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Supplemental Information

Functional Analysis of Dendritic Cells Generated from T-iPSCs from CD4+ T Cell Clones of Sjögren's Syndrome

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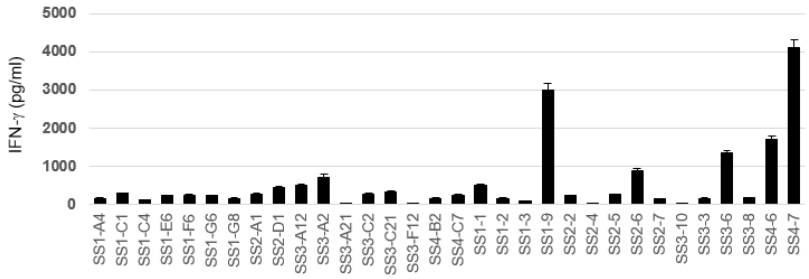
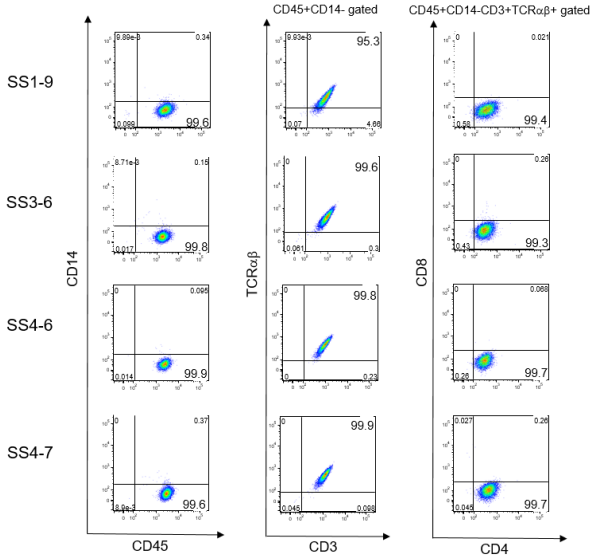
Figure S1

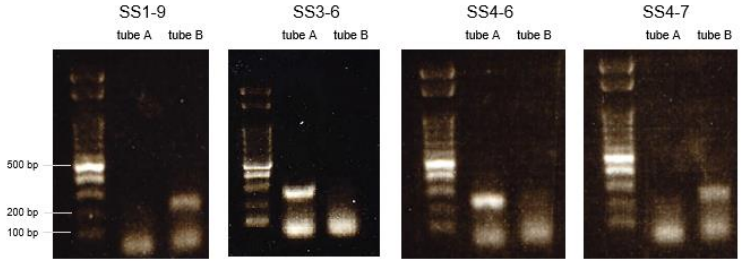
(A)



(B)



(C)

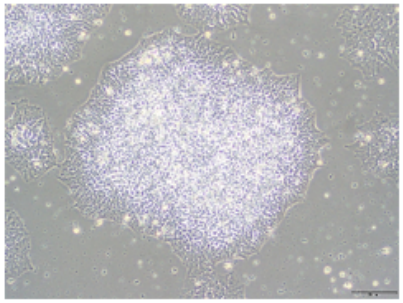


(D)

