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Identification of an immunogenic DKK1 long peptide for immunotherapy of human multiple myeloma

Rong Li,[1,2,3] Chengyun Zheng,[4] Qiang Wang,[1°] Enguang Bi,[1°] Maojie Yang,[1°] Jian Hou,[5] Weijun Fu,[2] Qing Yi[1°] and Jianfei Qian[1°]

[1]Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, OH, USA; [2]The Center of Lymphoma and Multiple Myeloma, ChangZheng Hospital, The Second Military Medical University, Shanghai, P. R. China; [3]Navy Medical Center of PLA, Shanghai, P. R. China; [4]Department of Hematology, Second Hospital of Shandong University, Jinan, P. R. China and [5]Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China

°Current address: Center for Translational Research in Hematologic Malignancies, Houston Methodist Cancer Center/Houston Methodist Research Institute, Houston, TX, USA

ABSTRACT

Dickkopf-1 (DKK1), broadly expressed by tumor cells from human multiple myeloma (MM) and other cancers but absent from most normal tissues, may be an ideal target for immunotherapy. Our previous studies have shown that DKK1 (peptide)-specific cytotoxic T lymphocytes can effectively lyse primary MM cells *in vitro*. To develop DKK1-based vaccines that can be easily and inexpensively made and used by all patients, we identified a DKK1 long peptide (LP), $DKK1_{3-76}$-LP, that contains 74 amino acids and epitopes that can potentially bind to all major MHC class I and II molecules. Using HLA-A*0201- and HLA-DR*4-transgenic mouse models, we found that DKK1-specific $CD4^+$ and $CD8^+$ T-cell responses, detected by DKK1 short peptide (P20 and P66v)-HLA-A*0201 tetramer staining and cytotoxic assay for $CD8^+$ T cells or by carboxyfluorescein diacetate succinimidyl ester (CSFE) dilution and IFN-γ secretion for $CD4^+$ T cells, respectively, can be induced *in vivo* by immunizing mice with the $DKK1_{3-76}$-LP. In addition, $DKK1_{3-76}$-LP also induced anti-DKK1 humoral immunity in the transgenic mice and the DKK1 antibodies were functional. Finally, $DKK1_{3-76}$-LP stimulated human blood T cells *ex vivo* to generate DKK1-specific $CD4^+$ and $CD8^+$ T-cell responses from 8 out of 10 MM patients with different MHC backgrounds. The generated DKK1-specific $CD8^+$ cells efficiently lysed autologous MM cells from these patients. Thus, these results confirm the immunogenicity of the $DKK1_{3-76}$-LP in eliciting DKK1-specific $CD4^+$ and $CD8^+$ T-cell responses *in vitro* and *in vivo*, and suggest that the $DKK1_{3-76}$-LP can be used for immunotherapy of MM and other cancers.

Correspondence:

RONG LI
lirong785@hotmail.com

CHENGYUN ZHENG
zhengchengyun186@126.com

JIANFEI QIAN
jqian2@houstonmethodist.org

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Introduction

Dickkopf-1 (DKK1) is highly expressed in tumor cells of multiple myeloma (MM) and other cancer types,[1,2] but is absent from normal tissues and organs, with the exception of the placenta and prostate.[3,4] Our previous studies have shown that DKK1 (peptide)-specific cytotoxic T lymphocytes (CTL) can effectively lyse primary myeloma cells *in vitro*, confirming that DKK1 may be a good tumor-associated antigen. We explored the efficacy of a murine DKK1 DNA vaccine in the murine MOPC-21 myeloma model and showed that active vaccination using the DKK1 vaccine was not only able to protect mice from developing myeloma, but was also therapeutic against established myeloma. Mechanistic studies revealed that the DKK1 vaccine elicited strong DKK1- and tumor-specific $CD4^+$ and $CD8^+$ immune responses.[5] Thus, our studies provide a strong rationale for targeting DKK1 for immunotherapy in myeloma patients.

There has been substantial progress in the clinical use of long peptide (LP) therapeutic vaccination in recent years.[6,7] Melief *et al.* reported an LP vaccine encompassing

a CTL epitope and possessing immunotherapeutic potential. Disis *et al.* reported that vaccination with a CTL-epitope LP derived from human epidermal growth factor receptor 2 (EGFR2, better known as HER2) generated robust and persistent tumor-specific T-cell immunity in patients with metastatic breast cancer.[8] Recent clinical studies using a telomerase-derived LP encompassing CTL-epitopes (GV1001) showed an increase in survival of cancer patients when given in combination with radio- and chemotherapy.[9] The success of LP therapeutic vaccines can be attributed to the fact that it could induce close collaboration between cells of the innate immune system, in particular antigen-presenting dendritic cells (DC) and cells of the adaptive immune system, especially $CD4^+$ T-helper (Th) cells and $CD8^+$ CTL.[10] LP must be taken up and processed by antigen-presenting cells (APC) before they are presented. Professional APC, such as DC, can manage pools of LP and are capable of properly excising multiple HLA class I and II peptide epitopes for presentation at the cell surface.[11-13] Therefore, injection of LP will ensure the induction of both $CD4^+$ and $CD8^+$ T cells to available epitopes, each of which can contribute to the anti-tumor response. $CD8^+$ CTL exert key cytotoxicity to tumor cells. $CD4^+$ T cells are necessary elements of cellular immunity for priming tumor-specific CTL and influencing the differentiation and expansion of tumor antigen-specific CTL. Thus, an ideal peptide vaccine for cancer immunotherapy may be optimally composed of a single LP spanning epitopes for both Th cells and CTL.

In this study, we identified a human DKK1-derived LP ($DKK1_{3-76}$-LP) and explored its potential as a vaccine to induce human DKK1-specific $CD4^+$ Th and $CD8^+$ CTL responses. We found that $DKK1_{3-76}$-LP successfully induced Th1-cell responses in individuals expressing several common HLA allelic variants, including *HLA-A, HLA-B, HLA-C, HLA-DR* alleles, and that an efficient cross-presentation of the $DKK1_{3-76}$-LP also induced DKK1-specific CTL response.

Methods

Patients and samples

Peripheral blood samples from patients with MM and healthy donors were used. This study was approved by the Institutional Review Board of the Cleveland Clinic, and informed consent was obtained in accordance with the Declaration of Helsinki.

