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Research paper

Isolation of tumor endothelial cells from murine cancer

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ABSTRACT

Tumor endothelial cells (TECs), which constitute the lining of the tumor blood vessels, have various characteristics as tumor constituent cells. In this study, we describe a novel method for the isolation of highly pure, fresh TECs, which form a small population within the tumor. Tumors were first dissected from tumor-bearing mice and digested to a single cell suspension with Collagenase Type II; the single cells were then separated by density gradient centrifugation. TECs were enriched by CD31-positive selection using magnetic activated cell sorting and subsequently purified by fluorescence activated cell sorting. The high purity of the obtained cells was verified by flow cytometry. Upon cell culture, the isolated cells showed a polygonal shape and a cobblestone appearance, which are features of the endothelial cells. Furthermore, a functional assay revealed that the TECs suppressed the proliferation of CD8+ T cells *in vitro*. We believe that the isolation method described in this study will enable the further elucidation of the characteristics of TECs.

1. Introduction

During the development of tumor microenvironment, various types of cells in the tumor stroma affect tumor growth. Tumor endothelial cells (TECs), which constitute the lining of the tumor blood vessels, not only contribute to supply blood to tumors, but have various characteristics as tumor constituent cells. Tumors construct an abnormal vasculature during tumor angiogenesis. It has been indicated that the morphology and gene expression of TECs differ from those of normal endothelial cells (St Croix et al., 2000; Jain, 2003; Lu et al., 2007; Nagy et al., 2009; Hida et al., 2011; Hida et al., 2013). TECs might also have aberrant growth, migratory, and differentiation abilities (Ghosh et al., 2008; Dunleavey et al., 2014), and they might acquire those characteristics as tumor microenvironment is established (Dudley, 2012; Ohga et al., 2012). Furthermore, there are some reports indicating that TECs might be able to acquire resistance to drugs (Bussolati et al., 2003; Grange et al., 2006; Xiong et al., 2009; Akiyama et al., 2012). Recently, it has been discussed that TECs might have important immunological roles in the regulating the trafficking of immune cells, controlling the microenvironment, and modulating the immune response (Mauge et al., 2014). Therefore, the understanding of these immunological mechanisms might help the development of advanced anti-cancer therapies, including immuno-therapies.

To study the dynamic functions of TECs, it is necessary to use TECs freshly isolated from tumors. Several isolating techniques of TECs from tumors, similar to the methods for isolating the endothelial cells from organs other than tumors (Marelli-Berg et al., 2000; Onoe et al., 2005a; Kajimoto et al., 2010), have been used to elucidate the characteristics of TECs (Amin et al., 2006; Dudley et al., 2008; Matsuda et al., 2010; Xiao et al., 2015). Often, however, we cannot obtain sufficient TECs using these techniques: TECs are indeed a small population within the tumor constituent cells, and their low number does not allow the purification of a sufficient number of cells of high purity. In contrast, for other types of tumor constituent cells with large numbers such as the tumor cells, the isolation techniques are mostly established: the protocols suggest the dissociation of target cells using enzymes and the subsequent cell isolation using magnetic sorting techniques. Even though a certain number of target cells is lost during these procedures, because of the

Abbreviations: ATCC, American Type Culture Collection; CFSE, carboxyflurescein diacetate succinimidyl ester; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; LSECs, liver sinusoidal endothelial cells; mAb, monoclonal antibody; MACS, magnetic activated cell sorting; PE, phycoerytrin; TECs, Tumor endothelial cells; TBST, Tris-buffered saline with Tween 20

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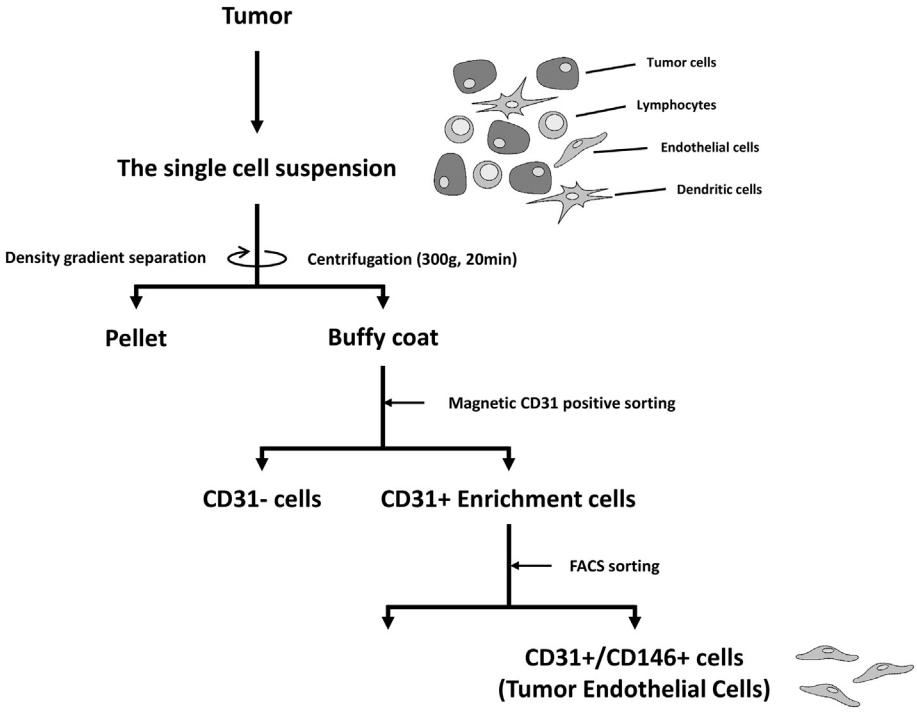


Fig. 1. Schematic illustration of the TECs purification method. Different tumor constituent cells, such as tumor cells, lymphocytes, endothelial cells and dendritic cells, are indicated. The fractions obtained at each step were subjected to fluorescence activated cell sorting (FACS) analysis to determine their purity (the data relative to the purification are in Fig. 3B).

large population of cells in the starting material, we can obtain a sufficient number of cells with high purity for use in various assays. As indicated above, the same does not apply to TECs. To characterize and study the immunological role of TECs, it is necessary to establish an effective method to isolate fresh and highly pure TECs. In this study, we established an effective method to isolate fresh and pure TECs from tumors. We describe a three-step method to obtain highly pure TECs from mouse tumors, based on a combination of the magnetic sorting method and subsequent fluorescence activated cell sorting (FACS). We then verified the purity of the isolated TECs by assessing their function in a suppression assay.

