

VEGF-C Promotes Immune Tolerance in B16 Melanomas and Cross-Presentation of Tumor Antigen by Lymph Node Lymphatics

Amanda W. Lund,^{1,2} Fernanda V. Duraes,³ Sachiko Hirose,¹ Vidya R. Raghavan,¹ Chiara Nembrini,¹ Susan N. Thomas,^{1,2} Amine Issa,¹ Stéphanie Hugues,^{3,*} and Melody A. Swartz^{1,2,*}

¹Institute of Bioengineering (IBI)

²Swiss Institute for Experimental Cancer Research (ISREC)

School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne 1015, Switzerland

³Department of Pathology and Immunology, University of Geneva Medical School, Centre Médical Universitaire (CMU), Geneva 1211, Switzerland

*Correspondence: stephanie.hugues@unige.ch (S.H.), melody.swartz@epfl.ch (M.A.S.)

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SUMMARY

Tumor expression of the lymphangiogenic factor VEGF-C is correlated with metastasis and poor prognosis, and although VEGF-C enhances transport to the draining lymph node (dLN) and antigen exposure to the adaptive immune system, its role in tumor immunity remains unexplored. Here, we demonstrate that VEGF-C promotes immune tolerance in murine melanoma. In B16 F10 melanomas expressing a foreign antigen (OVA), VEGF-C protected tumors against preexisting antitumor immunity and promoted local deletion of OVA-specific CD8⁺ T cells. Naive OVA-specific CD8⁺ T cells, transferred into tumor-bearing mice, were dysfunctionally activated and apoptotic. Lymphatic endothelial cells (LECs) in dLNs cross-presented OVA, and naive LECs scavenge and cross-present OVA in vitro. Cross-presenting LECs drove the proliferation and apoptosis of OVA-specific CD8⁺ T cells ex vivo. Our findings introduce a tumor-promoting role for lymphatics in the tumor and dLN and suggest that lymphatic endothelium in the local microenvironment may be a target for immunomodulation.

INTRODUCTION

It is now recognized that tumor cells can express immunogenic antigens through mutation and that adaptive immune responses can be mounted in tumor-bearing hosts (Baitsch et al., 2011; Munn and Mellor, 2006; Zippelius et al., 2004). However, while the functional activation of T cells by antigen-presenting cells usually occurs in secondary lymphoid organs, tumors and their draining lymph nodes (dLNs) can locally render such antigen-specific T cells ineffective (Baitsch et al., 2011; Munn and Mellor, 2006; Thauat et al., 2010; Zippelius et al., 2004).

Expression of the lymphangiogenic growth factor VEGF-C in the tumor promotes LN metastasis (Skobe et al., 2001; Tammela

and Alitalo, 2010; Yanai et al., 2001) and increased lymph drainage to the dLN (Harrell et al., 2007). While tumor-associated lymphangiogenesis was earlier considered to passively transport invasive tumor cells to the dLN, more recent studies demonstrate an active role for lymphatics and their associated chemokines in promoting tumor progression and metastasis to the dLN (Issa et al., 2009; Kerjaschki et al., 2011; Shields et al., 2010). Additionally, lymphatic vessels physically link the tumor immune status to its dLN through cell and antigen transport (Clement et al., 2010; Randolph et al., 2005) and the dLN plays an important role in balancing adaptive immunity and local peripheral tolerance (Cohen et al., 2010; Förster et al., 2008; Lund and Swartz, 2010; Reynoso et al., 2009). However, to date, the effects of VEGF-C and tumor-associated lymphangiogenesis on host antitumor immunity remain unexplored.

Here, we demonstrate that VEGF-C and associated LN lymphangiogenesis suppress antitumor immunity through mechanisms that include local deletional tolerance, which in turn drives disease progression and metastasis. VEGF-C-expressing (lymphangiogenic) tumors were protected from vaccine-induced immunity and promoted naive T cell deletion in sentinel dLN. Lymphatic drainage bathes the lymphatic vessels and dLN in soluble antigen (Clement et al., 2011; Sixt et al., 2005), and we show in B16 melanoma that VEGF-C-activated lymphatic endothelial cells (LECs) can take up and cross-present tumor antigen, leading to dysfunctional activation of CD8⁺ T cells. This mechanism of antigen cross-presentation by LECs is distinct from the promiscuous presentation of endogenous self-antigens by LN stromal cell subsets (Cohen et al., 2010; Fletcher et al., 2010) and may constitute a new target for cancer immunotherapy.

RESULTS

VEGF-C and Tumor Lymphangiogenesis Protects against Preexisting, Vaccine-Induced Immunity

Malignant melanoma growth and metastasis have been correlated with VEGF-C expression and lymphatic density in humans, and both human and murine melanomas are antigenic but employ active mechanisms of immune evasion (Anichini et al.,

