Cancer Research

# LSECtin Expressed on Melanoma Cells Promotes Tumor Progression by Inhibiting Antitumor T-cell Responses

Feng Xu[1], Jing Liu[1], Di Liu[1], Biao Liu[1], Min Wang[1], Zhiyuan Hu[2], Xuemei Du[3], Li Tang[1], and Fuchu He[1,4,5]

## Abstract

Therapeutic antibodies that target T-cell co-inhibitory molecules display potent antitumor effects in multiple types of cancer. LSECtin is a cell surface lectin of the DC-SIGN family expressed in dendritic cells that inhibits T-cell responses. LSECtin limits T-cell activity in infectious disease, but it has not been studied in cancer. Here we report the finding that LSECtin is expressed commonly in melanomas where it blunts tumor-specific T-cell responses. When expressed in B16 melanoma cells, LSECtin promoted tumor growth, whereas its blockade slowed tumor growth in either wild-type or LSECtin-deficient mice. The tumor-promoting effects of LSECtin were abrogated in *Rag1*$^{-/-}$ mice or in response to CD4$^+$ or CD8$^+$ T-cell depletion. Mechanistic investigations determined that LSECtin inhibited the proliferation of tumor-specific effector T cells by downregulating the cell cycle kinases CDK2, CDK4, and CDK6. Accordingly, as expressed in B16, tumor cells LSECtin inhibited tumor-specific T-cell responses relying upon proliferation *in vitro* and *in vivo*. Notably, LSECtin interacted with the co-regulatory molecule LAG-3, the blockade of which restored IFNγ secretion that was reduced by melanoma-derived expression of LSECtin. Together, our findings reveal that common expression of LSECtin in melanoma cells engenders a mechanism of immune escape, with implications for novel immunotherapeutic combination strategies. *Cancer Res; 74(13); 3418–28. ©2014 AACR.*

## Introduction

Although melanoma is substantially more immunogenic than other tumors, it usually remains refractory to immunologic manipulation, despite the fact that large numbers of tumor-infiltrating lymphocytes (TIL) are often found at melanoma sites. Most patients with melanoma, even those with advanced-stage disease, have circulating melanoma antigen-specific CD8$^+$ T cells (1). However, these T cells are likely ineffective at inducing disease regression or preventing progression (2). Much evidence has demonstrated that a large part of this problem is due to the immunosuppressive network at the tumor site. Despite tumor-specific CTLs and helper T cells generated, many of these effector cells are finally "turned off" at the tumor site by a number of immunosuppressive mechanisms, including tumor-induced antigen presentation impairment (3–5), downregulation of HLA molecules (4, 6), the elaboration of immunosuppressive factors (7–13), as well as the influence of regulatory cell populations that may contribute to this immunosuppressive network, including regulatory T cells (Treg; refs. 14, 15) and myeloid-derived suppressor cells (MDSC; refs. 16, 17). Recently, another immune resistance mechanism has gained much attention, specifically, the suppressive actions of co-inhibitory molecules (18, 19). Among these inhibitory receptors, monoclonal antibody targeted to CTLA4 (ipilimumab) and PD1 has demonstrated encouraging antitumor effects in initial clinical trials (20, 21). Accumulating studies have implied that combinatorial modulation is more effective (22). In one study, vaccine-treated mice were found to have significantly higher overall survival rates if they were also treated with antibodies against PD1 and CTLA4 (23). Thus, the identification of novel inhibitory receptors in antitumor immune responses is important to the development of more effective combinatorial strategies.

The role of lectin in pathogen detection has been extensively appreciated (24), but our awareness of lectin in immune homeostatic control is relatively new. A few studies have reported inhibitory control mechanisms that act on effector T cells in the context of antitumor immunity. For example, Tim-3/galectin-9 (Gal-9) pathway modulation affects tumor growth (25); LSECtin, which belongs to the C-type lectin receptor superfamily, is a type II transmembrane protein that is highly expressed in the liver (26). Our previous studies revealed that LSECtin, when expressed in the liver, acted as a co-inhibitory molecule and limited the ability of T-cell immunity to promote HBV tolerance (27, 28). Immunologically, tumors are quite similar to chronic viral infections. Because the potential role of LSECtin in antitumor immunity remains

---

**Authors' Affiliations:** [1]State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine; [2]National Center for Nanoscience and Technology; [3]Department of Pathology, Capital Medical University affiliated Beijing Shijitan Hospital; [4]College of Life Sciences, Peking University; and [5]Department of Biology Sciences and Biotechnology, Tsinghua University, Beijing, China

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

**Corresponding Authors:** Li Tang and Fuchu He, State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing 102206, China. Phone: 86-10-80705518; Fax: 86-10-80705518; E-mail: tangli08@aliyun.com or hefc@nic.bmi.ac.cn

**doi:** 10.1158/0008-5472.CAN-13-2690

©2014 American Association for Cancer Research.

unknown, we hypothesized that tumor cells might hamper antitumor T-cell immune responses through LSECtin and that this mechanism might help to shift the balance toward an immunosuppressive environment at the tumor site. In this study, we found that LSECtin expression was detected in human melanoma tissues and induced on melanoma cells within a tumor microenvironment. Both *in vivo* and *in vitro* evidence was provided for the relevance of LSECtin-mediated immunosuppression as a novel mechanism exploited by tumor cells to facilitate host immune system escape.

## Materials and Methods

### Cell lines, animals, and reagents

The B16 cell line was obtained from National Platform of Experimental Cell Resources for Sci-Tech and cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin. To guarantee cell line authenticity, cell line was aliquoted and banked, and cultures were grown and used for a limited number of passages before starting a new culture from stock. Cell lines were routinely tested for mycoplasma contamination. C57BL/6 female mice were used at the age of 6 to 8 weeks and purchased from Beijing HFK Bioscience Company and maintained in a specific pathogen-free facility. LSECtin KO mice are described (26). RAG1 KO mice were obtained from Model Animal Research Center (Nanjing, China). Information of cytokines and antibodies is available in Supplementary Methods.

### Patients

We studied previously untreated patients with melanoma at Beijing Cancer Hospital (Beijing, China). Patients gave written, informed consent and the study was approved by the Ethics Committee of Beijing Cancer Hospital. Clinical characteristics related to samples from 3 patients with melanoma are shown in Supplementary Table S4.

### Isolation of melanoma cell and TILs

Melanoma tissues were dissociated by finely mincing in complete medium followed by collagenase (Type I, Sigma) and DNase (Roche) digestion for 1 hour. The cell suspension was centrifuged on a discontinuous density gradient to remove tumor fragments, dead cells, and red blood cells. Tumor cells or TILs were collected and washed thoroughly for flow cytometry. Details can be found in Supplementary Methods.

### FACS reagents and staining

Information of antibodies used in FACS is available in Supplementary Methods. LSECtin monoclonal antibody (CCB059) was used as described previously (26). IFNγ patients cellular staining was performed according to the protocol provided by the manufacturers (eBioscience). Cells were analyzed using the FACSCalibur flow cytometer with data analyzed by FlowJo software (Treestar).

### Quantitative real-time PCR

Human tumor quantitative PCR tissue arrays were purchased from OriGene. Cancer survey array (CSRT104) contained 7 types of different cancers. Melanoma-specific array (MERT101) contained 40 melanoma samples. All relevant clinical information can be found in Supplementary Material or on the OriGene website. Detailed protocol and primers can be found in Supplementary Methods.

### Immunohistochemistry

Melanoma and normal tissue microarray (TMA) sections with stage and grade information (ME481a) were bought from US Biomax. All relevant clinical information can be found in Supplementary Material or on the US Biomax website. Detailed protocols can be found in Supplementary Methods.

