

Resuscitating Cancer Immunosurveillance: Selective Stimulation of DLL1-Notch Signaling in T cells Rescues T-cell Function and Inhibits Tumor Growth

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Abstract

Deficiencies in immune function that accumulate during cancer immunoediting lead to a progressive escape from host immunosurveillance. Therapies that correct or overcome these defects could have a powerful impact on cancer management, but current knowledge of the types and mechanisms of immune escape is still incomplete. Here, we report a novel mechanism of escape from T-cell immunity that is caused by reduction in levels of the Delta family Notch ligands DLL1 and DLL4 in hematopoietic microenvironments. An important mediator of this effect was an elevation in the levels of circulating VEGF. Selective activation of the DLL1-Notch signaling pathway in bone marrow precursors enhanced T-cell activation and inhibited tumor growth. Conversely, tumor growth led to inhibition of Delta family ligand signaling through Notch in the hematopoietic environment, resulting in suppressed T-cell function. Overall, our findings uncover a novel mechanism of tumoral immune escape and suggest that a soluble multivalent form of DLL1 may offer a generalized therapeutic intervention to stimulate T-cell immunity and suppress tumor growth. *Cancer Res*; 71(19); 6122–31. ©2011 AACR.

Introduction

Immunotherapy theoretically can specifically eliminate malignant cells with low toxicity. Unfortunately, although hints of activity have been observed, clinical immunotherapy efforts have proven largely ineffective to date (1, 2). We now recognize that at least part of this failure is due to the numerous immunoregulatory circuits influenced by cancers, which alter the differentiation and function of bone marrow-derived immune cells, including T cells. Both the Notch and VEGF signaling pathways are well known to regulate the function and differentiation of immune cells, but the interplay of these two and the therapeutic implications of this finding in cancer are poorly understood.

In mice, Notch signaling consists of 4 receptors (Notch1–Notch4), 2 serrate-like ligands (Jagged1, Jagged2), and 3 Delta-like ligands (DLL1, DLL3, and DLL4; refs. 3, 4). *DLL4* and *VEGF* are both genes in which loss of a single allele results in embryonic lethality due to failure to form a functional vas-

culature (5, 6). This fact provides genetic evidence for the importance of both VEGF and Notch signaling pathways in vascular development. Many other studies have shown the interplay of Notch and VEGF in angiogenesis (7). VEGF induces DLL4 ligand expression in tumor blood vessels (8, 9), and subsequent DLL4-mediated Notch signaling regulates the differentiation and proliferation of endothelial cells responsive to VEGF and thereby promotes the timely formation of a functional and well-organized vascular network (7).

In addition, Notch and VEGF signaling also plays essential roles in immune cell development and differentiation. Inactivation of Notch1 signaling results in impairment of T lymphopoiesis accompanied by promotion of B-cell development (10, 11). Conversely, retroviral transduction of bone marrow precursors with the Notch1 intracellular domain results in extrathymic T-cell development and a simultaneous block of bone marrow B-cell development (12, 13). Delta-like family ligands also play critical roles in T lymphopoiesis. Overexpression of Delta1 or Delta4 in bone marrow precursors *in vivo*, or expression of DLL1 or DLL4 in stromal cells *in vitro*, leads to induction of T-cell development and suppression of B-cell development (14–19). A recent study further shows that DLL4 is the essential and nonredundant Notch1 ligand responsible for T-cell lineage commitment (20). One important feature of Delta-like family ligands is that they act in a dose/density-dependent manner (5, 18, 21). Higher density of Delta1 promotes T-cell development and inhibits B-cell development, whereas relatively lower density of Delta1 promotes development of both T and B cells (22). The gain and loss of function studies clearly show that Notch signaling is necessary and sufficient for T- versus B-lineage commitment and that both DLL1 and DLL4 can modulate T-cell development (3, 4).

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

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VEGF and its receptors (VEGFR) are also widely expressed in the hematopoietic system and regulate hematopoiesis (6). In tumor-bearing hosts, elevated levels of VEGF affect the differentiation and maturation of immune cells, including T cells, and thus contribute to cancer-associated immunodeficiency (23–26). However, little is known about the interplay between Notch and VEGF signaling pathways in the immune system and specifically in cancer-associated immunosuppression.

Here, we show that tumor growth downregulates expression of DLL1 and DLL4 in the hematopoietic environment and elevated circulating VEGF is one causative factor. Selective stimulation of DLL1-Notch signaling rescues the observed tumor-associated T-cell alterations and dramatically slows tumor growth. Thus, this study suggests the novel concept that stimulation of DLL1-Notch signaling may be an effective strategy to overcome tumor-associated T-cell immunosuppression.

Materials and Methods

Cell lines

The murine Lewis lung carcinoma (LLC) cell line was obtained from the American Type Culture Collection less than 6 months ago. D459 cells are murine fibroblasts malignantly transformed in our laboratory by transfection of *ras* and *p53* (27). Thus, our laboratory is the primary source of these cells, and we regularly go back to reference stocks to ensure fidelity. No other identity testing was done; only routine sterility and mycoplasma testing were done.

Mice

Female Balb/c, C57BL/6, and *Rag1*^{-/-} mice (6- to 8-week-old) were purchased from Harlan and The Jackson Laboratory, respectively. The animals were housed in pathogen-free units at Vanderbilt University School of Medicine, in compliance with Institutional Animal Care and Use Committee regulations. Balb/c mice were inoculated s.c. with D459 (murine fibrosarcoma). C57BL/6 and *Rag1*^{-/-} mice were inoculated s.c. with LLC cells.

Reagents

DC101, a rat-neutralizing monoclonal antibody specifically against mouse VEGFR-2, was a generous gift from ImClone system. The matched control antibody rat IgG was purchased from Sigma-Aldrich. VEGF₁₆₅ and its mutants (KDR-sel and Flt-sel) were generous gifts from Genentech Inc. (28). Osmotic pumps were purchased from ALZET.

VEGF administration

VEGF₁₆₅, KDR-sel (VEGFR2-sel), or Flt-sel (VEGFR1-sel) was delivered into mice via ALZET osmotic pumps as previously described (26) for 28 days at 50 ng/h. Control pumps were filled with PBS. These mice were intraperitoneally injected with rat IgG or DC101 starting 1 day after pump implantation and every 3 days thereafter with a dose of 40 mg/kg (29).

