Development of therapeutic monoclonal antibodies against DKK1 peptide-HLA-A2 complex to treat human cancers

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ABSTRACT

Background Targeted immunotherapy with monoclonal antibodies (mAbs) is an effective and safe method for the treatment of malignancies. Development of mAbs with improved cytotoxicity, targeting new and known tumor-associated antigens, therefore continues to be an active research area. We reported that Dickkopf-1 (DKK1) is a good target for immunotherapy of human cancers based on its wide expression in different cancers but not in normal tissues. As DKK1 is a secreted protein, mAbs binding directly to DKK1 have limited effects on cancer cells in vivo.

Methods The specificity and antibody-binding capacity of DKK1-A2 mAbs were determined using indirect ELISA, confocal imaging, GiFKit antibody-binding capacity and cell surface binding assays. The affinity of mAbs was determined using a surface plasmon resonance biosensor. A flow cytometry-based cell death was performed to detect tumor cell apoptosis. Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays were used to evaluate the ability of DKK1-A2 mAbs to mediate ADCC and CDC activities against tumor cells in vitro. Flow cytometry data were collected with an FACSymphony A3 cell analyzer and analyzed with FlowJo V.10.1 software. Human cancer xenograft mouse models were used to determine the in vivo therapeutic efficacy and the potential safety and toxicity of DKK1-A2 mAbs. In situ TUNEL assay was performed to detect apoptosis in tumors and mouse organs.

Results We generated novel DKK1-A2 mAbs that recognize the DKK1 P20 peptide presented by human HLA-A*0201 (HLA-A2) molecules (DKK1-A2 complexes) that are naturally expressed by HLA-A2*DKK1+ cancer cells. These mAbs directly induced apoptosis in HLA-A2*DKK1+ hematologic and solid cancer cells by activating the caspase-9 cascade, effectively lysed the cancer cells in vitro by mediating CDC and ADCC and were therapeutic against established cancers in their xenograft mouse models. As DKK1 is not detected in most human tissues, DKK1-A2 mAbs neither bound to nor killed HLA-A2* blood cells in vitro nor caused tissue damage in tumor-free or tumor-bearing HLA-A2-transgenic mice.

Conclusion Our study suggests that DKK1-A2 mAbs may be a promising therapeutic agent to treat human cancers.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Dickkopf-1 (DKK1) is a good target for immunotherapy of human cancers based on its wide expression in different cancers but not in normal tissues.
- As DKK1 is a secreted protein, mAbs binding directly to DKK1 have limited effects on cancer cells in vivo.

WHAT THIS STUDY ADDS

- DKK1-A2 monoclonal antibodies (mAbs) recognizing DKK1-A2 complexes that are naturally expressed by HLA-A2*DKK1+ cancer cells were generated.
- DKK1-A2 mAbs can directly induce apoptosis in HLA-A2*DKK1+ cancer cells by activating the caspase-9 cascade, and effectively lyse cancer cells in vitro by mediating antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.
- DKK1-A2 mAbs were therapeutic against established cancers in their xenograft mouse models.
- DKK1-A2 mAbs neither bound to or killed HLA-A2+ blood cells in vitro nor caused tissue damage in tumor-free or tumor-bearing HLA-A2-transgenic mice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- DKK1-A2 mAbs may be a promising therapeutic agent to treat human cancers.

INTRODUCTION

Targeted immunotherapy with monoclonal antibodies (mAbs) is an effective and safe method for the treatment of malignancies. However, selected mAb-based cancer therapies have been approved for cancer treatment. In recent years, efforts have been made to identify potential therapeutic mAbs by defining alternative or novel target antigens, that is, CD40,1 2 IL6R,3 HM1.24,4 5 CD74,6 CD47,7 TRAIL-R1,8 CS1,9 CSF1R10 as well as by conjugating mAbs with classic or novel drugs to specifically kill cancer cells, that is, CD56-maytansinoid (DM1)11 and CD138-DM1/DM4.12 Development of mAbs with improved cytotoxicity, targeting new and
known tumor-associated antigens, therefore continues to be an active research area.

Dickkopf-1 (DKK1) is a secreted protein that specifically inhibits the Wnt/β-catenin signaling by interacting with the co-receptor Lrp-6. Previous studies have shown that the DKK1 gene has restricted expression in placenta and mesenchymal stem cells but not in other normal tissues, and DKK1 in patients with multiple myeloma (MM) was associated with the presence of lytic bone lesions. Immunohistochemical analysis of bone marrow (BM) biopsies showed that only myeloma cells contain detectable DKK1. Recombinant human DKK1 or plasma of BM aspirates from patients with MM containing an elevated level of DKK1 inhibited the differentiation of osteoblast precursor cells in vitro. Furthermore, anti-DKK1 mAb treatment was associated with reduced tumor growth in myeloma mouse model, and blocking DKK1 activity reduced osteolytic bone resorption, increased bone formation, and helped control myeloma progression. Similarly, DKK1 overexpression was also observed in breast cancer, ovarian serous carcinoma, esophageal squamous cell carcinoma, non-small cell lung cancer, and pancreatic ductal adenocarcinoma.

We determined whether DKK1 can be targeted as a tumor-associated antigen for immunotherapy of cancers such as MM. Our studies showed that DKK1 is widely expressed by various tumor cells including MM and other hematological malignancies but not normal tissues, and DKK1 peptide, such as P20 and P60v, which bind with HLA-A*0201 (HLA-A2) molecule, specific cytotoxic T cells effectively kill myeloma and other cancer cells that express DKK1 and HLA-A2, but not HLA-A2* normal cells. These results indicate that DKK1+ tumor cells naturally express these peptides in the context of HLA-A2 molecules on their surface. Therefore, we generated mAbs that recognize DKK1 (P20)-HLA-A2 (DKK1-A2) complex. In this study, we characterized the DKK1-A2 mAbs and examined their efficacy, toxicity, and safety in killing tumors and normal tissues or cells in vitro and in vivo using HLA-A2-transgenic SCID (A2-SCID) mice. Our study demonstrates that DKK1-A2 mAbs are potentially therapeutic mAbs for immunotherapy of human cancers.

**MATERIALS AND METHODS**

**Cell lines and primary tumor and normal cells**

Human MM cell lines used include U266, IM-9, XG1, ARP-1, ARK, CAG, MM1-144, RPMI-8226, MM.1R, and MM.1S. Human lymphoma cell lines used include Granta 519, Jeko-1, Mino and Sp53. Human leukemia cell line was ARH-77. Human breast cancer cell lines used include MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-453. Human prostate cancer cell lines used include LnCap-LN3, PC3-LN4, PC-3 and HLA-A2+ PC-3 (PC-3-A2+). Human pancreatic ductal adenocarcinoma (PDAC) cell lines include CFPAC-1 and PANCA-1. Human liver cancer cell line was Hep G2. All hematological cell lines were maintained in RPMI-1640 medium (Fisher Scientific, Herndon, Virginia, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia, USA). All solid cancer cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (Fisher Scientific, Herndon, Virginia, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia, USA). K562 cells (American Type Culture Collection; Rockville, Maryland) were used as natural killer (NK) cell-sensitive targets. Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by Ficoll-Hypaque density centrifugation. B cells were isolated from fresh PBMCs using EasySep Human B Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada). CD138+ MM cells were purified from BM aspirates of patients with MM using RoboSep Human Whole Blood and BM CD138 Positive Selection Kit (STEMCELL Technologies, Vancouver, Canada). Aliquots of purified MM cells were cryopreserved in liquid nitrogen until use. Written informed consent was obtained from patients and healthy donors for research use of their samples. The study was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center (UTMDACC) and Houston Methodist Hospital.