### B16 melanoma inoculation and animal treatment

C57BL/6 mice were injected subcutaneously in the flank with $2 \times 10^5$ tumor cells. Tumor growth was monitored every other day. For treatment experiments, mice were injected subcutaneously in the flank at day 0 with $5 \times 10^4$ B16 cells or B16-LSECtin cells and treated on days 3, 6, and 9 with PBS, 250 μg anti-LSECtin or rabbit IgG intraperitoneally (i.p.). For $CD4^+$/$CD8^+$ T-cell depletion experiments, mice were implanted with $1 \times 10^5$ B16 cells mice at day 0 and were injected i.p. with 200 μg of anti-CD4 or anti-CD8 every 4 days starting from day −2. Rat IgG was used as control antibody. Depletion of $CD4^+$ and $CD8^+$ T cells was confirmed by analyzing the splenocytes of the mice by flow cytometry.

### *In vitro* induction of B16-specific T cells and functional assays

Splenocytes from vaccinated mice were obtained and cultured with 10 μg/mL TRP2 180–188 peptide and 10 ng/mL IL2. After 5 days, B16-specific $CD4^+$or $CD8^+$ T cells were obtained from these splenocytes by FACS for further coculture assays. Protocols of T-cell proliferation, cell cycle, and apoptosis can be found in Supplementary Methods.

### ELISPOT and ELISA

Lymph node cells or splenocytes were harvested from vaccinated mice and subjected to ELISPOT analysis. Detailed protocols can be found in Supplementary Methods. Lymph node cells or B16-specific $CD4^+$ or $CD8^+$ T cells were restimulated *ex vivo* with irradiated B16-LSECtin and B16-mock cells for 48 hours. Cell-free supernatants were collected and subjected to cytokine determination.

### LAG-3 interaction and blockade

Protocols of SPR and protein ELISA can be found in Supplementary Methods. Anti-LAG-3 (C9B7W) was used to block interaction between LAG-3 and LSECtin. Details can be found in Supplementary Methods.

### Statistical analyses

The statistical differences between different groups were determined by the Student *t* test or 2-way ANOVA test in GraphPad Prism software. All results shown in the manuscript are representative of at least 2 independent experiments with similar results.

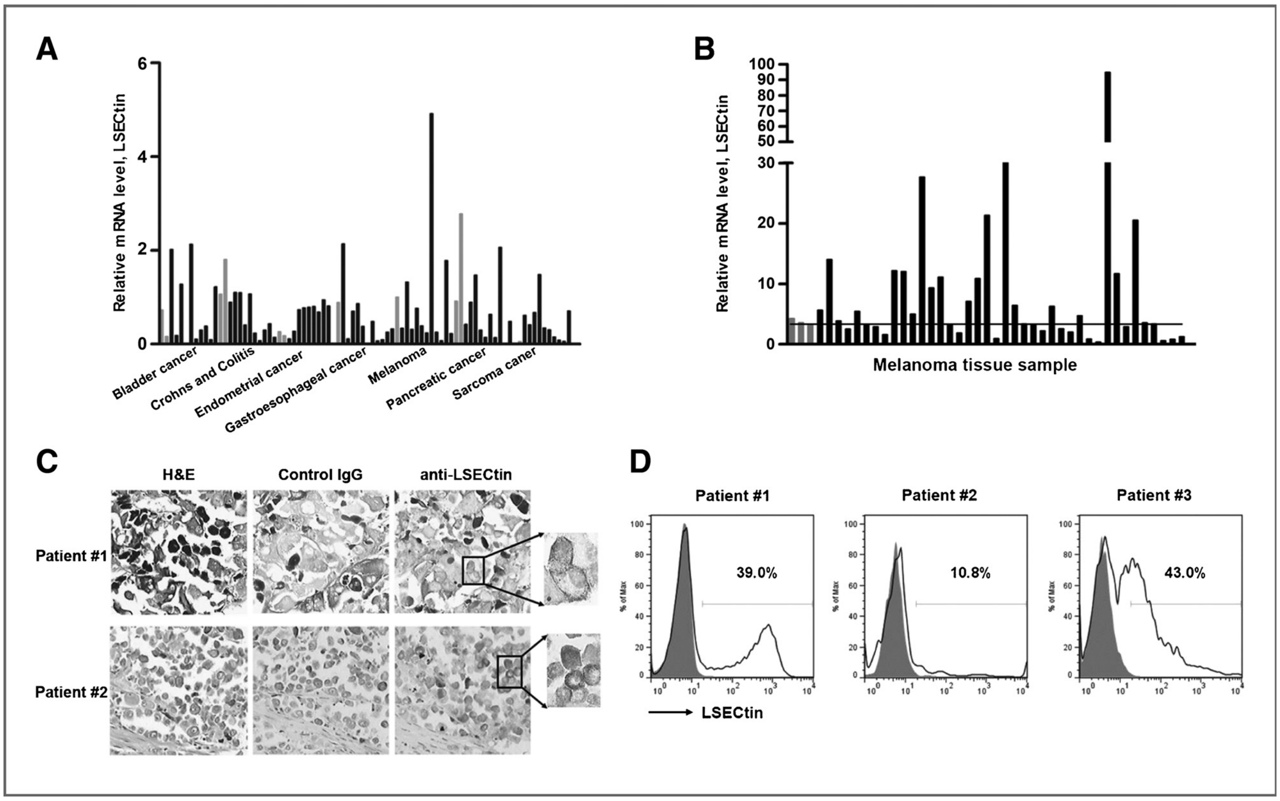

Figure 1. LSECtin is expressed in human melanoma tissues. A, quantitative PCR analysis of LSECtin expression using cDNA from seven different types of human tumor (black) and normal tissues (gray). B, quantitative PCR analysis of LSECtin expression using cDNA from 40 melanoma tumor tissues (black) and three normal tissues (gray). C, immunohistochemical analysis (200× or 400×) of LSECtin expression in human melanoma tissues. H&E, hematoxylin and eosin. D, white histograms demonstrate LSECtin surface expression on gated 7-aminoactinomycin–negative tumor cells from patients with melanoma. Negative controls are shown as gray histograms.

## Results

### LSECtin is expressed by human and murine melanoma tissues

LSECtin is a type II transmembrane protein that was initially found to be expressed in the liver. To determine whether LSECtin was expressed in human tumors, we performed real-time PCR (RT-PCR) with human tumor tissue cDNA (Fig. 1A). We detected increased expression of LSECtin in primary bladder (2 of 10), Crohn's (0 of 10), endometrial (8 of 10), gastroesophageal (1 of 11), melanoma (3 of 11), pancreatic (1 of 10), and sarcoma (4 of 10) tumor samples compared with average level of normal skin tissues. Melanoma, bladder, and pancreatic cancers had a higher LSECtin expression among these 7 types of tested tumors. Our previous studies indicated that LSECtin was a negative regulator of T-cell immune responses. Melanoma is a well-studied tumor type that shows a T-cell–inflamed phenotype consisting of infiltrating T cells. Therefore, we chose to determine whether LSECtin could play a role in anti-melanoma immunity. In addition, 20 of 40 human melanoma samples showed obvious LSECtin expression (Fig. 1B). An immunohistochemistry analysis also detected LSECtin expression in 20 of 32 melanoma samples. We found LSECtin immunoreactivity in the plasma membrane (Fig. 1C), cytoplasm, or both. Fresh isolated melanoma cells from patients expressed cell surface LSECtin protein (Fig. 1D). Previous studies showed that the B16 murine melanoma cell line on the C57BL/6 mouse strain background is a well-studied model of human melanoma (29); we next determined whether the B16 cells also expressed LSECtin. First, we analyzed LSECtin mRNA level in B16 cells and found that the band of LSECtin was feeble. This suggested that LSECtin expression in B16 cells was not constitutive and could possibly be induced *in vivo*. To test this, B16 cells and tumor cells extracted from B16-inoculated mice were obtained to analyze LSECtin expression. We found that tumor cells had a significantly higher induction of LSECtin expression at both mRNA and protein levels compared with B16 cells (Supplementary Fig. S1A and S1B).