Quantitative RT-PCR in cancer patient samples

We collected de-identified excess archived paraffin-embedded bone marrow samples resected for clinical indications

from lung cancer patients without bone marrow metastases before any treatment (4 squamous cell carcinoma, 3 adenocarcinoma, 1 transitional cell carcinoma, and 1 large cell carcinoma). We used de-identified archived excess bone marrow samples from femurs of age-matched individuals undergoing clinically indicated hip replacement as controls. We extracted RNA from formalin-fixed paraffin-embedded (FFPE) bone marrow samples by FFPE RNA isolation kit (Ambion) and used a set of specific primers described earlier (30) to analyze the transcription of *Delta1* and *Hes1* in bone marrow.

Quantitative RT-PCR in mouse samples

Total RNA was obtained using TRIzol (GIBCO-BRL, Invitrogen Corporation). cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR was done using SYBR Green PCR master mix (Qiagen) on an iCycler iQ system (Bio-Rad Laboratories). The primer sequences were shown in Supplementary Table S1.

Bone marrow transduction and transplantation

Retroviral constructs MigR1-DLL1 and Mig-R1 were generous gifts from Sunnybrook and Women's College Health Science Centre, Toronto, Ontario, Canada (17). Retroviral supernatants were generated using the Bosc23 packaging cell line. Bone marrow cells were infected with retrovirus as previously described (12, 14).

Enzyme-linked immunosorbent spot assay

Mice were inoculated s.c. with D459 tumor cells. Tumor volumes were assessed by bilateral Vernier caliper measurement every 3 days and calculated by the formula $[\text{length} \times (\text{width})^2]/2$. IFN- γ -producing T cells were measured by enzyme-linked immunosorbent spot (ELISPOT) assays according to the manufacturer's instructions (Becton Dickinson). Briefly, splenocytes (2×10^5 per well) were added in triplicate on anti-mouse IFN- γ -precoated 96-well plates and stimulated with anti-CD3 and anti-CD28 at 37°C in a 5% CO₂ humidified incubator overnight. The IFN- γ -secreting T cells were enumerated using a CTL ImmunoSpot analyzer (Cellular Technology Limited) and the supporting ImmunoSpot Software. Spots were counted by an automated system using a defined set of parameters for size, intensity, and gradient.

Soluble clustered DLL1 treatment and CD8⁺ T-cell depletion *in vivo*

DLL1-Fc fusion protein is composed of the extracellular domain of mouse DLL1 and the Fc part of mouse IgG_{2A}. To form clustered DLL1, DLL1-Fc, biotinylated donkey anti-mouse IgG antibodies (all from R&D Systems), and NeutrAvidin (Pierce) were mixed at a molar ratio of 1:4:10 in PBS (50 μ g/mL DLL1-Fc, 335 μ g/mL anti-mouse IgG antibodies, and 335 μ g/mL NeutrAvidin) and incubated at room temperature for 15 to 30 minutes. As a control in all applications, Fc fragment of mouse IgG₂ (Sigma) was used instead of DLL1-Fc. Six- to 8-week-old C57BL/6, *Rag1*^{-/-} mice, and Balb/c mice were inoculated s.c. with 0.5×10^6 LLC cells and 0.3×10^6 D459 tumor cells, respectively. Starting the same day, C57BL/6 mice were treated

with clustered DLL1, receiving 2.4 μg of DLL1-Fc protein in 100 μL of PBS intraperitoneally daily, and Balb/c mice were treated with clustered DLL1, receiving 5 μg of DLL1-Fc protein in 100 μL of PBS intraperitoneally every other day. The control group received control clusters with Fc fragments only. In some groups of mice, purified anti-mouse CD8a antibody (clone 53-6.7; BioLegend) or isotype-matched IgG antibody was administered intraperitoneally at a dose of 100 μg on days -1, 0, 7, 14, and 21 with respect to the day of tumor injection. Tumor volume was measured with calipers.

Western blotting

Proteins were extracted from splenocytes, separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes (Amersham Biosciences). Antibodies to DLL1 or DLL4 (sc-9102 or sc-18641; Santa Cruz Biotechnology) were used. Protein content was normalized using mouse monoclonal anti- β -actin (Sigma) or anti-mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH; sc-25778; Santa Cruz Biotechnology).

Flow cytometric analysis

Fresh single-cell suspensions of red blood cell-depleted splenocytes, bone marrow cells, or thymocytes (1×10^6 cells) were incubated with monoclonal antibodies in the dark on ice for 30 minutes. Flow cytometric data were acquired with a FACSCalibur flow cytometer (Becton Dickinson) and analyzed

by WinList 5.0. Matched fluorochrome-conjugated isotype IgG controls were used in all experiments. Monoclonal antibodies used were CD19-PE, CD3e-FITC, B220/CD45R-PerCP, CD3e-PerCP, CD4-FITC, CD8a-PE, CD3e-biotin, CD4-biotin, CD45-biotin, CD43-PE, streptavidin-PerCP, and streptavidin-APC (Becton Dickinson).

Statistical analysis

Results were presented as mean \pm SEM. Unpaired 2-tailed Student *t* test was used to analyze the difference between 2 groups. Values were considered statistically significant when *P* was less than 0.05.

Results

Tumor-derived factors attenuate the levels of Delta ligands in the bone marrow of cancer patients and tumor-bearing animals

Notch signaling is highly dose- and context-dependent and plays diverse roles in cancer (31). DLL1 and DLL4 are 2 critical Notch ligands involved in T-cell development and tumor angiogenesis (4, 7). We studied the transcriptional levels of *Delta1* (a Notch ligand) and *Hes1* (a Notch target gene) in the bone marrow of cancer patients and found them to be present at reduced levels compared with those from tumor-free donors (Fig. 1A), suggesting that tumors can attenuate Notch signaling in the bone marrow microenvironment. Consistent