**Generation of DKK1-A2 mAbs**

DKK1-P20 peptide (ALGGHPLLGV) was refolded with recombinant HLA-A2 and β-microglobulin (b2M) to produce DKK1-A2 monomer (MHC Tetramer Laboratory, Baylor College of Medicine, Houston, Texas, USA). Six-week-old Balb/c mice were immunized with DKK1-A2 monomer at a 2-week interval for a total of four times by intraperitoneal injection of the antigen plus adjuvant, followed by an intraperitoneal injection of the antigen alone 3 days before harvest of splenocytes. Lymphocytes from spleens were fused with SP2/0 myeloma cells, and positive hybridomas were screened with ELISA and surface staining by flow cytometry assay. Positive clones (n=156) were isolated by limiting dilution. Large-scale antibody production of selected clones, such as C2, HMB1 and HMB7 (all are IgG1) mAbs, was achieved by intraperitoneal injection of 2x10⁶ hybridoma cells into Balb/c mice to produce ascites, followed by purifying mAbs using a protein A Sepharose column (Amersham Biosciences, Piscataway, New Jersey, USA). Mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Animal care and use were approved by the Institutional Animal Care and Use Committee at MDACC (#A-3343-01) and Houston Methodist Research Institute (#A-4555-01).

**Apoptosis assays**

Tumor cells were incubated without or with various concentrations of DKK1-A2 mAbs or mouse IgG1 (mIgG1) for 48 hours. Apoptotic cells were detected by staining cells, suspended in annexin-V binding buffer (BD PharMingen, San Diego, California, USA), with Fluorescein isothiocyanate (FITC)-conjugated annexin-V and Propidium
iodide (PI), according to manufacturer’s instruction, and determined as annexin V-positive cells by flow cytometry. A flow cytometry-based cell death detection kit (APO-BrdU) was purchased from BD PharMingen, and experiments were performed according to the manufacturer’s protocol. Flow cytometry data were collected with an FACSymphony A3 cell analyzer (BD Biosciences, Franklin Lakes, New Jersey) and analyzed with FlowJo V.10.1 software (BD FlowJo, Ashland, Oregon, USA). In situ TUNEL assay was performed on tumor sections to detect apoptosis in tumors and mouse organs isolated from A2-SCID mice, according to the manufacturer’s instruction. MTT assay (Sigma-Aldrich, St. Louis, Missouri, USA) was used to examine the viability of cancer cells and performed according to the manufacturer’s recommendation (R&D Systems, Minneapolis, Minnesota, USA). Absorbance was measured at 570nm using a spectrophotometer.

**ADCC and CDC assays**

DKK1-A2 mAbs (C2, HMB1 and HMB7) were used in this study and mlgG1 (BioLegend, San Diego, California, USA) was used as control. Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) were measured by chromium (Cr) release assays. In ADCC assay, PBMCs from normal volunteers were used as effector cells, and in CDC assay, guinea pig complements (10 U/mL, Sigma-Aldrich) were used as the source of complements. Target cells (1×10⁶) including MM cells, lymphoma cells, leukemia cells, breast cancer cells, prostate cancer cells, PDAC cancer cells, or normal B cells were incubated with 200 µCi of Cr for 1 hour at 37°C with gentle resuspension of pellet at 15 min intervals. After washing, cells were plated at 10,000 cells/well in a 96-well U-bottom plate with PBMCs or guinea pig serum, followed by the addition of DKK1-A2 mAbs at final concentrations ranging from 5 µg/mL to 20 µg/mL. Cells were then incubated for 4 hours at 37°C, and cell-released Cr was measured using a gamma-counter. Spontaneous release was determined from target cells without the addition of DKK1-A2 mAbs, PBMCs or guinea pig serum, and maximum release was determined from target cells with 6% Triton X-100 without the addition of the mAbs, PBMCs or guinea pig serum. Per cent cytotoxicity was calculated as (counts in sample—spontaneous release)/(maximum counts—spontaneous release)×100%. All experiments were performed in triplicate.

In some experiments, ADCC and CDC were observed by the IncuCyte ZOOM live-cell imaging system (ESSEN BioScience, Ann Arbor, Michigan, USA). Briefly, target cells were labeled with NucLight Lentiviral Reagent (Sartorius Cat. No. 4475 or 4476) in ADCC assay. Target cancer cells (red cells, 3,000 cells in 100 µL per well) were seeded into an Incucyte-compatible flat-bottom 96-well plate at 37°C, 5% CO2 incubator overnight. PBMCs were added at a ratio of 10:1, or a guinea pig complement (10 U/mL, Sigma-Aldrich) was added into the appropriate wells of the cell plate to achieve a final assay volume of 200 µL. This was followed by the addition of DKK1-A2 mAbs at final concentrations ranging from 5 µg/mL to 20 µg/mL. Caspase-3/7 green reagent (20 µM) was used to detect apoptotic cells. The assay plates were placed into the IncuCyte Live-Cell Analysis System and scheduled for 4-hour repeat scanning. Adherent cell-by-cell analysis was used to detect the death of target cells (yellow cells for ADCC, green cells for CDC).

**Immunophenotyping, intracellular staining, confocal imaging, and antibody-binding capacity**

Affinity-purified C2 mAb was conjugated to fluoro-chromes Alexa Fluor 488, Alexa Fluor 647, and Phycocerythrin (PE) (Invitrogen, Carlsbad, California, USA). Alexa Fluor 488, Alexa Fluor 647, and PE-conjugated mAbs (2 µg/mL) were added to cell pellets, incubated for 30 min on ice, and washed three times before analysis. Intracellular DKK1 staining was confirmed using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s recommendation. Flow cytometry data were collected with an FACSymphony A3 cell analyzer (BD Biosciences) and analyzed with FlowJo V.10.1 software (BD FlowJo). For confocal imaging, cells were costained with C2 and anti-HLA-A2 mAb BB7.2 or C2 and anti-DKK1 mAb to confirm colocalization. Cells were fixed and stained with Alexa Fluor 488, Alexa Fluor 647, and dihydrochlo-ride (Invitrogen) was applied to cells before mounting on glass slides. Imaging was performed using a Leica Microsystems SP2 SE confocal microscope (Leica Microsystems, Wetzlar, Germany). QIFIKIT (Dako, Santa Clara, California, USA) was used for quantitative determination of cell surface antigen by flow cytometry using indirect immunofluorescence assay. Antibigen quantity was expressed as antibody-binding capacity units.

