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Research Article

Rational design of peptides for identification of linear epitopes and generation of neutralizing monoclonal antibodies against DKK2 for cancer therapy

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ABSTRACT

Dickkopf-related protein 2 (DKK2)is a member of the Dickkopf family in Wnt signaling pathway. Recently, we found that antibodies against DKK2 could activate natural killer (NK) and CD8+ T cells in tumors and inhibit tumor growth. In this paper, we report the rational design of peptides for identification of linear epitopes and generation of neutralizing monoclonal anti-DKK2 antibodies. To break the immune tolerance, we designed and chemically synthesized six peptides corresponding to different regions of DKK2 as immunogens and found five of them could generate mouse polyclonal antibodies that can bind to the active recombinant human DKK2 protein. Neutralizing mouse monoclonal antibodies (5F8 and 1A10) against human DKK2 were successfully developed by immunizing the mice with two different peptides (^{34}KLNSIKSSL^{42} and ^{240}KVWKDATYS^{248}) conjugated to Keyhole limpet hemocyanin (KLH). The monoclonal antibodies not only abolish DKK2's suppression of Wnt signaling in vitro but also inhibits tumor growth in vivo. Currently, those two mAbs are undergoing humanization as immunotherapy candidates and may offer a new drug for treatment of human cancers.

Statement of Significance: Using synthetic peptide as immunogen to break the immune tolerance. Antibodies against DKK2 could activate natural killer and CD8+ T cells in tumors and inhibit tumor growth. Generation of neutralizing monoclonal anti-DKK2 antibodies not only abolishes DKK2's suppression of Wnt signaling in vitro but also inhibits tumor growth in vivo.

KEYWORDS: DKK2; rational peptide design; neutralizing epitope; monoclonal antibody; immunotherapy

INTRODUCTION

Immunotherapy, using immune checkpoint inhibitors such as monoclonal antibodies against PD1, PD-L1 or CTLA4, has brought a great hope for thousands of cancer patients who had failed in the conventional chemotherapy and radiation therapy (1–6). However, the efficacy of these antibodies varies between different types of cancer. Colorectal cancers (CRCs) and some metastatic melanoma are largely refractory to immune checkpoint blockers (7–11). More than 80% of CRCs harbor the loss of function mutations in the adenomatosis polyposis coli (APC) gene (12), a suppressor of the Wnt-β-catenin pathway (13). The APC-loss in intestinal tumor cells or gene of phosphate and tension homology deleted on chromsome ten (PTEN)-loss in melanoma cells upregulate the expression of dickkopf-2 (DKK2).

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DKK2 belongs to a large family of the Dickkopf and it is involved in embryonic development through the interaction with Wnt signaling pathway (14). DKK2 can act as either the agonist or antagonist of Wnt/beta-catenin signaling depending on the cellular context and the presence of the cofactor kremen (15–20). Recently, we showed that the DKK2 antibody can activate natural killer (NK) and CD8+ T cells in tumors, which could cooperate with PD-1 blockade to impede tumor progression (21).

In this report, we present the data about the generation of neutralizing mouse monoclonal antibodies against human DKK2 using synthetic peptides as immunogens to break the immune tolerance. Human DKK2 and its mouse counterpart have very high homology with an identity score of 96% and similarity score of 98%, which created a technical barrier to raise mouse monoclonal antibody to human DKK2 due to the immune tolerance, resulted in failed attempt to generate mouse polyclonal antibodies against human DKK2 using the recombinant human DKK2 protein as immunogen. Using synthetic peptides conjugated to carrier proteins as immunogens has been successfully used by our group and many others to break immune tolerance (22).

To raise neutralizing monoclonal antibodies as candidates for therapy, we first have to identify the linear neutralizing epitope(s) on the outer surfaces of target proteins. One approach is called "peptide-walking." An example of this approach was carried out by Dr. Foo and others by immunizing 95 groups of mice with 95 overlapping synthetic peptides spanning the entire length of VP1 capsid protein (297 amino acids) of Hand-foot-mouth disease virus EV71, and two peptides (SP55 and SP70) containing amino acids 163–177 and 208–222 of VP1 were identified capable of eliciting neutralizing antibodies against EV71 (23). Active human DKK2 has 226 amino acids. Using the same "peptide-walking" approach to identify the good neutralizing linear antigen epitope(s) will be very costly and time-consuming.