2. Material and methods

2.1. Mice

Female C57BL/6 wild-type mice were used in this study at 6–12 weeks old. The mice were purchased from Clea Japan (Tokyo, Japan). We used age-matched mice for all experiments. All animals were maintained and handled under pathogen-free conditions and in compliance with the national and institutional animal guidelines. All protocols were approved by the Kure Medical Center Animal Ethics Committee (Japan).

2.2. Cell line and culture

B16-F10, a C57BL/6 mice-derived melanoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Eggenstein, Germany) at 37 °C with 5% CO_2. C57BL/6 mouse primary vein endothelial cells, isolated from the inferior vena cava tissue of C57BL/6 mice, were purchased from Cell Biologics (Chicago, IL, USA) and cultured in Endothelial Cells Growth Medium MV2 (Promocell, Heidelberg, Germany) onto gelatin-coated dishes (IWAKI, Tokyo, Japan) at 37 °C with 5% CO_2.

2.3. Tumor inoculation

To obtain TECs, were used three mice per experiment. The mice were anesthetized by exposure to 1–3% isoflurane. One million tumor cells were suspended in 0.1 mL of sterile PBS (Wako, Osaka, Japan) and subcutaneously inoculated in the left flank fold of the mice. Tumor sizes were measured with a caliper every 3 days and tumor volume was calculated according to the formula: Volume (mm^3) = length (mm) × width (mm) × height (mm). After two weeks, the tumors had reached 1.5 cm diameter; at this point they were excised and pulled together.

2.4. Isolation of TECs

The following subsections describe the procedure for obtaining highly pure TECs from tumors from mice. The method is illustrated in Fig. 1.

2.4.1. Digestion

Tumors were dissected from tumor-bearing mice and cut into small pieces (1 mm^3 fragments) with a scalpel on a sterile dish. Minced tumors were digested in 2 mL of HBSS with calcium and magnesium (Gibco) containing 10 mg/mL Collagenase Type II (Worthington, Lakewood, NJ) and 25 μg/mL DNase I (Roche Diagnostics, Basel, Switzerland) per tumor at 37 °C with shaking for 30 min. Digestion was stopped by adding 2 mL of heat-inactivated FBS. The suspension was then filtered through a 100-μm cell strainer placed on the top of a 50 mL conical tube, to collect the single cell suspension, and the cells were then washed with HBSS without calcium and magnesium (Gibco) at 4 °C. After centrifugation at 400 *g* for 10 min, the pellet was resuspended in HBSS without calcium and magnesium at 4 °C.

2.4.2. Density gradient separation (first separation)

Twenty milliliters of Histopaque-1077 (Sigma) were placed into a 50 mL conical tube. The single cell suspension obtained after digestion was resuspended in 20 mL of HBSS without calcium and magnesium and slowly layered over the Histopaque solution by gentle pipetting down the side of the tube containing it. After centrifugation at 300 *g* for

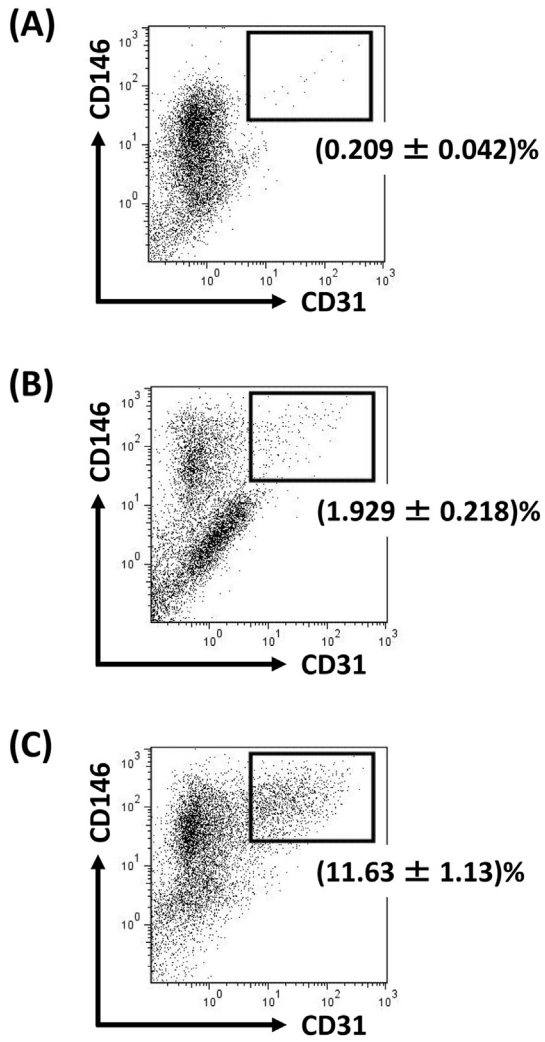


Fig. 2. Analysis of the purity of TECs at different stages of purification by magnetic sorting. The representative FCM profile of the cells obtained after the indicated purification was analyzed to evaluate the purity of TECs (CD31 + / CD146 +) within the whole population. (A) The percentage of CD31 + / CD146 + cells after the density gradient separation and before magnetic sorting were approximately 0.2%. Data are shown as the mean ± SEM (n = 10). (B) To purify TECs, the cells obtained from the density gradient separation were subjected to magnetic sorting using anti-mouse CD31 microbeads. The CD31 + / CD146 + cells were enriched approximately 10 times (2%) through magnetic CD31-positive sorting. Data are shown as the mean ± SEM (n = 8). (C) To improve the purification of TECs, the cells obtained from the density gradient separation were subjected to a two-step magnetic sorting in which a CD45-negative selection was followed by a CD31-positive selection. The CD31 + / CD146 + cells were further enriched approximately 10 times (10%). Data are shown as the mean ± SEM (n = 5).

