Tumor endothelial cells (TEC) play multiple roles in the regional specialization of vascular structure and physiology. Because TECs in the tumor microenvironment come in contact with circulating immune cells, they might influence not only trafficking but also the antitumor cellular immune response. In a mouse tumor implantation model with B16 melanoma cells, TECs expressed MHC class II, costimulating molecules, and programmed death-ligand 1 (PD-L1), suggesting that they are antigen (Ag)-presenting cells with suppressive activity. Furthermore, TECs were able to take up and present tumor-derived ovalbumin (OVA) peptide on MHC class I molecules. In functional assays, B16-OVA tumor-derived TECs significantly suppressed the proliferation and Ag-specific cytotoxicity of OVA-specific CD8+ T cells relative to those of B16 tumor-derived TECs. This suppressive activity required cell–cell contact and was abrogated by PD-L1 blockade. TECs impaired proinflammatory cytokine production of CD8+ T cells, including IL2, TNFα, and IFNγ. B16-OVA tumor-derived TECs induced immunosuppressive CD4+ T cells that suppressed OVA-specific CD8+ T-cell proliferation via inhibitory cytokines, including IL10 and TGFβ. Deficiency of PD-L1 in TECs, but not in hematopoietic cells, impaired suppression and apoptosis of tumor-infiltrating CD8+ T cells, resulting in inhibition of tumor development in vivo model. These data suggest that TECs might regulate the immune response of tumor Ag-specific CD8+ T cells via the PD-1/PD-L1 pathway and induce immune suppressive CD4+ T cells in an Ag-specific manner, contributing to tumor immune evasion.

**Implications:** The findings of this study might encourage the further development of novel anticancer therapies and strategies.
Materials and Methods

Mice and cell lines
Mice of ages 6 to 12 weeks were used in this study. All controls were age matched. All animals were maintained and handled under pathogen-free conditions, in compliance with ARRIVE and institutional animal guidelines. Female wild-type (WT) C57BL/6 mice were purchased from Clea Japan. C57BL/6 OT-I TCR transgenic mice were provided by Dartmouth College (Hanover, PA). B16 (B16-F10), a C57BL/6-derived melanoma, was obtained from the ATCC. B16-OVA (MO4), a chicken ovalbumin (OVA)–expressing subclone of B16-F10, was provided by Dartmouth College (Hanover, NH). Cell lines were authenticated by short tandem repeat test (GeneMapper, Applied Biosystems), checked negative for Mycoplasma every 2 months using the MycoAlert Mycoplasma Detection Kit (Lonza), according to the manufacturer’s instructions. Similarly, OVA-specific CD4+ T cells were purified from the spleens of C57BL/6 OT-I and C57BL/6 Pmel-1 mice, respectively, by magnetic-negative selection using a CD8a+ T Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer’s instructions. OVA-specific CD4+ T cells were purified from the spleens of C57BL/6 OT-II mice by magnetic-negative selection using a CD4+ T Cell Isolation Kit (Miltenyi Biotec). Cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) as described previously (25, 26).

Peptide Ags
The H-2Kb–restricted peptide SIINFEKL and the I-A–restricted peptide ISQAVHAAHAEINEAGR, which correspond to amino acid residues 257–264, and 323–339 of chicken OVA, respectively, and the H-2Db–restricted peptide KVPRNQDWL, which corresponds to amino acid residues 25–33 of human melanoma Ag gp100, were purchased from GenScript (Piscataway) and extensively purified (95%).

Ag uptake and processing using DQ-OVA
We incubated 0.5 × 10^6 CD31+ enriched cells in 24-well plates for 60 minutes at 4°C or 37°C, in the presence of 10 μg/mL DQ-OVA (Molecular Probes). After 15-minute intervals, cells were washed and stained to detect cell surface markers (anti-CD31 mAb). Highly fluorescent peptides of CD31+ TECs, which were released from DQ-OVA after proteolysis in TECs, were measured using the FITC channel by flow cytometry.

Preparation of responder cells and carboxyfluorescein diacetate succinimidyl ester labeling
OVA-specific CD8+ T cells and gp100-specific CD8+ T cells were purified from the spleens of C57BL/6 OT-I and C57BL/6 Pmel-1 mice, respectively, by magnetic-negative selection using a CD8a+ T Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer’s instructions. Similarly, OVA-specific CD4+ T cells were purified from the spleens of C57BL/6 OT-II mice by magnetic-negative selection using a CD4+ T Cell Isolation Kit (Miltenyi Biotec). Cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) as described previously (25, 26).

Preparation of Ag-pulsed bone marrow–derived dendritic cells for stimulating responders
To stimulate T cells, bone marrow–derived dendritic cells (BMDC) were generated by culturing bone marrow (BM) cells with recombinant murine GM-CSF (PeproTech), according to a method described previously with some modifications (27). On day 10 of culture, 1 × 10^6 cultured cells were seeded in each 100-mm dish in 40 mL of culture medium composed of RPMI1640 Medium (Sigma) supplemented with 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 2 mM L-1-glutamine (Sigma), 50 μM 2-mercaptoethanol (Gibco), 20 mM/L HEPES (Gibco), and 10% heat-inactivated FBS, and pulsed with 2.5 μM/L OVA257–264 (SIINFEKL), gp10025–33, or OVA323–339 at 37°C. After 18 hours, we used those cells for OT-I CD8+, Pmel-1 CD8+, and OT-II CD4+ T-cell stimulation, respectively (28). Ag-pulsed BMDC stimulation elicited the proliferation of Ag-specific responders in a dose-dependent manner, while nonpulsed BMDCs did not (Supplementary Fig. S1A).
Ag-specific T-cell suppression