Antibody–antigen interaction is like the interactions of a "key & lock." The first step of interaction is the docking of antibody complementarity-determining regions (CDRs) (key) into the cavity of the antigen epitope(s) of protein (lock). Different from small molecule drugs, antibody has a molecule weight of 150 Kda. To be bound by the antibody, the antigen epitope of the target protein has to be on the outer surface of the protein (water solubility). The second step of antibody–antigen interaction is the "locking," the interactions between the amino acids from the CDRs of antibodies and the contacts of antigen epitopes of the protein. The bigger the contact surfaces, the more of bonds, the higher the affinity of antibody (antigenicity). Based on these assumptions, we have developed proprietary software (AbEpiMax) to search for sequence(s) with high antigenicity and water solubility. We have designed tens of thousands of peptides as immunogens and successfully generated mAbs and/or pAbs against over 5 000 different proteins (22,24–26). For generation of therapeutic antibodies, factors such as species cross-reactivity, target protein-specificity (specificity) of the protein will also be taken into consideration.

Using this rational design approach, we identified six amino acid sequences of human DKK2 as the potential neutralizing epitopes and found that five of them are on the outer surfaces of human DKK2—evidenced by the binding of mouse antisera to the active human DKK2 protein. Neutralizing mouse monoclonal antibodies also been successfully developed showing with tumor inhibitory activity in vivo. Currently those two mAbs are undergoing humanization as immunotherapy candidates and may offer a new drug for treatment of human cancers.

MATERIALS AND METHODS

Materials

Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Polyethylene glycol 4000 (PEG4000), dimethyl sulfoxide (DMSO), tetramethylbenzidine (TMB) substrates and agarose were purchased from SIGMA (MO, USA). Female BALB/c mice were obtained from Vital River Co. (Beijing, China). Dulbecco's modified Eagle medium (DMEM) and FBS were from HyClone (CA, USA). Goat anti-mouse IgG Fc and Goat anti-human secondary antibodies are from Jackson Immune Lab (MA, USA). Recombinant DKK2 was purchased from R&D System (IL, USA). ApcMin/+ (C57BL/6J-ApcMin/J) mice were acquired from the Jackson Laboratory (MA, USA). YUMM1.7 was provided by Marcus Bosenberg. MC38 was purchased from Kerafast (MA, USA).

Synthesis of peptides and preparation of immunogens.

For peptides design, an undisclosed software AbEpiMax was used. The peptide position, formations of hydrogen bonds, salt bridge, van der Waals force between the amino acids from CDRs of antibodies and the contact surface areas of antigen epitopes were taken into consideration. Other factors such as species cross-reactivity, target protein-specificity, the functional domain(s) of the protein were also counted.

Peptides of DKK2 were synthesized by GLS Biochem (Shanghai, China). A cysteine residue was added at the end of peptides for conjugation. Each peptide was chemically linked to the carrier protein mcKLH through a sulfide-linker.

Generation of mouse monoclonal antibodies.

All experimental procedures involving mice in this study were in accordance with requirements and guidelines for treatment of experimental animals. The Ethics Committee on animal experiments of AbMax Biotechnology has approved all animal experiments conducted in this manuscript.

As previously described (22), 4- to 6-week-old female BALB/c mice were first immunized with immunogens in CFA and boosted with immunogens in IFA. Two to four weeks after the first immunization, bleeds obtained from the tails of the immunized mice were tested for titers by indirect enzyme-linked immunosorbent assay (ELISA). Spleens of the mice that showed the highest titers were removed, and spleen oocytes were fused with the mouse myeloma cell line SP2/0.

