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

Yang Yang[1,#], Zhen Liu[1,#], Xiaoqin He[2,3,#], Juanjuan Yang[4], Hailong Yang[5], Jing Wu[5], Min Li[1], Qian Qian[1], Ren Lai[2,*], Wei Xu[1,*], Lin Wei[1,*]

From the [1]Jiangsu Key Laboratory of Infection and Immunity, Institutes of Biology and Medical Sciences, Soochow University, Suzhou 215123, Jiangsu, China; [2]Key Laboratory of Animal Models and Human Disease Mechanisms, Chinese Academy of Sciences and Yunnan Province, Kunming Institute of Zoology, Kunming 650223, Yunnan, China; [3]National Health Commission Key Laboratory of Parasitic Disease Control and Prevention, Jiangsu Provincial Key Laboratory on Parasite and Vector Control Technology, Jiangsu Institute of Parasitic Diseases, Wuxi 214064, Jiangsu Province, China; [4]Institute of Pharmaceutical Biotechnology and Engineering, College of Biological Science and Technology, Fuzhou University, Fuzhou 350108, Fujian, China; [5]School of Basic Medical Sciences, Kunming Medical University, Kunming 650500, Yunnan, China.

Running Title: *Mycobacteriophage–derived anti–mycobacterial peptides*

Supplementary tables and table captions

Table S1. Anti–mycobacterial activities of mycobacteriophage–derived peptides against *M. tuberculosis* H37Rv *in vitro*

Peptide	Amino acid sequence	MIC (μg/ml)
RA14	RRERIEGDRRKEVA	>200
RP14	RRLDRKDYLRRVMP	150
AK15	AKKKLSRWWLRWWVK	37.5
II17	IEWGNVSRQPKPKATYI	>200
AQ17	AAHRAGRPIHDAGVVKQ	>200
AK18	ARNVSEENVDRLAKRWIK	150
KY19	KREMKRPGKGNRNNWKKEY	>200
RK19	RLIRVERDSVEALMRPIGK	>200
RR20	RPPGFYFEFRANIIPYLGRR	150
AK20	ARRLGMNPWKTPPAKPKGSK	>200
RE20	RKPRTTKPKPAPKQEPATEE	>200
AR21	ARRRELRARRKRPPERHPGRR	>200
RH22	RNRIREMKRPGKGNRNNWKKEH	>200
RQ23	RLFGLSIRQHEVMTGHTVKVKSQ	>200
RN30	RRMTFDADFELKVAQLNALIAIAELLKEKN	>200

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates.

Table S2. Antimicrobial activities of AK15 and its isomer AK15–6 against other Gram–negative, Gram–positive bacteria and fungi *in vitro*

Microorganisms	MIC (μg/ml)	
	AK15	AK15–6
Gram–negative bacteria		
E. coli ATCC 25922	>200	>200
E. coli ATCC 35218	>200	>200
E. coli 13A10022 (CI)	>200	150
E. coli 08A852 (CI)	>200	>200
P. aeruginosa ATCC27853	>200	>200
P. aeruginosa 08031205 (CI)	>200	>200
A. baumannii ATCC19606	>200	150
A. baumannii strain 1 (CI)	>200	>200
Gram–positive bacteria		
S. aureus ATCC 25923	>200	>200
S. aureus ATCC 6538	>200	>200
S. aureus 08A875 (CI)	>200	150
S. aureus 170805 (CI)	>200	150
S. aureus 181120 (CI)	>200	150
S. epidermidis 13A13730 (CI)	>200	>200
B. subtilis ATCC 6633	>200	150
E. faecalis 13U1964 (CI)	150	150
Fungi		
C. albicans ATCC 10231	>200	>200
C. albicans ATCC 2002	>200	>200
C. albicans 08A802 (CI)	150	150
C. albicans 08022710 (CI)	>200	>200

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates. CI: clinically isolated strain.

Table S3. Anti–mycobacterial activities of AK15, AK15–6 and other four well–studied small anti–mycobacterial peptides *in vitro*

Peptide	Amino acids sequence	MIC (μg/ml, μM) against *M. tuberculosis*			
		H37Rv	WXY	CAS3	FXY
AK15	AKKKLSRWWLRWWVK	37.5/18.1	9.1/18.75	75/36.2	37.5/18.1
AK15–6	AVKKLLRWWSRWWKK	18.75/9.1	9.38/4.5	18.75/9.1	18.75/9.1
Pin2 [14]	FWGLKGLKKFSKKL	18.75/11.2	37.5/22.3	18.75/11.2	18.75/11.2
Pin2 [17]	FWGLKGLKGPGKFSKKL	37.5/19.8	75/39.7	75/39.7	37.5/19.8
$M(LLKK)_2M$	MLLKKLLKKM	150/120.4	75/60.2	150/120.4	75/60.2
IDR–HH2	VQLRIRVAVIRA–NH_2	37.5/26.9	18.75/13.5	75/53.8	37.5/26.9

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates. *M. tuberculosis* WXY and CAS3 are clinically isolated strains. *M.*

tuberculosis FYX is clinically isolated rifampicin–resistant strain with MIC value higher than 32 μg/ml (rifampicin).