We cocultured $1 \times 10^5$ CFSE-labeled OT-I CD8+ T cells with $1 \times 10^5$ to $1 \times 10^6$ TECs isolated from B16 or B16-OVA tumors and simultaneously stimulated with $1 \times 10^5$ OVA257-264-pulsed BMDCs in culture medium in round-bottomed 96-well plates (BD Biosciences) at 37°C in a 5% CO2 incubator in the dark. After 5 days, proliferation of CD8+ T cells was measured via CFSE dilution, using flow cytometry. In this assay, permeable transwell inserts (0.4 μm; Corning) were used to prevent cellular contact between CD8+ T cells and TECs during coculture. In addition, 10 μg/mL of the rat IgG2a kappa isotype control (eB2a, ebioscience) or 10 μg/mL of anti-PD-L1 antibody (MIH5, ebioscience) was added during some assays. Similarly, we cocultured $1 \times 10^5$ CFSE-labeled Pmel-I CD8+ or OT-II CD4+ T cells with TECs isolated from B16 or B16-OVA tumors at the indicated ratios and simultaneously stimulated with $1 \times 10^5$ gp10025-33 or OVA233-359-pulsed BMDCs for 5 days, respectively, and T-cell proliferation was measured. The OT-I CD8+ T-cell suppression assay was also performed using OT-II CD4+ T cells. OT-II CD4+ T cells were cocultured with TECs isolated from B16 or B16-OVA tumors and simultaneously stimulated with OVA257-264-pulsed BMDCs for 5 days, as described above. CD8+ T cells were isolated from them by magnetic-positive selection, using a CD3e Microbead Kit (Miltenyi Biotec), to purify OT-II CD4+ T cells. Subsequently, $1 \times 10^5$ to $1 \times 10^6$ isolated OT-II CD4+ T cells were cocultured with $1 \times 10^5$ CFSE-labeled OT-I CD8+ T cells and simultaneously stimulated with $1 \times 10^5$ OVA257-264-pulsed BMDCs for 5 days, and the proliferation of CD8+ T cells was measured. In some experiments, anti-cytokine-neutralizing antibodies, including 10 μg/mL of the anti-TGF-beta1, 2, 3 antibody (1D11.16.8, ebioscience) or 10 μg/mL of the anti-IL10 antibody (JE33-9D7, ebioscience), were added to each well during coculture.

Quantification of T-cell proliferation levels

Mitotic indices that indicated OT-I CD8+ T-cell proliferation were quantified using their CFSE fluorescence intensities, using a previously described method (29). The stimulation ratios were calculated by dividing the mitotic indices of OT-I CD8+ T cells cocultured with TECs and simultaneously stimulated with OVA257-264-pulsed BMDC by those of positive controls that were not cocultured with TECs in the presence of OVA257-264-pulsed BMDC stimulation. The associated equation is shown in Supplementary Fig. S2A.

Ag-specific cell-mediated cytotoxic assay

B16 and B16-OVA cells were stained with 2 μmol/L and 0.2 μmol/L CFSE, respectively, and mixed in a 1:1 ratio. Mixed target cells were incubated with OT-I CD8+ T cells, which were cocultured with TECs (E:T ratio = 20:1) and simultaneously stimulated with $1 \times 10^5$ OVA233-359-pulsed BMDCs for 5 days, in round-bottomed 96-well plates at 37°C in a 5% CO2 incubator, for 4 hours. Cells were then incubated with PI for 10 minutes to stain dead cells. After the ratios of the targets were determined by flow cytometry, the percentage of specific killing was calculated by comparing the ratio of targets in sample wells to that in control wells (E:T ratio = 0:1), according to the equation: $[(1 - (B16-OVA cells in the sample well/B16 cells in the sample well))/(B16-OVA cells in the control well/B16 cells in the control well))] \times 100$ (Supplementary Fig. S2B).

In some experiments, B16 and B16-OVA target cells were stained to detect Annexin V and PI. The percentages of Annexin V–positive (apoptotic and necrotic cells) total cells were measured.

Intracellular staining (Foxp3 and cytokines)

CFSE-labeled cells were stimulated for 5 hours with 2 μL/mL of the Leukocyte Activation Cocktail with BD GolgiPlug (BD Biosciences; PMA, ionomycin, and Brefeldin A). Cells were first stained to detect CD4 and CD8, then subsequently stained to detect the presence of intracellular Foxp3 and cytokine, using the Transcription Factor Buffer Set (BD Biosciences), according to the manufacturer’s instructions.

Cytometric bead array for cytokines in cell culture supernatants

Supernatants were collected and analyzed to detect cytokines using the BD Flex Cytometric Bead Array System and the Mouse/Rat Soluble Protein Master Buffer Kit (BD Biosciences), both according to the manufacturer’s instructions.

ELISA for TGFβ1 in cell culture supernatants

OT-II CD4+ T cells were cocultured with TECs isolated from B16 or B16-OVA tumors for 5 days as described above. Supernatants were collected and TGFβ1 secretion levels were analyzed. TGFβ1 levels were measured using the Human/Mouse TGFβ1 ELISA Kit, according to the manufacturer’s instructions (Thermo Fisher Scientific).

In vivo tumor growth in PD-L1-knockout mice receiving syngeneic BM transplantation

BM cells were collected by flushing femurs and tibias of C57BL/6 CD45.1 congenic donors with a 25-gauge needle into PBS (Wako). Unselected BM cells ($20 \times 10^6$) were transplanted into syngeneic WT (CD45.2) or PD-L1-deficient mice (CD45.2) via tail vein injection. Recipients were irradiated 6 hours before transplantation with a lethal dose of 13 Gy. Mice were used for experiments 8 to 12 weeks after transplantation.

A total of $1 \times 10^6$ tumor cells (B16 or B16-OVA) suspended in PBS were inoculated in the left flank fold of syngeneic chimeric mice. At day 7 after tumor inoculation, mice with palpable tumors received a tail vein injection containing $10 \times 10^5$ CD8+ T cells purified from the spleens of C57BL/6 OT-I mice (CD45.2) via magnetic-negative selection, using a CD8a+ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. Tumor growth was evaluated by calculation of tumor volume using the formula: tumor volume (mm3) = (length) \times (width)^2 \times 0.43 (30).

