Published in final edited form as:

Cancer Res. 2011 October 1; 71(19): 6122–6131. doi:10.1158/0008-5472.CAN-10-4366.

Resuscitating cancer immunosurveillance: selective stimulation of DLL1-Notch signaling in T cells rescues T cell function and inhibits tumor growth

Yuhui Huang¹, Luping Lin¹, Anil Shanker^{1,2}, Anshu Malhotra², Li Yang¹, Mikhail M. Dikov¹, and David P. Carbone¹

¹Department of Cancer Biology, Vanderbilt University, Nashville, TN 37232, USA

²Laboratory of Lymphocyte Function, Department of Biochemistry and Cancer Biology, School of Medicine, Meharry Medical College, Nashville, TN 37208 USA

Abstract

Deficiencies in immune function that accumulate during cancer immunoediting lead to a progressive escape from host immune surveillance. 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 are 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 vascular endothelial growth factor (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.

Keywords

Notch; DLL1; DLL4; VEGF; immunosuppression

Introduction

Immunotherapy is theoretically able to specifically eliminate malignant cells with low toxicity. Unfortunately while 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 immune-regulatory 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.

Corresponding author: David P. Carbone, MD PhD, 685 PRB, Vanderbilt-Ingram Cancer Center, 2200 Pierce Avenue, Nashville, TN, 37232-6838, USA, Tel. 615-936-3321, Fax 615-936-3322, d.carbone@vanderbilt.edu.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

In mice, Notch signaling consists four receptors (Notch1-Notch4), two Serrate-like ligands (Jagged1, Jagged2), and three Delta-like ligands (DLL1, DLL3 and DLL4) (3, 4). DLL4 and VEGF are both genes where loss of a single allele results in embryonic lethality due to failure to form a functional vasculature (5, 6). This fact provides genetic evidence for the importance of both VEGF and Notch signaling in vascular development. Many other studies have demonstrated 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 play 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 non-redundant 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, while relatively lower density of Delta1 promotes both T and B cell development (22). The gain and loss of function studies clearly demonstrate that Notch signaling is necessary and sufficient for T- versus B-lineage commitment and that both DLL1 and DLL4 are able to modulate T cell development (3, 4).

VEGF and its receptors 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 down-regulates 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

Lewis Lung Carcinoma (LLC) cell lines were obtained from the American Type Culture Collection (Manassas, VA) less than 6 months ago. D459 cells are murine fibroblasts malignantly transformed in our lab by transfection of ras and p53 (34). Thus our lab 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.

Mice

Female Balb/c, C57BL/6 and RAG1^{-/-} mice (6–8 week old) were purchased from Harlan (Indianapolis, IN) and the Jackson Laboratory (Bar Harbor, ME), 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 (murine Lewis lung carcinoma cell line) 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 (27). 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 phosphate-buffered saline (PBS). Those mice were treated by intraperitoneal injection of rat IgG or DC101 starting 1 day after pump implantation and every 3 days thereafter with dose of 40 mg/Kg (28).

Quantitative RT-PCR in cancer patient samples

We collected de-identified excess archived paraffin-embedded BM 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 bone marrow samples by FFPE RNA isolation kit (Ambion) and used a set of specific primers described earlier (29) to analyze the transcription of Delta1 and Hes1 in BM.

Quantitative RT-PCR in mouse samples

Total RNA was obtained using TRIzol (GIBCO-BRL, Invitrogen Corp.). cDNA was synthesized using SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time 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 1.

Bone marrow transduction and transplantation

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

ELISPOT 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-gamma-producing T cells were measured by 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-gamma 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, Rockford, IL) 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 15–30 min. As a control in all applications, Fc fragment of mouse IgG₂ (Sigma, St. Louis, MO) was used instead of DLL1-Fc. 6- to 8-weeks old C57BL/6, RAG1^{-/-} mice and Balb/c mice were inoculated subcutaneously with 0.5 $\times$ 10⁶ Lewis Lung Carcinoma (LLC) cells and 0.3 $\times$ 10⁶ D459 tumor cells, respectively. Starting the same day, C57BL/6 mice were treated with clustered DLL1 receiving 2.4 μ g of DLL1-Fc protein in 100 μ l of PBS intraperitoneally daily, and Balb/c mice were treated with clustered DLL1 receiving 5 μ g of DLL1-Fc protein in 100 μ l of PBS intraperitoneally every other day. Control group received control clusters with Fc fragments only. In some groups of mice, purified anti-mouse CD8a antibody (clone 53–6.7, BioLegend, San Diego, CA) or isotype-matched IgG antibody was administered intraperitoneally at a dose of 100 μ g on days – 1, 0, 7, 14, and 21 with respect to the day of tumor injection. Tumor volume was measured using calipers.