**Immunohistochemistry analysis**

Formalin-fixed, paraffin-embedded sections of tissues or tumors from A2-SCID mice were deparaffinized with xylene and rehydrated to water through a graded alcohol series. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were incubated for 30min with DKK1-A2 mAb (C2, 10µg/mL) at room temperature after blocking with normal serum. Detection of the signal was achieved using secondary biotinylated antibodies and streptavidin/ horseradish peroxidase according to the manufacturer’s instructions (The VECTASTAIN Elite ABC Kit, Vector Laboratories, Newark, California, USA). Chromagen 3,3-diaminobenzidine/H2O2 (DaKo) was used and slides were counterstained with hematoxylin. All slides were observed with light microscopy, and images were captured with a SPOT RT camera (Diagnostic Instruments, Burlingame, California, USA).
Western blotting analysis

Western blot analysis was employed to detect signaling protein expression in tumor cells. Cells were cultured with or without DKK1-A2 mAbs (50 µg/mL), harvested, washed, and lysed with lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN3, 1% Triton-X-100, 1% NP-40, 1×protease inhibitor cocktail). Cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with antibodies (1–2 µg/mL) against caspase-3, -7, -8 to -9, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bad, Bax, or cytochrome c (Cell Signaling Technology, Beverly, Massachusetts, USA and Santa Cruz Biotechnology, Dallas, Texas, USA) overnight at 4°C. Membrane was stripped and reprobed with a β-actin-specific antibody (0.5 µg/mL, Sigma-Aldrich) to ensure equal protein loading. Secondary antibodies conjugated to horseradish peroxidase (1:2,000 dilution, Santa Cruz Biotechnology, Dallas, Texas, USA) were used for detection, followed by enhanced chemiluminescence (Pierce Biotechnology, Rockford, Illinois, USA) and autoradiography.

ELISA and surface plasmon resonance

HLA-A2-restricted DKK1 peptides P20 (ALGGHPLLGV) or P66v (ILYGGNKNV), influenza matrix protein-derived influenza (GILGFVFTL) and HIV pol protein-derived HIV (ILKPEVHGV) peptides were synthesized at the UTMDACC facility to a minimum 95% purity. The binding affinity of these peptides for HLA-A2 was described previously. Major histocompatibility complex (MHC)-peptide monomers were synthesized in the MHC Tetramer Laboratory at Baylor College of Medicine. Different antigens such as A2-P20, A2-P66v, A2-HIV, A2-Flu, A2, b2M, P20 and P66v were coated on a 96-well ELISA plate at the concentration of 0.5 µg/mL at 4°C overnight and the specificity of DKK1-A2 mAbs was detected using indirect ELISA assay. Briefly, the plate was washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature. Primary detection antibody (C2, HMB1, or HMB7, 2 µg/mL) was added to the plate and incubated for another 1 hour at room temperature, followed by a buffer wash. The secondary goat anti-mouse IgG-horseradish peroxidase (1:1,000 dilution, Santa Cruz Biotechnology, Dallas, Texas, USA) was added and incubated for another 1 hour at room temperature. The plate was rewashed and an OPD substrate (Sigma-Aldrich) was added to produce a color change. After stopping the enzymatic reaction, the plate was read at 490 nm with a plate reader.

The affinity of DKK1-A2 mAbs to P20/HLA-A2 was determined using a surface plasmon resonance (SPR) biosensor, Biacore T200 (GE HealthCare, Chicago, Illinois, USA) at GenScript USA (Piscataway, New Jersey). Antibody was immobilized on the sensor chip through the Fc capture method. Antigen was used as the analyte. The data of dissociation (kd) and association (ka) rate constants were obtained using Biacore evaluation software. The equilibrium dissociation constants (KD) were calculated from the ratio of kd over ka.

In vivo mouse studies

To generate human MM xenograft mouse model, 1×10⁶ luciferase-labeled U266 cells were intravenously inoculated into 6-week-old female SCID mice (The Jackson Laboratory, Bar Harbor, Maine, USA). Three weeks later when the tumor developed, mice (five per group) were intraperitoneally injected with 200 µg C2 mAb every 3 days for a total of six injections. Control mice received equal amounts of mlgG1. Serum was collected from mice weekly. Tumor burdens were evaluated by bioluminescence on the IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, Massachusetts, USA) and the level of human immunoglobulin light chain secreted by human MM cells was determined by ELISA. Mice were humanely sacrificed when moribund. Survival was evaluated from the day of tumor inoculation until death.

To generate human lymphoma, leukemia, breast cancer and PDAC cancer xenograft mouse models, 6-week-old SCID mice (The Jackson Laboratory) were injected subcutaneously in the right flank with 2×10⁶ luciferase-labeled Granta 519 mental cell lymphoma cells, ARH-77 leukemia cells, MDA-MB-231 breast cancer cells, or CFPAC-1 PDAC cells. Two to 3 weeks later when palpable tumors (5 mm in diameter) developed, mice (five per group) were intraperitoneally injected with 200 µg C2 mAb every 3 days for a total of six injections. Control mice received equal amounts of mlgG1. Tumor burdens were evaluated by bioluminescence on IVIS imaging system and were measured every 3 days with calipers. Tumor volume (mm³) was calculated as 4π/3×(tumor width/2)²×(tumor length/2). Mice were humanely sacrificed when moribund or subcutaneous tumors reached 15 mm in diameter. Survival was evaluated from the day of tumor inoculation until death.

For in vivo mechanism study, 6-week-old human A2-SCID mice (The Jackson Laboratory) were injected subcutaneously in the right flank with 2×10⁶ luciferase-labeled U266 MM cells. Three to 4 weeks later when palpable tumors (5 mm in diameter) developed, mice (five per group) were intraperitoneally injected with lenalidomide (10 mg/kg, for 5 days and 2 days off for 3 weeks), 200 µg C2 mAb every 3 days for a total of six injections, or their combination. Control mice received equal amounts of mlgG1 or DMSO. Tumor burdens and tumor volume were measured similarly as described earlier.

To deplete murine NK cells, mice were injected intraperitoneally with 25 µL of anti-asialo GM1 antiserum (Wako Chemicals, Richmond, Virginia, USA) 2 days before mAb treatment. This was repeated each week throughout the experiment in all mice. To deplete murine complement, mice were injected intraperitoneally with 10 µg cobra venom factor (CVF) (Sigma-Aldrich, St. Louis, Missouri, USA) 4 hours before mAb treatment. This was repeated each week throughout the experiment in all mice.
All mice were maintained in the American Association of Laboratory Animal Care-accredited facilities, and studies were approved by the Institutional Animal Care and Use Committee of UTMDACC (#A-3543–01), Cleveland Clinic (#A-3143–01), and Houston Methodist Research Institute (#A-4555–01).

**Statistical analysis**
The Student’s t-test was used to compare various experimental groups. A p value<0.05 was considered statistically significant. Unless otherwise indicated, means and SD are shown. Survival rate was evaluated using the Kaplan-Meier groups. A p value<0.05 was considered statistically significant. The Student's t-test was used to compare various experimental groups. A p value<0.05 was considered statistically significant. The Student's t-test was used to compare various experimental groups. A p value<0.05 was considered statistically significant.

