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

Mana Iizuka-Koga,[1,7,8] Hiromitsu Asashima,[1,8] Miki Ando,[2,3] Chen-Yi Lai,[4] Shinji Mochizuki,[4] Mahito Nakanishi,[5] Toshinobu Nishimura,[6] Hiroto Tsuboi,[1] Tomoya Hirota,[1] Hiroyuki Takahashi,[1] Isao Matsumoto,[1] Makoto Otsu,[2,4] and Takayuki Sumida[1,*]

[1]Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan
[2]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Tokyo 108-8639, Japan
[3]Department of Transfusion Medicine and Stem Cell Regulation, Juntendo University School of Medicine, Tokyo 113-8421, Japan
[4]Division of Stem Cell Processing/Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, Tokyo 108-8639, Japan
[5]Research Laboratory for Stem Cell Engineering, Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8565, Japan
[6]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, CA 94305, USA
[7]Present address: Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo 160-8582, Japan
[8]Co-first author
*Correspondence: tsumida@md.tsukuba.ac.jp
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SUMMARY

Although it is important to clarify the pathogenic functions of T cells in human samples, their examination is often limited due to difficulty in obtaining sufficient numbers of dendritic cells (DCs), used as antigen-presenting cells, especially in autoimmune diseases. We describe the generation of DCs from induced pluripotent stem cells derived from T cells (T-iPSCs). We reprogrammed CD4+ T cell clones from a patient with Sjögren's syndrome (SS) into iPSCs, which were differentiated into DCs (T-iPS-DCs). T-iPS-DCs had dendritic cell-like morphology, and expressed CD11c, HLA-DR, CD80, CD86, and also BDCA-3. Compared with monocyte-derived DCs, the capacity for antigen processing was similar, and T-iPS-DCs induced the proliferative response of autoreactive CD4+ T cells. Moreover, we could evaluate T cell functions of the patient with SS. In conclusion, we obtained adequate numbers of DCs from T-iPSCs, which could be used to characterize pathogenic T cells in autoimmune diseases such as SS.

INTRODUCTION

Sjögren's syndrome (SS) is an autoimmune disease characterized by infiltration of lymphocytes into lacrimal and salivary glands (Fox and Stern, 2002). Immunohistochemical studies have shown that most infiltrating lymphocytes are CD4+ αβT cells. With respect to the cytokine profile, overexpression of interferon gamma (IFN-γ) has been described in salivary glands of SS, and CD4+ T cells, which produce IFN-γ, activate salivary gland epithelial cells (Kawakami et al., 2012; Iizuka et al., 2012). Moreover, a high proportion of IFN-γ-producing CD4+ T cells is present among peripheral blood mononuclear cells (PBMCs), suggesting that they play an important role in the pathogenesis of SS (Koarada et al., 2006). Although many reports support the involvement of IFN-γ-producing CD4+ T cells in SS, there are only a few reports that provide precise analysis of CD4+ T cells, establishing monoclonal T cell lines from patients with SS.

Dendritic cells (DCs), used as antigen-presenting cells (APCs) in the analysis of CD4+ T cells, usually differentiate from monocytes in vitro. However, monocytes cannot be propagated, and obtaining sufficient number of these cells is not feasible since it requires an abundant amount of blood. Thus, versatile methods for obtaining sufficient numbers of APCs are needed.

Induced pluripotent stem cells (iPSCs) are generated from various cell types upon enforced expression of transcription factors, such as Oct4, Klf4, Sox2, and c-Myc (Takahashi et al., 2007). T cells could also convert into iPSCs (T-iPSCs), retaining rearranged TCR genes from the original T cell, and then re-differentiate into functional T cells (Nishimura et al., 2013; Vizcardo et al., 2013; Ando et al., 2015). Recently, differentiation of DCs from human iPSCs (iPS-DCs) derived from fibroblasts has been reported (Choi et al., 2009; Senju et al., 2011). There is no information, however, whether T-iPSCs, especially from patients with autoimmune diseases such as SS, could differentiate into functional DCs. For this reason, we tried to establish DCs from T-iPSCs via Sacs. This approach would be critically useful not only as a less invasive approach for patients but also due to the lower cost and less effort than other methods. Thus, it could replace conventional methods in which DCs are prepared from only a few monocytes or from iPS-DCs obtained from non-PBMCs such as fibroblasts.

In this study, we established DCs from T-iPSCs of patients with SS. This could be used in the functional examination of pathogenic T cells without any concern

regarding the preparation of monocytes or the background of donors.

RESULTS

Generation of T-iPSCs from T Cell Clones

First, to establish CD4+ T cell clones, we isolated single CD4+ T cells from PBMCs of a patient with primary SS. The patient had not been treated, and satisfied the Japanese Ministry of Health criteria for the diagnosis of SS (Fujibayashi et al., 2004).

From 384 wells of a single CD4+ cell, we obtained 32 clones. To rule out the possibility that clones were derived from feeder cells (irradiated allogenic PBMCs), we checked the haplotype of HLA-DRB1 and confirmed they were identical to that of the patient with SS. Since IFN-γ producing CD4+ T cells are thought to be involved in the pathogenesis of SS (Singh and Cohen, 2012), we checked IFN-γ production from the clones and selected four clones, SS1-9, SS3-6, SS4-6, and SS4-7, which produced more IFN-γ than the others (Figure S1A). These clones were all CD4+ T cells and did not express CD8 molecules (Figure S1B). Moreover, we confirmed their monoclonality from the TCR Vβ repertoire (Figures S1C and S1D).

