Tumor-Infiltrating Myeloid Cells Activate Dll4/Notch/TGF-β Signaling to Drive Malignant Progression

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Abstract
Myeloid cells that orchestrate malignant progression in the tumor microenvironment offer targets for a generalized strategy to attack solid tumors. Through an analysis of tumor microenvironments, we explored an experimental model of lung cancer that uncovered a network of Dll4/Notch/TGF-β1 signals that links myeloid cells to cancer progression. Myeloid cells attracted to the tumor microenvironment by the tumor-derived cytokines CCL2 and M-CSF expressed increased levels of the Notch ligand Dll4, thereby activating Notch signaling in the tumor cells and amplifying tumor-intrinsic Notch activation. Heightened Dll4/Notch signaling in tumor cells magnified TGF-β–induced pSMAD2/3 signaling and was required to sustain TGF-β–induced tumor cell growth. Conversely, Notch blockade reduced TGF-β signaling and limited lung carcinoma tumor progression. Corroborating these findings, by interrogating RNAseq results from tumor and adjacent normal tissue in clinical specimens of human head and neck squamous carcinoma, we found evidence that TGF-β/Notch crosstalk contributed to progression. In summary, the myeloid cell-carcinoma signaling network we describe uncovers novel mechanistic links between the tumor microenvironment and tumor growth, highlighting new opportunities to target tumors where this network is active. Cancer Res; 74(7); 1–12. ©2014 AACR.

Introduction
The tumor microenvironment is increasingly recognized as an enabling contributor to tumor progression (1, 2), and strategies that target the tumor microenvironment are effective at reducing tumor growth, despite persistence of genetically modified tumor cells (3–5).

The vasculature is a component of the tumor microenvironment that contributes to tumor growth through angiogenesis. Inhibition of angiogenesis by neutralization of VEGF is effective at reducing progression of certain tumors despite persistence of genetically modified tumor cells (5). "Inflammatory" cells, particularly "M2-type" myeloid cells (2) and stromal fibroblast-like cells/cancer-associated fibroblastic cells (1) are other protumorigenic components of the cancer microenvironment. Through integrin-mediated adhesion signaling and other mechanisms myeloid cells promote cancer cell survival (6). Acting directly or through effector molecules, including TGF-β, fibroblast growth factors (FGF), and epidermal growth factors, cancer-associated myeloid and fibroblastic cells supply mitogenic signals to tumors (1). By secreting VEGF, basic FGF, platelet-derived growth factor, placental growth factor, and BV8, myeloid cells promote tumor angiogenesis (5,7). By producing various proteases, myeloid cells induce the release VEGF and other mitogenic factors sequestered in the extracellular matrix and disrupt tissue integrity by cleaving homotypic and heterotypic cell adhesion molecules (8).

Pressing motivation to abrogate myeloid-derived protumorigenic signals has produced some encouraging results. Blocking macrophage recruitment with antagonists of colony-stimulating factor-1 receptor reduced mammary tumor progression and increased mice survival (4). TGF-β–targeted drugs are currently in clinical trials after showing encouraging ant cancerc activity in preclinical studies (9,10). Matrix metalloproteases inhibitors, which showed promising antitumor activities in mouse but not in human cancer trials, are being re-evaluated in light of emerging new understanding (11).

Despite these advances, the complexities of cell composition of tumor microenvironments and tendency to adaptive change present current obstacles. To overcome some of these difficulties, we have queried the "simpler" tumor microenvironment of growth factor independence-1 (Gfi1)-null mice, which lack mature granulocytes and harbor functionally impaired myeloid cells (12,13) to identify principal mechanisms that sustain reciprocal communications between tumor cells and cells of the tumor microenvironment. By focusing on Notch signaling, a crucial regulator of cell fate decisions activated by...
cell-to-cell interaction between Notch ligand (DLL1, DLL3, DLL4, Jagged1, and Jagged2) and Notch receptor (Notch1–Notch4)-expressing cells (14–16), we now uncovered a novel network of DLL4/Notch/TGF-β signals linking myeloid cells and cancer cells that drives lung carcinoma tumor progression. This network provides a mechanistic link between tumor-infiltrating myeloid cells and tumor cells with opportunities for intervention.

Materials and Methods

Cell culture and in vitro treatments

The EL4, LLC1, and B16F10 murine cell lines [American Type Culture Collection (ATCC); authentication confirmed by ATCC through depositor’s analysis of representative cultures from the master seed stock] were propagated in the laboratory for fewer than 6 months in culture medium (RPMI or Dulbecco’s Modified Eagle Medium with 1% BSA, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 50 μmol/L 2-ME, and 1 mmol/L sodium pyruvate); proliferation was measured by 3H thymidine incorporation (17). Recombinant human TGF-β1 (R&D Systems), DAPT [N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine tert-butyler ester; Sigma-Aldrich], DBZ [(2S)-2-[2-(3,5-difluorophenyl)-acetylamo]-N-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-propionamide; Millipore], and appropriate diluted controls were added to culture; recombinant mouse His tag-Dll4 (R&D Systems) and His control (Millipore) were immobilized (18 hours at 4°C) to culture vessels before cell addition.

