Effects of Notch Signaling on Regulation of Myeloid Cell Differentiation in Cancer

Pingyan Cheng[1], Vinit Kumar[2], Hao Liu[1], Je-In Youn[2], Mayer Fishman[1], Simon Sherman[3], and Dmitry Gabrilovich[1,2]

Abstract

Functionally altered myeloid cells play an important role in immune suppression in cancer, in angiogenesis, and in tumor cells' invasion and metastases. Here, we report that inhibition of Notch signaling in hematopoietic progenitor cells (HPC), myeloid-derived suppressor cells (MDSC), and dendritic cells is directly involved in abnormal myeloid cell differentiation in cancer. Inhibition of Notch signaling was caused by the disruption of the interaction between Notch receptor and transcriptional repressor CSL, which is normally required for efficient transcription of target genes. This disruption was the result of serine phosphorylation of Notch. We demonstrated that increased activity of casein kinase 2 (CK2) observed in HPC and in MDSC could be responsible for the phosphorylation of Notch and downregulation of Notch signaling. Inhibition of CK2 by siRNA or by pharmacological inhibitor restored Notch signaling in myeloid cells and substantially improved their differentiation, both *in vitro* and *in vivo*. This study demonstrates a novel mechanism regulation of Notch signaling in cancer. This may suggest a new perspective for pharmacological regulation of differentiation of myeloid cells in cancer. *Cancer Res; 74(1); 141–52. ©2013 AACR.*

Introduction

There is now ample evidence supporting the critical role of myeloid cells in tumor progression. The major changes that are observed in the myeloid compartment include accumulation of myeloid-derived suppressor cells (MDSC), defects in differentiation of dendritic cells, and accumulation and polarization of tumor-associated macrophages (1). Although some specific proteins factors such as STAT3, CEBP/β, PIR-B, NF-κB, and COX (2–12) were implicated in abnormal myeloid cell differentiation, this process remains unclear, which limits therapeutic targeting of myeloid cells.

Myeloid cell development in bone marrow is regulated by complex network of cytokines and by the direct physical interaction between hematopoietic progenitor/stem cells (HPC) and stromal cells (reviewed in; ref. 13). Signaling through the Notch family of transcriptional regulators plays a major role in the direct interaction between HPC and stroma. Each member of Notch family is a large, single heterodimeric receptor composed of noncovalently associated extracellular (ECN), transmembrane (TMN), and intracellular (ICN) sub-

units. The ICN region contains an RBPjκ association module (RAM) domain that binds the transcriptional repressor CSL (CBF-1, RBP-J), a series of cdc10/ankyrin repeats (ANK domain) that are involved in protein–protein interactions with CSL/CBF-1 and other polypeptides, and a C-terminal PEST sequence responsible for the degradation of the ICN signal. At present, 2 major Notch ligand families, Delta (Dll, Dll1-4) and Jagged (Jag, Jag1-4), have been described. Binding of the ECN domain of Notch by a Notch ligand results in the cleavage of ICN and its translocation to the nucleus where, together with number of recruited coactivators, it interacts with CSL. The binding of ICN with CSL displaces corepressor complexes, thereby activating transcription from promoters with CSL binding elements. In mammals, targets of ICN/CSL signals include genes of the Hairy/Enhancer of Split (HES) family, HRT/HERP genes, cyclin D1, p21, NF-κB, and many others (14–17).

Available data provide a consensus view on the critically important role of Notch signaling in myeloid cell differentiation. However, the exact nature of Notch effects remains controversial. Some existing data demonstrated the critical role of Notch in maintenance of progenitor cells and blockade of terminal differentiation of myeloid cells, whereas the other data showed requirements of Notch signaling for differentiation of mature myeloid cells (reviewed in ref. 18). It seems that the impact of Notch signaling depends on the stage of myeloid cell differentiation when Notch activation is triggered, the presence of specific cytokines, and on whether activation of Notch signaling was triggered by soluble or immobilized ligands. A number of studies have demonstrated that Notch signaling is important for dendritic cell differentiation (19–26). Notch signaling was shown to be also important for macrophage

Authors' Affiliations: [1]H. Lee Moffitt Cancer Center, Tampa, Florida; [2]The Wistar Institute, Philadelphia, Pennsylvania; and [3]University of Nebraska Medical Center, Omaha, Nebraska

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

Corresponding Author: Dmitry Gabrilovich, The Wistar Institute, Room 118C, 3601 Spruce Street, Philadelphia, PA 19104-4265. Phone: 215-495-6955; Fax: 215-573-2456; E-mail: dgabrilovich@wistar.org

localization and for interactions of endothelial cells during sprouting angiogenesis (27). Notch was also implicated in accumulation of immature myeloid cells (IMC) that can be defined as Gr-1$^+$CD11b$^+$, in mice. With overexpression of ADAM10, which is involved in the cleavage of ICN, systemic expansion of Gr-1$^+$ CD11b$^+$ was observed (28).