Table 1. Mouse titers against human DKK2 on different days

		Day 14					Day 20	
Titers		1:500	1:1000	1:5000	1:10000	1:50000	1:500	1:1000
Mouse No.	1#	0.27	0.101	0.05	0.046	0.05	0.296	0.239
	2#	0.125	0.065	0.049	0.046	0.046	0.207	0.101
	3#	0.363	0.123	0.047	0.048	0.047	0.337	0.171

Each well was coated with 0.1 μg/ml of the recombinant human DKK2. Then incubated with sera of mice tail bleeds at different dilutions, respectively. After washes, wells were then probed with either HRP-conjugated goat anti-mouse IgG Fc at 1:5000. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader.

Culture supernatants from individual hybridoma clones were screened by ELISA. To produce antibodies, the hybridoma clones were seeded in stationary bioreactors in DMEM plus 10% low-IgG FBS. The bioreactor fluids were collected every 3 days, and IgG fractions were affinity-purified using protein G agarose columns (GE). The concentrations of purified IgGs were determined by their absorbance at 280 nm.

Indirect ELISA

Each well of the 96-well high binding enzyme immunoassay (EIA) plates was coated with 100 μl of antigen (1 μg/ml), such as recombinant DKK2, at 4°C overnight in phosphate-buffered saline (PBS). After two washes with PBS and blocking with 5% skim-milk in PBS for 1 h at room temperature, wells were incubated with either the mouse sera or the culture supernatants or purified mAb in 5% skim-milk-PBS for another 1 h at room temperature. After two washes with PBS, wells were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc-specific secondary antibodies (Jackson Lab) in 5% skim-milk-PBS for 1 h at room temperature. After five washes with PBS plus 0.1% Tween20 (PBST), HRP substrate 3, 3′, 5, 5′-TMB solution was added. The reaction was stopped with stop solution (0.1 M H_2SO_4) after 30 min and absorbance was measured at 450 nm with a microplate reader. In some experiments, a chemiluminescence AP substrate was used and measured by an EnVision plate reader.

Wnt reporter gene assays

HEK293 cells were seeded at 8×10^4 cells per well in 48-well plate. The next day, cells were transfected by Lipofectamine 2000 (Invitrogen) with the TOPFlash and green fluorescent protein (GFP) plasmids using Lipofectamine Plus for 24 h. The cells were added 24 h after transfection with Wnt/DKK2/mAb complex. Six hours later, the cells were lysed and subjected to red fluorescent protein (RFP) fluorescence and luciferase luminescence measurement using an Envision plate reader. The reporter gene activity is shown after being normalized against RFP readings.

Tumor graft

MC38 tumor cells (0.5×10^6) were mixed in 100 μl BD Matrigel (Matrix Growth Factor Reduced) (BD 354230) and inoculated subcutaneously at the right flanks of the backs of female C57/BL mice (8–10 weeks old). Tumor growth was measured by calipers. For antibody treatment, control IgG antibody and anti-DKK2 antibody were diluted in PBS, and 100 μl was injected i.p. (200 μg/mouse every other day).

RESULTS

Recombinant human DKK2 failed to induce significant immune response in mice

To raise monoclonal antibody against DKK2, a group of three female Blab/c mice were immunized with the recombinant human DKK2. However, as shown in Table 1, after initial immunization and subsequent four boosts, no significant immune responses against human DKK2 were observed in all mice on Day 14. Two more boosts were given and the tail bleeds were examined again on Day 20 for titers against human DKK2. Unfortunately, no enhancement of immune response against the human DKK2 was achieved.

We did the blast of the human DKK2 amino acid sequence against its mouse counterpart as shown in Fig. 1. The two sequences have very high homology with an identity score of 96% and similarity score of 98%. It is most likely that the mouse immune system may not see the human DKK2 as foreign protein (immune tolerance) and, thus, would not develop antibody against it.

Break immune tolerance of mouse against human DKK2

To break the immune tolerance, we took the approach of using synthetic peptides conjugated to KLH as the immunogens. In this study, instead of the costly and time-consuming "peptide-walking" approach, we used a proprietary software program AbEpiMax to rational design the antigen peptide sequences.

