Supplementary Information

Selection of Threose Nucleic Acid Aptamers to Block PD-1/PD-L1 Interaction for Cancer Immunotherapy

Xintong Li[a,b,c], Zhe Li[a,d*], and Hanyang Yu[a,b,c*]

[a] Department of Biomedical Engineering, College of Engineering and Applied Sciences, Jiangsu Key Laboratory of Artificial Functional Materials, Nanjing University, Nanjing, Jiangsu 210023, China
[b] State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing, Jiangsu 210023, China
[c] Chemistry and Biomedicine Innovation Center (ChemBIC), Nanjing University, Nanjing, Jiangsu 210023, China
[d] State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University, Nanjing, Jiangsu 210023, China

* Corresponding author: Zhe Li and Hanyang Yu
Zhe Li: zheli@nju.edu.cn
Hanyang Yu: hanyangyu@nju.edu.cn

Experimental section

Chemicals and materials. DNA primers, templates and other chemicals were purchased from Sangon Biotechnology (Shanghai, China). TNA nucleoside triphosphates and phosphoramidites were purchased from Biosyntech (Suzhou, China). PD-1 and PD-L1 proteins were purchased from Sino Biological (Beijing, China). ELISA kit was purchased from MultiSciences (Hangzhou, China). Mouse PD-1-expressing plasmid was purchased from Genechem (Shanghai, China). Anti-human-IgG-Fc-APC antibody was purchased from Biolegend (USA). Balb/c mice were purchased from Qinglong Mountain Animal Farm (Nanjing, China). 5-Fluorouracil was purchased from Xudong Haipu Pharmaceutical (Shanghai, China). The murine monocyte cell line RAW264.7 was purchased from the American Type Culture Collection. Lipopolysaccharide was purchased from Solarbio (Beijing, China). The mouse TNF-α ELISA kit was purchased from Fcmacs (Nanjing, China).

Primer extension. The primer P2 was annealed to the DNA library in 1× ThermoPol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8) by heating at 95°C for 5 min and cooling at 4°C for 10 min. The Kod-RI DNA polymerase was pre-mixed with 1 mM $MnCl_2$ for 15 min, and then added to the reaction mixture. Primer extension reactions contained 1 μM primer–template complex, 100 μM tNTPs, and 1mg/mL Kod-RI DNA polymerase. Reactions were performed by adding the Kod-RI DNA polymerase and tNTP substrates to a solution containing all the other reagents, and heating the mixture at 55°C for 4 h. Primer extension products were analyzed by 12% denaturing polyacrylamide gel electrophoresis[1].

Reverse transcription. The primer P5 was annealed to the TNA library in 1× ThermoPol buffer by heating at 95°C for 5 min and cooling at 4°C for 10 min. Primer extension reactions contained 1 μM primer–template complex, 0.5 mM dNTPs, and 0.4 U/μL Bst DNA polymerase. Reactions were initiated by adding the dNTP substrates to a solution containing all the other reagents, and the reaction system was maintained at 55°C for 4 h. Reverse transcription reaction products were analyzed by 12% denaturing polyacrylamide gel electrophoresis[2].

***In vitro* selection.** For each round of selection, primer P3 was annealed to the 3'-biotin-labeled DNA library. The primer–template complex was extended with Kod-RI DNA polymerase using tNTP substrates. The extension product was loaded onto a streptavidin column, and the newly-synthesized TNA library strand was recovered by 200 mM NaOH elution and purified by ethanol precipitation. The resulting pool of single-stranded TNA molecules (10 μM in round 1) was allowed to fold by heating at 55°C for 5 min and cooling at 4°C for 10 min. The pool of folded TNA molecules was then incubated with target human PD-L1 protein (50 μM in round 1) in selection buffer (136 mM NaCl, 2.6 mM KCl, 4 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2 mM $MgCl_2$, pH 7.4) at 37°C for 1 h. Over the course of the selection, the target-to-library ratio was incrementally decreased from 5:1 in round 1 to 1:200 to round 4, to favor the enrichment of high-affinity aptamers. Protein-bound TNA molecules were separated from the unbound using capillary electrophoresis.
Capillary electrophoresis was performed on a Beckman ProteomeLab PA 800 Protein Characterization System. The glass capillary (inner diameter 50 μm; total length 60 cm) was first rinsed with water and equilibrated with selection buffer. A small portion (11 nL) of the TNA–PD-L1 mixture was injected onto the capillary using pressure injection (0.5 psi for 10 s) and electrophoresis was performed under a constant voltage of 10 kV for 27 min. Laser-induced fluorescence (LIF, excitation 488 nm, emission 520 nm) was used to monitor the separation of FAM-labeled TNA molecules. Five injections were performed for each round of *in vitro* selection. The PD-L1-bound TNA molecules were collected and reverse transcribed using primer P4 and dNTP substrates. The reverse transcription product was amplified by PCR using primer P1 and primer P6. The amplification product was loaded onto a streptavidin column, and the non-biotinylated strand was removed by 200 mM NaOH elution. The biotinylated strand was recovered using 95°C hot water and purified by ethanol precipitation. The recovered biotinylated DNA strand was used as template for the next round of selection. After four rounds of *in vitro* selection and amplification, the library was cloned and sequenced to examine the diversity of the molecules that remained in the pool.

Filter binding assay. Cy5-labeled chimeric DNA-TNA molecules were first synthesized by primer extension using primer P2 and appropriate templates, and separated from the template by on streptavidin column as described above. The isolated TNA molecules were annealed by first heating at 9°C for 5 min and cooling on ice for 10 min. The folded TNA molecules were incubated with human PD-L1 proteins of different concentrations at 37°C for 1 h. After incubation, the mixture was loaded onto the dot blot apparatus containing a double layer of nitrocellulose and nylon membranes. The protein-bound TNA molecules were partitioned away from the free TNA using vacuum. Both membranes were then imaged and quantified by for fluorescence intensity, and the dissociation constants were calculated using Origin.