In this study, we investigated possible changes in Notch signaling in myeloid cells in cancer and the consequences of those changes for differentiation of myeloid cells. We present evidence indicating that downregulation of Notch signaling plays a major role in abnormal myeloid cell differentiation in cancer. Furthermore, this inhibition could be caused by the disruption of physical interaction between Notch and CSL and implicated upregulation of casein kinase 2 (CK2) activity in that disruption.

Materials and Methods

Mice and tumor models

Six- to eight-week-old female BALB/c, C57BL/6, and CD45.1$^+$ mice were purchased from the National Cancer Institute. All animal experiments were approved by the University of South Florida Institutional Animal Care and Use Committee. EL4 lymphoma, CT26 colon carcinoma, and MethA sarcoma were established by subcutaneously injection of 5×10^5 cells into C57BL/6 (EL-4) or BALB/c (CT26, MethA) mice. In most experiments, cells were analyzed 3 weeks after tumor injection when tumor reached 1.2 cm in diameter.

Human subjects and isolation of cells

Six patients with renal cell carcinoma were enrolled to the study after signing informed consent form approved by the University of South Florida Internal Review Board. Samples of peripheral blood from 5 healthy volunteers were obtained from blood bank. Mononuclear cells were isolated using a Ficoll gradient density centrifugation. Monocytes (healthy donors) and MDSC (cancer patients) were collected from mononuclear fraction and CD14$^-$CD11b$^+$CD15$^+$ and CD14$^-$CD11b$^+$CD15$^-$ cells were sorted by FACSAria cell sorter (BD Biosciences). PMNs were collected after centrifugation on 63 of 72% Percoll gradient above 72% layer and CD14$^-$CD11b$^+$CD15$^+$ cells were sorted. The list of reagents is provided in supplementary data.

Isolation of mouse cells

Bone marrow cells were enriched for HPCs by depletion of lineage-specific cells with lineage depletion kit (Miltenyi Biotec). Isolation of the populations of myeloid cells was performed using biotinylated CD11c, Gr-1, or PE-conjugated CD34-specific antibodies followed by MiniMACS microbeads (Miltenyi Biotec). The purity of the cells was consistently more than 92% in all samples. In some experiments, Gr-1$^+$CD11b$^+$ cells were sorted using FACSAria cell sorter (BD Biosciences).

CK2α and Notch 1 constructs and transfection

CK2α plasmid pZW6 was obtained from Addgene. pcDNA3.1-ICN1-Myc plasmid was kindly provided by Dr. B. Osborne, University of Massachusetts, Amherst, MA. Preparation of retroviruses expressing CK2, ICN1-Myc, and mutant constructs is described in supplementary data. All retroviruses were generated by cotransfection of the constructs with pCL-Eco into the packaging cell line 293T using Geneporter-2 (Genlantis). The retroviral supernatants were harvested 48-hour posttransfection and used for infection of cells for 4 hours at 32°C in the presence of 4 μg/mL Polybrene (Sigma). After that time, viral supernatants were removed and complete culture medium supplemented with GM-CSF was added. Cells were cultured for 20 hours at 37°C, and then infection was repeated.

Cell cultures on Notch ligands and differentiation of dendritic cell

Fibroblast cell line expressing Jag1 was described previously (29). HPCs were cultured on the monolayer of irradiated (25 Gy) fibroblasts in the presence of 20 ng/mL GM-CSF. CD45$^+$ hematopoietic cells were isolated using microbeads and Mini-MACS columns with more than 93% purity. Culture of HPC on Dll1 immobilization on plastic was performed as described previously (29). Dendritic cells were generated from enriched bone marrow HPCs for 7 days with 20 ng/mL GM-CSF and tumor-conditioned media (TCM; 30%, v/v). For dendritic cell activation, LPS 1 μg/mL was added 24 hours before cell analysis. For human cell cultures, CD34$^+$ cells were cultured in medium supplemented with 20 ng/mL of GM-CSF and 20 ng/mL of IL-4. TCM from human breast carcinoma MCF7 and small lung cancer 81M1 cell lines were added at 30% (v/v).