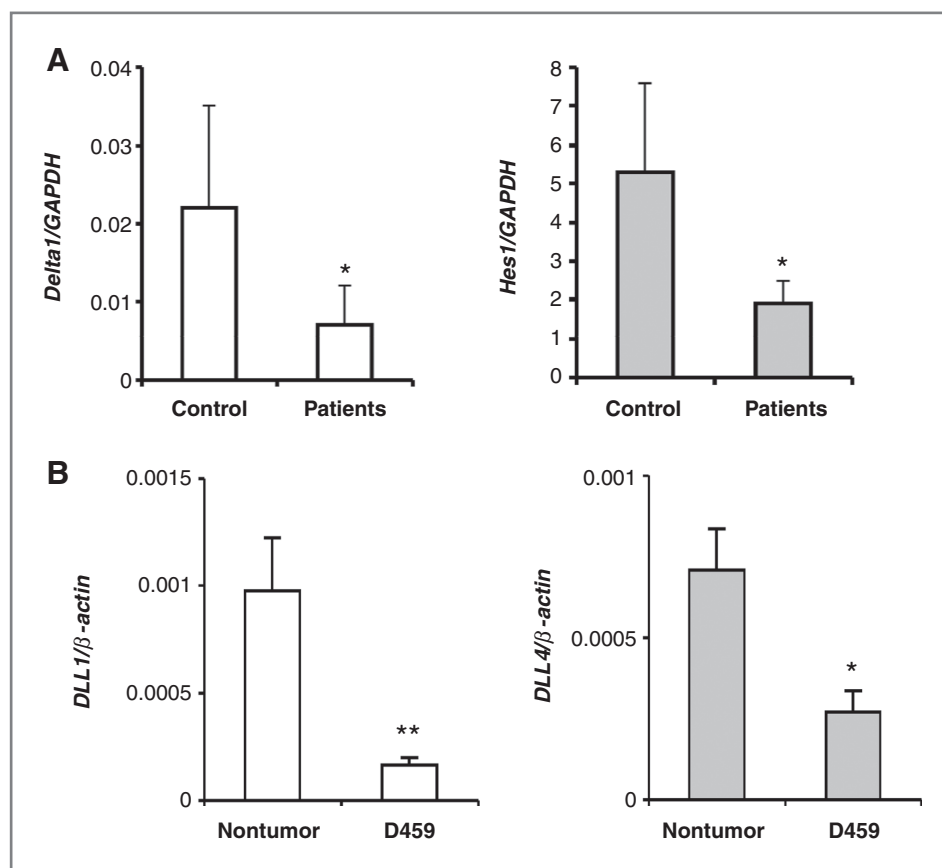


Figure 1. Tumors alter Notch ligand expression in cancer patients and tumor-bearing animals. A, tumors attenuate Notch signaling in the bone marrow of cancer patients. The transcriptional levels of *Delta1* and *Hes1* in bone marrow of lung cancer patients and tumor-free donors were determined by quantitative RT-PCR (*, $P < 0.05$, $n = 9$ patients and 10 control individuals). B, quantitative RT-PCR analysis of *DLL1* and *DLL4* in bone marrow cells from D459 tumor-bearing mice or non-tumor-bearing mice. Data were expressed as a relative ratio of target gene mRNA to housekeeping gene (β -actin) mRNA (*, $P < 0.05$; **, $P < 0.01$, 3 independent experiments). Data are mean \pm SEM.

with this observation, the transcriptional levels of *DLL1* and *DLL4* were also significantly decreased in bone marrow cells from tumor-bearing mice (Fig. 1B). Expression of *DLL1* and *DLL4* protein was also dramatically decreased in splenocytes from tumor-bearing mice (Supplementary Fig. S1). We then sought to determine the functional consequences of these alterations.

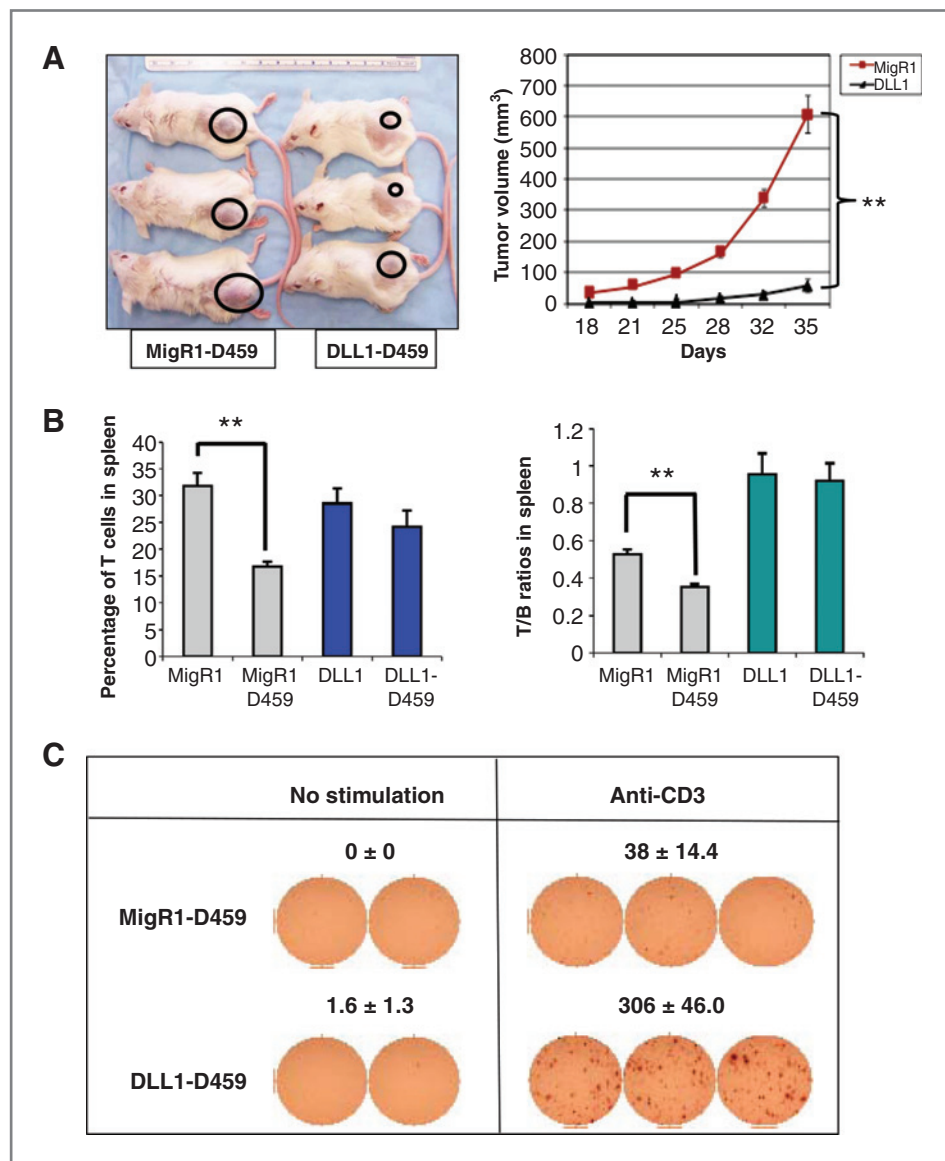
Overexpressing *DLL1* alone in bone marrow precursors significantly improves T-cell immune responses and inhibits tumor growth