Synthesis and purification of TNA truncated versions. Truncated versions of TNA aptamers were synthesized using TNA phosphoramidites on an automated ABI 349 DNA synthesizer, de-protected in concentrated NH_4OH at 55°C overnight, precipitated with ethanol and purified by high performance liquid chromatography (HPLC).

ELISA competition assay. The wells of ELISA plates were first coated with recombinant human PD-L1 protein (2 mg/mL) at 4°C for overnight and blocked at 37°C for 2 h. Fc-tagged human PD-1 protein (0.6 μg/mL) and TNA aptamers (200 nM)

were simultaneously added into wells to compete for PD-L1 binding. After washing, PD-1 proteins that remained in wells were detected by horseradish peroxidase-labeled anti-Fc antibody followed by colorimetric reaction and absorbance measurement. The amount of PD-1 protein was calculated by reference to a standard curve of absorbance vs. PD-1 protein concentration. Wells without PD-L1 coating was used as background control, and a previously reported PD-L1-binding DNA aptamer AptPD-L1 were used for comparison.

TNA stability assay. 1 pmole Cy5-labeled oligonucleotides were incubated with 20% or 50% human serum in selection buffer at 37℃ for various time. At specific time points, the reactions were stopped by adding stop buffer (8 M urea, 5 mM Tris-HCl, 20 mM EDTA, pH 7.5) and analyzed by 12% denaturing PAGE.

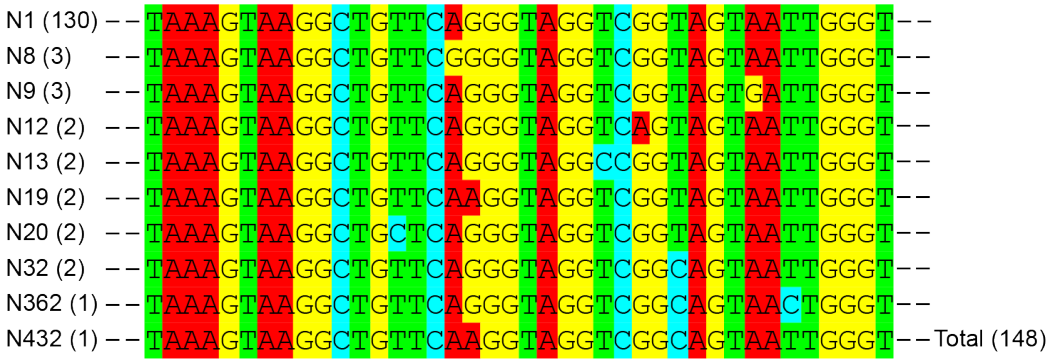
Flow cytometry analysis. Human normal liver cells L02 (5×10^4) were transfected with a DNA plasmid (1 μg) expressing mouse PD-1 with Lipofectamine 2000. Positive cells were screened by 2 μg/mL puromycin in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C with 5% CO_2 under fully humidified conditions. Cells were collected and re-suspended in phosphate-buffered saline (PBS) containing 10% FBS at 37°C for 1 h. About 1×10^5 cells were incubated with 100 μL recombinant Fc-tagged PD-L1 protein (96 μg/mL) and TNA aptamer (2 μM) at 37°C for 30 min. Subsequently, cells were washed twice by PBS and stained with APC-conjugated anti-Fc antibody for 30 min. After washing twice by PBS, cells were analyzed on a Verse cytometer. Groups with no added PD-L1 protein and with no added TNA aptamer were included as controls[3].

***In vivo* fluorescence imaging.** All the mice experiments were performed in compliance with Nanjing University's policy on animal use and ethics. The mouse colon cancer cell CT26 was maintained at 37°C in 5% CO_2 in RPMI-1640 media supplemented with 10% FBS. The six-week-old female Balb/c mice were injected with 5×10^6 CT26 cells subcutaneously in the left flank. When the tumor volume reached 200 mm^3, mice were injected with 200 μL (2 nmole/mouse) Cy5-labeled TNA sequence via tail vein. The mice were then imaged 2 h after injection on an IVIS Spectrum image acquisition system. After 24 h the mice were sacrificed and the distribution of TNA molecules in various tissues and organs were determined.

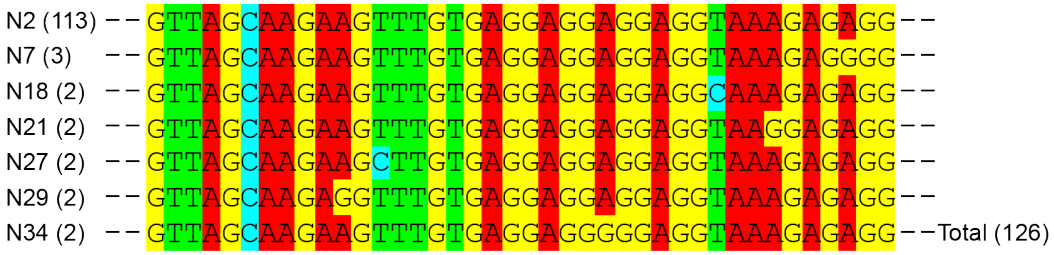
Tumor growth inhibition. Four-week-old female Balb/c mice were injected with 5×10^6 CT26 cells subcutaneously in the back. When the tumor volume reached 50-100 mm^3, TNA aptamer (2 mg/kg), 5-fluorouracil (10 mg/mL), or saline solution was injected intraperitoneally once every day for 6 days. Tumor major diameter a and minor diameter b were measured, and the tumor volume was calculated using the formula $\pi/6 \times a \times b^2$.