**RESULTS**

Specificity and antibody-binding capacity of DKK1-A2 mAbs
We generated a panel of mouse mAbs against the human DKK1-A2 complex by immunizing Balb/c mice with the DKK1-A2 monomer. After several rounds of fusion and screening, positive clones (n=156) were obtained by limiting dilution assay. Three (C2, HMB1, and HMB7) of these clones were selected because they secreted IgG1-isotype mAbs. Two of the mAbs, C2 and HMB1, had high titers against A2-P20, but not A2-HIV, A2-Flu, or A2-P66v complexes, nor against HLA-A2, b2M molecules, P20, or P66v peptide in an ELISA assay (figure 1A). All three mAbs, C2, HMB1 and HMB7, bound to cell surface of DKK1-HLA-A2+ MM cell line U266 but not to DKK1-HLA-A2+ MM cell line ARP-1, and C2 mAb had the strongest binding to cell surface (figure 1B). DKK1-A2 mAbs are also bound to DKK1-HLA-A2+ lymphoma cell line Granta 519 and leukemia cell line ARH-77 (online supplemental figure 1A). To further determine the specificity of these mAbs, immunofluorescent confocal imaging was used. Figure 1C showed that C2 mAb displayed strong surface staining on U266 and Granta 516 but not ARP-1 cells, while anti-human DKK1 mAb stained intracellular DKK1 in all three cell lines. On U266, Granta 519, and T2 cells pulsed with the P20 peptide, but not unpulsed T2 or T2 pulsed with the P66v peptide, C2 mAb and HLA-A2 colocalized together (figure 1D), indicating that all three mAbs bind specifically to the DKK1-A2 complexes on the tumor cells. SPR was used to measure antibody binding affinity and confirmed that C2 mAb had the highest binding affinity (KD=1.82 nM) among all three mAbs (online supplemental table 1). Therefore, C2 mAb was used for most experiments in the study.

Next, we determined the optimal concentration of C2 mAb for surface binding to U266 to be 2.0 µg/mL (figure 1E). Using the optimal concentration of C2 mAb, we detected the best concentration of P20 loading on T2 cells to be 50 µg/mL (figure 1F). Furthermore, antibody-binding capacity of C2 mAb to normal PBMC, monoclonal gammopathy of undetermined significance (MGUS) or patient with MM tumor samples, and U266 was detected by QIFIKIT kit (Agilent, Santa Clara, California, USA). C2 mAb strongly bound to HLA-A2+ MGUS and patient with MM tumor samples and U266 but not to HLA-A2+ PBMC, and the antibody-binding capacity on MGUS and patient with MM tumor samples and U266 was 12,000, 25,000 and 45,000, respectively (figure 1G and H). We also detected DKK1-A2 complex expression on patient with MM tumor samples. DKK1 protein was expressed in all tested patient with MM samples. Among them, 4/8 tumor samples were HLA-A2+, and DKK1-A2 complex expression was detected on all four patient with HLA-A2+ MM tumor samples (figure 1I). Together, these data demonstrate that our DKK1-A2 mAbs have high specificity, binding affinity, and antibody-binding capacity to the DKK1-A2 complex on tumor cells.

**DDK1-A2 mAbs at high concentrations directly induce apoptosis in DKK1+HLA-A2+ cancer cells**
We investigated whether DKK1-A2 mAbs could kill cancer cells in vitro. As shown in figure 2A, after 48 hours of culture, apoptosis of U266 cells was induced by C2 mAb at high concentrations (25–100 µg/mL), while control mlgG1 had no effect. To determine whether there was a correlation between the killing efficiency and the level of DKK1-A2 complex expression on cell surface, T2 cells were loaded with different concentrations of DKK1 P20 peptide overnight, treated with C2 mAb or mlgG1 for 48 hours, and analyzed for apoptosis. The results showed that T2 cell apoptosis was induced by C2 mAb, but not mlgG1, along with increased concentrations of loading P20 peptide, with the highest killing achieved at 50 µg/mL (figure 2B). Next, we examined apoptosis of T2 cells loaded without or with different peptides in the presence of C2, HMB1 or HMB7 mAb, or mlgG1. All three DKK1-A2 mAbs, but not control mlgG1, induced apoptosis in T2 cells loaded with P20, but not P66v, influenza or HIV peptide nor in T2 cells without peptide loading (figure 2C). Furthermore, we examined apoptosis in different hematological tumor cells including DKK1+HLA-A2+ primary MM cells from two patients (P05 and P06) and cell lines U266, Granta 519 and ARH-77, DKK1-HLA-A2+ ARP-1 or HLA-A2+ normal human B cells. DKK1-A2 mAbs induced significant cell death in DKK1+HLA-A2+ U266, Granta 519, ARH-77, and primary MM cells. No effects were observed in DKK1+HLA-A2+ ARP-1 or HLA-A2+ human B cells (figure 2D). To verify that the cell surface DKK1-A2 complex was the target, small interfering RNA for DKK1 gene was synthesized and used to knockdown DKK1 expression in U266 MM cells (online supplemental figure 2A,B). After knocking down DKK1, U266 MM cells were no longer sensitive to DKK1-A2 mAb-induced apoptosis (figure 2D). We further showed that DKK1-A2 mAbs induced cell death in DKK1+HLA-A2+ solid tumor cells such as CFPAC-1 and PANC-1 PDAC cells, MDA-MB-231 and MCF-7 breast cancer cells, and LnCap-LN-3 and PC-3-A2+ prostate cancer cells (online supplemental figure 3A–C). These results indicate that...
Figure 1  Specificity and antibody-binding capacity of DKK1-A2 mAb. (A) The binding of DKK1-A2 mAbs to different molecules or immune complexes coated on the plate was measured by ELISA. (B) Surface binding ability of DKK1-A2 (C2, HMB1, or HMB7) mAbs to MM cell lines U266 (HLA-A2+) and ARP-1 (HLA-A2-). Confocal immunofluorescent microscopy images showing (C) U266, Granta 519, and ARP-1 cells costained with Alexa Fluor 488 (A488)-conjugated C2 mAb (green), Alexa Fluor 647 (A647)-conjugated DKK1 mAb (red), and DAPI (blue). Merged images are depicted on the right; and (D) T2 cells, T2 cells loaded with P20, T2 cells loaded with P66v, U266, or Granta 519 cells costained with Alexa Fluor 488 (A488)-conjugated C2 mAb (green), Alexa Fluor 594 (A594)-conjugated HLA-A2 mAb (red), and DAPI (blue). Merged images are depicted on the right. (E) Surface binding affinity of C2 mAb at indicated concentrations to U266 cells. (F) T2 cells were loaded with different concentrations of DKK1 P20 or P66v peptide overnight and analyzed for binding affinity (MFI) of C2 mAb to the surface DKK1-A2 complex. (G) The binding capacity of C2 mAb to normal PBMC and plasma cells from MGUS or patients with MM, and U266, and (H) Summary of results. (I) The surface expression of HLA-A2 and DKK1-A2 complex (stained by C2 mAb), and intracellular expression of DKK1 protein in primary MM cells from eight patients (Pt01–Pt08) was measured by flow cytometry. Error bars=SD. ***p<0.001; ****p<0.0001. DAPI, 4', 6-diamidino-2-phenylindole, dihydrochloride; DKK1, Dickkopf-1; mAb, monoclonal antibodies; MFI, mean fluorescent intensity; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PBMC, peripheral blood mononuclear cell.
Figure 2  DKK1-A2 mAbs at high concentrations induce apoptosis in cancer cells in vitro. (A) Apoptosis of U266 cells induced by C2 mAb at different concentrations. Mouse IgG1 (mlgG1) served as control. (B) T2 cells were loaded with different concentrations of DKK1 P20 peptide overnight and then washed and treated with 50 μg/mL C2 mAb or mlgG1 for 48 hours, followed by analysis for apoptosis. (C) Apoptosis of T2 cells loaded without or with different peptides and then treated with 50 μg/mL DKK1-A2 C2, HMB1 or HMB7 mAb or mlgG1. (D) Apoptosis of MM cell lines U266, DKK1-knockdown U266 (U266-DKK1-KD), and ARP-1, lymphoma cell lines Granta 519, leukemia cell line ARH-77, primary MM cells from two patients (Pt05 and Pt06), and normal B cells in 48-hour culture with 50 μg/mL C2 mAb or mlgG1. (E) The expression of total and cleaved caspases, PARP, and cytochrome c (Cyto c), Bax, and phosphorylated (P) Bcl2 and Bad in U266 and Granta 519 cells treated with 50 μg/mL C2 mAb for different times measured by western blot. Error bars=SD. *p<0.05; **p<0.01. DKK1, Dickkopf-1; mAb, monoclonal antibodies; MM, multiple myeloma; PARP, poly(ADP-ribose) polymerase; pBad, phosphorylated Bad; pBcl2, phosphorylated Bcl-2.
DKK1-A2 mAbs have a higher affinity for the surface DKK1-A2 complex and can directly induce apoptosis in tumor cells in vitro.