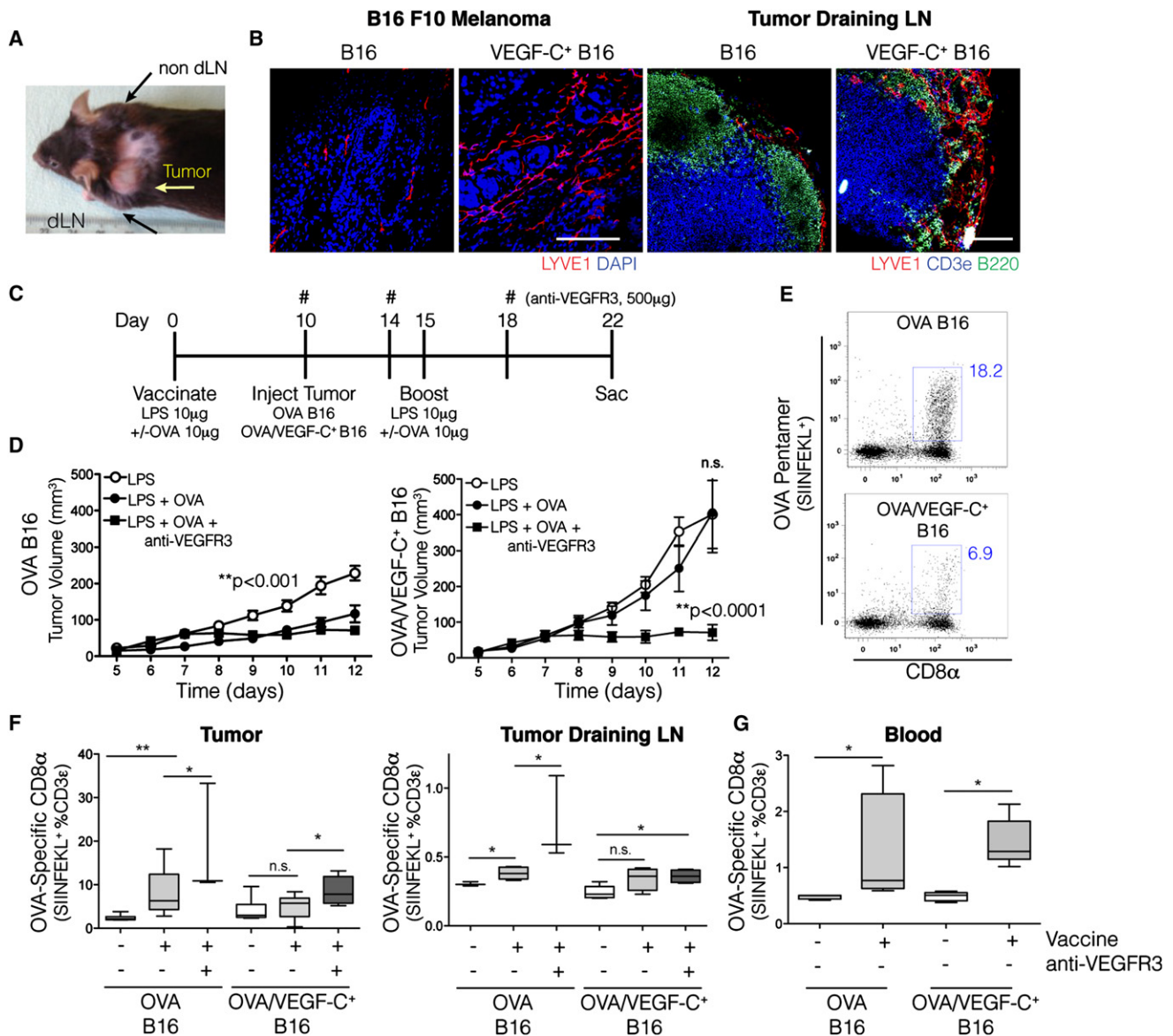


Figure 1. VEGF-C Protect against Preexisting Antitumor Immunity in B16-F10 Melanomas

(A) Location of tumor inoculation with associated draining lymph node (dLN, axillary, and brachial) and contralateral non-dLN.

(B) Lymphatic vessels in the tumor (left) and dLN (right) at day 12. Bar, 50 μm.

(C) OVA vaccination scheme with lipopolysaccharide (LPS) adjuvant and 500 μg anti-VEGFR3 (mF4-31C1 #).

(D) Growth profiles for control OVA and OVA/VEGF-C⁺ B16 tumors after vaccination and anti-VEGFR3 treatment.

(E–G) Representative plots (E) and quantification (F) of OVA-specific CD8α⁺ T cells infiltrating the tumor (left) or draining lymph node (right) and in the blood (G). Shown are medians and whiskers show range.

*p ≤ 0.05, **p ≤ 0.01, n ≥ 8.

2004; Ferguson et al., 2008). To address the potential roles of VEGF-C in modulating host antitumor immunity, we expressed VEGF-C along with chicken ovalbumin (OVA) in B16-F10 melanoma cells (Figures S1A–S1C) and compared host immune responses in immune competent, syngenic C57BL/6 mice.

Tumor cells were implanted intradermally (i.d.) and dorsolaterally to define drainage to a single set of sentinel lymph nodes

(axillary and brachial, Figure 1A). Though primary tumor growth was largely unaffected by VEGF-C expression (Figures S1D and S1E), as expected, VEGF-C⁺ tumors induced lymphangiogenesis around the tumor and in the dLN and drove increased metastasis to the dLNs (Figure 1B; Figures S1F and S1G). Furthermore, VEGF-C⁺ B16 tumors demonstrated increased fluid drainage and dendritic cell (DC) migration from the tumor to the dLN (Figures S1H and S1I).

VEGF-C⁺ tumors contained more CD45⁺ immune cell infiltrates, including T cells (Figures S2A and S2B). These immune infiltrates, however, contained more regulatory T (T_{Reg}) cells but fewer antigen-specific CD8 T cells (Figures S2C and S2D). Other immune regulatory subsets preferentially recruited to VEGF-C⁺ tumors included myeloid-derived suppressor cells (CD11b⁺F4/80[−]GR1^{hi}) and CD11c⁺CD11b⁺CD8α[−] DCs (Figure S2E).

Next, we wondered whether VEGF-C could provide protection against a strong immune response and what role, if any, local lymphatics may play. LN stromal cells, including LECs, promiscuously express peripheral tissue antigens (such as tyrosinase) to maintain peripheral tolerance (Cohen et al., 2010; Fletcher et al., 2010), making it difficult to assess new mechanisms of tolerance above this background. Therefore, we introduced a foreign antigen (OVA) into the tumor cells, and vaccinated mice prior to implantation to generate systemic cytotoxic T lymphocytes (CTLs) against the tumor. In this way, we could distinguish newly formed tolerance to a tumor-associated antigen from preexisting tolerance due to endogenous self-antigen presentation.

To determine the potential protective effects of tumor VEGF-C against preexisting immunity, we vaccinated mice with OVA and lipopolysaccharide (LPS) as an adjuvant 10 days before tumor implantation (Figure 1C). As expected, this vaccination strongly inhibited the growth of OVA tumors (Figure 1D, left). Remarkably, however, vaccination had no effect on the growth of OVA/VEGF-C⁺ tumors (Figure 1D, right). Both in the tumor and dLN, mice bearing OVA/VEGF-C⁺ tumors showed a decrease in the local infiltration of OVA (pentamer)-specific CD8⁺ T cells (Figures 1E and 1F), even while these antitumor CTLs were circulating at similar levels in all vaccinated mice (Figure 1G). This VEGF-C-mediated local suppression of vaccine-induced immunity was dependent upon VEGFR3 signaling, since mice that were additionally treated with mF4-31C1, a neutralizing antibody against VEGFR-3, during tumor growth were susceptible to the vaccine (Figure 1D). Additionally, VEGFR-3 inhibition further enhanced antitumor CTLs in the tumor and dLN in control OVA tumors (Figure 1F), suggesting that endogenous levels of VEGF-C found in the stroma, likely due to CD11b⁺ macrophage infiltrates (Figure S2F; Kluger and Colegio, 2011), may also help suppress host immunity.