CCK8 cell viability assay. Colon carcinoma cell CT26 were cultured in RPMI-1640 media supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C with 5% CO_2 under fully humidified conditions. Cells were seeded at 1×10^4 cells/well in 96-well plates and allowed to grow for 12 hours. Cells were treated with TNA aptamers of different concentrations for 24 h. Compound WST-8 was added to monitor cell viability. After 4 h, the absorbance at 450 nm was measured, and the percent cell viability was calculated.

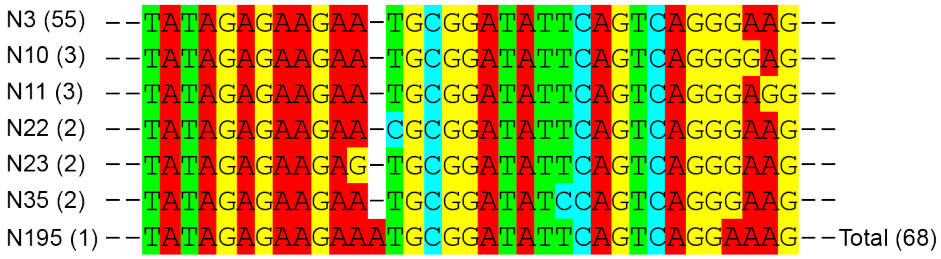
Immune stimulation assay. The murine monocytes RAW264.7 was cultured at 37°C in a 5% CO_2 humidified incubator, and maintained in DMEM media supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C with 5% CO_2 under fully humidified conditions. 2×10^5 cells were planted in 24-well plates and incubated overnight. The cells were treated with different agents including TNA aptamer N5 (pre-incubated with lipofectamine 3000), N5, lipopolysaccharide (LPS) and lipofectamine 3000 at 37°C for 12 h, using non-treated cells as a negative control. The concentrations of N5 and LPS were 1 or 10 μg/mL. Culture supernatants from each well were collected, and the secreted TNFα levels were quantified by enzyme-linked immunosorbent assay (ELISA) using 96-well ELISA kit.



Family 1



Family 2



Family 3

Figure S1. Three families of consensus sequences revealed by sequence alignment of NGS results. Of the 1,000 sequences that appear most often, 148 sequences contain a unique consensus sequence motif and are grouped into family 1. Similarly, 126 and 68 sequences are grouped into family 2 and 3, respectively. Representative sequences N1, N2 and N3 were chosen for further affinity measurement.

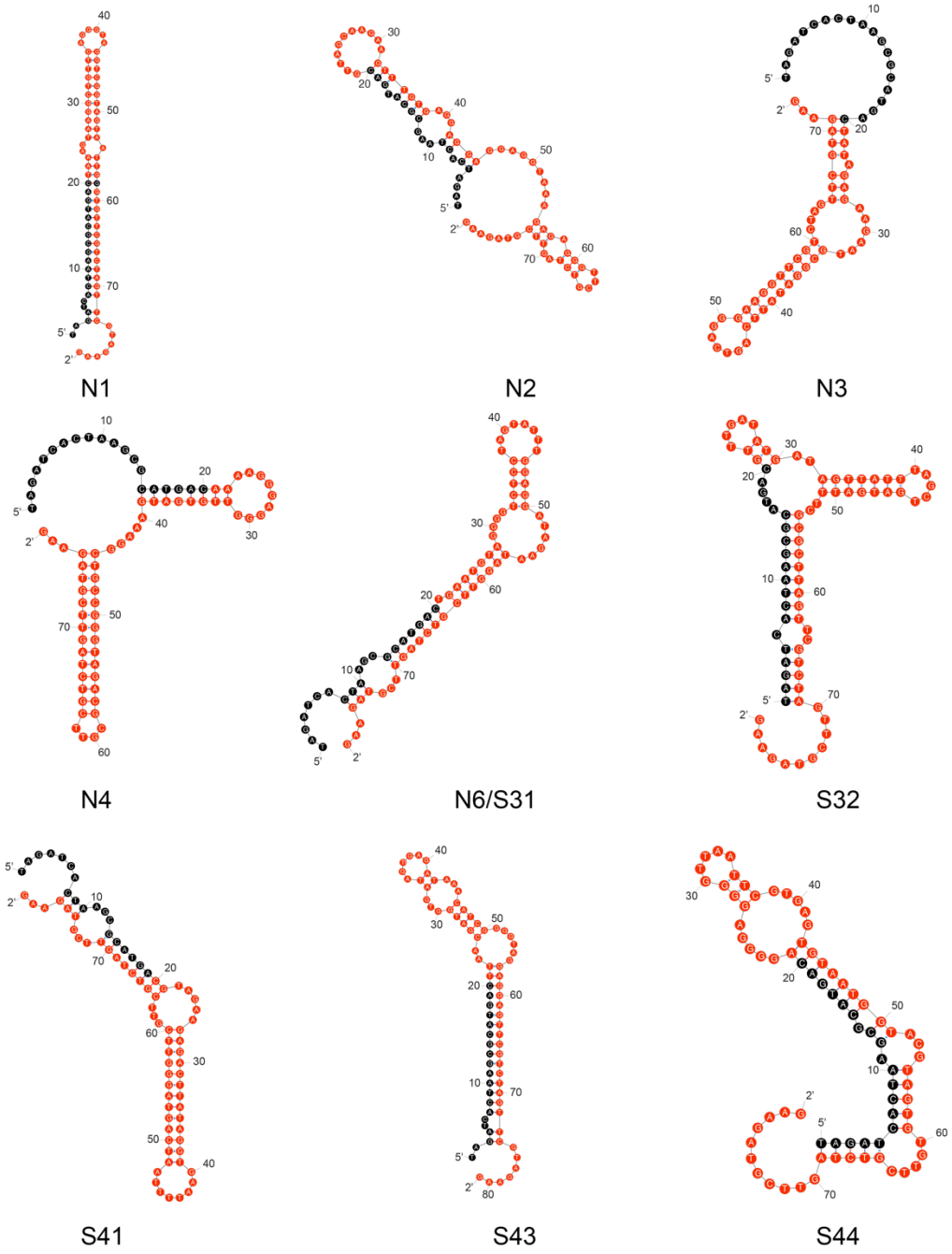


Figure S2. Predicted secondary structures of TNA aptamers. The secondary structures were predicted using RNA backbone in Mfold. DNA and TNA are shown in black and red, respectively.