Animal studies

Animal experiments were approved by the NCI-Bethesda Institutional Animal Care and Use Committee and conducted according to protocol. Gf11−/−, Gf11−/+, and Gf11− mice (18) were used between 4 and 8 weeks of age. Mouse tumor cell lines were implanted (10 × 106) subcutaneously in the left abdominal quadrant. DAPT (10 mg/kg, i.p. 5 days/week) or diluent control treatment was initiated 24 hours after tumor-cell injection. Tumors were removed in toto after 12 and 16 days. Spleens and bone marrows were obtained from tumor-bearing and control mice.

Flow cytometric analysis and cell sorting

Single-cell suspensions from bone marrow, spleen, and tumor tissues were incubated with mouse Fc block CD16/32 antibody (2.4G2; BD Biosciences) for 20 minutes at 4°C in PBS containing 2%BSA (PBS/BSA) to reduce nonspecific antibody binding. After washing in PBS/BSA, cells were incubated with control Ig or fluorophore-conjugated antibodies in PBS with 1%BSA and 2 mmol/L EDTA. Cell sorting and data collection were performed on a FACSVantage SE or FACSARia (BD Biosciences); data analysis used Flowjo software. Details on antibodies are found in Supplementary Experimental Procedures.

Immunohistochemistry and immunoblotting

Tissues were fixed with 2% or 4% paraformaldehyde overnight or 4 hours at 4°C (19). Tissue immunostaining and quantification was performed as described previously (19). Protein extracts prepared as described (19) were run through 4% to 12% bis-Tris gels (Invitrogen) or 10% to 20% polyacrylamide gels (Novex), transferred to proton BA83 cellulose membranes (Whatman) and stained with the primary and secondary antibodies as detailed in Supplementary Experimental Procedures.

Bioinformatics and statistical analysis

All bioinformatic analyses were conducted on the publicly available gene expression data (normalized values from Illumina RNAseq version 2, level 3) from The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/). The data were downloaded from TCGA matrix and was evaluated by box plot analysis and the Mann–Whitney U test using the R system (2.14.1) for statistical computation and graphics. In all other experiments, group differences were analyzed by using the 2-tailed Student t test with equal variance assumption and the Fisher exact test (Microsoft Excel). P ≤ 0.05 were considered significant.

Results

Host dependency of LLC1 carcinoma and EL4 T-cell lymphoma progression

To explore contributions of the tumor microenvironment to tumor progression, we utilized Gf11-null mice that lack mature granulocytes and have functionally defective monocyes, while displaying a mostly intact lymphoid system (12,13,18). Gf11-heterozygote mice are indistinguishable from wild type (12,13). By analysis of syngeneic subcutaneous transplant systems, we evaluated tumor growth induced by cell lines representative of T-cell lymphoma (EL4), lung carcinoma (LLC1), and melanoma (B16F10; Fig. 1A and Supplementary Fig. S1). EL4 cells generated tumors that grew more aggressively (Fig. 1A and Supplementary Fig. S1) in Gf11-null (KO) mice compared with Gf11−/− (wild type, WT) or Gf11−/+/− heterozygous (Het) mice. By contrast, LLC1 cells generated tumors that grew more aggressively (Fig. 1B and Supplementary Fig. S1) in Gf11-null KO mice compared with Gf11 KO. B16F10 cells generated tumors that grew similarly in Gf11-WT/Het and KO mice (Fig. 1C and Supplementary Fig. S1). We concluded that EL4 and LLC1 tumor progression is significantly affected by host factors.