To investigate whether and which caspase cascades participated in DKK1-A2-mAb-induced apoptosis in tumor cells, activation and cleavage of caspase-9, -8, -3, or -7, and subsequent cleavage of PARP were investigated by western blotting analysis. C2 mAb resulted in the activation and cleavage of caspase-9, -3, -7 to -8 and PARP in MM and lymphoma cells (figure 2E). Since these results indicated that the intrinsic apoptosis pathway was used, we next investigated the involvement of mitochondria and its associated pro-apoptosis and anti-apoptosis proteins. C2 mAb upregulated the expression of proapoptotic proteins Bax and phosphorylated Bcl-2, but decreased phosphorylated Bad expression (figure 2E); which favored the induction of apoptosis due to increased permeability of mitochondria, leading to the release of cytochrome c into the cytosol. Indeed, an increased level of cytosolic cytochrome c was detected in C2 mAb-treated tumor cells at 24 hours and onward (figure 2E).

**DKK1-A2 mAbs mediate ADCC and CDC to kill tumor cells**

Since the therapeutic efficacy of mAbs depends on ADCC and CDC, we first evaluated the ability of DKK1-A2 mAbs to mediate ADCC using in vitro assay. PBMCs from healthy donors were used as effector cells. DKK1-A2 mAbs at 20 µg/mL were able to mediate significant ADCC activities against DKK1+HLA-A2+ U266, Granta 519, ARH-77, and primary MM cells from patients (p<0.05 to p<0.01, compared with mIgG1 control). Among the three mAbs, C2 mAb mediated the highest ADCC activity against DKK1+HLA-A2+ tumor cells, followed by HMB1 and HMB7. No significant effect was observed with DKK1+HLA-A2+ tumor cells, indicating that DKK1-A2 mAbs are therapeutic against established cancers.

To confirm the results, we determined DKK1-A2 mAb-mediated ADCC against T2 cells loaded without or with P20, P66, influenza or HIV peptide. Compared with mIgG1, all three DKK1-A2 mAbs induced effective lysis of T2 cells pulsed with P20, but not T2 cells pulsed with P66, influenza or HIV peptide or unpulsed T2 cells (figure 3B; p<0.05 to p<0.01). We further confirmed that all three DKK1-A2 mAbs induced ADCC against DKK1+HLA-A2+ solid tumor cells such as MCF-7, MDA-MB-231, PC-3-A2+, LNCap-LN3, CFPA-1, and PANC-1 (figure 3C; p<0.05 to p<0.01). ADCC against CFPA-1 or MDA-MB-231 cells mediated by DKK1-A2 mAbs was further observed in real time under Live-Cell IncuCyte analysis, which demonstrated significant killing of CFPA-1 or MDA-MB-231 cells by C2 mAbs (figure 3D; p<0.01, compared with mIgG1 control). Taken together, these results indicate that DKK1-A2 mAbs are effective at mediating ADCC activities against DKK1+HLA-A2+ tumor cells in vitro.

Next, we evaluated DKK1-A2 mAb-mediated CDC activities against tumor cells. In CDC assay, guinea pig complements (10 U/mL) were used as the source of complements. As shown in figure 3E and F, DKK1-A2 mAbs mediated significant CDC activities against DKK1+HLA-A2+ U266, Granta 519, ARH-77, MDA-MB-231, PC-3-A2+, LNCap-LN3, CFPA-1, PANC-1, and primary MM cells from patients, but not DKK1+HLA-A2+ ARP-1 or HLA-A2+ human B cells compared with mIgG1 control (p<0.05 to p<0.01). When heat-inactivated guinea pig serum was used as a negative control, no CDC activities were detected (online supplemental figure 4). We also examined CDC activity against the wild-type PC-3 cells which are HLA-A2+ (PC-3-A2+) and HLA-A2-transduced PC-3-A2+ cells mediated by DKK1-A2 mAbs in real-time under Live-Cell IncuCyte and confirmed significant killing of DKK1+HLA-A2+ PC-3-A2+, but not (DKK1+HLA-A2+) PC-3-A2+ cells by C2 mAb (figure 3G; p<0.01, compared with mIgG1 control). These results indicate that DKK1-A2 mAb-mediated CDC activity is specific against DKK1+HLA-A2+ tumor cells. Taken together, these results demonstrate the ability of DKK1-A2 mAbs to kill tumor cells by mediating ADCC and CDC in vitro.

**In vivo therapeutic effects of DKK1-A2 mAbs against established cancers**

To determine the in vivo therapeutic efficacy of DKK1-A2 mAbs, SCID mice were inoculated intravenously with luciferase-labeled U266, followed by treatment with intraperitoneal injections of C2 mAb or mIgG1, or PBS when mice developed MM (positive in vivo image and/or human M proteins or its light chain reaching >5 µg/mL). C2 mAb significantly reduced tumor burden and retarded tumor growth, as evaluated by bioluminescence images (figure 4A), and levels of circulating M-protein secreted by MM cells (figure 4B; p<0.05 to p<0.01, compared with controls). Within the study period, all control SCID mice died, whereas ≥60% of mice treated with C2 mAb survived with minimal tumor burden (figure 4B; p<0.01, compared with controls). MM cell growth in the BM was verified by in vivo bioluminescence combined with X-ray imaging (figure 4C). We observed that the number of CD138+ MM cells in the BM and spleen was significantly reduced in C2 mAb-treated mice (figure 4D; p<0.01, compared with mIgG1 control), indicating that DKK1-A2 mAbs are therapeutic against established MM in vivo.

To confirm the results, SCID mice were inoculated subcutaneously with luciferase-labeled Granta 519 or ARH-77 cells and treated with C2 mAb when mice developed palpable tumors (R=5 mm in diameter). The bioluminescence images in figure 4E and G and the tumor sizes in figure 4F and H show the strong therapeutic efficacy of C2 mAb compared with mIgG1 control (p<0.05 to p<0.01). All control mice died within 65 days after tumor inoculation, whereas ≥40% C2 mAb-treated mice survived with minimal tumor burden (figure 4F and H; p<0.05 to p<0.01, compared with controls).