VEGF-C⁺ Tumors Suppress Naive T Cell Activation in the Draining Lymph Node

Given the observed suppression of antigen-specific T cell responses locally within the tumor and dLN, we then asked whether these CD8⁺ T cells were being inhibited during their activation. We injected carboxyfluorescein succinimidyl ester (CFSE)-labeled naive CD8⁺ T cells bearing the receptor for the OVA peptide (SIINFEKL)-loaded major histocompatibility class I (MHC I) complex (OT-I) into tumor-bearing mice (CD45.1) and assessed their activation in the dLN (Figure 2A). When transferred into unvaccinated tumor-bearing hosts at day 9, OT-I cells demonstrated altered homing to OVA/VEGF-C⁺ tumors compared to OVA tumors (Figures 2B and 2C). The presence of highly proliferated CFSE[−]CD45.2⁺CD8α⁺ cells, and absence of CFSE⁺ (unproliferated) cells, in OVA B16 tumors suggested that proliferation and activation of transferred OT-I cells primarily occurred

distal to the tumor. Significantly fewer proliferated but more unproliferated cells were detected in OVA/VEGF-C⁺ compared to OVA B16 tumors (Figure 2C). These infiltrates were functionally assessed two days later and those accumulating in OVA/VEGF-C⁺ tumors produced significantly less IFN-γ (Figure S3A) and had lower expression of T cell activation markers CD25 and CD69 (Figure S3B).

Next, we asked whether naive OT-I cells were differentially activated in dLNs of OVA and OVA/VEGF-C⁺ tumors, thereby leading to the observed changes in tumor infiltration. We found that while the extent of OT-I cell proliferation in OVA/VEGF-C⁺ dLN was similar to that in OVA dLNs (Figures 2D and 2E), their functional activation was impaired. Specifically, after 3 or 5 days, activated OT-I cells in the dLNs of OVA/VEGF-C⁺ tumors secreted less IFN-γ, were progressively more apoptotic compared to those in the dLN of OVA tumors, and expressed lower levels of the activation markers CD25 and CD69 (Figures 2F and 2G; Figures S3C and S3D). In contrast, transferred naive OT-I T cells were activated and less apoptotic when mice were treated with anti-VEGFR-3 blocking antibodies (Figure 2G). These data indicate a dysfunctional T cell response in dLN of OVA/VEGF-C⁺ tumors compared to OVA tumors.

Tumor-Associated Lymphatics Directly Interact with T Cells and Present Tumor Antigen Peptides on MHC I In Vivo

Tumors were sectioned and stained 3 days following adoptive transfer to localize the T cell infiltrate within the stroma. We observed an increased infiltration of transferred OT-I T cells, particularly localized and clustered around peritumoral lymphatic vessels, in OVA/VEGF-C⁺ versus OVA tumors (Figure 3A), suggesting that VEGF-C activated LECs might directly alter the CD8⁺ T cell response. Since LN LECs present endogenous peripheral tissue antigens on MHC I molecules (Cohen et al., 2010), we asked whether VEGF-C-activated LECs in the tumor and dLN might extend this tolerogenic function to scavenge and cross-present exogenous antigen for T cell deletion. To address this, we implanted OVA tumors into chimeric mice whose CD45⁺ cells lacked MHC I (Figures S4A and S4B), and then adoptively transferred naive OT-I cells after 9 days. Despite the lack of cross-presentation by professional APCs in these mice, OT-I cells still proliferated in the dLNs of OVA-expressing tumors (Figures 3B and 3C), indicating that the exogenous MHC I peptide of OVA was presented on MHC I molecules of stromal (CD45[−]) cells in the dLN.

To determine specifically which LN stromal cells were presenting antigen, we sorted the stromal cell populations from the dLNs in tumor-bearing wild-type mice after 14 days (Figure S4C). Ex vivo, LECs (gp38⁺CD31⁺) and blood endothelial cells (BECs, gp38[−]CD31⁺) significantly induced OT-I proliferation, while fibroblastic reticular cells (FRCs, gp38⁺CD31[−]) did not (Figures 3D and 3E). Consistent with these results, LECs in OVA-expressing tumors and their dLNs were positive for 25d1.16 (an antibody that specifically recognizes the MHC I-SIINFEKL peptide complex), and presented more SIINFEKL per cell in VEGF-C-expressing tumors (Figure 3F). These data suggest that LECs may take up and process OVA for cross-presentation on MHC I molecules.