Dendritic cell differentiation *in vivo*

Bone marrow cells obtained from naïve C57BL/6 mice (CD45.2) were depleted from lineage-positive cells using lineage cell depletion kit (Miltenyi Biotec) followed by sorting of lineage negative and c-kit positive cells using a FACSAria. Lin$^-$c-Kit$^+$ progenitor cells were cultured in myeloid long-term culture medium for primitive mouse hematopoietic cells, supplemented with 20 ng/mL of SCF, FLT3L, IL-6, and IL-11 as previously described (30). On days 1 and 2, cells were infected with retroviruses expressing ICN1-GFP. On day 4, 10^6 cells were injected intravenously into lethally irradiated (9.5 Gy) naïve or EL4 tumor-bearing (TB) CD45.1 congenic mice with 0.5×10^6 host whole bone marrow cells. Analysis was performed on day 12 after the cell transfer with CD45.2 and GFP double positive cell used for the analysis.

Allogeneic mixed leukocyte reaction

Splenocytes from naïve or TB Balb/c mice were irradiated at 20 Gy and mixed in triplicates with 2×10^5 T cells from naïve C57BL/6 mice at different ratios. [^{3}H]-thymidine (1 μCi) was added 18 hours before cell harvesting. Radioactivity was counted on a liquid scintillation counter (Packard Instrument).

TBCA treatment *in vivo* and *in vitro*

CT26 TB mice were treated with tetrabromocinnamic acid [(E)-3-(2,3,4,5-tetrabromophenyl)acrylic acid, TBCA; 1.7 mg in 200 μL dimethyl sulfoxide (DMSO)/water (1:4), i.p. daily for 6 days] or vehicle alone. Next day after the last treatment, mice were sacrificed and analyzed. For *in vitro* treatment, HPCs from naïve bone marrow were cultured in the presence TCM. TBCA (0.6–2.5 μmol/L) was added and medium was changed every day.

Western blot assay, immunoprecipitation, and electrophoretic mobility shift assay

Nuclear extracts were prepared and Western blotting was performed as described previously (29). Immunoprecipitation was carried out using 800 µg to 1.2 mg of whole cell lysates. Electrophoretic mobility shift assay (EMSA) was performed as previously described (31) and details are provided in supplementary data.

siRNA transfection

The cells were mixed with 100 nmol/L Silencer Select Pre-Designed siRNA (*Csnk2a1*) specific to sequences encoding CK2α exon 8, and another siRNA for exon 9 for confirmation and scrambled siRNA (Invitrogen) for control. The transfection was carried out using an Amaxa Nucleofactor Kit (Lonza). After 24 to 48 hours, the cells were collected and *Hes 1* expression was analyzed by quantitative real-time PCR.

Real-time quantitative PCR

PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems), and target gene assay mix containing sequence-specific primers for hes1, hes5, notch 1, notch 2, and 6-carboxyfluorescein (6-FAM) dye-labeled TaqMan minor groove binder (MGB) probe (Applied Biosystems). Amplification with 18S endogenous control assay mix was used for controls. Data quantitation was performed using the relative standard curve method or ΔCT. Expression levels of the genes were normalized by 18S rRNA.

Luciferase reporter assay

For the analysis of CSL luciferase activity, we used Notch reporter retroviral construct containing CSL responsive element (31). Activity was measured 18 hours after infection in duplicates on a Lumat LB 9501 luminometer (Berthold) and normalized to protein concentration.

CK2 activity

CK2 activity of whole cell lysates was measured using Millipore Assay Kit after precipitation of 750 µg of protein with CK2α antibody.

Statistical analysis

Statistical analysis was performed using a 2-tailed Student *t* test and GraphPad Prism 5 software (GraphPad Software Inc.), with significance determined at $P < 0.05$.

Results

Downregulation of Notch signaling in HPC and myeloid cells in tumor-bearing hosts

Impairment of differentiation of myeloid cells from HPC in the presence of TCM manifests in decreased proportion of CD11c+IA^d+ dendritic cells and accumulation of Gr-1+CD11b+ MDSC (Fig. S1A). In TB mice, the proportion of CD11c+IA^d+ dendritic cells in spleens was substantially reduced, whereas the proportion of Gr-1+CD11b+ MDSC was dramatically increased (Supplementary Fig. S1B).