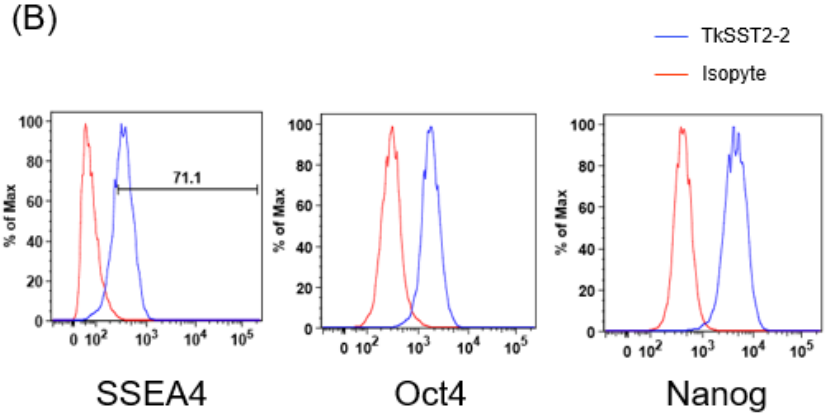
Clone	Productivity	Rearrangement			Sequence of Junctional region		
		Vβ	Dβ	Jβ	3'Vβ	N1-Dβ-N2	5'Vβ
SS1-9	**Productive**	TRBV7-2*01	TRBD2*01	TRBJ2-3*01	TGTGCCAGCAGCTTAG	GCGGGGGGTA	CACAGATACGCAGTATTTT
SS3-6	**Productive**	TRBV6-2*01	TRBD1*01	TRBJ2-7*01	TGTGCCAGCAGTTAC	CGTACCCAA	TACGAGCAGTACTTC
	Unproductive	TRBV7-3*01	TRBD1*01	TRBJ1-5*01	TGTGCCAGCAGCT	CCGATCGGGGGACAGGGCCTGTCCGGTCAG	AGCATTTT
SS4-6	**Productive**	TRBV4-3*04	TRBD2*02	TRBJ2-7*01	TGCGCCAGCAGCC	TAGGACTAGCGGGAATT	TCCTACGAGCAGTACTTC
	Unproductive	TRBV19*01	TRBD2*01	TRBJ1-1*01	TGTGCCA	CCCTCCGGGGGATTGCAG	AGTCTTTCTTT
SS4-7	**Productive**	TRBV20-1*01	TRBD2*02	TRBJ2-3*01	TGCAGTGCTAG	TCCTAGCGGGATGAAAAG	TACGCAGTATTTT

Figure S2

(A)



(B)



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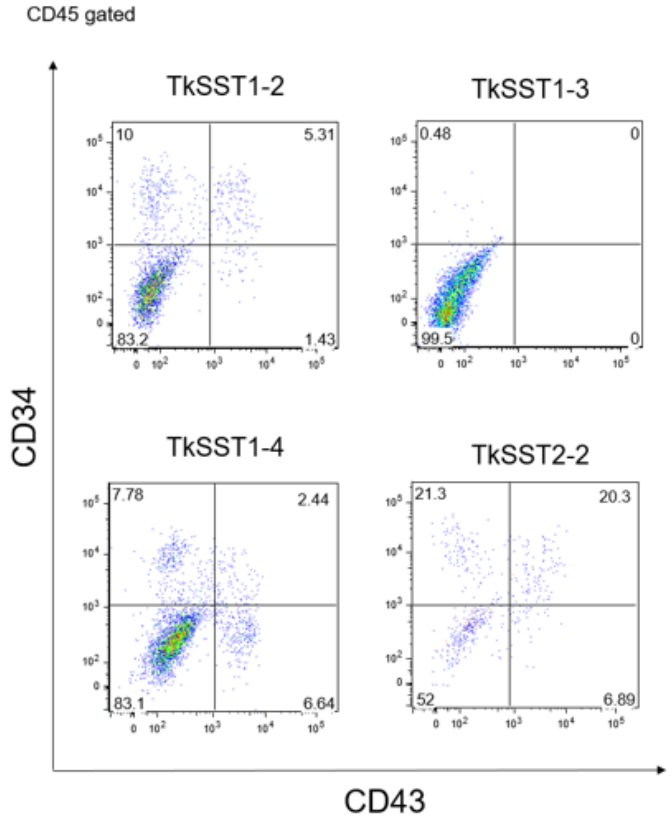

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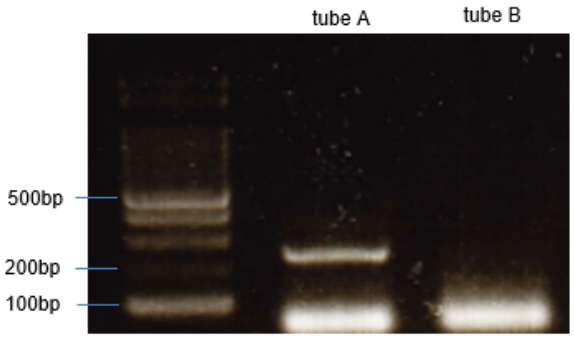

(E)