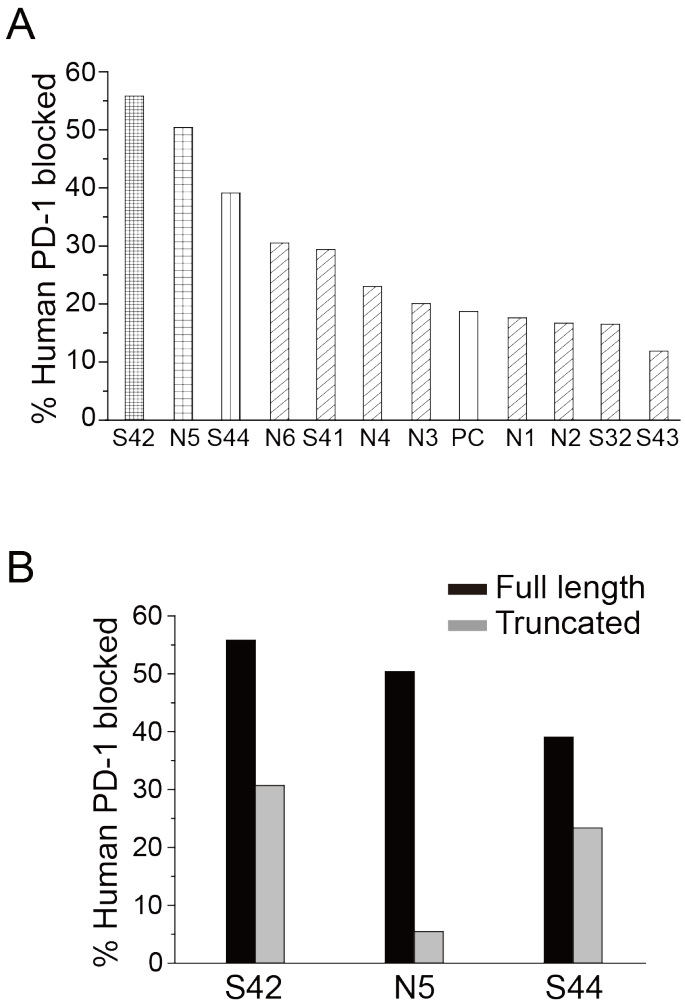


Figure S3. TNA aptamers blocked PD-1/PD-L1 interaction in ELISA competition assay. (A) Eleven TNA aptamers showed different levels of blockade to PD-L1/PD-1 interaction. PC: positive control using a previously reported PD-L1-binding DNA aptamer AptPD-L1. (B) Truncated versions of three best TNA aptamers S42, N5 and S44 showed compromised PD-1/PD-L1 inhibition, compared to their full-length counterparts.

