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メタデータ	言語: English 出版者: 公開日: 2021-04-05 キーワード (Ja): キーワード (En): 作成者: Mitani, Kosuke, Ito, Yuki, Takene, Yukio, Hatoya, Shingo, Sugiura, Kikuya, Inaba, Toshio メールアドレス: 所属:
URL	http://hdl.handle.net/10466/00017315

Long-Term Trypsin Treatment Promotes Stem Cell Potency of Canine Adipose-Derived Mesenchymal Stem Cells

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A brief running title: Long-term trypsin treatment-tolerant cells

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Keywords: canine, mesenchymal stem cells, adipose tissue-derived stem cells, ADSCs

20 **Abbreviations**

21 ADSCs Adipose-Derived Mesenchymal Stem Cells

22 CTAC Canine Thyroid Adenocarcinoma Cells

23 CXCR-4 C-X-C chemokine Receptor type 4

24 LTT Long-Term Trypsin Treatment

25 MSCs Mesenchymal Stem Cells

26 Muse cells Multilineage-differentiating Stress-Enduring cells

27 R/T Room Temperature

28 SDF-1β Stromal cell Derived Factor 1β

29 S1P Sphingosine-1-Phosphate

30 S1PR2 Sphingosine-1-Phosphate Receptor 2

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Abstract

Mesenchymal stem cells (MSCs) isolated from adipose tissue (adipose-derived stem cells, ADSCs) are considered one of the most promising cell types for applications in regenerative medicine. However, the regenerative potency of ADSCs may vary due to heterogeneity. Long-term trypsin treatment (LTT) is known to significantly concentrate multilineage-differentiating stress-enduring (Muse) cells from human MSCs. In this study, we aimed to generate cells with high stem cell potency from canine ADSCs using LTT. After 16 h of treatment with trypsin, surviving ADSCs (LTT-tolerant cells) had significantly enhanced expression of stage-specific embryonic antigen (SSEA)-1, a mouse embryonic stem cell marker, and fucosyltransferase 9, one of several fucosyltransferases for SSEA-1 biosynthesis. However, LTT-tolerant cells did not enhance the expression of SSEA-3, a known human Muse cell marker. LTT-tolerant cells, however, showed significantly higher self-renewal capacity in the colony-forming unit fibroblast assay than ADSCs. In addition, the LTT-tolerant cells formed cell clusters similar to embryoid bodies and expressed undifferentiated markers. Moreover, these cells differentiated into cells of all three germ layers and showed significantly higher levels of α 2–6 sialic acid (Sia)-specific lectins, known as differentiation potential markers of human MSCs, than ADSCs. LTT-tolerant cells had a normal karyotype and had low telomerase activity, showing little carcinogenetic potency. LTT-tolerant cells also showed significantly increased activity of transmigration in the presence of chemoattractants and had increased expression of migration-related genes compared to ADSCs. In

58 addition, LTT-tolerant cells had stronger suppressive activity against mitogen-stimulated
59 lymphocyte proliferation than ADSCs. Overall, these results indicated that the LTT-tolerant cells
60 in canine ADSCs have similar properties as human Muse cells (although one of the undifferentiated
61 markers is different) and are expected to be a promising tool for regenerative therapy in dogs.
62
63

64 ## Introduction

65 The clinical application of stem cells in regenerative medicine is expected to result in tissue
66 repair and functional recovery of lost tissues in many intractable diseases. Regenerative research
67 using embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, and mesenchymal stem
68 cells (MSCs) have been actively conducted.

69 Although ES cells and iPS cells have the ability to differentiate into three germ layers, these
70 cells have a tumorigenic risk *in vivo* [1-3]. Hence only differentiated cells must be directly
71 transplanted to damaged tissues [4]. On the contrary, MSCs have some advantages, such as low
72 risk of malignant transformation and homing ability to damaged tissues, despite their limited
73 differentiation potential [5-7]. In addition, MSCs have been reported to secrete a variety of growth
74 factors and cytokines, inhibit T cell activation, and exhibit trophic effects of angiogenesis [6-10].
75 Hence, MSCs are viable cells with high potential for clinical application.

76 MSCs are typically isolated as adherent cells from the mesenchymal tissues. Owing to the

77 simple methods of isolation, MSCs are a heterogeneous population of cells containing cells other
78 than stem cells such as endothelial cells, fibroblasts and progenitor cells [4,5,11]. In addition, it
79 has been indicated that there are some subpopulations of MSCs with a variety of stemness [12-15].
80 Therefore, MSCs are expected to be purified to enhance the high therapeutic effects.

81 Multilineage-differentiating stress-enduring (Muse) cells that are double-positive for stage-
82 specific embryonic antigen (SSEA) -3/CD105. They are rare pluripotent subpopulations within
83 human MSCs and have the ability to differentiate into three germ layers [1]. Some of the
84 advantages of Muse cells are that they have the high homing capacity to damaged tissues, can
85 differentiate into specific or a variety of cells depending on the environment, and can contribute to
86 tissue-repair and functional recovery by intravenous (IV) administration [16-18]. On the contrary,
87 ES cells and iPS cells under undifferentiated state cannot be administered to patients. Therefore,
88 Muse cells are the ideal cell-type and superior to ES cells and iPS cells for clinical application [1,
89 3].

90 A first step in the clinical application of Muse cells is to validate the scientific method and to
91 collect the data using animal models. Dogs are unrivaled animal models for research of human
92 diseases [19]. They share a common living environmental and lifestyle with humans. It has been
93 reported that similar and spontaneous diseases such as intervertebral disc herniation, inflammatory
94 bowel disease, Sjogren's disease, and osteoarthritis occur in dogs, unlike in laboratory rodents [20].
95 The results from this study and the development of Muse cell therapy in dogs might have the

potential to serve as an animal model with future applications in human medicine.

Muse cells are collected by cell sorting from human MSCs [21] and by putting lipoaspirated human fat under severe cellular stress treatment [22]. However, these methods are not convenient and reasonable for clinical application because specific equipment and a large quantity of fat are necessary. On the contrary, long-term trypsin treatment (LTT) is an effective method of collecting Muse cells from human MSCs [1] and is easy to use. Adipose-derived stem cells (ADSCs) consist a type of MSCs that are accessible and useful for clinical applications because fat tissues can be collected easily and compared to bone marrow-derived stem cells (BMSCs), ADSCs have high proliferative ability [23]. The aim of this study was to generate the cells with the high stem cell potency from canine ADSCs with LTT compared with ADSCs without LTT.

Materials and Method

Ethics statement

The animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka Prefecture University. The animals were neither operated on, nor sacrificed for this study. The uses of tissues were approved by the ethics committee of a local veterinary clinic. All dogs were privately owned and the owners provided consent before the samples were collected."

115 ***Isolation and culture of canine ADSCs and LTT-tolerant cells***

116 Adipose tissues were obtained from 10 female healthy dogs between 10 and 18 months of age
117 during surgery for spaying by clinical veterinarians. All collected samples were stored at 4 °C and
118 processed within 4 h of sampling. ADSCs were isolated from abdominal adipose tissues according
119 to a previously published method [24] with modifications, using the Canine Adipose-Derived Stem
120 Cell Culture Kit (J-ARM Co., Ltd., Osaka, Japan). In brief, fat tissues were incubated in
121 collagenase (FUJIFILM, Tokyo, Japan) solution (2 mg/mL) for 1 h at 37 °C, and then centrifuged
122 at 340 *g* for 5 min. The cell pellet was resuspended in 5 mL Dulbecco's Phosphate-Buffered Saline
123 (DPBS) (FUJIFILM) and centrifuged again under the same conditions. Finally, the pellet was
124 resuspended in prewarmed Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal
125 bovine serum and 1% antibiotic and incubated at 37 °C with 5% CO_2 and 95% humidity for 48 h.
126 Subsequently, the adherent cells were washed with DPBS and placed in fresh culture medium.
127 Upon reaching 70% confluency, cells were trypsinized, and the cells were subcultured until
128 passage 1. The cells were then cryopreserved, thawed, and cultured at the time of use for this
129 experiment. Following previously described methods [1], expanded ADSCs were exposed under
130 0.05% trypsin solution for 16 h without CO_2 exchange at 37 °C. LTT-tolerant cells were collected
131 by centrifugation at 536 *g* for 15 min, cultured in suspension in a 75T Flask for 5–7 days, and
132 subcultured in adherent according to previously described methods [1].

133

134 ***Flow cytometry***

135 Flow cytometric analysis was performed to evaluate the cell surface markers according to
136 previously described methods [21, 25]. 1×10^6 LTT-tolerant cells or ADSCs from four dogs were
137 washed and resuspended in 100 μL FACS buffer and incubated with fluorescent isothiocyanate
138 (FITC)-labeled antibody for CD14 and CD45 phycoerythrin-labeled antibody for CD44,
139 nonlabelled antibody for CD90, SSEA-1, and SSEA-3. Isotype control was used in a negative
140 control sample. And then, the cells were incubated with corresponding secondary antibodies. Data
141 were measured using flow cytometry (10,000 events; Attune™ NxT; Thermo Fisher Scientific,
142 Waltham, USA), repeated twice, and analyzed using the Attune NxT software (Thermo Fisher
143 Scientific). The antibodies are described in Supplementary Table S1.