At day 21 after adoptive OT-I CD8+ T-cell transfer, tumors were dissected from tumor-bearing mice and digested to a single-cell suspension using collagenase Type II. We analyzed tumor-infiltrating cells within tumors. The cells were stained with CD45.1, and the populations of OT-I CD8+ T cells among total CD8+ T cells were measured. Among OT-I CD8+ T cells, the percentages of apoptotic cells were measured using Annexin V. In addition, the absolute number of Annexin V–positive CD8+ T cells per volume of the tumor was measured.

Statistical analysis

Results are shown as means ± SEM. We performed statistical analysis between two experimental groups using an unpaired Student two-tailed t test. One-way ANOVA was used with the Tukey test for the comparison of three groups. P values that were less than or equal to 0.05 were considered to be statistically significant.

Study approval

Animal experiment protocols were consistent with national and institutional animal guidelines. All studies were approved by the Kure Medical Center Animal Ethics Committee.
Results

TECs have the immunologic phenotype of suppressive APCs

Histopathology of excised tumors showed that TECs constituted the lining of tumor vessels (Fig. 1A, left). IHC showed that TECs were positive for CD31, an endothelial marker (Fig. 1A, right). Flow cytometry indicated that TECs that were simultaneously positive for endothelial markers CD31 and CD146, constituted <0.1% of the total population of isolated tumor constituent cells (Fig. 1B, left). They were CD45 negative (Fig. 1B, right). TECs expressed MHC class II and costimulating molecules, such as CD40 (Fig. 1C). Notably, they expressed PD-L1, however, not PD-L2, both which are apoptosis-inducing molecules. These results suggest that they are suppressive APCs.

Uptake and presentation of tumor Ags by TECs

To assess the capacity of TECs for uptake and processing of Ags, TECs were incubated with DQ-OVA. During the incubation, the mean fluorescence intensity (MFI) of FITC in TECs increased with time at 37°C, under conditions in which proteolysis could occur, in contrast to the MFI of FITC in TECs observed at 4°C (Fig. 1D and E), indicating that TECs had the capacity for taking up OVA and processing it into peptides.

To evaluate exogenous Ag presentation by TECs such as tumor Ags, B16-OVA cells were inoculated into mice and TECs from the resulting tumors were analyzed for the presence of an OVA-derived Ag (SIINFEKL)/MHC class I (H2Kb) complex. The expression of the complex of these TECs was significantly higher than that of B16 tumor (non-OVA–transfected tumor)-derived TECs (Fig. 1F and G). Of note, the expression of costimulating molecules such as CD80, CD86, CD40, and PD-L1 on endothelial cells isolated from B16 and B16-OVA tumors were not different (data not shown). These results suggest that TECs could take up, process, and present tumor-derived Ags on MHC class I molecules in vitro and in vivo.

TECs regulate the proliferation and cytotoxicity of CD8+ T cells in an antigen-specific manner

To evaluate the immunosuppressive capacity of TECs against antigen-specific CD8+ T cells, we performed a suppression assay in which CFSE-labeled OT-1 CD8+ T cells were cocultured with TECs and simultaneously stimulated by OVA257–264-pulsed syngeneic BMDCs in vitro. OT-1 CD8+ T cells showed almost no proliferation in the absence of stimulation with OVA257–264-pulsed BMDCs (negative control), while a robust level of proliferation was observed after stimulation with OVA257–264-pulsed BMDCs (positive control, Fig. 2A). Notably, the addition of TECs isolated from B16-OVA tumors caused a significantly greater suppression of OT-1 CD8+ T-cell proliferation after stimulation with OVA257–264-pulsed BMDCs, relative to that observed with the addition of TECs from non-OVA–transfected B16 tumors (Fig. 2A and B). This suppression by B16-OVA tumor–derived TECs was dose dependent (Fig. 2B). Furthermore, the proliferation of OT-1 CD8+ T cells was significantly more suppressed by B16-OVA tumor–derived TECs than by Pmel-1 CD8+ T cells (Fig. 2C). These results suggest that TECs suppressed the proliferation of CD8+ T cells in an antigen-specific manner. Of note, suppression of OT-1 T cells by B16-OVA tumor–derived TECs was not observed with high-dose OVA257–264-pulsed BMDC stimulation (Supplementary Fig. SI8). Furthermore, B16-OVA tumor–derived TECs alone did not elicit OT-1 CD8+ T-cell proliferation in the absence of stimulation with OVA257–264-pulsed BMDCs (Supplementary Fig. S1C).

Next, we performed an Ag-specific, cell-mediated cytotoxicity assay. The proportion of B16-OVA target cells was lower in the presence of OT-1 CD8+ T cells that had not been cocultured with TECs relative to that observed for the control (E:T ratio = 0:1; Fig. 2D). Furthermore, the proportion of B16-OVA target cells incubated with OT-1 CD8+ T cells that were preliminarily cocultured with B16-OVA tumor–derived TECs was higher than that of cells incubated with OT-1 CD8+ T cells that were preliminarily cocultured with B16 tumor–derived TECs or cells not cocultured with TECs. The specific killing is summarized in Fig. 2E; the differences in specific killing were statistically significant. In addition, cytotoxic assays evaluated by Annexin V and PI staining were performed. The percentage of Annexin V–positive cells among B16-OVA cells incubated with OT-1 CD8+ T cells that were preliminarily cocultured with B16-OVA tumor–derived TECs was significantly lower than that with B16 tumor–derived TECs or without TECs (Fig. 2F). The percentages Annexin V–positive cells (total apoptotic and necrotic cells) are shown in Fig. 2G. The difference was statistically significant. These results suggest that TECs suppressed the cytotoxicity of CD8+ T cells in an Ag-specific manner.