Next, we transduced these clones with reprogramming factors via Sendai virus vectors, and developed iPSCs (T-iPSCs) (Nishimura et al., 2013). We obtained 12 colonies from the above four T cell clones, and of these, four colonies could be stably cultured without feeder cells (TkSST1-2, TkSST1-3, TkSST1-4 from SS3-6, and TkSST2-2 from SS4-6). These T-iPSCs had embryonic stem cell (ESC)-like morphology (Figure S2A, and data not shown) and overexpressed pluripotency markers of SSEA4, Oct4, and Nanog (Figure S2B, and data not shown). They were cultured on C3H10T1/2 cells and checked on CD34+CD43+ early hematopoietic progenitor cells, which had comparable capacity to differentiate into myeloid cells (Vodyanik et al., 2006) (Figure S2C). Since the highest proportion of CD34+CD43+ cells were TkSST2-2 compared with the other three T-iPSCs, they were selected for the following experiments. The usage of TCR Vβ and the sequence of the CDR3 region in TkSST2-2 were consistent with those of the original T cell clone (SS4-6) (Figures S1D, S2D, and S2E), demonstrating that T-iPSCs were developed from the above-mentioned single CD4+ T cell clone.
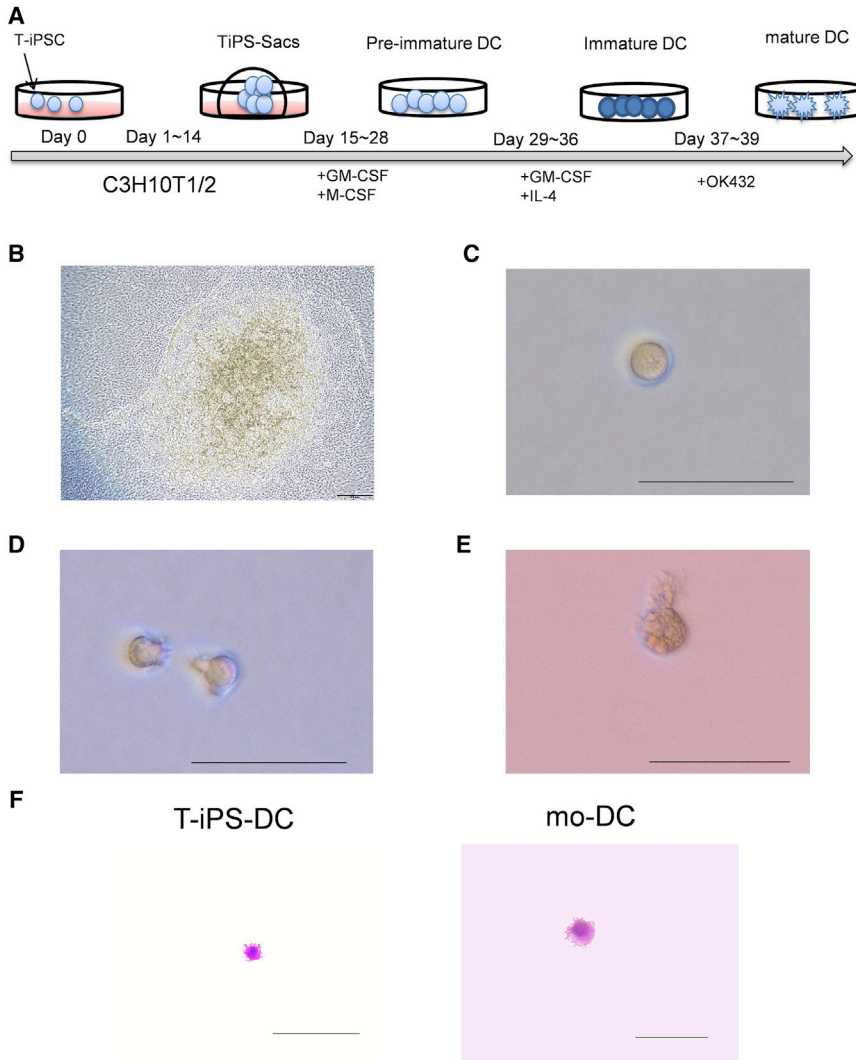
Differentiation of T-iPSCs into DCs

We induced the differentiation of T-iPSCs into DCs (T-iPS-DCs) (Figure 1A). T-iPSCs were cultured on C3H10T1/2 cells to differentiate into hematopoietic cells through TiPS-Sacs (Figure 1B). TiPS-Sacs consisted of a morphologically distinct external layer that enveloped thousands of spherical cells. TiPS-Sacs were dissociated into single cells and cultured in the presence of GM-CSF and M-CSF without feeder cells (pre-immature cells of T-iPS-DCs [preDCs]) (Figure 1C). To acquire more CD11c+ mature DCs, we evaluated the culture period (Figure S3A). As a culture period of 14 days with GM-SCF and M-SCF induced the greatest numbers of CD11c+ DCs constantly, we used that for the following protocol (Figure S3B). The expression of CD117, also known as c-kit, in the cells cultured with these cytokines for 14 days had already disappeared, and the cells did not fit the phenotype of precursor DCs (Breton et al., 2015) (Figures S3C and S3D). We added GM-CSF and interleukin-4 (IL-4) to generate immature T-iPS-DCs (Figure 1D), and finally, OK432 was added (Figure 1E). Mature T-iPS-DCs, and mo-DCs by way of comparison, were analyzed with May-Grunwald-Giemsa staining and found to have DC-like morphology (Figure 1F). Thus, mature DCs were assumed to be established from T-iPSCs.

Characterization of T-iPS-DCs

We examined cell surface markers specific for DCs. The cells within TiPS-Sacs expressed CD34, CD43 (hematopoietic cells), and CD31 (endothelial cells) (Figure 2A). This result suggests that the cells were multipotent hematopoietic progenitors. In TiPS-Sacs, HLA-DR was not detected, but CD11b, CD11c, and HLA-ABC were slightly expressed. In preDCs, CD11b and CD45 expressed higher and CD14 was detected, which is related to myeloid precursor cells (Figure 2B). In addition, CD80, CD83, and CD86 were slightly expressed but not HLA-DR. When advancing the step of immature T-iPS-DCs, not only co-stimulatory molecules (CD40, CD80, and CD86) but also HLA-DR expression were clearly observed (Figures 2C and S4A). Mature T-iPS-DCs expressed a higher level of CD40 than immature T-iPS-DCs (Figure 2D). As for the proportion of CD11c+ cells, mature and immature T-iPS-DCs were significantly higher than preDCs and TiPS-Sacs (Figure 2E). To further evaluate the phenotype of T-iPS-DCs, we checked the expression of both BDCA-1 and BDCA-3 (Figure 2F). Intriguingly, T-iPS-DCs expressed BDCA-3 but had little BDCA-1.

We also analyzed the chemokines and transcription factors specific for DCs. Although CCL17, CCL13, and MMP12 were rarely expressed in TiPS-Sacs and preDCs, they were significantly upregulated in the mature T-iPS-DCs and mo-DC (Figure 3A). The expression of MMP12 was significantly higher on mature T-iPS-DCs than immature DCs. PU.1, which is higher in myeloid DCs (mDC) compared with plasmacytoid DCs (pDC), was also detected in mature T-iPS-DCs (Nutt et al., 2005). On the other hand, pre-T cell receptor α expression, expressed in pDCs, but not in mDCs, was not detected (Figure 3B). It has been reported that BDCA-3+ DCs expressed the signature marker of CLEC9A (Haniffa et al., 2012); T-iPS-DCs expressed it

Figure 1. Differentiation of T-iPS-DCs from T-iPSCs

(A) Schematic illustration of differentiation of T-iPSCs into T-iPS-DCs.

(B–E) Phase-contrast images of TiPS-Sacs on day 13 on C3H10T1/2 feeder cells (B), pre-immature cells of T-iPS-DCs (C), immature T-iPS-DCs (D), and mature T-iPS-DCs (E). Original magnification (B) 200×, bars represent 20 μm; (C–E) 400×, bars represent 50 μm. Data are representative of ten cultured samples.