To examine the effect of alterations in *DLL1* expression in the hematopoietic environment on tumor growth, we transduced bone marrow precursors with a *DLL1*-carrying retrovirus (Mig-DLL1) or a control retrovirus (MigR1), both of which also express a green fluorescent protein (GFP) marker (Supplementary Fig. S2). The transduced bone marrow

precursors were then transplanted into lethally irradiated recipient mice, which are referred to here as *DLL1*-mice and *MigR1*-mice. Thirteen weeks posttransplantation, we routinely observed 60% to 70% GFP-positive cells in peripheral blood cells, confirming successful transduction and reconstitution (data not shown). We then inoculated *MigR1*-mice or *DLL1*-mice with D459 tumor cells 8 weeks after bone marrow transplantation. We observed a remarkable inhibition of tumor growth in *DLL1*-mice compared with *MigR1*-mice (Fig. 2A). This inhibition was evident as early as day 21, and the tumor size remained nearly unchanged through day 35 (Fig. 2A). These data show that overexpressing *DLL1* alone significantly inhibits tumor growth.

Notch signaling is known to play a critical role in T- versus B-cell fate decisions, and both *DLL1* and *DLL4* can promote T-cell development (3, 4). We have previously shown that tumor-derived factors inhibit T-lymphocyte development and

Figure 2. Overexpressing *DLL1* alone in bone marrow precursors significantly enhances T-cell functions and inhibits tumor growth. *MigR1*-mice and *DLL1*-mice were inoculated with D459 tumor cells and spleens were harvested on day 35 after tumor inoculation. A, overexpressing *DLL1* significantly reduces D459 tumor growth. Left, representative D459 tumor-bearing *MigR1*-mice and *DLL1*-mice. Right, D459 tumor growth curves in *MigR1*-mice and *DLL1*-mice. The circles indicate the D459 tumor growth (**, $P < 0.01$, $n = 12$ mice per group). Y-axis, tumor volume; X-axis, days after tumor inoculation. B, D459 tumor burden reduces the fraction of T cells (left) and the T/B ratios (right) in splenocytes, whereas overexpressing *DLL1* reverses these effects (**, $P < 0.01$, $n = 10$ –12 mice per group). C, overexpressing *DLL1* enhances T cells to produce IFN- γ upon anti-CD3 stimulation (ELISPOT assays). One of 3 representative experiments was done. Numbers ($P < 0.01$) indicate the spots of IFN- γ -producing T cells from 10^5 T (CD3e) cells. Data are mean \pm SEM, $n = 3$.



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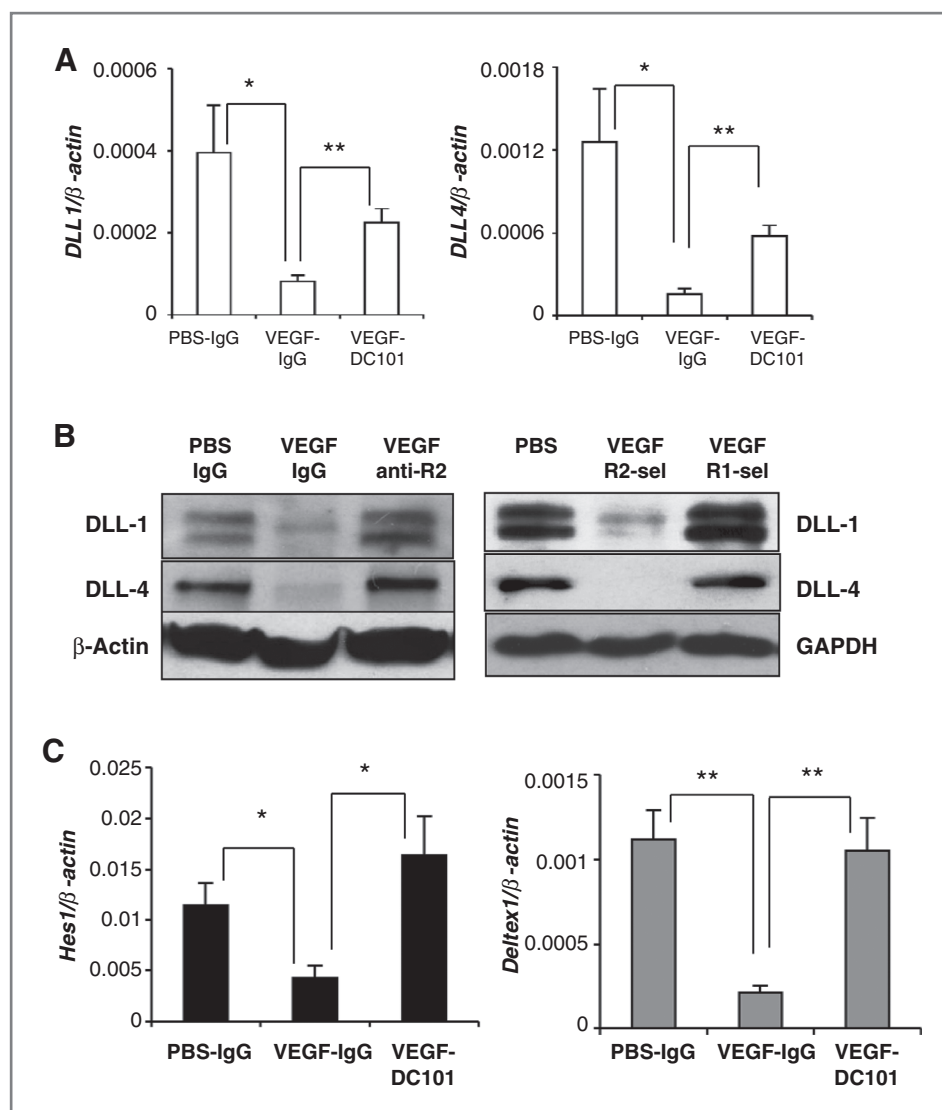