TECs regulate the proliferation and cytotoxicity of CD8+ T cells in an Ag-specific manner by cell–cell contact via the PD-1/PDL1 pathway

To determine whether cell–cell contact was necessary for achieving the suppressive effects of TECs, we used permeable transwell inserts in the suppression assay or cell-mediated cytotoxicity assay. B16-OVA tumor–derived TECs suppressed OT-1 CD8+ T-cell proliferation in a dose-dependent manner with cell–cell contact, whereas the physical separation of TECs from OT-1 CD8+ T cells abrogated suppression and Ag-specific cytotoxicity (Fig. 3A and B). Thus, these results suggest that TECs require cell–cell contact to suppress OT-1 CD8+ T cells. Even if cell–cell contact was available, when PD-L1 on TECs was blocked with the anti-PD-L1 mAb, TEC-induced Ag-specific suppressive effects on OT-1 CD8+ T cells were also abrogated in both suppression (Fig. 3C) and cytotoxicity (Fig. 3D) assays. These results indicated that PD-1/PD-L1 signals play a significant role in Ag-specific suppression.

TECs impair proinflammatory cytokine production in Ag-specific CD8+ T cells in an Ag-nonspecific manner

Next, we determined the cytokine profile of these OT-1 CD8+ T cells by intracellular staining in a suppression assay. CD8+ T-cell activity can be evaluated on the basis of the pattern of production of the proinflammatory cytokines IL2, TNFα, and IFNγ (31, 32). The proportion of CD8+ T cells producing these cytokines was significantly decreased in the presence of TECs, relative to that observed in the absence of TECs, while the proportion was not significantly different between B16 and B16-OVA tumor–derived TECs (Fig. 4A and B). IL2 levels in supernatants were significantly lower after the coculture of OT-1 CD8+ T cells with TECs than that without TECs. Furthermore, IL10 levels were significantly higher after the coculture of OT-1 CD8+ T cells with TECs than that of a solution without TECs. However, levels of IL2 and IL10 produced were similar, irrespective of whether the TECs were B16 tumor–derived or B16-OVA tumor–derived (Fig. 4C).

TECs induced inhibitory CD4+ T cells in an Ag-specific manner

To evaluate the interaction between TECs and Ag-specific CD4+ T cells, CFSE-labeled OT-II CD4+ T cells were cocultured with TECs and simultaneously stimulated with OVA253–339-pulsed
Figure 1.
TECs constituting the lining of tumor blood vessels exhibit immunologic properties, and are involved in exogenous Ag uptake and presentation of Ag peptides bound to MHC class I molecules. A, Tumor histology showing tumor vessels and the endothelial cells lining them (left). These cells were CD31⁺ in an IHC study (right). Scale bar, 50 μm. B, Flow cytometry of tumor cells after density gradient separation; CD31/CD146 double-positive cells indicative of TECs were detected in a small population within tumor constituent cells, which constituted <0.1% of the total population (left). TECs are CD45⁻ (right). C, TECs express MHC class II and costimulating molecules such as CD40 and PD-L1. B and C are representative results from three independent experiments. D and E, In vitro OVA-uptake assay. TECs incubated with OVA Ag with a self-quenching conjugate (BODIPY) release highly fluorescent peptides upon proteolysis during Ag processing. D, MFI of FITC in TECs increased with time at 37°C, in contrast to that at 4°C. E, Percentages of TECs involved in OVA uptake; at 37°C, the percentage was increased and significantly higher than that at 4°C, indicating that TECs take up OVA and process them into peptides. n = 6. Unpaired Student two-tailed t test. *, P < 0.05; **, P < 0.01. F and G, OVA peptide expression on MHC class I molecules of TECs. F, TECs in B16-OVA (OVA-transfected) or B16 (nontransfected) tumors were analyzed for an antibody reacting specifically to a complex of an OVA-derived Ag presented with a C57BL6 mouse MHC class I molecule (anti-H2Kb/SIINFEKL antibody). Representative data from eight independent experiments are shown. B16-OVA tumor-derived TECs expressing OVA peptide/MHC class I complexes were greater than that of B16 tumor-derived TECs. G, Percentages of expression of OVA peptides/MHC class I complexes of B16-OVA tumor-derived TECs were significantly higher than that of B16 tumor-derived TECs. n = 8. Unpaired Student two-tailed t test (*, P < 0.05; **, P < 0.01; NS, not significant).
Figure 2.
TECs regulate the proliferation and cytotoxicity of CD8$^+$ T cells in an Ag-specific manner. A–C, Ag-specific CD8$^+$ T-cell suppression assay. CFSE-labeled OT-I CD8$^+$ T cells were cocultured with TECs and simultaneously stimulated by OVA$_{257-264}$-pulsed BMDCs for 5 days. A, Representative histograms of CFSE dilution and stimulation ratios in the top and bottom panels, respectively. OT-I CD8$^+$ T cells showed almost no proliferation without stimulation with pulsed BMDCs (negative control), but proliferated robustly upon stimulation with BMDCs (positive control). B, Increasing the number of TECs showed that suppression was dose dependent. $n = 15$. Unpaired Student two-tailed t test, $^* P < 0.01$. C, OT-I CD8$^+$ T-cell proliferation was significantly more suppressed by B16-OVA tumor-derived TECs than by Pmel-I CD8$^+$ T cells. $n = 4$. Unpaired Student two-tailed t test, $^* P < 0.01$. D–G, Ag-specific cytotoxic assay. B16 and B16-OVA stained with CFSE at high and low concentrations, respectively, were incubated with OT-I CD8$^+$ T cells cocultured with TECs of indicated tumors. D, Representative histograms of CFSE fluorescence intensities. The proportion of B16-OVA cells was higher in the presence of OT-I CD8$^+$ T cells with B16-OVA tumor-derived TECs than with B16 tumor-derived TECs or those without TECs. E, Specific killing by OT-I CD8$^+$ T cells cocultured with B16-OVA tumor-derived TECs was significantly lower than those without TECs or those with B16 tumor-derived TECs. $n = 5$. One-way ANOVA with Tukey test, $^* P < 0.05$; $^{**} P < 0.01$. F, Cytotoxicity evaluated by Annexin V and PI staining for both B16 and B16-OVA cells. Representative results from five independent experiments are shown. The percentage of B16-OVA Annexin V-positive (Annexin V$^+$) cells was lower in the presence of OT-I CD8$^+$ T cells with B16-OVA tumor-derived TECs than with B16 tumor-derived TECs or those without TECs. G, Percentages of Annexin V$^+$ positive cells (total apoptotic and necrotic cells) from B16-OVA target cells. $n = 5$. One-way ANOVA with Tukey test ($^{**} P < 0.01$). NS, not significant.
TECs Suppress Antitumor T-cell Responses