144

145 ***Induced differentiation of ADSCs and LTT-tolerant cells in vitro***

146 Cells from five dogs were evaluated for differentiation. For osteoblast and adipocyte
147 differentiation, 5×10^4 LTT-tolerant cells (at passage 3) or ADSCs (at passage 2 or 3) were seeded
148 onto each well of a six-well culture plate in canine induction medium for 14 days to osteoblasts
149 and for 21 days to adipocytes. For chondrocyte differentiation, 2×10^5 ADSCs (at passage 2) were
150 seeded into each a well of a 96V-well culture plate by induction medium for 21 days [21, 26]. For
151 hepatocyte differentiation, 2×10^4 LTT-tolerant cells (at passage 3) were seeded into each well of
152 a collagen coated 48-well cell culture plate by the induction medium for 14 days [21]. For neural

153 cell differentiation, 1×10^5 LTT-tolerant cells (at passage 3) were seeded as non-adherent cells in
154 polyHEMA coated 25T Flask in the presence of neural induction medium for 7 days. The cells
155 were then transferred to recombinant laminin-coated PLL 48-well cell culture plate and cultured
156 for another 10 days as adherent cells in the induction medium to differentiate into neurons [21].
157 Medium change was performed every other day. After induction, the cells were stained with von
158 Kossa staining for osteoblasts, oil Red O staining for adipocytes, Alcian blue staining for
159 chondrocytes and immunocytochemistry for hepatocytes and neural cells and repeated twice
160 according to previously described methods [1, 26].
161
162 ***Immunocytochemistry for pluripotent stem cell, hepatocyte and neural cell markers***
163 Cells from five dogs were evaluated for immunocytochemistry. Immunocytochemistry was
164 performed to confirm their pluripotency in LTT-tolerant cells according to previously described
165 methods [1]. LTT tolerant cells placed in suspension culture at days 5–7 and induced for neural
166 precursor cells were collected by centrifugation, fixed, embedded in OCT compound, and frozen
167 at -80 °C. Subsequently, the cryosections were cut to 5 μm thickness using a cryostat (HM525NX7;
168 Thermo Fisher Scientific). LTT-tolerant cells induced for hepatocytes and neural cells in adherent
169 culture were fixed. After washing, the cells were treated with 0.5% Triton™ X-100 and blocked
170 with blocking solution. After another wash, the cells were incubated with primary antibodies
171 overnight at 4 °C. Anti-SSEA-1, SSEA-3, NANOG, OCT3/4, SOX2, and TRA-1 antibodies for

172 pluripotency stem cell markers; anti-albumin and alpha 1 fetoprotein antibodies for endodermal
173 lineage; and anti-NESTIN, Musashi-1, NeuroD 2, and MAP-2 antibodies for ectodermal lineage
174 were used as the primary antibodies. The cells were washed again and incubated with secondary
175 antibodies under protection from light at room temperature (R/T) for 2 h. Finally, cells were
176 washed and treated with DAPI for nuclear staining (Sigma-Aldrich, St. Louis, USA). Cells were
177 repeated two times for immunocytochemistry. The primary and secondary antibodies are described
178 in Supplementary Table S1. Images were taken with a fluorescence microscope (Keyence, Osaka,
179 Japan).
180
181 ***Colony-forming unit fibroblast (CFU-F) assay***
182 Cells from seven dogs were evaluated for Colony-forming unit fibroblast (CFU-F) assay. A
183 total of 500 cells were seeded onto each well of a six-well culture plate for 14 days in a culture
184 medium. After 14 days, the adherent cells were washed with DPBS and fixed with 4%
185 paraformaldehyde phosphate buffer solution at R/T for 15 min. The cells were stained with 1mL
186 Giemsa stain solution at R/T for 30 min. The average number of cells in each colony in three wells
187 was determined by counting, and colonies containing > 50 cells were scored.
188
189 ***Alkaline phosphatase (ALP) Activity***
190 Cells from five dogs were evaluated for alkaline phosphatase (ALP) assay. 1×10^5 LTT-

191 tolerant cells (days 5–7 in suspension culture) were washed three times with a sufficient volume
192 of saline. These cells in 1 ml of the buffer were stained using a leukocyte alkaline phosphatase kit
193 (Sigma-Aldrich) according to the manufacturer's instructions.
194
195 ***Karyotype analysis***
196 Cells from three dogs were evaluated for karyotype analysis. The karyotype of LTT-tolerant
197 cells was analyzed to examine their influence on LTT according to previously described methods
198 [27]. These cells were cultured in suspension until day 6 and subcultured in an adherent culture.
199 Colcemid (0.1 mg/mL, Thermo Fisher Scientific) was added to the 75T flask for 5 h. The cells
200 were treated with trypsin and collected by centrifugation, and 0.075 M potassium chloride was
201 added in the pellets at 37 °C for 15 min. Subsequently, Carnoy's fluid was added. The pellets were
202 dropped to a glass slide, and the number of chromosomes was evaluated every two times and
203 analyzed by Giemsa staining.
204
205 ***Lectins and antibodies***
206 PSL1a lectin (FUJIFILM), which is known to be a marker of the differentiation potential of
207 human MSCs, was labeled using fluorescein labeling kit-NH_2 (Dojindo, Kumamoto, Japan)
208 according to the manufacturer's protocol. ADSCs and LTT-tolerant cells in suspension from five
209 dogs were incubated at 4 °C for 30 min with FITC-labeled PSL1a lectin (6 μg/mL), according to

210 previously described methods [28]. Cells were washed several times with FACS buffer and
211 measured using flow cytometry, repeated twice, and analyzed using the AttuneTM NxT software.
212
213 ***Telomerase activities***
214 *Telomere reverse transcriptase (TERT)*, which is correlated with tumorigenic activities, of
215 LTT-tolerant cells in suspension culture and ADSCs in adherent culture at day 7 from five dogs
216 were examined by quantitative reverse transcription-PCR (RT-qPCR) analysis. Canine thyroid
217 adenocarcinoma cells (CTAC) were used as a positive control.
218
219 ***Migration ability***
220 Cells from six or seven dogs were evaluated for migration assay. Migration was assayed in
221 24-well cell culture companion plates (Corning, Glendale, USA) with 8-μm pore inserts (Corning),
222 according to previously described methods [29]. LTT-tolerant cells in suspension or ADSCs in
223 adherence at 7 days were collected and washed with DPBS. After that, 1×10^4 LTT-tolerant cells
224 or ADSCs in 300 μL of serum-free medium were seeded onto the apical surface of the insert.
225 Chemoattractants were added to the basal chamber (500 μL of basal medium) as follows:
226 recombinant feline/human stromal cell-derived factor 1β (SDF-1β; R&D Systems, Minneapolis,
227 MN) and sphingosine 1-phosphate (S1P; Sigma-Aldrich). After overnight incubation at 37 °C, the
228 cells were washed and wiped out on the top surface of the insert membrane using a cotton swab.

229 After staining with the Diff-Quik stain kit (Sysmex, Hyogo, Japan), the cells were air-dried. Cells
230 outside of the insert membrane were evaluated by counting the total number of observations in
231 four different areas using a 10× objective lens. In another experiment, we stimulated LTT-tolerant
232 cells or ADSCs from eight dogs by tumor necrosis factor (TNF)-α, which is an inflammatory
233 cytokine that stimulates SDF, to clarify the migration process. LTT-tolerant cells in suspension or
234 ADSCs in adherence at 7 days were stimulated by a final concentration of 10 ng/mL TNF-α for 24
235 h. Subsequently, the stimulated cells were collected by centrifugation and total RNA was isolated.
236

237 ***Inhibition of lymphocyte proliferation***

238 LTT-tolerant cells or ADSCs from nine dogs were evaluated for flow cytometry analysis.
239 Immune suppression assays were performed according to previously described methods [30].
240 Peripheral blood mononuclear cells (PBMCs) were isolated from five healthy donor dogs by
241 density gradient centrifugation. For further experiments, a portion of the PBMCs were
242 cryopreserved by CELLBANKER® 1 (TAKARA, Shiga, Japan). LTT-tolerant cells or ADSCs
243 were seeded onto each well for 1 day before coculture with PBMCs. Subsequently, the culture
244 media was exchanged, and PBMCs were cocultured for 3 days. Lymphocyte proliferation in a
245 lymphocyte gate with forward-scatter (FSC) and side-scatter (SSC) was analyzed using flow
246 cytometry.
247

248 ***Quantitative reverse transcription (RT-qPCR)***

249 Total RNA was isolated using NucleoSpin® RNA Plus kit (TAKARA). cDNA was
250 synthesized using a PrimeScript™ RT Master Mix (Perfect Real Time) (TAKARA). qRT-PCR was
251 performed with the SYBR Green assay using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus;
252 TAKARA) analyzed with a real-time PCR detection system (Thermal Cycler Dice® Real Time
253 System; TAKARA), with β-actin used for normalization. All primers used are listed in
254 Supplementary Table S2.