Figure 3. VEGF decreases expression of Notch ligands and suppresses Notch signaling in the bone marrow microenvironment. PBS-IgG, Balb/c mice infused with PBS and then treated with rat IgG. VEGF-IgG, mice infused with VEGF and then treated with rat IgG. VEGF-DC101 or VEGF-anti-R2, mice infused with VEGF and then treated with DC101 (anti-VEGFR-2). A, quantitative RT-PCR analysis of *DLL1* and *DLL4* in bone marrow cells (*, $P < 0.05$; **, $P < 0.01$, 3 independent experiments). B, Western blot analysis of expression of *DLL1*, *DLL4*, β -actin, or GAPDH proteins in splenocytes. PBS, mice infused with PBS; VEGFR2-sel, mice infused with VEGFR2-specific ligand; VEGFR1-sel, mice infused with VEGFR1-specific ligand. Results shown are representative from 3 experiments carried out. C, quantitative RT-PCR analysis of Notch target genes (*Hes1* and *Deltex1*) in bone marrow cells (*, $P < 0.05$; **, $P < 0.01$, 3 independent experiments). The quantitative RT-PCR data were expressed as a relative ratio of target gene mRNA to housekeeping gene (β -actin) mRNA. Data are mean \pm SEM.

function (25, 26); thus, we hypothesized that reduced tumor growth in DLL1-mice could be mediated by enhanced T-cell activity. We observed a significant decrease in the fraction of T cells as well as the T(CD3e)/B(CD19) ratios in the spleens of MigR1-mice inoculated with D459 tumors (Fig. 2B). Conversely, overexpression of DLL1 alone in bone marrow precursors rescued these T-cell alterations, as tumor-bearing DLL1 animals showed no significant decrease in the proportion of splenic T cells and actually a significant increase in splenic T(CD3e)/B(CD19) ratios (Fig. 2B).

Production of IFN- γ is a critical indicator of Th1-type cytotoxic T-cell function that is important for antitumor immune responses. Upon anti-CD3 stimulation, we found that the number of IFN- γ -producing T cells (per 10^5 T cells) was dramatically increased in DLL1-mice bearing D459 tumors compared with MigR1-mice bearing D459 tumors (Fig. 2C). We also observed more tumor-infiltrating T cells in DLL1-mice than in MigR1-mice (data not shown). Importantly,

these infiltrating T cells (and the peripheral blood T-cell fractions) had the same fractional GFP expression as did bulk circulating nucleated cells, suggesting that this was not a cell-autonomous effect (requiring DLL1 expression in the T cells themselves) but rather a consequence of increased expression in the hematopoietic environment in which they develop (data not shown). Together, our data suggest that overexpressing DLL1 alone significantly enhances T-cell immune responses and inhibits tumor growth.

Elevated circulating VEGF inhibits T-cell development by downregulating the levels of DLL1 and DLL4 in the hematopoietic environment

Higher levels of serum VEGF have been reported in patients with many types of cancers, such as non-small cell lung cancer (32). In animal models, circulating VEGF has been found to be derived not only from tumor cells but also from stromal cells, such as immune cells and endothelial cells (33, 34).

We have previously shown that elevated circulating VEGF alters host hematopoiesis including the inhibition of T lymphopoiesis (26). We therefore hypothesized that VEGF is one of the mediators to inhibit the expression of DLL1 and DLL4 in tumor-bearing hosts. As we observed in tumor-bearing mice, we found that transcriptional levels of *DLL1* and *DLL4* in bone marrow cells of non-tumor-bearing mice were significantly decreased in mice infused with recombinant VEGF at levels observed in tumor-bearing mice (Fig. 3A). Simultaneous administration of a VEGFR-2-specific antibody (DC101) reversed this effect (Fig. 3A). Expression of DLL1 and DLL4 protein in splenocytes was also dramatically decreased in VEGF-infused mice as was seen in tumor-bearing animals, and again DC101 treatment reversed this effect (Fig. 3B). Similarly, a VEGFR-2 but not a VEGFR-1-specific ligand dramatically decreased expression of DLL1 and DLL4 proteins in the spleen (Fig. 3B). Together, these data indicate that VEGFR-2 signaling decreases the levels of DLL1 and DLL4 in the hematopoietic environment, consistent with the effects observed in tumor-bearing mice.

In experiments designed to determine whether lower levels of DLL1 and DLL4 in the bone marrow microenvironment altered Notch signaling, we found that transcriptional levels of *Hes1* and *Deltex1* (Notch target genes) were significantly decreased in VEGF-infused mice compared with those of PBS-infused mice (Fig. 3C) and DC101 treatment totally reversed this effect. We then implanted osmotic pumps containing recombinant VEGF or PBS diluent into MigR1-mice and DLL1-mice. The T-cell data from spleen and thymus indicate that overexpressing DLL1 alone overcomes the inhibition of VEGF on T-cell development (Fig. 4). Collectively, our data show that elevated circulating VEGF inhibits T-cell development by downregulating the levels of DLL1 and DLL4 in the hematopoietic environment.

T cells contribute to the tumor growth inhibition induced by selectively activated DLL1-Notch signaling

We also developed a novel reagent we refer to as clustered DLL1, which is a complex of DLL1-Fc, anti-Fc antibody, and avidin. Activation of Notch receptor proteolytic cleavage and signaling requires a multivalent interaction between Notch-expressing cells and Notch ligands. Whereas soluble forms of Notch ligands including DLL1 act as inhibitors of Notch signaling, soluble clustered DLL1 acts as an activator of Notch receptors (ref. 27; Fig. 5A and Supplementary Fig. S3A). To determine its effect on tumor growth *in vivo*, we treated D459 tumor-bearing mice with soluble clustered DLL1 or anti-Fc antibody-avidin complex as control. Like the DLL1 overexpression data shown in Fig. 2A, we observed the inhibition of tumor growth as early as day 20 and the tumor growth rate remained much slower through day 30 in the soluble clustered DLL1 treatment mice than in the control group (Fig. 5B). A similar effect was observed in a different tumor model of Lewis lung carcinoma in a different strain of mice (C57BL/6), showing that this effect is not model specific (Fig. 5C). Together, our data show that soluble clustered DLL1 treatment dramatically inhibits tumor growth.