TiPSC	Productivity	Rearrangement			Sequence of Junctional region		
		Vβ	Dβ	Jβ	3'Vβ	N1-Dβ-N2	5'Vβ
TkSST 2-2	**Productive**	TRBV4-3*04	TRBD2*02	TRBJ2-7*01	TGCGCCAGCAGCC	TAGGACTAGCGGGAATT	TCCTACGAGCAGTACTTC
	Unproductive	TRBV19*01	TRBD2*01	TRBJ1-1*01	TGTGCCA	CCCTCCGGGGGATTGCAG	AGTCTTTCTTT

Figure S3

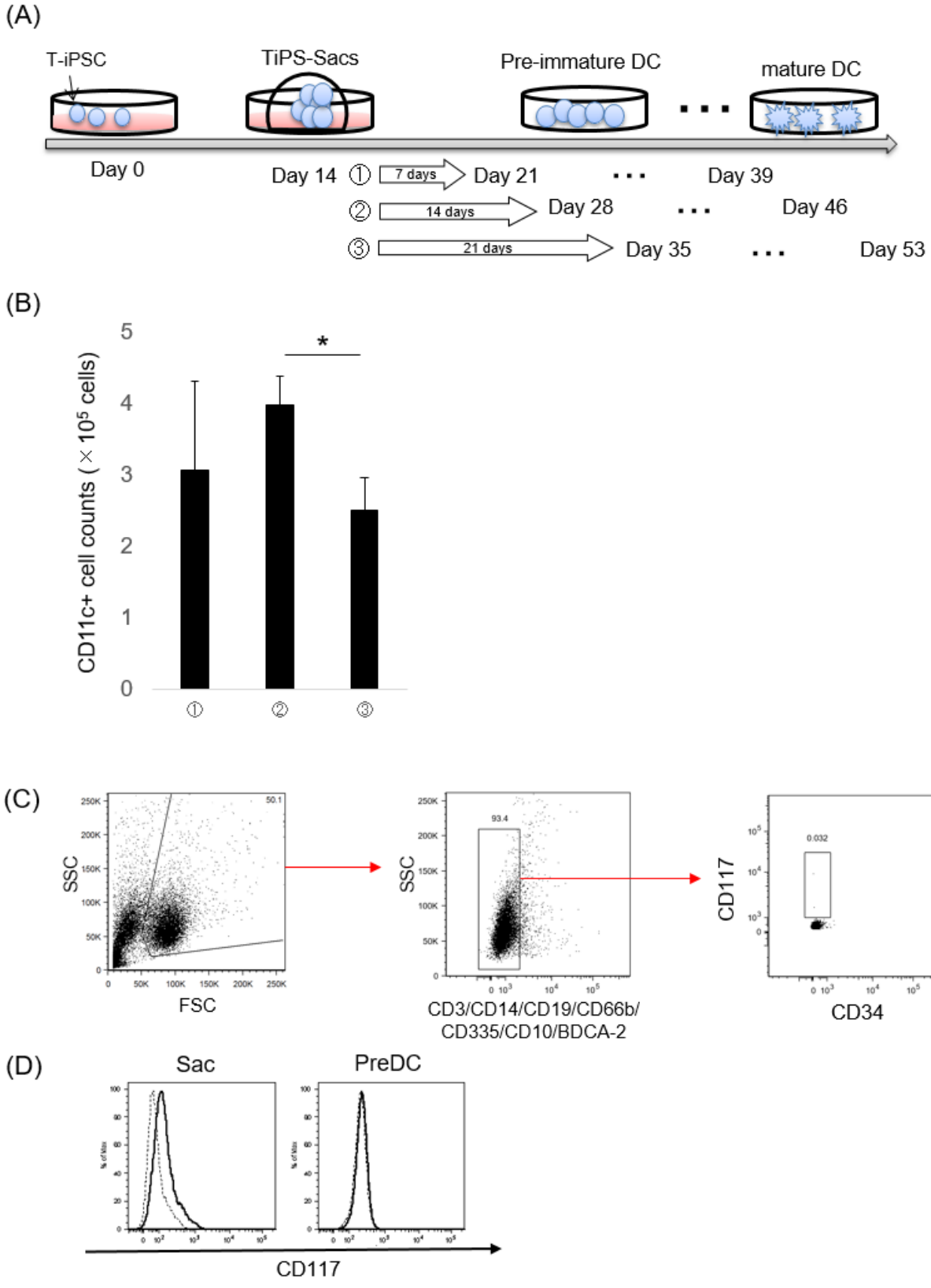
(A)
T-iPSC
TiPS-Sacs
Pre-immature DC
mature DC
Day 0
Day 14
① 7 days
Day 21
Day 39
② 14 days
Day 28
Day 46
③ 21 days
Day 35
Day 53
(B)
CD11c+ cell counts (×10⁵ cells)
*
①
②
③
(C)
SSC
FSC
CD3/CD14/CD19/CD66b/
CD335/CD10/BDCA-2
CD117
CD34
(D)
Sac
PreDC
CD117