Table S4. Performance comparison of mycobacteriophage–derived anti–mycobacterial peptides and other small cationic anti–mycobacterial peptides

Peptide	Modifications	Source	Mechanism/anti–mycobacterial activity
AK15[a]	–	Derived from mycobacterium phage Che12	Direct anti–*M. tb* activity: TDM–binding, membrane disruption/pore formation Immunomodulatory activity: anti–inflammatory activity and pro–inflammatory activity *In vitro*: MIC(rifampicin–resistant and rifampicin–susceptible *M. tb* H37Rv, *M. tb* H37Ra, clinically isolated *M. tb* and MDR–*M. tb*):18.75–75 μg/ml (9.05–36.20 μM) *In vivo*: (*M. tb* H37Rv–infected mice): about 63.3% inhibition at 10 mg/kg (4.83 μM, i.v.)
AK15–6[a]	Rearrangement of the amino acid residues of the helix of AK15	Derived from AK15	Direct anti–*M. tb* activity: TDM–binding, membrane disruption/pore formation Immunomodulatory activity: anti–inflammatory activity and pro–inflammatory activity *In vitro*: MIC(rifampicin–resistant and rifampicin–susceptible *M. tb* H37Rv, clinically isolated *M. tb*):9.38–37.5 μg/ml (4.53–9.05 μM) *In vivo*: (*M. tb* H37Rv–infected mice): about 79.5% inhibition at 10 mg/kg (4.83 μM, i.v.)
PK34[a]	–	Derived from mycobacterium phage D29	Direct anti–*M. tb* activity: TDM–binding Immunomodulatory activity: anti–inflammatory activity *In vitro*: MIC(*M. tb* H37Rv):50 μg/ml (12.63 μM) *In vivo*: (*M. tb* H37Rv–infected mice): about 53.6% inhibition at 10 mg/kg (2.53 μM, i.v.)
1–$C13_{4mer}$	Tetrameric form; oligo–N–substituted glycines (peptoid) and alkylation	Design *de novo*	Direct anti–*M. tb* activity: Pore formation MIC (*M. tb* H37Rv): 6.6 μM
A18G5, A24C1ac, A29C5FA, and A38A1guan	D–enantiomer, alkylation, tetramethylguanidinilation, and polyethylene glycol	Derived from the insect proline–rich peptide Apidaecin	Direct anti–*M. tb* activity: bacterial membrane permeation/inhibition of protein synthesis MIC: not obtained

	conjugation		
CAMP/PL–D	–	Short cationic peptides (10 AA) rich in W and R selected from peptide libraries	Direct anti–*M. tb* activity: pore formation MIC (*M. tb* H37Rv): 1.1–141 μM
CP26	–	Derived from cecropin A: mellitin	Direct anti–*M. tb* activity: cell wall disruption MIC (*M. tb* H37Rv): 2 μg/ml (0.70 μM)
D–LAK 120	D–enantiomer	Synthetic α–helical peptides	Direct anti–*M. tb* activity: pore–formation/Inhibition of protein synthesis MIC (*M. tb* H37Rv): not determined
D–LL37	D–enantiomer	Derived from LL–37	Direct anti–*M. tb* activity: pore–formation Immunomodulatory activity *In vitro*: MIC(*M. tb* H37Rv): 100 μg/ml (22.26 μM)
E2 and E6	–	Derived from bactenecin (bovine cathelicidin) Bac8c (8 AA)	Direct anti–*M. tb* activity: cell wall disruption MIC (*M. tb* H37Rv): 2.6–3.2 μg/ml (1.92–2.20 μM)
HHC–10	–	Derived from bactenecin	Direct anti–*M. tb* activity MIC (*M. bovis* BCG): not determined
hLFcin1–11/ hLFcin17–30	D –enantiomer	Derived from lactoferricin (All–R and All–K substitutions)	Direct anti–*M. tb* activity: bacterial cell wall and membrane lysis IC90 (*M. avium*): 15–30 μM
IDR–1002, –HH2, IDR–1018[a]	–	Derived from macrophage chemotactic protein–1 (MCP–1)	Direct anti–*M. tb* activity: mechanism not determined Immunomodulatory activity: anti–inflammatory activity MIC (*M. tb* H37Rv): 16–29.3 μg/ml (10.42–21.03 μM) *In vivo*: [Mtb H37Rv and multidrug resistant TB strain (MDR–TB) infected mice]: 10–71% killing at 32 μg/mouse (3×week intra–tracheal administration, 30 days)
LLAP	Hyaluronic acid nanogel conjugation	Derived from LL–37	Direct anti–*M. tb* activity: inhibition of ATPase MIC (*M. smegmatis* mc^2 155): 600 μg/ml (357.33 μM)
LLKKK18[a]	Hyaluronic acid nanogel conjugation	Derived from LL–37	Direct anti–*M. tb* activity: pore formation Immunomodulatory activity *In vivo* (*M. tb* H37Rv–infected mice): 1.2–log reduction at 100 μM (10 intra–tracheal administrations)