Figure 3.
TECs regulate proliferation and cytotoxicity of CD8+ T cells in an Ag-specific manner by cell–cell contact via the PD-1/PD-L1 pathway. A and B, Transwell migration assays with CD8+ T cells and TECs. A, B16-OVA tumor–derived TECs suppressed OT-I CD8+ T-cell proliferation dose dependently (black solid line), but this suppression was not observed in the absence of cell–cell contact between CD8+ T cells and TECs (black broken line). n = 3. Unpaired Student two-tailed t test compared with positive control. *, P < 0.05. B, Specific killing assay. Similar to the suppression assay, in the Ag-specific cytotoxic assay, specific killing in the presence of OT-I CD8+ T cells cocultured with B16-OVA tumor–derived TECs was significantly lower than that for cells not cocultured with TECs (solid bars), but suppression was not observed in the absence of cell–cell contact between CD8+ T cells and TECs (shaded bars). n = 3. Unpaired Student two-tailed t test. *, P < 0.05. C and D, The PD-L1-blocking assay. C, The suppression of CD8+ T-cell proliferation by B16-OVA tumor–derived TECs was not abrogated by isotype antibody (Ab), but by the anti-PD-L1 (aPD-L1) antibody. The summary of the stimulation ratios is shown. n = 4. One-way ANOVA with Tukey test compared with positive control. *, P < 0.05. NS, not significant. D, Specific killing when anti-PD-L1 antibody was introduced during coculture of OT-I CD8+ T cells and TECs, and suppression of Ag-specific cytotoxicity by B16-OVA tumor–derived TECs was abrogated by PD-L1 blockade. n = 4. Unpaired Student two-tailed t test (*, P < 0.05). NS, not significant.

syngeneic BMDCs in vitro. Increasing the number of TECs (between 1 × 10^3 and 1 × 10^4) significantly promoted the proliferation of OT-II CD4+ T cells; however, the proliferation of OT-II CD4+ T cells was not significantly different, irrespective of whether the TECs were B16 or B16-OVA tumor derived (Fig. 5A). Next, we performed a suppression assay, in which CFSE-labeled OT-I CD8+ T cells were cocultured with TEC-inducing OT-II CD4+ T cells, to evaluate the suppressive effects of TEC-inducing tumor Ag-specific CD4+ T cells on tumor Ag-specific CD8+ T cells. OT-II CD4+ T cells that had been cocultured with TECs and simultaneously stimulated with OVA333–339-pulsed syngeneic BMDCs were separated (purity was >96%; Supplementary Fig. S3). Subsequently, separated OT-II CD4+ T cells were cocultured with CFSE-labeled OT-I CD8+ T cells and simultaneously stimulated with OVA327–264-pulsed syngeneic BMDCs in vitro. OT-II CD4+ T cells that were preliminarily cocultured with B16-OVA tumor–derived TECs showed significantly higher suppression than those that were preliminarily cocultured with B16 tumor–derived TECs or those that were not preliminarily cocultured with TECs (Fig. 5B). IL6 and IL10 levels in supernatants were significantly higher during the coculture of OT-I CD8+ T cells and OT-II CD4+ T cells that were preliminarily cocultured with B16-OVA tumor–derived TECs and simultaneously stimulated with OVA327–264-pulsed syngeneic BMDCs than those observed during the coculture of OT-I CD8+ T cells and OT-II CD4+ T cells that were not preliminarily cocultured with B16-OVA tumor–derived TECs (Fig. 5C). These results suggest that TECs could induce immunosuppressive CD4+ T cells in an Ag-specific manner.

Next, we analyzed supernatants obtained after the coculture of OT-II CD4+ T cells and TECs simultaneously stimulated with OVA327–339-pulsed syngeneic BMDCs. TGFβ1, IL6, IL10, and TNF levels were significantly higher during the coculture of OT-I CD4+ T cells and B16 or B16-OVA tumor–derived TECs than those observed without TECs (Fig. 6A). Furthermore, TGFβ1 levels were higher during the coculture of OT-II CD4+ T cells with B16-OVA tumor–derived TECs than with B16 tumor–derived TECs (Fig. 6A). In
our model, the population of Foxp3-positive CD4$^+$ T cells significantly increased in coculture with B16-OVA tumor-derived TECs, which suggests that TECs induced Foxp3-positive regulatory CD4$^+$ T cells in an Ag-specific manner to a certain extent (Fig. 6B). IL10 and TGFβ have been reported to be tolerogenic cytokines that induce immunosuppressive CD4$^+$ T-cell production (4). Therefore, we performed cytokine blocking of IL10 and TGFβ using neutralizing mAbs during the coculture of TECs and OT-I CD8$^+$ T cells. IL2, TNFα, or IFNγ producing cells were significantly fewer in the presence of TECs than in their absence. n = 4. One-way ANOVA with Tukey test. *, P < 0.05; **, P < 0.01. C, Cytokines in cocultured supernatants of OT-I CD8$^+$ T cells and TECs simultaneously stimulated with OVA257-264-pulsed BMDCs. n = 9. One-way ANOVA with Tukey test (*, P < 0.05; **, P < 0.01). NS, not significant.

