescent dye into *M. tuberculosis* H37Ra cells. *M. tuberculosis* H37Ra suspension (400 μl, approximately $5 \times 10^5$ CFU/ml) was incubated with an equal volume of PBS solution containing AK15 or AK15–6 (75 μg/ml) and FITC–dextran (150 kDa, 250 μg/ml, Sigma) at 37°C for 1 h with constant shaking at 200 rpm. The images were acquired using confocal microscope after mycobacterial cells were washed three times with PBS to remove the free FITC–dextran.
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Figure 7. AK15 and its isomer AK15–6 impaired the surface morphology of *M. tuberculosis* H37Ra. *M. tuberculosis* H37Ra cells (approximately $2 \times 10^7$ CFU/ml) were incubated with AK15 or AK15–6 (187.5 μg/ml) or an equal volume of PBS (vehicle) at 37 °C for 4 h. The surface morphology was observed with a Hitachi S–4800 SEM. The typical alterations of surface morphology were marked by arrows.

Figure 8. AK15 (A) and its isomer AK15–6 (B) induced the leakage of intracellular ATP from *M. tb*. A series of peptide dilutions and *M. tb* suspension (OD$_{600}$=0.4) were prepared in 10 mM phosphate buffer, respectively, and peptide dilutions or PBS were added to an equal volume of *M. tb* suspension (300 μl). After incubation at 37°C for 2 h, extracellular ATP concentrations were determined using ATP assay kit (Beyotime, Shanghai, China) according to the manufacturer’s instruction. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 9. AK15 and its isomer AK15–6 restrained TDM–stimulated inflammatory response in murine BMDMs. (A) AK15 and AK15–6 inhibited TDM–induced inflammatory signaling activation in murine BMDMs. BMDMs (1×10⁶/well) were incubated with TDM (5 μg/ml) in the presence or absence of peptide (20 μg/ml). After incubation for 1 h, cells were lysed for western blot analysis. (B) AK15 and AK15–6 attenuated TDM–induced inflammatory cytokine production in murine BMDMs. BMDMs (2.5×10⁵/well) were incubated with TDM (5 μg/ml) in the presence or absence of peptide. After incubation for 24 h, supernatants were harvested for determination of cytokine levels by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 10. AK15 and its isomer AK15–6 efficiently protected mice from M. tb infection. (A) Viable CFU counts in lung. (B) Protein levels of inflammatory cytokines in lung. (C) Granulomatous response (granuloma number and granuloma size) in lung. BALB/c mice (20 ± 2 g, n=6) were intravenously injected of 2×10⁶ CFU of M. tuberculosis H37Rv. After M. tb infection, mice were intravenously injected with peptides (10 mg/kg) or an equal volume of PBS (vehicle) once a day from day 1 to day 7. Sham mice received the same volume of vehicle (PBS and 7H9 medium). Mice were sacrificed by cervical dislocation at 4 weeks post infection, and the CFUs, inflammatory cytokine levels and granulomatous response were evaluated, respectively. *P < 0.05, **P < 0.01.
Figure 11. AK15 and its isomer AK15–6 enhanced IFN–γ/TNF–α–secreting CD4+ and CD8+ T cell responses in the lung. Statistical analysis of the frequency of IFN–γ or TNF–α–secreting CD4+ and CD8+ T cells. BALB/c mice were injected with peptide (10 mg/kg, dissolved in PBS, i.v.) once a day at week 1. Control mice received the same volume of vehicle (PBS), M. bovis BCG (10⁶ CFU/mouse) or TP–5 (10 mg/kg). Mice were sacrificed at week 1, 2, 3 and 4, respectively. Pulmonary lymphocytes were isolated, intracellularly stained with FITC–IFN–γ and APC–TNF–α antibody, and assayed by flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001.
A small mycobacteriophage–derived peptide and its improved isomer restrict mycobacterial infection via dual mycobactericidal–immunoregulatory activities
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