Six potential neutralizing epitopes (YAL008-1 ^{34}KLNSIKSSL^{42}, YAL008-2 ^{148}RDRNHGHYS^{156}, YAL008-3 ^{166}GRPHTKMSH^{174}, YAL008-4^{215}TKQRKKGSHGLE^{226}, YAL008-5 ^{240}KVWKDATYS^{248} and YAL008-7 ^{195}CARHFWTKIC^{204}) were identified and their corresponding sequences in human DKK2 were shown in Fig. 2. The peptides were chemically synthesized and conjugated to KLH as immunogens to generate anti-human DKK2 antibodies.

Peptides YAL008-1, -2, -3, -4 and-5 were all synthesized as linear ones. The sequence of YAL008-7 was located in the cysteine-rich domain mediating DKK2 interaction with

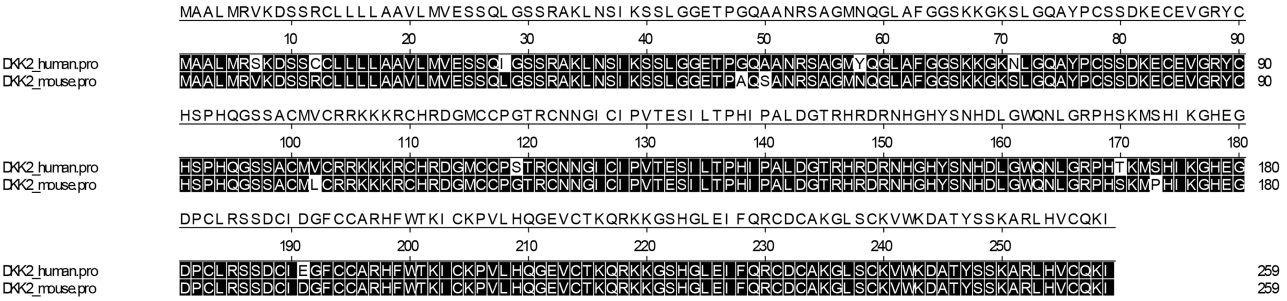
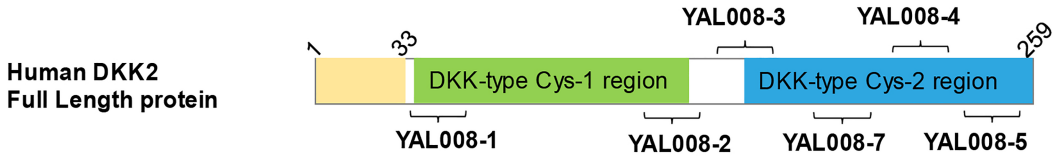


Figure 1. Sequence alignment of human and mouse DKK2. The protein sequences of mouse (UniProt ID: Q9QYZ8) and human (UniProt ID: Q9UBU2) DKK2 were aligned. Two sequences show an identity of 96% and similarity of 98%. The differential amino acids are distinguished in black and white.

A



B

Seq. ID	Amino acids of sequence	Antigenicity Score	Rotation (degree)	Solubility (g/100ml)	Specificity
YAL008-1	34 KLNSIKSSL 42	10	217	45	DKK2-specific
YAL008-2	148 RDRNHGHYS 156	20	221	20	DKK2-specific
YAL008-3	166 GRPHTKMSH 174	15	260	50	DKK2-specific
YAL008-4	215 TKQRKKGSHGLE 226	20	161	50	DKK1/2
YAL008-5	240 KVWKDATYS 248	14	120	45	DKK2-specific
YAL008-7	195 CARHFWTKIC 204	15	145	45	DKK2-specific

Figure 2. Diagram and antigen epitopes of DKK2. (A) Bar diagram of DKK2 domain structure. Residues' numbers refer to the human DKK2 (Uniprot entry Q9UBU2-1). The green and blue regions represent the DKK-type Cys-1 region and DKK-type Cys-2 region, respectively. (B) Designed specific peptides were numbered with the detail amino acids referred to the full-length sequence of human DKK2. The Cys residues were added for conjugation and all 6 peptides were conjugated to KLH via the Cys at the N or C-terminal of peptides.