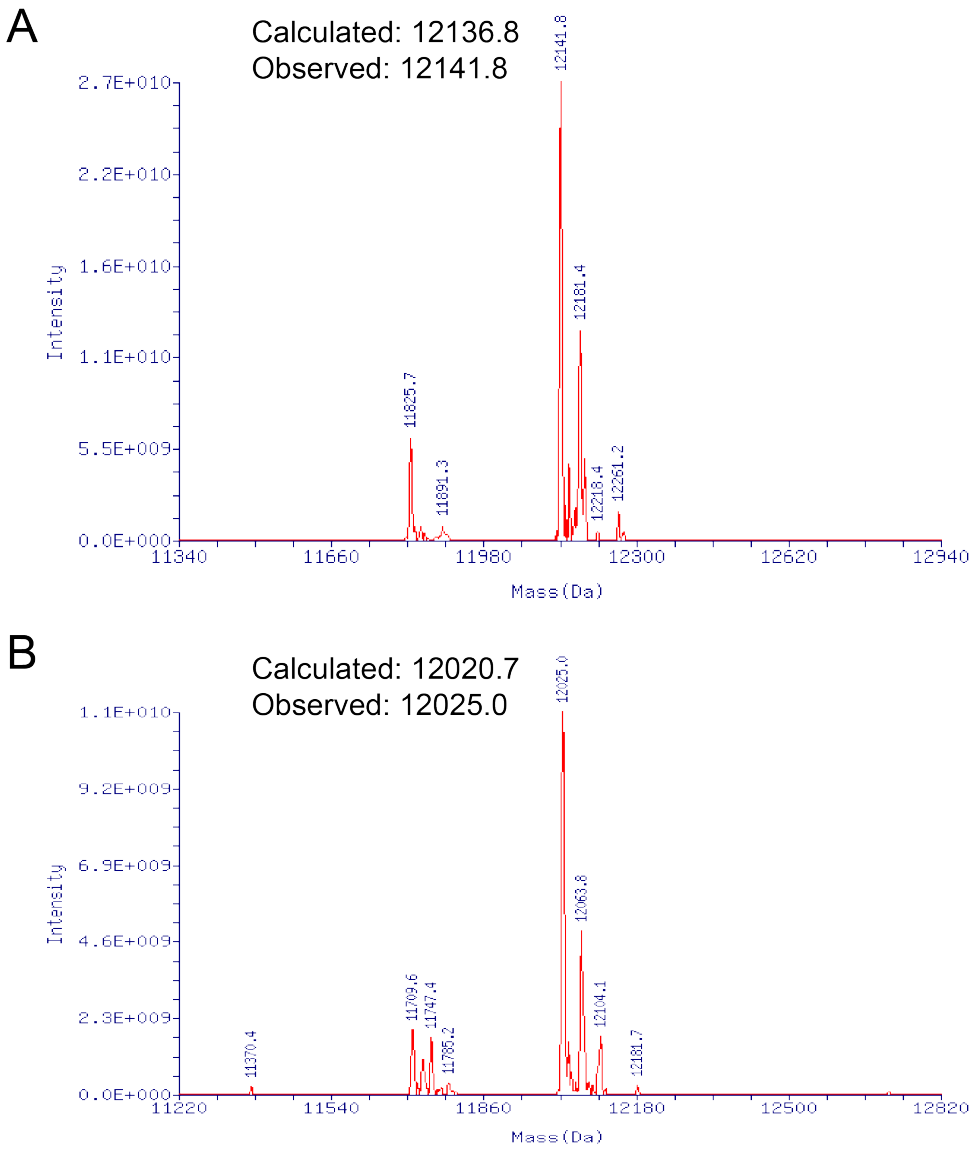


Figure S4. Electrospray ionization mass spectrometry of truncated TNA sequences. The truncated versions of TNA aptamers S42 (A) and S44 (B) were prepared by solid phase synthesis.

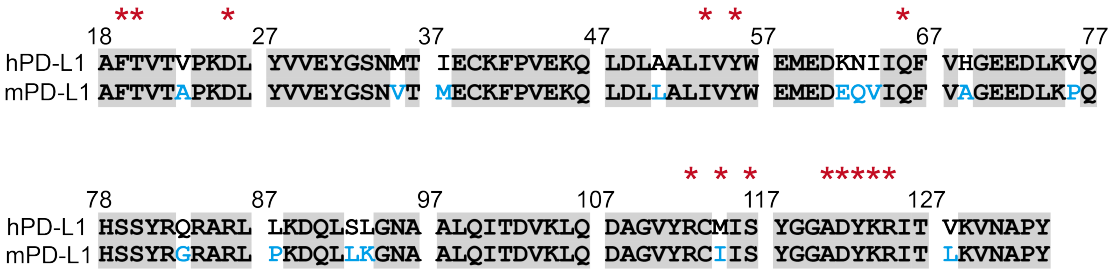


Figure S5. Sequence homology of human and murine PD-L1 proteins. Residues that interact to form PD-1/PD-L1 complex are marked with asterisks.

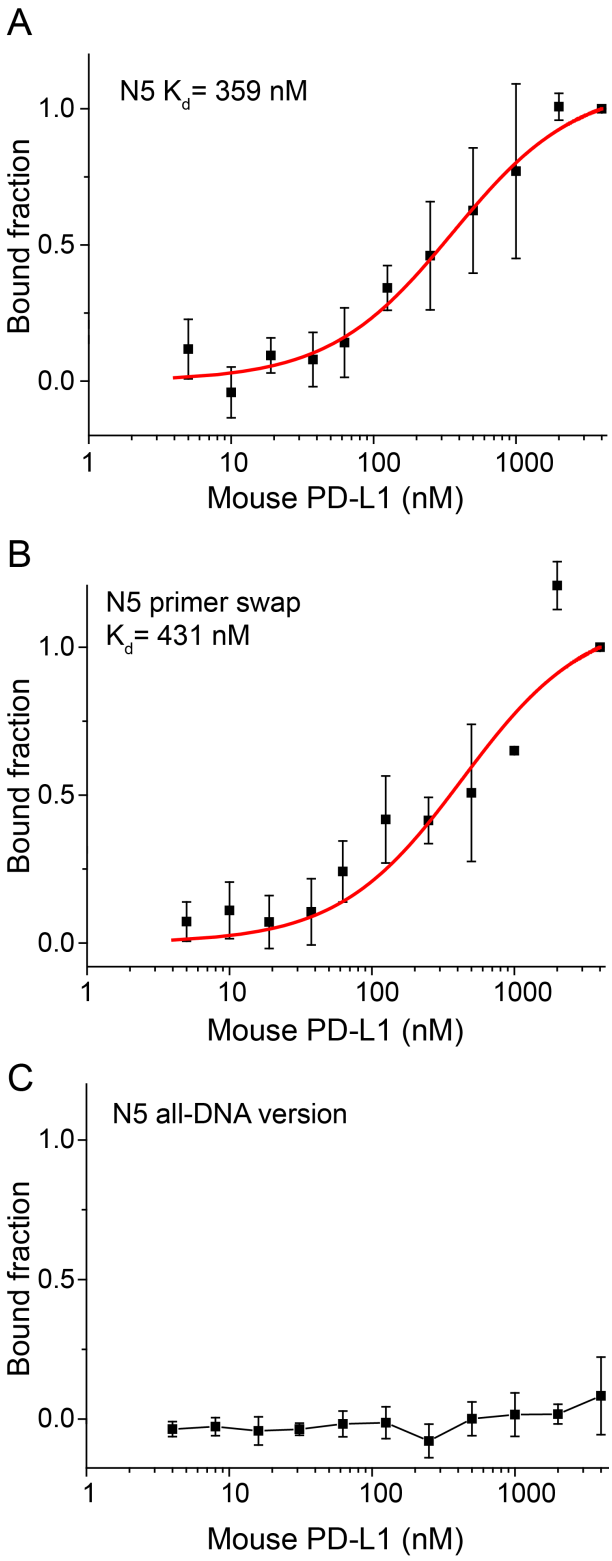


Figure S6. The dissociation constants of (A) TNA aptamer N5, (B) TNA aptamer N5 with swapped DNA primer and (C) all-DNA version of N5 to murine PD-L1 protein, determined by a nitrocellulose filter binding assay (n = 3).