Selection of HLA class I- and class II-binding peptides

To predict possible promiscuous HLA class I and II-binding peptides on human DKK1, the amino acid sequence of the human DKK1 protein was analyzed by Immune Epitope Database (IEBD) recommended methods.[14] The program identified a 74 amino acid LP, $DKK1_{3-76}$, that contains multiple peptide motifs (Figure 1) with high affinity for common and major MHC class I and class II molecules, representing 95% of humans.[14]

All peptides, including long and short MHC class I and class II binding peptides, were synthesized by Biosynthesis (Lewisville, TX, USA). The purity of synthetic peptides, confirmed by reversed-phase high-performance liquid chromatography and mass spectrometry, was over 98%. Synthetic peptides were dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA), and stored at -20°C until use.

Generation of dendritic cells

Monocyte-derived mature DC were generated from human peripheral blood mononuclear cells (PBMC).[11,15] The quality of DC was judged based on their expression of CD11c, CD40, CD80, CD86, and MHC class II molecules.[16] Detailed information is provided in the *Online Supplementary Appendix*.

Determination of *in vivo* immunogenicity of DKK1 peptides

HLA-A*0201-transgenic (Tg[HLA-A2.1])[17] and HLA-DR*4-transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).[18] Mice were maintained at the animal facility and studies were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

For immunization, peptides were diluted in phosphate buffered saline at room temperature, mixed, and emulsified with an equal volume of incomplete Freund's adjuvant (Sigma). Groups of three mice were immunized subcutaneously at the tail base with 100 L of emulsion containing 100 g of peptides. All of the mice were immunized at least three times. Two weeks after the immunization, mice were killed and splenocytes were isolated for *in vitro* studies. The same experiments were repeated three times.

Generation of DKK1-specific $CD4^+$ and $CD8^+$ T-cell responses

DKK1-specific T cells were generated from PBMC of HLA-$A*0201^+$ and HLA-$DR*4^+$ blood donors and patients with MM by repeated stimulations of autologous T cells with DKK1 peptide-loaded mature DC. Further details are available in the *Online Supplementary Appendix*.

Cytotoxicity assay

The standard 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit was used to measure the cytolytic activity of T cells on target cells. Further details are available in the *Online Supplementary Appendix*.

Assessment of DKK1-specific T-cell responses

The frequency of peptide-specific, IFN-γ-secreting $CD4^+$ T cells was analyzed using $3x10^4$ bulk $CD4^+$ T cells stimulated with equal numbers of peptide-pulsed autologous PBMC or, alternatively, $5x10^4$ bulk $CD4^+$ T cells stimulated with $1x10^4$ peptide-pulsed DC expressing HLA-DR or -DP molecules. Further details are available in the *Online Supplementary Appendix*.

Statistical analyses

Statistical analysis was performed with Student *t*-test. $P<0.05$ was considered statistically significant. Results are presented as mean ± standard deviation unless otherwise indicated.

Results

Identification of long peptide containing multiple T-cell epitopes on Dickkopf-1 protein

To identify LP comprising the most potential MHC class I and II binding epitopes on human DKK1 protein, we examined the amino acid sequence of DKK1 using the following websites: *http://www.bimas.cit.nih.gov/molbio/hla bind/, http://www.imtech.res.in/raghava/propred/, www.immuneepitope.org* to predict the epitopes. We focused on regions with multiple MHC class I and class II epitope binding prospects. As a result, we identified an LP, $DKK1_{3-76}$, that contains 74 amino acids and multiple epitopes that can potentially bind with all major MHC class I (e.g., HLA-A, B, or C) and class II molecules (e.g., HLA-

Table 1. Potential Dickkopf-1 peptides for different MHC molecules.

MHC class I HLA	Name	Sequence	Position	Predictive binding[a,b,c]
A0201	P3	ALGAAGATRV	3	70
	Py3	YLGAAGATRV	3	320
	Py11v	RVFVAMVAA	11	238
	P20	ALGGHPLLGV	20	160
	Py20	YLGGHPLLGV	20	736
	Py25	YLLGVSATL	25	364
	Py32v	YLNSVLNSNV	32	320
	P36	VLNSNAIKNL	36	84
	P36v	VLNSNAIKNV	36	226
	P66v	ILYPGGNKV	66	378
A1	Py35v	YVLNSNAIV	35	42.5
A0205	Py11v	YVFVAMVAV	11	72
	P13	FVAMVAAAL	13	42
A24	P12	VFVAmVAAAL	12	42
B62	P66	ILYPGGNKY	66	124.8
B7	P24	HPLLgVSATL	24	80
	P59	AVSAAPGIL	59	60
B5101	P68	YPGGnKYQTI	68	692
B5102	P68	YPGGnKYQTI	68	1064.8
B5103	P5	GAAGaTRVFV	5	121
	P51	GAAGhPGSAV	51	110
B5201	P4	LGAAgATRVF	4	74.3
CW0401	P12	VFVAmVAAAL	12	240
	P24	HPLLgVSATL	24	96
MHC class II				
DRB1*0101	P10	TRVFVAMVAAALGGH	10	33
DRB1*0301	P26	LLGVSATLNSVLNSN	26	25
DRB1*0401	P30	SATLNSVLNSNAIKN	30	26
DRB1*0701	P26	LLGVSATLNSVLNSN	26	30
DRB1*1501	P7	AGATRVFVAMVAAAL	7	24

[a]http://www.bimas.cit.nih.gov/molbio/hla_bind. [b]http://www.imtech.res.in/raghava/propred/. [c]http://www.immuneepitope.org.

DR1, -DR4, or -DR7) (Table 1 and Figure 1). $DKK1_{3-76}$-LP contains our previously identified HLA-A*0201-restricted T-cell epitopes DKK1-P20 and DKK1-P66v.[19]

In vivo immunogenicity of the Dickkopf-1_{3-76}-long peptide in activating Dickkopf-1-specific $CD8^+$ cytotoxic T lymphocytes