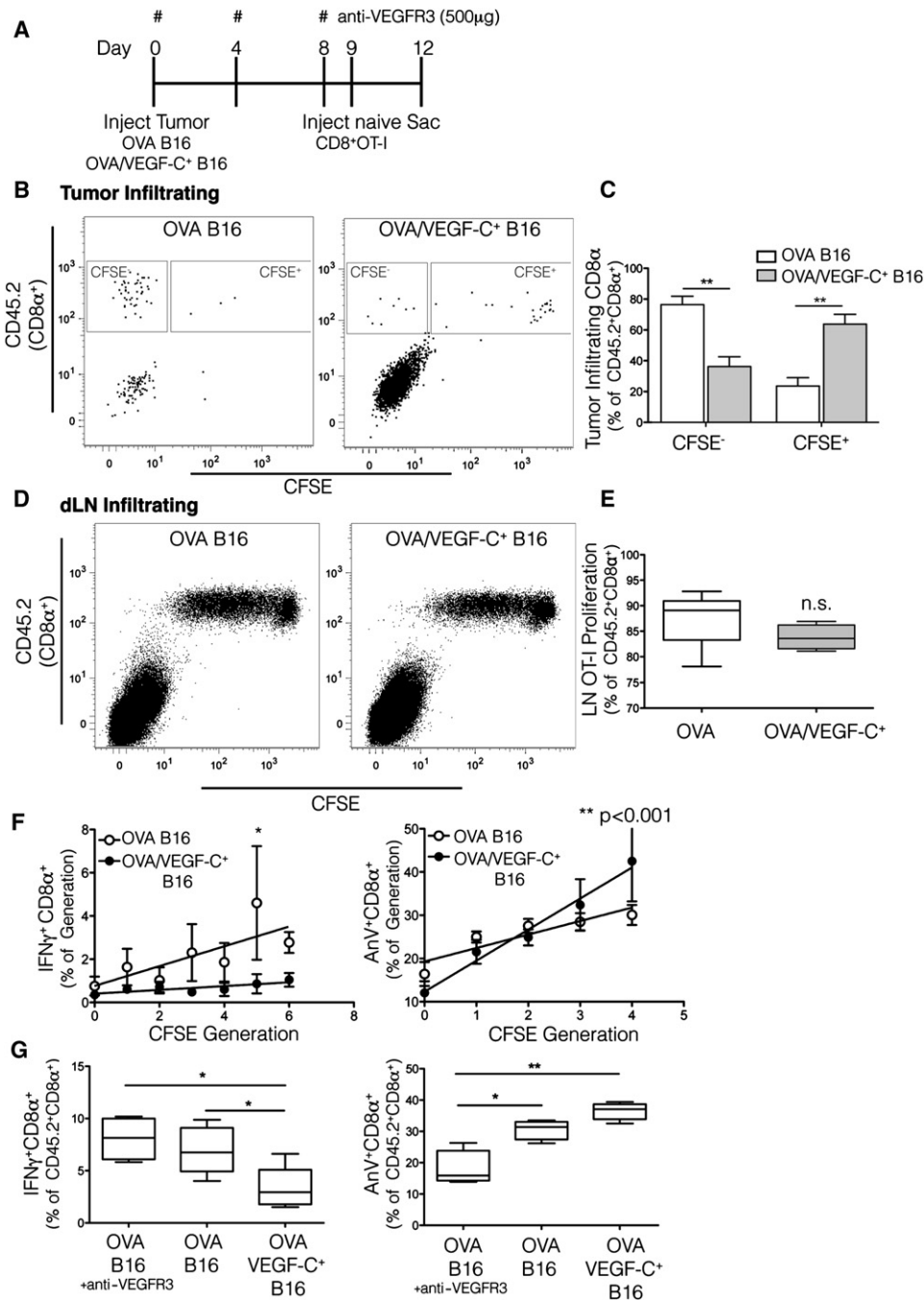


Figure 2. CD8⁺ T Cells Activated in Lymph Nodes Draining VEGF-C⁺ Tumors Are Dysfunctional and Apoptotic

(A) Scheme for OT-I adoptive transfer. After 9 days tumor growth, 10⁶ naive CD8⁺ OT-I cells were injected i.v. with 500 μg mF4-31C1 (#).

(B–E) Representative plots (B and D) and quantification (C and E) of CFSE-labeled OT-I cells (CD45.2⁺CD8α⁺) 3 days after transfer in the tumor (B and C) or draining LN (D and E).

(F) IFN-γ production (left) and annexin V (AnV, right) binding by transferred OT-I cells with proliferation.

(G) IFN-γ (left) and AnV (right) as % OT-I cells present within the draining LN. Shown are medians and whiskers show range.

*p < 0.05, **p < 0.01, n ≥ 8.

Lymphatic Endothelial Cells Cross-Present Antigen and Cross-Tolerize T Cells In Vitro

Pre-nodal lymph contains preprocessed peptides from peripheral tissues that can bind directly to MHC I molecules expressed on

the cell surface (Clement et al., 2011). In order to demonstrate that LECs can also directly process and cross-present antigens we exposed ex vivo LN stromal cell cultures (Fletcher et al., 2010), which were predominantly FRCs and LECs (Figures S4D

and S4E), to nanoparticles coupled with a longer version of the OVA MHC I peptide (SIINFEKL) designed to require uptake and processing for presentation on MHCI (NPssCOVA₂₅₀₋₂₆₄) (Hiro-sue et al., 2010). The MHCI-SIINFEKL complex was detected on B16 F10 melanoma cells (negative control) only when exposed to the peptide but not the nanoparticle-coupled peptide. However, LECs and bone marrow-derived DCs (BMDCs, positive control) displayed the complex after exposure to the nanoparticle-coupled peptide (Figures 4A and 4B), indicating a capacity for antigen processing and cross-presentation. These LECs, when sorted and rinsed following NPssCOVA₂₅₀₋₂₆₄ exposure, could induce significant proliferation of naive OT-I cells (Figures 4C and 4D). As expected, proliferation was significantly less than that induced by BMDCs; however, LEC-stimulated T cells expressed less IFN- γ (Figure 4E) and bound more annexin V than BMDCs or splenocytes (Figures 4F and 4G). Together, these data demonstrate that LN LECs can cross-present tumor antigen and directly suppress tumor-specific CD8 T cells by inhibiting cytotoxic function and inducing apoptosis.

DISCUSSION

Here we introduce VEGF-C as a protumor immunomodulatory factor and identified a new immunosuppressive role for lymphatic endothelial cells, to scavenge and cross-present antigens for direct suppression of CTLs. **This adds to our understanding of VEGF-C as a tumor metastasis promoter by linking it to tumor immune escape, often considered critical to tumor progression.**

Hundreds of studies have now established the clinical relevance of VEGF-C in the tumor microenvironment (summarized by Tammela and Alitalo, 2010). In human cancer, VEGF-C expression correlates with lymph node metastasis and poor prognosis (Padera et al., 2002; Wong et al., 2005), while VEGF-C overexpression in murine tumors induces metastasis (Mandriota et al., 2001; Padera et al., 2002; Skobe et al., 2001; Wong et al., 2005; Yanai et al., 2001) and blocking VEGFR-3 signaling decreases tumor invasion and metastasis (He et al., 2002; Roberts et al., 2006). However, although tumor immunologists have long appreciated the importance of the sentinel LN in suppressing antitumor immune responses (Munn and Mellor, 2006), the roles of tumor VEGF-C and lymphangiogenesis, which enables and enhances communication between the tumor and the dLN, in modulating tumor immunity remain largely unexplored.

The local inhibition of activated CD8⁺ T cells by LECs in the dLN is consistent with recent studies in human melanoma, where exhausted CD8⁺ T cells were found in primary and secondary tumors while active CTLs remained in circulation (Baitsch et al., 2011). A major function of the LN is to maintain and regulate immune homeostasis and peripheral tolerance (Förster et al., 2008; Turley et al., 2010), and LN stromal cells delete autoreactive T cells through promiscuous presentation of peripheral self-antigens by stromal cells (Cohen et al., 2010; Fletcher et al., 2010). In addition, lymphatic drainage carries soluble antigen, membrane bound vesicles, cytokines and cells to the LN where it flows through an extensive collagen conduit system, providing

antigen to the largely tolerogenic and immature local APC population (Munn and Mellor, 2006; Sixt et al., 2005). This constant sampling of peripheral antigens in the local dLN is likely important for maintaining tolerance, as when lymphatic drainage was locally blocked in the skin, tolerance to skin contact hypersensitizing agents could not be induced (Friedlaender and Baer, 1972). Therefore it is possible that tumors may hijack such tolerance-maintaining functions of the dLN to suppress cytotoxic immune responses.