LRP5 and LRP6, so it was synthesized as circular one (a disulfide bond was artificially formed between C^{195} and $C^{204}$to best mimic the native conformation.

Three mice per group were immunized with the six KLH-conjugated peptides respectively. Two weeks after the first immunization, tail bleeds were tested for titers against the peptide-BSA conjugates by ELISA. As shown in Table 2, all of the six peptides induced very strong immune responses in all mice against the corresponding peptides respectively, indicating that our antigenicity scoring was pretty accurate.

Nevertheless, as shown in Table 3, not all six peptides could generate mouse polyclonal antisera that recognize the recombinant human DKK2 protein. For example, the antisera of YAL008-3 reacted with the synthetic peptide strongly but showed no reactivity toward the DKK2 protein. This suggests that the antigen epitope of ^{166}GRPHTKMSH^{174} is either not a linear epitope or not on the outer surface that could be bound by antibodies.

For the other 5 peptides, the mouse polyclonal antiserum can recognize the recombinant DKK2, suggesting those peptide-corresponded epitopes were linear and on the outer surface of DKK2 protein.

Generation of neutralizing mouse monoclonal antibody against human DKK2

One mouse from each group immunized with peptide YAL008-1, -2, -5 and -7 were scarified and the spleen cells from them were used to fuse with mouse myeloma SP2/0 cells separately to generate hybridoma cell lines.

As summarized in Table 4, using the peptides of DKK2 (YAL008-1/2/5/7) as immunogen, thousands of monoclonal hybridomas were obtained. Hundreds of them showed positive reactions against the corresponding peptides in the initial ELISA screening (readings > 2 times than negative control). Based on the ELISA data, the best monoclonal antibodies including clones 3F7, 3G2 and 5F8 from mice immunized with peptide YAL008-1, clones 4B2 and 6C1 from mice immunized with peptide YAL008-2, and clones 1A10, 3B8, 5C2 and 7B5 mice immunized with

Table 2. Mouse sera against the corresponding peptide-BSA conjugates

Coating		**YAL008–1-BSA**					
Dilution rate		1:500	1:1000	1:5000	1:10000	1:50000	NC
Mouse No.	1#	3.499	3.419	2.87	2.194	0.468	0.112
	2#	>3.5	>3.5	>3.5	>3.5	0.855	0.152
	3#	>3.5	>3.5	>3.5	3.465	1.209	0.156
Coating		**YAL008–2-BSA**					
Dilution rate		1:500	1:1000	1:5000	1:10000	1:50000	NC
Mouse No.	1#	3.346	3.295	3.295	3.133	1.003	0.268
	2#	3.269	3.379	3.222	3.268	1.089	0.124
	3#	3.126	3.059	3.058	2.787	0.592	0.093
Coating		**YAL008–3-BSA**					
Dilution rate		1:500	1:1000	1:5000	1:10000	1:50000	NC
Mouse No.	1#	>3.5	>3.5	2.712	2.38	0.6	0.074
	2#	3.173	3.271	2.587	2.365	0.6	0.063
	3#	3.196	>3.5	1.824	2.182	0.31	3.031
Coating		**YAL008–4-BSA**					
Dilution rate		1:500	1:1000	1:5000	1:10000	1:50000	NC
Mouse No.	1#	3.404	3.345	2.412	1.459	0.265	0.05
	2#	3.378	3.377	3.179	3.141	0.723	0.064
	3#	3.058	3.058	1.962	1.232	0.245	0.06
Coating		**YAL008–5-BSA**					
Dilution rate		1:500	1:1000	1:5000	1:10000	1:50000	NC
Mouse No.	1#	3.417	>3.5	2.358	2.157	0.427	0.095
	2#	3.269	3.269	3.055	2.585	0.476	0.12
	3#	>3.5	>3.5	2.41	1.557	0.283	0.111
Coating		**YAL008–7-BSA**					
Dilution rate		1:500	1:1000	1:5000	1:10000	1:50000	NC
Mouse No.	1#	>3.5	>3.5	>3.5	2.954	0.903	0.055
	2#	>3.5	>3.5	2.939	2.486	0.599	0.05
	3#	3.182	3.182	3.035	2.901	1.382	0.045

Each well was coated with 1 μg/ml of peptide-BSA conjugates. Then incubated with sera of mice tail bleeds at different dilutions. After washes, wells were then probed with HRP-conjugated GAM IgG Fc. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm. NC referred to the negative control with 5% milk-PBS instead of mice tail bleeds.