**Figure 4.** TECs impaired proinflammatory cytokine production of Ag-specific CD8$^+$ T cells in an Ag-nonspecific manner. A and B, Intracellular staining for cytokines in OT-I CD8$^+$ T cells during suppression. OT-I CD8$^+$ T cells were cocultured with 1 x 10^6 TECs and simultaneously stimulated with OVA257-264-pulsed BMDCs. A, Representative dot plots of OT-I CD69$^+$ T cells from three independent experiments are shown. The proportions of indicated cytokine-producing CD8$^+$ T cells decreased with TEC addition, as compared with those observed without TEC addition. B, Proportions of indicated cytokine-positive cell among OT-I CD8$^+$ T cells. IL2, TNFα, or IFNγ producing cells were significantly fewer in the presence of TECs than in their absence. n = 4. One-way ANOVA with Tukey test. *, P < 0.05; **, P < 0.01. C, Cytokines in cocultured supernatants of OT-I CD8$^+$ T cells and TECs simultaneously stimulated with OVA257-264-pulsed BMDCs. n = 4. One-way ANOVA with Tukey test (*, P < 0.05; **, P < 0.01). NS, not significant.

**PD-L1 in TECs attenuates antitumor effect of Ag-specific CD8$^+$ T cells in an Ag-specific manner by inducing apoptosis to tumor-infiltrating lymphocytes in vivo**

To evaluate whether PD-L1 in TECs affects tumor growth, we performed in vivo tumor growth experiments using PD-L1-deficient mice whose hematopoietic cells were replaced with PD-L1-positive cells by BM transplantation from WT donors. In these syngeneic
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chimeras, the expression of PD-L1 in TECs was still absent (Supplementary Fig. S4). At day 7 after B16 or B16-OVA inoculation, OT-I CD8+ T cells were transferred (Fig. 7A). Tumor growth of B16-OVA cells was significantly reduced in chimeric relative to WT mice, while tumor growth of B16 cells was not different between these groups (Fig. 7B). This result suggests that PD-L1 in TECs attenuates the antitumor effect of OT-I CD8+ T cells in an Ag-specific manner. For tumor-infiltrating lymphocytes (TIL), the proportions of OT-I CD8+ T cells in tumors were significantly lower in syngeneic chimeras than in WT mice (Fig. 7E–G). This result suggests that PD-L1 in TECs contribute to suppression of Ag-specific T cells by inducing apoptosis.

Discussion

In tumor vasculature, TECs come in contact with circulating immune cells when immune cells infiltrate tumors; this has attracted attention to the mechanism by which TECs control the antitumor reaction of the immune system. TECs have been studied primarily as tumor barriers that regulate antitumor T-cell trafficking (11, 33). Recent studies suggest that TECs not only prevent the trafficking and infiltration of antitumor T cells, but also actively regulate immune reactions by suppressing T-cell function (10, 34). In support of this hypothesis, Motz and colleagues reported that TECs regulated the infiltration of T cells into tumors and promoted the development of an immunosuppressive and tolerogenic environment by expressing Fas ligand and T-cell apoptosis mediator in an in vivo model (10). In addition, Mulligan and colleagues reported that TECs release soluble factors that influence T-cell responses and the outcome of treatment, including IL6, TGFβ, VEGF, and prostaglandin E2 (35, 36). However, it has not been investigated how TECs interact directly with lymphocytes. In this study, we showed that TECs expressed MHC class I and II molecules, the costimulating molecule CD40, and PD-L1, an apoptosis-inducing molecule; this suggests that they have the suppressive properties of APCs. It has been reported that freshly isolated mouse endothelial cells, including heart and microvascular endothelial cells, do not express costimulating molecules as well as PD-L1 (37, 38). Similarly, human endothelial cells including human umbilical vein
endothelial cells (HUVEC), which are frequently used as normal endothelial cells, do not express MHC class II and costimulating molecules (39). These reports also show that IFN-γ stimulation induces MHC class II and PD-L1 expression in both mouse and human endothelial cells. Therefore, we considered that the phenotypes of TECs might be induced in tumor microenvironment, which could contribute to inflammatory circumstance. Of note, it has been reported that endothelial cells in certain organs, including lung and liver, express MHC class II and costimulatory molecules (20, 40, 41). However, this expression of MHC class II and costimulatory molecules in endothelial cell is likely unique to those organs because both organs frequently contact exogenous Ags.

Furthermore, the suppression assay revealed that TECs suppressed the proliferation and cytotoxicity of OT-I CD8+ T cells in an Ag-specific manner. Furthermore, this suppressive activity was not observed if TECs could not come into contact with CD8+ T cells. These results suggest that TECs exhibit an immunosuppressive function against CD8+ T cells by the direct presentation of tumor Ags to CD8+ T cells during cell–cell contact. Remarkably, these suppressive activities were abrogated by IL10 or TGF-β blockade during initial coculture of OT-I CD8+ T cells and TECs, while it was not abrogated by isotype-specific antibodies. n = 4. One-way ANOVA with Tukey test (⁎, P < 0.05; *, P < 0.01). NS, not significant.

Figure 6.
TECs induce inhibitory CD4+ T cells via changes in IL10 and TGFβ levels. A, Cytokine levels in coculture supernatants of OT-II CD4+ T cells and TECs. TNF, IL6, IL10, and TGFβ levels were significantly higher in supernatant of coculture of OT-II CD4+ T cells and TECs than that in coculture in the absence of TECs. TGFβ levels were higher in supernatant of coculture of OT-II CD4+ T cells and B16-OVA tumor–derived TECs than in cocultures of OT-II CD4+ T cells and B16 tumor–derived TECs. n = 9. One-way ANOVA with Tukey test. **, P < 0.01. B, Intracellular staining of OT-II CD4+ T cells that were cocultured with TECs and simultaneously stimulated with OVA257-264-pulsed BMDCs. Representative dot plots of OT-II CD4+ T cells in indicated combinations from eight independent experiments are shown (top). The proportion of Foxp3-positive CD4+ T cells was significantly increased by B16-OVA tumor–derived TEC addition. n = 8. One-way ANOVA with Tukey test. **, P < 0.01. C, Cytokine blocking during coculture of OT-II CD4+ T cells and TECs and subsequent OT-I CD8+ T-cell suppression assay by OT-II CD4+ T cells with stimulation by OVA257-264-pulsed BMDCs. Suppression of CD8+ T-cell proliferation by OT-II CD4+ T cells cocultured with B16-OVA tumor–derived TECs was abrogated by IL10 or TGF blockade during initial coculture of OT-I CD4+ T cells and TECs, while it was not abrogated by isotype-specific antibodies. n = 4. One-way ANOVA with Tukey test (**, P < 0.05).
represent a rudimentary ability of TECs to present OVA Ags by insufficient expression of costimulating molecules. In our experimental system, Ag-specific T cells were activated by DCs; TECs obviously show active suppression of those T cells in an Ag-specific manner through PD-L1 signaling. This result suggests that once T cells are activated by professional APCs like DCs, TECs can present Ag to...