VEGF-C-enhanced lymph flow may also promote tumor progression in other ways. First, it may increase interstitial flow in the tumor stroma, which itself can promote invasion through fibroblast activation (Shieh et al., 2011), matrix stiffening (DuFort et al., 2011), and biasing chemokine gradients (Fleury et al., 2006; Shields et al., 2007). Additionally, it may help to prime the premetastatic niche, which prepares the dLN for tumor cell invasion. Importantly, premetastatic tumor-dLNs, but not normal LNs, in humans were found to contain bone marrow progenitor cells associated with the metastatic niche (Peinado et al., 2011), implicating lymphatic drainage from the tumor in driving these changes. Furthermore, enhanced flow and delivery of peripheral antigens to LN LECs (Clement et al., 2011) may promote their newly proposed suppressive functions (Cohen et al., 2010; Liao et al., 2011; Lukacs-Kornek et al., 2011).

Our finding that dLN LECs cross-present draining, exogenous antigen for CD8 T cell deletion is reminiscent of liver sinusoidal endothelial cells (LSECs). Self-antigen presentation on MHCI is defined for many cell types for “self” identification, but scavenging, processing, and cross-presentation of exogenous antigens are functions attributed typically to specialized antigen-presenting cells. LSECs are constantly exposed to foreign antigens from food and they scavenge and cross-present these antigens to promote CD8 T cell clustering and suppression (Schurich et al., 2010; von Oppen et al., 2009). An important mechanism of LSEC suppression is T cell adhesion to the endothelium and migratory arrest (von Oppen et al., 2009), and we frequently observed OVA-specific T cells adhering to lymphatic vessels surrounding OVA/VEGF-C⁺ tumors (Figure 3A).

Lymphatic antigen presentation is a newly described mechanism by which tumors evade host immunity through the direct suppression of tumor-specific CD8 T cells by LECs. This immunomodulatory function may have evolved to help maintain peripheral tolerance after injury and to prevent autoimmunity in chronic inflammation, where macrophages produce VEGF-C that can drive local and dLN lymphangiogenesis. In such conditions, lymphatic vessels can actively modulate flow to the dLN (Harrell et al., 2007; Miteva et al., 2010), thereby linking lymphatic transport function to host immunity. A recent study demonstrated that IFN- γ and cytotoxic T cells negatively regulate lymphangiogenesis (Kataru et al., 2011). Our observed enhancement of antigen-specific CD8 tumor infiltration following anti-VEGFR3 treatment is consistent with this, suggesting that anti-lymphangiogenic therapy, even with endogenous levels of VEGF-C, may enhance antitumor responses. Therefore a dichotomy of function seems to exist whereby lymphangiogenesis inhibits CTLs, while CTLs and their proinflammatory cytokines inhibit lymphangiogenesis leading to a balance between inflammation and peripheral tolerance.

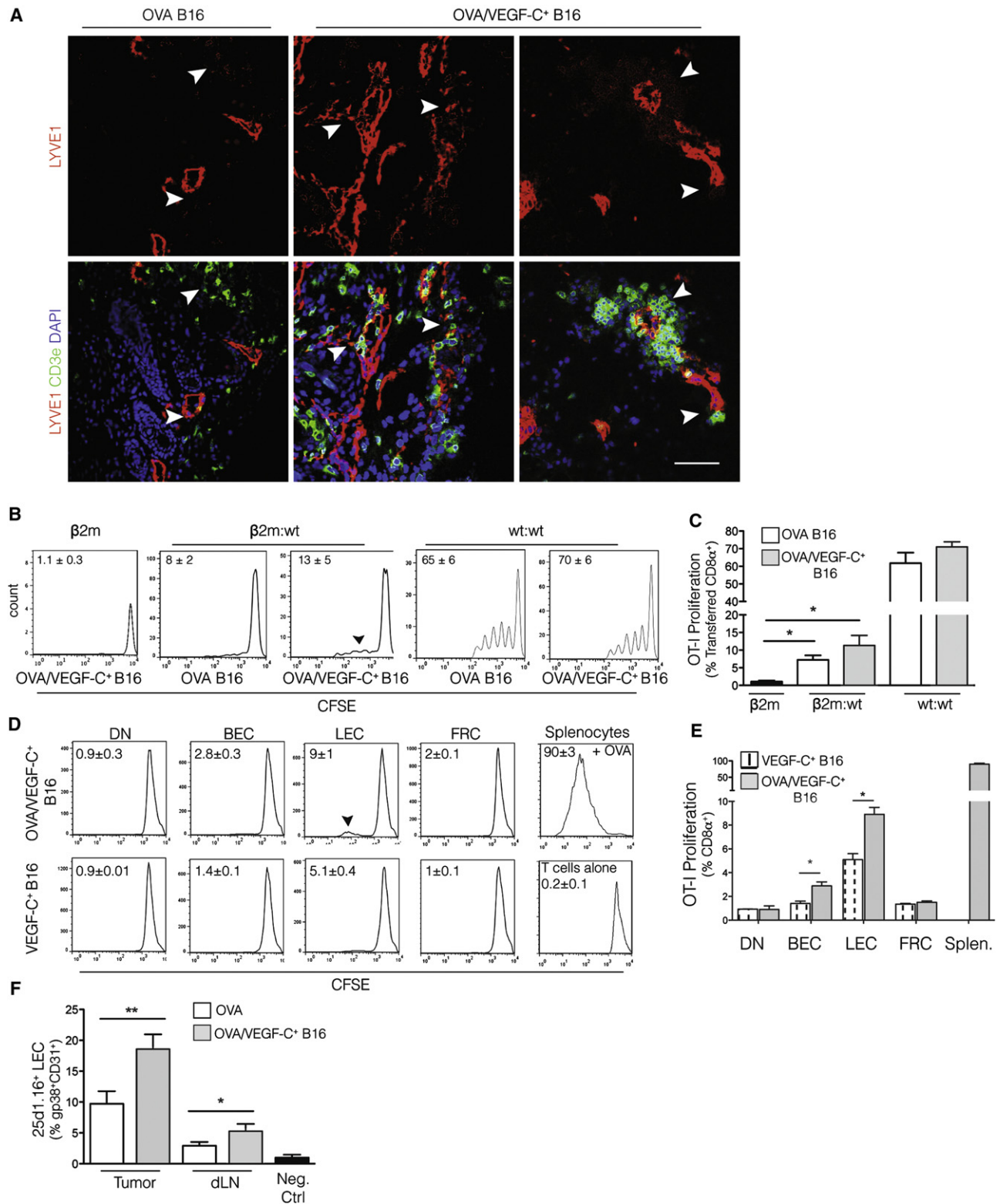


Figure 3. Lymphatic Endothelial Cells Interact with CD8⁺ T Cells In Vivo and Cross-Present Tumor Antigen

(A) Immunofluorescence showing OVA-specific T cells interacting with LECs (arrows) in the stroma of OVA and OVA/VEGF-C⁺ tumors. Bar, 100 μ m.