255

256 ***Statistics***

257 The data was tested for normality in this study. Experimental groups were compared using
258 one-way analysis of variance followed by Fisher's protected–least significance–post hoc analysis
259 (Microsoft® Excel® for Microsoft 365 MSO (16.0.13426.20270)) because that data did not have a
260 normal distribution. Quantitative data are expressed as mean ± standard error. The significance
261 levels were set at $P < 0.01$ and $P < 0.05$.

262

263

264 **Results**

265 ***Isolation and characterization of canine ADSCs and LTT-tolerant cells***

266 We morphologically compared canine ADSCs and LTT-tolerant cells to examine their

267 characteristics. ADSCs typically showed a spindle shape (Fig. 1A), had an osteogenic phenotype
268 for 14 days, and adipogenic and chondrogenic phenotypes for 21 days after induction (Fig. 1B).
269 We showed the result of cell survival rate after LTT. About 9.3% ± 0.5% cells in canine ADSCs
270 survived. In this study, we defined the survived cells as LTT-tolerant cells. LTT-tolerant cells in
271 suspension culture formed cell clusters (～50 μm in diameter), similar to the embryoid body (Fig.
272 1C). After passaging to adherent culture, LTT-tolerant cells were adherent and exhibited a spindle
273 shape without becoming flattened or large (Fig. 1C). There were no clear morphological
274 differences between ADSCs and LTT-tolerant cells in adherent cultures. We evaluated the CD
275 antigen surface using flow cytometry to examine the established MSC markers. ADSCs expressed
276 for CD44 and CD90 known as positive markers of human MSCs and those that lacked for CD14
277 and CD45 known as negative markers of human MSCs (Fig. 2A). Similar results were obtained
278 for LTT-tolerant cells for these markers (Fig. 2B). However, RT-PCR analysis revealed that just
279 after LTT, the relative messenger RNA (mRNA) expression of *FUT9* in LTT-tolerant cells was ～
280 74 times higher than that in ADSCs without LTT (Fig. 2C). The expression intensity of SSEA-1
281 was significantly enhanced by LTT (Fig. 2C). On the other contrary, the expression intensity of
282 SSEA-3, a known human Muse cell marker, did not change by LTT (Fig. 2C).
283

284 ***Self-renewal capacity and pluripotency in LTT-tolerant cells***

285 We conducted CFU-F assays *in vitro* to compare the self-renewal capacity of ADSCs and

286 LTT-tolerant cells. LTT-tolerant cells in suspension generated a significantly ($P < 0.01$) higher
287 number of CFU-F than ADSCs when assayed 14 days later (Fig. 3A, B).

288 We evaluated the cell clusters in LTT-tolerant cells in suspension culture to examine their
289 pluripotency. LTT-tolerant cells were positive for ALP activity (Fig. 3C) and expressed pluripotent
290 stem cell markers such as SSEA-1, SSEA-3, NANOG, SOX2 and OCT3/4, and TRA-1-60 (Fig.
291 3D).

292 We analyzed the karyotype of LTT-tolerant cells to examine their influence on LTT. LTT-
293 tolerant cells had a normal 78, XX karyotype, with 38 matched pairs of autosomes at passage 4,
294 with no identifiable gross structural rearrangements (Fig. 4).

295

296 ***In vitro differentiation of LTT-tolerant cells into the three germ layers***

297 The *in vitro* differentiation capacity of LTT-tolerant cells was evaluated by von Kossa, Oil
298 Red O and immunocytochemical staining to examine the three germ layers. Seven days after
299 adipocyte and osteoblast induction, LTT-tolerant cells had osteogenic and adipogenic phenotypes
300 (endodermal), faster than that observed in ADSCs (Fig. 5A, B). Fourteen days after hepatocyte
301 induction, LTT-tolerant cells were positive for albumin and α-fetoprotein (endodermal) (Fig. 5C).
302 Seven days after neural induction, LTT-tolerant cells formed neurospheres and were positive for
303 NESTIN, Musashi-1, and NeuroD 2 (Fig. 5D). Ten days after additional induction, LTT-tolerant
304 cells were positive for MAP-2 (ectodermal) (Fig. 5E).

305

306 ***Distinct differentiation ability of LTT-tolerant cells***

307 To compare the differentiation potential of ADSCs and LTT-tolerant cells, we conducted an
308 evaluation with PSL1a lectin using flow cytometry. PSL1a lectin produced stronger signals to LTT-
309 tolerant cells than to ADSCs. The expression intensity of FITC-labeled PSL1a lectin was higher
310 than that of ADSCs (Fig. 6A, B). To confirm this result, we morphologically analyzed the
311 differentiation ability of ADSCs and LTT-tolerant cells into osteoblasts and adipocytes. A minority
312 of ADSCs in representative areas differentiated into osteoblasts and adipocytes after induction. On
313 the other hand, the majority of LTT-tolerant cells in representative areas differentiated into
314 osteoblasts and adipocytes under the same differentiation conditions (Fig. 6C).

315

316 ***In vitro nontumorigenic activities***

317 To confirm tumorigenic activities in LTT-tolerant cells, we examined telomerase activity
318 using RT-PCR analysis. The mRNA expression of *TERT* in LTT-tolerant cells and ADSCs was
319 significantly lower than in CTAC, the positive control (Fig. 7).

320

321 ***In vitro migration ability***

322 To examine the migration ability of LTT-tolerant cells and ADSCs, we conducted a migration
323 assay and investigated the mRNA expression of the migrated receptors by RT-PCR analysis.

324 Compared to ADSCs, LTT-tolerant cells had migrated significantly to S1P and SDF-1β and were
325 upregulated in a concentration-dependent manner (Fig. 8A). The mRNA expressions of
326 Sphingosine-1-Phosphate Receptor 2 (*S1PR2*) and C-X-C chemokine Receptor type 4 (*CXCR-4*)
327 were higher in LTT tolerant cells than in ADSCs. Moreover, the mRNA expression of *CXCR-4*
328 was upregulated by tumor necrosis factor-alpha (TNF-α) in both LTT-tolerant cells and ADSCs
329 (Fig. 8B).
330
331 ***Inhibition of phytohaemagglutinin-stimulated PBMC proliferation in LTT-tolerant cells***
332 To determine the immunomodulatory properties of LTT-tolerant cells, we examined the
333 effects of LTT-tolerant cells and ADSCs on PBMC proliferation by flow cytometry. We found that,
334 compared to ADSCs, LTT-tolerant cells significantly inhibited phytohemagglutinin (PHA)-
335 stimulated PBMC proliferation (Fig. 9).
336
337
338 **Discussion**
339 We showed here that LTT-tolerant cells from canine ADSCs had high stem cell potency. This
340 is the first report on the generation of pluripotent stem cells from canine ADSCs. To date, few
341 studies have reported a subpopulation of canine ADSCs by cell sorting. In a previous study, the
342 aldehyde dehydrogenase high-activity ($ALDH^{Hi}$) subpopulation was shown to have a high capacity

343 for adipogenic and osteogenic differentiation [12]. The subpopulation with cystine transporter
344 expression (xCT^{Hi})/$CD44^{+}$ is reported to have higher proliferation capacity and show higher gene
345 expression of stem cell markers than the xCT^{Lo}/$CD44^{+}$ subpopulation [13]. However, their
346 pluripotency has not been investigated in detail [12,13]. The LTT-tolerant cells we isolated had
347 similar properties to human Muse cells, such as high self-renewal capacity, formation of cell
348 clusters in suspension culture, expression of pluripotent stem cell markers, and differentiation into
349 three germ layers *in vitro*. However, cells from different species often have unique characteristics
350 that are absent in cells from other species. Further studies are needed to examine whether LTT-
351 tolerant cells from canine ADSCs have more unique characteristics.

352 Our ADSCs were spindle-shaped, showed adherence to plastic, displayed self-renewal,
353 expressed CD44 and CD90 surface markers, lacked expression of CD14 and CD45, and exhibited
354 adipogenic, osteogenic, and chondrogenic differentiation, similar to canine MSCs [31-33]. We
355 conducted severe stress stimulation, that is, LTT, to generate cells with high stem cell potency from
356 canine ADSCs, similar to the collection of human Muse cells [1]. The presence of ～1% Muse
357 cells in human BMSCs using flow cytometry analysis [1, 21], and 3% survival of human BMSCs
358 after LTT [1] has been reported. These results suggest that LTT-tolerant cells from human BMSCs
359 are enriched in Muse cells. In addition, Muse cells from human ADSCs were found to be present
360 at 8.8 ± 1.3% in flow cytometry analysis [34]. In this study, LTT-tolerant cells were isolated at 9.3
361 ± 0.5%. These results indicate that the ratio of LTT-tolerant cells from canine ADSCs might be

362 similar to that of human Muse cells. Further studies should be performed to determine the presence
363 of pluripotent stem cells in canine ADSCs without LTT.