To assess the immunity of the $DKK1_{3-76}$-LP in inducing $CD8^+$ CTL response *in vivo*, we used HLA-A*0201-transgenic mice and immunized them four times with either $DKK1_{3-76}$-LP or DKK1-P20 short peptide. The capacity of $DKK1_{3-76}$-LP to prime DKK1-specific CTL response was examined using HLA-A*0201-P20- (Figure 2A) or HLA-A*0201-P66v tetramer staining (Figure 2B). The results clearly showed that mice immunized with DKK1-P20 short peptide (Figure 2A and B, top panels) had increased percentages of DKK1-P20 (P<0.01, compared with control), but not DKK1-P66v, tetramer$^+$ $CD8^+$ T cells in the spleen after each round of immunization, whereas mice immunized with $DKK1_{3-76}$-LP (Figure 2A and B, bottom panels) generated $CD8^+$ T-cell response against both DKK1-P20 and DKK1-P66v (P<0.01, compared with control). Moreover, $CD8^+$ T cells isolated from mice immunized with $DKK1_{3-76}$-LP were not only able to kill syngeneic DC pulsed with $DKK1_{3-76}$-LP, but also DC pulsed with DKK1-P20 short peptide (P<0.01, compared with controls) (Figure 2C). Furthermore, these $CD8^+$ T cells also killed HLA-A*0201+ U266, but not HLA-A*0201 ARP-1 myeloma cells or K562 cells (to exclude natural killer [NK]-cell activity) (P<0.01, compared with controls) (Figure 2D). Hence, these results demonstrate that DC, after uptake of $DKK1_{3-76}$-LP, efficiently cross-present T-cell epitopes on the $DKK1_{3-76}$-LP and activate CTL specific for various T-cell epitopes, including DKK1-P20- and DKK1-P66v, *in vivo*.

In vivo immunogenicity of the Dickkopf-1_{3-76}-long peptide in activating Dickkopf-1-specific $CD4^+$ T-helper cells and antibody production

Next, we assessed whether $DKK1_{3-76}$-LP could also elicit DKK1-specific $CD4^+$ Th cell response. HLA-DR*4-transgenic mice were available commercially and immunized

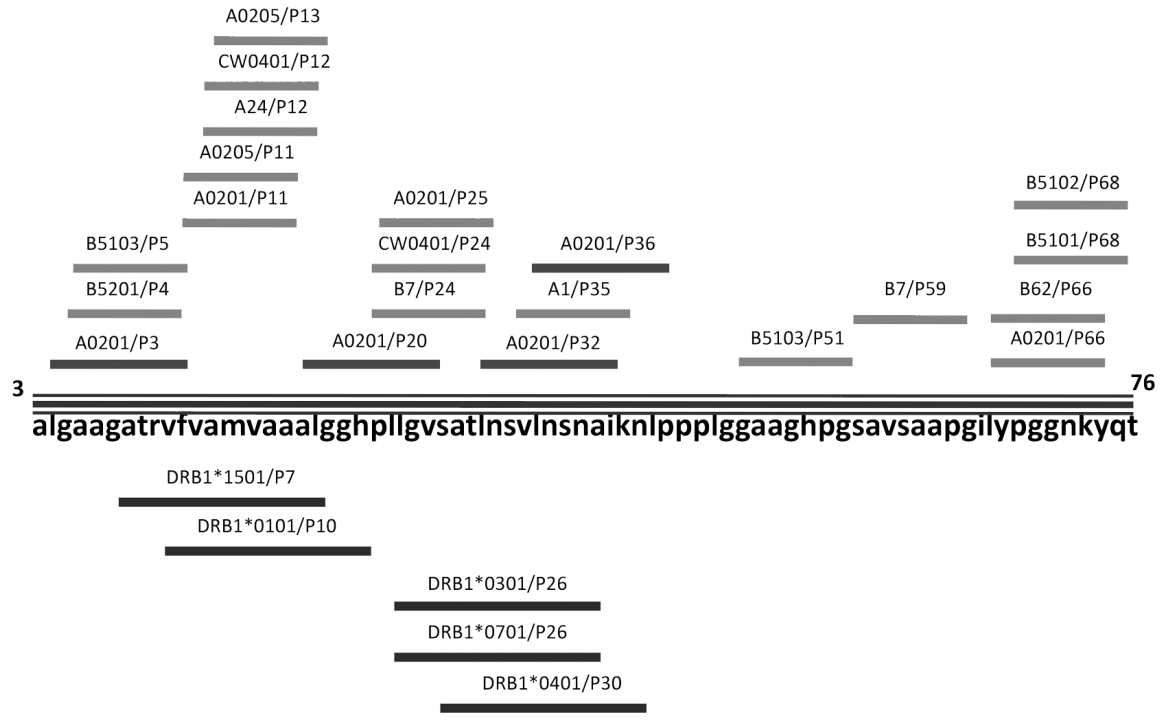

Figure 1. Dickkopf-1 (DKK1)$_{3-76}$-long peptide (LP). Schematic presentation of DKK1$_{3-76}$-LP and epitopes for MHC class I and class II molecules.

four times with DKK1$_{3-76}$-LP or a HLA-DR*4-restricted and -binding DKK1-P30 short peptide (Table 1). CD4$^+$ T-cell response was detected by CFSE dilution and IFN-γ secretion. The results clearly showed that mice immunized with either DKK1$_{3-76}$-LP or DKK1 P30 short peptide had significantly higher percentages of proliferating CD4$^+$ T cells in the spleen after *ex vivo* re-stimulation with DC pulsed, but not unpulsed, with DKK1$_{3-76}$-LP or DKK1-P30 short peptide (P<0.01, compared with non-immunized mice) (Figure 3A). Moreover, splenocytes isolated from DKK1$_{3-76}$-LP - or DKK1 P30-immunized mice contained significantly more CD4$^+$ IFN-γ-expressing cells after *ex vivo* re-stimulation with DKK1$_{3-76}$-LP - or DKK1 P30-, respectively, pulsed, but not unpulsed, DC than non-immunized mice (P<0.01, compared with those from non-immunized mice) (Figure 3B). Interestingly, the percentages of Foxp3$^+$CD4$^+$ T cells were similarly low in mice with or without peptide immunization (Figure 3B), indicating that DKK1$_{3-76}$-LP vaccination induced DKK1-specific CD4$^+$ T-cell responses without promoting regulatory T-cell (Treg) formation *in vivo*. Taken together, these results demonstrate that the DKK1$_{3-76}$-LP is immunogenic *in vivo* to induce DKK1-specific CD4$^+$ and CD8$^+$ T-cell responses.

We also investigated whether the DKK1$_{3-76}$-LP could induce a DKK1-specific humoral immune response and examined whether there were DKK1-specific antibodies in the sera of DKK1$_{3-76}$-LP-immunized HLA-A*0201- or HLA-DR*4-transgenic mice. ELISA results showed that high titers of DKK1-specific IgG antibodies were detected in HLA-A*0201-transgenic mice immunized with DKK1$_{3-76}$-LP, but not with DKK1 P20 short peptide, and the titers of the antibodies increased after each cycle of immunization (P<0.01, compared with DMSO control) (Figure 4A). Similarly, DKK1-specific antibodies were also detected in the sera of HLA-DR*4-transgenic mice immunized with DKK1$_{3-76}$-LP but not the DKK1-P30 short peptide (P<0.01, compared with DMSO control) (Figure 4B).