MIAP	–	Derived from Magainin–I	Direct anti–*M. tb* activity: inhibition of ATPase MIC (*M. tb* H37Ra):300 μg/ml (191.58 μM)
Pin2 variants	–	Derived from pandinin2 (short helical peptides)	Direct anti–*M. tb* activity: membrane disruption MIC (*M. tb* H37Rv and MDR–*M. tb*): 6–33 μM
RN3(1–45) RN6(1–45) RN7(1–45)	–	Derived from human RNases N–terminus	Bacterial cell wall disruption/cell agglutination and intracellular macrophage killing *In vitro*: MIC *(M. vacae*; *M. aurum*; *M. smegmatis* mc^2 155; *M bovis* BCG) *In vivo*: 10–20 μM and *ex vivo* (*M. aurum*): 5–10 μM
Synthetic AMPs (SAMPs–Dma)	Dimethylamination and imidazolation	Design *de novo*	Direct anti–*M. tb* activity: cell penetration and DNA binding synthetic antimicrobial peptide–Dma10: MIC (*M. smegmatis* mc^2 155): <20 μM
X(LLKK) 2X:II–D, II–Orn, IIDab, and IIDap	Peptide D–enantiomer, ornithination, 2,4–diaminobutyric acidation, and 2,3–diaminopropionic acidation	Short stabilized –helical amphipatic peptides	Direct anti–*M. tb* activity: pore formation M(LLKK)2M: MIC (*M. smegmatis* mc^2 155, *M. tb* H37Rv: 62.5–125 μg/ml (50.21–100.42 μM) $I(LLKK)_2I$: effective against MDR–TB

[a]These peptides also showed anti–mycobacterial activities against *M. tb in vivo* using murine infection models.
MIC: minimal inhibitory concentration.