Figure S4

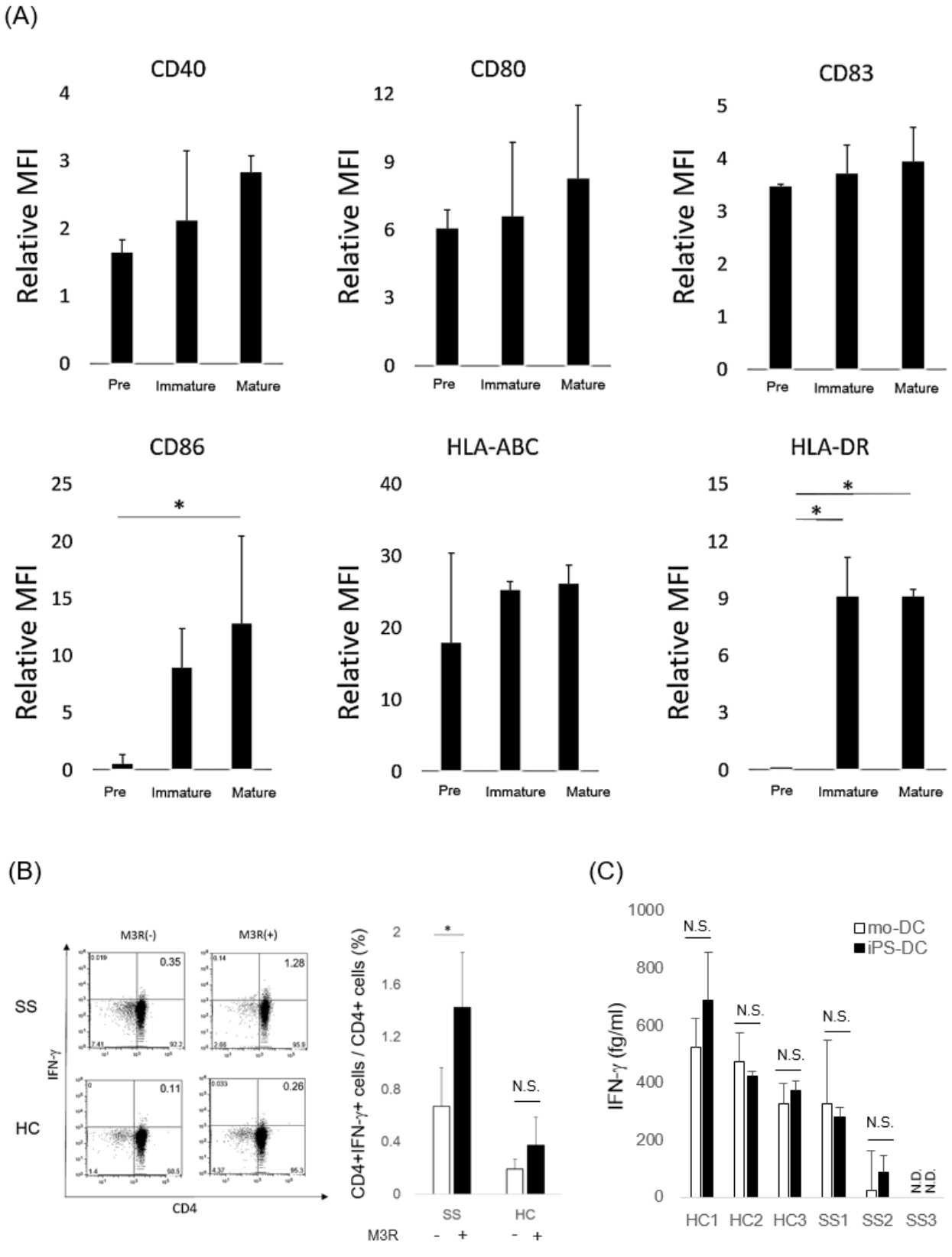
(A)
CD40
CD80
CD83
CD86
HLA-ABC
HLA-DR
Relative MFI
Pre
Immature
Mature
*
(B)
M3R(-)
M3R(+)
SS
HC
IFN-γ
CD4
0.35
1.28
0.11
0.26
CD4+IFN-γ+ cells / CD4+ cells (%)
N.S.
M3R
(C)
mo-DC
iPS-DC
IFN-γ (fg/ml)
HC1
HC2
HC3
SS1
SS2
SS3
N.D.