Table 3. Mouse sera reactivity against recombinant human DKK2 protein

Tail bleeds		**YAL008–1**			**YAL008–2**			**YAL008–3**			**YAL008–4**			**YAL008–5**			**YAL008–7**		
Dilution rate		1:500	1:1000	1:5000	1:500	1:1000	1:5000	1:500	1:1000	1:5000	1:500	1:1000	1:5000	1:500	1:1000	1:5000	1:500	1:1000	1:5000
Mouse No.	1#	0.131	0.06	0.058	0.936	0.777	0.196	0.059	0.103	0.049	0.29	0.176	0.099	0.06	0.06	0.052	0.344	0.234	0.049
	2#	0.313	0.283	0.097	0.312	0.285	0.09	0.129	0.101	0.038	0.358	0.436	0.188	0.996	1.086	0.354	0.072	0.073	0.038
	3#	1.737	1.076	0.182	0.223	0.106	0.115	0.111	0.067	0.041	0.425	0.526	0.113	0.191	0.057	0.046	0.273	0.181	0.041

Each well was coated with 1 μg/ml of the recombinant human DKK2 protein. Then incubated with sera of mice tail bleeds at different dilutions. After washes, wells were then probed with HRP-conjugated goat anti-mouse IgG Fc. HRP substrate TMB was added to develop the color and absorbance was determined at 450 nm with a plate reader. NC referred to the negative control with 5% milk-PBS instead of mice tail bleeds.

peptide YAL008-5. Some of the clones were selected to small batch production using stationed Bioreactors. The purified mAbs were tested again at various concentrations against each peptide by ELISA, and mAbs YAL008-1-5F8, YAL008-5-1A10 mAb and YAL008-7-1A10 had the best titers at 0.5 ng/ml (Table 4).

Anti-DKK2 mAbs bind to DKK2 specifically

To study their specificity, 96-wells plate were coated with either recombinant DKK2 or recombinant DKK1 and incubated with various concentrations of anti-DKK2 mAbs YAL008–1-5F8 and YAL008–5-1A10. As shown in Fig. 3, both anti-DKK2 mAbs YAL008–1-5F8 (1 nM) and YAL008–5-1A10 (15 nM) were specific for DKK2 with no cross-reactivity to DKK1. However, mAb YAL008–1-5F8 has better affinity to full length of DKK2 than mAb YAL008–5-1A10. The disproportion of binding activities of mAb YAL008–5-1A10 to peptides and proteins suggest the epitope of ^{240}KVWKDATYS^{248} (YAL008–5) may not be fully exposed on the outer surface of DKK2 protein.

Anti-DKK2 mAbs inhibit DKK2-mediated antagonism of Wnt signaling

To demonstrate their inhibitory activity, a cell-based assay was performed. Briefly, HEK293 cells transfected with

Table 4. Summary of mAbs generated against different peptides

Peptide	Hybridoma cells screened	Initial positive	Further expanded	Best clones	Best titers
YAL008–1	600	67	18	3F7, 3G2, 5F8	0.5 ng/ml
YAL008–2	700	200	31	4B2, 6C1	50 ng/ml
YAL008–5	700	658	29	1A10, 3B8,5C2,7B5	0.5 ng/ml
YAL008–7	600	40	6	1A10,6E3	0.5 ng/ml

The culture supernatants of hybridomas or the purified mAb IgGs were screened against their immunizing peptide by ELISA. Clones were screened using peptides according to each number.