(B and C) Representative plots (B) and quantification (C) of OT-I CD8⁺ T cell proliferation in draining lymph nodes (dLN) of tumor-bearing mice after adoptive transfer. $\beta 2m^{-/-}:wt$ ($\beta 2m:wt$) chimeric mice lack MHC I expression in hematopoietic but not stromal cells.

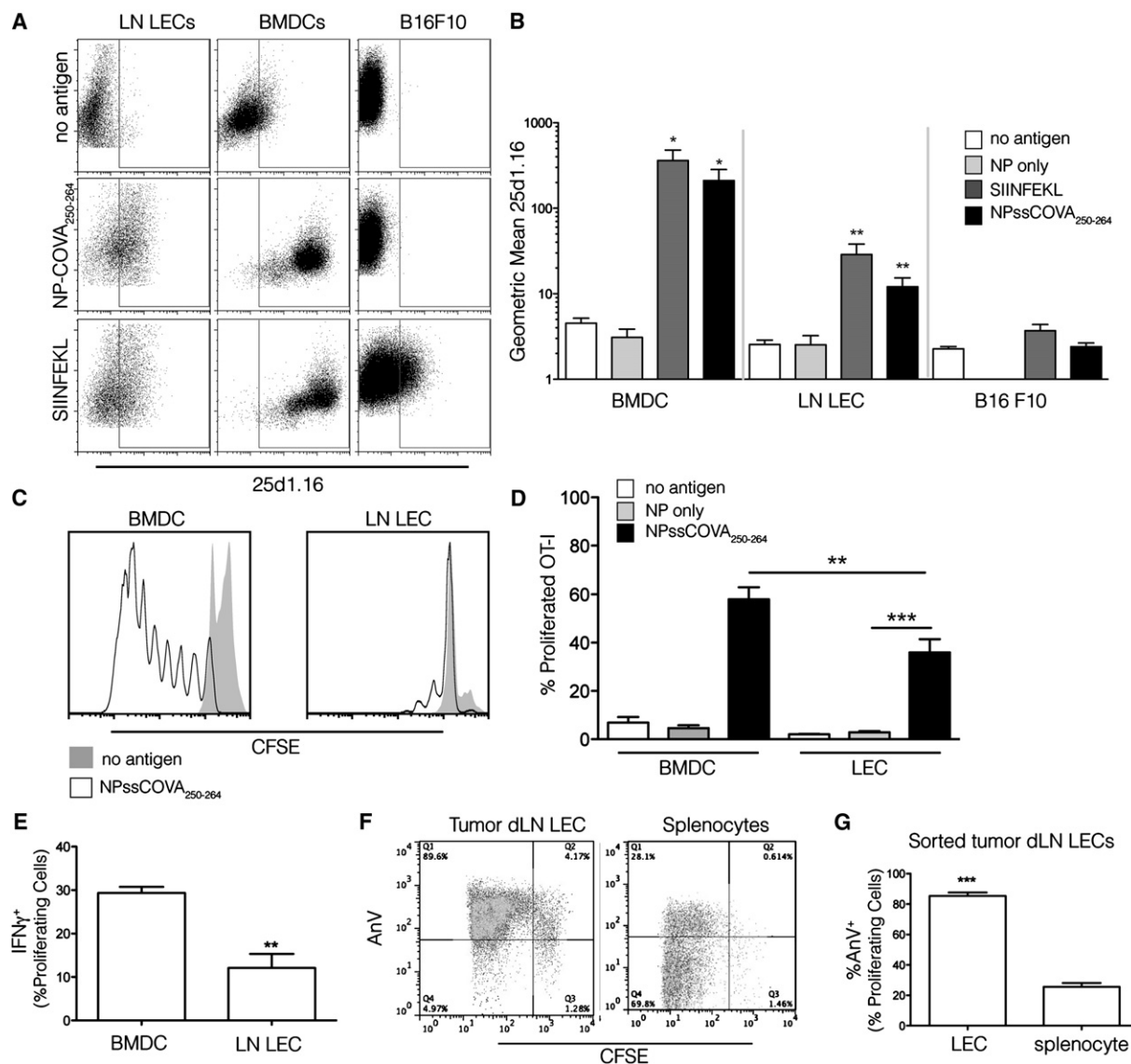


Figure 4. Lymphatic Endothelial Cells Cross-Present Antigen to Induce Dysfunctional T Cell Activation

(A and B) Representative plots (A) and geometric means (B) of MHC/IIINFEKL complex (25d1.16) on cultured lymph node (LN) LECs, dendritic cells (BMDCs), and B16-F10 tumor cells after 24 hr pulse with NPssCOVA₂₅₀₋₂₆₄ or SIINFEKL.

(C and D) Representative plots (C) and quantification (D) of OT-I CD8⁺ T cell proliferation after coculture with antigen-pulsed LECs.

(E) IFN- γ production (by intracellular cytokine staining) in OT-I cells stimulated by antigen-presenting BMDCs or LECs.

(F and G) Representative plots (F) and quantification (G) of Annexin V (AnV) binding on OT-I cells after coculture with freshly sorted tumor-draining LN LECs or splenocytes reexposed to SIINFEKL antigen ex vivo.

Shown are means \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, $n \geq 3$.

The relative importance of antigen presentation by LECs in VEGF-C-driven tumor tolerance remains to be determined, as well as whether LECs and VEGF-C play similar immunomodulatory functions in contexts other than the unique microenviron-

ment of aggressive tumors. Nevertheless, we introduce the lymphatic endothelium as an active modulator of antitumor immunity and suggest that it may be targetable in strategies to improve cancer immunotherapy.

