Supplementary Methods

Reagents

Human and canine recombinant GM-CSF and IL-4 were purchased from R&D systems (Minneapolis, MN). IL-2 was purchased from Shionogi & Co., LTD (Osaka, Japan). Human recombinant IL-7 and IL-15 were purchased from Pepro TECH, Inc. (Rocky Hill, NJ). α–GalCer was synthesized by Dr. Ishii in RIKEN. α–GalCer and vehicle (0.4% DMSO) were diluted in PBS. LPS-free OVA was purchased from Seikagaku Corp. MART-1/MelanA:26-35(27L) analog peptide (EAAGIGILTV) was obtained from Toray Research Center, Inc. (Tokyo, Japan). The following monoclonal antibodies (mAbs) were purchased: anti-human CD1d (CD1d42), CD8 (RPA-T8), CD11c (B-ly6), CD86 (2311), human invariant NKT Cell (6B11) from BD (San Diego, CA) and anti-human Vα24 (C15) , Vβ11 (C21) from Beckman Coulter, anti-human CD3 (UCHT1) from e-Bioscience, anti-mouse CD8α (53-6.7), CD11c (HL3), CD19 (1D3), CD45.1(A20), CD86 (B7-2), IFN-γ (XMG1.2) and mouse CD1d-Dimerix from BD, and anti-dog CD3 (CA17.2A12), CD4 (YKIX302.9), CD8 (YCATE55.9) from AbD Serotec (Oxford, UK). H-2Kb/OVA tetramer-SIINFEKL-PE and HLA-A*0201 Mart-1 tetramer-ELAGIGILTV-PE were purchased from MBL (Nagoya, JAPAN). For analysis, a FACSCalibur™ instrument and CELLQuest™ (BD Biosciences**)** or FlowJo

(Tree Star, San Carlos, CA) software were used.

Murine iNKT IFN-γ assay

Splenic cells were incubated in the presence of Golgi Plug (BD Bioscience) for 12 hours with or without 100 ng/mL α-GalCer and then preincubated with an anti-CD16/32 Ab to block non-specific binding of antibodies to FcγR, washed, and incubated with CD1d-dimerix/Gal-APC and CD19-FITC. The cells were then permeabilized in Cytofix-Cytoperm Plus (BD Biosciences) and stained with anti-IFN-γ-PE.

Isolation of human PBMCs

Human PBMCs were obtained from buffy coats from healthy blood donors (Tokyo Red Cross, Tokyo, Japan) and separated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density centrifugation. PBMCs and in some cases, $CD14^+$ monocytes purified by magnetic beads separation (Miltenyi Biotec Inc.) were washed three times with PBS, and stored using serum-free freezing medium Cellbanker2 (JUJI Field Inc., Tokyo, Japan) in liquid nitrogen until use. All studies were approved by the RIKEN institutional review board.

Generation of human DCs

$CD14^+$ cells, isolated using magnetic beads (Miltenyi), were used for the generation of imDCs. Monocytes were cultured in the presence of GM-CSF (100 ng/mL) and IL-4 (25 ng/mL) for 4 days to generate imDCs.

***In vitro* generation of human iNKT cell lines**

To prepare NKT cell lines, PBMCs were pulsed with α-GalCer (100 ng/mL) in the presence of 100 U/mL IL-2. After 10–14 days, human iNKT cells were stained using FITC-labeled anti-Vα24 mAb and selected with anti-FITC magnetic beads (Miltenyi Biotec). Human iNKT cells were maintained in the presence of 100 U/mL IL-2, 5 ng/mL IL-7, and 10 ng/mL IL-15 and stimulated with mouse BM-DC pulsed with α-GalCer every other week.

Canine IFN-γ assays

ELISPOT assays for IFN-γ secreting iNKT cells were performed on 96-well filtration plates (Millipore, Bedford, MA) coated with mouse anti-canine IFN-γ capture antibody (R&D System) for 20 hours. Canine PBMCs were cultured with or without α-GalCer for 16 hours as previously described (24). Then, biotinylated goat anti-canine

IFN-γ detection antibody was added (R&D System) for 24 hours and spots were developed with streptavidin-AP (R&D System) and BCIP/NBT substrate (R&D System). The number of ligand-dependent IFN-γ spots was analyzed with the series 3B ImmunoSpot Image Analyzer (Cellular Technology, Cleveland, OH).

Generation of canine DCs and electroporation of OVA protein

Canine $CD14^+$ cells were isolated from canine peripheral blood using mouse anti-CD14 monoclonal antibodies (clone M-M9; VMRD, Inc.) and goat anti-mouse IgG microbeads (Miltenyi Biotec). To generate DCs, $CD14^+$ cells were cultured at a concentration of 1×10^6 cells/mL in a medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, and canine GM-CSF (100 ng/mL) and canine IL-4 (25 ng/mL) at 37°C for 10 days.

After washing, DCs were preincubated in OPTI-MEM containing 5 mg/mL OVA (Seikagaku corporation, Tokyo, Japan) at a concentration ~$2x10^7$ cells/mL on ice for 10 minutes and then a volume of 400 μL of cell suspension was transferred into a 4-mm cuvette. Pulse conditions were square-wave pulse, 500 V, 0.5 msec. Immediately after electroporation, the cells were transferred to culture medium and then frozen until use.

Flow cytometric CTL assay

Target cells were stained with PKH26 membrane dye (Sigma-Aldrich) and added to wells containing candidate CTLs. After 6 hours, cells were harvested, exposed to a membrane-impermeable DNA stain (TO-PRO-3 iodide, 1 μM final concentration; Invitrogen), and analyzed by flow cytometry. Target cells were gated for PKH26 red fluorescence, and lysed targets were costained with TO-PRO-3. Unlysed targets excluded this membrane-impermeable stain. Background and maximum TO-PRO-3 staining were obtained by incubation with medium and detergent-exposed targets, respectively.

Measurement of tumor size

Tumor growth was monitored by measuring three perpendicular diameters. Tumor volume was calculated according to the formula $V = L \times W^2 \times 0.52$, where V is the volume, L is the length, and W is the width.

Preparation of TCR-transduced PBLs

T cells from healthy HLA-$A2^+$ donors were transduced with a retroviral vector carrying the MART-1 DMF5 TCR gene (DMF5TCR) as previously described (38). In

brief, retroviral supernatants were prepared using the pMSGV DMF5 furin 2-A vector and plat GP provided by Dr. T. Kitamura (Tokyo University, Tokyo, Japan). PBL were stimulated *in vitro* at 10^6 cells/mL with 50 ng/mL anti-CD3 mAb OKT3 (Janssen Pharmaceutical, Inc., Tokyo, Japan) in DMEM medium (Invitrogen) supplemented with 10 % FCS-containing DMEM (Sigma-Aldrich) and 100 IU IL-2. Two days later, TCR-encoding retroviral supernatant was rapid-thawed, diluted 1:1 in 20% DMEM media, and added to plates that had been coated overnight with 25 μg/mL retronectin (Takara Bio Inc). Supernatant was spin-loaded onto plates by centrifuging 2 hours at 2000g at 32^{o}C. The stimulated PBLs were added to the wells of the retrovirus-loaded plates. Plates were spun at 1000g at 32^{o}C for 10 minutes and incubated overnight at 37^{o}C, 5% CO_2. The next day, PBLs were transferred to freshly prepared retro-viral coated 6-well plates as on day 2. The following day, transduced PBLs were washed, resuspended in fresh TCR media, and transferred to flasks at 37^{o}C, 5% CO_2 for 5 days and then were sorted.