We confirmed the results in solid cancer xenograft mouse models. SCID mice were inoculated subcutaneously with luciferase-labeled CFPA-1 PDAC cells and treated with C2 mAb when mice developed palpable tumors (R=5 mm in diameter). The bioluminescence images in figure 5A and the tumor sizes in figure 5B show...

the strong therapeutic efficacy of C2 mAb (p<0.05 to p<0.01, compared with mlgG1 control). All control mice died within 50 days after tumor inoculation, whereas ≥60% C2 mAb-treated mice survived with minimal tumor burden (Figure 5C, p<0.05 to p<0.01, compared with control).

Figure 3 Dickkopf-1-A2 mAbs mediate ADCC and CDC activities to kill cancer cells in vitro. ADCC-mediated cytotoxicity detected by 51Cr-release assay against (A) primary MM cells from two patients (Pt05 and Pt06), MM, lymphoma, or leukemia cell lines, and normal B cells, (B) T2 cells loaded with different peptides, or (C) breast cancer cell lines (MCF-7 and MDA-MB-231), prostate cancer cell lines (PC-3-A2+ and LnCap-LN3), or PDAC cell lines (CFPAC-1 and PANC-1), in culture with human peripheral blood mononuclear cell and 20µg/mL C2, HMB1, or HMB7 mAb, or mlgG1. (D) ADCC-mediated cytotoxicity detected by IncuCyte ZOOM live imaging system against PDAC cell lines CFPAC-1 and breast cancer cell line MDA-MB-231 induced by C2 mAb or mlgG1. (E) CDC-mediated cytotoxicity detected by 51Cr-release assay against (E) primary MM cells from two patients (Pt05 and Pt06), MM, lymphoma, or leukemia cell lines, and normal B cells, or (F) breast cancer cell lines (MCF-7 and MDA-MB-231), prostate cancer cell lines (PC-3-A2+ and LnCap-LN3), or PDAC cell lines (CFPAC-1 and PANC-1), in culture with guinea pig complement and 20µg/mL C2, HMB1, or HMB7 mAbs or mlgG1. (G) Time-dependent CDC-mediated cytotoxicity detected by IncuCyte ZOOM live imaging system against prostate cancer cell lines PC-3-A2+ or PC-3-A2− induced by C2 mAb or mlgG1. Error bars=SD. *p<0.05; **p<0.01. ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; mAb, monoclonal antibodies; mlgG1, mouse IgG1; MM, multiple myeloma; PDAC, pancreatic ductal adenocarcinoma; 51Cr, 51Chromium.
Dickkopf-1 -A2 mAbs are therapeutic against established tumors in vivo. (A–D) SCID mice were injected intravenously with luciferase-labeled U266 MM cells, followed by treatment with intraperitoneal injections (every 3 days for a total of six injections) of 200 µg C2 mAb or mIgG. PBS served as a control. Tumor burdens were monitored by (A) in vivo imaging, (B) levels of circulating human M-protein or its light chain, and (C) in vivo bioluminescence plus X-ray imaging. Mouse survival is also shown in (B). (D) Three weeks after the treatment, bone marrow cells and splenocytes were collected for analyzing human CD138+ MM cells using flow cytometry. Summary data is shown. (E–F) SCID mice were injected subcutaneously with Granta 519 lymphoma cells, followed by treatment with intraperitoneal injections (every 3 days for a total of six injections) of 200 µg C2 mAb or mIgG. PBS served as a control. Tumor burdens were measured twice every week by (E) IVIS imaging system and (F) tumor sizes measured by caliper. Mouse survival is shown in (F). (G–H) SCID mice were injected subcutaneously with ARH-77 leukemia cells, followed by treatment with intraperitoneal injections (every 3 days for a total of six injections) of 200 µg C2 mAb or mIgG. PBS served as a control. Tumor burdens were measured twice every week by (G) IVIS imaging system and (H) caliper. Mouse survival is shown in (H). Error bars=SD. *p<0.05; **p<0.01. mAb, monoclonal antibodies; mIgG, mouse IgG1; MM, multiple myeloma; PBS, phosphate-buffered saline.
Figure 5  DKK1-A2 mAbs are therapeutic against established tumors in vivo. (A–C) SCID mice were injected subcutaneously with CFPAC-1 PDAC cells, followed by treatment with intraperitoneal injections (every 3 days for a total of six injections) of 200 µg C2 mAb or mlgG. PBS served as a control. Tumor burdens were measured twice every week by (A) IVIS imaging system and (B) caliper. Mouse survival is shown in (C). (D–F) SCID mice were injected subcutaneously with MDA-MB-231 breast cancer cells, followed by treatment with intraperitoneal injections (every 3 days for a total of six injections) of 200 µg C2, HMB1 or HBM7 mAb or mlgG1. Tumor burdens were measured twice every week by (D) IVIS imaging system and (E) caliper. Mouse survival is shown in (F). Error bars=SD. *p<0.05; **p<0.01. mAb, monoclonal antibodies; mlgG1, mouse IgG1; PBS, phosphate-buffered saline; PDAC, pancreatic ductal adenocarcinoma.
To compare the therapeutic efficacy of three DKK1-A2 mAbs in mouse models, SCID mice were inoculated subcutaneously with luciferase-labeled MDA-MB-231 breast cancer cells and treated with C2, HMB1, or HMB7 mAb when mice developed palpable tumors (R=5 mm in diameter). Treatment with C2 or HMB1 mAb, but not HMB7, significantly retarded tumor growth monitored by bioluminescence images (figure 5D) or tumor size measurements (figure 5E; p<0.05 to p<0.01, compared with mIgG1 control). All control mice and HMB7-treated mice died within 40 and 45 days, respectively, after tumor inoculation, whereas ≥40% C2 or HMB1 mAb-treated mice survived with minimal tumor burden (figure 5F; p<0.05 to p<0.01, compared with controls). These results indicate that DKK1-A2 mAbs can effectively control established cancers in vivo.

To determine the therapeutic mechanism of DKK1-A2 mAbs in vivo, SCID mice were inoculated subcutaneously with U266, followed by treatment with mIgG1 or C2 mAb, C2 mAb combined with NK depletion using anti-NK cell antibody, or C2 mAb combined with complement depletion using CVF. Tumor burdens were significantly reduced in C2 mAb-treated mice (figure 6A and B; p<0.01, compared with mIgG1 control). Interestingly, depleting NK cells compromised the therapeutic efficacy (figure 6A and B; p<0.05 to p<0.01, compared with C2 treatment), but depleting complements did not (figure 6A and B; p<0.01, compared with mIgG1 control). Within the experimental period, all control mice died, while mice treated with C2 mAb alone, C2 mAb with NK depletion or complement depletion had 60%, 20% and 60% survival rates, respectively (figure 6C). Taken together, these findings indicate that DKK1-A2 mAbs may be a potent therapeutic agent for the treatment of cancers via the mechanisms of directly killing tumor cells and indirectly lysing tumor cells by mediating ADCC activity in vivo.