(F) May-Grunwald-Giemsa staining of mature T-iPS-DCs and mo-DCs. Original magnification 400×, bars represent 100 μm. Data are representative of three stained samples.

significantly higher than pDCs and mo-DCs (Figure 3C). These findings indicated that T-iPS-DCs, typical of the mDC phenotype, were generated.
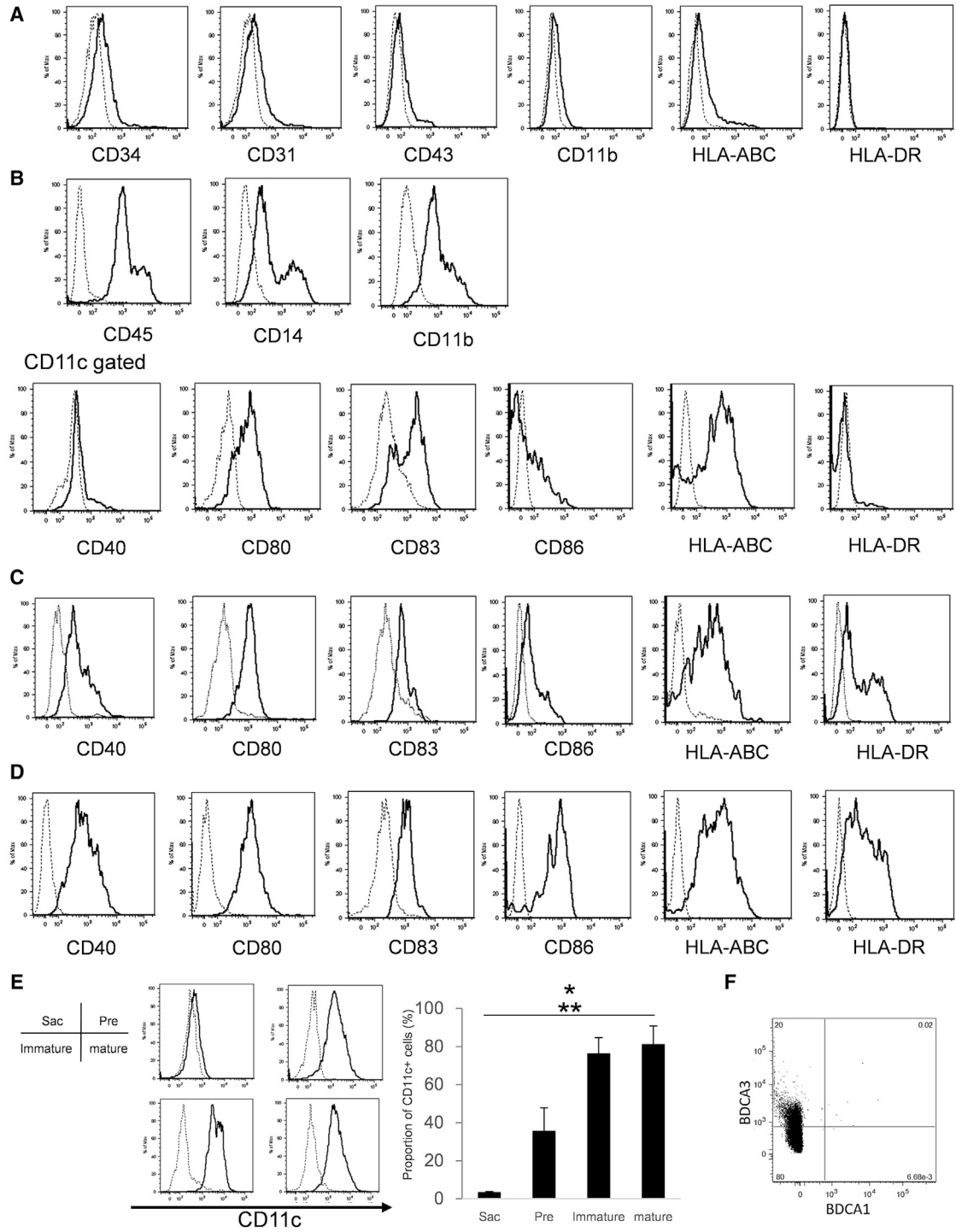
Functional Analysis of T-iPS-DCs

Since mo-DCs produced pro-inflammatory cytokines such as TNF-α and IL-6, we checked these cytokines. Although the levels of TNF-α and IL-6 from T-iPS-DCs were comparatively different from those in mo-DCs with the kind of stimulations, we detected them in mature T-iPS-DCs (Figures 4A and 4B). On another front, they, unexpectedly, did not produce IL-12p70 with any stimulations (Figure 4C).

To check the ability of phagocytosis, mature T-iPS-DCs were treated with green-labeled zymosan. It was phagocytosed by mature T-iPS-DCs (Figure 4D), and there was no significant difference in phagocytosis properties between T-iPS-DCs and mo-DCs obtained from the same patient

(Figure 4E). These findings indicated that the ability of phagocytosis in T-iPS-DCs was equal to that in mo-DCs.

We also examined allogeneic T cell responses, the capacity to stimulate naive T cells. Mo-DCs could stimulate allogenic T cells even with a lower numbers of cells. Meanwhile, mature T-iPS-DCs induced a robust proliferative response of allogeneic T cells in proportion to the cell number, and at greater numbers (1/15 ratio of T cells), there was no significant difference between T-iPS-DCs and mo-DCs (Figure 4F). This implied that when evaluating the functional studies of T cells using T-iPS-DCs, especially the reactions of allogenic T cells, we need to take care of the number of T-iPS-DCs, and over a certain level, they might have the capacity to stimulate T cells the same as mo-DCs.

Finally, we evaluated the antigen-presenting capacity of T-iPS-DCs to autologous CD4+ T cells (Figure 4G). M3R is known as one of the etiologic auto-antigens of SS, and

Figure 2. Characterization of Differentiated T-iPS-DCs

(A–D) Flow cytometric analysis of surface molecules on (A) Sacs, (B) pre-immature cells of T-iPS-DCs, (C) immature T-iPS-DCs, and (D) mature T-iPS-DCs. (C) and (D) were gated on CD11c+ cells.

(E) The expression levels and the proportion of CD11c+ cells. Error bars denote SD.