Supplementary figures and figure legends

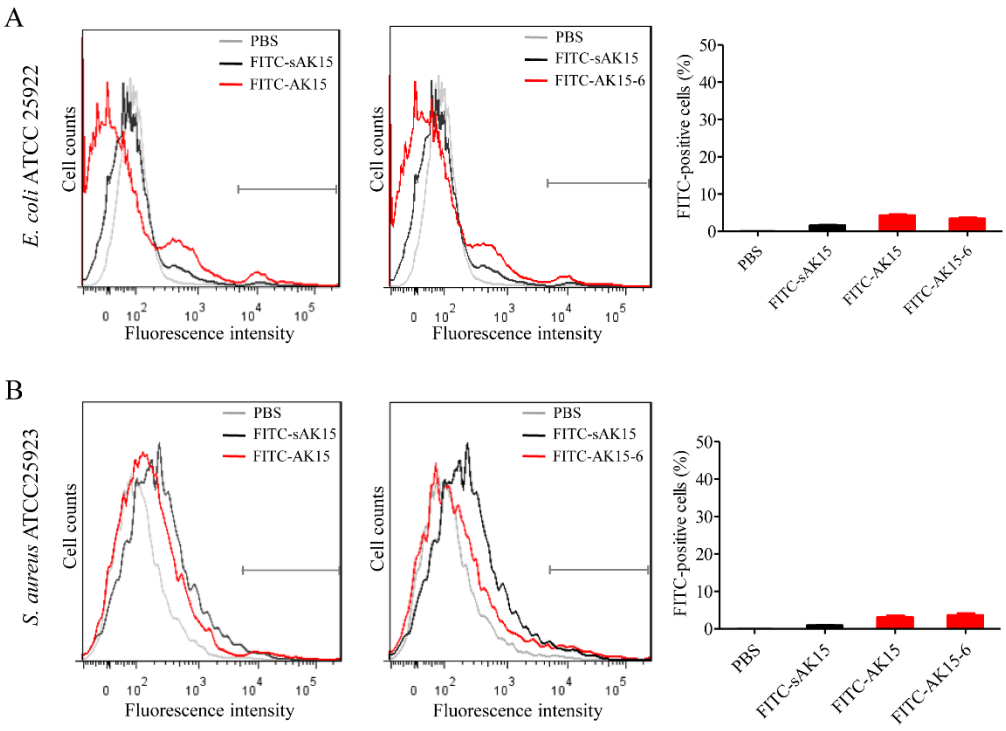

Figure S1. **AK15 and its isomer AK15–6 showed weak binding capacity to *E. coli* (A) and *S. aureus* (B) as compared to *M. tb*.** Bacteria were washed twice with PBS and exposed to FITC–labeled AK15 or AK15–6 (1 μg/ml) at 37 °C. PBS and FITC–labeled sAK15 were used as control, respectively. After incubation for 5 min, bacteria were washed twice with PBS, assayed on a FACS calibur flow cytometer and analyzed by Cell Quest software (BD Immunocytometry). Results are represented as mean ± SEM of three independent experiments.

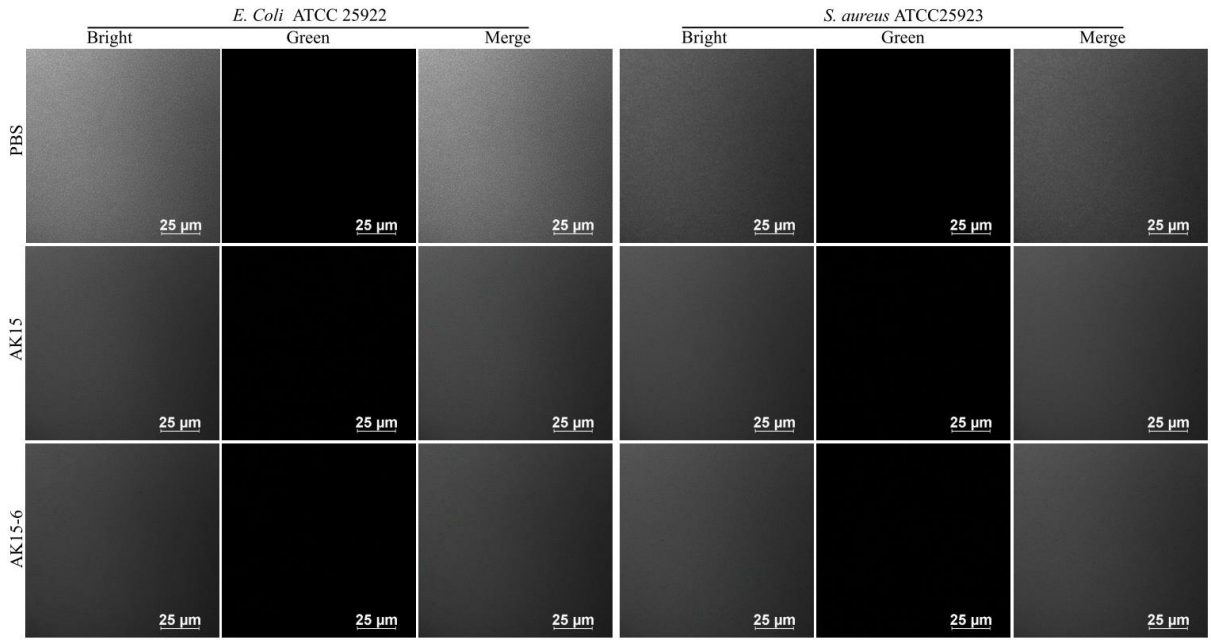

Figure S2. **AK15 and its isomer AK15–6 showed no significant membrane–permeating capacity to *E. coli* and *S. aureus*.** Bacteria were washed twice with PBS and exposed to FITC–labeled AK15 or AK15–6 (1 μg/ml) at 37 °C. PBS and FITC–labeled sAK15 were used as control, respectively. After incubation for 5 min, Bacteria were washed twice with PBS, assayed on a FACS calibur flow cytometer and analyzed by Cell Quest software (BD Immunocytometry).