We hypothesized that the Gf11 and wild-type tumor microenvironment differed in EL4 and LLC1 tumors, but not in B16F10 tumors. Because neutrophils, a source of the proangiogenic Bv8 factor (7), are absent in Gf11-null mice, we examined tumor vascularization. We found that vascularization of EL4 and LLC1 tumors from WT/Het and Gf11-null mice was quantitatively and morphologically similar, as assessed by CD31 immunostaining (Supplementary Fig. S2A and S2B). A comprehensive analysis of major cell types revealed a significantly greater infiltration of CD11bLy6C Ly6G− cells in LLC1 tumors from Gf11-null mice compared with control, whereas this population was similarly represented in EL4 tumors from Gf11-null and wild-type hosts, and was rare in B16F10 tumor tissues (Fig. 1D and E). By contrast, the CD11bLy6C Ly6G− cells were significantly more abundant in EL4 tumors from wild type compared with Gf11-null mice;
Figure 1. The Gfi1-null microenvironment regulates tumor progression. A–C, tumor weight from control (WT Gfi1 þ/ þ or het Gfi1 þ/–) and Gfi1-null (KO Gfi1 2/2) mice analyzed 12 to 15 days postsubcutaneous injection of EL4, LLC1, and B16F10 tumor lines. Data are averages ± SD from individual experiments, each representative of three experiments performed: A, EL4 tumors WT/Het, n = 12; KO, n = 10; B, LLC1 tumors WT/Het, n = 15; KO, n = 12; C, B16F10 tumors WT/Het, n = 12; KO, n = 10; P values from the Student t test. D–G, monocytes and granulocytes infiltrate tumors from control and Gfi1-null mice. In the bar graphs (D and F), flow cytometry data are expressed as average percentage of total cells from tumor ± SD; EL4, n = 5; LLC1, n = 6; B16F10, n = 3. In the representative flow cytometry plots (E and G), the numerical values are expressed as percentages of total CD11b þ leukocytes in the tumor; P values from the Student t test. H and I, distribution of CD4 þ and CD8 þ lymphocytes in tumors. The data are expressed as average percentage of total cells from tumor ± SD; EL4, n = 5; LLC1, n = 6; B16F10, n = 3; P values from the Student t test. J, frequency of tumor development in wild-type mice injected with EL4 cells alone or with splenocytes unfractionated (wild-type or KO) or depleted of Ly6G þ cells (WT). Splenocytes were from EL4-bearing mice. EL4 alone, EL4 þ WT cells, EL4 þ KO cells, n = 10; EL4 ‘WT LyG’ cells, n = 6; wild-type or KO cells alone, n = 3. Data indicate the percentage of mice injected that developed tumors over a period of 14 to 16 days; P values from the Fisher exact test. K, tumor weight in wild-type mice injected with LLC1 cell alone or with wild-type monocytes sorted from bone marrow of LLC1-bearing wild-type or KO mice; n = 6/group. Data are averaged ± SD; P values from the Student t test.
this population was virtually absent in LLC1 and B16 tumor tissues from wild-type and Gf1-null hosts (Fig. 1F and G). CD4+ and CD8+ T lymphocytes (Fig. 1H and I) were similarly represented in EL4, LLC1, and B16F10 tumor tissues from wild-type and Gf1-null mice. CD25+ T cells; CD4+ FOXP3+ T cells, B220+ B (and other) cells; CD11c+ B220+ dendritic cell populations; SMA+ myofibroblasts; CD49b NK cells and CD11b+F4/80 macrophages were also similarly represented in EL4, LLC1, and B16F10 tumor tissues from wild-type and Gf1-null mice (not shown).

Reflecting the Gf1-null phenotype, spleens (Supplementary Fig. S2C and S2D) and bone marrows (Supplementary Fig. S2D and S2E) from Gf1-null mice (control and tumor-bearing) showed an increase of CD11b+Ly6C+Ly6G− cells and a decrease of CD11b+Ly6C+Ly6G+ cells compared with wild-type mice. Spleens of EL4 tumor-bearing wild-type mice displayed a significant increase of granulocytes compared with naive spleens, and spleens of LLC1 tumor-bearing wild-type mice displayed a significant increase of monocytes compared with naive spleens (Supplementary Fig. S2C). Given the predominant differences in infiltrates distinguishing EL4 and LLC1 tumors growing in the Gf1 and wild-type hosts and changes developing in the spleens of tumor-bearing mice, we focused on the role of CD11b+Ly6C+Ly6G− and CD11b+Ly6C−Ly6G+ cells in these models.

Adoptive transfer experiments supported a tumor-inhibitory activity of wild-type granulocytes in the EL4 system, because depletion of Ly6G+ cells reduced the antitumor activity of wild-type splenocytes (Fig. 1J), and a tumor-promoting function of monocytes in the LLC1 tumor model, because CD11b+Ly6C+Ly6G− cells enhanced LLC1 tumor growth whereas this cell population from Gf1-null mice did not (Fig. 1K).

LLC1 cells express CCL2/MCP1 mRNA (Fig. 2A; ref. 20) and protein present in LLC1 culture supernatant (2.8 ng/mL). Control and Gf1-null CD11b+Gr1+ cells similarly migrated to recombinant CCL2 (Fig. 2B), suggesting that LLC1-derived CCL2 may recruit myeloid cells to the tumor. LLC1 cells also express M-CSF/CSF1 mRNA (Fig. 2A), but protein was undetected in culture supernatant. EL4 cells express GM-CSF/CSF2 mRNA (Fig. 2A) and protein detected in the culture supernatant at 21 pg/mL, suggesting that it may recruit granulocytes to EL4 tumors (21).