Figure 7.

PD-L1 in TECs attenuates antitumor effect of Ag-specific CD8+ T cells in an Ag-specific manner by inducing apoptosis of TILs in vivo. A, Experimental scheme. Hematopoietic cells in the recipient mice (CD45.2) were completely replaced by donor cells (CD45.1) in 60 days. After tumor inoculation, 10^6 OT-I CD8+ T cells (CD45.2) were injected into the syngeneic chimera PD-L1-KO mice and WT mice. TBI, total body irradiation; BMT, bone marrow transplantation. B, Adoptive transfer of OT-I CD8+ T cells showed antitumor effects against B16-OVA tumors; the antitumor effect was significantly stronger in syngeneic chimeric PD-L1-KO mice than that in WT control mice. n = 4. One-way ANOVA with Tukey test (\( P < 0.05 \)). NS, not significant. C–G, TIL analyses. C and D, Proportions of OT-I CD8+ T cells infiltrating in B16-OVA tumors compared with those in B16 tumors. n = 4. One-way ANOVA with Tukey test. \( P < 0.05 \). E–G, Proportions of Annexin V–positive cells among OT-I CD8+ T cells infiltrating in tumors and absolute number of Annexin V–positive CD8+ T cells per volume of the tumors were significantly lower in syngeneic chimera PD-L1-KO mice than that in WT mice. n = 4. One-way ANOVA with Tukey test (\( P < 0.05 \); \( P < 0.01 \)). NS, not significant.
reactive T cells without a costimulation signal and actively suppress T cells through the PD-1/PD-L1 pathway simultaneously. Therefore, a rudimentary ability of TECs to present OVA Ags likely represents a parallel trait that is different from TECs’ property of active suppression. Nonetheless, this phenotype might contribute to tumor immune evasion by immunologic neglect and/or anergy in the absence of stimulation with DCs.

To evaluate the impact of PD-L1 expression in TECs on tumor growth in vivo, it was necessary to develop mice that lack PD-L1 expression specifically in TECs, but not in other cells, especially hematopoietic cells. Purbon and colleagues reported that angiogenesis during tumor growth neither involves nor requires a contribution from BM-derived circulating progenitors for vascular endothelial cells (42). Therefore, we established a syngeneic, chimeric PD-L1– knockout (KO) mouse model. In these syngeneic chimeras with adoptive transfer of congenic OT-I CD8+ T cells, we demonstrated that B16-OVA tumor growth was significantly reduced. This result suggests that PD-L1 in TECs is crucial for inducing apoptosis of OT-I CD8+ T cells and enhancing tumor growth.

Cytokine assays revealed that TECs suppressed the production of proinflammatory cytokines in CD8+ T cells, including IL2, TNF, and IFNγ. These results indicate that CD8+ T cells were exhausted by TECs. Interestingly, the levels of exhaustion were not significantly different between addition of B16 tumor–derived TECs and B16-OVA tumor–derived TECs, suggesting that TECs suppressed the production of proinflammatory cytokines in CD8+ T cells in a non-Ag–specific manner. Endothelial cells from a mouse model of lung carcinoma reportedly secreted high levels of PGE2, IL6, TGFβ, and VEGF, and conditioned medium containing these endothelial cells disrupted T-cell cytokine production in response to anti-CD3 stimulation (43). It is conceivable that such immune suppressive cytokines secreted by TECs might have suppressed proinflammatory cytokines produced by CD8+ T cells in a non-Ag–specific manner, while MHC/TCR ligation and PD-L1/PD-1 between TECs and CD8+ T cells were dominantly involved in the suppression of CD8+ T-cell cytotoxicity. Of note, Gabrilovich and colleagues reported that VEGF dramatically affects DC maturation, resulting in inadequate presentation of tumor antigens (44). It is also reported that TECs secrete various suppressive mediators including VEGF (35, 36). Thus, it is conceivable that VEGF secreted by TECs could suppress the function of professional APCs and tumor Ag–specific immune stimulation. It remains unclear whether VEGF directly affects Ag presentation by TECs, however, endothelium–T cell adhesion is reportedly attenuated by the VEGF through the downregulation of adhesion molecules, including VCAM-1 and ICAM-1, in TECs (12, 13). Taken together, these evidences indicate that VEGF could act as immunosuppressive mediator for professional APCs and TECs, and probably for the crosstalk between those cells and other immune cells.

We found that TECs did not suppress the proliferation of OT-II CD4+ T cells. However, OT-II CD4+ T cells that were preliminarily cocultured with TECs suppressed OT-I CD8+ T-cell proliferation, which indicated that TECs induce inhibitory CD4+ T cells. IL10 and TGFβ levels were significantly elevated during the coculture of TECs and CD4+ T cells; moreover, the neutralization of IL10 or TGFβ during the coculture of TECs and CD4+ T cells abrogated the suppressive effect of CD4+ T cells on the proliferation of CD8+ T cells. Therefore, inhibitory cytokines such as IL10 and TGFβ might be crucial for the induction of immunosuppressive CD4+ T cells. This result is consistent with a previous report, in which CD4+ T cells produced significantly increased levels of IL10 and TGFβ after coming into contact with endothelial cells; this indicates that TECs might provide an environment for inhibitory CD4+ T cells (45). Furthermore, the numbers of Foxp3+ CD4+ T cells, which are commonly considered as Tregs, were increased during the coculture of CD4+ T cells and TECs, especially in Ag–specific combinations. Endothelial cells can reportedly induce CD4+ Foxp3+ Tregs via TGFβ and augment their suppressive function both in mice and humans (46–49). Thus, Tregs might be one of the immunosuppressive CD4+ T cells induced by TECs.