20 min at 22 °C with no brake, the interface layer between the upper layer of PBS and the bottom layer of Histopaque (the so-called "buffy coat"), containing the endothelial cells, was aspirated, transferred to a 50 mL conical tube, diluted with HBSS without calcium and magnesium (volume up to 50 mL) and centrifuged at 400 *g* for 10 min at 4 °C. The supernatant was discarded and the cell pellet was further purified.

2.4.3. Magnetic sorting (second separation)

We performed magnetic activated cell sorting (MACS) to enrich the TECs population. The cells were suspended in MACS buffer (0.5% bovine serum albumin [BSA], and 2 mM EDTA in PBS, pH 7.2; 1×10^7 cells/100 µL) and incubated with anti-mouse CD31 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4 °C, according to manufacturer's instructions. Subsequently, the cells were washed and applied to equilibrated MACS LS columns (Miltenyi Biotec) and washed with 3 mL of MACS buffer 3 times. Then, the CD31-positive cells retained on the LS columns were flushed out by firmly applying the plunger after washing.

2.4.4. Flow cytometry

2.4.4.1. Antibodies for flow cytometry. The following reagents were used for cell-surface staining: anti-CD8-PE (53–6.7), anti-CD31-FITC (390) and anti-CD45-PE (30-F11) mAbs were purchased from BD Biosciences (Mountain View, CA, USA). Anti-CD146-APC (ME-9F1) mAbs was purchased from Miltenyi Biotec. Enriched CD31-positive cells were analyzed using the Navious flow cytometer (Beckman Coulter, Brea, CA, USA) and evaluated using the FlowJo v7.6.5 software (Tree Star, Ashland, OR, USA). Nonspecific Fc gamma-receptor binding of labeled mAbs was blocked with the anti-mouse Fc gamma-receptor mAb (2.4G2) (BD Biosciences). Dead cells were excluded from the analysis through forward scatter gating and propidium iodide (PI) incorporation.

2.4.4.2. FCM staining and cell sorting protocol (third separation). After enrichment of the CD31-positive cells by MACS, the cells were stained with anti-mouse CD31-FITC and anti-mouse CD146-APC mAb for 30 min at 4 °C. After washing, CD31/CD146 double positive cells were sorted using the FACSAria Cell Sorting System (BD Biosciences) equipped with the FACS Diva v4.1.2 software (BD Biosciences). Cell sorting was performed using the 100 µm nozzle with 100 psi (pounds per square inch) of sheath fluid pressure. After cell sorting, we checked the purity of sorted cells with the FACSAria Cell Sorting System. At the end of the separation, we had obtained highly pure CD31/CD146 double positive cells (> 95% of purity).

2.5. Morphological characteristics of isolated cells

We cultured the freshly purified TECs to study their morphological characteristics.

2.5.1. Primary culture of the isolated cells

The isolated cells were washed and resuspended in Endothelial Cells Growth Medium MV2 and then seeded (4×10^4 in 200 µL) onto 8 well-culture slides coated with collagen type I (0.69 cm^2/well, BD Biosciences). Non-adherent cells were removed on day 3 and 5 by washing with sterile PBS. On day 7, the cells reached 80–90% of confluency.

2.5.2. Immunofluorescent staining

On day 7, the adherent cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing adherent cells with Tris-buffered saline with Tween 20 (TBST) 3 times, the cells were blocked with 5% BSA in TBST for 30 min at room temperature (20–25 °C). After blocking, the cells were incubated with rabbit anti-mouse CD31 mAb (EPR17260–263, Abcam, Cambridge, MA, USA) in blocking solution (1:100) for 1 h at room temperature. After washing with TBST 3 times, the cells were incubated with Alexa 555-labeled anti-rabbit IgG (H + L) secondary antibody (Thermo Fisher Scientific, San Jose, CA, USA) and 4′6′-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific - for nuclei staining) in blocking solution (1:100) for 1 h at room temperature. After washing, micro-coverglasses were placed on the slides and the cells were observed with the inverted fluorescence phase contrast microscope BZ-X710 (Keyence, Osaka, Japan).

We also investigated the expression of tumor endothelial markers (TEM) in the isolated cells by immunofluorescence. The isolated cells were seeded and, after three days, they were incubated with rabbit anti-TEM1/CD248 polyclonal Ab, anti-GPR124/TEM5 polyclonal Ab or anti-TEM8 polyclonal Ab (Bioss antibodies, Woburn, MA, USA) in blocking