(D and E) Representative plots (D) and quantification (E) of ex vivo OT-I cell proliferation after coculture with cells isolated dLN of VEGF-C⁺ tumors. Splenocytes from naive mice with SIINFEKL were used as a positive control.

(F) MHC I-SIINFEKL complex (25d1.16) on LECs (gp38⁺CD31⁺) in the tumor and dLN.

Shown are means \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, $n \geq 8$.

EXPERIMENTAL PROCEDURES

More detailed information can be found in the [Extended Experimental Procedures](#). All procedures were approved by the Veterinary Authorities of the Canton Vaud according to Swiss Law.

Ovalbumin Immunization

Mice received 10 μ g lipopolysaccharide (LPS) as a negative control or 10 μ g LPS plus 10 μ g OVA (grade IV, Sigma) in two i.d. doses 25 μ l per foreleg. Ten days later, 5×10^5 B16-F10 OVA or OVA/VEGF-C⁺ tumor cells in 50 μ l were inoculated i.d. and dorsolaterally, and a vaccine boost (identical to the first) was administered again on day 15. In some mice, 500 μ g anti-VEGFR-3 neutralizing antibody (mF4-31C1, ImClone/Eli Lilly) was administered at days 0, 4, and 8 days after tumor inoculation.

OT-I Adoptive Transfer and Coculture

Splenic CD8⁺ T cells were isolated by negative magnetic cell sorting (CD8 α ⁺ T cell isolation kit II, Miltenyi Biotech). Purified CD8⁺ OT-I cells were labeled with 1 μ M CFSE and injected i.v. into tumor-bearing mice (1×10^6 cells in 200 μ l). To measure IFN- γ production, mice were given 250 μ g brefeldin A intraperitoneally (i.p.) 6 hr prior to sacrifice ([Thompson et al., 2010](#)). In ex vivo experiments, naive OT-I CD8⁺ T cells were directly cocultured with sorted lymph node stromal cells at a 1:3 ratio for 3–4 days in vitro.

Ex Vivo LEC Sorting and Coculture

On day 7 of LEC/FRC culture, cells were stimulated 24 hr prior to sorting with (1) synthetic nanoparticles disulfide-conjugated to an N-terminally elongated SIINFEKL peptide (CSGLEQLSIINFEKL ([Hirosue et al., 2010](#)), (2) SIINFEKL, or (3) empty nanoparticles as controls; in all cases, 10 nM SIINFEKL was delivered. LECs (CD45⁺ gp38⁺ CD31⁺) were sorted with a FACS Vantage SE. DIVA were cocultured with CFSE-labeled naive CD8⁺ OT-I T cells (1:10) for 4 days, which were then analyzed by flow cytometry for IFN- γ and Annexin-V binding (see [Extended Experimental Procedures](#)).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at [doi:10.1016/j.celrep.2012.01.005](https://doi.org/10.1016/j.celrep.2012.01.005).

LICENSING INFORMATION

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REFERENCES

Anichini, A., Vegetti, C., and Mortarini, R. (2004). The paradox of T-cell-mediated antitumor immunity in spite of poor clinical outcome in human melanoma. *Cancer Immunol. Immunother.* 53, 855–864.

Baitsch, L., Baumgaertner, P., Dev  vre, E., Raghav, S.K., Legat, A., Barba, L., Wieckowski, S., Bouzourene, H., Deplancke, B., Romero, P., et al. (2011). Exhaustion of tumor-specific CD8⁺ T cells in metastases from melanoma patients. *J. Clin. Invest.* 121, 2350–2360.

Clement, C.C., Cannizzo, E.S., Nastke, M.D., Sahu, R., Olszewski, W., Miller, N.E., Stern, L.J., and Santambrogio, L. (2010). An expanded self-antigen peptidome is carried by the human lymph as compared to the plasma. *PLoS ONE* 5, e9863.

Clement, C.C., Rotzschke, O., and Santambrogio, L. (2011). The lymph as a pool of self-antigens. *Trends Immunol.* 32, 6–11.

Cohen, J.N., Guidi, C.J., Tewalt, E.F., Qiao, H., Rouhani, S.J., Ruddell, A., Farr, A.G., Tung, K.S., and Engelhard, V.H. (2010). Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J. Exp. Med.* 207, 681–688.

DuFort, C.C., Paszek, M.J., and Weaver, V.M. (2011). Balancing forces: architectural control of mechanotransduction. *Nat. Rev. Mol. Cell Biol.* 12, 308–319.

Ferguson, A.R., Nichols, L.A., Zarling, A.L., Thompson, E.D., Brinkman, C.C., Hargadon, K.M., Bullock, T.N., and Engelhard, V.H. (2008). Strategies and challenges in eliciting immunity to melanoma. *Immunol. Rev.* 222, 28–42.

Fletcher, A.L., Lukacs-Kornek, V., Reynoso, E.D., Pinner, S.E., Bellemare-Pelletier, A., Curry, M.S., Collier, A.R., Boyd, R.L., and Turley, S.J. (2010). Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J. Exp. Med.* 207, 689–697.

Fleury, M.E., Boardman, K.C., and Swartz, M.A. (2006). Autologous morphogen gradients by subtle interstitial flow and matrix interactions. *Biophys. J.* 91, 113–121.

F  rster, R., Davalos-Misslitz, A.C., and Rot, A. (2008). CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* 8, 362–371.

Friedlaender, M.H., and Baer, H. (1972). Immunologic tolerance: role of the regional lymph node. *Science* 176, 312–314.

Harrell, M.I., Iritani, B.M., and Ruddell, A. (2007). Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. *Am. J. Pathol.* 170, 774–786.

He, Y., Kozaki, K., Karpanen, T., Koshikawa, K., Yla-Herttuala, S., Takahashi, T., and Alitalo, K. (2002). Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J. Natl. Cancer Inst.* 94, 819–825.

Hirosue, S., Kourtis, I.C., van der Vlies, A.J., Hubbell, J.A., and Swartz, M.A. (2010). Antigen delivery to dendritic cells by poly(propylene sulfide) nanoparticles with disulfide conjugated peptides: Cross-presentation and T cell activation. *Vaccine* 28, 7897–7906.

Issa, A., Le, T.X., Shoushtari, A.N., Shields, J.D., and Swartz, M.A. (2009). Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype. *Cancer Res.* 69, 349–357.