Because LSECtin expression was induced in B16 cells, we hypothesized that intratumoral factors may stimulate LSECtin expression on B16 cells. When treated with tumor extract solution from fresh tumors, B16 cells were induced to express LSECtin (Fig. 2A and B). Addition of a neutralizing monoclonal antibody against IL6 or IL10 significantly blocked tumor extract solution–mediated LSECtin upregulation (Fig. 2C), suggesting that intratumoral IL6 and IL10 could stimulate LSECtin expression. In support, IL6 and IL10 proteins were detected in the tumor extract solution (Supplementary Fig. S2). Furthermore, when treated with recombinant IL6 and IL10, B16 cells were also induced to express LSECtin (Fig. 2D and E). Taken together, we determined that LSECtin was expressed in

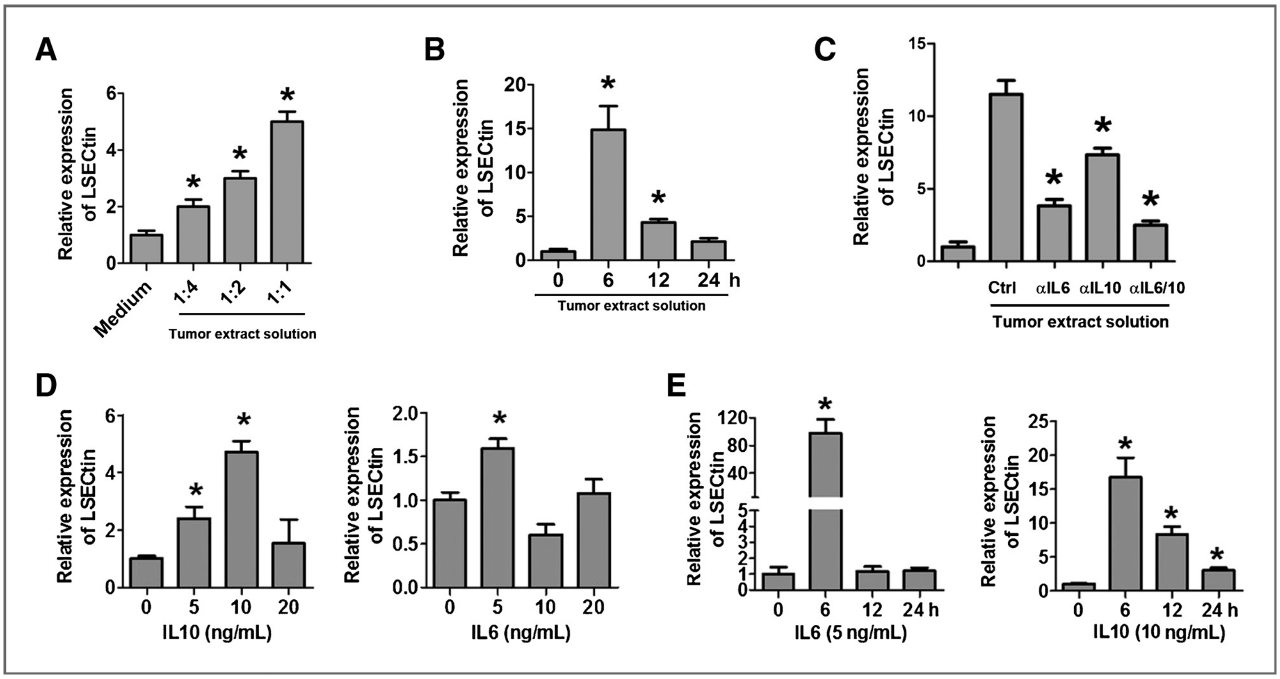

Figure 2. Intratumoral IL6 and IL10 upregulate LSECtin expression on B16 cells. A, intratumoral cytokines extracted from fresh melanomas induced LSECtin expression on B16 cells. The number indicates the ratio of the tumor extract solution to the total culture medium. $^{*}$, $P < 0.05$. B, the regulation of LSECtin expression on B16 cells in response to stimulation by tumor extract solution for indicated times. $^{*}$, $P < 0.05$. C, intratumoral IL6 and IL10 stimulated LSECtin expression on B16 cells. B16 cells were cultured with tumor extract solution in the presence or absence of anti-mouse IL6 and/or anti-mouse IL10. $^{*}$, $P < 0.05$. D and E, the regulation of LSECtin expression in response to stimulation by serial concentrations of recombinant cytokines for different times was determined using RT-PCR. B16 cells were cultured for 24 hours at the indicated cytokine concentrations (D) or for indicated times with a certain concentration of cytokine (E). $^{*}$, $P < 0.05$; error bars, SEM.

human melanoma tissues, and its expression could be induced in B16 cells within tumor microenvironment.

### Tumor-derived LSECtin promotes B16 melanoma progression

Because LSECtin was not constitutively expressed by B16 cells, we obtained a stable transfectant B16-LSECtin (Supplementary Fig. S3) to study the role of tumor-derived LSECtin. First, all stable transfectants exhibited similar growth rates (Fig. 3A), indicating that B16-LSECtin cells had no growth advantage over B16-mock cells. Remarkably, B16-LSECtin cell administration resulted in a more rapid melanoma growth in both wild-type (WT) and LSECtin KO mice (Fig. 3B and C; ref. 27). This indicated that tumor-derived LSECtin could promote B16 melanoma progression in mice. Furthermore, we explored the impact of LSECtin blockade on B16 melanoma progression by injecting C57BL/6 mice with LSECtin antibody (Supplementary Fig. S4) to investigate whether tumor cells could overwhelm antitumor cell responses *in vivo* through LSECtin-dependent mechanisms. WT C57BL/6 mice were inoculated with B16 (Fig. 3D) or B16-LSECtin (Supplementary Fig. S5) cells and treated with anti-LSECtin or control antibody. We found that anti-LSECtin significantly prevented B16 tumor progression. Furthermore, LSECtin KO mice were used to exclude the potential cross-reactivity of anti-LSECtin against host cell–expressed LSECtin. We found that anti-LSECtin could delay the progression of B16 melanoma in the absence of host LSECtin (Fig. 3E). Collectively, our results demonstrated that tumor-derived LSECtin actually promotes B16 tumor growth *in vivo*.

### LSECtin, when expressed by B16 cells, reduces the number of tumor-infiltrating effector T cells

To determine which mechanisms are involved in LSECtin-mediated tumor promotion, tumor tissue histology was assessed. Ki67 was used to detect tumor cell proliferation (30). There was no difference in cell proliferation between the two groups, indicating that, in this model, tumor cell growth arrest was not a main component of tumor control (Fig. 4A). To determine whether apoptosis could contribute to tumor promotion in our model, a terminal-deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay was performed. Only marginal apoptosis and no difference was observed between the two groups (Fig. 4B). Furthermore, to determine whether immune cells, particularly T and B cells, were involved in LSECtin-mediated tumor promotion, we inoculated R*ag1*$^{-/-}$ (31) and WT mice with B16-LSECtin and B16-mock cells. Interestingly, LSECtin had no effect on B16 melanoma growth promotion when T and B cells were absent (Fig. 4C). Because LSECtin was previously shown to dampen T-cell–mediated antiviral immune responses (28), we first hypothesized that tumor-derived LSECtin could promote B16 melanoma growth by inhibiting T-cell–mediated antitumor immunity. To test this, we depleted CD4$^{+}$ and CD8$^{+}$ T cells in mice before treatment. As expected, the LSECtin-mediated melanoma promotion was abrogated in response