To evaluate the role of Notch signaling in myeloid cell differentiation in cancer, we studied 3 populations of cells, which represent sequential stages of myeloid cell differentiation: (1) bone marrow CD34+ HPC; (2) spleen Gr-1+CD11b+ cells, which in naïve mice represent mixed population of precursors of myeloid cells, IMC; and in TB mice are characterized as MDSC (32); and (3) spleen CD11c+ MHC class II+ dendritic cells.

Expression of Notch target genes *hes1* and *hes5* was dramatically reduced in all 3 populations of cells isolated from TB mice as compared with the cells from naïve mice (Fig. 1A and B). In recent years, 2 subsets of MDSC were identified (1): CD11b+Ly6C^hiLy6G− monocytic MDSC (M-MDSC) representing ~10% of all MDSC and CD11b+Ly6C^loLy6G+ polymorphonuclear MDSC (PMN-MDSC) representing ~90% of these cells (Supplementary Fig. S1C). We compared the expression of Notch target genes in these populations sorted from bone marrow of TB mice with monocytes and PMN with the same phenotype sorted from bone marrow of control mice. PMN-MDSC but not M-MDSC had significantly lower expression of *hes1* and *hes5* than their control counterparts (Fig. 1C).

The activity of Notch signaling was further analyzed by measurement of the binding of transcriptional factor CSL/CBF1 to its DNA consensus sequence using EMSA. The CSL/CBF1-specific binding in HPCs from TB mice was substantially lower than the binding in HPCs from control mice (Fig. 1D). The CSL/CBF1 DNA binding activity was also evaluated in luciferase reporter assay. Notch activity in HPCs from TB mice was significantly ($P < 0.01$) lower than in HPCs from naïve mice. This effect was observed also when Notch signaling was activated by culturing HPCs on a monolayer of fibroblasts expressing Jag1 (Fig. 1E). Similar experiments were performed with Gr-1+CD11b+ cells isolated from spleens of TB and naïve mice. MDSC from TB mice had significantly ($P < 0.001$) lower Notch activity than was observed in the IMC from the control mice (Fig. 1F).

Notch activity (*HES1* expression) was evaluated in CD14− CD11b+CD15+ granulocytes isolated from peripheral blood of healthy donors and PMN-MDSC with the same phenotype isolated from patients with renal cell carcinoma. Expression of *HES1* in the cells from patients with cancer was significantly lower than in those from the healthy donors (Fig. 1G). A similar effect, albeit less pronounced, was observed in testing the population of CD14−CD11b+CD15− monocytic cells (Fig. 1G). Three-day culture of human CD34+ HPC with TCM from breast carcinoma MCF7 and small cell lung cancer cell line 81M1 caused significant ($P < 0.01$) decrease in the expression of *HES1* (Fig. 1H). These data indicate that Notch activity was strongly decreased in HPC and myeloid cells in TB mice, and in myeloid cells obtained from patients with kidney cancer.

The role of ligands and receptors in causing decreased Notch signaling in HPC and in myeloid cells in cancer