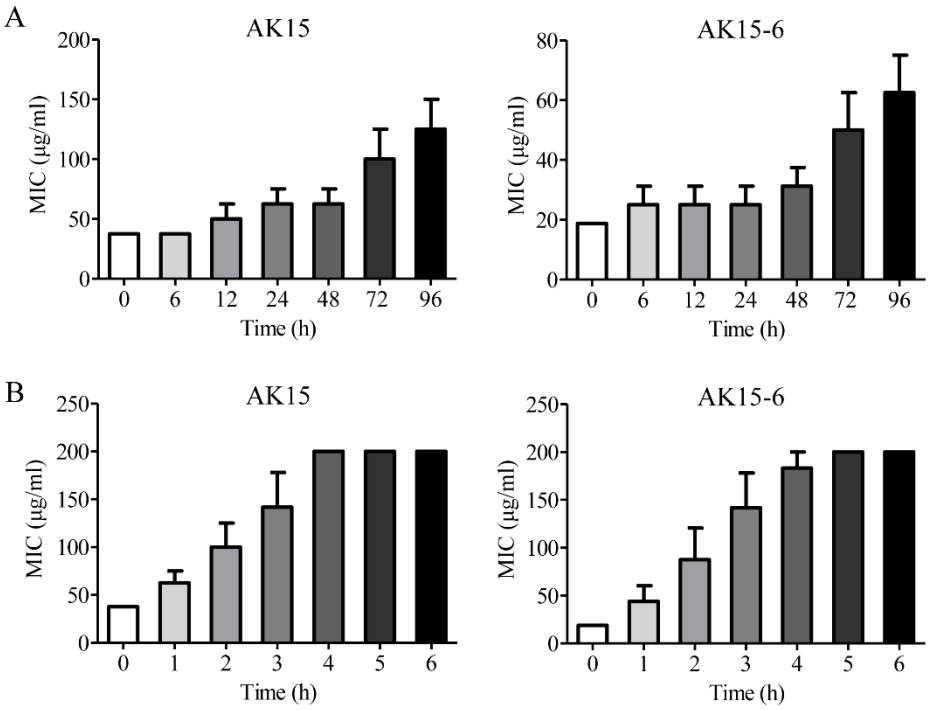

Figure S3. Stability of AK15 and its isomer AK15–6. (A) Thermal stability. Peptide solution (2 mg/ml) was incubated at 37 ℃ for 0, 6, 12, 24, 48, 72 and 96 h. After incubation, thermal stability of the peptide was evaluated by detection the MIC value of the peptide against *M. tuberculosis* H37Rv. (B) Serum stability. Peptide solution (10 mg/ml) was mixed with human serum at a volume ratio of 1:4 to reach a final concentration of 2 mg/ml, and incubated at 37 ℃ for 0–6 h. After incubation, serum stability of the peptide was evaluated by determination of the MIC value of the peptide against *M. tuberculosis* H37Rv.

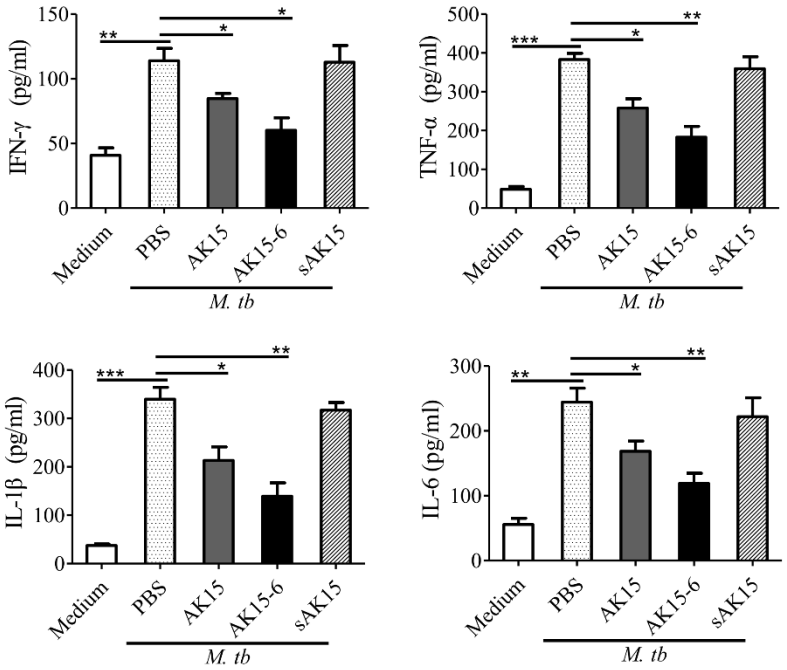

Figure S4. **AK15 and its isomer AK15–6 attenuated the pro–inflammatory cytokine production in *M. tb*–infected murine BMDMs.** BMDMs (1×10^5/well) were infected or not with *M. tuberculosis* H37Rv (M.O.I. = 2) and further incubated in the presence of peptide (20 μg/ml) or an equal volume of PBS. After incubation for 72 h, supernatants were harvested for determination of cytokine levels by ELISA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