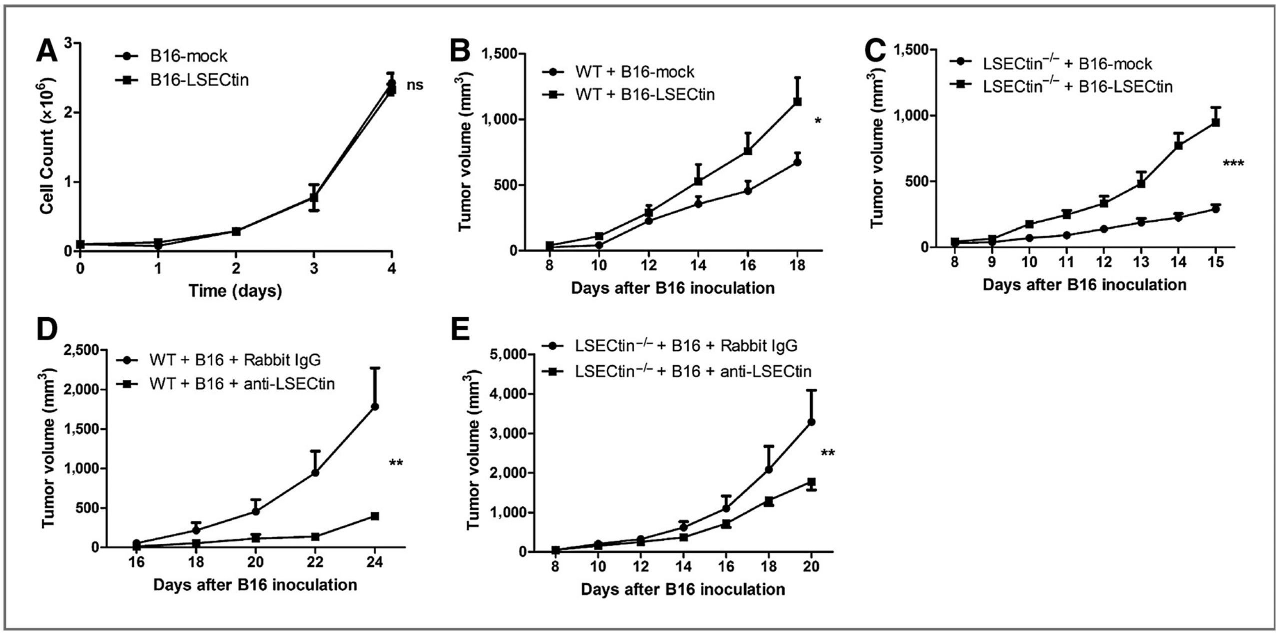

Figure 3. LSECtin promotes B16 melanoma growth *in vivo*. A, the *in vitro* growth of B16-LSECtin and B16-mock transfectants did not significantly differ. WT C57BL/6 mice (B) or LSECtin KO mice (C) were inoculated with $2 \times 10^5$B16-LSECtin and B16-mock cells. *, $P < 0.05$; ***, $P < 0.001$. Data represent the mean ± SEM of three different experiments. WT C57BL/6 mice (D) or LSECtin KO mice (E) were inoculated with $5 \times 10^4$ B16 cells on day 0 and treated on day 3, 6, and 9 with 200 μg/mL of anti-LSECtin or control antibody i.p. **, $P < 0.01$. Data represent the mean ± SEM of three different experiments.

to the depletion of both $CD4^+$ and $CD8^+$ T cells (Fig. 4D). Taken together, these data imply that LSECtin-mediated melanoma promotion was mainly due to weakened antitumor T-cell immunity.

To assess the impact of LSECtin on antitumor T-cell immune responses, we first isolated tumors and assayed them for T-cell infiltration using flow cytometry. Consistently, B16-LSECtin cell inoculation resulted in tumor growth promotion, and the tumor weights were significantly higher in mice inoculated with B16-LSECtin cells (Fig. 5A); this difference also corresponded to a decrease in the absolute number of $CD3^+$ T cells (Fig. 5B). In addition, the absolute number of $CD8^+$ T cells/gram tumor (Fig. 5C) decreased significantly by approximately 3-fold in B16-LSECtin mice, compared with B16-mock mice. Moreover, the absolute number of $CD4^+$ T cells (Fig. 5D) also decreased, but it was not statistically significant.

Because tumor-derived LSECtin reduced the number of tumor-infiltrating T cells, we next examined the impact of LSECtin on the proliferation of tumor-infiltrating T cell. B16-specific $CD4^+$ and $CD8^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cocultured with irradiated B16-LSECtin and B16-mock cells in the presence of coated anti-CD3 and soluble IL2. The CFSE dilution assay results (Fig. 5E) demonstrated that B16-expressed LSECtin could significantly inhibit the proliferation of B16-specific $CD8^+$ T cells, but did not have such an obvious effect on B16-specific $CD4^+$ T cells. We also quantified the expression of key genes that control the cell cycle. In agreement with the above CFSE dilution analysis, we found that the expression level of the cyclin-dependent kinase inhibitor p21 was increased and those of the $G_1$–S phase-associated genes CDK2, CDK4, and CDK6 were decreased in $CD8^+$ T cells that were cocultured with B16-LSECtin versus those cocultured with B16-mock cells (Fig. 5F), suggesting that $CD8^+$ T cells cocultured with B16-LSECtin cells failed to enter cell cycle. There was no difference in the expression of another inhibitor p27 between these two groups (Fig. 5F). It indicated that p21 might be a key factor in LSECtin-mediated T-cell proliferation.

Because tumor-specific T cells are more prone to apoptosis in the tumor microenvironment (32, 33), we next investigated whether LSECtin could affect the apoptosis of B16-specific T cells. B16-specific $CD4^+$ and $CD8^+$ T cells were cocultured with B16-LSECtin and B16-mock cells, and the percentages of Annexin $V^+$ T cells were analyzed at the indicated time points. We found that B16-expressed LSECtin did not affect the apoptosis of either B16-specific $CD4^+$or $CD8^+$ T cells *in vitro* (Fig. 5G). Taken together, B16-expressed LSECtin inhibits the proliferation and reduces the numbers of tumor-infiltrating B16-specific effector T cells.

### LSECtin, when expressed by B16 cells, inhibits tumor-specific T-cell immune responses *in vivo* and *in vitro*

The immunoregulatory effect of LSECtin and its ability to promote B16 melanoma growth in mice prompted us to investigate whether LSECtin could inhibit tumor-specific immune responses in the tumor microenvironment. Tumor-draining lymph nodes (TDLN) were harvested from mice that had been inoculated with B16-LSECtin or B16-mock cells. Lymph node cells were examined for antigen-specific IFNγ production using intracellular staining (Fig. 6B) and an ELISPOT assay (Fig. 6C) following TRP2 180–188 stimulation for 6 and 24 hours, respectively. We found that $CD8^+$ T cells from mice inoculated with B16-LSECtin cells secreted lower amounts of IFNγ than did control mice. Because LSECtin

