Supporting Information

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SI Materials and Methods

Antibodies. BD Horizon V500 (V500)-conjugated anti-CD8 (RPA-T8) mAb, FITC-conjugated anti-CD45RA (HI100) mAb, allophycocyanin-cyanine7 (APC-Cy7)-conjugated anti-CD14 (MφP9) mAb, biotin-conjugated anti-CD194 (C-C chemokine receptor 4, CCR4) (1G1) mAb, phycoerythrin-cyanine7 (PE-Cy7)-conjugated anti-CD4 (SK3) mAb and purified anti-CD25 (M-A251, no azide/low endotoxin) mAb were purchased from BD Biosciences. Alexafluor 700-conjugated anti-CD3 (OKT3) mAb, PE-Cy7–conjugated anti-CD16 (eBioCB16) mAb, APC-conjugated anti-CD56 (MEM188) mAb, PE-Cy7–conjugated TNF-α (MAb11) mAb, PE-conjugated anti-FOXP3 (Forkhead Box P3, 236A/E7) mAb, PE-conjugated anti-IFN-γ (4S.B3) mAb, and eFluor 780-conjugated fixable viability dye were purchased from eBioscience. PE-conjugated CD19 (HIB19) mAb, peridinin chlorophyll protein complex-cyanine5.5 (PerCP-Cy5.5)-conjugated CD11c (3.9) mAb, Brilliant violet 421-conjugated streptavidin and biotin-conjugated CD25 (BC96) mAb were obtained from BioLegend. Purified anti-CCR4 mAb (KM2160) was kindly provided by Kyowa Hakko Kirin (Shizuoka, Japan).

Peptides. Synthetic peptides of NY-ESO-$1_{1–20}$ (MQAEGRGTGGSTGDADGPGG), NY-ESO-$1_{11–30}$ (STGDADGPGGPGIPDGPGGN), NY-ESO-$1_{21–40}$ (PGIPDGPGGNAGGPGEAGAT), NY-ESO-$1_{31–50}$ (AGGPGEAGATGGRGPRGAGA), NY-ESO-$1_{41–60}$ (GGRGPRGAGAARASGPGGGA), NY-ESO-$1_{51–70}$ (ARASGPGGGAPRGPHGGAAS), NY-ESO-$1_{61–80}$ (PRGPHGGAASGLNGCCRCGA), NY-ESO-$1_{71–90}$ (GLNGCCRCGARGPESRLLEF), NY-ESO-$1_{81–100}$ (RGPESRLLEFYLAMPFATPM), NY-ESO-$1_{91–110}$ (YLAMPFATPMEAELARRSLA), NY-ESO-$1_{101–120}$ (EAELARRSLAQDAPPLPVPG), NY-ESO-$1_{111–130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119–143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131–150}$ (NILTIRLTAADHRQLQLSIS), NY-ESO-$1_{139–160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151–170}$ (SCLQQLSLLMWITQCFLPVF), NY-ESO-$1_{161–180}$ (WITQCFLPVFLAQPPSGQRR), NY-ESO-$1_{87–98}$ (LLEFYLAMPFAT), NY-ESO-$1_{121–132}$ (VLLKEFTYSGNI), NY-ESO-$1_{143–154}$ (RQLQLSISSCLQ), NY-ESO-$1_{157–170}$ (SLLMWITQCFLPVF), NY-ESO-$1_{157–165}$ (SLLMWITQC), NY-ESO-$1_{92–100}$ (LAMPFATPM) were obtained from Invitrogen.

In Vitro Sensitization of NY-ESO-1–Specific $CD4^+$ T Cells. $CD8^+$ T cells were depleted from peripheral blood mononuclear cells (PBMCs) with CD8 Microbeads (Miltenyi Biotec). The remaining cells were subjected to negative selection of $CD4^+$ T cells with $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec). These $CD4^+$ T cells were further separated into $CD4^+CD25^-$ or $CD4^+CCR4^-$ T cells. $CD4^-CD8^-$ cells were used as antigen-presenting cells (APCs) after pulsing with pooled peptides (10 μM) overnight at 37 °C. After irradiation (35 Gy), 3–5 × 10^5 APCs were added to cultures containing 1–3 × 10^5 $CD4^+$ T cells, and were fed with IL-2 (10 U/mL; Roche Diagnostics) and IL-7 (20 ng/mL; R&D Systems) in round-bottom 96-well plates (Thermo Fisher Scientific). Subsequently, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/mL) and IL-7 (40 ng/mL) twice per week.