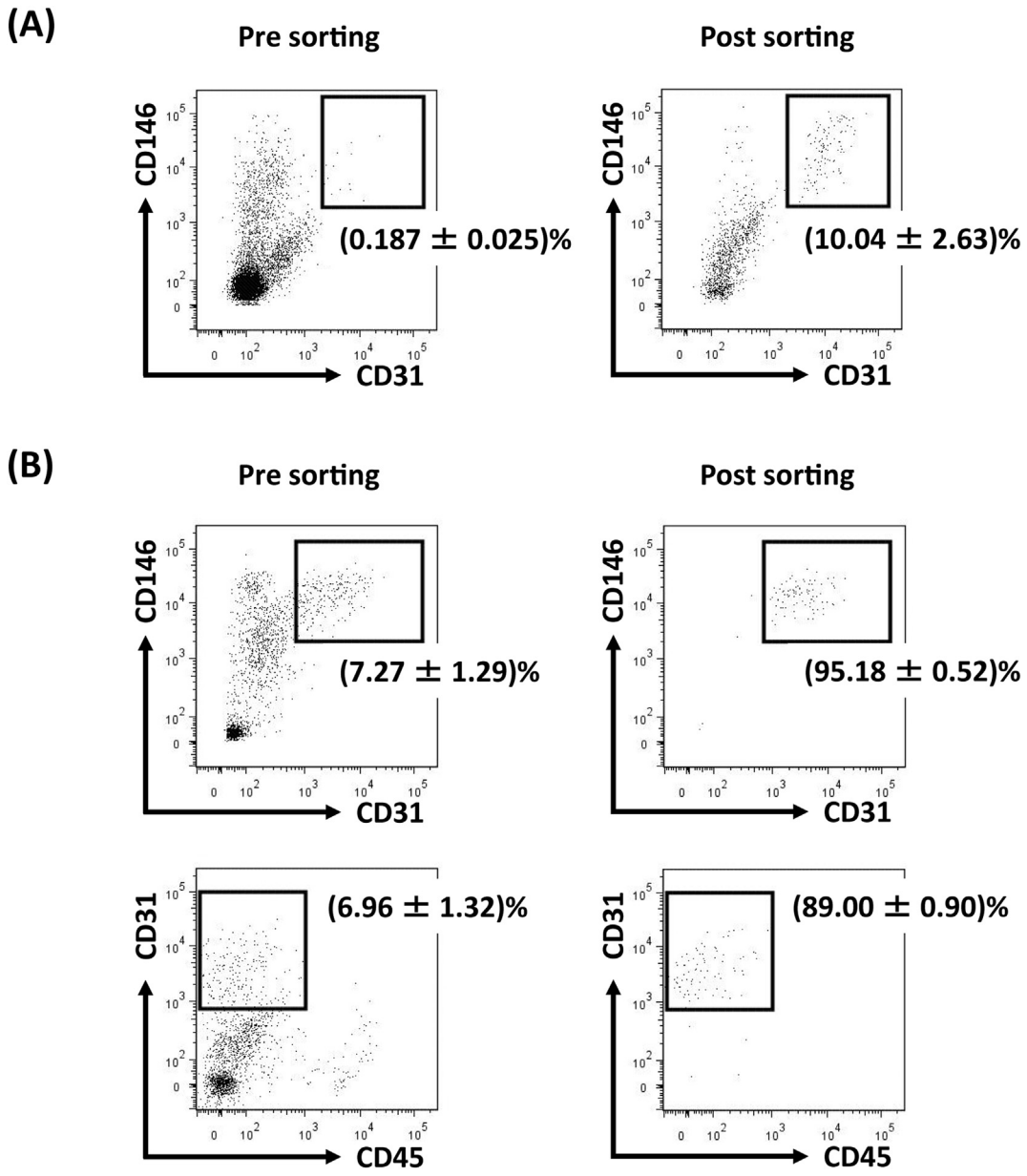


Fig. 3. Analysis of the purity of TECs at different stages of purification by FACS or magnetic sorting followed by FACS. (A) FCM profile of CD31 + /CD146 + cells before and after FACS, which was performed following the density gradient separation without precedent enrichment of CD31 + cells by magnetic sorting: the purity of CD31 + /CD146 + cells increased from 2% to 10% by FACS (CD31/CD146 double positive cells sorting). Data are shown as the mean ± SEM (n = 4). (B) With magnetic CD31-positive sorting after the density gradient separation, the percentage of CD31 + /CD146 + cells was similar to that obtained after magnetic sorting (10%); these cells could be further purified by FACS (CD31/CD146 double positive cells sorting), which increased their purity to > 95% (upper panels). The purified cells were CD45-negative (bottom panels). Data are shown as the mean ± SEM (n = 4).

Table 1
Yield of TECs after each step (three-step isolation).

	Collected cells	TECs	
	(million)	(%)	(million)
Single cell suspension from three tumors (n = 10)	515 ± 49		
1st separation (n = 10): Density gradient separation	187 ± 17	0.209 ± 0.042	0.39 ± 0.09
2nd separation (n = 8): Magnetic CD31 + sorting	11.2 ± 1.1	1.929 ± 0.218	0.21 ± 0.03
3rd separation (n = 5): FACS CD31/CD146 sorting	0.11 ± 0.05	95.18 ± 0.52	0.10 ± 0.05

solution (1:100) as primary Ab and subsequently incubated with secondary Ab and DAPI same as described above. Similarly, C57BL/6 mouse primary vein endothelial cells were stained with the same primary antibodies (anti-TEM1/CD248, anti-GPR124/TEM5 or anti-TEM8 polyclonal Ab). Those cells were observed with the inverted fluorescence phase contrast microscope BZ-X710 (Keyence, Osaka, Japan).

Table 2
Yield of TECs after each step (two-step isolation with magnetic cell sorting after density gradient separation).

	Collected cells	TECs	
	(million)	(%)	(million)
Single cell suspension from three tumors (n = 5)	521 ± 22		
1st separation (n = 5): Density gradient separation	213 ± 25	0.229 ± 0.020	0.49 ± 0.07
2nd separation (n = 5): Magnetic CD45− sorting and CD31+ sorting	1.88 ± 0.08	11.63 ± 1.13	0.22 ± 0.02

Table 3
Yield of TECs after each step (two-step isolation with fluorescence activated cell sorting after density gradient separation).