**Lenalidomide enhances the therapeutic efficacy of DKK1-A2 mAbs against established tumors in vivo**

To determine whether lenalidomide may enhance the therapeutic effect of the mAbs, we investigated the therapeutic effects of DKK1-A2 mAbs in combination with lenalidomide in vivo. SCID mice were inoculated subcutaneously with U266 and treated with intraperitoneal injections of C2 mAb or mIgG1, lenalidomide, or lenalidomide plus C2 mAb after tumor development. As shown in figure 6D and E, although mice treated with C2 mAb or lenalidomide alone had significantly reduced tumor burdens (p<0.05 to p<0.01, compared with mIgG1 control), combination treatment with C2 mAb and lenalidomide was more efficacious than the single agent treatment (p<0.05, compared with mAb or lenalidomide alone). Within the experimental period, all control mice died, while mice treated with C2 mAb or lenalidomide alone or lenalidomide plus C2 had 60%, 40% and 80% survival rates, respectively (figure 6F). These data indicate that combination therapy using DKK1-A2 mAb and lenalidomide displays an enhanced therapeutic efficacy against established tumors in vivo.

**Potential toxicity and safety of DKK1-A2 mAbs**

To determine the potential safety and toxicity of DKK1-A2 mAbs in humans, we first analyzed DKK1 expression profiles using the Oncomine database. As expected, significantly increased DKK1 messenger RNA (mRNA) expression was observed in most human cancers compared with their counterpart normal tissues (figure 7A), especially in large-cell lung carcinoma, pancreatic carcinoma, and fibrous histiocytoma. For hematological malignancies, high DKK1 mRNA expression was detected in B-cell childhood acute lymphoblastic leukemia (figure 7B, p<0.0001, compared with PBMCs from healthy donors), follicular lymphoma (figure 7C, p<0.028, compared with B lymphocyte from healthy donors), and MM (figure 7D, p<0.027, compared with normal plasma cells from healthy donors). Next, human A2-SCID mice were used to examine the potential toxicity of DKK1-A2 mAbs on human-like tissues and organs. MM-bearing A2-SCID mice were examined after a high dose (400 µg per mouse per injection for a total of six injections) C2 mAb treatment and showed no normal tissue damage (figure 7E). Immunohistochemical examination of tumors and organs from the A2-SCID mice showed that the DKK1-A2 complexes were only expressed in tumors (figure 7E and F). To confirm the results, tumors and other tissues from the treated MM-bearing mice were removed to detect apoptotic cells by TUNEL assay. As shown in figure 7G and H, significantly higher numbers of apoptotic tumor cells but not normal tissue cells were detected in mice treated with C2 mAb (p<0.01, compared with mIgG1 control). Furthermore, tumor-free A2-SCID mice were also treated in the same way as tumor-bearing mice and were sacrificed 3 days after the final injection of DKK1-A2 mAbs. Organs including heart, lung, liver, spleen, kidney, and bone from treated A2-SCID mice were removed for histological examination. Treatment with C2 mAb or mIgG1 did not change the body weight of mice (online supplemental figure 5A) nor cause cell or tissue damage in the murine organs that express human HLA-A2 molecules (online supplemental figure 5B). Taken together, these results demonstrate that DKK1-A2 mAbs are therapeutic against established tumors but cause no damage to HLA-A2-expressing murine tissues and organs in vivo. Next, we examined the expression of DKK1-A2 complexes in HA-A2 normal human tissues. DKK1-A2 complexes were not detected in normal human BM, breast, lung or pancreas (figure 7I and J), while HLA-A2 was detected in normal human tissues (figure 7I). These results suggest that DKK1-A2 mAbs should have a low or no on-target toxicity against human tissues and organs.

**DISCUSSION**

In this study, we report the generation and characterization of novel DKK1-A2 mAbs that not only bind specifically...
Figure 6  In vivo therapeutic effects of Dickkopf-1-A2 mAbs depend on NK cells and are enhanced by lenalidomide. (A–C) SCID mice were xenografted subcutaneously with U266 MM cells followed by treatment with 200 µg mlgG1 or C2 mAb, C2 mAb combined with NK cell depletion by injection of 100 µg specific antibodies (NK depletion), or C2 mAb combined with complement depletion by injection of cobra venom factor (CVF; CP depletion). Tumor burdens were measured twice every week by (A) IVIS imaging system and (B) caliper. Mouse survival is shown in (C). (D–F) SCID mice were challenged subcutaneously with U266 MM cells, followed by treatment with intraperitoneal injections of 200 µg C2 mAb or mlgG1 (every 3 days for a total of six injections), lenalidomide (10 mg/kg, for 5 days and 2 days off for 3 weeks) or lenalidomide plus C2 mAb. Tumor burdens were measured twice every week by (D) IVIS imaging system and (E) caliper. Mouse survival is shown in (F). Representative results of one out of two independent experiments performed are shown. Error bars=SD. *p<0.05, compared with mlgG1 control. mAb, monoclonal antibodies; mlgG1, mouse; NK, natural killer.
Figure 7  Evaluation of in vivo toxicity and safety of DKK1-A2 mAbs. (A) DKK1 mRNA expression profile in human solid tumors compared with their normal tissues or organs based on data in the Oncomine database. (B) DKK1 mRNA expression profile in leukemias compared with normal human PBMC based on GSE13159 in the Oncomine database. (C) DKK1 mRNA expression in lymphomas compared with normal human BM plasma cells (PC) based on GSE13591 in the Oncomine database. A2-SCID mice were inoculated subcutaneously with U266 MM cells, followed by treatment with intraperitoneal injections (every 3 days for a total of six injections) of 400 µg C2 mAb or mlgG1. On day 3 after the final injection, mice were sacrificed, and tumor and normal tissues were harvested for the following examinations; (E, upper panel) H&E staining, (E, lower panel) detection of the DKK1-A2 complex by C2 mAb staining, (F) mean fluorescent intensity (MFI) showing the expression level of HLA-A2 and DKK1-A2 complex (detected by C2 mAb) on different tissues and tumors, and (G and H) detection of apoptotic cells by TUNEL assay. (I) The surface expression of HLA-A2 and DKK1-A2 complex (stained by C2 mAb) in normal human bone marrow, breast, lung and pancreas was measured by flow cytometry. (J) Normal HLA-A2 positive human breast (n=5), lung (n=5) and pancreas (n=5) were stained by IHC staining with C2 antibody. Representative results of one out of two independent experiments are shown. Error bars=SD. **p<0.01 (compared with controls). ACC, adrenocortical carcinoma; AML, acute myeloid leukemia; BALL, B-cell acute lymphoblastic leukemia; BC, breast cancer; BCALL, B-cell childhood acute lymphoblastic leukemia; BL, B-lymphocyte; BLCA, bladder urothelial carcinoma; BM, bone marrow; BRCA, breast cancer; CC, cervical cancer; CLL, chronic lymphocyte leukemia; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; CML, chronic myelomonocytic leukemia; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; DKK1A, Dickkopf-1; ESCA, esophageal carcinoma; FHC, fibrous histiocytoma; FL, follicular lymphoma; FSA, fibrosarcoma; GBM, glioblastoma multiforme; HCCA, hepatocellular carcinoma; HD, health donor; HL, Hodgkin’s lymphoma; HNSC, head and neck squamous cell carcinoma; IHC, Immunohistochemistry; MCI, Metadata Coverage Index; MS, myelodysplastic syndrome; OV, ovarian serous cystadenocarcinoma; PBALL, pro-B acute lymphoblastic leukemia; PC, plasma cells; PCA, pancreatic carcinoma; PCL, plasma cell leukemia; PCPG, pheochromocytoma and paraganglioma; PDAC, pancreatic ductal adenocarcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; RCC, renal cell carcinoma; SARC, sarcoma; SCLC, squamous cell lung carcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TALL, T-cell acute lymphoblastic leukemia; TCL, T-cell lymphoma; TCPL, T-cell prolymphocytic leukemia; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; TT, testicular teratoma; TYST, testicular yolk sac tumor; UCEC, uterine corpus endometrial carcinoma, UCS, uterine carcinosarcoma; YST, yolk sac tumor. IHC, Immunohistochemistry; MCI, Metadata Coverage Index
to cell surface of DKK1-expressing, HLA-A2* cancer cells but also induce apoptosis in all tested DKK1*HLA-A2* human cancer cells, including established cancer cell lines and primary MM cells isolated from patients. We demonstrated that DKK1-A2 mAbs effectively lyse cancer cells via mediating ADCC and CDC activities in vitro and are therapeutic in vivo against established MM, mantle cell lymphoma, leukemia, breast cancer, and PDAC in their xenografted mouse models. More importantly, the mAbs do not cause significant toxicity against normal tissues in vitro and in vivo, indicating that DKK1-A2 mAbs may be a promising therapeutic agent for immunotherapy of human cancers.