Enzyme-Linked Immunospot Assay. Flat-bottomed, 96-well nitrocellulose plates (MAHAS4510; Millipore) were coated with anti–IFN-γ mAb (4 μg/mL, 1-D1K; MABTECH) and incubated overnight at 4 °C and washed and blocked with RPMI with 10% (vol/vol) FCS. Presensitized 2–5 × 10^4 $CD4^+$ T cells and 5 × 10^4 target cells (peptide-pulsed autologous activated-T-cell APCs, or protein-pulsed dendritic cells, DCs) were added to each well and incubated for 20–22 h at 37 °C. Spots were developed using biotinylated anti–IFN-γ mAb (0.2 μg/mL, 7-B6-1-biotin; MABTECH), alkaline phosphatase conjugated streptavidin (Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) and counted with a C. T. L. Immunospot S5 Micro Analyzer (Cellular Technologies).

Tetramer Assay. Presensitized $CD8^+$ T cells were stained with PE-labeled HLA-A*0201 HLA-B*3501 or Cw*0304/NY-ESO-1 tetramer for 10 min at 37 °C before additional staining with cell surface markers for 15 min at 4 °C. After washing, results were analyzed by flow cytometry and FlowJo v9.6.2 software.

Preparation of DCs. $CD14^+$ cells were isolated from PBMCs using CD14 Microbeads (Miltenyi Biotec). Next, 1 × 10^6 $CD14^+$ cells were cultured in X-VIVO 15 (Lonza) with IL-4 (20 ng/mL) and GM-CSF (20 ng/mL, R&D) in 37 °C. One-half of the medium was replaced by fresh medium containing IL-4 (40 ng/mL) and GM-CSF (40 ng/mL) at day 3 and day 5. Cells were collected 6 d later and used as DCs.

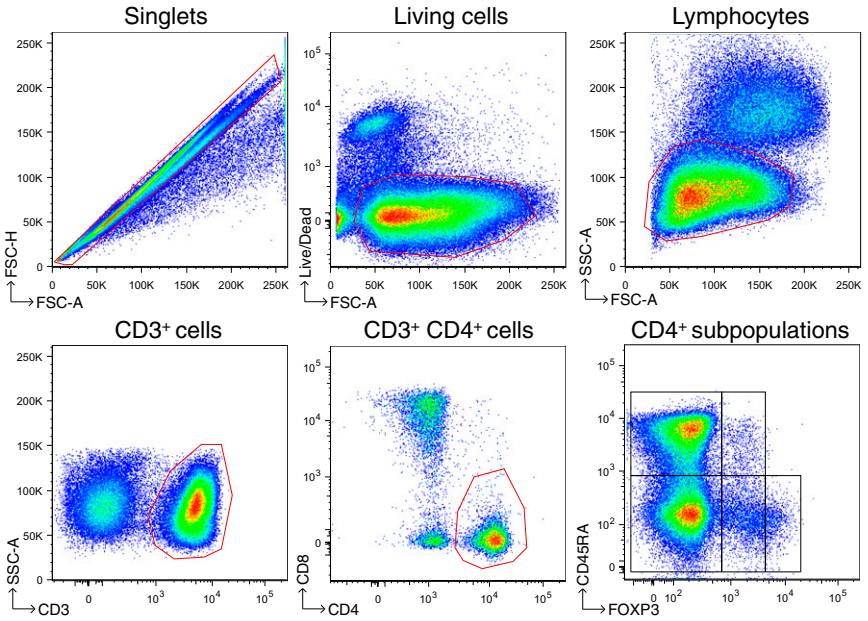

Fig. S1. Flow cytometric analysis of one representative healthy donor with detailed gating strategy. Staining for $CD4^+$ T-cell subpopulation based on the expression of CD45RA and FOXP3. Singlets were identified by exclusion of doublets using an FSC-A vs. FSC-H plot. Within the singlets living lymphocytes were identified by gating live/dead marker negative cells, and lymphocytes were defined by gating as a FSC-A vs. SSC-A plot. Then, gates were set on $CD3^+$ cells and $CD3^+CD4^+$ cells, and CD45RA and FOXP3 stained cells were visualized to identify each $CD4^+$ T-cell subpopulation.