Next, we determined whether the detected DKK1-specific antibodies in DKK1$_{3-76}$-LP-immunized mice were biologically functional. As DKK1 was shown to inhibit human osteoblast differentiation,[20] we used an *in vitro* osteoblast culture system to determine the function of the immunized sera on osteoblast formation in the presence of recombinant human DKK1. The results showed that commercially obtained DKK1-specific antibodies and sera from DKK1$_{3-76}$-LP-immunized transgenic mice abolished DKK1-induced inhibition of human osteoblast differentiation (Figure 4C). These results indicate that the DKK1$_{3-76}$-LP encompasses naturally processed B-cell epitopes, and antibodies generated by DKK1$_{3-76}$-LP immunization are able to neutralize DKK1.

Immunogenicity of the Dickkopf-1$_{3-76}$-long peptide in priming human Dickkopf-1-specific T cells *ex vivo*

Next, we investigated whether DKK1$_{3-76}$-LP was able to induce human DKK1-specific T-cell responses *ex vivo*. Freshly prepared PBMC from healthy donors or myeloma patients were stimulated with DKK1$_{3-76}$-LP every week. The presence and frequency of DKK1-specific T cells were detected by flow cytometry. Figure 5A shows an increased frequency of DKK1-specific, IFN-γ-secreting CD4$^+$ and CD8$^+$ cells in cultured T cells during *in vitro* (re)-stimulation with the LP. Figure 5B shows the percentages of HLA-A*0201-DKK1-P20 tetramer$^+$ CD8$^+$ T cells in cultures after repeated stimulations with DKK1$_{3-76}$-LP. By using monoclonal antibodies (mAb) specific to HLA-DR or -DQ or HLA-ABC added to T-cell cultures before assay, we showed that MHC class II-restricted CD4$^+$ T cells were

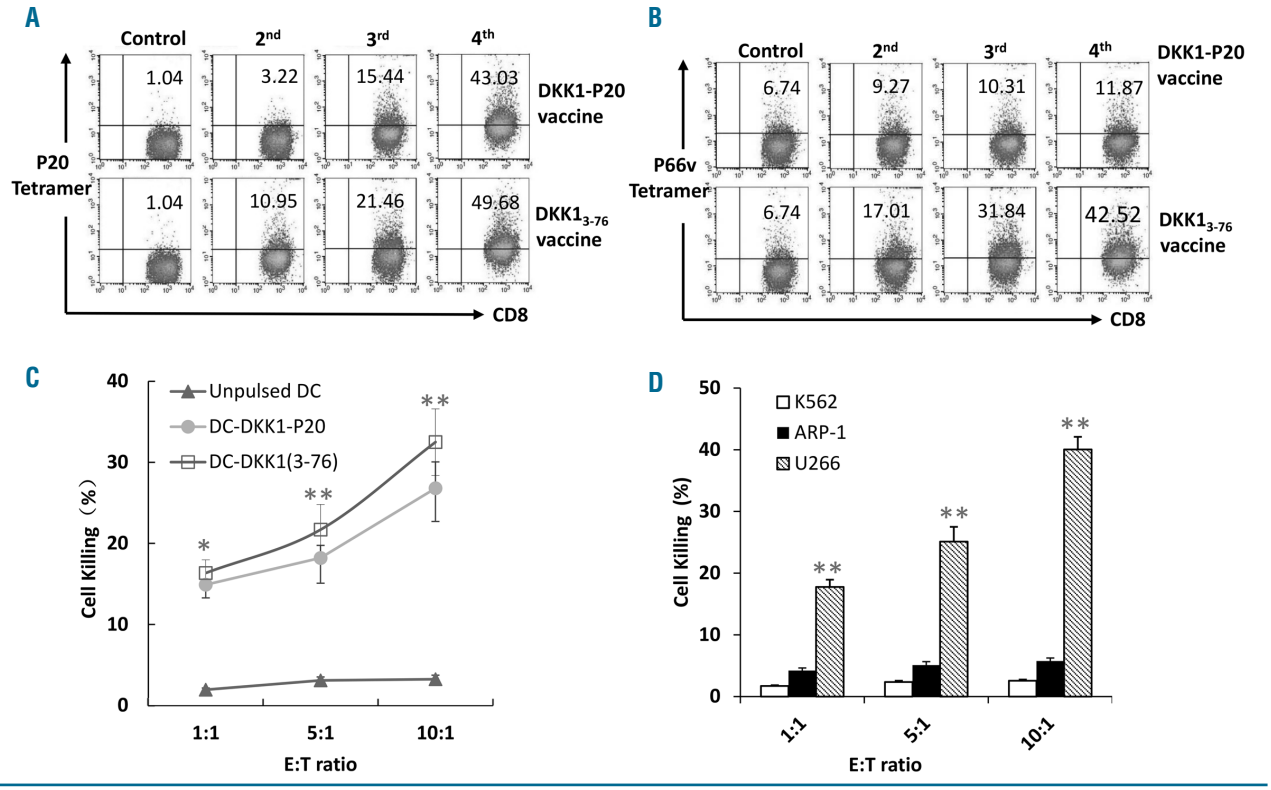

Figure 2. Cross-presentation of Dickkopf-1 (DKK1)$_{3-76}$-long peptide (LP) efficiently primes DKK1-specific CD8$^+$ T cells *in vivo*. Shown are CD8$^+$ T-cell responses induced in HLA-A*0201-transgenic mice after immunization with DKK1$_{3-76}$-LP or HLA-A*0201-restricted P20 short peptide. (A) DKK1-P20-specific tetramer staining showing the frequency of DKK1 P20-specific CD8$^+$ T cells in the spleen of a HLA-A*0201-transgenic mouse. Representative results from one of three mice are shown. (B) DKK1-P66v-specific tetramer staining showing the frequency of DKK1 P66v-specific CD8$^+$ T cells in the spleen of an HLA-A*0201-transgenic mouse. These results indicate that DKK1$_{3-76}$-LP was cross-presented and efficiently primed DKK1-P66v-specific CD8$^+$ T cells in HLA-A*0201-transgenic mice. Representative results from one of three mice are shown. (C) Cytolytic activity of CD8$^+$ T cells isolated from mice immunized with DKK1$_{3-76}$ LP against unpulsed dendritic cells (DC) or DC pulsed with DKK1$_{3-76}$ LP or P20 short peptide. Representative results of three experiments are shown. (D) Cytolytic activity of CD8$^+$ T cells isolated from mice immunized with DKK1$_{3-76}$ LP against U266 and ARP-1 human myeloma cell lines, and K562. Representative results of three experiments are shown. *P<0.05; **P<0.01.