(F) BDCA-1 and BDCA-3 on mature iPS-DCs. *p < 0.05 versus mature T-iPS-DCs, **p < 0.05 versus immature T-iPS-DCs by Kruskal-Wallis test. N = 3 independent experiments. The dotted line represents the isotype control.
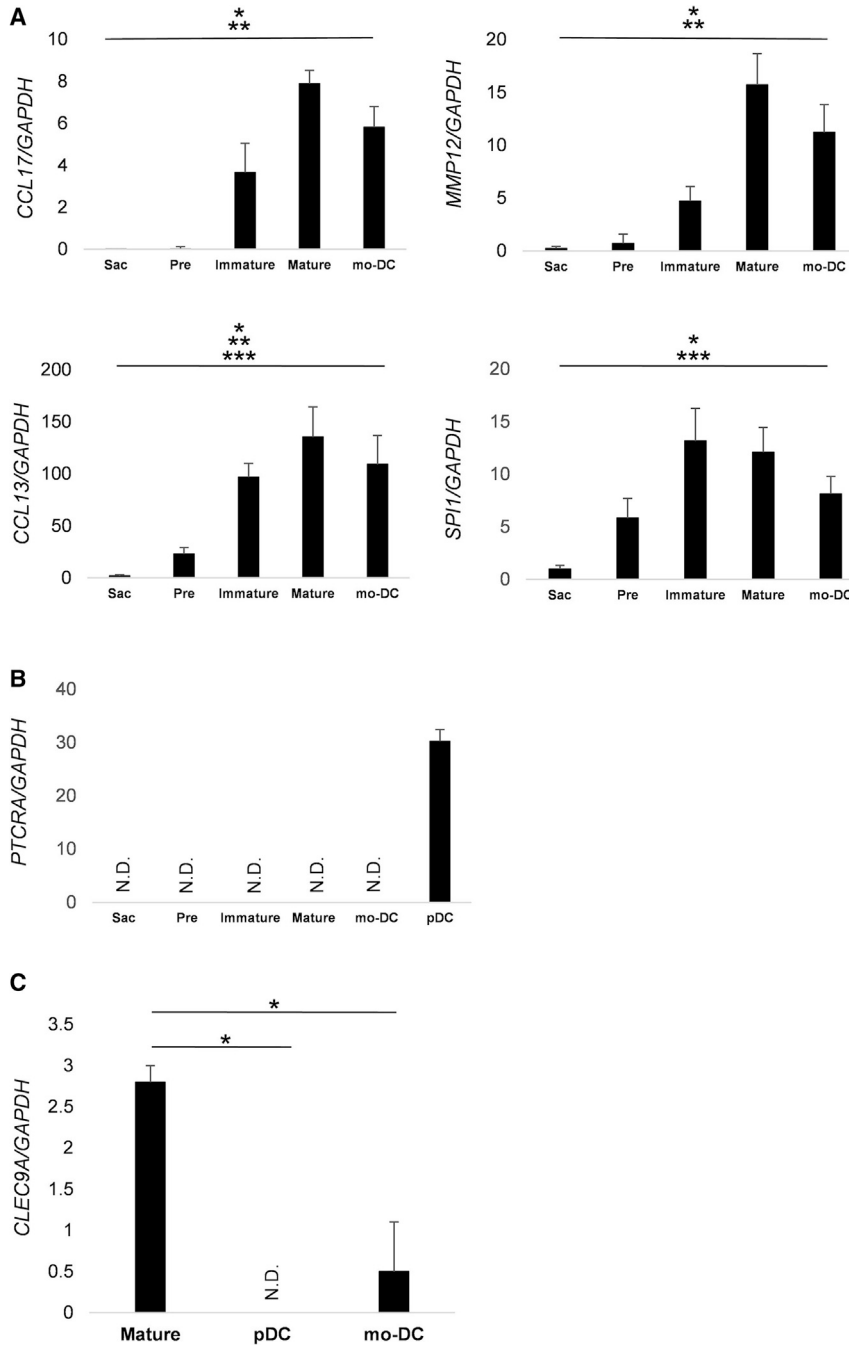
Figure 3. Gene Expression Analysis of T-iPS-DCs

(A and B) qPCR analysis in Sacs, pre-immature cells of T-iPS-DCs (Pre), immature T-iPS-DCs (Immature), mature T-iPS-DCs (Mature), mature monocytes-derived DCs (mo-DC), and plasmacytoid DCs (pDCs). Data were normalized to the reference gene, *GAPDH*. Values are means ± SD. *p < 0.05 versus mature T-iPS-DCs, **p < 0.05 versus mo-DCs, ***p < 0.05 versus immature T-iPS-DCs by Kruskal-Wallis test.

(C) qPCR analysis of CLEC9A. *p < 0.05 by Mann-Whitney's test. N = 3 independent experiments.

Error bars denote SD.

the important role of the autoimmune response against M3R has been clarified (Naito et al., 2006; Iizuka et al., 2010). M3R reactive CD4+ T cells were detected in PBMCs of patients with SS, and the proportion of these cells was significantly higher than in HLA-DRB1-matched healthy donors (Figure S4B). Thus, we checked IFN-γ production from M3R reactive CD4+ T cell lines under M3R peptide stimulation using T-iPS-DCs. A significantly higher level of IFN-γ was produced when cultured with M3R antigens,

the same as mo-DCs. These results indicated that T-iPS-DCs were able to process antigens.

The Analyses of T Cell Function in SS Using T-iPS-DCs

Although T cells from patients with systemic lupus erythematosus (SLE) were reported to be poorer responders to allogenic non-T cells (Russell et al., 1983), the response in patients with SS remains unknown. Thus, we evaluated the response of allogenic T cells, using T-iPS-DCs. As for patients
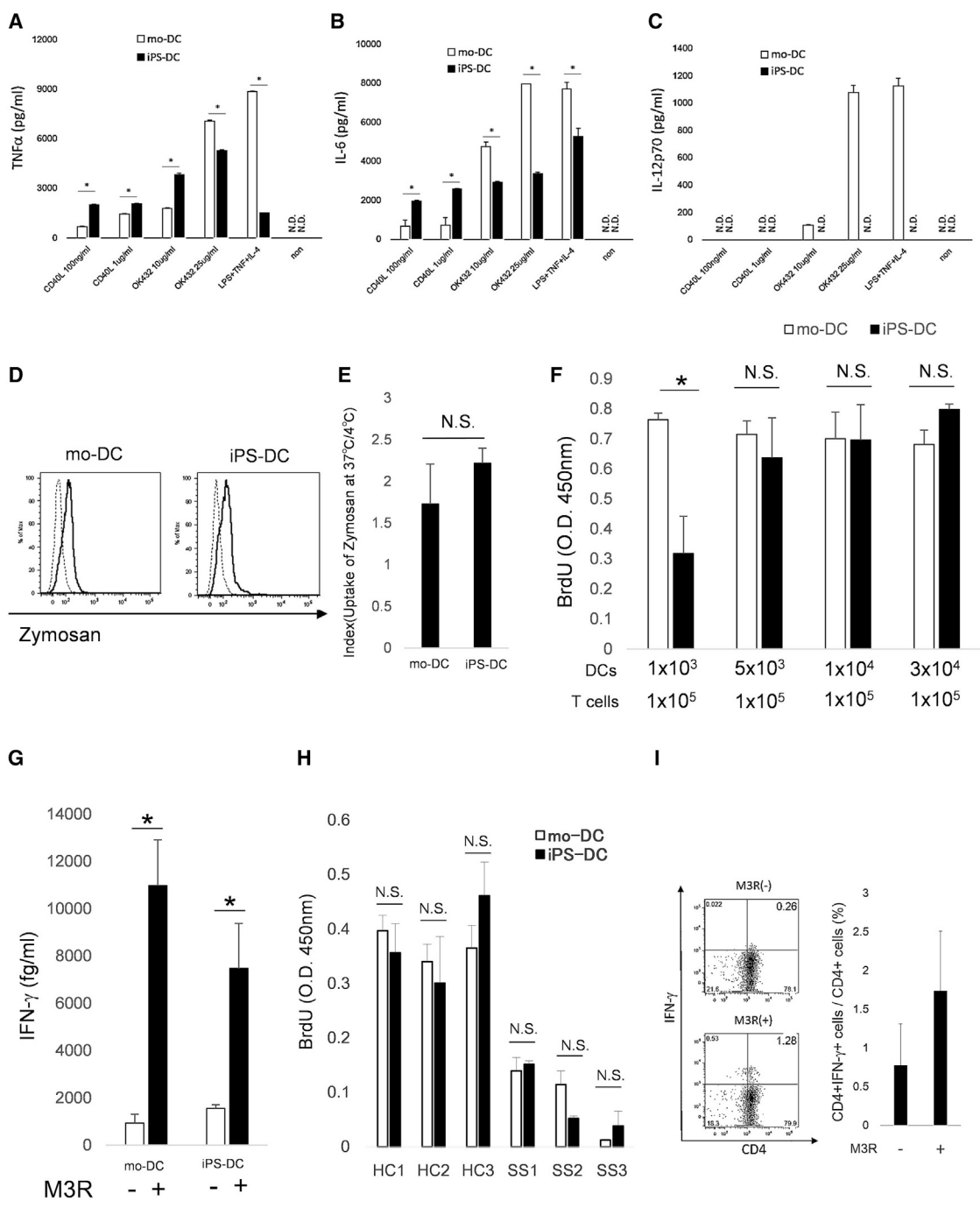
Figure 4. Functional Analysis of T-iPS-DCs