364 This study revealed that LTT-tolerant cells from canine ADSCs were not distinguishable
365 from ADSCs without LTT in adherent culture, retained the typical surface markers of ADSCs and
366 differentiated into osteocytes and adipocytes. Furthermore, our LTT-tolerant cells had similar
367 properties to human Muse cells, such as being LTT-tolerant, possessing self-renewal abilities,
368 being able to form cell clusters in suspension culture, being positive for ALP activity and
369 pluripotency stem cell markers, being able to differentiate into three germ layers, possessing a
370 normal karyotype, and having low telomerase activity *in vitro*. SSEA-1 and SSEA-3 are well-
371 known as a cell marker of pluripotent stem cell. These markers are effectively-used to distinguish
372 pluripotent stem cells from other cells and to track the differentiation process in stem cells and
373 generally disappear once the pluripotent stem cells differentiate. Therefore, they are useful to
374 check whether stem cells maintain the stemness. Interestingly, our study also demonstrated that
375 the expression of *FUT9* mRNA and the expression intensity of SSEA-1 in LTT-tolerant cells were
376 significantly increased, and that the expression intensity of SSEA-3 in LTT-tolerant cells did not
377 change immediately after LTT. In pigs, it has been reported that SSEA-1 expressing enhances
378 reprogramming of cells, which are negative for SSEA-3, in embryonic fibroblasts [35]. In rats, it
379 has also been reported that SSEA-1 and SSEA-3 positive cells are mixed in LTT-tolerant cells from
380 rat BMSCs, and ～ 58% of LTT-tolerant cells are double positive for SSEA-1 and SSEA-3 [36].

381 Our results indicated that SSEA-1 and SSEA-3 positive cells might be mixed in LTT-tolerant cells
382 from canine ADSCs because the expression of SSEA-1 was increased and that of SSEA-3 was
383 slightly decreased. Moreover, LTT-tolerant cells in dogs might be characterized by *FUT9* mRNA
384 and SSEA-1, unlike human Muse cell, although the details of the differences in the two pluripotent
385 stem cell markers have not been clarified. Further studies are needed to analyze the ratio of
386 expression of both SSEA-1 and SSEA-3 using a cell sorting. SSEA-3 is a glycolipid, whereas
387 SSEA-1 is a glycoprotein. Therefore, the gene analysis and the investigation in a cell origin using
388 SSEA-1 is possible in LTT-tolerant cells from canine ADSCs. Further studies are necessary using
389 progressive methods such as the analysis of single-cell levels in cell sorting by SSEA-1, the
390 formation of cell clusters, and analysis using other FACS antibodies to confirm specific cross-
391 reactivity.

392 Human Muse cells have been reported to have superior differentiation ability to that of
393 MSCs [23]. Human Muse cells with BODIPY-C16 (which is a lipid marker) were present at 80%
394 ± 4% for 3 days after induction, although ADSCs did not yet possess a signal for the marker,
395 according to a previous report [22]. In the present study, we evaluated the differentiation ability of
396 LTT-tolerant cells and ADSCs on PSL1a lectin. The morphology of osteogenic and adipogenic
397 differentiation after induction correlated well with that of PSL1a lectin. These results indicated
398 that, compared to ADSCs like human Muse cells, LTT-tolerant cells had a superior ability to
399 differentiate into mesodermal lineage cells. In addition, human Muse cells have been reported to

400 have spontaneous differentiation ability into the three germ layers *in vitro* [1, 22]. More studies on
401 canine LTT-tolerant cells are necessary to investigate the spontaneous *in vitro* differentiation ability
402 without induction into three germ layers and into a variety of damaged cells *in vivo* using animal
403 models of disease.

404 In the migration of MSCs, the interaction between SDF-1β, which is a small chemotactic
405 cytokine that is often induced by pro-inflammatory stimulation, such as TNF-α and interleukin-1
406 produced inflammatory cells and damage tissues, and CXCR-4, which is the receptor of this
407 chemokine in MSCs, has been reported to play an important role [6, 37]. Human Muse cells have
408 been reported to have a high migration ability to damaged tissues through S1P-S1PR2 interaction
409 [18]. In our study, LTT-tolerant cells exhibited migration ability to S1P and high expression of
410 *S1PR2* mRNA like human Muse cells *in vitro*. This result indicated that LTT-tolerant cells might
411 have a similar migration mechanism as that of human Muse cells. In addition, LTT-tolerant cells
412 showed migration ability to SDF-1β and high expression of *CXCR-4* mRNA, similar to human
413 MSCs *in vitro*. Overall, these results suggest that the LTT-tolerant cells may have two processes
414 for migration. This ability in LTT-tolerant cells can be advantageous in case IV administration for
415 clinical applications similar to human Muse cells. Further studies are necessary to investigate
416 migration abilities to damaged tissues *in vivo* after IV administration of LTT-tolerant cells using
417 animal models of disease.

418 The immunosuppressive ability of Muse cells has not yet been reported. In one previous

419 report, human Muse cells were described as possessing immunosuppressive capacity, such as the
420 inhibition of lymphocyte and macrophage proliferation by producing transforming growth factor-
421 β1[38]. The immunosuppressive function of Muse cells is expected to play a useful role in MSCs.
422 In this study, LTT-tolerant cells were superior to ADSCs in the immunosuppression ability of PHA-
423 stimulated PBMC proliferation. We believe that LTT-tolerant cells will be useful for the treatment
424 of immune-mediated diseases. MSCs have been reported to have immunosuppressive ability to
425 immune cells. In T cells, MSCs suppress the proliferation of $CD4^+$ and $CD8^+$ T cells and promote
426 the proliferation of Treg. In B cells, MSCs suppress the production of antibodies and their
427 proliferation. In NK cells, MSCs suppress the proliferation and production of cytokine. In
428 macrophages and DCs, MSCs suppress maturation [8]. Further research in LTT-tolerant cells is
429 necessary to investigate their interaction with immune cells.

430 In conclusion, we successfully generated LTT tolerant cells with high stem cell potency
431 from ADSCs of adult dogs. These cells had similar properties as human Muse cells, that is, they
432 were LTT-tolerant, non-tumorigenic, expressed pluripotent stem cell markers, differentiated into
433 the three germ layers, and had superior migration and immunosuppressive capacities compared to
434 ADSCs. We believe that these cells are not only useful cell sources for clinical application in
435 veterinary regenerative medicine but also meaningful for human medicine.

436

437

Acknowledgments

The authors would like to thank Nippon Lighthouse Guide Dog Training Center (Osaka, Japan) for providing blood samples and fat tissues. The authors also thank Editage for English language editing.

Author Disclosure Statement

Y. T. is a Chief Executive Officer and a stock-holder of J-ARM Co., Ltd., the company that markets the cell culture kit that have been used in the study reported herein. K. M. and Y. I. are employees of J-ARM Co., Ltd. The other authors declare no conflicts of interest.

Funding Information

This study was supported by the Japan Society for the Promotion of Science (Grant No.: 18K19274).

Supplementary Material

Supplementary Table S1

Supplementary Table S2

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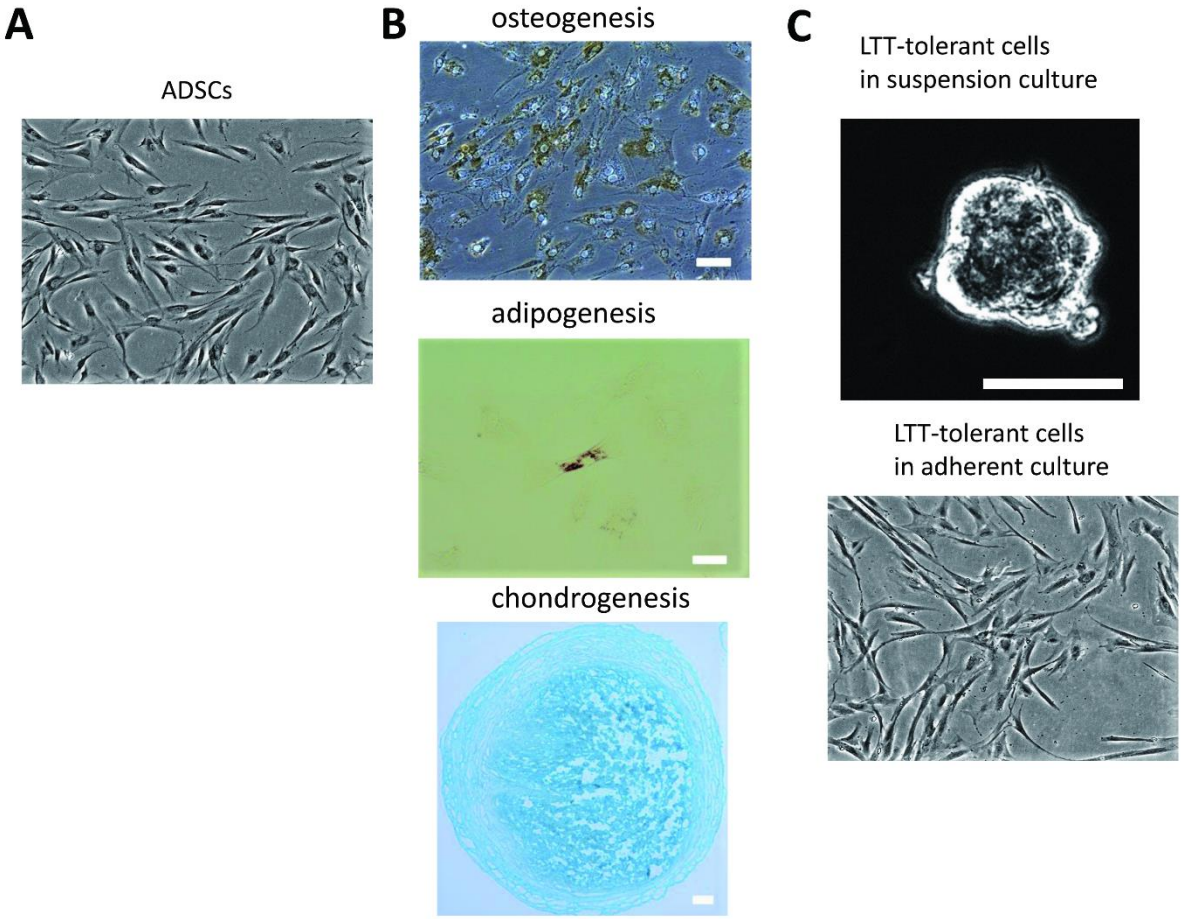

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579 **FIG. 1.** The morphologies of canine ADSCs and LTT-tolerant cells. (A) Canine ADSCs were

typically observed as spindle-shaped cells. (B) Canine ADSCs were able to differentiate osteogenic (*upper*), adipogenic (*middle*) and chondrogenic (*lower*) lineages. (C) LTT-tolerant cells formed cell clusters in suspension culture (*upper*) and spindle-shaped cells in adherent culture (*lower*). scale bar, 50 μm.