	Collected cells	TECs	
	(million)	(%)	(million)
Single cell suspension from three tumors (n = 4)	522 ± 27		
1st separation (n = 4): Density gradient separation	211 ± 22	0.203 ± 0.021	0.44 ± 0.09
2nd separation (n = 4): FACS CD31/CD146 sorting	0.43 ± 0.03	10.04 ± 2.63	0.04 ± 0.01

2.6. Immunological characterization of the isolated TECs

Using the isolated highly pure TECs, we performed a functional assay based on the hypothesis that TECs have an immune-suppressive function.

2.6.1. Preparation of responder CD8+ T cells

CD8+ T cells were purified from the spleen of wild type C57BL/6 mice by the magnetic negative selection. Briefly, after harvesting of the spleens and lysis of the erythrocytes with an ammonium chloride/potassium solution, the splenocytes were suspended in MACS buffer and CD8+ T cells were isolated using a CD8a + T cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. The cell suspension was applied to equilibrated MACS LS columns and the flow-through, containing the unlabeled, purified CD8a + T cells, was collected.

2.6.2. Carboxyflurescein diacetate succinimidyl ester (CFSE)-suppression assay

CD8+ T cells were stained with CFSE (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. A solution of CFSE (2 μM final concentration) was added, and the cells were gently mixed and incubated for 15 min at 37 °C in a CO_2 incubator protected from light. Labeling of cells was stopped by addition of cold heat-inactivated FBS for 10 min; the cells were then washed. Freshly purified TECs (1×10^5) were co-cultured with 1×10^5 CD8+ T cells in RPMI-1640 medium supplemented with 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 2 mM L-glutamine (Sigma), 50 μM 2-mercaptoethanol (Gibco), 20 mM HEPES (Gibco) and 10% heat-inactivated FBS in flat-bottom 96-well plates (BD Biosciences). CD8+ T cells were stimulated with solid-phased anti-CD3e (145-2C11, eBioscience, San Diego, CA, USA) and liquid-phased anti-CD28 (37.51, eBioscience) mAbs. Before seeding the cells, 50 μL of anti-CD3e solution (2 μg/mL final concentration) were dispensed to each well and the plates were incubated at 37 °C for 4 h. The anti-CD3e solution was then removed

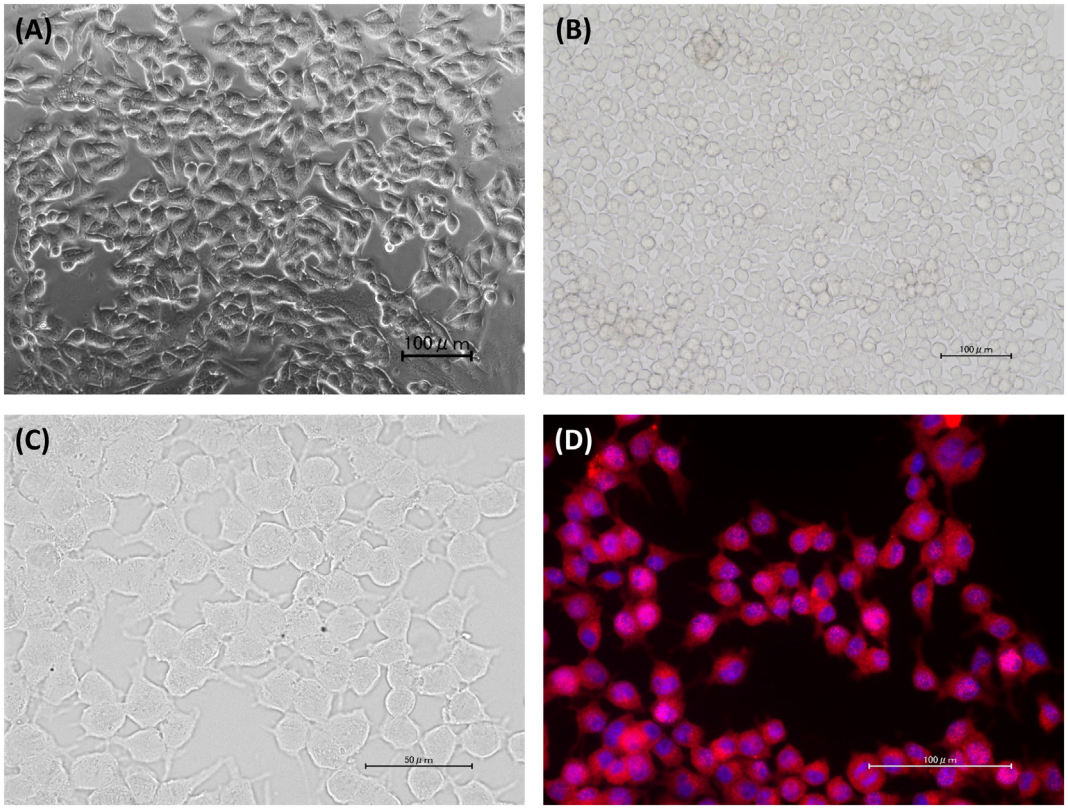


Fig. 4. Imaging of the purified TECs. Representative phase contrast (A-C) and immunofluorescent staining (D) images. (A) On day 5 of culture, the cells formed clusters. Scale bar: 100 μm. (B-D) On day 7 of culture, the adherent cells were fixed in 4% paraformaldehyde: they had polygonal shape and cobblestone appearance in phase contrast images (B and C). Scale bars: 100 μm (B) and 50 μm (C). (D) Immunofluorescent staining of cultured cells. Red: CD31 staining; blue: nuclei stained with DAPI. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