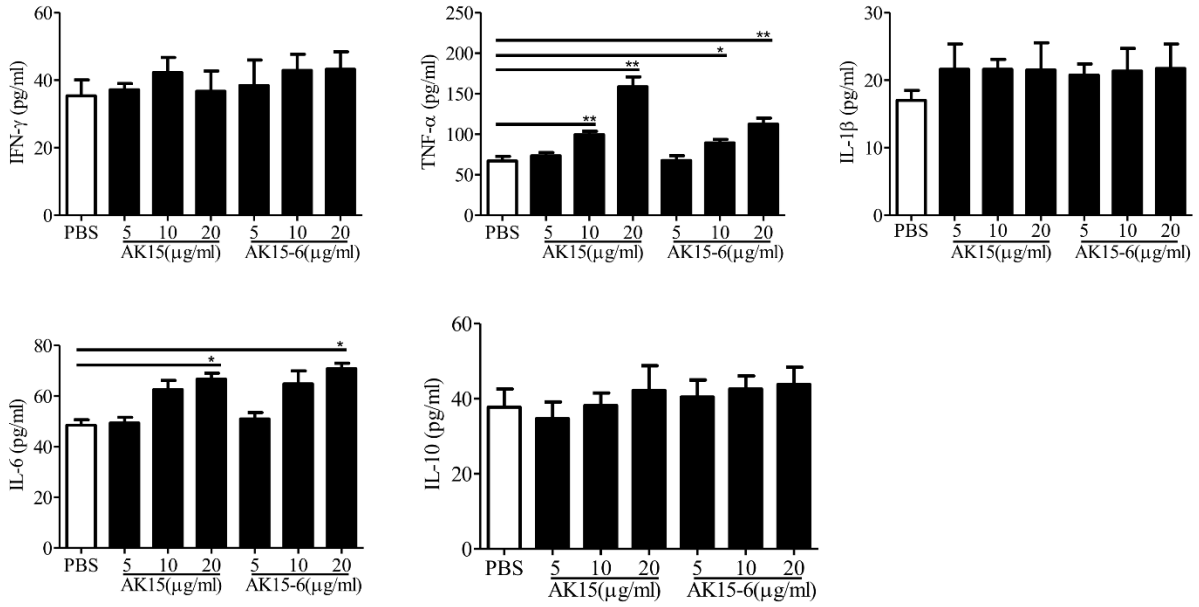

Figure S5. **AK15 and its isomer AK15–6 elicited modest level**s **of TNF–α and IL–6 production in murine BMDMs.** BMDMs (2.5×10^5/well, 24–well culture plate) were incubated with peptide (20 μg/ml) or an equal volume of PBS in RPMI–1640 (2% FBS). After incubation for 24 h, cytokine levels in the supernatant were measured by ELISA.