Western Blotting

Proteins were extracted from splenocytes, separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Antibodies to Delta-like 1 or Delta-like 4 (sc-9102 or sc-18641, Santa Cruz Biotechnology) were used. Protein content was normalized using mouse monoclonal anti- β -actin (Sigma) or anti-mouse GAPDH (sc-25778, Santa Cruz Biotechnology).

Flow cytometric (FCM) analysis

Fresh single cell suspensions of RBC-depleted splenocytes, BM cells or thymocytes (1 $\times$ 10⁶ cells) were incubated with mAbs in the dark on ice for 30 min. FCM data were acquired using a FACSCalibur™ (Becton Dickinson) and analyzed by WinList 5.0. Matched fluorochrome-conjugated isotype IgG controls were used in all experiments. Monoclonal antibodies used: 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 $\pm$ s.e.m (standard error of the mean). Unpaired two-tailed student's t-test was used to analyze the difference between two groups. Values were considered statistically significant when *P* values were less than 0.05.

Results

Tumor-derived factors attenuate DLL1 and DLL4 levels in the BM of cancer patients and tumor-bearing animals

Notch signaling is highly dose- and context-dependent and plays diverse roles in cancer (30). DLL1 and DLL4 are two 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 (BM) of cancer patients and found them to be present at reduced levels compared to those from tumor-free donors (Fig. 1A), suggesting that tumors can attenuate Notch signaling in the BM microenvironment. Consistent 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. 1). We then sought to determine the functional consequences of these alterations.

Over-expressing DLL1 alone in BM 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 BM precursors with a DLL1-carrying-retrovirus (Mig-DLL1), or a control retrovirus (MigR1) both of which also express a GFP marker (Supplementary Fig. 2). The transduced BM precursors were then transplanted into lethally irradiated recipient mice, which are referred to here as DLL1-mice and MigR1-mice. Thirteen weeks post-transplantation, 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 BM 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 over-expressing 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 are able to promote T cell development (3, 4). We have previously shown that tumor-derived factors inhibit T lymphocyte development and 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 of 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, over-expression of DLL1 alone in BM 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 anti-tumor 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 over-expressing 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 no-small-cell lung cancer (31). In animal models, circulating VEGF has been found to be derived from not only tumor cells, but also stromal cells, such as immune cells and

endothelial cells (32, 33). 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 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 BM 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 protein 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 to the effects observed in tumor-bearing mice.

In experiments designed to determine whether lower levels of DLL1 and DLL4 in the BM microenvironment altered Notch signaling, we found that transcriptional levels of Hes1 and Deltex1 (Notch target genes) were significantly decreased in VEGF-infused mice compared to 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 (34) (Fig 5A and Supplementary Fig. 3A). 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 over-expression data shown in Figure 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, compared with control group (Fig. 5B). A similar effect was observed in a different tumor model of Lewis Lung Carcinoma (LLC) in a different strain of mice (C57BL/6), demonstrating that this effect is not model-specific (Fig. 5C). Together, our data show that soluble clustered DLL1 treatment dramatically inhibits tumor growth.

We then analyzed the effects of soluble clustered DLL1 treatment on T cell function. As observed for the DLL1 vector transduction in Figures 2B and 2C, soluble clustered DLL1 treatment increases the transcriptional levels of Hes1 and Deltex1 (Notch target genes) in the BM (Fig. 6A), prevents the decrease in the T cell fraction in tumor-bearing animals (Supplementary Fig. 3B) and significantly improves T cell 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 (Fig. 2A and 5), we performed two kinds of experiments in two different tumor models (Fig. 5). First, we implanted LLC in both immune competent C57BL/6 mice and immune deficient RAG1^{-/-} mice. Then, we treated those 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 didn't affect LLC tumor growth in the immune deficient syngeneic RAG1^{-/-} mice (Fig. 5C). We also treated WT 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 interfering with the induction and function of these immune responses, both systemically and in the tumor microenvironment. 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 immune surveillance 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 WT but not syngeneic C57BL/6 Rag1^{-/-} mice. Both T and B cells are deficient in Rag1^{-/-} mice, and B cells have been reported to have pro-tumor effects (38). Although B cells may be reduced upon DLL1 stimulation, we did not address the role of B cells in our study. By contrast, our data showed that depletion of CD8⁺ T cell *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 two 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 over-expressing DLL1 alone in BM 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).