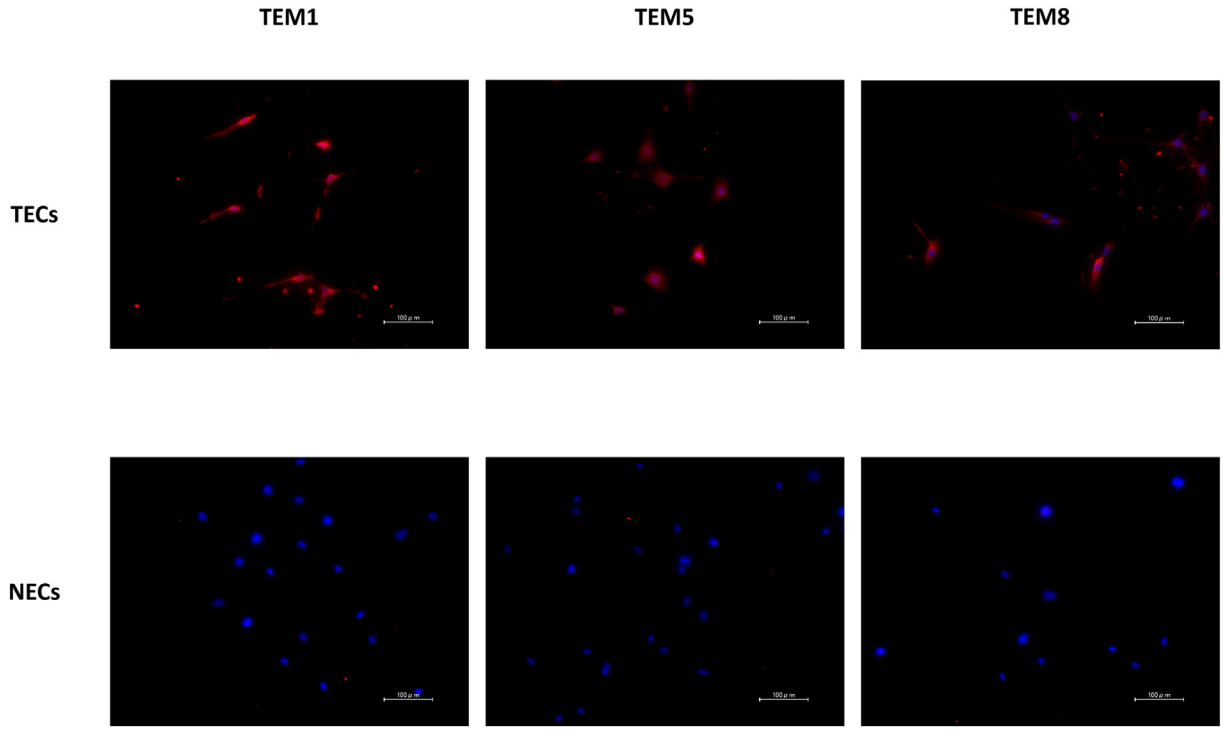


Fig. 5. Phenotype of the purified TECs. On day three of culture, the adherent cells were fixed and stained for TEMs. TECs were positive for TEM1, TEM5 and TEM8 (upper panels). Normal endothelial cells did not express the TEM investigated. Red: TEMs staining (left: TEM1, middle: TEM5, right: TEM8); blue: nuclei stained with DAPI. Scale bar: 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the wells were washed with PBS. The cells were then seeded with soluble anti-CD28 (1 µg/mL final concentration). C57BL/6 mouse primary vein endothelial cells were used as normal endothelial cells (NECs), and they were compared with TECs. After 3 days of co-culture, the proliferation of CD8+ T cells was measured based on CFSE dilution, using flow cytometry.

2.7. Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). Statistical analysis among experimental groups was performed by ANOVA, and Tukey's test was used to compare individual groups. A value of $p < .05$ was considered statistically significant.

3. Results

3.1. Detection of TECs in a mouse cancer-model

We first established a mouse cancer model using the mouse B16-F10 melanoma cell line. We then dissected the tumors from the mice and digested them with Collagenase Type II to obtain a single cell suspension. We obtained approximately 500 million cells from three tumors. After the density gradient separation (first separation), we collected approximately 200 million cells. Analysis by flow cytometry of the pre-sorted cells indicated that TECs, which are positive for the endothelial markers CD31 and CD146, were approximately 0.2% of the total population (Fig. 2A).

3.2. The combination of magnetic sorting and FACS allows the isolation of highly pure TECs

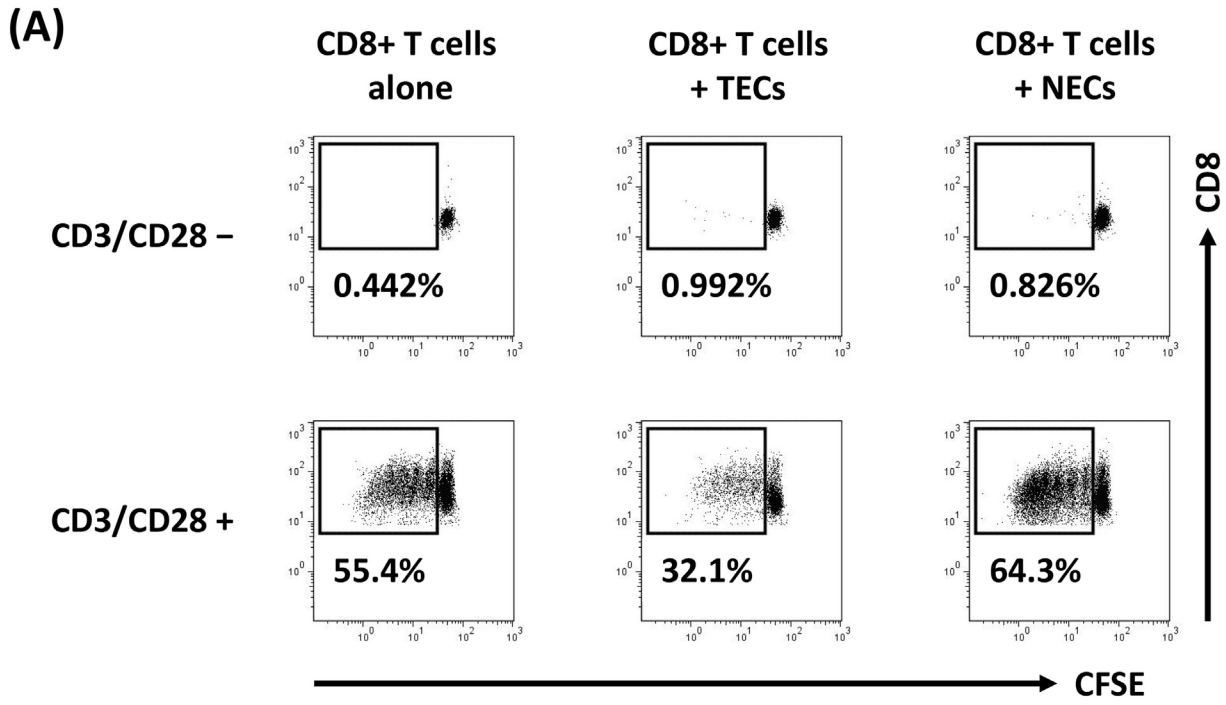
To purify TECs, we first carried out a magnetic sorting using anti-mouse CD31 microbeads on the cells obtained from the density gradient separation. After CD31 positive sorting (second separation), we obtained approximately 10 million cells. The population of CD31/CD146 positive cells increased to approximately 2% of the total, indicating a 10-times enrichment (Fig. 2B). To improve the purity of TECs, we depleted CD45 positive cells by magnetic sorting using anti-mouse CD45 microbeads before CD31 positive enrichment – CD45 is a hematopoietic cell marker. However, the percent of the CD31/CD146 double positive cells was only up to 10 after the two-step magnetic sorting (Fig. 2C). Therefore, we decided to perform FACS instead of the magnetic sorting.