Identification of myeloid cell-derived signals that modulate tumor cell growth

Results from adoptive transfer experiments suggested that myeloid cell types recruited by EL4 and LLC1 tumor cells might produce paracrine signals that modulate cancer cell growth/survival. To identify such signals, we profiled gene expression in EL4, LLC1, and B16F10 tumors from wild-type and Gf1-deficient mice. To distinguish signals derived from the tumor microenvironment from others derived from the tumor cells, we profiled in parallel EL4, LLC1, and B16F10 cell lines from culture. Focusing on genes previously linked to modulation of tumor growth, we measured 57 mRNAs in 10 EL4 tumors (5 each from wild-type and Gf1-null mice) and in 15 LLC1 tumors (10 from wild type; 5 from Gf1-null mice) from two to four different experiments (Table 1 and Supplementary Table S1). From this pool, we identified 10 mRNAs at significantly different levels in EL4 and/or LLC1 tumors arising in wild type as opposed to Gf1-null mice (Table 1). Extending analysis of these 10 mRNAs to B16F10 tumors, we found no expression difference between tumors from wild-type and Gf1-null mice (Table 1).

All but 1 (Cxc94f) of the 10 candidate genes fulfilled the criteria of being likely host induced in that expression was higher in the EL4 or LLC1 tumors compared with the tumor cell line. For the remainder, we looked for myeloid-derived genes preferentially expressed in the wild-type host that might be linked to suppression of EL4 and stimulation of LLC1 tumor growth. Tgf1 (encodes TGF-β1) and Tgff2 (encodes TGF-β2) fulfilled these criteria (Table 1). TGF-β1/2 is a monocyte and neutrophil product that can inhibit and stimulate cell growth dependent on context: it is a tumor suppressor in early tumor development, but a tumor enhancer in more advanced tumors (3). We confirmed that TGF-β1 mRNA is expressed at higher levels in the LLC1 tumor microenvironment of wild type compared with Gf1-null mice by sorting the CD11b+Gr1+ cells (Fig. 2C). Similarly, we confirmed that TGF-β2 mRNA is expressed at significantly higher levels in the EL4 tumor microenvironment of wild-type mice compared with Gf1-null mice (not shown). Naïve spleens from wild-type mice constitutively express higher levels of TGF-β1 (Fig. 2D) and TGF-β2 (not shown) mRNA compared with Gf1-null mice, and naïve CD11b+Gr1+ from wild-type bone marrow secrete higher levels of TGF-β1 compared with Gf1-null bone marrow, both constitutively and after activation with M-CSF/CSF1 or GM-CSF/CSF2 (Fig. 2E). We also found that levels of the TGF-β signaling mediator pSMAD3 were higher in tumors arising in wild-type mice than in Gf1-null mice (Fig. 2F and G), indicative of greater TGF-β activity in vivo. We tested the effects of TGF-β on tumor cell growth. TGF-β1 significantly and dose-dependently reduced EL4 proliferation but enhanced LLC1 proliferation (Fig. 2H). Increased LLC1 cell proliferation by TGF-β1 is cell density dependent, suggesting a requirement for cell–cell interaction (Fig. 2I); no such cell dose dependency was observed with EL4 cells (not shown).

To investigate this cell dose dependency of LLC1 proliferation to TGF-β, we examined the potential involvement of Notch signaling, because it is induced by cell–cell interaction and can establish cooperative crosstalk with TGF-β signaling in other systems (14–16). We found that the Notch ligand Dll4 and the Notch signaling mediator Hey2 were expressed at higher levels in LLC1 tumors from wild type compared with Gfi1-KO (Table 1). To identify the cell sources of the differentially expressed Dll4 and Hey2, we characterized Notch receptors/ligands expression in tumor cells and myeloid cells. LLC1 cells express Notch1 and Notch4 receptors mRNAs at higher levels than the EL4 and B16F10 cells (Fig. 3A); primary wild-type monocytes sorted from bone marrow express higher levels of Dll4 mRNA (Notch1 and Notch4 ligand) than Gf1-null monocytes (Fig. 3B). LLC1 cells also express endogenous Dll1 mRNA (Notch1/2/3 ligand) and Dll4 mRNA (Fig. 3A). We sorted wild-type and Gf1-null CD11b+Ly6C+Ly6G- cells from LLC1 tumor cell suspensions to measure expression levels of Notch1,
Figure 2. TGF-β inhibits EL4 cell proliferation and increases LLC1 cell proliferation. A, relative cytokine mRNA expression in cell lines; results are from qPCR (averages from duplicate measurements). B, mouse CCL2 induces migration of bone marrow CD11b^+ Gr1^+ cells. Data are averages ± SD; n = 3 experiments. C, relative TGF-β1 expression in CD11b^+ Gr1^+ cells sorted from tumor cell suspensions. Data from qPCR are averages ± SD; n = 5 to 6 tumors/group. D, relative TGF-β1 expression in naïve splenocytes; qPCR data are means ± SD; n = 5. E, TGF-β in supernatant of bone marrow CD11b^+ Gr1^+ cells; M-CSF (20 ng/mL), GM-CSF (40 ng/mL); ELISA results are means ± SD; triplicate cultures. F, representative images of pSMAD3-expressing cells in LLC1 tumor; nuclei are visualized with DAPI. G, quantification of pSMAD3-expressing cells in EL4 and LLC1 tumors. Data are averages ± SD cells/field (×40); 5 fields/tumor; n = 5 tumors/group. H, proliferation of EL4 and LLC1 cells to TGF-β1. Representative results (of 3–5 experiments) are means ± SD (triplicate cultures). I, LLC1 proliferation to TGF-β is cell-density dependent. Representative results (of three experiments) are means ± SD of (triplicate cultures). All P values are from the Student t test.
Crosstalk between Notch and TGF-β signaling regulates carcinoma cell growth