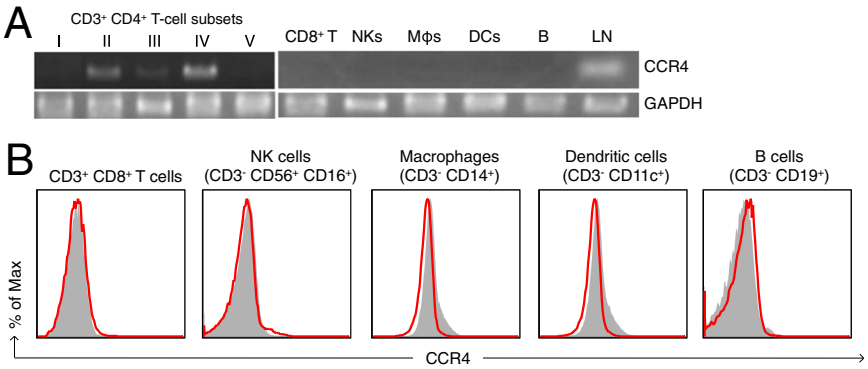

Fig. S2. CCR4 expression in T cells, NK cells, macrophage/monocytes, DCs, and B cells. (*A*) RT-PCR for CCR4 mRNA of $CD4^+$ T-cell subpopulation, $CD8^+$ T cells, NK cells, macrophage/monocytes, DCs, and B cells in PBMCs from three healthy donors (cDNA of lymph node was used as a positive control). Total RNA was isolated from cells with RNeasy Micro Kit (Qiagen). cDNA was synthesized from 0.2 μg of total RNA using a SuperScript III reverse-transcriptase kit (Invitrogen) and the Oligo (dT) primer in a total volume of 40 μL. cDNA was amplified in a final volume of 25 μL containing 10 μM of each CCR4 primer (sense, 5′-TTCCTCA-GAGCCGCTTTCAG-3′; antisense, 5′-CATCGAGGGTGGTGTCTGCTAT-3′) and 0.125 μL of Ex-Taq polymerase (TaKaRa Bio) according to the instructions provided by the manufacturer. (*B*) PBMCs from three healthy donors were stained for cell type-specific markers and CCR4 expression by each cell population was analyzed by flow cytometry. Representative data from a healthy donor are shown. These experiments were performed at least twice with similar results.