The cells collected after the density gradient separation were stained with anti-mouse CD31-FITC and anti-mouse CD146-APC mAbs and the CD31/CD146 double positive cells were sorted using the FACS Aria Cell Sorting System. CD31/CD146 double positive cells were only 10% of the total number of cells (Fig. 3A). Similarly to magnetic sorting, FACS was not the best method for TECs purification, because of the low number of these cells in the original population. Therefore, we decided to combine magnetic sorting and FACS: we first enriched the cell population of CD31 positive cells by magnetic sorting using anti-mouse CD31 microbeads. Next, we further sorted the cells by FACS, using anti-mouse CD31-FITC and anti-mouse CD146-APC mAbs and isolating the CD31/CD146 double positive cells. Using this three-step method, we obtained approximately 0.1 million CD31/CD146 double positive cells whose purity was higher than 95% (Fig. 3B, upper panels). Furthermore, purified cells did not show expression of CD45 (Fig. 3B, lower panels).

Table 1 summarizes the number of collected cells and the percent of TECs within the total population at each step during the three-step isolation described above. Table 2 describes the number of collected cells and the percent of TECs after density gradient separation and magnetic sorting (CD45 negative selection and CD31 positive enrichment) and Table 3 reports the same data after density gradient separation and CD31/CD146 FACS sorting.

3.3. Isolated cells show the morphological characteristics of endothelial cells

To confirm that the isolated cells were endothelial, we seeded them onto culture slides coated with collagen type I and cultured them in the



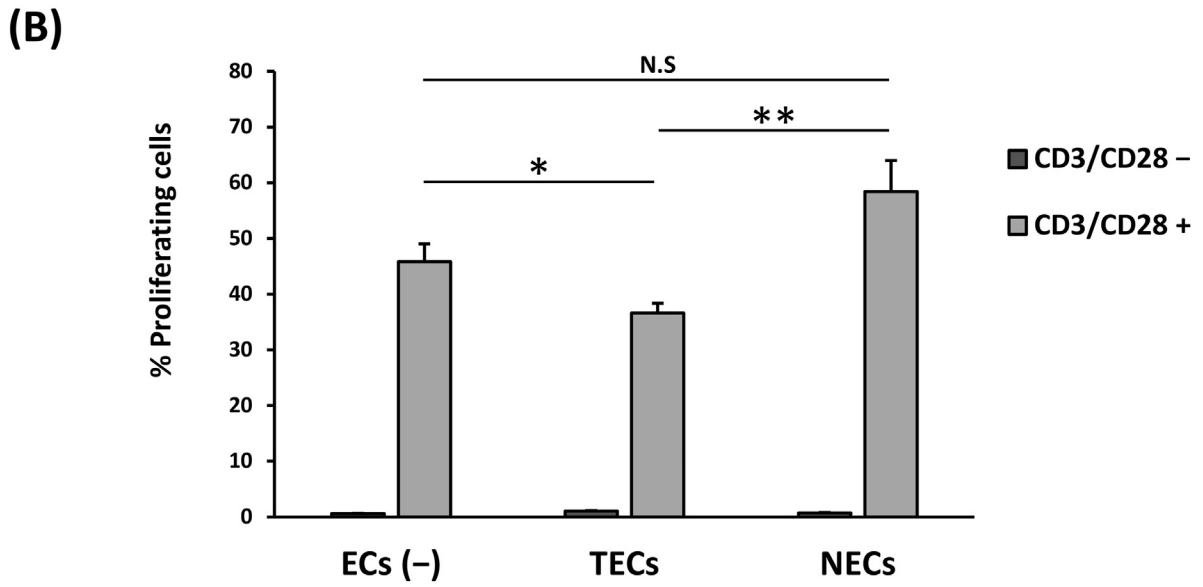


Fig. 6. CFSE suppression assay using purified TECs. The TECs were co-cultured with fluorescent dye (CFSE)-labeled responder CD8+ T cells from spleens of C57BL/6 mice either with or without stimulation by CD3/CD28 monoclonal antibodies (mAbs). After 3 days, harvested CD8+ T cells were stained with a phycoerytrin (PE)-conjugated anti-CD8 mAb. We measured the proliferation of CD8+ T cells based on CFSE dilution using flow cytometry. (A) Representative FCM results were shown. Without the stimulation, most of CD8+ T cells did not proliferate. With the stimulation, CD8+ T cells showed robust proliferation. In the presence of TECs, the proliferation of CD8+ T cells was suppressed. Compared to TECs, NECs did not suppress the proliferation of CD8+ T cells. Without the stimulation, most of CD8+ T cells did not proliferate. (B) Mean of the ratio of the proliferating cells in the whole CD8+ T cell population. The ratio of the proliferating cells was significantly lower in the presence of TECs than in the absence of TECs or in the presence of NECs. The mean ± SEM of 4 independent experiments is shown. *, $p < 0.05$. **, $p < 0.01$.