Kataru, R.P., Kim, H., Jang, C., Choi, D.K., Koh, B.I., Kim, M., Gollamudi, S., Kim, Y.K., Lee, S.H., and Koh, G.Y. (2011). T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity* 34, 96–107.

Kerjaschki, D., Bago-Horvath, Z., Rudas, M., Sexl, V., Schneckenleithner, C., Wolbank, S., Bartel, G., Krieger, S., Kalt, R., Hantusch, B., et al. (2011). Lipoxigenase mediates invasion of intrametastatic lymphatic vessels and propagates lymph node metastasis of human mammary carcinoma xenografts in mouse. *J. Clin. Invest.* 121, 2000–2012.

Kluger, M.S., and Colegio, O.R. (2011). Lymphangiogenesis linked to VEGF-C from tumor-associated macrophages: accomplices to metastasis by cutaneous squamous cell carcinoma? *J. Invest. Dermatol.* 131, 17–19.

Liao, S., Cheng, G., Conner, D.A., Huang, Y., Kucherlapati, R.S., Munn, L.L., Ruddell, N.H., Jain, R.K., Fukumura, D., and Padera, T.P. (2011). Impaired lymphatic contraction associated with immunosuppression. *Proc. Natl. Acad. Sci. USA* 108, 18784–18789.

Lukacs-Kornek, V., Malhotra, D., Fletcher, A.L., Acton, S.E., Elpek, K.G., Tayaia, P., Collier, A.R., and Turley, S.J. (2011). Regulated release of nitric oxide by

- nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. *Nat. Immunol.* 12, 1096–1104.
- Lund, A.W., and Swartz, M.A. (2010). Role of lymphatic vessels in tumor immunity: passive conduits or active participants? *J. Mammary Gland Biol. Neoplasia* 15, 341–352.
- Mandriota, S.J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D.G., et al. (2001). Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J.* 20, 672–682.
- Miteva, D.O., Rutkowski, J.M., Dixon, J.B., Kilarski, W., Shields, J.D., and Swartz, M.A. (2010). Transmural flow modulates cell and fluid transport functions of lymphatic endothelium. *Circ. Res.* 106, 920–931.
- Munn, D.H., and Mellor, A.L. (2006). The tumor-draining lymph node as an immune-privileged site. *Immunol. Rev.* 213, 146–158.
- Padera, T.P., Kadambi, A., di Tomaso, E., Carreira, C.M., Brown, E.B., Boucher, Y., Choi, N.C., Mathisen, D., Wain, J., Mark, E.J., et al. (2002). Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 296, 1883–1886.
- Peinado, H., Lavotshkin, S., and Lyden, D. (2011). The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin. Cancer Biol.* 21, 139–146.
- Randolph, G.J., Angeli, V., and Swartz, M.A. (2005). Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.* 5, 617–628.
- Reynoso, E.D., Lee, J.W., and Turley, S.J. (2009). Peripheral tolerance induction by lymph node stroma. *Adv. Exp. Med. Biol.* 633, 113–127.
- Roberts, N., Kloos, B., Cassella, M., Podgrabska, S., Persaud, K., Wu, Y., Pytowski, B., and Skobe, M. (2006). Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res.* 66, 2650–2657.
- Schurich, A., Berg, M., Stabenow, D., Böttcher, J., Kern, M., Schild, H.J., Kurts, C., Schuette, V., Burgdorf, S., Diehl, L., et al. (2010). Dynamic regulation of CD8 T cell tolerance induction by liver sinusoidal endothelial cells. *J. Immunol.* 184, 4107–4114.
- Shieh, A.C., Rozansky, H.A., Hinz, B., and Swartz, M.A. (2011). Tumor cell invasion is promoted by interstitial flow-induced matrix priming by stromal fibroblasts. *Cancer Res.* 71, 790–800.
- Shields, J.D., Fleury, M.E., Yong, C., Tomei, A.A., Randolph, G.J., and Swartz, M.A. (2007). Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. *Cancer Cell* 11, 526–538.
- Shields, J.D., Kourtis, I.C., Tomei, A.A., Roberts, J.M., and Swartz, M.A. (2010). Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science* 328, 749–752.
- Sixt, M., Kanazawa, N., Selg, M., Samson, T., Roos, G., Reinhardt, D.P., Pabst, R., Lutz, M.B., and Sorokin, L. (2005). The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* 22, 19–29.
- Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., and Detmar, M. (2001). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat. Med.* 7, 192–198.
- Tammela, T., and Alitalo, K. (2010). Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* 140, 460–476.
- Thaunat, O., Patey, N., Caligiuri, G., Gautreau, C., Mamani-Matsuda, M., Mekki, Y., Dieu-Nosjean, M.C., Eberl, G., Ecochard, R., Michel, J.B., et al. (2010). Chronic rejection triggers the development of an aggressive intra-graft immune response through recapitulation of lymphoid organogenesis. *J. Immunol.* 185, 717–728.
- Thompson, E.D., Enriquez, H.L., Fu, Y.X., and Engelhard, V.H. (2010). Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *J. Exp. Med.* 207, 1791–1804.
- Turley, S.J., Fletcher, A.L., and Elpek, K.G. (2010). The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nat. Rev. Immunol.* 10, 813–825.
- von Oppen, N., Schurich, A., Hegenbarth, S., Stabenow, D., Tolba, R., Weiskirchen, R., Geerts, A., Kolanus, W., Knolle, P., and Diehl, L. (2009). Systemic antigen cross-presented by liver sinusoidal endothelial cells induces liver-specific CD8 T-cell retention and tolerization. *Hepatology* 49, 1664–1672.
- Wong, S.Y., Haack, H., Crowley, D., Barry, M., Bronson, R.T., and Hynes, R.O. (2005). Tumor-secreted vascular endothelial growth factor-C is necessary for prostate cancer lymphangiogenesis, but lymphangiogenesis is unnecessary for lymph node metastasis. *Cancer Res.* 65, 9789–9798.
- Yanai, Y., Furuhashi, T., Kimura, Y., Yamaguchi, K., Yasoshima, T., Mitaka, T., Mochizuki, Y., and Hirata, K. (2001). Vascular endothelial growth factor C promotes human gastric carcinoma lymph node metastasis in mice. *J. Exp. Clin. Cancer Res.* 20, 419–428.
- Zippelius, A., Batard, P., Rubio-Godoy, V., Bioley, G., Liénard, D., Lejeune, F., Rimoldi, D., Guillaume, P.M., Meidenbauer, N., Mackensen, A., et al. (2004). Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Cancer Res.* 64, 2865–2873.