the main IFN-γ-secreting cells, because anti-HLA-DR mAb significantly reduced the percentage of IFN-γ-secreting CD4$^+$ cells (Figure 5C). Moreover, HLA-A2 antibody significantly reduced the percentages of IFN-γ-secreting CD8$^+$ T cells in cultures after repeated stimulations with DKK1$_{3-76}$-LP. The results showed that DKK1$_{3-76}$-LP-specific CD8$^+$ T-cell responses were HLA*0201-restricted (Figure 5D). Taken together, the results demonstrate that human DKK1-specific CTL and Th1 cells can be induced by DKK1$_{3-76}$-LP *ex vivo*.

Finally, we determined whether the DKK1$_{3-76}$-LP could induce DKK1-specific HLA-A*0201-restricted and HLA-DR*4-restricted T-cell responses from MM patients. After 3-4 weeks of stimulating patient-derived PBMC with DKK1$_{3-76}$-LP *in vitro*, the frequency of DKK1-specifc CD8$^+$ CTL and CD4$^+$ Th1 cells was detected by intracellular IFN-γ staining. Figure 6A is a representative flow cytometry analysis showing the percentages of IFN-γ-secreting CD8$^+$ and CD4$^+$ T cells after re-stimulation with DKK1$_{3-76}$-LP from a MM patient (MM1). Low percentages of IFN-γ-secreting CD8$^+$ and CD4$^+$ T cells were observed in T-cell cultures re-stimulated with unpulsed DC or DC pulsed with DKK1-P20 short peptide. Figure 6B shows the percentages of IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells from a total of ten patients with MM (with different MHC backgrounds) after a 4-week *in vitro* stimulation of blood T cells with DKK1$_{3-76}$-LP-pulsed autologous DC. Furthermore, we generated CD8$^+$ T-cell lines from HLA-A*0201$^+$ patients by *in vitro* repeated stimulations with DKK1$_{3-76}$-LP and these DKK1-specific T cells killed autologous patient (MM1) myeloma cells, primary myeloma cells from another HLA-A*0201$^+$ patient (MM2), and HLA-A*0201$^+$ myeloma cell line U266. No killing was observed on HLA-A*0201$^-$ primary myeloma cells (MM3) or myeloma cell line ARP-1, normal B cells, or K562 cells (to exclude NK-cell activity) (Figure 6C).

Discussion

In this study, we identified a 74aa DKK1$_{3-76}$-LP thast contains multiple epitopes for CD4$^+$ and CD8$^+$ T cells and explored the potential of using this LP for immunotherapy of human MM. We showed that DKK1-specific CTL, detected by DKK1 short peptide (P20 and P66v)-HLA-A*0201 tetramer staining, and DKK1-specific Th cells, detected by IFN-γ secretion and CSFE-dilution assay, can be induced by (cross)-presentation of DKK1$_{3-76}$-LP *in vitro* and *in vivo*. We also verified the presence of DKK1-specific Th1 responses in MM patients.

Recent studies evaluating the CTL repertoire of HPV-16 e6 and e7 oncogenic protein showed complete and lasting regression of end-stage cervical cancer patients after melanoma antigen A3 (MAGE-A3) vaccination with peptide[21,22,29-31] After immunization, a new wave of antigen-specific CTL clones arose in the peripheral blood, provid-