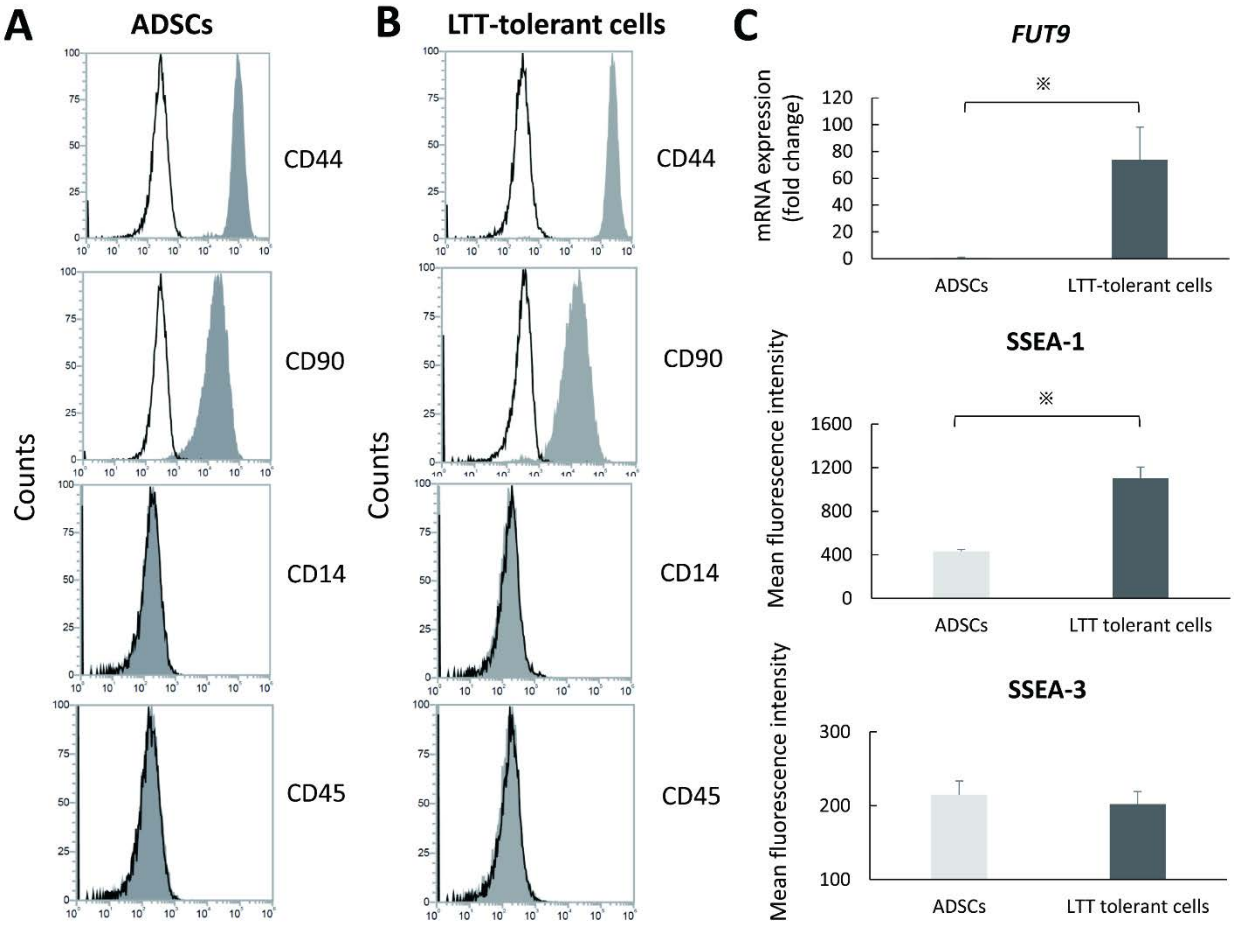
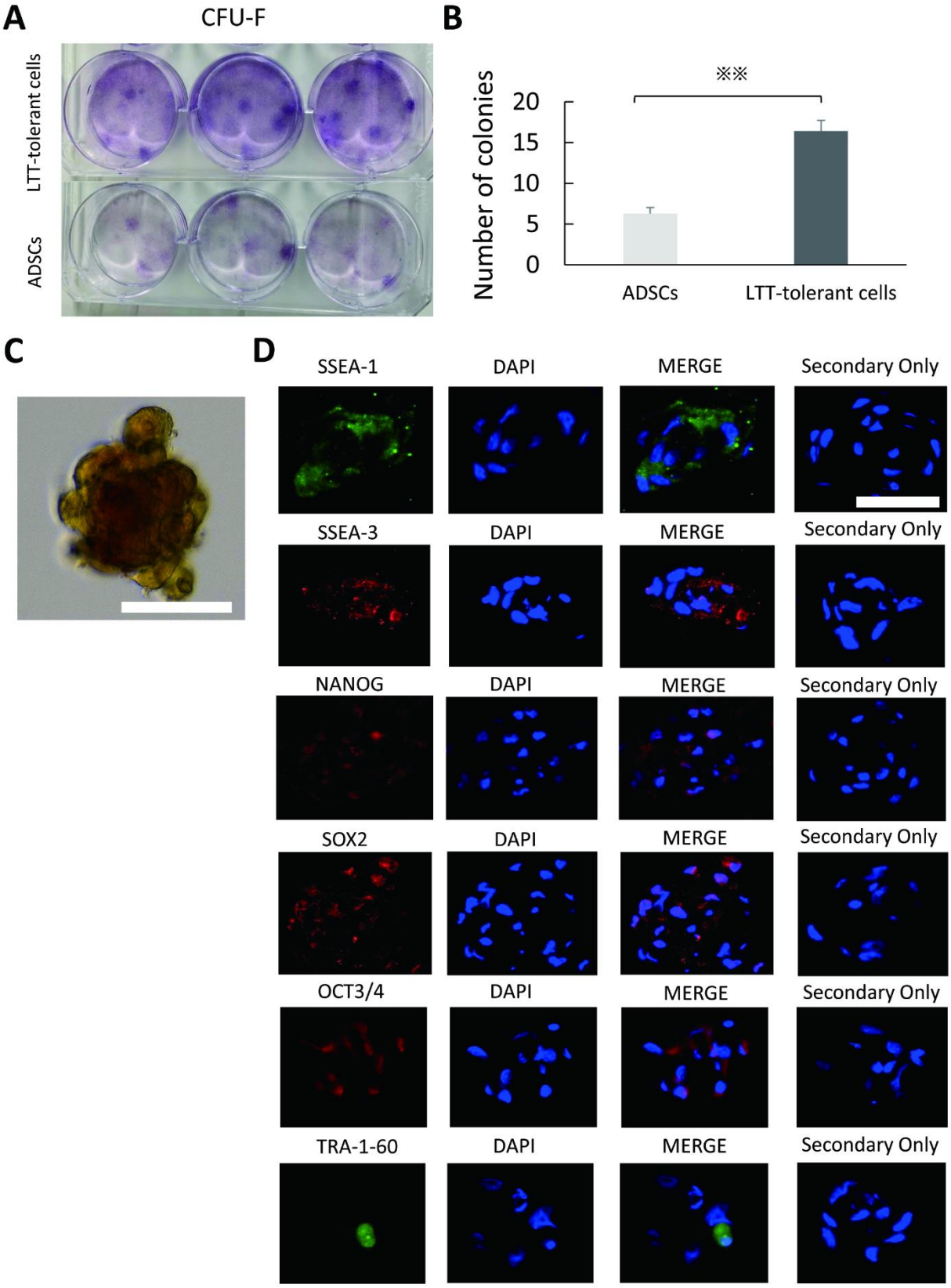


FIG. 2. The characteristics of ADSCs and LTT-tolerant cells. (A, B) ADSCs and LTT-tolerant cells