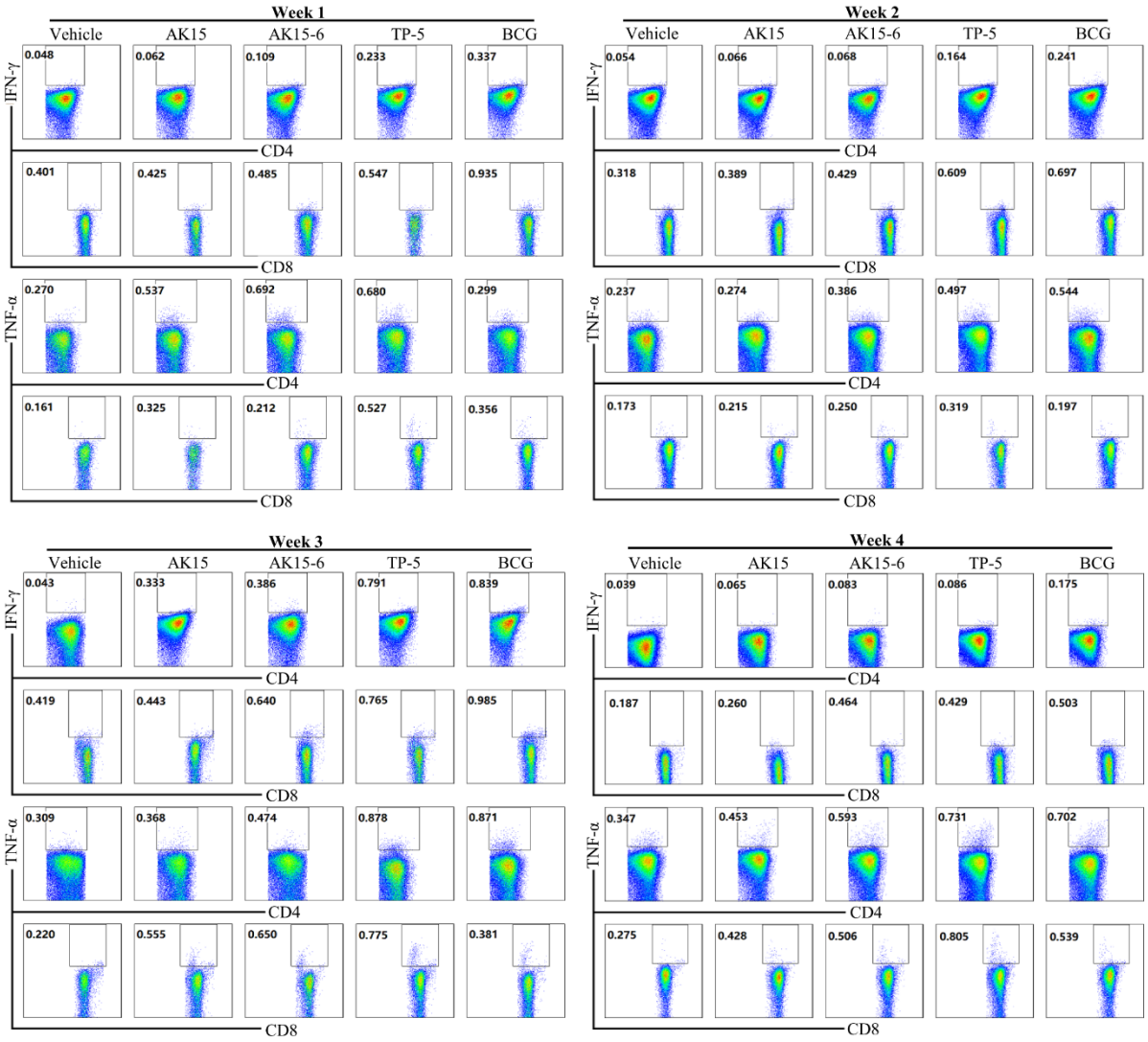

Figure S6. AK15 and its isomer AK15–6 enhanced IFN–γ/TNF–α–secreting CD4$^+$ and CD8$^+$ T cell responses in the lung. The frequency of CD4$^+$ and CD8$^+$ T cells in the lung producing IFN–γ and TNF–α in response to AK15, AK15–6, TP–5 (thymopentin, peptide control, a clinically used immunomodulatory peptide) or BCG (positive control). Numbers in each quadrant represent percentages of IFN–γ or TNF–α–positive cells in CD4$^+$ or CD8$^+$ T population. 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 (10^6 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.