Figure S1. Generation and selection of CD4+ T cell clone. Related to Figure 1

Analyses of the established CD4+ T cell clones. (A) IFN-γ production by CD4+ T cell clones. Each T cell clone was stimulated with anti-CD3/28 antibodies (each 1 μg/ml) for 72 hrs and IFN-γ concentration was evaluated by ELISA. (B) Flow cytometric analysis of the surface molecules of SS1-9, SS3-6, SS4-6, and SS4-7. (C) Multiplex-PCR analysis to detect TCRB gene rearrangements. Tubes A and B contained Vb-Jb assemblies. (D) Sequence result of TCRB gene rearrangements.

Figure S2. The establishment of T-iPSCs and the proportion of hematopoietic cells in Sacs. Related to Figure 1

(A) Colony shape analysis and (B) flow cytometric analysis for the expression of SSEA4, Oct4 and Nanog in TkSST2-2. Scale bars, 200 μm. (C) Flow cytometric analysis of CD34+ hematopoietic cells on each TiPS-Sac. The cells were gated on CD45+ cells. (D) Multiplex-PCR analysis to detect TCRB gene rearrangements. Tubes A and B contained Vb-Jb assemblies. (E) Sequence result of TCRB gene rearrangements.

Figure S3. The analysis of the optimal culture period. Related to Figure 1

(A) Schematic illustration of the differentiation into T-iPS-DCs. (B) the numbers of CD11c+ mature T-iPS-DCs. *P < 0.05 by Kruskal-Wallis test. N=3 independent experiments. (C) Flow cytometric analysis of surface molecules on pre-immature cells of T-iPS-DCs (D) The expression level of CD117 on TiPS-Sacs and pre-immature cells of T-iPS-DCs.

Figure S4. The expressions of surface molecules, and functional analysis using T-iPS-DCs. Related to Figure 2, 4

Relative expression levels of CD40, CD80, CD83, CD86, HLA-ABC, HLA-DR in TiPS-Sacs, pre-immature cells of T-iPS-DCs (Pre), immature T-iPS-DCs (Immature), and mature T-iPS-DCs (Mature). Values are mean ± SD. *P < 0.05 by Kruskal-Wallis test. (B) Mo-DCs of a patient with SS or those of an HLA-DRB1-matched healthy donor were pre-incubated with or without M3R peptide (10 μg/ml) and co-cultured with auto-CD4+ T cells (1×10^5 cells/well). M3R reactive CD4+ T cells were evaluated. (C) Mature T-iPS-DCs (5×10^3 cells/well) were co-cultured with allogeneic T cells (3×10^4 cells/well) of healthy donors (HC1-3) or patients with SS (SS1-3) for 5 days. IFN-γ production was measured by CBA. Values are the mean ± SD. *P < 0.05 by Mann-Whitney's test. N=3 independent experiments.

Experimental Procedures

Generation of CD4+ T cell clones

CD4+ T cell clones were established from PBMCs of patients with SS as described in detail previously (Nishimura T et al., 2013). Briefly, CD4+ T cells were separated from PBMCs by magnetic-activated cell sorting (Miltenyi Biotec). The obtained cells were single-cell sorted and mixed with feeder cells (irradiated allogenic PBMCs) stimulated with 1% phytohemagglutinin (PHA) (Sigma Aldrich) and 100 U/ml IL-2, and cultured in RPMI-1640 supplemented with 10% human AB Serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin (Gibco). The single-cell derived CD4+ T cell clone was stimulated and expanded every 2-4 weeks with gradual increase in the number of feeder cells. This study was approved by the local ethics committees of each participating institution and a signed informed consent was obtained from each subject.

