**Supplementary methods**

**Human Treg cells isolation**

Human PBMCs were prepared using specific gravity centrifugal methods from peripheral blood, and CD4+ T cells were isolated as previously described ([20](#_ENREF_20)). CD4+ T cells were stained with the following anti-human antibodies, all of which were purchased from BioLegend: CD4 (RPA-T4), CD45RA (HI100), CD25 (BC96), and CD127 (A01905). Treg cells were further sorted using FACS Aria according to the method previously described ([49](#_ENREF_49)). This study was approved by the Ethics Committee of Keio University.

**Plasmid construction**

Human Nr4a2 was amplified through PCR and subcloned into eMIGR1 or pCMV plasmids. Because human Nr4a2 is highly similar to mouse Nr4a2 (595 of 598 aa are identical), human Nr4a2 is believed to act identically to mouse Nr4a2. Genes encoding mouse nuclear receptors were PCR-amplified from the mouse cDNA, and subcloned into pCMV vectors. Oligonucleotides containing repeats of nuclear receptor response element sequences were synthesized by Eurofin Genomics (Tokyo, Japan). Annealed oligos were cloned into pGL4-luciferase plasmids.

**ELISA (enzyme-linked immunosorbent assay)**

For analysis of IFN-γ in a cultured medium, the supernatants were analyzed with a Mouse IFN-γ ELISA MAX (BioLegend), according to the manufacturer’s protocol.

**Luciferase assay-based drug screening**

293T cells were transfected with NBRE-luciferase reporter construct (containing three copies of NBRE [Nr4a response element] sequence) and Nr4a2-expressing plasmids. Luciferase assay was performed as described in **Methods**. Chemicals in a SCADS inhibitor kit (consisting of 4 plates/363 chemicals) were initially screened at 1μM.If the value of the internal control (β-gal) was less than 50% of the DMSO control, the relevant drugs were re-examined at concentrations of 100 nM or 10 nM because secondary effects such as drug cytotoxicity were of concern. Compounds that reduced Nr4a2-induced transactivation of NBRE-luciferase reporter to 50% or less of control DMSO treatment were considered to be positive.

**Study approval**

This study was approved by the Institutional Review Board of Keio University School of Medicine (Approval number: 20120039), and conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from all individuals.

**Supplementary Figure Legends**

**Supplementary Figure S1. Expression of Nr4a factors in human Treg cells**

**(A)** A representative flow-cytometric profile of PBMC from healthy donors and the gating strategy used to separate the indicated fractions of Tregs. Here we used CD127 as a marker for purifying Foxp3+ cells ([49](#_ENREF_49)). **(B)** *Nr4a1*, *Nr4a2*, and *Foxp3* mRNA expression in the separated Tregs fraction. Expression of each gene was normalized to 18S rRNA levels.

**Supplementary Figure S2. Treg-specific deletion of Nr4a1 and Nr4a2 attenuated Treg cell suppressive activities**

WT and Nr4a-DcKO mice (6-8 weeks old) were analyzed. **(A)** CD4+ and CD8+ compartments in the thymus (top), spleens (middle) and inguinal lymph nodes (iLNs; bottom). **(B)** CD4+ T cells in **(A)** were further analyzed for Foxp3 expression. **(C)** Quantitative PCR analysis of mRNA for the indicated genes in WT and Nr4a-DcKO Tregs. Gene expression was normalized to 18S rRNA levels. Data were representative of three independent experiments each performed in triplicate. **(D)** Flow cytometry profiles of the indicated proteins in CD4+Foxp3+ cell population in the spleens (top) and iLNs (bottom). Numbers in the histograms indicate mean fluorescence intensities (MFIs). **(E)** *In vitro* suppression assay. Suppression of CFSE-labeled CD4+CD25- CD45.1+ cells (responder T cells; Tresp) by CD45.1- WT or Nr4a-DcKO Treg cells. Cells were stimulated with anti-CD3/28 beads for 96 h. Numbers in the histograms represent percentages of undivided cells gated on CD4+CD45.1+. Data were representative of two independent experiments. **(F)** Cytokine production from CD4+ and CD8+ T cells in pooled spleens and lymph nodes after stimulation with PMA/ionomycin in the presence of Brefeldin A for 4 h. **(A,B)(D-F)** Representative FACS plots and bar graphs summarizing the FACS data are shown (*n*= 4-6/ group). \**P* < 0.05 compared with WT; n.s., not significant; data are presented as the mean+SD.

**Supplementary Figure S3. Potent anti-tumor immune responses were induced in Nr4a-DcKO mice**

MC38 **(A-C, E,G)** or 3LL **(D, F)** tumor-bearing WT and Nr4a-DcKO mice (*n*= 5-7/group) were analyzed on day 22 or 20, respectively. **(A)** Frequency of tumor-infiltrating CD4+/CD8+ (top) and CD4+ Foxp3+ Tregs (bottom). The lower panels show gated CD4+ T cells. **(B)** Ratio of tumor-infiltrating CD8+ T cells to Treg cells. **(C)** Frequency of tumor-infiltrating effector T cells (CD8+-IFN-γ+, -TNF-α+, -CD107a+ and CD4+IFN-γ+). The panels show either gated CD8+ or CD4+ T cells. **(D)** *In vitro* recall responses. Single-cell suspensions were prepared from TDLNs and re-stimulated with mitomycin-C-treated 3LL or MC38 tumor cells in an 8:1 ratio. Four days later, the culture-supernatant was collected, and IFN-γ production was measured by ELISA. Cells were stimulated in triplicate wells for each condition, and SD was calculated from the values determined by ELISA. Results from two independent mice (#1, #2) per genotype are shown. **(E,F)** CD80 and CD86 expression on CD45+CD11b+CD11c+ tumor-infiltrating DCs. Numbers in the histograms indicate mean fluorescence intensities (MFIs). **(G)** CTLA-4 expression on CD4+Foxp3+ tumor-infiltrating Tregs. **(A, C, E, G)** Representative FACS plots or histograms, and bar graphs summarizing the FACS data are shown. \**P* < 0.05, \*\**P* < 0.01 compared with WT; n.s., not significant; data are presented as the mean+SD.