Next, we examined whether Notch/Hey2 signaling in LLC1 cells, attributable at least in part to activation by wild-type myeloid cell-derived Dll4, modulates TGF-β signaling and function in LLC1 cells. The Notch signaling inhibitors DAPT and DBZ reduced TGF-β-induced LLC1 proliferation (Fig. 4A) but minimally affected TGF-β-induced repression of EL4 proliferation (Fig. 4B). These results support a functional requirement for Notch signaling in sustaining TGF-β-induced LLC1 growth stimulation.

Notch4, Dll4, Hey1, and Hey2 (Supplementary Fig. S3A). LLC1 tumor-infiltrating CD11b^Ly6C^-Ly6G^-cells from wild-type mice expressed more Dll4 than this population sorted from Gf1-null mice (Fig. 3C), and more than LLC1 cells from culture (Supplementary Fig. S3B). Notch1 and Notch4 were expressed in LLC1 tumor-infiltrating CD11b^Ly6C^-Ly6G^- cells from wild-type mice and Gf1-null mice (Supplementary Fig. S3B) at somewhat lower levels than found in LLC1 cells (Supplementary Fig. S3B). Because Hey1 and Hey2 mRNAs were at the limit of detection in tumor-infiltrating CD11b^Ly6C^-Ly6G^- cells from wild-type and Gf1-null mice (not shown), we concluded that Hey2 mRNA detected at higher levels in LLC1 tumor tissues from wild type as opposed to KO mice was likely tumor-cell derived. Collectively, these results suggested a model in which Dll4-expressing tumor-infiltrating wild-type myeloid cells, stimulate Notch1 and Notch4 signaling in LLC1 cells inducing Hey2 expression (Fig. 3D and Supplementary Fig. S3C). Supporting this model, immobilized Dll4-his specifically induced Hey1 and Hey2 expression in LLC1 cells, but not the expression of TGF-β (Fig. 3E). By contrast, Dll4 did not induce Hey1 and Hey2 expression in EL4 cells, which express Notch1 and Notch4 at considerably lower levels than LLC1 cells (Fig. 3E).

DAPT and DBZ block early steps in the Notch signaling cascade by preventing the γ-secretase-dependent proteolytic cleavage of Notch intracellular domain. To investigate points of potential integration of Notch and TGF-β signaling, we focused on SMADs phosphorylation induced by TGF-β binding to type I and type II serine–threonine kinase receptors. SMAD2 and SMAD3 receptor–regulated SMADs are recognized by type I TGF-β receptors, which are expressed by EL4 and LLC1 cells. We found that TGF-β similarly induces the phosphorylation of SMAD2 and SMAD3 in LLC1 and EL4 cells, despite the different biologic outcomes (Fig. 4C). However, the Notch inhibitor DAPT reduces this phosphorylation in LLC1, but not in EL4 cells (Fig. 4C), indicating that TGF-β–induced SMADs phosphorylation is dependent, in part, upon Notch signaling in LLC1, not EL4. cells. This crosstalk of TGF-β and Notch signaling pathways in LLC1 cells is consistent with the previously recognized binding of the active Notch intracellular domain to SMAD2/3 (14, 15). Based on experiments showing that active Notch promotes cMyc transcription (22), whereas TGF-β inhibits cell-cycle progression by transcriptional and nontranscriptional cMyc repression in many cell types (23, 24), we examined cMyc expression. We found that cMyc levels increase in LLC1 cells after TGF-β activation and that the Notch inhibitor DAPT reduces this effect, whereas cMyc levels are unaffected by TGF-β and/or DAPT in EL4 cells (Fig. 4C). This provides additional evidence for cooperative crosstalk between TGF-β and Notch signaling resulting in increased cMyc expression in LLC1 cells. Strengthening this evidence, we found that TGF-β induces cMyc mRNA expression in LLC1 cells, which is reduced by DAPT, and that TGF-β promotes expression of the Notch target gene Hey1 and Hey2, not shown) while minimally affecting expression of SMAD2, SMAD3 (not shown), and TbRI (Fig. 4D). TGF-β did not change expression levels of Hey1 or cMyc in EL4 cells (Fig. 4D).