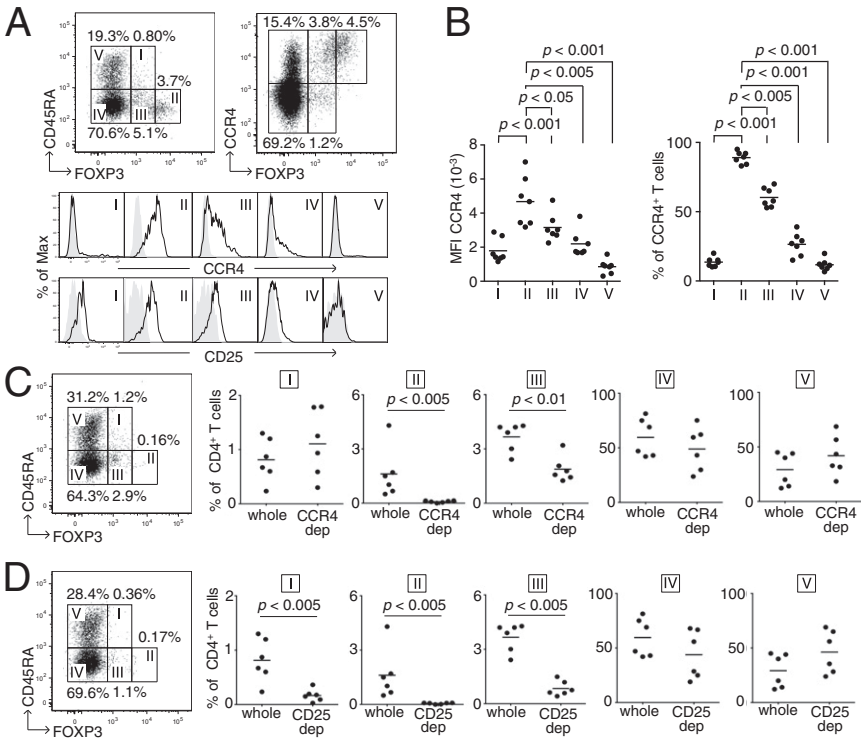

Fig. S3. In vitro CCR4$^+$ T-cell depletion efficiently reduces effector Treg cells in melanoma patients. (*A*) CCR4 expression by FOXP3$^+$ subpopulations. CD4$^+$ T cells prepared from PBMC of melanoma patients were fractionated into subpopulations based on the expression of CD45RA and FOXP3. Representative data from one patient are shown. (*B*) Median fluorescent intensity (MFI, *Left*) and frequency (*Right*) of CCR4 expression by each fraction of T cells from PBMCs of melanoma patients ($n = 7$). (*C* and *D*) Depletion of CCR4$^+$ cells (CCR4 dep) (*C*) or CD25$^+$ cells (CD25 dep) (*D*) ($n = 6$ each). These experiments were performed at least twice with similar results.

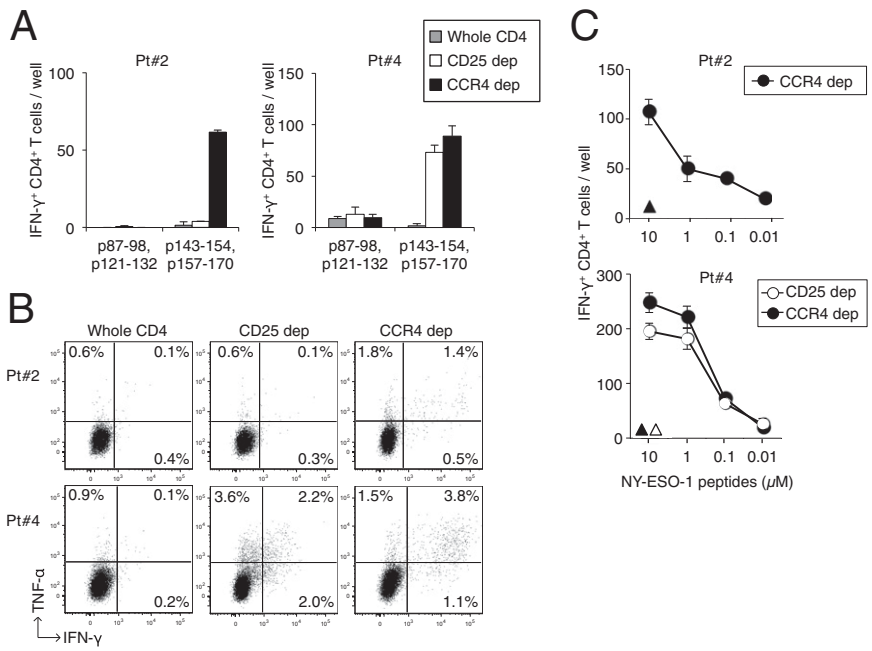

Fig. S4. In vitro CCR4$^+$ T-cell depletion efficiently induces NY-ESO-1–specific CD4$^+$ T cells in melanoma patients. (*A* and *B*) CD4$^+$ T cells were prepared from PBMCs of melanoma patients ($n = 8$) and presensitized with CD4$^-$CD8$^-$ PBMCs pulsed with NY-ESO-1 peptide covering the hot spots of NY-ESO-1 sequence as previously described (1). Induction of NY-ESO-1–specific CD4$^+$ T cells was analyzed by enzyme-linked immunospot (ELISpot) assays (*A*) and by intracellular cytokine staining upon recognition of autologous activated–T-cell APCs pulsed with NY-ESO-1 peptide that induced specific T-cell response (*B*). Data from two representative melanoma patients are shown in *A* and *B*. (*C*) Recognition of graded amounts of NY-ESO-1 peptides by NY-ESO-1–specific CD4$^+$ T cells. NY-ESO-1-specific CD4$^+$ T cells from CCR4$^+$ or CD25$^+$ T-cell–depleted cells (CCR4-dep and CD25-dep, respectively) were cultured with autologous activated T-cell APCs pulsed with graded amounts of NY-ESO-1 peptides and assessed for the number of IFN-γ–secreting cells, as in *A*. Triangles indicate response to control peptide at 10 μM. These experiments were performed independently twice with similar results.