589 in adherent culture expressed CD90 and CD44 and lacked CD14 and CD45 surface markers.
590 Isotype control samples are included in each panel (black line). (C) Relative mRNA expression of
591 *FUT9* in LTT-tolerant cells just after LTT and ADSCs (*upper*). The expression intensity of SSEA-
592 1 just after LTT was significantly increased compared to ADSCs in flow cytometry (*middle*). The
593 expression intensity of SSEA-3 just after LTT did not change by LTT (*lower*). $n = 4$. $^{*}P < 0.05$.
594


A
CFU-F
LTT-tolerant cells
ADSCs
B
※※
Number of colonies
20
15
10
5
0
ADSCs
LTT-tolerant cells
C
D
SSEA-1
DAPI
MERGE
Secondary Only
SSEA-3
DAPI
MERGE
Secondary Only
NANOG
DAPI
MERGE
Secondary Only
SOX2
DAPI
MERGE
Secondary Only
OCT3/4
DAPI
MERGE
Secondary Only
TRA-1-60
DAPI
MERGE
Secondary Only

595

596 **FIG. 3.** Self-renewal capacity and pluripotency of LTT-tolerant cells in suspension culture. (A)
597 Self-renewal capacity for colony-forming unit fibroblasts (CFU-F) assay in LTT-tolerant cells
598 (*upper*) and ADSCs (*lower*). (B) Number of colonies in LTT-tolerant cells was significantly higher
599 than that of ADSCs. $n = 7$. $^{**}P < 0.01$. (C) Cell cluster was positive for ALP activity. (D) Cell
600 cluster expressed pluripotent stem cell markers such as SSEA-1, SSEA-3, NANOG, SOX2,
601 OCT3/4 and TRA-1-60 by immunocytochemistry. Nuclei was stained with DAPI (blue). scale bar,
602 50 μm.
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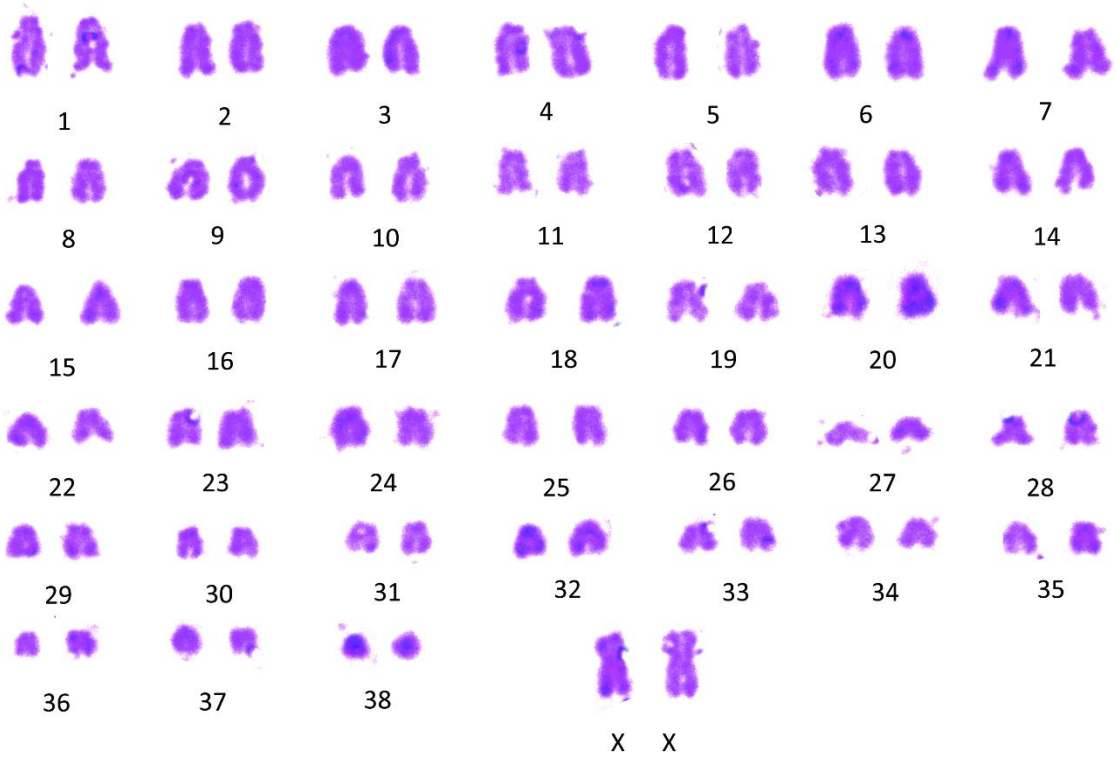

615

616 **FIG. 4.** Karyotype analysis of LTT-tolerant cells. Normal: 78, XX with 38 matched pairs of

617 autosomes. Magnification, 1,000X.

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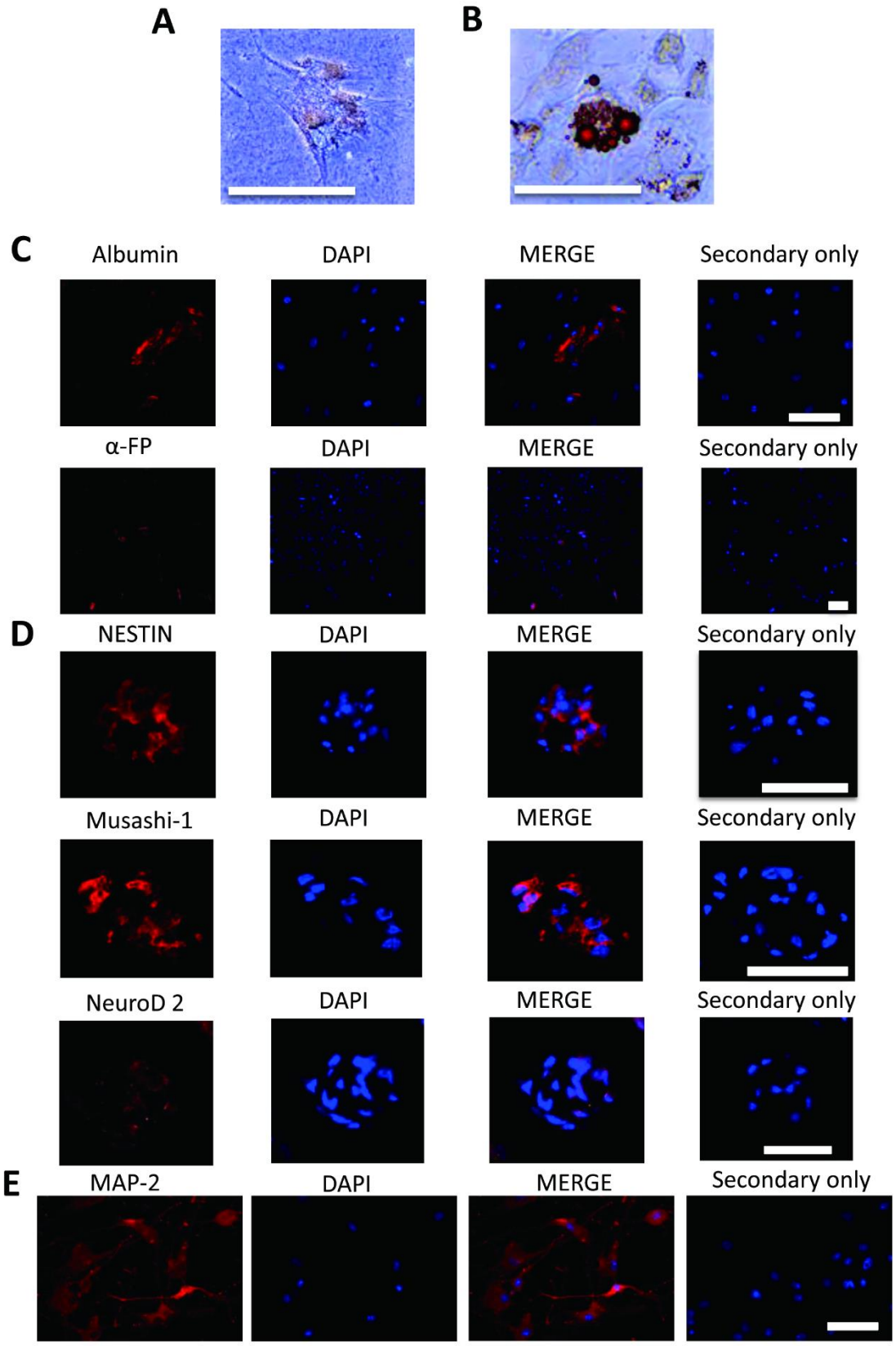
A
B
C
Albumin
DAPI
MERGE
Secondary only
α-FP
DAPI
MERGE
Secondary only
D
NESTIN
DAPI
MERGE
Secondary only
Musashi-1
DAPI
MERGE
Secondary only
NeuroD 2
DAPI
MERGE
Secondary only
E
MAP-2
DAPI
MERGE
Secondary only