Pharmacological 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 chronic treatment with a γ -secretase inhibitor also caused thymic atrophy, 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. 1). The difference is certainly due to the different cell types and tissues studied – local effects on vascular endothelial cells vs. 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).

While 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 over-expression DLL4, not DLL1, results in T cell leukemia (14–17). Consistent with these reports, we didn't observe ectopic T cell development and T cell leukemia in these DLL1 over-expressing 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 haven't 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank, Eugene Oltz, Pierre Massion and Claudio Mosse for the critical reading and suggestions, Juan Carlos Zúñiga-Pflücker (University of Toronto) for providing MigR1 and Mig-DLL1 constructs, Warren S. Pear (University of Pennsylvania) and Xiaolan Chen for their technical assistances in bone marrow transplantation. We would also like to thank ImClone system for providing the DC101 antibody, and Genentech Inc. for providing rhVEGF and receptor-specific VEGF mutants.

Grant support: This work was supported by grants CA76321 (DPC), CA138923 (MMD), and CA100562 (MMD) from the Vanderbilt Lung Cancer SPORE CA090949.

References

1. Peggs KS, Segal NH, Allison JP. Targeting immunosuppressive cancer therapies: accentuate the positive, eliminate the negative. *Cancer Cell*. 2007; 12:192–9. [PubMed: 17785201]

2. Dougan M, Dranoff G. Immune therapy for cancer. *Annu Rev Immunol.* 2009; 27:83–117. [PubMed: 19007331]
3. Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. *Immunity.* 32:14–27. [PubMed: 20152168]
4. Maillard I, Fang T, Pear WS. Regulation of lymphoid development, differentiation, and function by the notch pathway. *Annu Rev Immunol.* 2005; 23:945–74. [PubMed: 15771590]
5. Gale NW, Dominguez MG, Noguera I, et al. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A.* 2004; 101:15949–54. [PubMed: 15520367]
6. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.* 1996; 380:439–42. [PubMed: 8602242]
7. Thurston G, Noguera-Troise I, Yancopoulos GD. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer.* 2007; 7:327–31. [PubMed: 17457300]
8. Patel NS, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL. Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res.* 2005; 65:8690–7. [PubMed: 16204037]
9. Ridgway J, Zhang G, Wu Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature.* 2006; 444:1083–7. [PubMed: 17183323]
10. Radtke F, Wilson A, Stark G, et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity.* 1999; 10:547–58. [PubMed: 10367900]
11. Wilson A, MacDonald HR, Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med.* 2001; 194:1003–12. [PubMed: 11581321]
12. Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med.* 1996; 183:2283–91. [PubMed: 8642337]
13. Pui JC, Allman D, Xu L, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity.* 1999; 11:299–308. [PubMed: 10514008]
14. Yan XQ, Sarmiento U, Sun Y, et al. A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. *Blood.* 2001; 98:3793–9. [PubMed: 11739188]
15. de La Coste A, Six E, Fazilleau N, et al. In vivo and in absence of a thymus, the enforced expression of the Notch ligands delta-1 or delta-4 promotes T cell development with specific unique effects. *J Immunol.* 2005; 174:2730–7. [PubMed: 15728481]
16. Hozumi K, Negishi N, Suzuki D, et al. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat Immunol.* 2004; 5:638–44. [PubMed: 15146182]
17. Schmitt TM, Zuniga-Pflucker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity.* 2002; 17:749–56. [PubMed: 12479821]
18. Delaney C, Varnum-Finney B, Aoyama K, Brashem-Stein C, Bernstein ID. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood.* 2005; 106:2693–9. [PubMed: 15976178]
19. Dorsch M, Zheng G, Yowe D, et al. Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood.* 2002; 100:2046–55. [PubMed: 12200365]
20. Koch U, Fiorini E, Benedito R, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J Exp Med.* 2008; 205:2515–23. [PubMed: 18824585]
21. Duarte A, Hirashima M, Benedito R, et al. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev.* 2004; 18:2474–8. [PubMed: 15466159]
22. Dallas MH, Varnum-Finney B, Delaney C, Kato K, Bernstein ID. Density of the Notch ligand Delta1 determines generation of B and T cell precursors from hematopoietic stem cells. *J Exp Med.* 2005; 201:1361–6. [PubMed: 15851488]
23. Adkins B, Charyulu V, Sun QL, Lobo D, Lopez DM. Early block in maturation is associated with thymic involution in mammary tumor-bearing mice. *J Immunol.* 2000; 164:5635–40. [PubMed: 10820238]

24. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med*. 1996; 2:1096–103. [PubMed: 8837607]
25. Ohm JE, Gabrilovich DI, Sempowski GD, et al. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood*. 2003; 101:4878–86. [PubMed: 12586633]
26. Huang Y, Chen X, Dikov MM, et al. Distinct roles of VEGFR-1 and -2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood*. 2007
27. Li B, Fuh G, Meng G, et al. Receptor-selective variants of human vascular endothelial growth factor. Generation and characterization. *J Biol Chem*. 2000; 275:29823–8. [PubMed: 10878003]
28. Witte L, Hicklin DJ, Zhu Z, et al. Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy. *Cancer Metastasis Rev*. 1998; 17:155–61. [PubMed: 9770111]
29. Konishi J, Kawaguchi KS, Vo H, et al. Gamma-secretase inhibitor prevents Notch3 activation and reduces proliferation in human lung cancers. *Cancer Res*. 2007; 67:8051–7. [PubMed: 17804716]
30. Roy M, Pear WS, Aster JC. The multifaceted role of Notch in cancer. *Curr Opin Genet Dev*. 2007; 17:52–9. [PubMed: 17178457]
31. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev*. 1992; 13:18–32. [PubMed: 1372863]
32. Liang WC, Wu X, Peale FV, et al. Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. *J Biol Chem*. 2006; 281:951–61. [PubMed: 16278208]
33. Poon RT, Fan ST, Wong J. Clinical implications of circulating angiogenic factors in cancer patients. *J Clin Oncol*. 2001; 19:1207–25. [PubMed: 11181687]
34. Heinzel K, Benz C, Martins VC, Haidl ID, Bleul CC. Bone marrow-derived hemopoietic precursors commit to the T cell lineage only after arrival in the thymic microenvironment. *J Immunol*. 2007; 178:858–68. [PubMed: 17202347]
35. Hamzah J, Jugold M, Kiessling F, et al. Vascular normalization in Rgs5-deficient tumours promotes immune destruction. *Nature*. 2008; 453:410–4. [PubMed: 18418378]
36. DeNardo DG, Brennan DJ, Rexhepaj E, et al. Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discovery*. 2011
37. Blattman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. *Science*. 2004; 305:200–5. [PubMed: 15247469]
38. Andreu P, Johansson M, Affara NI, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell*. 2010; 17:121–34. [PubMed: 20138013]
39. Jaleco AC, Neves H, Hooijberg E, et al. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med*. 2001; 194:991–1002. [PubMed: 11581320]
40. de La Coste A, Freitas AA. Notch signaling: Distinct ligands induce specific signals during lymphocyte development and maturation. *Immunol Lett*. 2006; 102:1–9. [PubMed: 16140393]
41. Lehar SM, Dooley J, Farr AG, Bevan MJ. Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood*. 2005; 105:1440–7. [PubMed: 15486060]
42. Mancini SJ, Mantei N, Dumortier A, Suter U, Macdonald HR, Radtke F. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood*. 2005; 105:2340–2. [PubMed: 15550486]
43. Jiang R, Lan Y, Chapman HD, et al. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev*. 1998; 12:1046–57. [PubMed: 9531541]
44. Schmitt TM, Ciofani M, Petrie HT, Zuniga-Pflucker JC. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med*. 2004; 200:469–79. [PubMed: 15314075]
45. Wong GT, Manfra D, Poulet FM, et al. Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem*. 2004; 279:12876–82. [PubMed: 14709552]