1. Nishikawa H, et al. (2006) Influence of CD4+CD25+ regulatory T cells on low/high-avidity CD4+ T cells following peptide vaccination. *J Immunol* 176(10):6340–6346.

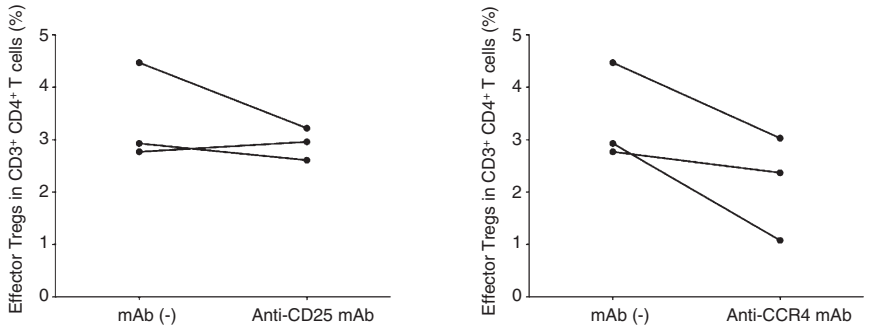

Fig. S5. Changes in the ratio of effector Treg cells in the presence of anti-CD25 mAb or anti-CCR4 mAb in cell culture. PBMCs were cultured in the presence of anti-CD25 mAb or anti-CCR4 mAb. Seven days later, cells were stained for CD4, CD45RA, and FOXP3, and analyzed with flow cytometry. These experiments were performed at least twice with similar results.

Table S1. Summary of ELISpot data in healthy donors

	Peptide p61–100			Peptide p91–120			Peptide p111–150			Peptide p141–180		
Donor	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$
HD#1	—	—	—	—	—	—	—	—	+++	—	—	—
HD#2	—	—	—	—	—	—	—	—	—	—	—	—
HD#3	—	—	—	—	—	—	—	—	—	—	—	—
HD#4	—	—	—	—	—	—	—	—	—	—	—	—
HD#5	—	—	—	—	—	—	—	—	—	—	—	—
HD#6	ND	ND	ND	—	—	—	—	—	—	—	++	—
HD#7	—	++	—	—	+	—	—	+	+	—	—	—
HD#8	—	—	++	—	—	—	—	—	—	—	—	—
HD#9	—	—	—	—	—	—	—	—	—	—	—	—
HD#10	—	—	—	—	—	—	—	—	++	—	—	—
HD#11	—	—	—	—	—	—	—	—	—	—	—	—
HD#12	—	—	++	—	—	—	—	—	++	—	—	—
HD#13	—	—	—	—	—	—	—	—	—	—	—	—
HD#14	—	—	++	—	—	—	—	—	—	—	—	—
HD#15	—	—	—	—	—	—	—	—	—	—	—	—
HD#16	—	—	—	—	—	—	—	—	—	—	—	—

For numbers of IFN-γ–secreting cells, symbols in the table are as follows: $0 <$ — < 50, $50 \leq$ + < 100, $100 \leq$ ++ < 200, $200 \leq$ +++. None of patients developed $CD4^+$ T-cell responses against N-terminal (1–60 amino acids) peptide of NY-ESO-1 protein. ND, not determined.

Table S2. Summary of ELISpot data in malignant melanoma patients

	Peptide p87–98			Peptide p121–132			Peptide p143–154			Peptide p157–170		
Patient	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$	Whole CD4	$CD4^+$ $CD25^-$	$CD4^+$ $CCR4^-$
Pt#1	—	—	—	—	—	—	—	—	—	—	—	—
Pt#2	—	—	+	—	—	—	—	—	—	—	—	—
Pt#3	—	—	—	—	—	—	—	—	—	—	—	—
Pt#4	—	—	—	—	—	—	—	+	++	—	—	—
Pt#5	—	—	—	—	—	—	—	—	—	—	—	—
Pt#6	—	—	—	—	—	—	—	—	—	—	—	—
Pt#7	—	—	—	—	—	—	—	—	—	—	—	—
Pt#8	—	—	—	—	—	—	—	—	—	—	—	++

For number of IFN-γ–secreting cells, symbols in the table are as follows: $0 <$ — <50, $50 \leq$ + < 100, $100 \leq$ ++ < 200.