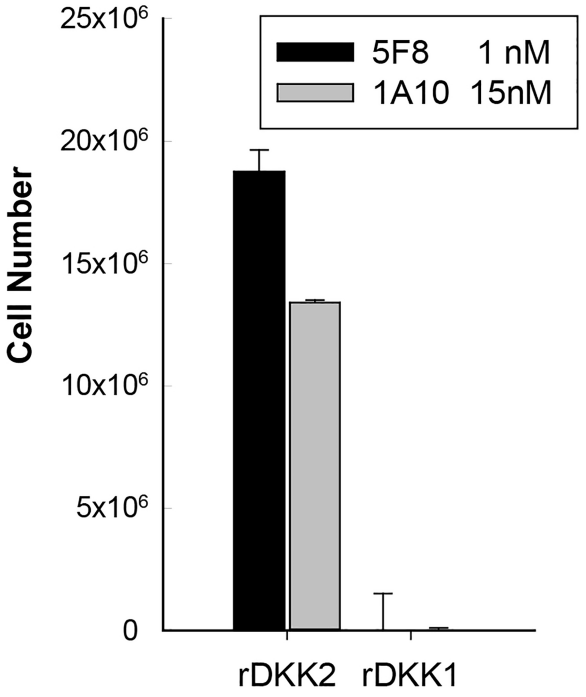


Figure 3. Specificity test of anti-DKK2 mAbs. Each well was coated with 1 μg/ml of either recombinant human DKK2 or DKK1. Then incubated with the anti-DKK2 antibody 5F8 (1 nM) and 1A10 (15 nM). A chemiluminescence AP substrate was used and measured by an EnVision plate reader.

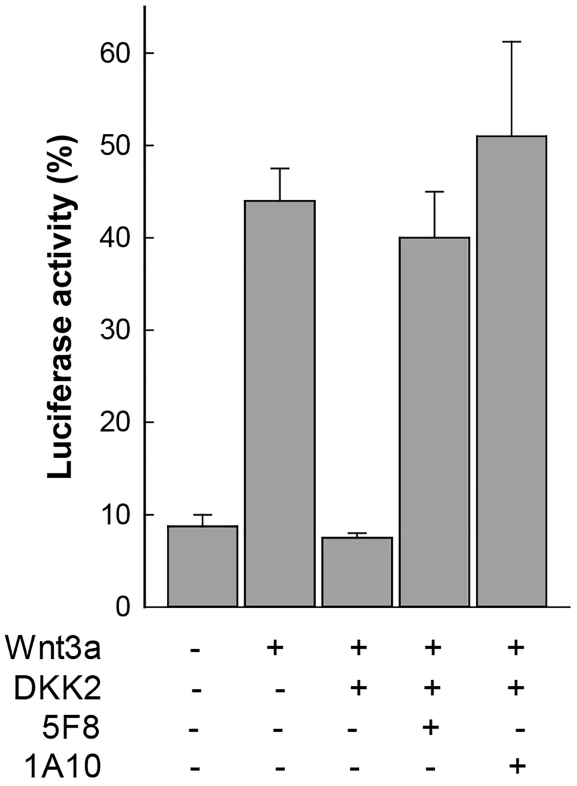


Figure 4. 5F8 and 1A10 inhibit DKK2-mediated antagonism of Wnt signaling. HEK293 cells transfected with the TOPFlash and GFP plasmids were incubated with different combinations of Wnt/DKK2/mAb complex. About 6 h later, the cells were lysed and subjected to RFP fluorescence and luciferase luminescence measurement using an Envision plate reader. The reporter gene activity is shown after being normalized against RFP readings.

the Wnt reporter gene were incubated with different combinations of Wnt 3a/DKK2/anti-DKK2 mAb complex. Six hours later, the cells were lysed and subjected to RFP fluorescence and luciferase luminescence measurement. As shown in Fig. 4, recombinant Wnt 3a alone activated the reporter gene/Luciferase activity. Presence of DKK2 suppressed the Wnt 3a-induced Luciferase activity. Addition of either mAbs YAL008-1-5F8 or YAL008-5-1A10 could reverse the DKK2-mediated antagonism of Wnt signaling. In a separate experiment, YAL008-7-1A10 also has been shown to inhibit DKK2 activity on Wnt 3a signaling (data not shown).