624

625 **FIG. 5.** Differentiation of LTT-tolerant cells into the three germ layers *in vitro*. (A) After induction,
626 the cells were estimated by von Kossa staining for mineralized matrix deposition and (B) Oil Red
627 O staining for fat droplets. (C) After hepatocyte induction, the cells were positive for albumin
628 (*upper*) and α-fetoprotein (α-FP) (*lower*). (D) After neural induction, the neurospheres were
629 positive for the neural progenitor markers such as NESTIN (*upper*), Musashi-1 (*middle*) and
630 NeuroD 2 (*lower*). (E) After additionally induction to Neuro cells, the cells were positive for neural
631 cell markers like MAP-2. Nuclei was stained with DAPI (blue). scale bar, 50μm.
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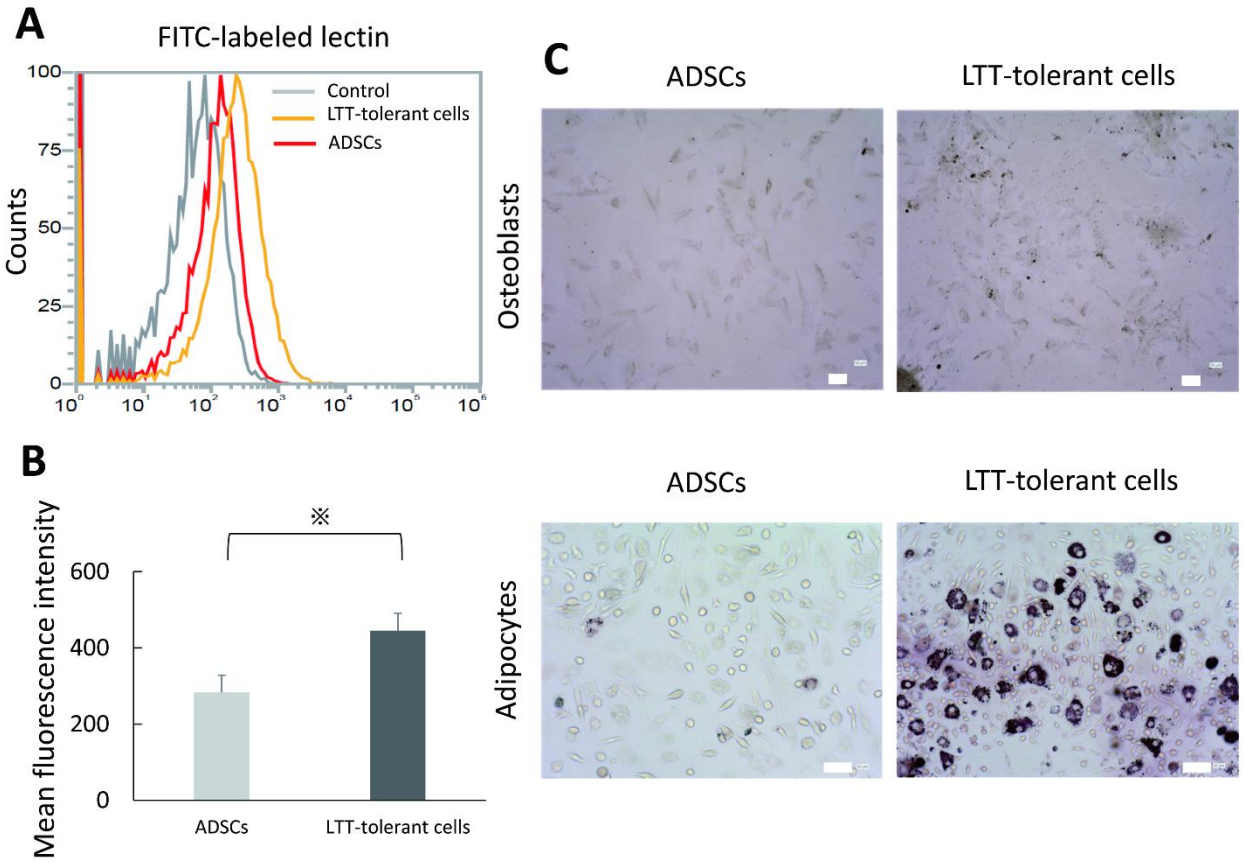

636

637 **FIG. 6.** Distinct differentiation ability of LTT-tolerant cells and ADSCs. (A) LTT-tolerant cells

638 (yellow line) and ADSCs (red line) stained with FITC-labeled PSL1a lectins and unstained sample

639 (gray line) are shown. (B) The expression intensity of binding of FITC-labeled lectins to LTT-

640 tolerant cells was significantly higher than that of ADSCs. $n = 5$. $^*P < 0.05$. (C) ADSCs and LTT-

641 tolerant cells were differentiated into osteoblasts and adipocytes, which stained with von Kossa

642 and Oil Red O, respectively. LTT-tolerant cells showed distinct differentiation ability. scale bar,

643 50μm.

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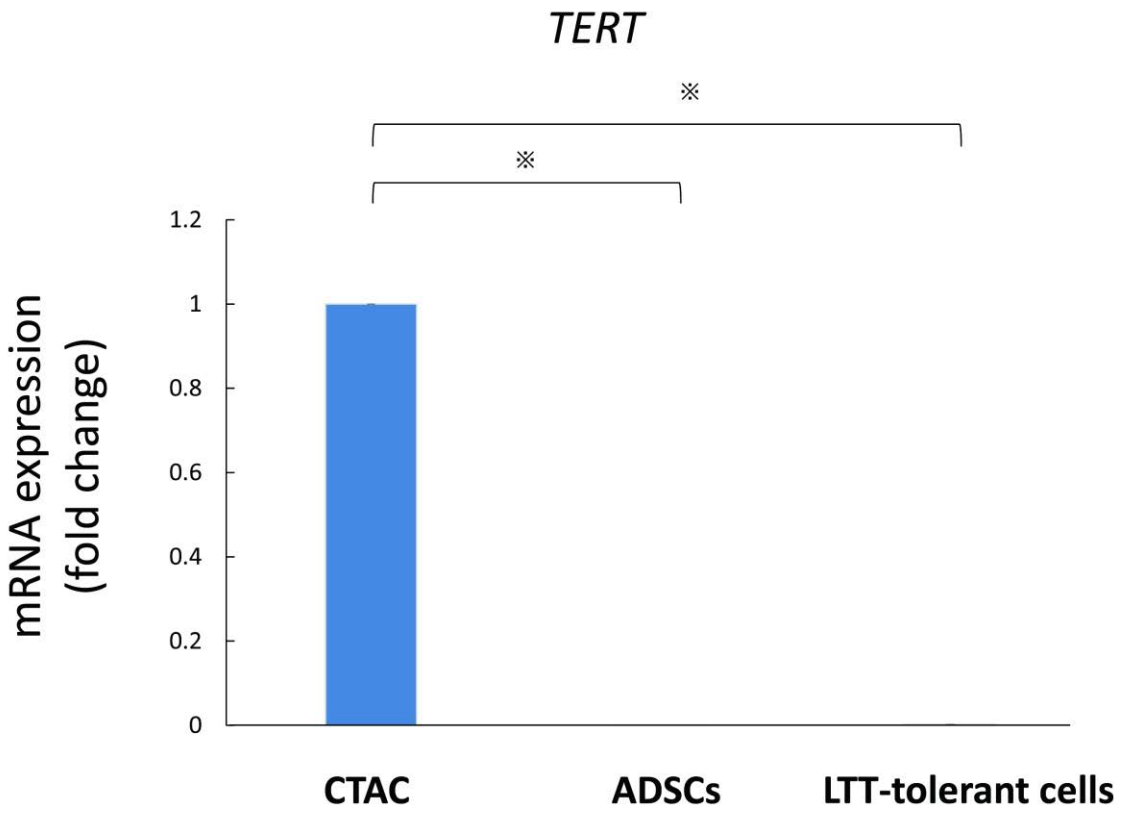

645

646 **FIG. 7.** Non-tumorigenic characteristics of LTT-tolerant cells. Relative mRNA expression of

647 *telomerase reverse transcriptase (TERT)* in LTT-tolerant cells and ADSCs. CTACs were used as

648 the positive control. $n = 5$. $^{*}P < 0.05$.