In our study, induction of Ag–specific Tregs by TECs was significant, although the population was relatively small. Although, Foxp3+ Tregs are a representative immunosuppressive subset of CD4+ T cells, another suppressive subset has been reported. T regulatory cells 1 (Tr1), which are known to represent a Foxp3–negative CD4+ T-cell subset, are induced by IL10, and suppress Ag–specific immune responses by the production of IL10 (50). Tr1 CD4+ T cells reportedly produce high levels of IL10, moderate levels of IFNγ and TGFβ, and low levels of IL2 and IL4 (51). These features of Tr1 cells are consistent with those observed in the results of cytokine assay, although the difference in IFNγ levels was not statistically significant. TECs might support the conversion of naïve CD4+ T cells to immunosuppressive populations or the expansion of immunosuppressive CD4+ T-cell populations.

Considerable levels of IFNγ and TGFβ were secreted during the coculture of TECs and CD4+ T cells in Ag–nonspecific combinations. TEC–secreting cytokines such as TGFβ might have affected CD4+ T cells in an Ag–specific manner, such as that observed with a bystander response. However, CD4+ T cells that were cocultured with TECs in an Ag–specific combination strongly suppressed CD8+ T-cell proliferation. These results suggest that the Ag–specific combination of TECs and CD4+ T cells might shape an immune synapse and activate each other.

TECs constitutively express CD40, which is a receptor of CD154 that is expressed on T cells. CD154 can activate APCs, which in turn provide signals that costimulate T cells. Therefore, Ag presentation and costimulation with TECs and T cells might activate TECs, which would induce the generation of immunosuppressive CD4+ T cells.

Considerable levels of proinflammatory cytokines, including IL6, TNF, or IFNγ were observed during the coculture of TECs. Proinflammatory cytokines reportedly induced and enhanced MHC class I and II expression, and costimulated molecules on TECs (52–54). Furthermore, endothelial cells can reportedly support the proliferation of Tregs (49). Therefore, proinflammatory cytokines might have supported the induction of immunosuppressive CD4+ T cells by TECs in our settings. The immunologic cross-talk that directs the activity of those cytokines needs to be investigated further.

In this study, we consistently used pure, freshly isolated TECs from tumors, because the immunologic status of TECs might easily change after manipulation in vitro. Furthermore, the immunologic status of endothelial cell lines such as HUVEC might be different from that of fresh TECs, although they have been commonly used as an alternative to fresh TECs. Therefore, our strategy of using fresh TECs matured in fresh TECs, although they have been commonly used as an alternative to fresh TECs.
This study confirmed that PD-L1 molecules expressed on TECs decrease the cytotoxicity of Ag-specific lymphocytes, which is a key signal that defines their immunosuppressive property. Recent studies suggest that many factors in tumor microenvironments, including proinflammatory cytokines, such as IFNγ and TNFα, cell growth factors such as EGF and TGFβ, and hypoxia, support PD-L1 expression (56), and therefore might lead to the expression of PD-L1 on TECs. We investigated immunosuppressive activities of mouse TECs, however, not those of human TECs. Motz and colleagues reported that human TECs exhibit similar suppressive activities, which would support the hypothesis that TECs contribute to tumor evasion in humans also (10). Notably, in this study, murine TECs express PD-L1 but not PD-L2, which is also an apoptosis-inducing molecule. This result is consistent with a previous report, in which human, but not murine, endothelial cells express PD-L2, even after stimulation (38). There might be some differences in the regulation of PD-L1 and PD-L2 expression between different endothelial cell subsets and/or species. In addition, endothelial cells reportedly express multiple other inhibitory molecules, including B7-H3 (57), B7-H4 (22), and Fas ligand (10, 58). These inhibitory molecules might also contribute to the immunosuppressive function of TECs. These aspects need to be investigated further.

In this study, we used the OVA system both for in vitro and in vivo experiments because it is suitable for investigating Ag-specific responses. Moreover, OVA represents a newly developed tumor Ag that could elicit strong immune responses. In fact, in Fig. 3C, we found that the proliferation of OT-I CD8+ T cells was significantly more suppressed than that of Pmel-1 CD8+ T cells, which respond to the naturally occurring tumor cell–derived Ag gp100, by B16-OVA tumor–derived TECs. This result also suggests that suppressive activity of TECs is not OVA-associated artifact, at least. Of note, the proliferation of Pmel-1 CD8+ T cells seemed to be slightly suppressed by B16-OVA tumor–derived TECs. It is therefore possible that TECs might present naturally occurring gp100 Ag as well as OVA in B16-OVA melanoma to Pmel-1 CD8+ T cells. This aspect also needs to be investigated further in future using other types of tumor Ags.

In summary, we have demonstrated that TECs exhibit several characteristics of APCs, including Ag uptake and presentation. They regulate the immune response of tumor Ag–specific cytotoxic T cells by cell–cell contact via the PD-1/PD-L1 pathway. Furthermore, TECs induced immunosuppressive CD4+ T cells that regulated CD8+ T cells by modifying IL-10 and TGFβ levels. These findings elucidate how TECs contribute to one of the mechanisms underlying tumor immune evasion and might help us to establish novel strategies to combat it.

Disclosure of Potential Conflicts of Interest
T. Onoe reports grants from JSPS KAKENHI (JP 17K10573) and AMED (17h0210207) during the conduct of the study. H. Ohdan reports grants from JSPS (KAKENHI grant no.: JP19H01057) during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
K. Taguchi: Conceptualization, resources, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. T. Onoe: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. T. Yoshida: Resources, data curation, software, validation, investigation, visualization. Y. Yamashita: Supervision, funding acquisition. Y. Tanaka: Conceptualization, formal analysis, validation, investigation. H. Ohdan: Conceptualization, supervision, funding acquisition, project administration.

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