We evaluated the major components of ternary complex responsible for Notch signaling. The amounts of ICN1, ICN2 (2 major Notch receptors in HPC), and CSL in nuclear extracts from bone marrow HPCs were similar in control and TB mice (Fig. 2A). For both control and TB mice, similar levels of expression of *notch1* and *notch2* mRNA were observed in the bone marrow HPC and spleen-derived dendritic cells.
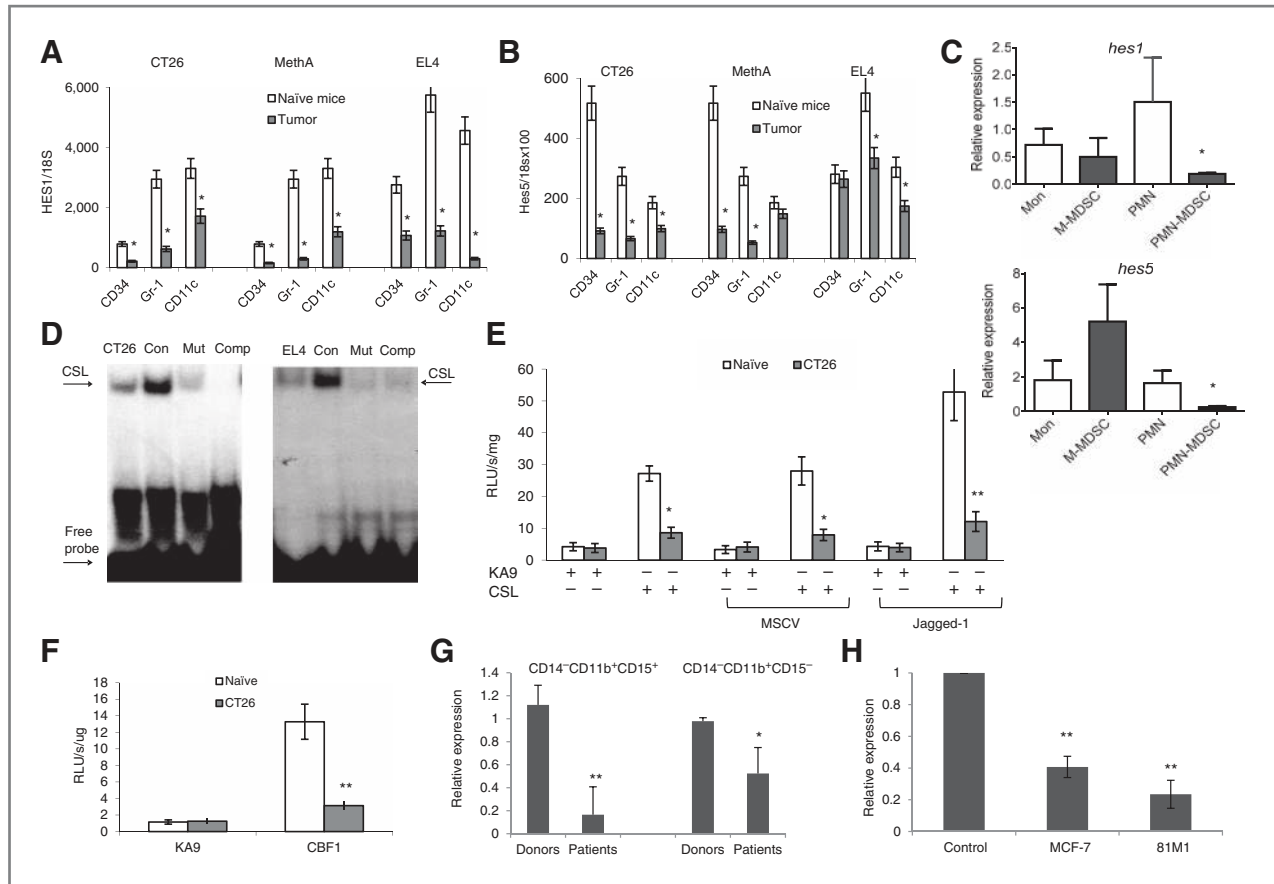
Figure 1. Downregulation of Notch signaling in myeloid cells. A and B, expression of *hes1* (A) and *hes5* (B) in CD34^{+} bone marrow cells, Gr-1^{+}, or CD11c^{+} splenocytes isolated from naïve or TB mice by quantitative PCR. Cumulative results of four experiments are shown. *, statistically significant differences between groups ($P < 0.05$). C, expression of *hes1* and *hes5* in bone marrow M-MDSC and PMN-MDSC from EL-4 TB mice and bone marrow Mon and PMN from naïve mice ($n = 3$; *, statistically significant differences between groups ($P < 0.05$)). D, CSL EMSA of nuclear proteins from enriched HPCs (naïve and indicated TB mice). Two experiments with the same results were performed. E, CSL/CBF1 reporter activity in HPCs from bone marrow of naïve or CT26 TB mice. Cells were infected with CSL-IL-2-Luc or control KA9 viruses, and cultured on monolayer of 3T3-Jag1–expressing fibroblasts or control MSCV fibroblasts. Three experiments (each in duplicates) were performed. Significant differences between groups: *, $P < 0.05$; **, $P < 0.001$. F, CSL reporter assay in Gr-1^{+} cells isolated from spleen of naïve or CT26 TB mice. Three experiments were performed. **, $P < 0.01$ between groups. G, expression of *HES1* in human cells isolated from patients with renal cell carcinoma and healthy donors. Four samples were analyzed by quantitative PCR. Statistically significant differences between groups: *, $P < 0.05$; **, $P < 0.01$. H, effect of indicated TCM on the expression of *HES1* in CD34^{+} cells isolated from blood of healthy donors. Cells were analyzed after 3 days of culture. Three experiments were performed. **, $P < 0.01$ from control.