Generation of T-iPSCs

Human iPSCs were also established from lines of patients with SS using the method described in detail previously (Nishimura T et al., 2013; Nishimura K et al., 2011). Briefly, CD4+ T cells were transduced with reprogramming factors via SeV vectors and were cultured in RPMI-1640 medium with 10% human AB Serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, which were gradually replaced with human iPS cells medium (Nishimura T et al., 2013). The derived iPS cells were maintained on feeder-free condition in Essential 8 medium (Gibco).

Analysis of TCR gene rearrangement in genomic DNA

Genomic DNA was extracted using QIAamp DNA kits (Qiagen) according to the instructions provided by the manufacturer. For TCRB gene rearrangement analysis, PCR was performed according to the protocol described previously (van Dongen JJ et al., 2003). In Brief, we used multiplex-PCR primers for analysis of TCRB gene assemblies designed by the BIOMED-2 consortium. The dominant band within the expected size range was purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega). The PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced. The V, D, and J segments were identified by using the imMunoGeneTics (IMGT) database (http://www.imgt.org/). Gene-segment nomenclature follows IMGT usage.

Analysis of TCR gene rearrangement in mRNA

Total RNA was extracted from CD4+ T cell clones by ISOGEN method, and cDNA was synthesized by cDNA synthesis kit (TAKARA, Japan). For TCRB rearrangement analysis, PCR was performed and also V, D, and J segments were identified as described above.

Differentiation of monocytes derived DCs

CD14+ cells were isolated from PBMCs of patients with SS using CD14+ cell isolation kit (Miltenyi Biotec). The isolated monocytes were cultured with GM-CSF (100 ng/ml) and IL-4 (10 ng/ml) for 5 days, and then

treated with LPS (3 μg/ml), IL-4 (20 ng/ml), and TNF-α (10 ng/ml) for 3 days in RPMI-1640 medium containing 10% FBS.

Analysis of gene expression levels

Total RNA was extracted and cDNA was synthesized from DCs as described above. The expression levels of CCL13, CCL17, MMP-12, PU.1, pTCRα and CLEC9A were determined by quantitative PCR. Plasmacytoid DCs were extracted using plasmacytoid Dendritic Cells isolation kit II (Miltenyi Biotec).

Fluorescence-activated cell sorting (FACS) staining

The cells were washed in FACS buffer (2% FBS in PBS) and incubated with FACS antibodies. All antibodies were used at a concentration of 5-10 μg/ml. The anti-human antibodies used included antibodies against CD3 (catalog number: 300430), CD4 (317416, 317418), CD8 (300926), CD10 (312215), CD11b (301329), CD11c (301614), CD14 (325604, 367110), CD19 (302230), CD31 (313103), CD34 (343526, 343608), CD40 (334306), CD43 (343204), CD45 (304014, 304058), CD66b (305108), CD80 (305218), CD83 (305325), CD86 (305406), CD117 (313216), CD335 (331919), TCRαβ (306720), BDCA-1 (331519), BDCA-2 (354209), BDCA-3 (344109), HLA-ABC (311426), HLA-DR (307606), and SSEA-4 (330406) (all from Biolegend). The antibodies for mouse IgM (401624), mouse IgG1 (400108, 400112, 400120, 400126, 400128, 400144, 400150, 400158), mouse IgG2a (400208, 400212, 400222, 400230, 400251), mouse IgG2b (400320), and mouse IgG3 (401320) were used as the isotype controls (all from Biolegend). The 7-AAD Viability Staining Solution (Biolegend) was also used. For intracellular staining of Oct4 and Nanog, cells were fixed in 2-4% folmaldehyde, and permeabilized in 90% methanol. Anti-human OCT4A (2840S) and NANOG (D73G4) antibodies (Cell Signal Technology) were used for the primary antibodies, and Alexa Fluor 647-conjugated anti-rabbit IgG (A31573) (Thermo Fisher) was used for the secondary antibody. Rabbit IgG (3900S) antibodies (Cell Signal Technology) were used as the isotype control.