46. Beckers J, Caron A, Hrabe de Angelis M, Hans S, Campos-Ortega JA, Gossler A. Distinct regulatory elements direct delta1 expression in the nervous system and paraxial mesoderm of transgenic mice. *Mech Dev.* 2000; 95:23–34. [PubMed: 10906447]
47. Shutter JR, Scully S, Fan W, et al. Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.* 2000; 14:1313–8. [PubMed: 10837024]
48. Sugimoto K, Maekawa Y, Kitamura A, et al. Notch2 signaling is required for potent antitumor immunity in vivo. *J Immunol.* 184:4673–8. [PubMed: 20351182]

A **B**

Figure 1. Tumors alter Notch ligand expression in cancer patients and tumor-bearing animals
A, Tumors attenuate Notch signaling in the BM of cancer patients. The transcriptional levels of Delta1 and Hes1 in BM 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 BM cells from D459 tumor-bearing mice or non-tumor-bearing mice. The data were expressed as a relative ratio of target gene mRNA to housekeeping gene (β -actin) mRNA (* $P < 0.05$; ** $P < 0.01$, three independent experiments). Data are mean $\pm$ s.e.m.

Figure 2. Over-expressing DLL1 alone in BM 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 post tumor inoculation. **A**, Over-expressing 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, while over-expressing DLL1 reverses these effects (** $P < 0.01$, $n = 10-12$ mice per group). **C**, Over-expressing DLL1 enhances T cells to produce IFN- γ upon anti-CD3 stimulation (ELISPOT assays). One representative experiment is shown from three performed. Numbers ($p < 0.01$) indicate the spots of IFN- γ producing T cells from 10^5 T (CD3e) cells. Data are mean $\pm$ s.e.m, $n = 3$.

Figure 3. VEGF decreases expression of Notch ligands and suppresses Notch signaling in the BM 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 BM cells (* $P<0.05$; ** $P<0.01$, three 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 three performed. **C**, Quantitative RT-PCR analysis of Notch target genes (Hes1 and Deltex1) in BM cells (* $P<0.05$; ** $P<0.01$, three 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 $\pm$ s.e.m.

Figure 4. Over-expressing DLL1 alone in BM 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 (100ng/hr) over a period of 28 days. **A**, Flow cytometric analysis of splenocytes expressed CD3e and CD19. The representative plots are shown from four performed. 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±s.e.m, * P<0.05, ** P<0.01, n=3–4, four independent experiments). **C**, Flow cytometric analysis of thymocytes expressed CD4 and CD8a in CD3e^{low/-} cells. The representative plots are shown from three performed. **D**, The percentage of the T cell subtypes in thymus (mean±s.e.m, n≥3).

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 co-transfected with Notch1, Hes1-luciferase reporter and Renilla-luciferase expression constructs. Cells were treated as indicated for 24 hrs and luciferase assay was performed using a Dual Luciferase Assay with Renilla luciferase used as an internal control. In “Cell-bound DLL1”, DLL1 expression construct was also included in co-transfection without further treatments. Data are mean $\pm$ s.e.m., $n=3$; * $p<0.05$, unpaired t -test. **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 received anti-CD8 antibody. (5–10 mice per group; * $p<0.05$, ** $p<0.01$). Data are mean $\pm$ s.e.m. **C**, Soluble clustered DLL1 attenuates LLC tumor growth in C57BL/6 mice, but not RAG1^{-/-} mice (5 mice per group, * $p<0.05$, ** $p<0.01$).

A **B**

Figure 6. Treatment with soluble clustered DLL1 stimulates Notch signaling in BM cells and improves T cell immune response

A, Soluble clustered DLL1 treatment stimulates Notch signaling in BM cells (* $p < 0.05$, $n = 3$). Data are mean $\pm$ s.e.m. **B**, Soluble clustered DLL1 treatment enhances IFN- γ production of T cells responding to anti-CD3 and anti-CD28 stimulation (** $p < 0.01$, $n = 3$). Spleens were harvested on Day 29 post D459 tumor inoculation. Numbers indicate the spots of IFN-gamma producing T cells from 10^5 T (CD3e) cells.