Expression of *notch1* in MDSC from TB mice was even higher than in IMC from control mice (Fig. 2B and C). Similar results were obtained in experiments *in vitro* where bone marrow HPC were cultured in the presence of TCM. Significant reduction in the expression of *hes1* was observed, whereas no differences were seen in the expression of *notch1* and *notch2* (Fig. 2D). Thus, these data indicated that decrease in Notch signaling was not the result of downregulation of the receptors, their cleavage and nuclear translocation, or the amount of CSL.

We next addressed whether downregulation of Notch ligands could contribute to a reduced Notch signaling in HPCs. Dramatic downregulation of all studied Notch ligands (Jag1, Jag2, Dll1, 3, and 4) was found in bone marrow from CT26 TB mice as compared with naïve tumor-free mice (Fig. 2E).

We hypothesized that if decreased expression of Notch ligands was responsible for downregulation of signaling, then Notch signaling and dendritic cell differentiation should be

restored by incubating cells with immobilized ligand. To test this hypothesis, HPCs were cultured overnight with Dll1 immobilized on plastic. In the absence of TCM, Dll1 caused 6-fold increase in *hes1* expression. However, this upregulation was completely abrogated in the presence of TCM (Fig. 2F). Similar results were obtained in CSL luciferase reporter assay (Fig. 2G). Thus, Notch ligand could not restore the blockade of the Notch signaling caused by TCM, which indicated that this block was downstream of the receptor.

We next tested the effect of activation of Notch signaling on dendritic cell differentiation. HPC were cultured for 6 days with GM-CSF on immobilized Dll1 in the presence of TCM. In the absence of TCM, Dll1 promoted dendritic cell differentiation. However, Dll1 was not able to overcome the inhibitory effect of TCM on dendritic cell differentiation (Fig. 3A and Supplementary Fig. S2). The presence of dendritic cells was tested functionally using allogeneic mixed leukocyte reaction (MLR), the
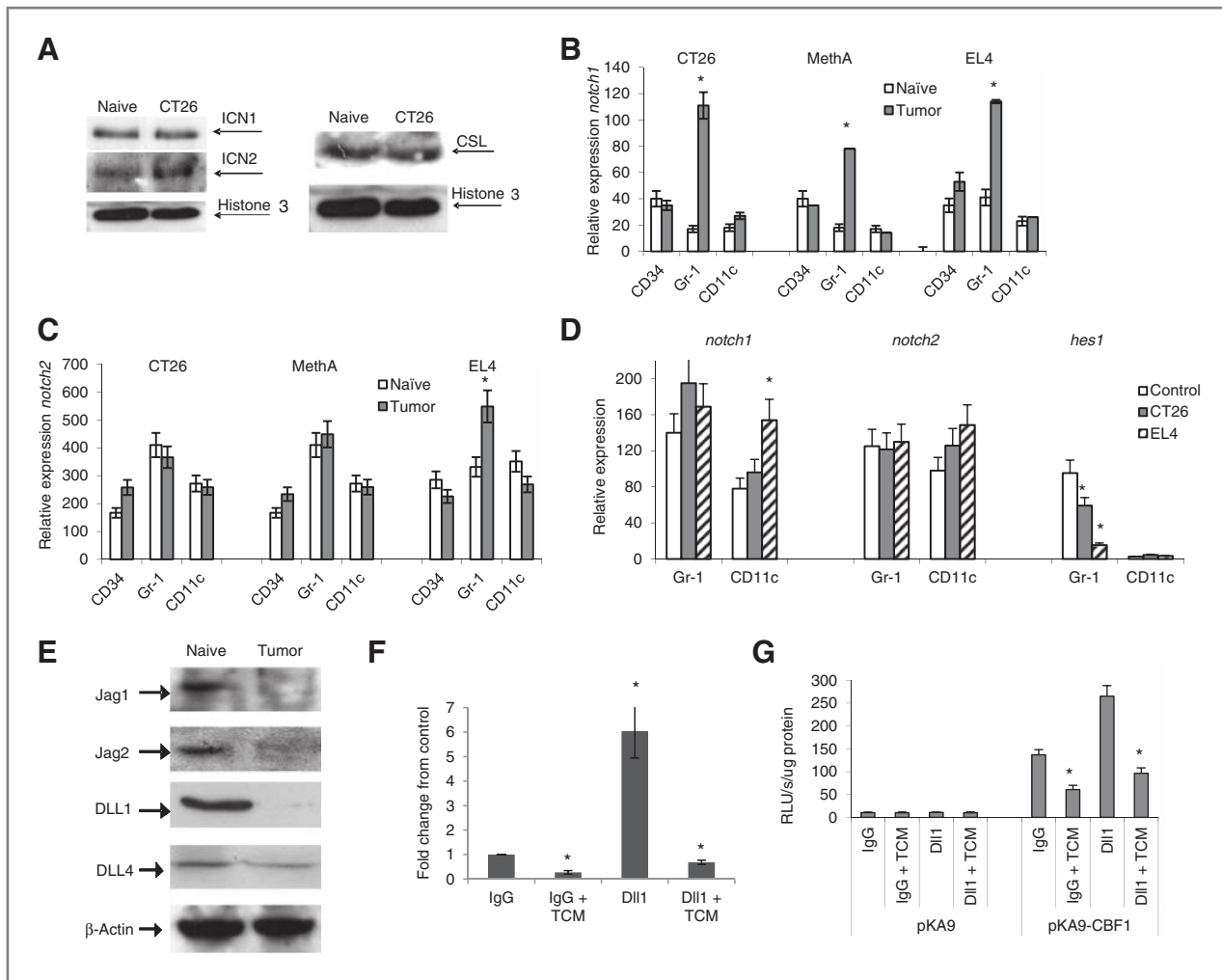
Figure 2. The role of Notch receptors and ligands in downregulation of Notch signaling. A, nuclear localization of Notch1, Notch2, and CSL. Nuclear proteins were analyzed by Western blot in HPCs from naïve or TB mice. Two experiments with the same results were performed. B and C, expression of *Notch1* (B) and *Notch2* (C) in indicated cells isolated from naïve or CT26 TB mice and assessed by quantitative PCR. Each experiment was performed in triplicate and includes three mice. *, statistically significant difference from control ($P < 0.05$). D, expression of *notch1*, *notch2*, and *hes1* in indicated cells generated *in vitro* from HPCs. Each experiment was performed in triplicate and includes three mice. E, notch ligands in bone marrow of naïve and CT26 TB mice. Two experiments with the same results were performed. F, expression of *hes1* evaluated by quantitative PCR in HPCs cultured on immobilized Dll1 with and without TCM. Each experiment was performed in triplicate. Cumulative results of three experiments are shown. G, luciferase reporter assay in HPC cultured on Dll1 with and without TCM. pKA9, control luciferase virus; pKA9-CBF1, virus containing CSL binding site. Each experiment was performed in duplicate. Combined results of three experiments are shown. In all experiments: *, statistically significant difference from control ($P < 0.05$).