Analysis of cytokine production

The CD4+ T cell clones (2.0×10^5 cells/well) were stimulated with anti-CD3 antibodies (Biolegend) and anti-CD28 antibodies (BD Bioscience) (each 1 μg/ml) under 5% CO_2 at 37°C. After 72 hrs, IFN-γ levels were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

Zymosan uptake

The pHrodo Green Zymosan Bioparticles (about 3 μm) conjugate for Phagocytosis (ThermoFisher Scientific) was dissolved in RPMI-1640 medium with 10% FBS, and added 100 μl to mature T-iPS-DCs or monocytes-derived DCs (mo-DCs) (5×10^4 cells/well). The cells were incubated for 60 min at 37°C or 4°C, washed three times with FACS buffer, and then stained with CD11c.

Quantitative analysis of cytokine production

The cells ($1x10^5$ cells/well) were cultured in the presence of LPS (3 μg/ml), IL-4 (20 ng/ml), TNF-α (10 ng/ml) or OK432 (10 μg/ml or 25 μg/ml) or cross-linked CD40L (100 ng/ml or 1 μg/ml) for 72 hrs. The supernatant was collected, and then the concentration of TNF-α, IL-6, and IL-12p70 was measured using ELISA (R&D systems).

Allogeneic T cell stimulation assay

CD3+ T cells ($1x10^5$ cells/well) were purified from PBMCs obtained from allogeneic healthy donors or patients with primary SS by using Pan T cell isolation kit (Miltenyi Biotec) and co-cultured with graded numbers of irradiated (45 Gy) T-iPS-DCs or mo-DCs in RPMI-1640 medium with 10% human AB Serum for 5 days. Cell proliferation was evaluated by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation (Roche). As indicated above, this study was also approved by the local ethics committees of each participating institution and a signed informed consent was obtained from each subject.

Generation of M3 muscarinic acetylcholine receptor (M3R) reactive CD4+ T cell line

The development of M3R reactive CD4+ T cell lines was performed as described (Tomita Y et al., 2013). CD4+ T cells were purified from PBMCs obtained from the patient with SS, whose T-iPSCs were generated, and mo-DCs induced from CD14+ cells were used as APC. From the immune epitope database of MHC binding (http://www.iedb.org/home_v2.php?Clear=Clear), $M3R_{125-144}$ peptide (FTTYIIMNRWALGNLACDLW) was selected as the HLA-DRB1 epitopes (14:54/15:02) of the patient. At day 0, mo-DCs (5×10^3 cells/well) pulsed with M3R peptide (10 μg/ml) for 3 hrs, were irradiated and co-cultured with CD4+ T cells (3×10^4 cells/well) in AIM-V supplemented with 5% FBS, adding rhIL-7 (5 ng/ml). At day 2, rhIL-2 (10 U/ml) was also added. This cycle was repeated for 4 times and established M3R reactive CD4+ T cell lines.

Antigen presentation assay

Established M3R reactive CD4+ T cell lines (5×10^4 cells/well) were co-cultured with irradiated T-iPS-DCs or mo-DCs (1×10^4 cells/well) in AIM-V medium with 5% FBS. After 4 days, the level of IFN-γ was measured by Cytometric Beads Array (CBA) (BD Bioscience).

Detection of M3R reactive CD4+ T cells

CD4+ T cells (1×10^5 cells/well), derived from the patient with SS or an HLA-DRB1-matched (HLA-DRB1 14:54) healthy donor, were co-cultured with irradiated DCs of respective donors, pulsed with M3R peptide (10 ug/ml) for 3 hrs. After 6 hrs, the M3R reactive CD4+ T cells were detected by MACS cytokine secretion assay (Miltenyi Biotec).

References

Nishimura K, Sano M, Ohtake M, Furuta B, Umemura Y, Nakajima Y, Ikehara Y, Kobayashi T, Segawa H, Takayasu S, et al. (2011). *Development of defective and persistent Sendai virus vector: a unique gene delivery/expression system ideal for cell reprogramming.* J Biol Chem. 286, 4760-71.

van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, Garcia-Sanz R, et al. (2003). *Design and standarlization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936.* Leukemia. 17, 2257-317.

Tomita Y, Yuno A, Tsukamoto H, Senju S, Yoshimura S, Osawa R, Kuroda Y, Hirayama M, Irie A, Hamada A, et al. (2013). *Identification of CDCA1- derived long peptides bearing both CD4+ and CD8+ T-cell epitopes: CDCA1-specific CD4+ T-cell immunity in cancer patients.* Int J Cancer. 134, 352–366.