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

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Supplementary Methods

Generation of dendritic cells

Monocyte-derived mature DCs were generated from human peripheral blood mononuclear cells (PBMCs). In brief, PBMCs were allowed to adhere in culture flasks for 2 hours, and nonadherent cells were collected and cryopreserved for future use. The adherent cells were cultured in Aim-V medium (Invitrogen, Carlsbad, CA) supplemented with granulocyte macrophage-colony-stimulating factor (GM-CSF) (10 ng/mL) and interleukin (IL-4; 10 ng/mL; both from R&D Systems, Minneapolis, MN), with further addition of cytokines every other day. After 5 days of culture, DCs were induced to maturation by addition of tumor necrosis factor-α (TNF-α; 10 ng/mL), IL-1β (10 ng/mL); IL-6 (10 ng/mL) and PEG2 (5 ng/mL, both from R&D Systems) for 48 hours.

Murine DCs were generated from bone marrow stem cells as described previously.[1] Briefly, bone marrow cells were cultured, at a density of 2×10^5 cells/mL in 6-well plates, in RPMI-1640 complete medium supplemented with 20 ng/mL GM-CSF (R&D Systems, Minneapolis, MN). At day 4, medium was replaced with fresh medium containing 10 ng/mL GM-CSF. At day 8, immature DCs were collected, pooled, and pulsed with DKK1 peptides at a concentration of 100 μg per 10^6 DCs.

TNF-α (10 ng/mL) and IL-1β (10 ng/mL) (R&D Systems) were added, and after 48 hours of culture, mature DCs were collected and used. The quality of DCs was judged based on their expression of CD11c, CD40, CD80, CD86, and MHC class II molecules.

Immunophenotyping and intracellular cytokine staining

Phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) were added to cell pellets, incubated for 30 minutes on ice, and washed 3 times before analysis. Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to the manufacturer's recommendation. Samples were analyzed using a flow cytometer (FACSCalibur; BD Biosciences).

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

DKK1-specific T cells were generated from PBMCs 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 DCs. In brief, the non-adherent cells of PBMCs (2×10^6/mL; used as T-cell population) were cocultured in 50-mL tissue-culture flasks at 37°C in 5% CO_2 for 7 to 10 days in Aim-V medium supplemented with 10% pooled human serum (T-cell medium) with mature DCs (2×10^5/mL) that were preincubated with DKK1 peptides at a final concentration of 50 μg/mL at 37°C for 48 hours. After culture, T cells were collected and restimulated with DKK1 peptide-pulsed autologous mature DCs every week, and the cultures were fed every 5

days with fresh medium containing recombinant IL-2 (20 IU/mL), IL-7 (5 ng/mL), and IL-15 (5 ng/mL; all from R&D Systems). Induction of DKK1-specific $CD8^+$ T cells was monitored weekly by DKK1 peptide-HLA-A*0201 tetramer (synthesized by MHC Tetramer Laboratory, Baylor College of Medicine, Houston, TX) staining. Induction of DKK1-specific $CD4^+$ T cells was monitored weekly using T-cell proliferation assay and intracellular IFN-γ staining.

Proliferation assays

T cells were first labeled with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 μM; Invitrogen) for 10 minutes at 37°C, washed and then seeded (5×10^4/100 μL/well) into 96-well U-bottomed tissue culture plates (Corning Glassworks, Corning, NY) in T-cell medium. Various numbers of autologous mature DCs pulsed with or without DKK1 peptides were added to the plates and cultured for 4 days at 37°C in 5% CO_2. Flow cytometry analysis was used to detect dilution of CFSE.[2] Results are expressed as mean counts per minute of triplicate cultures.

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 including autologous DCs loaded with or without peptides and MM cells (primary MM cells from patients or cell lines). Target cells were incubated with CFSE for 15 minutes, washed extensively, seeded (1×10^4 cells/well) into 96-well U-bottomed plates in T-cell

medium, and cocultured for 4 hours with various numbers of T cells. All assays were performed in triplicates.

To determine whether the cytolytic activity was restricted by MHC class I or II molecules, 20 μg/mL mAbs against HLA-ABC (W6/32) or HLA-A*0201 (BB7.2; both from Serotec Ltd., Oxford, UK), HLA-DR (B8.12.2; Immunotech, Marseilles, France), or isotypic controls (Immunotech) were added to the cultures at the start of the assay.[3]

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assay (ELISA) was used to measure the production and titer of antibodies against human DKK1. Serum derived from HLA-A*0201- and HLA-DR*4-transgenic mice before and after DKK1 peptide immunization was collected, and the titer of DKK1 antibodies was quantified according to the protocol.

Examination of the effect of anti-DKK1 antibodies on osteoblast formation

Human mesenchymal stem cells were cultured in human osteoblast growth medium (Sigma) for 7-14 days to generate human osteoblasts.[16] In some cultures, recombinant DKK1 (R&D Systems) was used to inhibit osteoblasts formation, in the presence or absence of control mouse IgG, anti-DKK1 antibody (R&D Systems), or serum from $DKK1_{3\text{-}76}$-LP vaccinated (after the 4^{th} vaccination) HLA-A*0201-transgenic mice.

Assessment of DKK1-specific T-cell responses

The frequency of peptide-specific, IFN-γ-secreting $CD4^+$ T-cells was analyzed using

3×10^4 bulk $CD4^+$ T-cells stimulated with equal numbers of peptide-pulsed autologous PBMCs, or alternatively, 1×10^4 bulk $CD4^+$ T-cells stimulated with 5×10^4 peptide-pulsed L-cells expressing HLA-DR or -DP molecules. To determine the HLA molecules involved in antigen presentation, antigen-induced IFN-γ production was blocked by adding mAbs against HLA-DR (L243, IgG2a, BioLegend), HLA-DP mAb (B7/21, IgG3, Abcam), HLA-DQ mAb (SPV-L3, IgG2a, Abcam), HLA-A2 mAb (BB7.2, IgG2b, Abcam) or the broad spectrum anti-HLA class I mAb (W6/32, IgG2a, Abcam). All mAbs were used at a final concentration of 5 μg/mL. Cells cultured with HIV-derived peptides were used as negative control. All IFN-γ assays were performed in triplicate and results are presented as mean ± SD.