(A–C) Production of (A) TNF-α, (B) IL-6, and (C) IL-12p70 in mature T-iPS-DCs and mo-DCs stimulated for 72 hr with TNF-α, lipopolysaccharide, and IL-4 or OK432 (10 μg/mL, 25 μg/mL) or CD40L (100 ng/mL, 1 μg/mL). Values are means ± SD. *p < 0.05 by Mann-Whitney test.

(D) Flow cytometric analysis of CD11c gated T-iPS-DCs incubated with green-labeled zymosan for 60 min at 37°C (solid line) or 4°C (dotted line).

(E) Index mean value of the ratio of zymosan incorporation at 37°C and 4°C. Values are means ± SD. N.S., not significant.

(F) T-iPS-DCs or mo-DCs were co-cultured with allogeneic T cells for 5 days. Proliferation of T cells was measured based on bromodeoxyuridine uptake in the last 20 hr of the culture. *p < 0.05 by Mann-Whitney's test. Data were run in triplicate.

(legend continued on next page)

with SS, not only proliferation but also activation, such as IFN-γ production, of allogenic T cells had a propensity to be lower than in healthy donors (Figures 4H and S4C).

Moreover, we tried to detect M3R-reactive CD4+ T cells. As expected, we were able to detect M3R-reactive T cells using T-iPS-DCs, and they were comparable with mo-DCs (Figures 4I and S4B).

These results indicate that, using T-iPS-DCs, we can analyze some of the T cell functions of SS, which could elucidate the steps in its pathology.

DISCUSSION

The results indicate that T-iPSCs were developed from a single CD4+ T cell from a patient with SS, through the establishment of CD4+ T cell clones, and were differentiated into an adequate amount of functional DCs via TiPS-Sacs.

First, we established CD4+ T cell clones from PBMCs of a patient with SS. Although various T cell clones have been established from other autoimmune diseases (Greidinger et al., 2004), as far as we know, there are only a few reports on the development of CD4+ T cell clones from SS. SS is characterized by the appearance of lymphopenic conditions and a decrease in T cell response to IL-2 (Goules and Tzioufas, 2016; Alcocer-Varela et al., 1984). Under these unfavorable conditions, we were able to develop CD4+ T cell clones smoothly. Since IFN-γ-producing CD4+ T cells are thought to be involved in the pathogenesis of SS (Singh and Cohen, 2012), we checked IFN-γ production and selected clones.

When evaluating the immune responses, especially in patients with uncommon diseases such as SS, it is critical to acquire sufficient numbers of mature DCs periodically. Haruta et al. (2013) developed a method to amplify monocytes by lentivirus-mediated transduction of cMYC along with BMI1. This method is easy and solid, but is dependent on the efficiency of virus transduction. In fact, it was difficult to transduce these genes into CD14 cells from our patient with SS efficiently (data not shown). Their development from ESCs or iPSCs derived from human fibroblasts of healthy donors has been also reported (Choi et al., 2009; Senju et al., 2007, 2011). In the latter case, mouse embryonic fibroblasts were used to maintain human iPSCs, and feeder layers of mouse OP9 were used to induce hematopoi-

etic cells. In our study, T-iPSCs were maintained under feeder-free conditions, and they were transferred on C3H10T1/2 feeder cells to generate TiPS-Sacs. Takayama et al. (2008) found sac-like structures after human ESCs were cultured on C3H10T1/2 cells, which contained multipotent hematopoietic progenitors. Our data showed that TiPS-Sacs also included CD34-, CD31-, and CD43-positive cells. Unexpectedly, the slight expression of CD11c, which is evident on both mature and precursor DCs (Diao et al., 2006), was confirmed on TiPS-Sacs.

Under the maturation of T-iPS-DCs, we detected high expression levels of CCL17, CCL13, and MMP12, which were comparable with mo-DCs. They are selectively produced by mDCs, but not by pDCs, and are upregulated after the immature state of mDCs (Penna et al., 2002; Tang and Saltzman, 2004). PU.1, which is required for the generation of myeloid-derived DCs, was also detected (Guerriero et al., 2000). On the other hand, T-iPS-DCs did not express pre-T cell receptor α, expressed with pDCs (Spits et al., 2000). Furthermore T-iPS-DCs expressed BDCA-3 but not BDCA-1, and in fact higher expression of CLEC9A was detected (Haniffa et al., 2012). Considered together, our results indicate that T-iPS-DCs were myeloid lineage cells, consistent with those of previous studies on the generation of DCs from ESCs or iPSCs (Slukvin et al., 2006; Senju et al., 2011), and differentiated into BDCA-3+ DCs.

The ability of antigen uptake in T-iPS-DCs was equal to that of mo-DCs, but there was a slight difference in cytokine production. T-iPS-DCs produced much more IL-6 and TNF-α than mo-DCs, especially when cultured with CD40L stimulation, but much less with other stimulations. T-iPS-DCs had a tendency to express CD40 higher than mo-DCs, and also express other co-molecules such as CD80 and CD86 less (data not shown), which might explain the differences. Moreover, T-iPS-DCs did not produce IL-12p70 at all. As discussed above, T-iPS-DCs expressed BDCA-3, and it was reported that BDCA-3+DCs produced very little or no IL-12 (Jongbloed et al., 2010; Haniffa et al., 2012), which might be one of the reasons.

There was also a slight difference in allogenic T cell stimulation. Although T-iPS-DCs in a ratio of more than 1/15 to T cells could stimulate them as well as mo-DCs, the lower number of T-iPS-DCs (1/100 of T cells) was insufficient compared with that of mo-DCs. One of the reasons for this difference might be the expression levels of co-stimulatory

(G) T-iPS-DCs or mo-DC pre-incubated with or without M3R peptide (10 μg/mL) were co-cultured with the M3R-reactive T cell line (5 × 10^4 cells/well). Autoreactive CD4+ T cell responses were evaluated. *p < 0.05 by Mann-Whitney test. N = 3 independent experiments.

(H) T-iPS-DCs or mo-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. Proliferation of T cells was measured.

(I) T-iPS-DCs (1 × 10^4 cells/well) 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.