MHC class I and II molecules may be unique targets for induction of apoptosis in cancer cells. Both murine and fully human HLA-DR-specific mAbs have been shown to inhibit the growth and induce programmed death of MHC class II-expressing tumor cells.29–31 By secondary cross-linking via plastic immobilization32 33 or using secondary antibodies,34 35 MHC class I-specific mAbs induced apoptosis in B-cell lymphoma and other cells in vitro. Our previous studies showed that b2M-specific mAbs target surface b2M/MHC class I, and binding of b2M-specific mAbs to tumor cells result in internalization and downmodulation of these molecules and induction of apoptosis of tumor cells without requiring secondary cross-linking.25 35–39 In the present study, DKK1-A2 mAbs at high concentrations could directly induce apoptosis in cancer cells in a caspase-dependent manner. We showed that DKK1-A2 mAbs bind to surface DKK1-A2 complex, cleave caspase-9, –3, –7 to –8, and PARP, upregulate Bad and Bax protein expression and induce phosphorylation of Bcl-2, and decrease phosphorylation of Bad, resulting in the release of cytochrome c into the cytosol and induction of cell apoptosis in treated cancer cells.

Therapeutic mAbs are emerging as a major new class of drugs that confer great benefits to patients with cancer. Enhancing ADCC and CDC activities is one of the most promising ways to improve the clinical efficacy of mAbs, and this concept is actively being examined in the clinic, especially in the field of hematological malignancies.40 41 In this study, we observed ADCC and CDC activities of DKK1-A2 mAbs against established cancer cell lines and primary MM cells from patients. The ADCC and CDC activities of DKK1-A2 mAbs are dependent on the level of surface expression of the DKK1-A2 complex and are more prone to tumor cells in comparison with normal B cells or PBMCs. Although in vitro CDC experiments showed that DKK1-A2 mAbs killed more cancer cells in the presence of complements, in vivo depletion of complements did not compromise the therapeutic efficacy of DKK1-A2 mAbs. The reason for this discrepancy is not obvious. It is possible, as Siao-Yi Wang, et al.2 demonstrated, that complement depletion may activate NK cells and enhance the efficacy of mAb therapy in vivo model. Thus, these findings indicate that DKK1-A2 mAbs may be a useful agent for immunotherapy of patients with cancer with low side effects.

To enhance the therapeutic efficacy of DKK1-A2 mAbs, we investigated the effect of combination treatment of DKK1-A2 mAbs with lenalidomide. Lenalidomide has been shown to modulate the activity of NK cells and macrophages in vitro and in vivo, thus providing a scientific rationale for combining it with mAb-based cancer therapies.29 42 Furthermore, there is evidence indicating that chemotherapy drugs such as thalidomide and lenalidomide upregulate DKK1 mRNA expression in MM cells.43 DKK1-A2 mAbs, with its enhanced effector cell interaction capability, are expected to have strong anti-cancer activity in combination with lenalidomide. Indeed, our results showed that there is an additive therapeutic effect between DKK1-A2 mAbs and lenalidomide in vivo, underscoring a potential clinical development strategy for combining DKK1-A2 mAb with lenalidomide to treat patients with cancer.

Novartis has a humanized anti-DKK1 mAb (BHQ880) that binds with and neutralizes DKK1 protein. This product has been under clinical investigation in cancers.45 46 Another mAb, DKN-01, produced promising results in phase II study.47–49 DKN-01 is an anti-DKK1 mAb that has demonstrated antitumor activity in patients with advanced gastroesophageal adenocarcinoma with low tumor programmed death-ligand 1 (PD-L1) expression, a subset of patients with limited therapeutic options. DKN-01 has immunomodulatory activity, stimulates a pro-inflammatory tumor microenvironment, and upregulates PD-L1 expression. However, as DKK1 is a secreted protein, DKN-01 and BHQ880 mAbs cannot bind with cancer cells and thus may not induce direct or indirect (via CDC or ADCC) killing against cancer cells. At relatively high concentrations, our DKK1-A2 mAbs were able to kill cancer cells via directly inducing apoptosis and indirectly by mediating ADCC and/or CDC activities in vitro and in vivo. However, by linking them to anti-CD3 mAb to become T-cell engagers or toxins to become antibody-drug conjugates, significantly lower doses of DKK1-A2 mAbs will be needed to effectively kill tumor cells in patients.

In this study, we confirmed in A2-SCID mice that DKK1-A2 mAbs cause no damage to HLA-A2-expressing murine tissues and organs. These results suggest that therapeutical targeting of the DKK1-A2 complex on cancer cells may be safe and efficacious, although further preclinical and clinical testing will be required to confirm the safety and efficacy of the mAbs in patients with cancer.

In conclusion, our study demonstrates that DKK1-A2 mAbs are able to induce apoptosis in human tumor cells in vitro and regress established tumors in the xenograft mouse models of human hematological and solid cancers. The mAbs can directly induce cell death via cytochrome c release and activation of the caspase-9 cascade and kill more cancer cells in vitro and in vivo by mediating ADCC, and CDC activities. Furthermore, lenalidomide potentiates DKK1-A2 mAb-induced tumor cell killing in vivo, which provides a rationale for combining these agents/drugs to improve patient outcome in hematological malignancies.
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Contributors JG and QY initiated the work, designed the experiments, and wrote the paper. JQ, QW, LX, WX, MX, MY, PS, MY, CZ, YL and LZ performed the experiments and statistical analyses. SG and YZ provided samples and critical suggestions for this study. QY is responsible for the overall content as guarantor.

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Competing interests JQ, WX, and QY are inventors on patents in the field of DKK1-A2 targeted therapies. The remaining authors declare no competing financial interests.

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