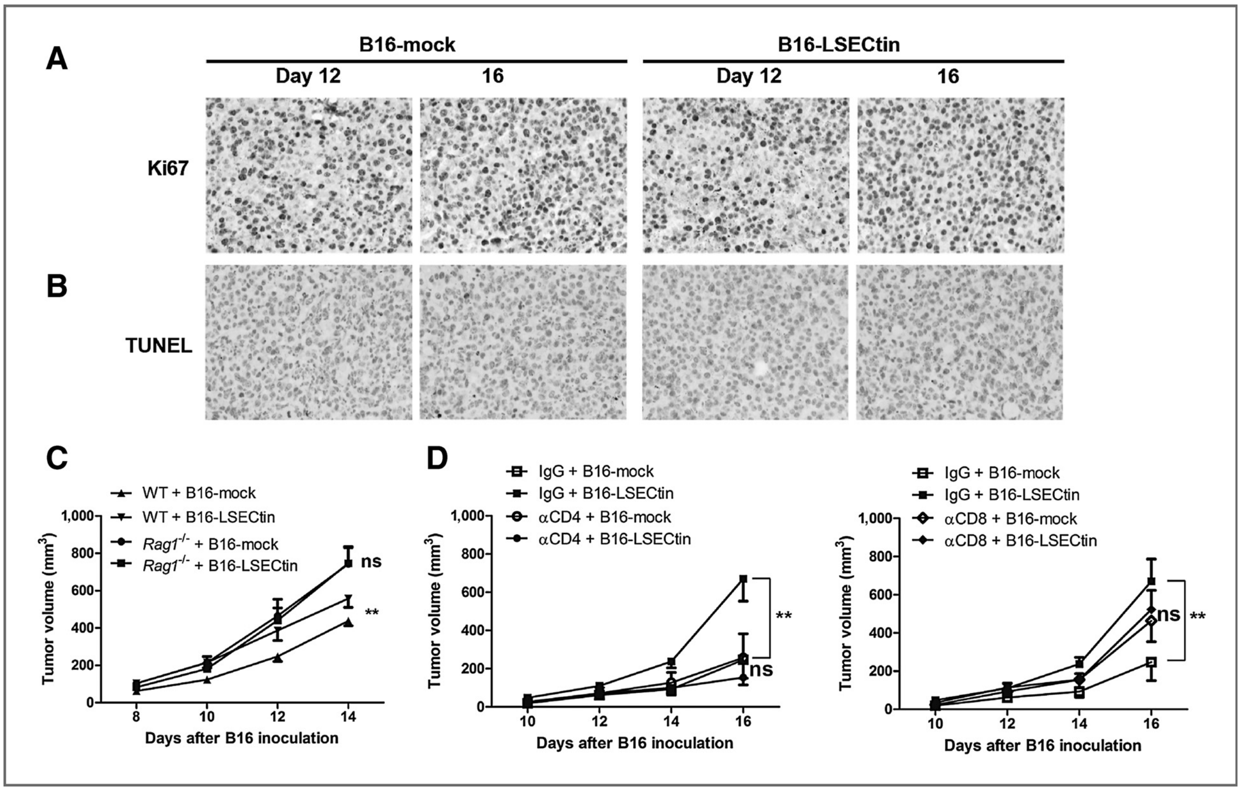

Figure 4. LSECtin-mediated tumor promotion is associated with T cells. A and B, tumor tissues were collected and immunohistochemistry was performed with Ki67 staining (A) and TUNEL assay (B). C, a total of $2 \times 10^5$ B16-LSECtin and B16-mock cells were implanted in C57BL/6 WT and RAG1 KO mice. $^{**}$, $P < 0.01$. Data represent the mean ± SEM of two different experiments. D, a total of $1 \times 10^5$ B16-LSECtin and B16-mock cells were implanted in C57BL/6 WT mice treated with anti-CD4 (left) and anti-CD8 (right). $^{**}$, $P < 0.01$. Data represent the mean ± SEM of two different experiments. ns, not significant.

could inhibit the tumor-specific T-cell immune responses in the TDLNs, we next investigated whether B16-expressed LSECtin could also inhibit peripheral antitumor immune responses. B16-LSECtin and B16-mock cells were subcutaneously injected into C57BL/6 mice. We then performed ELISPOT assays to detect TRP2-specific, IFNγ-producing CD8$^+$ T cells in splenocytes isolated from these mice. Substantially lower levels of TRP2-specific IFNγ production were observed in splenocytes from mice injected with B16-LSECtin cells (Fig. 6D). Collectively, we demonstrated that LSECtin-expressing B16 cells could inhibit the effector functions of B16-specific T cells, both in the TDLNs and the periphery.

Next, WT C57BL/6 mice were implanted with irradiated B16 cells, after which the lymph node cells were isolated and then restimulated *ex vivo* with irradiated B16-LSECtin and B16-mock cells. IFNγ ELISA showed results consistent with the intracellular IFNγ staining. TDLN cells that were cocultured with B16-LSECtin cells produced significantly lower levels of IFNγ (Fig. 6F). Similarly, we obtained B16-specific CD8$^+$ T cells, as described above, and cocultured these cells with irradiated B16-LSECtin and B16-mock cells. Consistently, CD8$^+$ T cells cocultured with B16-LSECtin cells also secreted smaller amounts of IFNγ (Fig. 6F). Furthermore, we obtained B16-specific effector CD4$^+$ T cells (CD4$^+$CD25$^-$) and Treg-enriched cells (CD4$^+$CD25$^+$) and cocultured them with irradiated B16-LSECtin and B16-mock cells in the presence of coated anti-CD3. We found that LSECtin-expressing B16 cells could inhibit IFNγ secretion from effector CD4$^+$ T cells (Fig. 6G) and induce more IL10 production from Treg cells (Fig. 6G). Collectively, B16-expressed LSECtin inhibits the generation of tumor-specific T-cell immune responses both *in vivo* and *in vitro*.

### LSECtin interacts with LAG-3 to inhibit IFNγ secretion of effector T cells

Because LSECtin is a transmembrane protein with an obvious role as a regulator of T-cell immune responses, studies that aim to identify the binding partner for LSECtin are warranted. Because of its co-inhibitory function, we used the bioinformatics tool PRINCESS (34) to screen for LSECtin's interacting partner among some known co-inhibitory molecules reported by a series of literature. Among these candidates, LAG-3 obtained the highest score. Interestingly, LAG-3 was previously thought to contribute to tumor-mediated immunosuppression (35–37). Preclinical studies that used a LAG-3 blocking antibody for cancer treatment revealed enhanced activation of antigen-specific T cells at the tumor site and disruption of tumor growth (38). To determine whether LSECtin could interact with LAG-3, we first performed surface plasmon resonance (SPR) to determine the interactions between LSECtin and LAG-3, BTLA (another candidate with the highest score) or LSECtin itself, using a monoclonal antibody as a positive control. We immobilized these candidate proteins and