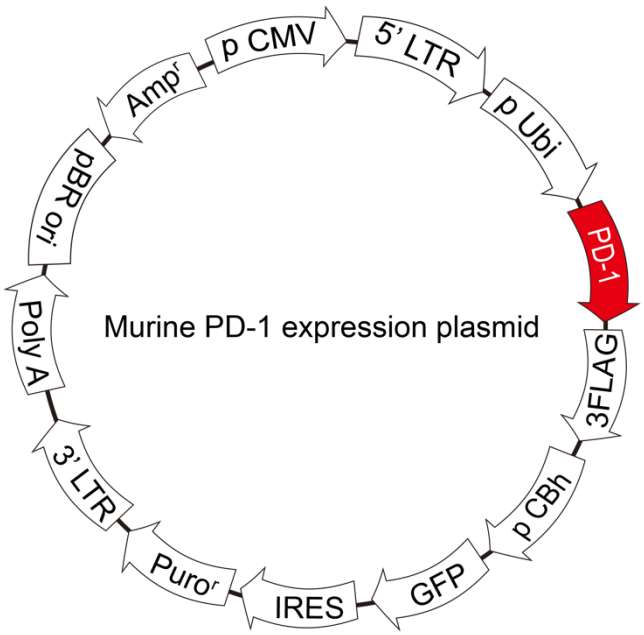


Figure S7. Murine PD-l expression plasmid map.

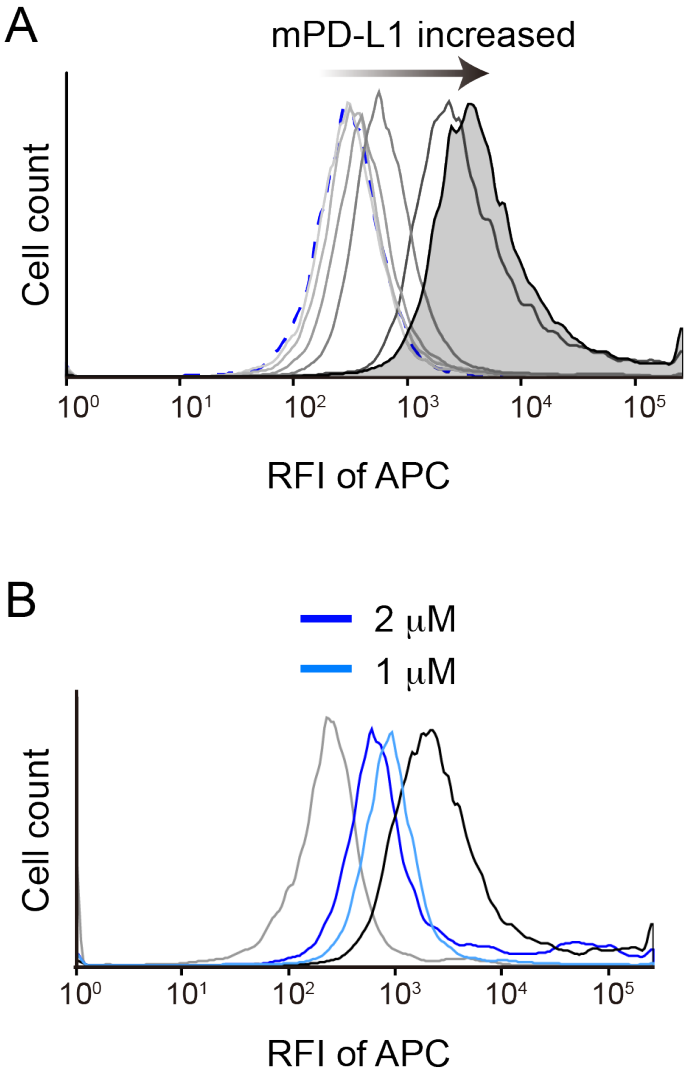


Figure S8. TNA aptamer N5 blocked PD-L1 binding to PD-1 protein on cell surface. (A) Flow cytometry analysis showed that more cells became fluorescently labeled as PD-L1 protein concentration increased. RFI: relative fluorescence intensity. (B) Addition of TNA aptamer N5 inhibited PD-1/PD-L1 interaction, and resulted in lower levels of cellular fluorescence intensities. Using higher concentration of N5 led to a greater reduction in fluorescence intensity.