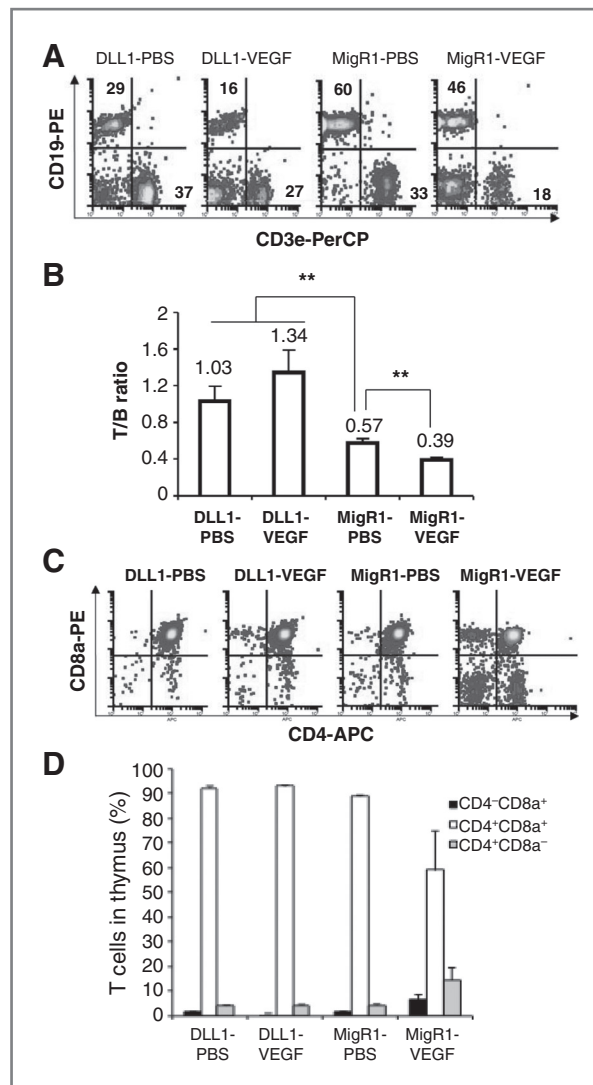


Figure 4. Overexpressing DLL1 alone in bone marrow precursors prevents the suppression of VEGF on T-cell development. MigR1-mice or DLL1-mice were given a continuous infusion of PBS or of rhVEGF (100 ng/h) over a period of 28 days. A, flow cytometric analysis of splenocytes expressed CD3e and CD19. The representative plots are shown from 4 experiments carried out. Numbers in quadrants indicate the average percentage of cells in the respective population. B, the averages of T/B ratios in spleen, which were calculated by dividing the fraction of CD3e by the fraction of CD19 (mean \pm SEM, **, $P < 0.01$, $n = 3-4$, 4 independent experiments). C, flow cytometric analysis of thymocytes expressed CD4 and CD8a in CD3e^{low/-} cells. The representative plots are shown from 3 experiments carried out. D, the percentage of the T-cell subtypes in thymus (mean \pm SEM, $n \geq 3$).

We then analyzed the effects of soluble clustered DLL1 treatment on T-cell function. As observed for the DLL1 vector transduction in Fig. 2B and C, soluble clustered DLL1 treatment increases the transcriptional levels of *Hes1* and *Deltex1* (Notch target genes) in the bone marrow (Fig. 6A), prevents the decrease in the T-cell fraction in tumor-bearing animals (Supplementary Fig. S3B), and significantly improves T-cell