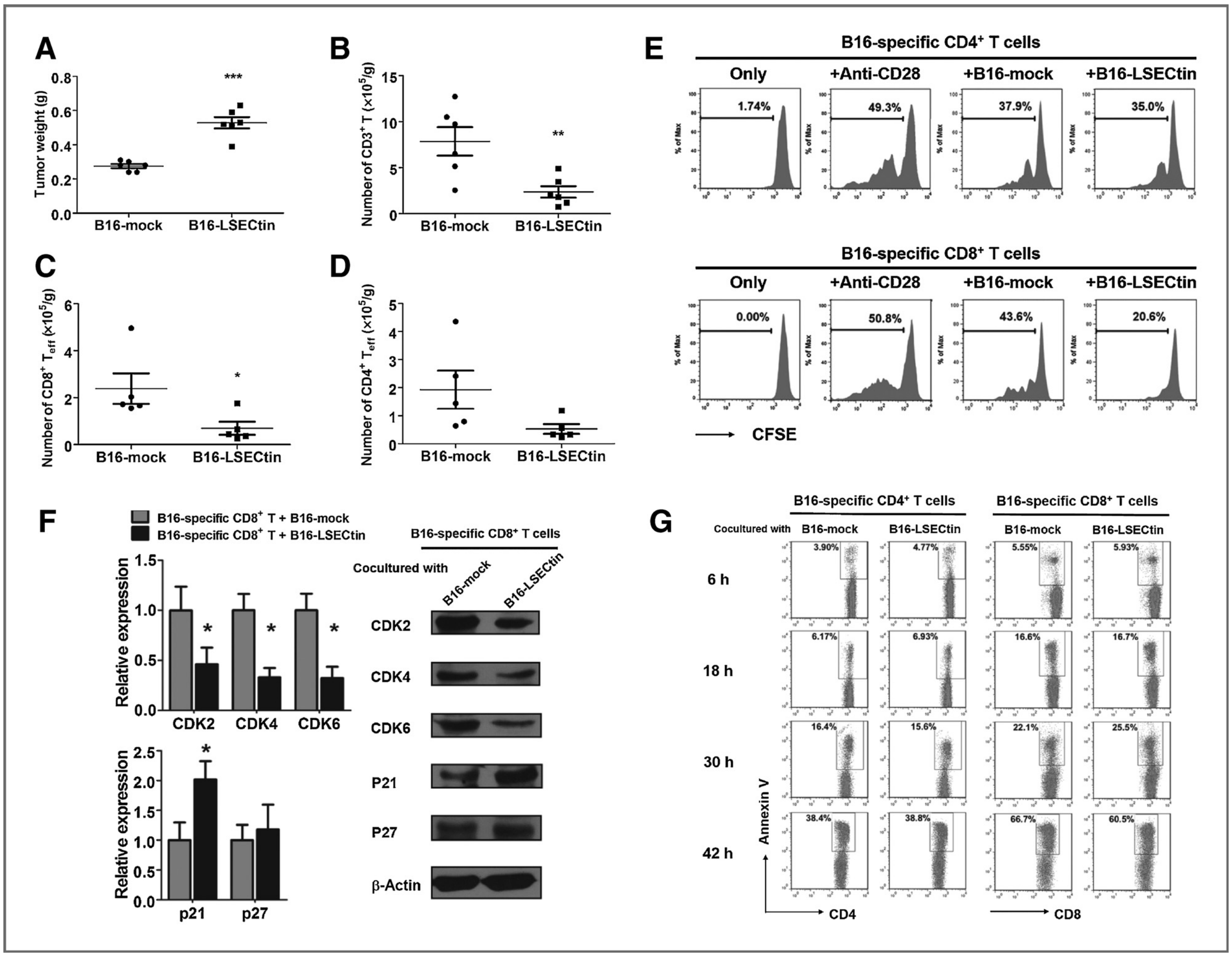

Figure 5. LSECtin, expressed on B16 cells, reduces the number of tumor-infiltrating effector T cells. A, the weights of tumor extracted from mice inoculated with B16-LSECtin and B16-mock cells. ***, $P < 0.001$. B–D, the numbers of $CD3^+$ (B; **, $P < 0.01$), $CD8^+$ (C; *, $P < 0.05$), and $CD4^+$ (D; $P = 0.082$) T cells in the TILs. Individual samples are represented as dots, and bars represent mean. E, B16-specific $CD4^+$ and $CD8^+$ T cells were labeled with CFSE and then co-cultured with irradiated B16-LSECtin or B16-mock cells at a ratio of 5:1 in the presence of plate-bound anti-CD3 and soluble IL2. CFSE dilution was analyzed after 48 hours. Graphs are representative of three independent experiments. F, cell-cycle–associated gene expression in B16-specific $CD8^+$ T cells co-cultured with B16-LSECtin (black) or B16-mock (gray) cells was determined using RT-PCR and Western blotting. *, $P < 0.05$. Data represent the mean ± SEM of two different experiments. G, B16-specific $CD4^+$ and $CD8^+$ T cells were cocultured with B16-LSECtin and B16-mock cells. T cells were harvested at the indicated time points, and the percentages of Annexin $V^+$ in the $CD4^+$ and $CD8^+$ T cells were analyzed using flow cytometry. Graphs are representative of three independent experiments.

injected LSECtin at a concentration of 500 ng/mL. Among the tested candidates, LAG-3 (Fig. 7A) showed obvious binding to LSECtin with a relative affinity (Fig. 7B) slightly lower than that of LSECtin itself and monoclonal antibody against LSECtin, whereas the other candidate BTLA did not bind. Next, a fusion protein-binding ELISA was performed to confirm the interaction between LSECtin and LAG-3. We coated the plate with LAG-3 and BTLA (1 μg/mL) and incubated the plate with LSECtin at various concentrations. The results revealed that LSECtin bound to LAG-3 in a dose-dependent way (Fig. 7C). In addition, a co-immunoprecipitation assay was used to further verify the interaction between LSECtin and LAG-3 (Fig. 7D). Finally, we performed a cell surface staining assay to further determine whether LSECtin could bind to LAG-3 on the surface of live cells. We found that LSECtin could bind to 293T cells that had been transiently transfected with LAG-3 (Fig. 7E).

We used *in vitro* T-cell activation assays to investigate the functional significance of the LSECtin/LAG-3 interaction. LSECtin or control IgG was coated onto plates, and T cells sorted from WT C57BL/6 mice were cultured with or without LAG-3 blocking antibodies (C9B7W) in the presence of coated anti-CD3 and soluble anti-CD28. The expression of LAG-3 on T cells was confirmed by flow cytometry (Supplementary Fig. S7). As expected, LSECtin dramatically inhibited IFNγ secretion from the T cells, which was consistent with our observations regarding antitumor T-cell immune responses. In addition, LAG-3 blocking antibodies partially restored IFNγ secretion (Fig. 7F). To understand the functional significance of the LSECtin/LAG-3 interaction in antitumor immunity, we

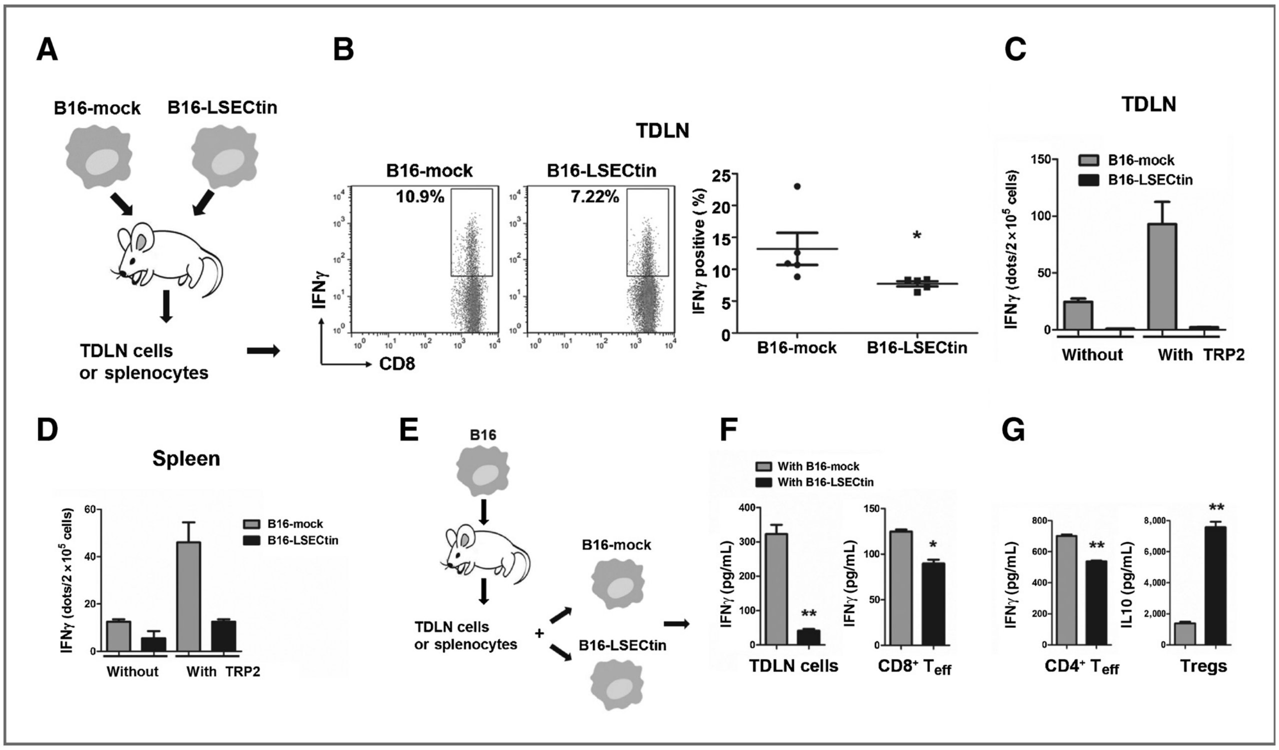

Figure 6. LSECtin, expressed by B16 cells, inhibits the tumor-specific immune responses both *in vivo* and *in vitro*. A, WT C57BL/6 mice were inoculated with B16-LSECtin and B16-mock cells, and the TDLN cells or splenocytes were isolated for IFNγ intracellular staining and ELISPOT assays. B, representative dot plots for intracellular IFNγ staining and the percentages of IFN$\gamma^+$ T cells in total CD8$^+$ T cells are shown. $^*$, $P < 0.05$. C and D, IFNγ ELISPOT assays of TDLN cells (C) and splenocytes (D) from mice inoculated with B16-LSECtin (black) and B16-mock (gray) cells in the presence or absence of TRP2 180–188. E, WT C57BL/6 mice were inoculated with irradiated B16 cells, and TDLN cells or splenocytes were isolated for coculture assays. F, TDLN cells (left) or CD8$^+$ T cells (right) were subjected to restimulation *ex vivo* with irradiated B16-LSECtin (black) and B16-mock (gray) cells. $^*$, $P < 0.05$; $^{**}$, $P < 0.01$. G, CD4$^+$ T cells (CD4$^+$CD25$^-$) or Treg-enriched cells (CD4$^+$CD25$^+$), sorted from restimulated splenocytes, were also subjected to restimulation *ex vivo* with irradiated B16-LSECtin (black) and B16-mock (gray) cells in the presence of 2 μg/mL coated anti-CD3. $^{**}$, $P < 0.01$. These results are representative of three independent experiments.

obtained B16-specific effector T cells from immunized mice and cocultured them with irradiated B16-LSECtin and B16-mock cells with or without a LAG-3 blocking antibody. Consistently, blockade of LAG-3 could restore IFNγ secretion from effector T cells (Fig. 7G). In summary, LSECtin interacted with LAG-3 to inhibit IFNγ secretion from effector T cells during antitumor immune responses.