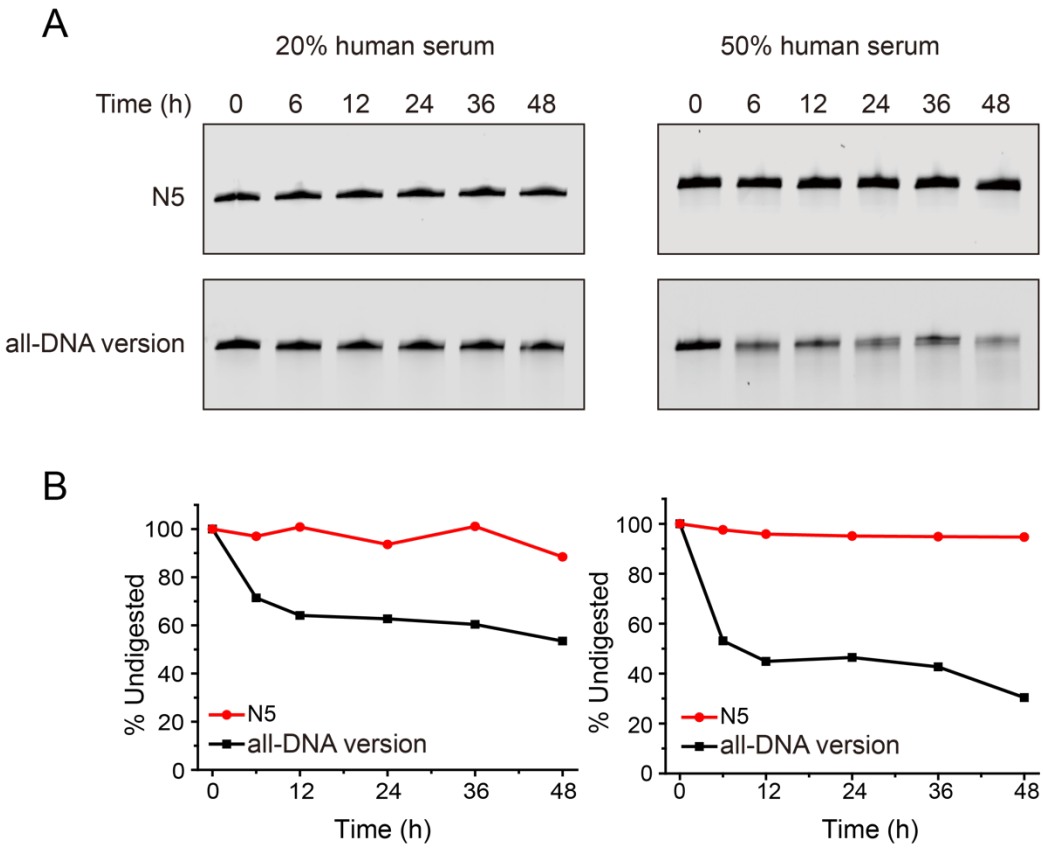


Figure S9. Biological stability of aptamer N5. TNA aptamer N5 was incubated in 20% and 50% human sera at 37°C for up to 48 hours. An all-DNA version of N5 was included as a control.

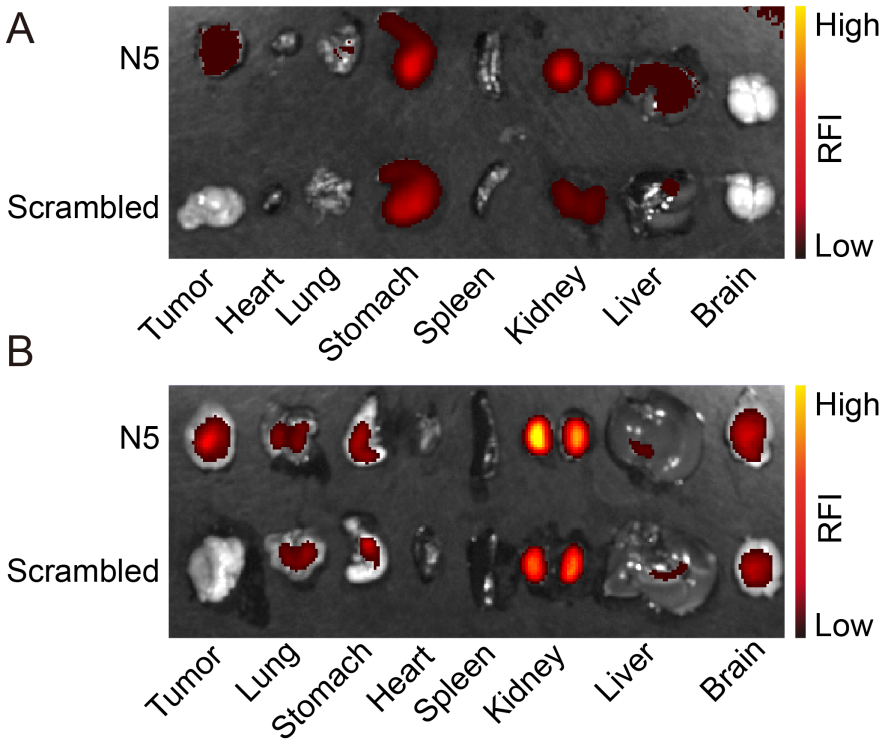


Figure S10. Tissue distribution of TNA aptamer. Two additional repeats of tissue distribution of TNA aptamer N5 or scrambled sequence 24 hours after tail vein injection.

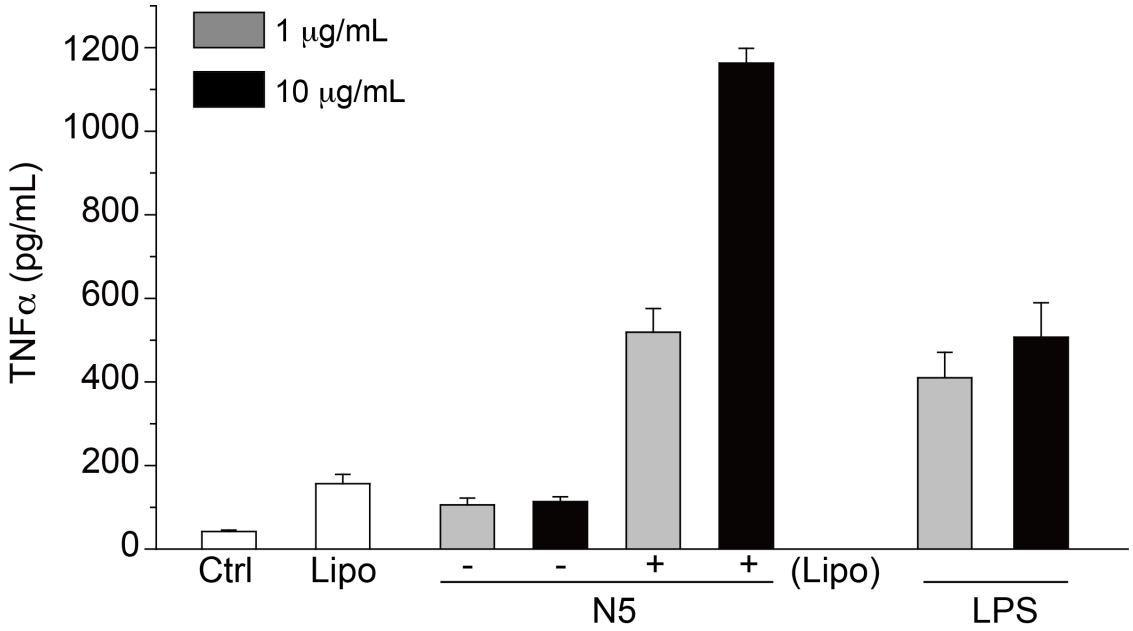


Figure S11. Immune stimulatory effect of TNA aptamer N5. Without transfection TNA aptamer N5 showed minimal immune stimulatory effect. In the presence of lipofectamine 3000 (Lipo), the immune stimulatory effect of N5 was comparable to or even stronger than the positive control lipopolysaccharide (LPS). The TNFα levels were measured 12 hours after treatment.