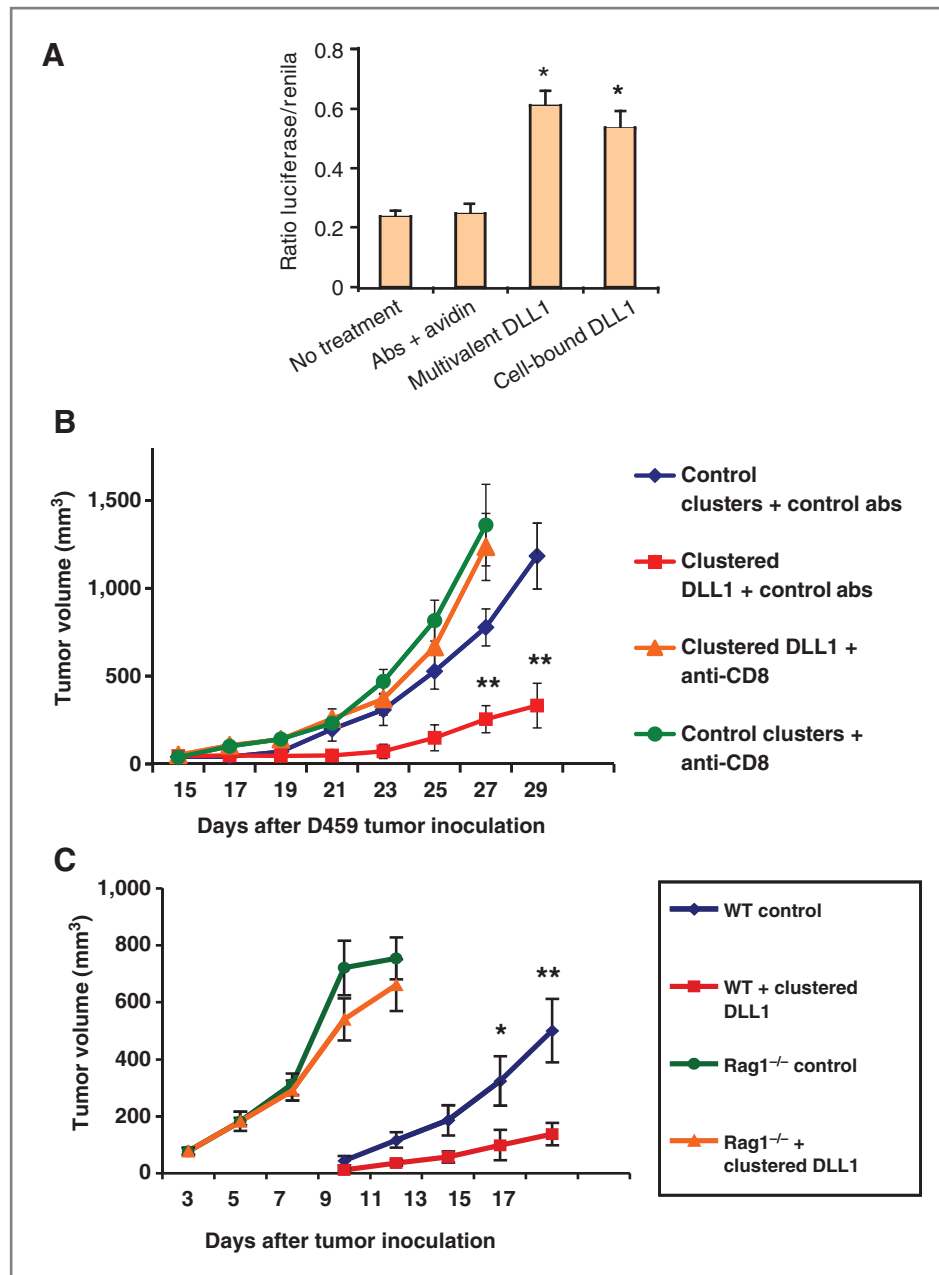


Figure 5. Soluble clustered DLL1 activates Notch signaling *in vitro* and significantly inhibits tumor growth *in vivo* in a T-cell-dependent manner. A, NIH3T3 cells were transiently cotransfected with *Notch1*, *Hes1*-luciferase reporter, and *Renilla*-luciferase expression constructs. Cells were treated as indicated for 24 hours, and luciferase assay was conducted using a dual luciferase assay with *Renilla* luciferase used as an internal control. In "Cell-bound DLL1," DLL1 expression construct was also included in cotransfection without further treatments. Data are mean \pm SEM, $n = 3$; *, $P < 0.05$, unpaired t test. Abs, antibodies. B, soluble clustered DLL1 inhibits D459 tumor growth in a T-cell-dependent manner. Note that tumor inhibitory effect of clustered DLL1 is lost in mice that received anti-CD8 antibody ($n = 5$ –10 mice per group; **, $P < 0.01$). Data are mean \pm SEM. C, soluble clustered DLL1 attenuates LLC tumor growth in C57BL/6 mice but not in *Rag1*^{-/-} mice ($n = 5$ mice per group; *, $P < 0.05$; **, $P < 0.01$). WT, wild type.

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IFN- γ production (Fig. 6B). This suggests that DLL1 treatment improves T-cell immune responses.

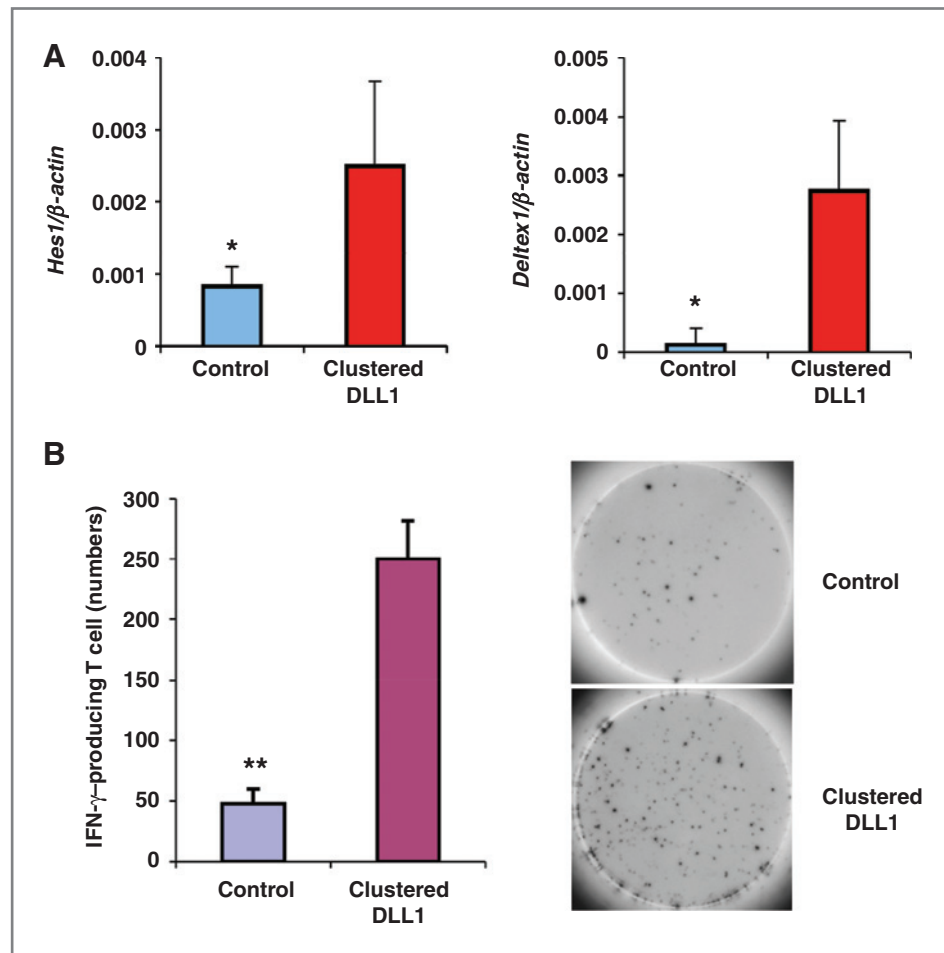
To test the contribution of T cells in tumor growth inhibition (Figs. 2A and 5), we carried out 2 kinds of experiments in 2 different tumor models (Fig. 5). First, we implanted LLC in both immunocompetent C57BL/6 mice and immunodeficient *Rag1*^{-/-} mice. Then, we treated these LLC tumor-bearing mice with soluble clustered DLL1 or anti-Fc antibody-avidin complex as control. Soluble clustered DLL1 treatment significantly inhibited tumor growth in C57BL/6 mice but did not affect LLC tumor growth in the immunodeficient syngeneic *Rag1*^{-/-} mice (Fig. 5C). We also treated wild-type mice

bearing D459 tumor with clustered DLL1-Fc together with anti-CD8 antibody. CD8 T-cell depletion in mice abrogated tumor growth inhibition induced by soluble clustered DLL1 treatment (Fig 5B). Together, our data suggest that CD8 T cells contribute to tumor growth inhibition induced by soluble clustered DLL1 treatment.