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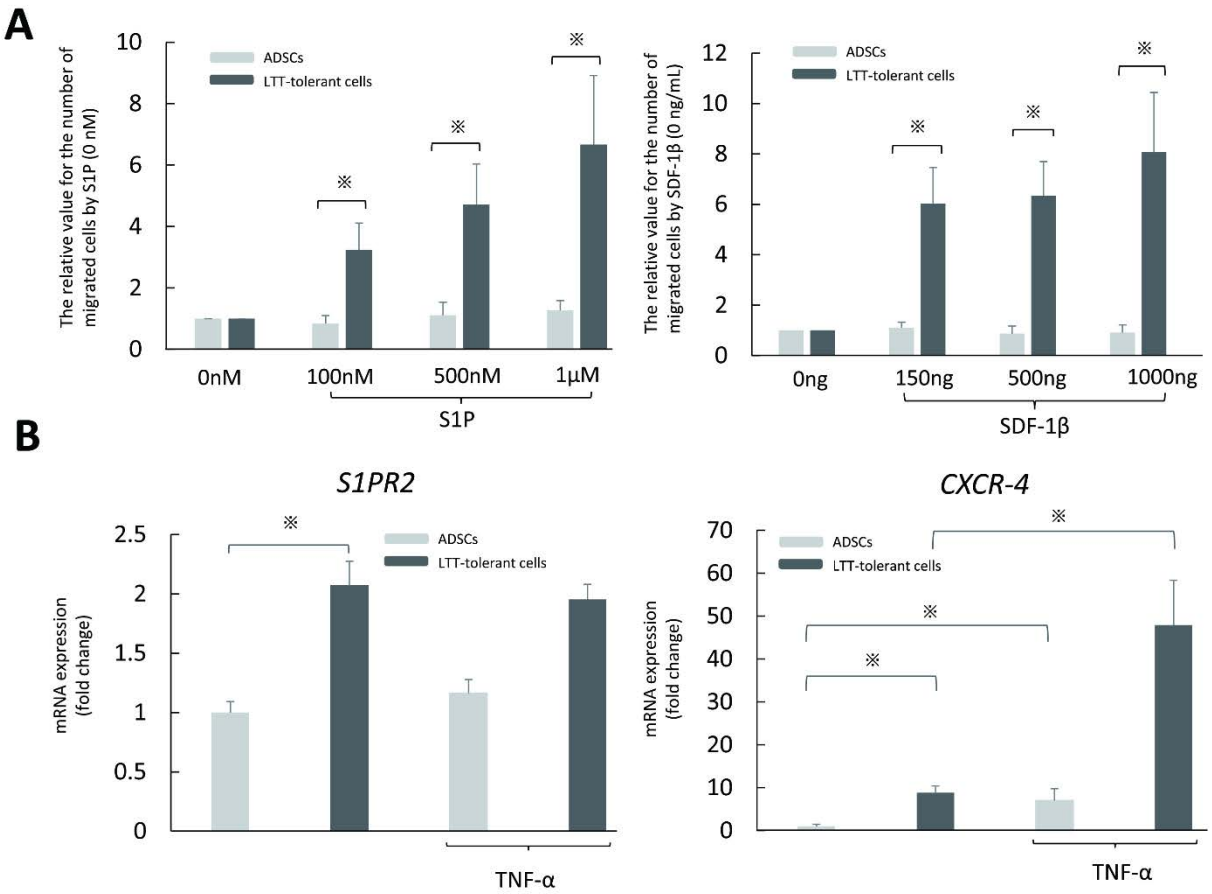

652

653 **FIG. 8.** Migration Ability of LTT-tolerant cells and ADSCs *in vitro*. (A) The relative value for

654 number of migrated cells by S1P (0 nM) (*left*) and SDF-1β (0 ng/ml) (*right*). LTT-tolerant cells

655 were increased in a concentration-dependent manner. (*left*) $n = 6$. $*P < 0.05$. (*right*) $n = 7$. $*P <$

656 0.05. (B) Relative mRNA expression of *S1PR2* (*left*) and *CXCR-4* (*right*) in LTT-tolerant cells and

657 ADSCs. $n = 8$. $*P < 0.05$.

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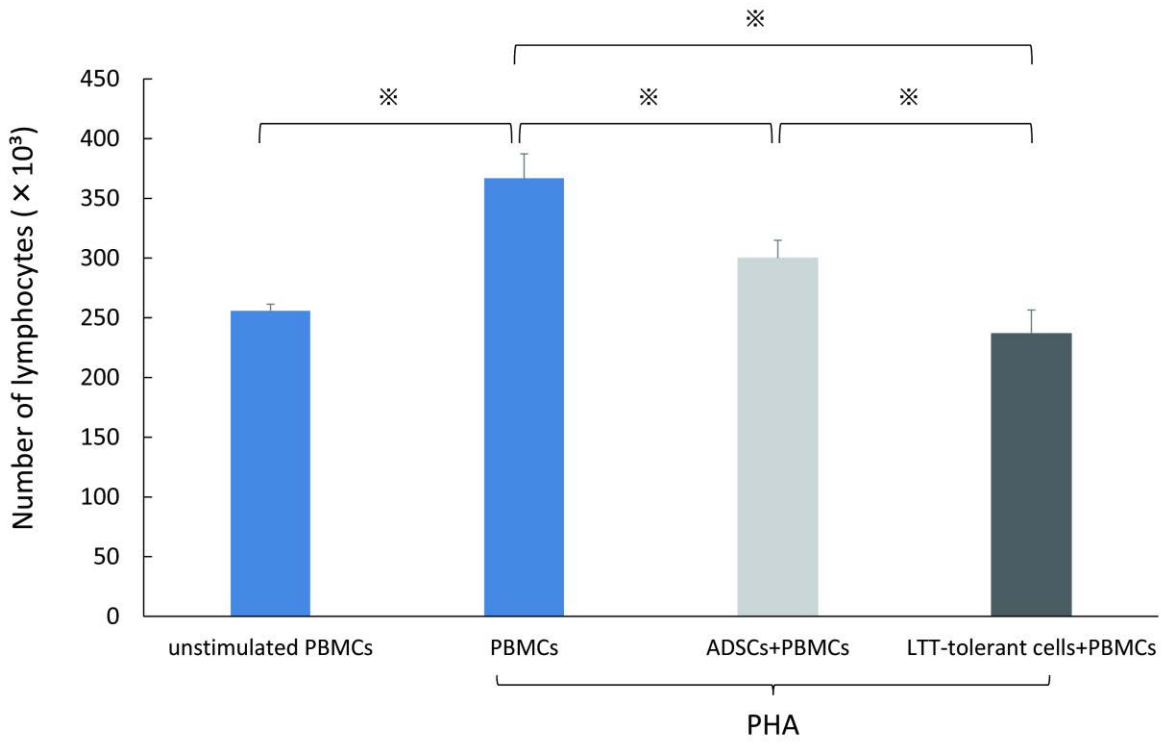

661

662 **FIG. 9.** Suppression of phytohaemagglutinin (PHA) -stimulated PBMC proliferation in LTT-
663 tolerant cells and ADSCs. PBMCs were stimulated with PHA and then incubated with LTT-tolerant
664 cells and ADSCs. LTT-tolerant cells had stronger suppressive activity against the proliferation of
665 mitogen-stimulated lymphocytes than that of ADSCs. $n = 9$. $^{*}P < 0.05$.

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670 Supplementary Table 1

671 Antibody list

Antigen	Antibody host and type [a]	Dilution	Source [b]
CD14	Mouse monoclonal (FITC)	10	Thermo Fisher Scientific
CD45	Rat monoclonal (FITC)	20	Thermo Fisher Scientific
CD44	Rat monoclonal (PE)	100	Thermo Fisher Scientific
CD90	Rat monoclonal	100	Abcam
SSEA-1	Mouse monoclonal	100	Thermo Fisher Scientific
SSEA-3	Rat monoclonal	100	BioLegend
SSEA-3	Rat monoclonal	100	GeneTex
NANOG	Rabbit polyclonal	100	GeneTex
OCT3/4	Mouse monoclonal	500	Abcam
SOX2	Rabbit polyclonal	100	GeneTex
TRA-1-60	Mouse monoclonal	1000	Abcam
ALBUMIN	Rabbit polyclonal	2000	LSBio
alpha 1 Fetoprotein	Rabbit polyclonal	300	Abcam
NESTIN	Rabbit polyclonal	800	Merck Millipore
Musashi-1	Rabbit polyclonal	400	Merck Millipore
NeuroD 2	Rabbit polyclonal	200	Merck Millipore
MAP-2	Rabbit polyclonal	60	Bio-Rad
IgG, rat	Goat (APC)	50	BioLegend
IgM, rat	Alexa Fluor® 647	100	BioLegend
IgM, rat	Goat (DyLight 555)	100	Thermo Fisher Scientific
IgM, mouse	Goat (FITC)	300	Abcam
IgM, mouse	Rat (PE)	100	Thermo Fisher Scientific
IgG, mouse	Donkey (Alexa Fluor® 546)	500	Thermo Fisher Scientific

IgG, rabbit	Goat (Alexa Fluor® 546)	500	Thermo Fisher Scientific

672 [a] FITC, fluorescein isothicyanate; PE, phycoerythrin; APC, Allophycocyanin.

673 [b] Thermo Fisher Scientific (Waltham, MA); Abcam (Cambridge, MA); BioLegend (San Diego, CA); GeneTex

674 (Irvine, CA); LSBio (Seattle, WA); Merck Millipore (Darmstadt, Germany); Bio-Rad (Hercules, CA)

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677

678 Supplementary Table 2

679 Real-time RT-PCR and RT-PCR Primer sequences

Real-time RT-PCR Primers	Sequences (5'-3')
Canine *FUT9*	TCCAATGGAATCAGCCAGCTC
	AGGTCAAAGGTCTGCCCAAATG
Canine *TERT*	CGCACCTTTGTGCTACGCATAC
	CACATTGGCAATCACCTCTACCAG
Canine *S1PR2*	TCGAGGCCTGTTCCACTGTTC
	GAGCGACGATGGCCAATAAGA
Canine *CXCR-4*	TTGAGGCTGTGGCAAACTGG
	GACTGTTGGTGGCATGGACAATA
Canine *beta ACTIN*	GACGACATGGAGAAGATCTG
	GAAGGTCTCGAACATGATCTG

680

681