Error bars denote SD.

molecules, which are known to play critical roles in T cell activation (Orabona et al., 2004). In addition, BDCA3+ DCs were reported to stimulate allogenic T cells less than mo-DCs, depending on the number of DCs (MacDonald et al., 2002; Chu et al., 2012). Although further studies are needed, these data might support the slight difference in T cell stimulation between T-iPS-DCs and mo-DCs.

It is not difficult to prepare a large amount of T-iPS-DCs because there are no limitations to expanding T-iPSCs. From only one well of 80% confluent T-iPSCs (6-well plate; 9.6 cm^2), we obtained about $3–5 \times 10^5$ cells of DCs, repeatedly (Figure S3B). On the other hand, about 10 mL of blood must be collected to obtain the same number of DCs from CD14+ monocytes, each time. Thus, we were able to obtain stable and functional DCs from only a small blood sample from the donor.

Taking into account the functional analyses of T-iPS-DCs, we evaluated T cell functions of SS. As expected from the genetic similarities between SLE and SS (Teruel and Alarcon-Riquelme, 2016), we confirmed that T cells from patients with SS, like SLE, were poorer responders to allogenic non-T cells than T cells from healthy donors (Russell et al., 1983). Furthermore, we could evaluate M3R-reactive CD4+ T cells, which are thought to be one of the pathogenic T cells for SS.

The present study describes the generation of functional DCs from T-iPSCs prepared from a single patient with SS. T-iPS-DCs could be useful for characterization of pathogenic T cells in autoimmune diseases such as SS and elucidate the pathogenicity of autoreactive T cells.

EXPERIMENTAL PROCEDURES

Differentiation of T-iPS-DCs from T-iPSCs

T-iPSCs were differentiated into hematopoietic cells as previously reported (Nishimura et al., 2013). They were first cultured in αMEM with 20% fetal bovine serum (FBS), GM-CSF (100 ng/mL), and M-CSF (50 ng/mL), and cultured again in RPMI-1640 medium with 10% FBS, GM-CSF (100 ng/mL), and IL-4 (10 ng/mL) to generate immature T-iPS-DCs. OK432 (10 μg/mL) was added to induce mature T-iPS-DCs. This study was approved by the local ethics committees of each participating institution and a signed informed consent was obtained from each subject.

Statistical Analysis

Data are expressed as the mean ± SD. Differences between groups were examined using the Mann-Whitney test and Kruskal-Wallis test. A p value <0.05 denotes the presence of a statistically significant difference.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.04.010.

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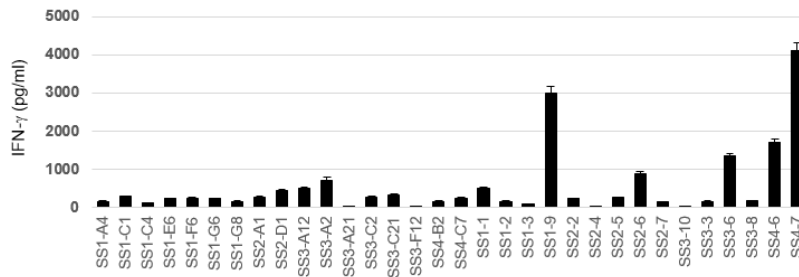
Supplemental Information

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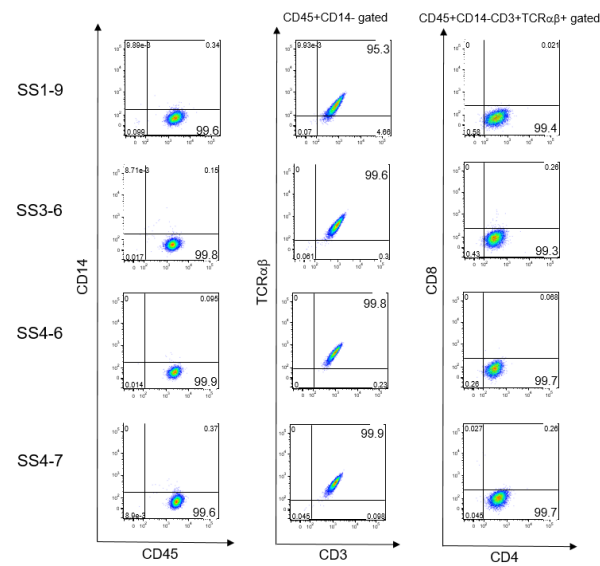
T Cell Clones of Sjögren's Syndrome

Mana Iizuka-Koga, Hiromitsu Asashima, Miki Ando, Chen-Yi Lai, Shinji Mochizuki, Mahito Nakanishi, Toshinobu Nishimura, Hiroto Tsuboi, Tomoya Hirota, Hiroyuki Takahashi, Isao Matsumoto, Makoto Otsu, and Takayuki Sumida
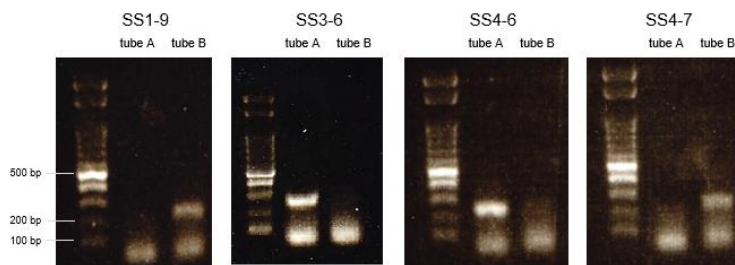
(A)