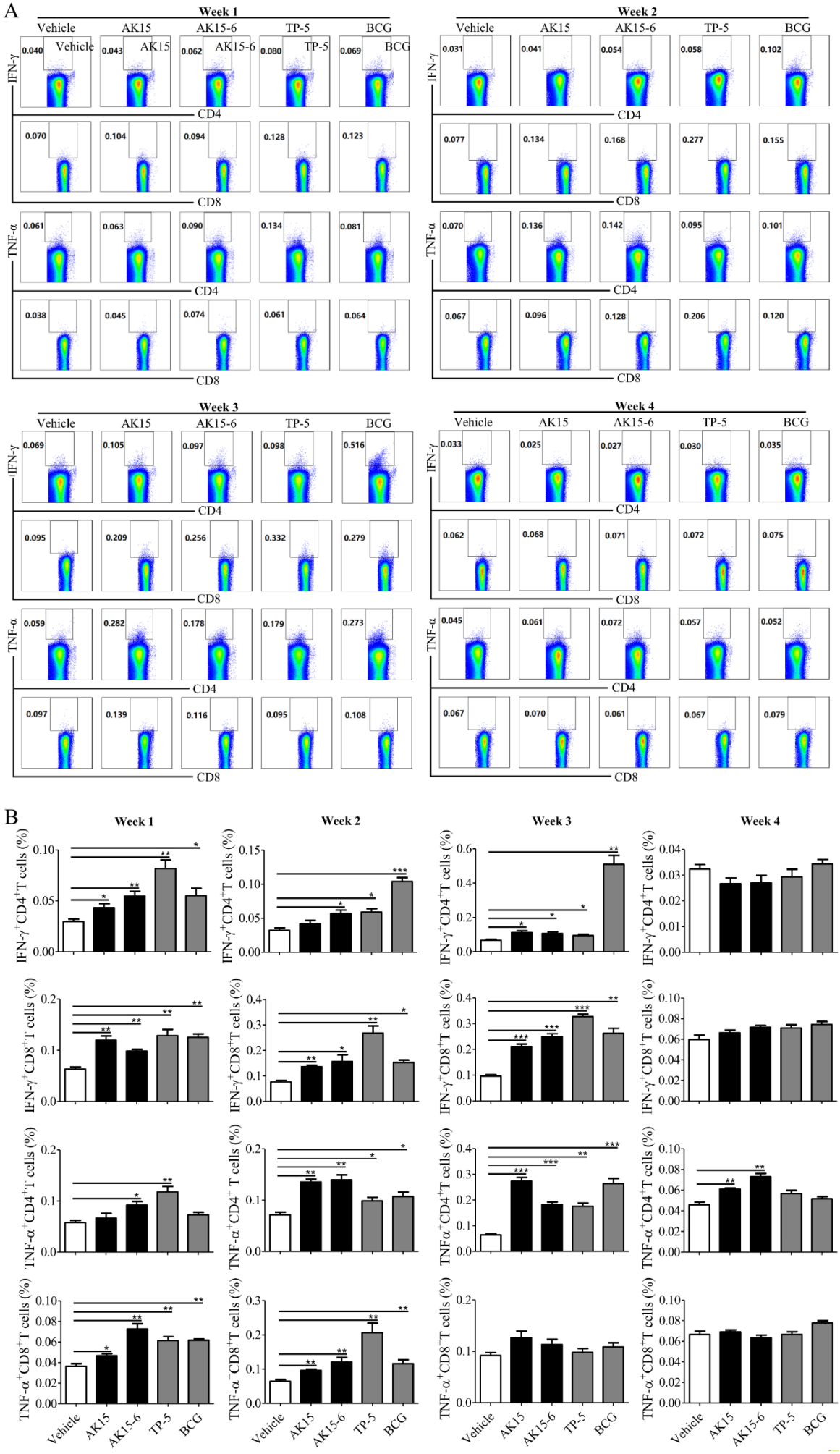
A
Week 1
Week 2
Week 3
Week 4
Vehicle
AK15
AK15-6
TP-5
BCG
IFN-γ
TNF-α
CD4
CD8
B
IFN-γ+CD4+T cells (%)
IFN-γ+CD8+T cells (%)
TNF-α+CD4+T cells (%)
TNF-α+CD8+T cells (%)

Figure S7. AK15 and its isomer AK15–6 enhanced IFN–γ/TNF–α–secreting CD4$^+$ and CD8$^+$ T cell responses in the spleen. (A) The frequency of CD4$^+$ and CD8$^+$ T cells in the spleen producing IFN–γ and TNF–α in response to AK15, AK15–6, TP–5 (thymopentin, peptide control, a clinically used immunomodulatory peptide) or BCG (positive control). Numbers in each quadrant represent percentages of IFN–γ or TNF–α–positive cells in CD4$^+$ or CD8$^+$ T population (B) 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 (10^6 CFU/mouse) or TP–5 (10 mg/kg). Mice were sacrificed at week 1, 2, 3 and 4, respectively. Splenocytes 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.

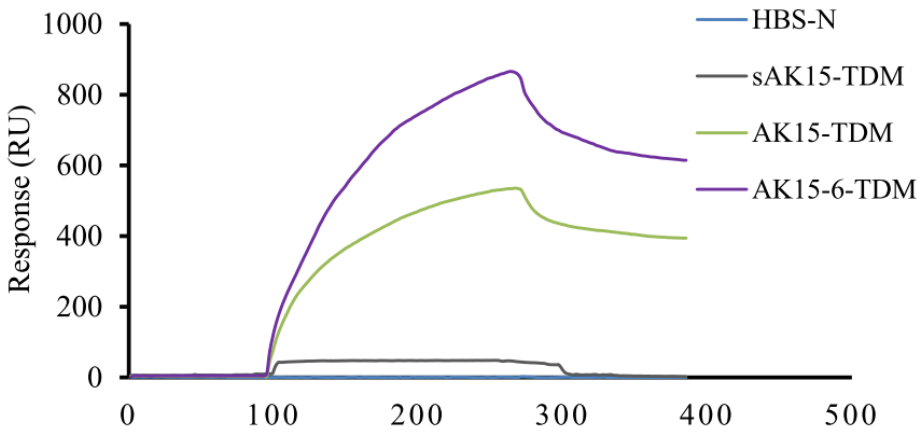

Figure S8. Analysis of the binding specificity of TDM to the peptides. Interaction between peptide (AK15, AK15–6, scrambled AK15: sAK15) and TDM were determined by surface plasma resonance. Peptide was immobilized on a CM5 sensor chip as ligand, and TDM was diluted in HBS–N buffer. Response (resonance units, RU) are recorded for 40 nM TDM.