In summary, three neutralizing linear antigen epitopes containing ^{34}KLNSIKSSL^{42}, ^{240}KVWKDATYS^{248} and ^{195}CARHFWTKIC^{204} in human DKK2 were discovered by using rational design of synthetic peptides as immunogens.

Anti-DKK2 mAb 5F8 can inhibit tumor growth inC57Bl mice xenograft model

As shown in Fig. 1, the antigen epitope (^{34}KLNSIKSSL^{42}) of anti-human DKK2 mAb YAL008-1-5F8 is 100% homologous to mouse DKK2. Making it feasible to test the therapeutic potential of the anti-DKK2 mAb YAL008-1-5F8 for treating advanced cancers in a MC38 tumor cells grafted mouse model. MC38 cells were derived from mouse colon carcinoma in a C57Bl mouse, and progress very fast when grafted to immune-competent WT C57Bl mice. Six days later after the inoculation of MC38 cells, the C57Bl mice ($n = 5$ per group) were treated with either

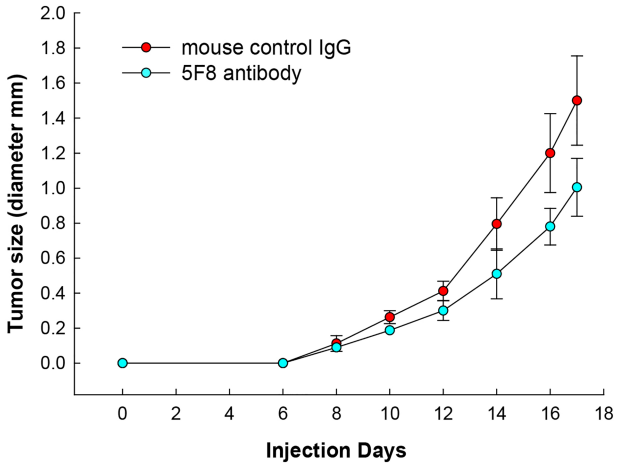


Figure 5. mAb 5F8 inhibits tumor growth in vivo. C38 tumor cells (0.5×10^6) were inoculated subcutaneously at the right flanks of the backs of female C57/BL mice. For antibody treatment, 100 µl of control IgG antibody or anti-DKK2 antibody were injected i.p. (200 µg/mouse every other day). Tumor sizes were measured by calipers.

anti-DKK2 mAb YAL008-1-5F8 or same isotype control mouse IgG at 200 µg/mouse every other day for another 12 days. As shown in Fig. 5, anti-DKK2 mAb YAL008-1-5F8 significantly inhibits tumor growth. This *in vivo* anti-tumor activity suggests that anti-DKK2 mAb YAL008-1-5F8 could be a good lead molecule for development of monoclonal antibody drug for cancer treatment.

DISCUSSION

Human DKK2 and its mouse counterpart have very high homology with an identity score of 96% and similarity score of 98%. Immunization of mice with human DKK2 failed to induce strong immune response. In this report, we showed that we can break the immune tolerance by using synthetic peptide-KLH conjugates as immunogens to raise mouse antibodies which can recognize the highly homologous human DKK2. Using proprietary software called AbEpiMax, we also demonstrated that by rational design of just six peptides we had identified three linear neutralizing antigen epitopes. With such high accuracy and success rate, it is a much more cost-effective and less time-consuming way than the "Peptide-walking" approach. In the future, we could use such approach to identify linear neutralizing epitopes of cancer biomarkers and pathogens for development of peptide vaccines.

Using the anti-DKK2 mAbs we developed here, our group reported the discovery of a new role of DKK2 as an immune-suppressor in our previous report. The loss of APC or PTEN in cancer cells upregulates the expression of DKK2, which in turn leads the suppression of the immune system by inactivation of NK and CD8+ T cells. It has been showed that mouse anti-DKK2 mAb 5F8 can cooperate with anti-PD-1 antibody by impede tumor progression (21). Successful humanization of this mouse anti-DKK2 mAb 5F8 may offer a new drug for treatment of human cancers.

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CONFLICT OF INTEREST

Patents of the antigenic epitopes and anti-DKK2 mAbs have been filed.

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