**Supplementary Figure S4. Screening of drugs that inhibit transcriptional activity of Nr4a**

**(A)** Schematic design of the drug-screening system in this study. **(B)** Brief overview of the screening procedure. **(C)** Screening results. Experiments were performed in duplicate, and the average value was calculated. Luciferase activity of the DMSO control was set as one, and the relative value is shown for each drug. Blank wells indicate missing numbers in the inhibitor kit. **(D)** Various topoisomerase inhibitors were tested in our screening system. All chemicals were tested at a 200 nM. cpt, camptothecin; dau, daunorubicin; dox, doxorubicin; ida, idarubicin; topo, topotecan; eto, etoposide. **(E)** Target specificity of CPT. 293T cells were transfected with various expression plasmids encoding nuclear receptor genes (NRs), and luciferase reporter constructs containing the response element sequence of the corresponding genes. The luciferase activity value of the DMSO control was set as one, and the relative value is shown.

**Supplementary Figure S5. Validation of the chemotherapeutic agent Camptothecin as a functional Nr4a inhibitor**

**(A,B)** mRNA expression of the indicated genes was measured by quantitative RT-PCR. Gene expression was normalized to 18SrRNA levels. **(A)** Effects of CPT on *Foxp3* (left) and *Ifng* (right) mRNA expression under iTreg and Th1 conditions, respectively. **(B)** Effects of CPT on *Foxp3* (left) and *Ifng* (right) mRNA expression under Th1-skewing condition in the presence or absence of TGF-β. **(C)** Effects of CPT on Foxp3 expression under iTreg conditions in the presence of Z-VAD-fmk (50 μM). **(D)** Effects of SN-38 on NBRE-luciferase activity transactivated by Nr4a2. **(E)** Flow cytometry profiles of the indicated proteins in WT CD4+CD25+Tregs, cultured with or without SN-38 in the presence of Z-VAD-fmk (50 μM) and thymidine (2 mM) for 72 h. Numbers in the histograms indicate mean fluorescence intensities (MFIs). **(F-I)** CPT-derivative CPT-11 (75 mg/kg) was administrated intraperitoneally to WT naive mice twice at intervals of 3 days (days 0 and 4), and analyzed on day 10 (*n*= 5/group). **(F)** Body weight curve throughout the experimental period. **(G)** Spleen weights were measured on day 10. **(H)** CD4+ and CD8+ compartments in thymus (top) and spleens (bottom). **(I)** CD4+ T cell fraction in **(H)** was further analyzed for Foxp3 expression (top, thymus; bottom, spleens). **(A-E)** The indicated concentration of CPT **(A-C)** or SN-38 **(D,E)** was added to the culture throughout the experiment. Under culture conditions without CPT or SN-38, an equivalent volume of DMSO was added only as a control. Data represent three **(A-C)** or two **(D-E)** independent experiments. As for **(A,B, D),** eachexperiment was performed in triplicate. **(C, E, H, I)** Representative FACS plots and bar graphs summarizing the FACS data are shown. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P*< 0.001 compared with the untreated group unless otherwise indicated; n.s., not significant; data are presented as the mean+SD.

**Supplementary Figure S6. Camptothecin and a COX-2 inhibitor induced anti-tumor effects in a MC38 tumor model**

**(A-C)** WT mice were subcutaneously injected with 5.0×105 MC38 tumor cells (day 0), and inhibitors were administered according to the schedule shown in the **Fig. 5A**. Mice were analyzed on day 14 (*n*= 4-6/group). **(A)** Tumor weights. **(B)** Frequency of CD4+/CD8+ (top) and CD4+Foxp3+ cells (bottom) in TDLNs. The panels show either gated CD3+ or CD4+ cells, respectively. **(C)** Frequency of tumor-infiltrating effector T cells (CD4+IFN-γ+, CD8+IFN-γ+). The panels show either gated CD4+ or CD8+ cells. **(B,C)** Representative FACS plots and bar graphs summarizing the FACS data are shown. \**P* < 0.05; \*\**P* < 0.01 compared with the untreated group; n.s., not significant; data are presented as the mean+SD.

**Supplementary Figure S7. Camptothecin and a COX-2 inhibitor regulate Treg-mediated down-regulation of costimulatory molecules on tumor-infiltrating DCs**

**(A)** *In vitro* suppression assay. Suppression of CFSE-labeled CD4+CD25- CD45.1+ cells (responder T cells; Tresp) by CD45.1- Tregs from tumor-free (No tumor), and 3LL tumor-bearing mice left untreated or treated with the indicated inhibitors. Tresp and Tregs were cultured at a 2:1 ratio, and stimulated with anti-CD3/28 beads for 96 h. Numbers in histograms represent percentages of undivided cells gated on CD4+CD45.1+. Data are representative of two independent experiments involving four mice per group. **(B)** CD80 and CD86 expression on CD45+CD11b+CD11c+ tumor-infiltrating DCs in 3LL-tumor bearing mice described in **Fig. 6C,D**. Bar graphs show quantification of FACS data using the mean fluorescence intensities (MFIs) as an index (*n=*5-8/group). \**P* < 0.05 compared with the untreated group; n.s., not significant; data are presented as the mean+SD.

**Supplementary reference**

49. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional

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