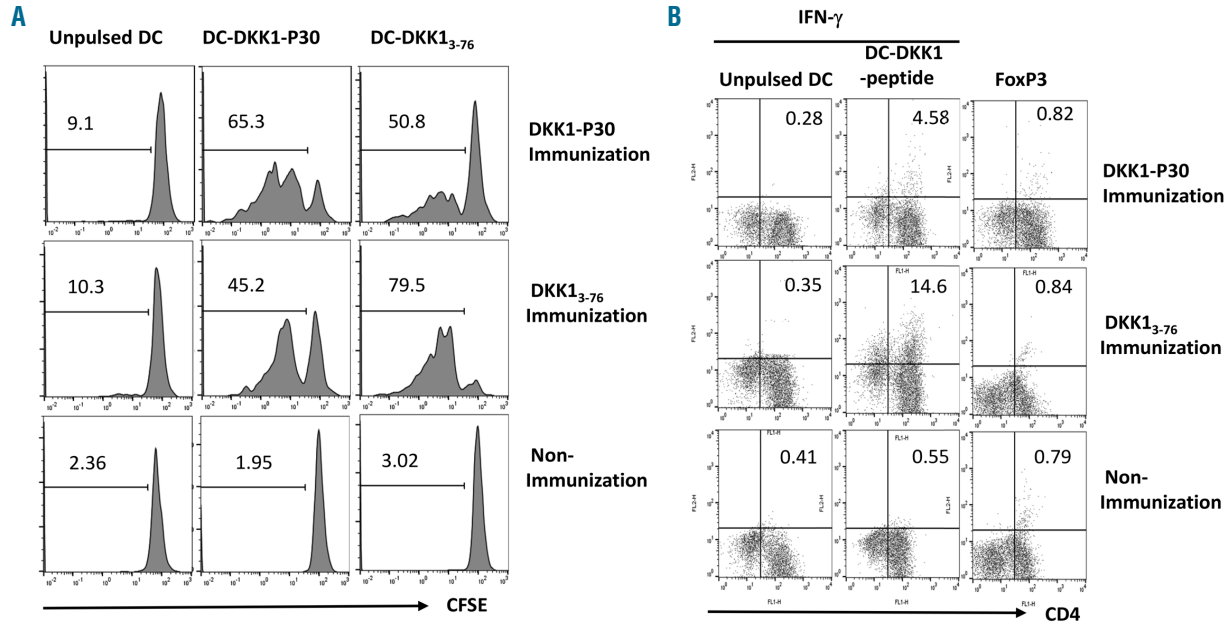

Figure 3. Dickkopf-1 (DKK1)$_{3-76}$-long peptide (LP) efficiently induces DKK1-specific CD4$^+$ T-cell response *in vivo*. Shown are CD4$^+$ T-cell responses induced in HLA-DR*4-transgenic mice after immunization with DKK1$_{3-76}$-LP or HLA-DR-restricted P30 short peptide. (A) Carboxyfluorescein succinimidyl ester (CFSE) dilution assay showing the percentages of proliferating CD4$^+$ T cells from the spleen of mice after *ex vivo* re-stimulation with dendritic cells (DC) pulsed, but not unpulsed, with DKK1$_{3-76}$ LP or DKK1-P30 short peptide. Representative results from one of three independent experiments are shown. (B) Flow cytometry analysis showing the percentages of IFN-γ-expressing or FoxP3$^+$ CD4$^+$ T cells from the spleen of mice after *ex vivo* re-stimulation with DC pulsed, but not unpulsed, with DKK1$_{3-76}$ LP or DKK1-P30 short peptide. Representative results from one of four experiments are shown.

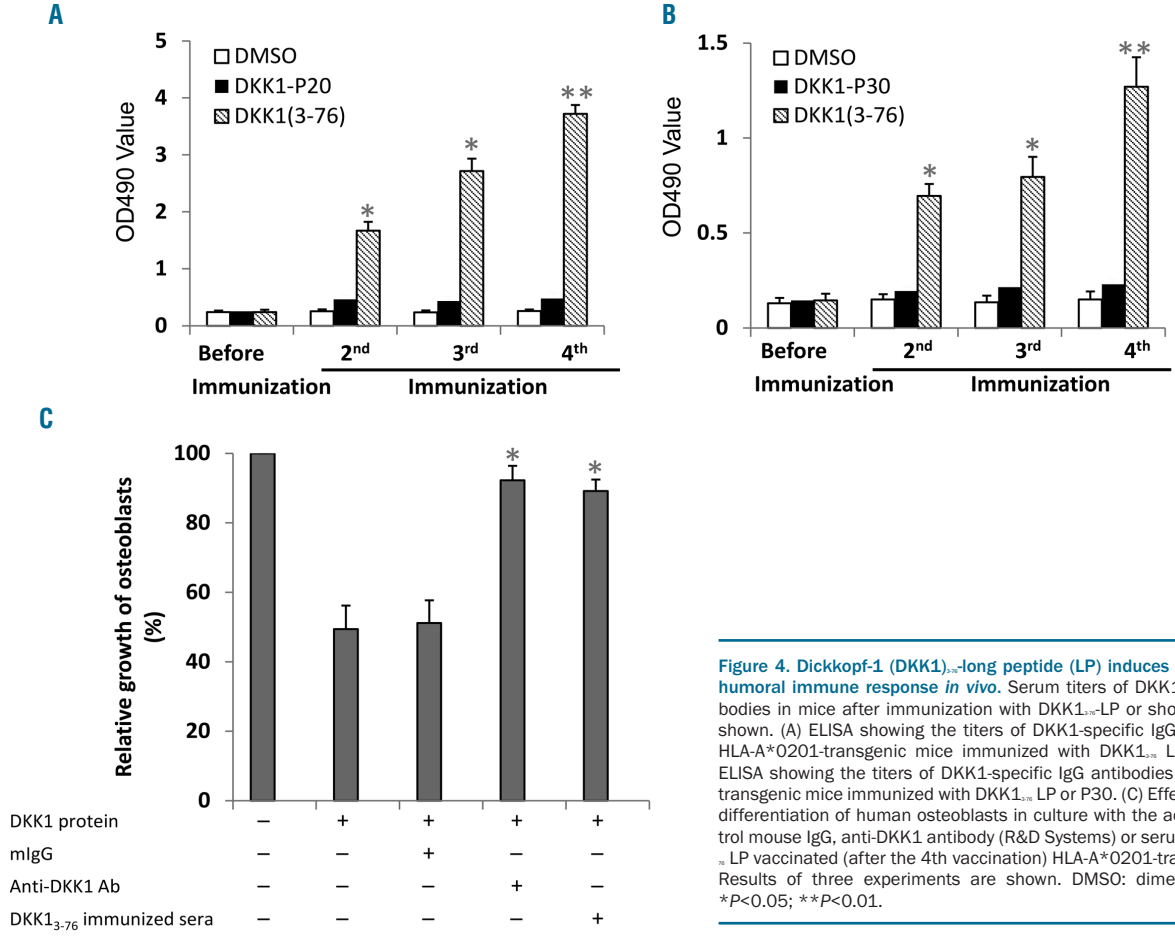

Figure 4. Dickkopf-1 (DKK1)$_{3-76}$-long peptide (LP) induces DKK1-specific humoral immune response *in vivo*. Serum titers of DKK1-specific antibodies in mice after immunization with DKK1$_{3-76}$-LP or short peptide are shown. (A) ELISA showing the titers of DKK1-specific IgG antibodies in HLA-A*0201-transgenic mice immunized with DKK1$_{3-76}$ LP or P20. (B) ELISA showing the titers of DKK1-specific IgG antibodies in HLA-DR*4-transgenic mice immunized with DKK1$_{3-76}$ LP or P30. (C) Effect of DKK1 on differentiation of human osteoblasts in culture with the addition of control mouse IgG, anti-DKK1 antibody (R&D Systems) or serum from DKK1$_{3-76}$ LP vaccinated (after the 4th vaccination) HLA-A*0201-transgenic mice. Results of three experiments are shown. DMSO: dimethyl sulfoxide. *P<0.05; **P<0.01.