Because Hey2 mRNA levels are 8- to 11-fold higher in LLC1 tumors from wild-type mice compared with LLC1 tumors

Table 1. Differentially expressed genes in tumors from Gf1-null and control mice

<table>
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<tr>
<th>Gene</th>
<th>Relative expression^a</th>
<th>Relative expression^b</th>
<th>Relative expression^c</th>
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<td>EL4 WT KO</td>
<td>LLC1 WT KO</td>
<td>B16 WT KO</td>
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<tr>
<td>Tgif1</td>
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^aResults from qPCR analysis of RNAs from cell lines (EL4, LLC1, B16) and tumor tissues from WT/Het or Gf1-null (KO) mice injected with these cell lines. Data from tumors in WT/Het and KO mice are expressed as relative mRNA levels ± SD compared with the injected cell line when expression was detected or compared with each other when not detected in the injected cell line.

^bValues were determined by the Student t test; n = 4 to 10 tumor tissues per group; values in bold reflect significant differences.

Abbreviation: nd, not detected.
from Gfi1-null mice and to LLC1 cells from culture (Table 1), we hypothesized that this might be because of a contribution by wild-type tumor-infiltrating monocytes that express Dll4 at higher levels than Gfi1-null monocytes (Fig. 3C and Supplementary Fig. S3B). We therefore investigated whether increased Hey2 levels in LLC1 tumors are attributable to Dll4-expressing wild-type monocytes in infiltrating the tumor, and whether this increased Notch signaling accelerates LLC1 tumor cell growth. To mimic the monocyte effect, we first used immobilized Dll4 to modulate Notch signaling intensity in LLC1 cells seeded at low density to reduce cell–cell contact expected to activate Notch through membrane-anchored Dll1 and Dll4 ligands. When activated with Dll4-his, low-density LLC1 cells responded to TGF-β with increased proliferation, which was absent from control cultures lacking Dll4-his (Fig. 4E). DAPT and DBZ both reduced this proliferation (Fig. 4E), providing evidence of functional cooperation between TGF-β and Notch signaling in promoting LLC1 proliferation. We then tested wild-type monocytes, which express Dll4. TGF-β enhanced the proliferation of low-density LLC1 cells cocultured with bone marrow CD11b+Ly6C+Ly6G− cells from wild-type, but not Gfi1-null mice. Hey1 mRNA levels were significantly (P < 0.05) higher in cocultures of LLC1 cells with wild-type monocytes compared with cocultures of LLC1 cells with KO monocytes, confirming that wild-type monocytes

Figure 3. Tumor-infiltrating myeloid cells express Dll4, which activates Notch signaling in LLC1 tumor cells that express Notch1 and Notch4. A and B, Notch receptors and ligands expression in tumor cell lines (A) and in CD11b+Ly6C−Ly6G−/CD11b+Ly6C+Ly6G− populations (B). Data from qPCR are averages from triplicates measurements (variation <12%); the sorted populations are pools from three mice per group. C, Dll4 expression in CD11b+Gr1− cells infiltrating LLC1 tumors. Data are averages ± SD; n = 3 mice/group. D, a model for tumor/tumor-infiltrating myeloid cell interaction. E, Hey1 and Hey2 expression in tumor cells activated with immobilized Dll4-his; controls, uncoated and His-coated wells. Data from qPCR are averages ± SD; n = 4 to 5. P values are from the Student t test.
activate Notch in LLC1 cells better than KO monocytes. DAPT reduced this stimulatory effect of wild-type monocytes (Fig. 4F). Collectively, these results show that TGF-β enhances LLC1 tumor cell growth in the presence of Notch signaling from either LLC1 cell–cell contact and/or through interaction with myeloid cells expressing Dll4.