## Discussion

The data presented herein demonstrate that tumors can overwhelm effector T-cell immune responses via LSECtin-mediated mechanisms. We found LSECtin was expressed by human melanoma and induced to be expressed by B16 cells in response to tumor microenvironmental stimuli. Using a combination of *in vivo* and *in vitro* experiments, we have revealed a link between LSECtin-mediated immunoregulation and its contribution to tumor immune escape. LSECtin expression resulted in enhanced B16 tumor growth and inhibited the generation of tumor-specific T-cell immune responses. Moreover, blockade of the inhibitory effects of LSECtin resulted in a reduced tumor mass.

Tumor cells can grow unfettered by escaping host immune system surveillance, which is progressively suppressed as a result of tumor progression and metastasis. Many immunosuppressive strategies can be used by tumor cells to disrupt the immune system. In previous years, the suppressive actions of co-inhibitory molecules have gained considerable attention. Tumor cells can evade immune control by upregulating various co-inhibitory ligands, thereby limiting the therapeutic potential of current anticancer immunotherapies. Herein, we first reported that LSECtin is expressed on melanoma cells and is used by tumor cells to damper T-cell antitumor immune responses. Previous studies revealed that LSECtin was also expressed on other immune cells, including macrophages and monocyte-derived dendritic cells (MDDC; ref. 39). We used LSECtin-deficient C57BL/6 mice to develop a B16 melanoma model and found that tumor-derived LSECtin could promote melanoma growth in the absence of host LSECtin. Meanwhile, the role of LSECtin expression on immune cells in antitumor immunity should be investigated in the future.

To decipher the underlying mechanism by which tumor-derived LSECtin inhibited tumor-specific T-cell immune responses, we sought to identify the binding partner of LSECtin on the T-cell surface. Combing with an informatics tool and literature mining, we identified the lymphocyte activation gene-3 (LAG-3) as a putative receptor of LSECtin on the T-cell surface. Previous studies revealed that LAG-3 could inhibit

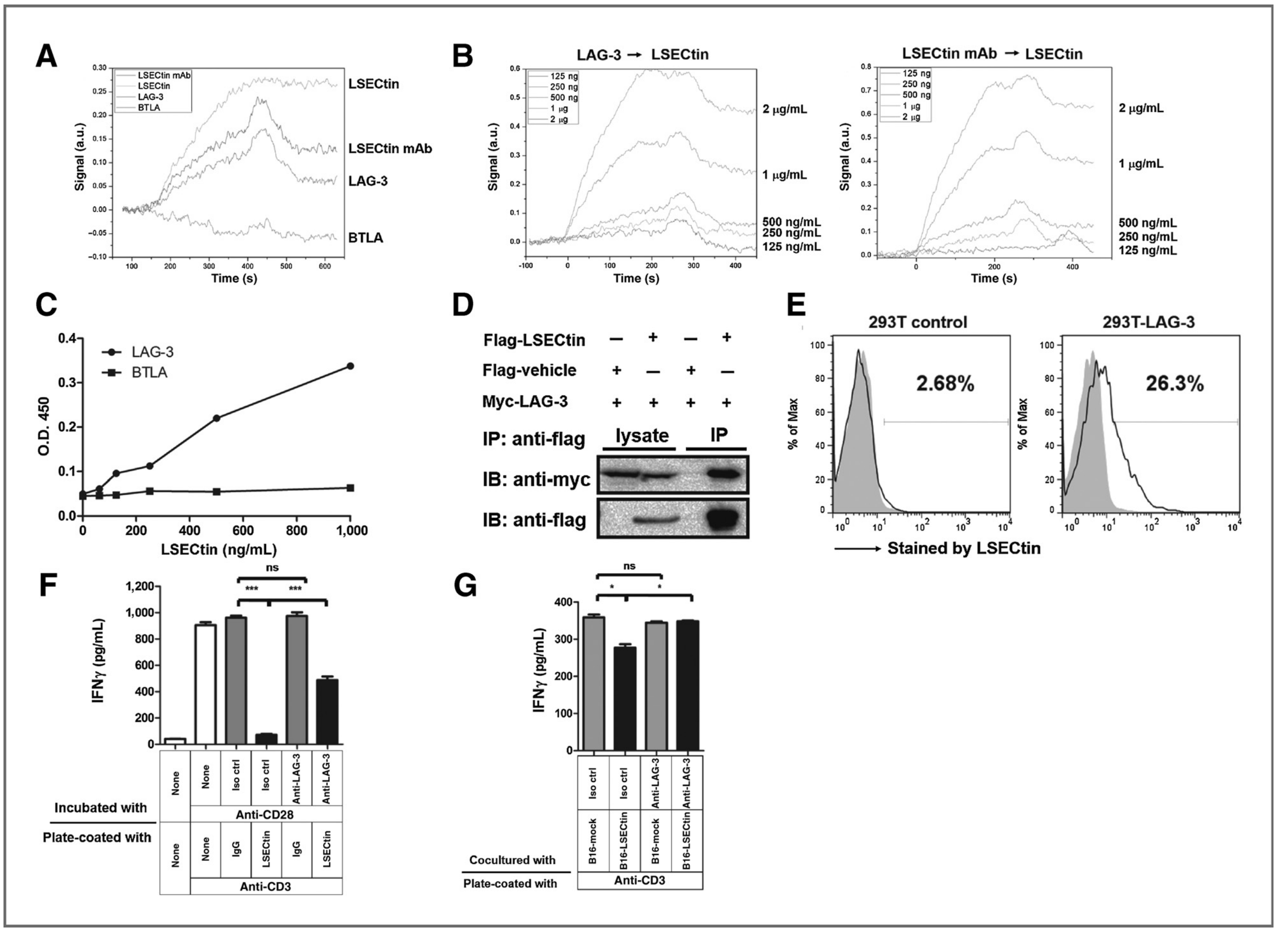

Figure 7. LSECtin interacts with LAG-3 to inhibit IFNγ secretion from effector T cells. A, an SPR analysis of the binding of 500 ng/mL injected LSECtin to 500 ng/mL immobilized LAG-3, BTLA, recombinant LSECtin, and LSECtin mAb on a sensor chip. B, the specificity and affinity were determined using SPR analysis. Various concentrations of LAG-3 or LSECtin mAb were flowed over 500 ng/mL of immobilized LSECtin on a sensor chip. The $K_d$ values for LAG-3/LSECtin and LSECtin mAb/LSECtin were 5.65 and 2.46 μmol/L, respectively. SPR data are presented as signals (a.u.) over time (seconds). Data are representative of three independent experiments. C, ELISA plates were coated with 1 μg/mL of LAG-3 or BTLA and then incubated with LSECtin at various concentrations. D, confirmation of the LAG-3/LSECtin interaction via coimmunoprecipitation (IP) assays. IB, immunoblotting. E, HEK293T cells that had been transfected with LAG-3 or control plasmid were stained with an LSECtin (solid line) or control IgG (shaded). F, plates were coated with 5 μg/mL of anti-CD3 and 10 μg/mL of LSECtin or control IgG. Sorted T cells from WT C57BL/6 mice were cultured with 2 μg/mL of soluble anti-CD28 and 50 μg/mL of anti-LAG-3 (C9B7W) or isotype control. ***, $P < 0.001$. Data are representative of three independent experiments. G, plates were coated with 2 μg/mL of anti-CD3. B16-specific T cells, obtained from immunized mice as described, were cocultured with irradiated B16-LSECtin (black) and B16-mock (gray) cells in the presence of 50 μg/mL of anti-LAG-3 (C9B7W) or isotype control. *, $P < 0.05$. Data are representative of two independent experiments.

the infiltration and expansion of both CD4$^+$ and CD8$^+$ T cells (40). Like other co-inhibitory molecules, LAG-3 was also used by tumors to disrupt antitumor T-cell immune responses (35, 36, 38). In addition, LAG-3 protects A375 melanoma cells from Fas-mediated apoptosis through its ligand MHC II signaling capacity in melanoma cells (37). Herein, we found that the inhibitory role of LSECtin in B16 immunity was significantly similar to that of LAG-3 and identified for the first time that LSECtin, expressed on the surface of B16 melanoma cells, was another putative ligand for LAG-3. Functionally, antibody blockade of LAG-3 restored the reduced IFNγ secretion mediated by both recombinant and tumor-expressed LSECtin. Taken together, in contrast to the apoptosis resistance mediated by the MHC II/LAG-3 axis, we found that LSECtin could interact with LAG-3 to damper antitumor immunity by inhibiting IFNγ secretion from antigen-specific effector T cells.