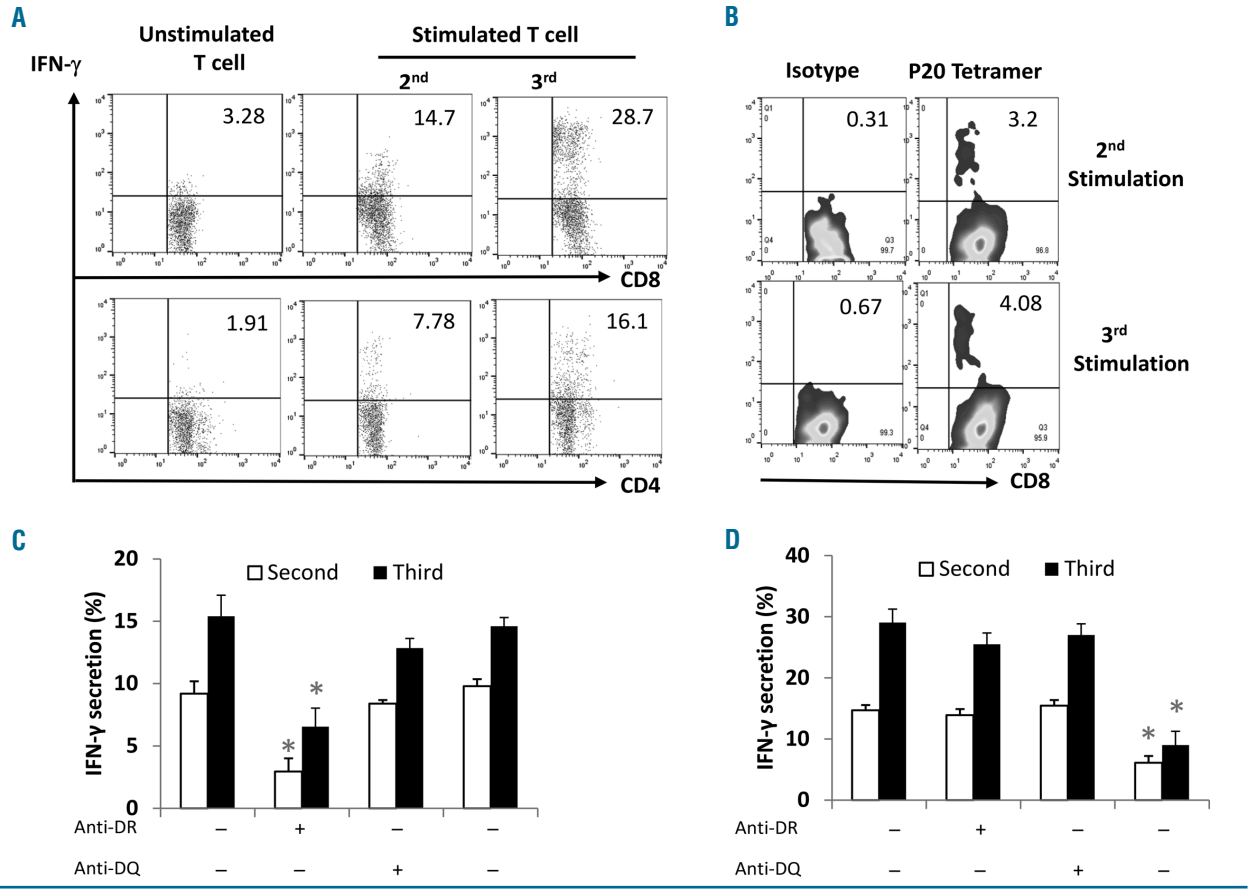

Figure 5. Dickkopf-1 ($DKK1_{3-76}$)-long peptide (LP) induces DKK1-specific human T cells *in vitro*. Fresh peripheral blood mononuclear cells (PBMC) derived from healthy donors (HLA-A0201$^+$ or HLA-DR*4$^+$) were stimulated with $DKK1_{3-76}$-LP plus IL-2 and IL-7 weekly *in vitro*. (A) Frequency of DKK1-specific CD4$^+$ and CD8$^+$ IFN-γ-secreting cells detected by intracellular staining assay. (B) Percentages of HLA-A*0201-DKK1-P20 tetramer$^+$CD8$^+$ T cells in culture after second or third *in vitro* stimulation with $DKK1_{3-76}$-LP. (C) MHC class II-restriction of $DKK1_{3-76}$-LP-specific CD4$^+$ T-cell response. PBMC stimulated with $DKK1_{3-76}$-LP for 1 week were re-stimulated with $DKK1_{3-76}$-LP in the presence of different monoclonal antibodies (mAb) specific for HLA-DR, -DQ or HLA-ABC. The results showed that HLA-DR antibody significantly reduced the percentages of IFN-γ-secreting CD4$^+$ T cells. (D) HLA-A*0201-restriction of $DKK1_{3-76}$-LP-specific CD8$^+$ T-cell response. PBMC stimulated with $DKK1_{3-76}$-LP for 1 week were re-stimulated with $DKK1_{3-76}$-LP in the presence of different mAb specific for HLA-DR, -DQ or HLA-A2. The results showed that HLA-A*0201 antibody significantly reduced the percentages of IFN-γ-secreting CD8$^+$ T cells. Representative results of three experiments are shown. *P<0.05; **P<0.01.

ing solid evidence that the phenomenon of epitope spreading is critical to the development of effective anti-cancer immunity elicited by peptide vaccination. These results further implicate functional interactions between vaccine-induced CTL and malignant cells that facilitate the induction of large numbers of tumor-specific CTL, the cytolytic effector immune cells that subsequently destroy tumor cells.

Disis *et al*. reported that vaccination with a herceptin-2 (HER-2/neu)-derived LP encompassing an HLA-A*0201-restricted CTL epitope elicited embedded CTL-epitope specific CD8$^+$ T cells in cancer patients.[8] They showed that tumor-specific CTL can be elicited *in vivo* via cross-presentation of HER-2/neu-derived LP. Such T-cell responses are considered to be crucial for tumor eradication and for generating long-term memory.[23] With this premise in mind, we identified an immunogenic $DKK1_{3-76}$-LP that encompasses both Th epitopes and CTL-epitopes and demonstrated that cross-presentation of $DKK1_{3-76}$-LP induced priming and expansion of DKK1-specific CTL *in vitro* and *in vivo*. Vaccination with $DKK1_{3-76}$-LP can potentially elicit combined Th and CTL responses. A recent clinical trial showed that targeting Th cells with DC pulsed with both HLA class I and II-restricted epitopes effectively enhanced vaccine-specific immune responses and improved clinical outcome.[24] $DKK1_{3-76}$-LP bolstered the induction of DKK1-P20-specific CTL derived from both healthy donors and MM patients *in vitro*. Thus, $DKK1_{3-76}$-LP administered in combination with DKK1-P20 immunotherapy may be able to augment the elicitation of antigen-specific CTL.