Endothelial Cells Growth Medium MV2. After 2–3 days, adherent cells were detected and some of them formed clusters (Fig. 4A). After one week, the cells reached 80–90% confluency and showed a cobblestone morphology, which is characteristic of the endothelial cells. Furthermore, immunofluorescent staining analysis showed that they were CD31 positive (Fig. 4B-D).

3.4. TECs express TEMs

To further investigate the nature of the isolated cells, we performed immunofluorescent staining of TEMs. It has been reported that TEM1, TEM5 and TEM8 are expressed on endothelial cells of normal tissues (Carson-Walter et al., 2001). C57BL/6 mouse primary vein endothelial cells, used as NECs, did not express TEM1, TEM5 and TEM8 (Fig. 5, lower panels), while the purified TECs expressed them (Fig. 5, upper panels).

3.5. TECs have immuno-suppressive potential

Next, we performed a CFSE suppression assay to assess the immunological function of the TECs isolated with the method described. CFSE-labeled CD8+ T cells were stimulated with anti-CD3 and CD28 mAbs and co-cultured with isolated TECs. The proliferation of CD8+ T cells was evaluated based on CFSE dilution, measured using flow cytometry, and the ratio of the proliferating cells in the whole CD8+ T cell population was analyzed. Most of the CD8+ T cells did not proliferate without stimulation, and proliferated in the presence of anti-CD3 and CD28 mAbs. Notably, the proliferation of CD8+ T cells was suppressed in the presence of the TECs. On the other hand, the proliferation of CD8+ T cells was not suppressed in the presence of NECs (Fig. 6A). The summary of the percentage of the proliferating cells within CD8+ T cells is shown in Fig. 6B.

4. Discussion

The purpose of this study was to establish an effective isolation method of highly pure TECs, which represent a small population among tumor constituent cells. Endothelial cells lines such as human umbilical vein endothelial cells (HUVEC) have been used instead of TECs in many studies (Burrows et al., 1995; Hellebrekers et al., 2007; Thijssen et al., 2008; Motz et al., 2014), due to the difficulties in obtaining highly pure TECs. However, it has been found that TECs and normal endothelial cells have some differences: in this study, we focused on the immunological properties of TECs. The use of TECs in functional assays, as the one we wanted to perform to investigate the immunological function of TECs, requires a high number of fresh and highly pure TECs. For this purpose, we tested and optimized strategies for the isolation and purification of these cells.

First, we tried to purify TECs directly from the cells obtained after density gradient separation of tumors by magnetic CD45 negative and CD31 positive selection, modifying a protocol that has been published (Kajimoto et al., 2010). We found that this method was not very efficient. Alternatively, we tried to purify TECs (CD31/CD146 double positive cells) by FACS. Once more, the purity of the TECs isolated was not sufficient for the studies we wanted to perform. The difficulty encountered was caused by the low number of TECs, compared with that of the expanding tumor cells. To obtain purified TECs, it was necessary to deplete the cell population of their most abundant constituent, but this goal was difficult to obtain using conventional procedures. Therefore, we used the combination of magnetic sorting and subsequent FACS cell sorting, and optimized a protocol for the three-step purification of TECs that allowed us to obtain highly pure TECs, which could subsequently be used in functional assays.

By flow cytometry, we confirmed that the TECs isolated were CD31/CD146 double positive cells and were CD45 negative (CD45 is not expressed on the surface of the endothelial cells). The cells were propagated on culture dishes to assess their morphology. The isolated cells attached to the dishes, showed a cobblestone morphology, and expressed CD31, suggesting they were endothelial cells. Thus, we confirmed the effectiveness of the sequential purification procedures for isolating TECs developed in this study. In addition, we performed a functional assay using the purified TECs. We found that TECs suppressed the proliferation of CD8+ T cells stimulated with CD3/28 mAbs. This result suggests that TECs have an immune-suppressive potential. It has been reported that immuno-suppressive endothelial cells are induced in some unique environments such as liver sinusoidal endothelial cells (LSECs) (Onoe et al., 2005b). Furthermore, some reports have already suggested that TECs might have immune-suppressive potential (Mauge et al., 2014; Motz et al., 2014; Hendry et al., 2016): TECs seemed to suppress the intra-tumoral adaptive immunity and therefore, the development of novel therapies that regulate the immunological functions of TECs has been considered. However, no *in vitro* study has yet demonstrated the immunological functions of TECs in functional assays using fresh and highly pure TECs. Therefore, the immune-suppressive functions of TECs remain unknown. On the other hand, the understanding of the immunological roles of TECs is important to establish effective strategies targeting TECs in anti-cancer therapy. Our purification method allowed us to obtain highly pure TECs that could be used to investigate the immunological functions of TECs in an *in vitro* assay. Additionally, we found that TECs which was purified by our three-step isolation method can suppress T cell proliferation *in vitro*.

5. Conclusions

The isolation method of TECs described in this study might help the elucidation of the characteristics and functions of TECs in the tumor microenvironment and provide important insights on TECs. Importantly, the functional characterization of TECs might allow the development of novel and effective anti-cancer therapies targeting TECs.

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Declarations of interest

None.

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