IFN-γ (pg/ml)
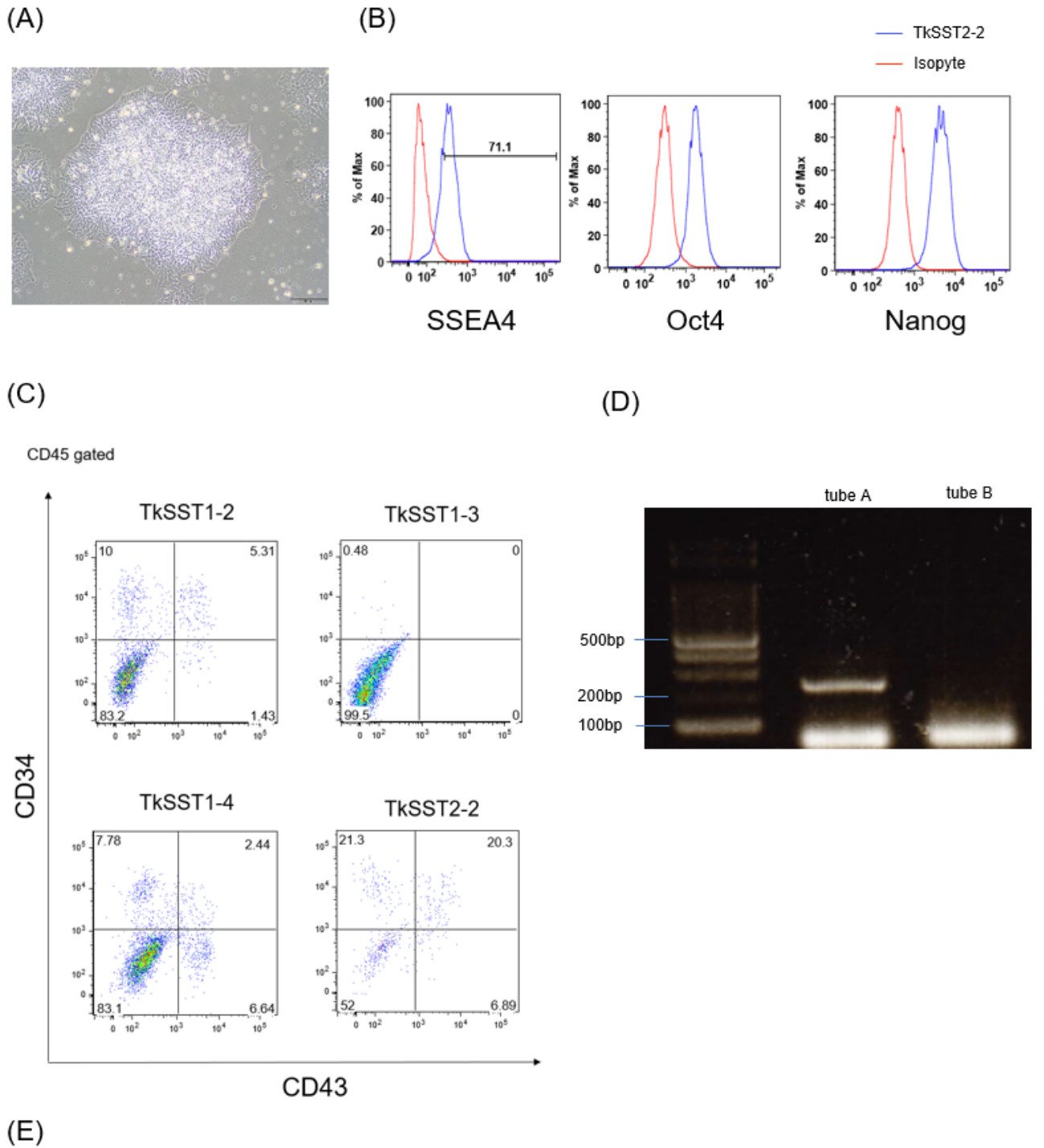
5000
4000
3000
2000
1000
0

SS1-A4 SS1-C1 SS1-C4 SS1-E6 SS1-F6 SS1-G6 SS1-G8 SS2-A1 SS2-D1 SS3-A12 SS3-A2 SS3-A21 SS3-C2 SS3-C21 SS3-F12 SS4-B2 SS4-C7 SS1-1 SS1-2 SS1-9 SS2-2 SS2-4 SS2-5 SS2-6 SS2-7 SS3-10 SS3-3 SS3-6 SS3-8 SS4-6 SS4-7

(B)

CD45+CD14- gated   CD45+CD14-CD3+TCRαβ+ gated

SS1-9
SS3-6
SS4-6
SS4-7

CD14
CD45
TCRαβ
CD3
CD8
CD4

(C)

SS1-9   SS3-6   SS4-6   SS4-7
tube A tube B   tube A tube B   tube A tube B   tube A tube B

500 bp
200 bp
100 bp

(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	TCCTACGACGAGTACTTC
	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

(A)

(B)

TkSST2-2
Isopyte

71.1

SSEA4

Oct4

Nanog

(C)

CD45 gated

TkSST1-2

10   5.31

83.2   1.43

TkSST1-3

0.48   0

99.5   0

TkSST1-4

7.78   2.44

83.1   6.64

TkSST2-2

21.3   20.3

52   6.89

CD34

CD43

(D)

tube A   tube B

500bp

200bp

100bp

(E)