Immunotherapy for cancer has now become one of the most successful strategies for the treatment of patients with tumors. This cancer treatment strategy aims to turn on co-stimulators and/or turn off co-inhibitors of T-cell immunity. Interventions that would enhance tumor-specific immunity by modulating co-inhibitors have demonstrated some clinical successes. For example, the anti-CTLA4 and anti-PD1 monoclonal antibodies showed promising antitumor activity against various types of tumors in clinical trials (20–22). However, preclinical studies have indicated that blockade of the ligands of these co-inhibitors, which are expressed on various tumor cells, like PD-L1, also delivered promising clinical impacts. In a phase I trial, the anti-PD-L1 antibody BMS-936559 showed modest antitumor activity in patients with non–small cell lung cancer (NSCLC), renal cell carcinoma (RCC), and melanoma (41). Our preliminary blocking experiment also showed that the administration of LSECtin polyclonal antibody could slow melanoma progression in mice. In the future, a specific monoclonal LSECtin-blocking antibody should be developed, and details of the immune responses after the administration of this blocking antibody should be further evaluated. Meanwhile, accumulating evidence suggests that optimizing immunotherapy requires treatments that affect multiple facets of the immune response. In a phase I trial, deeper and more rapid clinical antitumor responses were observed in patients with advanced melanoma who were treated with the combination therapy of ipilimumab (anti-CTLA4) and nivolumab (anti-PD1; ref. 22). The suppressive role of LSECtin in antitumor immunity, presented herein, will provide a foundation for potential combinatorial immunotherapy strategies in combination with the blockade of other co-inhibitory molecules.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** L. Tang, F. He
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** F. Xu, Z. Hu
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** F. Xu, Z. Hu
**Writing, review, and/or revision of the manuscript:** L. Tang
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J. Liu, D. Liu, B. Liu, M. Wang, X. Du
**Study supervision:** L. Tang, F. He

## Grant Support

The study was supported by Chinese National Natural Science Foundation Projects (31270953), Chinese State Key Program in Basic Research (2010CB911902, 2013CB910802, 2013ZX10002009), and the Chinese National High-tech Program (2012AA020206-2).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 17, 2013; revised March 12, 2014; accepted March 26, 2014; published OnlineFirst April 25, 2014.

## References

1. Letsch A, Keilholz U, Schadendorf D, Nagorsen D, Schmittel A, Thiel E, et al. High frequencies of circulating melanoma-reactive CD8+ T cells in patients with advanced melanoma. Int J Cancer 2000;87:659–64.
2. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol 2006;24:175–208.
3. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 2007;25:267–96.
4. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol 2000;74:181–273.
5. Spiotto MT, Rowley DA, Schreiber H. Bystander elimination of antigen loss variants in established tumors. Nat Med 2004;10:294–8.
6. Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. Adv Immunol 2006;90:51–81.
7. Waldmann TA. Effective cancer therapy through immunomodulation. Annu Rev Med 2006;57:65–81.
8. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99–146.
9. Gerlini G, Tun-Kyi A, Dudli C, Burg G, Pimpinelli N, Nestle FO. Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumor lesions. Am J Pathol 2004;165:1853–63.
10. Kurte M, Lopez M, Aguirre A, Escobar A, Aguillon JC, Charo J, et al. A synthetic peptide homologous to functional domain of human IL-10 down-regulates expression of MHC class I and Transporter associated with Antigen Processing 1/2 in human melanoma cells. J Immunol 2004;173:1731–7.
11. Akasaki Y, Liu G, Chung NH, Ehtesham M, Black KL, Yu JS. Induction of a CD4+ T regulatory type 1 response by cyclooxygenase-2-overexpressing glioma. J Immunol 2004;173:4352–9.
12. Agrawal B, Krantz MJ, Reddish MA, Longenecker BM. Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. Nat Med 1998;4:43–9.
13. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281:1191–3.
14. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531–62.
15. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5:263–74.
16. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother 2002;51:293–8.
17. Nagaraj S, Gabrilovich DI. Tumor escape mechanism governed by myeloid-derived suppressor cells. Cancer Res 2008;68:2561–3.
18. Sotomayor EM, Borrello I, Tubb E, Allison JP, Levitsky HI. *In vivo* blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. Proc Natl Acad Sci U S A 1999;96:11476–81.
19. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother 2005;54:307–14.
20. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711–23.
21. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 2013;369:134–44.
22. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;369:122–33.
23. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci U S A 2010;107:4275–80.
24. Zelensky AN, Gready JE. The C-type lectin-like domain superfamily. FEBS J 2005;272:6179–217.
25. Nagahara K, Arikawa T, Oomizu S, Kontani K, Nobumoto A, Tateno H, et al. Galectin-9 increases Tim-3+ dendritic cells and CD8+ T cells and enhances antitumor immunity via galectin-9-Tim-3 interactions. J Immunol 2008;181:7660–9.
26. Liu W, Tang L, Zhang G, Wei H, Cui Y, Guo L, et al. Characterization of a novel C-type lectin-like gene, LSECtin: demonstration of carbohydrate binding and expression in sinusoidal endothelial cells of liver and lymph node. J Biol Chem 2004;279:18748–58.
27. Tang L, Yang J, Liu W, Tang X, Chen J, Zhao D, et al. Liver sinusoidal endothelial cell lectin, LSECtin, negatively regulates hepatic T-cell immune response. Gastroenterology 2009;137:1498–508.e1–5.
28. Liu B, Wang M, Wang X, Zhao D, Liu D, Liu J, et al. Liver sinusoidal endothelial cell lectin inhibits CTL-dependent virus clearance in mouse models of viral hepatitis. J Immunol 2013;190:4185–95.
29. Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. Curr Protoc Immunol 2001;Chapter 20:Unit 20.1.

30. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133: 1710–5.
31. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 1992;68:869–77.
32. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002;8:793–800.
33. Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, et al. Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. Science 1996;274:1363–6.
34. Li D, Liu W, Liu Z, Wang J, Liu Q, Zhu Y, et al. PRINCESS, a protein interaction confidence evaluation system with multiple data sources. Mol Cell Proteomics 2008;7:1043–52.
35. Gandhi MK, Lambley E, Duraiswamy J, Dua U, Smith C, Elliott S, et al. Expression of LAG-3 by tumor-infiltrating lymphocytes is coincident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients. Blood 2006;108:2280–9.
36. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, et al.Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad Sci U S A 2010;107:7875–80.
37. Hemon P, Jean-Louis F, Ramgolam K, Brignone C, Viguier M, Bachelez H, et al. MHC class II engagement by its ligand LAG-3 (CD223) contributes to melanoma resistance to apoptosis. J Immunol 2011;186:5173–83.
38. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. J Clin Invest 2007;117: 3383–92.
39. Dominguez-Soto A, Aragoneses-Fenoll L, Martin-Gayo E, Martinez-Prats L, Colmenares M, Naranjo-Gomez M, et al. The DC-SIGN-related lectin LSECtin mediates antigen capture and pathogen binding by human myeloid cells. Blood 2007;109:5337–45.
40. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. Eur J Immunol 2003; 33:970–9.
41. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012;366:2455–65.