Based on the HLA-subtypes capable of antigen presentation from studies using healthy donors, DKK1-P20 and $DKK1_{3-76}$-LP are predicted to be useful in approximately 80% of the total population. We showed that $DKK1_{3-76}$-LP induced HLA-DP5-, HLA-DR8-, or HLA-DR15-restricted Th cells in healthy donors and also induced HLA-DR- or HLA-DQ-restricted Th cells in MM patients. However, these MM patients were negative for HLA-DP5, -DR8, or -DR15 alleles. We also showed that $DKK1_{3-76}$-LP induced HLA-DR15- or HLA-DQ-restricted Th cells in healthy donors. These results suggest that $DKK1_{3-76}$-LP may encompass Th cell epitopes not previously identified in experiments involving cells derived from healthy donors and $DKK1_{3-76}$-LP may be broadly useful in the majority of MM patients.

Weide *et al*. reported that the presence of circulating Th cells responding to melanoma antigens Melan-A or NY-ESO-1 has a strong independent prognostic impact on survival among chemotherapy-treated advanced melanoma patients.[25] Another study has shown a possible

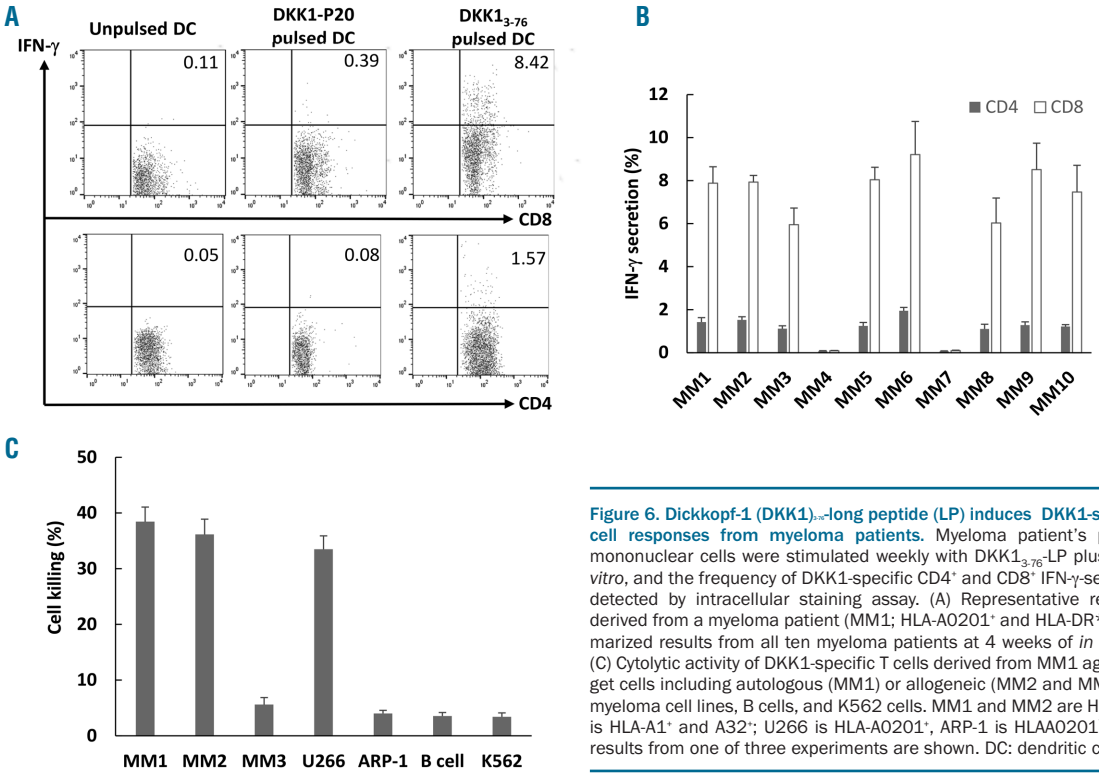

Figure 6. Dickkopf-1 (DKK1)$_{3-76}$-long peptide (LP) induces DKK1-specific human T-cell responses from myeloma patients. Myeloma patient's peripheral blood mononuclear cells were stimulated weekly with DKK1$_{3-76}$-LP plus IL-2 and IL-7 *in vitro*, and the frequency of DKK1-specific CD4$^+$ and CD8$^+$ IFN-γ-secreting cells was detected by intracellular staining assay. (A) Representative results of T cells derived from a myeloma patient (MM1; HLA-A0201$^+$ and HLA-DR*4$^+$) and (B) summarized results from all ten myeloma patients at 4 weeks of *in vitro* stimulation. (C) Cytolytic activity of DKK1-specific T cells derived from MM1 against various target cells including autologous (MM1) or allogeneic (MM2 and MM3) plasma cells, myeloma cell lines, B cells, and K562 cells. MM1 and MM2 are HLA-A0201$^+$; MM3 is HLA-A1$^+$ and A32$^+$; U266 is HLA-A0201$^+$, ARP-1 is HLAA0201$^-$. Representative results from one of three experiments are shown. DC: dendritic cells.

synergy between the telomerase-specific Th responses with chemotherapy in lung cancer.[26] The introduction of immunotherapy in clinical practice also emphasized the influence of immune responses on cancer prognosis and chemotherapy effectiveness.[27,28] Politou *et al.*[2] and Terpos *et al.*[29,30] reported that serum concentration of DKK1 protein were increased in patients with MM and were correlated with severe bone disease. Autologous stem cell transplantation and chemotherapies with bortezomib, melphalan, dexamethasone and intermittent thalidomide significantly reduced serum DKK1 level and led to normalization of bone remodeling in relapsed myeloma. These pieces of evidence support the hypothesis that induction or augmentation of DKK1-specific Th1 cells by vaccination with DKK1$_{3-76}$-LP may improve the clinical outcome of cancer patients when combined with chemotherapy or other standard therapies.[31,32] DKK1$_{3-76}$-LP-specific Th responses in MM patients may positively influence overall survival. The impact of DKK1-specific Th responses on clinical outcome will be evaluated in future studies.

In conclusion, DKK1$_{3-76}$-LP provides a useful tool for propagation of both DKK1-specific Th1 cells and CTL, and may synergize with CTL-epitopes to enhance cancer cell killing. These findings provide a rationale for a clinical trial of DKK1$_{3-76}$-LP -based immunotherapy against a broad spectrum of cancer types, as DKK1 is widely expressed by human cancer cells.[19]

Disclosures

No conflicts of interest to disclose.

Contributions

JQ, RL and QY initiated the study. JQ, RL and CZ designed the experiments and wrote the paper; RL and JQ performed most of the experiments and statistical analyses; QW prepared human samples; EB helped with animal experiments; MY assisted in generating mice; JH and WF provided important suggestions.

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