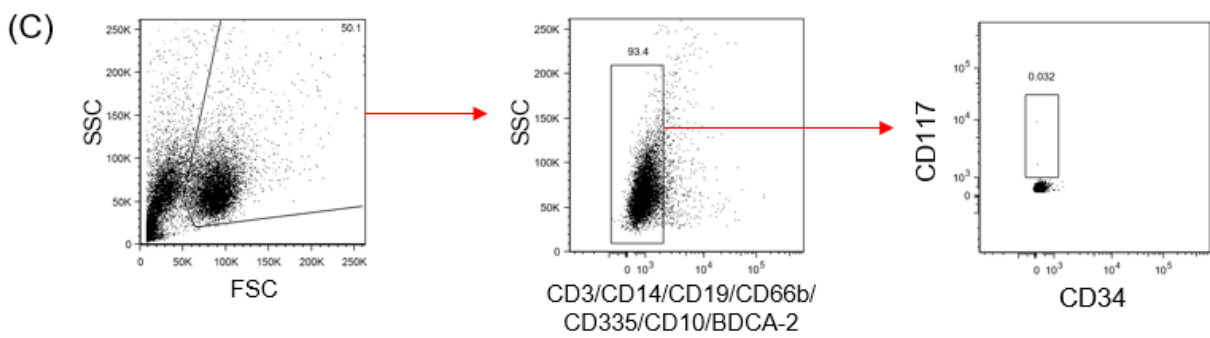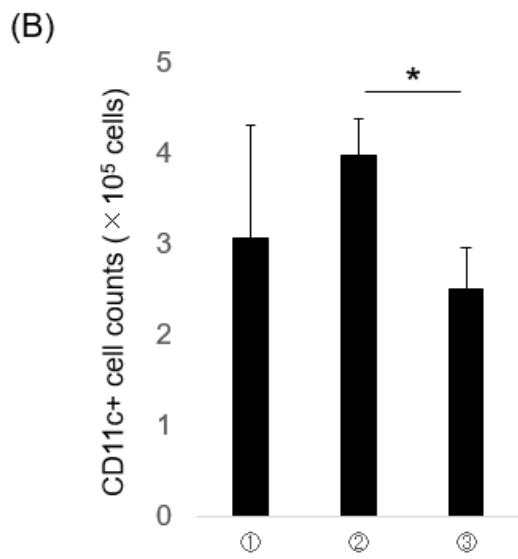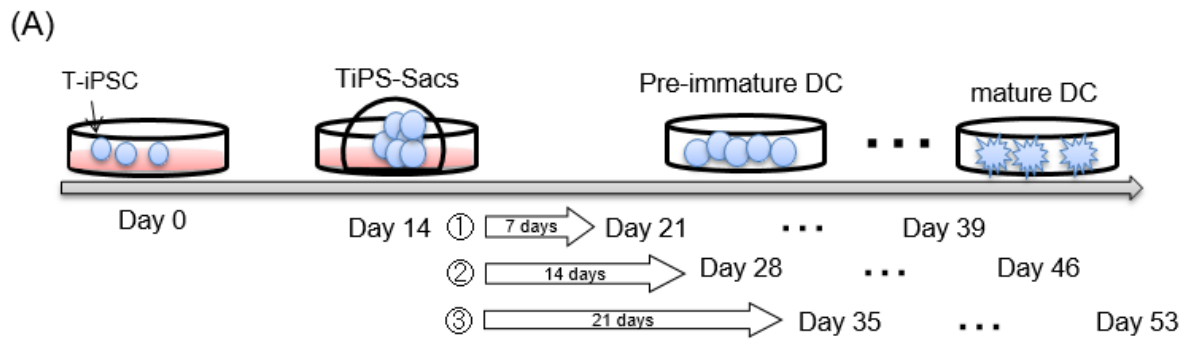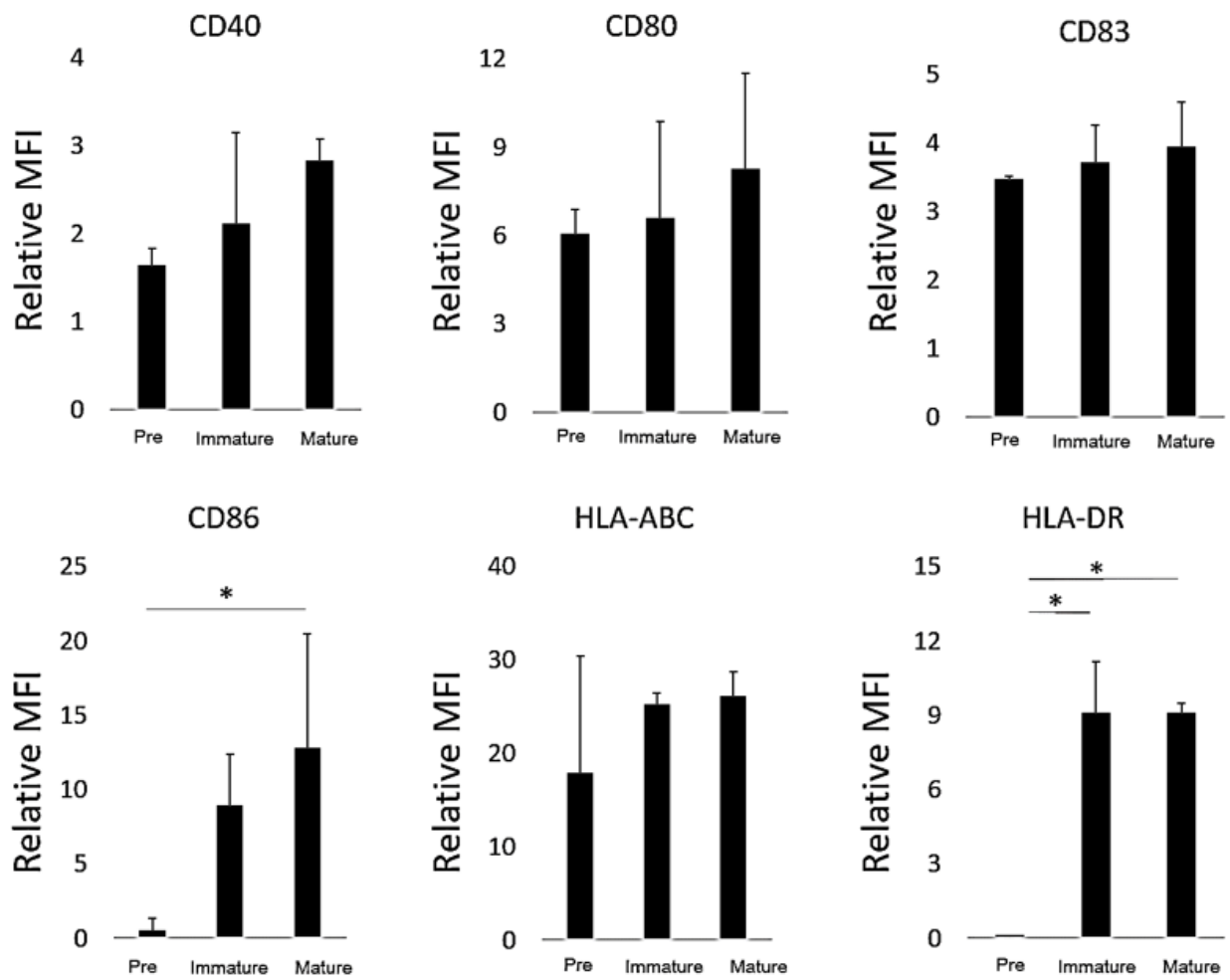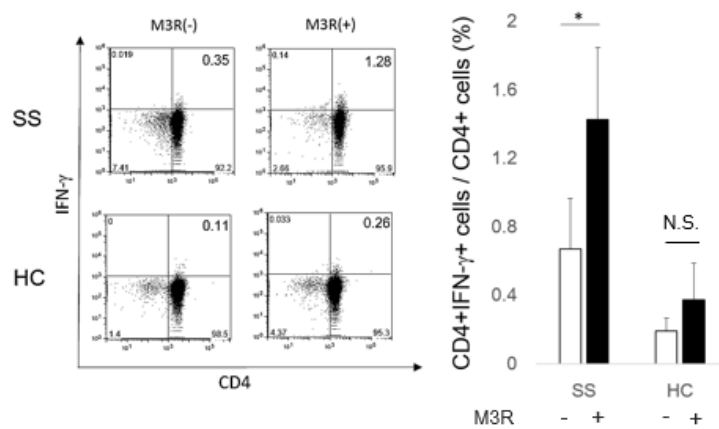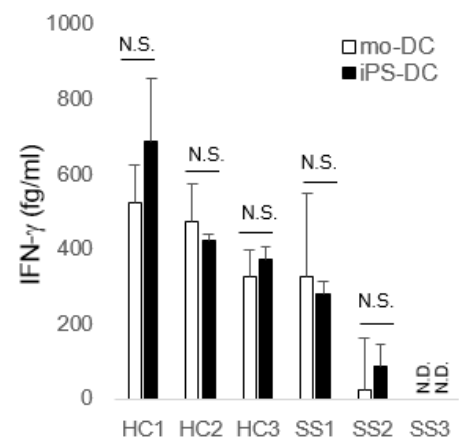| TiPSC	Productivity	Rearrangement			Sequence of Junctional region		
		Vβ	Dβ	Jβ	3'Vβ	N1-Dβ-N2	5'Vβ
TkSST 2-2	**Productive**	TRBV4-3*04	TRBD2*02	TRBJ2-7*01	TGCGCCAGCAGCC	TAGGACTAGCGGGAATT	TCCTACGAGCAGTACTTC
	Unproductive	TRBV19*01	TRBD2*01	TRBJ1-1*01	TGTGCCA	CCCTCCGGGGGATTGCAG	AGTCTTTCTTT

Figure S4

- 4 -
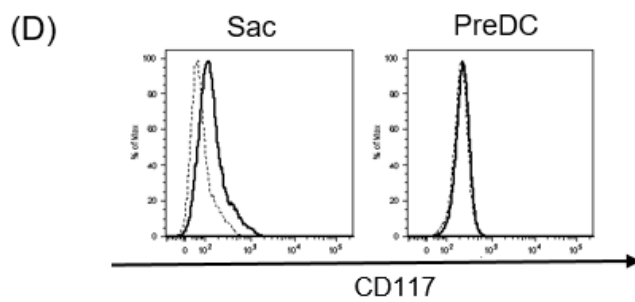
Figure S1. Generation and selection of CD4+ T cell clone. Related to Figure 1

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 x 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) ($5x10^4$ 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 (1×10^5 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 (1×10^5 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).

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