Contribution of Notch signaling to carcinoma progression

Next, we investigated whether Notch signaling contributes to increased LLC1 tumor progression. Wild-type mice bearing LLC1 tumors showed a significant reduction in tumor progression when treated with the Notch inhibitor DAPT, whereas KO mice did not (Fig. 5A and Supplementary Fig. S4). Treatment with DAPT reduced Hey1 and Hey2 mRNA expression in the tumor tissue, indicative of Notch signaling inhibition in vivo (Fig. 5B). Instead, mRNA levels of SMAD2,
Notch signaling promotes LLC1 tumor progression. A, groups of littermate mice were treated intraperitoneally 5 days per week (7 total injections) with the Notch inhibitor DAPT (10 mg/kg) or vehicle alone, beginning 24 hours after inoculation of LLC1 cells. Tumors were removed 16 days after LLC1 inoculation. Individual tumor weights and average weight ± SD. B, relative gene expression in LLC1 tumors after treatment with vehicle alone or DAPT. Data are from individual tumors and group averages ± SD. C, model showing the contribution of myeloid cells to Notch signaling and tumor cell density is low, Dll4-expressing tumor-infiltrating edge of the tumor, where tumor infiltrating myeloid cells activate Notch1 and Notch4 (Fig. 5C). We envision that the contribution of myeloid cells is most critical at the invasive edge of the tumor where myeloid cells accumulate (27), providing an opportunity for myeloid–tumor cell interactions resulting in Notch activation. Tumor cells are mobile at the tumor invasive edge and tumor cell density is reduced (28), likely limiting Notch signaling from tumor-intrinsic cell–cell interactions (Fig. 5C).

Prompted by the current findings showing a role of tumor-infiltrating monocytes in enhancing TGF-β/Notch signaling and LLC1 tumor progression, we sought evidence for this link in clinical samples. We queried TGCA, focusing on head and neck squamous cell carcinoma, which resembles subcutaneous LLC1 in having significant monocyte infiltration correlating with increased tumor aggressiveness and reduced survival, increased expression CCL2, deregulated TGF-β and Notch signaling components, and evidence of active TGF-β signaling (29–32). We also examined lung squamous cell carcinoma based on the lung carcinoma derivation of LLC, albeit LLC1 is a cloned line adapted to culture. Remarkably, head and neck squamous cell carcinomas display significantly greater expression of Dll4, Notch4, Hey1, TGF-β1, and TGF-βR1, compared with normal tissue, much alike LLC1 tumors in wild-type mice (Fig. 6A). By contrast, lung squamous cell carcinoma display significantly lower expression of Dll4, Notch4, TGF-β1, and TGF-βR1, and TGF-βR2 compared with normal tissue (Fig. 6A). This discordant pattern of gene expression is strengthened by comparing ratios of tumor/normal control from paired samples of individual patients (Fig. 6B). The gene expression signature emerging from this analysis highlights the potential for activation of TGF-β/TGF-βR1 and Dll4/Notch4/Hey1 signaling in head and neck squamous cell carcinoma and contribution to tumor progression. Additional studies are planned to measure TGF-β/TGF-βR1 and Notch signaling activity in head and neck squamous cell tumors and correlate signaling levels with disease outcome.

Discussion

One of the emerging challenges to the successful treatment of cancer is the tumor microenvironment that variously

SMAD3, and TGF-β were similar in untreated and DAPT-treated tumors (Fig. 5B). Although this was not predicted by the results of short-term experiments in vitro, we found that TGF-βR1 mRNA levels were reduced in DAPT-treated tumors and that cMyc mRNA levels were similar in DAPT-treated mice compared with controls (Fig. 5B). This could be explained by observations linking Notch and its downstream mediator RPB-jk to the regulation of TGF-β receptors expression (25), and the complexities of Myc transcriptional regulation by many signaling pathways besides Notch and TGF-β (26). Reduced tumor growth with DAPT treatment could not be attributed to reduced accumulation of protumorigenic myeloid cells, because similar infiltration of CD11b+Ly6C+ cells was present in LLC1 tumors treated or not treated with DAPT (not shown). Overall, these results support a model in which wild-type myeloid cells recruited to the tumor accelerate tumor progression by secreting mitogenic TGF-β and enhancing TGF-β signaling in the tumor cells via expression of the ligand Dll4, which activates Notch1 and Notch4 (Fig. 5C).

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contributes to cancer progression through reciprocal communication with the tumor cells. In addressing this challenge, our work unveils the Notch and TGF-β signaling pathways as functional partners in a network of cancer cells and tumor-infiltrating CD11b^+Ly6C^+Ly6G−/C0 cells that promotes cancer cell growth and tumor progression. This new understanding offers an opportunity for the targeting of tumors where TGF-β and Notch signaling are linked protumorigenic partners. Head and neck squamous cell carcinoma may represent such setting as we find that this tumor generally displays increased expression of TGF-β and its receptors, in conjunction with evidence of Notch signaling activity.