Discussion

Compelling evidence suggests that immune cells play an important role in the control of malignant tumor growth (35, 36). However, tumors circumvent this control by

Figure 6. Treatment with soluble clustered DLL1 stimulates Notch signaling in bone marrow cells and improves T-cell immune response. **A**, soluble clustered DLL1 treatment stimulates Notch signaling in bone marrow cells (*, $P < 0.05$, $n = 3$). Data are mean \pm SEM. **B**, soluble clustered DLL1 treatment enhances IFN- γ production of T cells responding to anti-CD3 and anti-CD28 stimulation (**, $P < 0.01$, $n = 3$). Splensens were harvested on day 29 after D459 tumor inoculation. Numbers indicate the spots of IFN- γ -producing T cells from 10^5 T (CD3e) cells.



interfering with the induction and function of these immune responses, both systemically and in the tumor micro-environment. Thus, a major challenge for cancer immunotherapy is to use advances in cellular and molecular immunology to develop strategies that effectively and safely augment antitumor immune responses (37).

In this study, we describe a novel observation that tumors can systemically suppress host T-cell development and function by decreased levels of DLL1 and DLL4 in the hematopoietic environments. Selective stimulation of DLL1-Notch signaling enhances host tumor immunosurveillance and dramatically inhibits tumor growth. Importantly, we developed a potential therapeutic strategy using soluble clustered DLL1 ligand, which selectively stimulates DLL1-Notch signaling, augments T-cell function, and inhibits tumor growth.

To evaluate the roles and elements of the immune system in the inhibition of tumor growth responding to DLL1-Notch signaling activation, we showed that soluble clustered DLL1 treatment inhibited LLC tumor growth in wild-type mice but not in syngeneic C57BL/6 *Rag1*^{-/-} mice. Both T and B cells are deficient in *Rag1*^{-/-} mice, and B cells have been reported to have protumor effects (38). Although B cells may be reduced upon DLL1 stimulation, we did not address the role of B cells in our study. In contrast, our data showed that depletion of

CD8⁺ T cells *in vivo* abrogated tumor growth inhibition induced by soluble clustered DLL1.

In non-tumor-bearing hosts, it is well established that Notch signaling plays essential roles in immune cell development and differentiation (3, 4). There are 2 functional classes of Notch ligands in mammals: the Jagged family and Delta-like family. There are different functional consequences of Notch signaling stimulated by Jagged family members and stimulated by Delta-like family members (39–41), but the exact function of each Notch ligand in T lymphopoiesis is still not fully understood. Both *in vitro* and *in vivo* experiments suggest that the functions of Jagged1 and Jagged2 do not seem to play an important role in lymphopoiesis (39, 42, 43).

On the contrary, there are abundant data supporting a specific role for the Delta family ligands in T lymphopoiesis. Stromal cell overexpression of DLL1 or DLL4 is sufficient to induce T-cell development and inhibit B-cell development *in vitro*, but stromal cells expressing Jagged1 do not (16, 17, 39). Consistent with these observations, our data showed that overexpressing DLL1 alone in bone marrow precursors promotes T-cell development while suppressing B-cell development (Fig. 4). The effects may be different depending on signal intensity as well, as relatively lower densities of Delta1^{ext-IgG} enhanced the generation of both T and B precursor cells and

higher densities of Delta1^{ext-IgG} prompted the development of T precursor cells but inhibited B precursor cell development (22).

Pharmacologic inhibitors of Notch signaling have similar effects to those we have observed with tumors and systemic VEGF infusion. Inhibition of Notch signaling by increasing concentrations of a presenilin1/2 inhibitor converted T-cell development to B-cell development from hematopoietic progenitor cells cultured on OP9-DLL1 (44). Reduced Notch signaling *in vivo* by long-term treatment with a γ -secretase inhibitor also caused thymic atrophy and decreased total thymocytes as well as T-cell subtypes in thymus (45).

All of these results indicate that stronger Notch signaling by Delta family ligands induces T-cell development and inhibits B-cell development, whereas reduction in Notch signaling inhibits T-cell development and favors B-cell differentiation. This is consistent with a causative relationship that we find in this study between the inhibition of T-cell development and decreased Notch signaling in tumor-bearing animals and VEGF-infused mice.

In contrast to the induction of DLL4 in tumor vasculature (9), we observed a striking reduction of DLL1 and DLL4 in the hematopoietic environment (Fig. 1 and Supplementary Fig. S1). The difference is certainly due to the different cell types and tissues studied—local effects on vascular endothelial cells versus systemic effects on the hematopoietic environments we studied here. Dynamic, temporally, and spatially regulated expression of the mouse *DLL1* gene in distinct tissues and cell types is frequently observed in embryonic development (46).

Although DLL4 shows substantial homology with DLL1, others also have shown that they have distinct functions and cannot completely substitute for each other (47). Heterozygous *DLL4*, but not *DLL1*, knockouts have a vascular embryonic lethal phenotype (5). Either DLL1 or DLL4 alone is sufficient to support T-cell development, but overexpression of DLL4, not DLL1, results in T-cell leukemia (14–17). Consistent with these reports, we did not observe ectopic T-cell

development and T-cell leukemia in these DLL1-overexpressing mouse models during our experimental period. DLL4 thus potentially affects both vascular development and T-cell development, but DLL1 appears more selectively important for T-cell maturation. Currently, we have not dissected which Notch receptor mediates the effect of DLL1 in our models. Notch1 may mediate the restoration of T-cell development (12, 13), and Notch2 seems to mediate the improvement of T-cell function (48).

Tumors suppress expression of both DLL1 and DLL4 in the bone marrow and spleen and induce DLL4 in tumor-adjacent endothelial cells (7), reflecting the distinct and regional immunologic and vascular effects of Notch signaling. Our data suggest that selectively promoting DLL1-mediated signaling reverses T-cell-mediated immunosuppression while avoiding the promotion of tumor angiogenesis proposed for DLL4.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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