Table S1. Oligonucleotide sequences used in this study

Note: The TNA sequences are shown in red. N40 indicates a 40-nucleotide randomized region.

Name	Sequence (DNA written from 5' to 3'. TNA written from 3' to 2')	Length (nt)
P1	TAGATCACTAAGCGCATGAC	20
P2	Cy5-TAGATCACTAAGCGCATGAC	20
P3	FAM-TAGATCACTAAGCGCATGAC	20
P4	CTTCTACGAACTAGACGAAC	20
P5	Cy5-CTTCTACGAACTAGACGAAC	20
P6	Biotin-CTTCTACGAACTAGACGAAC	20
DNA library	Biotin-CTTCTACGAACTAGACGAAC-N40-GTCATGCGCTTAGTGATCTA	80
TNA library	FAM-TAGATCACTAAGCGCATGAC-N40-GTTCGTCTAGTTCGTAGAAG	80
Truncated S42	GAGTGGGATGGATAGAGAATAAGTGTGGAGTGGAAGGTGA	40
Truncated S44	AGGGGAGGGGTTAATTCGTGAGTGTAATGGTACGTAGTGT	40
Truncated N5	GATTGAGTAGATAGTGGTTCTGTACGTAGTGAAAGAGTGG	40
Scrambled N5	Cy5-TAGATCACTAAGCGCATGACGAGACAGGTGCGTGCTAGCTAA TTATGTGAATGTTAGTGCTTGTAGATATGGATGGAGTG	80
N5 primer swap	TAGATCACTAAGCGCATGACGATTGAGTAGATAGTGGTTCTGTACGTAGTG AAAGAGTGGGTTCGTCTAGTTCGTAGAAG	80

Table S2. Sequence and binding affinity of TNA aptamers.

Clone	Sequence (DNA written from 5' to 3'. TNA written from 3' to 2')	K_d (nM)	Copy
N1	TAGATCACTAAGCGCATGACTAAAGTAAGGCTGTTCAGGGTAGGTCGGTAGTAATTGGGTGTTCGTCTAGTTCGTAGAAG	364	130
N2	TAGATCACTAAGCGCATGACGTTAGCAAGAAGTTTGTGAGGAGGAGGAGGTAAAGAGAGGGTTCGTCTAGTTCGTAGAAG	217	113
N3	TAGATCACTAAGCGCATGACTATAGAGAAGAATGCGGATATTCAGTCAGGGAAGGTTCGTCTAGTTCGTAGAAG	441	55
N4	TAGATCACTAAGCGCATGACAAAAGGGAGGGTTGTGATGAAAGGCTGCCGGGTAGACGCGTTCGTCTAGTTCGTAGAAG	121	45
N5	TAGATCACTAAGCGCATGACGATTGAGTAGATAGTGGTTCTGTACGTAGTGAAAGAGTGGGTTCGTCTAGTTCGTAGAAG	441	4
N6/S31	TAGATCACTAAGCGCATGACTGAATGTAGGGGTCTCCTAGTATTTGGAGGATAGAATAGGTTCGTCTAGTTCGTAGAAG	1080	4
S32	TAGATCACTAAGCGCATGACGTTTGATATGATAGTTATTTAGCTGATGATTTCGCGCTTAGTTCGTCTAGTTCGTAGAAG	664	--
S41	TAGATCACTAAGCGCATGACGTAGAAGAGACTTATAGGTGAATTTTAATCAGTAGGGTTCGTTCGTCTAGTTCGTAGAAG	1091	--
S42	TAGATCACTAAGCGCATGACGAGTGGGATGGATAGAGAATAAGTGTGGAGTGGAAGGTGAGTTCGTCTAGTTCGTAGAAG	360	--
S43	TAGATCACTAAGCGCATGACTTAACGATGGTGATAGTGAGATAAACATCGGGGTAGGAGGAGTTCGTCTAGTTCGTAGAAG	937	--
S44	TAGATCACTAAGCGCATGACAGGGGAGGGGTTAATTCGTGAGTGTAATGGTACGTAGTGTGTTCGTCTAGTTCGTAGAAG	1025	--

Note: The TNA sequences are shown in red and the constant primer-binding regions are underlined. Sequences N1-N6 are derived from next-generation sequencing (NGS) results of round 4 library. Sequences S31-S32 and S41-S44 are derived from Sanger sequencing (SS) results of rounds 3 and 4 library clones, respectively.

References

1 M. R. Dunn, C. Otto, K. E. Fenton, J. C Chaput, *ACS Chem. Biol.* 2016**, 11**, 1210-1219.
2 J. C. Chaput, J. K. Ichida, J. W. Szostak, *J. Am. Chem. Soc.* 2002, **125**, 856-857.
3 H. N. Chang, B. Y. Liu, Y. K. Qi, Y. Zhou, Y. P. Chen, K. M. Pan, W. W. Li, X. M. Zhou, W. W. Ma, C. Y. Fu, Y. M. Qi, L. Liu, Y. F. Gao, *Angew. Chem. Int. Ed.* 2015, **54**, 11760-11764.