TGF-β, a product of myeloid-lineage cells in many tumor microenvironments, plays a well-recognized role in tumor progression and resistance to treatment (33, 34). TGF-β has cytostatic and proapoptotic effects for normal cells and premalignant lesions (33). In advanced cancer, TGF-β promotes tumor progression (33). Such functional change is attributed to inactivating mutations of TGF-βR2 and SMAD4 preventing TGF-β tumor-suppressive signaling in gastrointestinal and pancreatic tumors (33, 34). In many other cancer types, including the LLC1 tumor model in our study, TGF-β receptors and SMAD signaling are intact (33). It remains a puzzle how TGF-β can exert contextually different functions. Here we show that crosstalk between the Notch and TGF-β pathways is critical to TGF-β signaling and function as a tumor enhancer in the LLC1 model. Blocking Notch signaling significantly reduces SMADs phosphorylation and cell growth induced by TGF-β. It also slows LLC1 tumor progression, which is accelerated by TGF-β-producing tumor-infiltrating myeloid cells.

Figure 6. Analysis of gene expression in human head and neck squamous cell carcinomas reveals similarities to gene expression in mouse LLC1 tumors. A, gene expression in head and neck normal tissue (n = 37) and squamous carcinoma (n = 303); and in lung normal tissue (n = 50) and squamous carcinoma (n = 369). C, control tissue; T, tumor. Results from RNAseq (TGCA) are expressed as transcript expression levels; data distribution is displayed as box plots (the box limits first and third quartiles; band inside the box is median value; the vertical dotted lines indicate variability; outlier values are shown as dots). Statistical significance of group differences from the Mann–Whitney U test. B, distinct patterns of gene expression in head and neck and lung squamous cell carcinomas. Results depict ratios of gene expression in tumor tissue and corresponding normal tissue in paired patient samples with head and neck (black bars; n = 35) or lung (gray bars; n = 50) squamous cell carcinomas.
TGF-β and Notch signaling converge in regulating a number of developmental processes (35). Notch signaling modulates expression of BMP family members (36) and TGF-β target genes (37). Conversely, TGF-β induces expression of the Notch ligand Jagged1 and target gene Hey1 (38). Interestingly, Notch signal transducers can physically interact with components of the TGF-β signaling pathway contributing more directly to TGF-β and BMP function (14–16, 39). The present work shows that crosstalk between the TGF-β and Notch signaling pathways converge on SMAD2/3 activation, which sustains TGF-β tumor-promoting function.

Our current findings further identify a previously unrecognized role of tumor-infiltrating CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>−</sup> cells as activators of Notch signaling in tumor cells enabling paracrine TGF-β growth stimulation. CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>−</sup> myeloid cells constitute a functionally heterogeneous population (2, 40), which includes tumor-promoting myeloid-derived suppressor cells (MDSC) with T-cell suppressive functions via expression of inducible forms of nitric oxide and arginase, and in some cases TGF-β (1, 2, 41). MDSCs have been identified in many tumor types (41), including experimental EL4 and LLC1 tumors. In this study, T-cell immunity is not identified a major contributor to tumor growth modulation, and the tumor-promoting CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>−</sup> cells do not express high-level Nos2 and Arg1 typical of MDSCs. Rather, the cells resemble phenotypically tumor-derived M2-type macrophages (2), which express the Notch ligand Dll4, similar to monocytes exposed to proinflammatory signals (42).

Recent reports and the current work show that Notch activity is a contributor to progression of some cancers (43), albeit not all cancers (44–48). It is currently unclear whether Notch-activating signals from the tumor microenvironment contribute to tumor progression. This was suggested in myeloma through tumor–stromal cells interactions (49). Using genetic, biochemical, and functional approaches our results show that Dll4-expressing myeloid cells induce Notch stimulation in LLC1 tumor cells, which allows paracrine TGF-β signaling and growth in the tumor cells. This provides evidence for yet another mechanism of tumor progression by tumor-infiltrating myeloid cells. Our results argue that this function of tumor-infiltrating myeloid cells is most critical at the locally invasive edge of the tumor where single cells migration is a principal mode of invasion (28), and there is opportunity for myeloid–tumor cells interaction (27, 50).

In conclusion, our results provide mechanistic insights into TGF-β tumor promotion by linking TGF-β and Notch signaling, and disclose an activating Dll4/Notch signaling axis linking tumor-promoting myeloid cells and tumor cells. This raises the possibility of dual targeting of Notch and TGF-β signaling in cancers where TGF-β plays Notch-dependent protumorigenic functions, as in head and neck squamous cell carcinoma, which expresses components of the TGF-β and Notch signaling pathways at abnormally high levels. It is fortunate that inhibitors of TGF-β and Notch signaling (43) have been developed and hold promise in clinical development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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