hallmark of dendritic cell activity. TCM dramatically reduced allogeneic MLR in the cells generated from HPCs and abrogated stimulatory effect of Dll1 (Fig. 3B). These data indicated that the Notch ligand Dll1 was not able to rescue inhibited dendritic cell differentiation and MDSC expansion caused by TCM.

To bypass receptor–ligand interaction, we induced Notch signaling by overexpressing ICN1 using a retroviral construct containing ICN1 and GFP. The GFP served to track cells with ICN1 expression. In the absence of TCM, expression of ICN1 significantly increased the proportion of dendritic cells (Fig. 3C). However, the overexpression of ICN1 was not able to overcome the defect in dendritic cell differentiation or accumulation of IMC caused by TCM (Fig. 3C).

We also tested possible effect of ICN1 overexpression on HPC differentiation *in vivo*. Lin⁻c-kit⁺ myeloid progenitor cells were sorted from bone marrow of naïve CD45.2⁺ mice, infected with retrovirus expressing ICN1, and then injected intravenously into lethally irradiated naïve or EL4 TB CD45.1 congenic mice (Fig. 3D). Two weeks after the transfer, the donor cells (CD45.2⁺) transfected with ICN1 (GFP⁺) cells were evaluated in spleens of recipient mice. We observed that HPC transfected with control virus and transferred to TB mice produced significantly smaller proportion of dendritic cells but higher proportion of MDSC than HPC transferred to control mice (Fig. 3D and E). Expression of ICN1 in HPC transferred to naïve recipients resulted in upregulation of dendritic cells and of macrophages' differentiation. However, in TB mice no
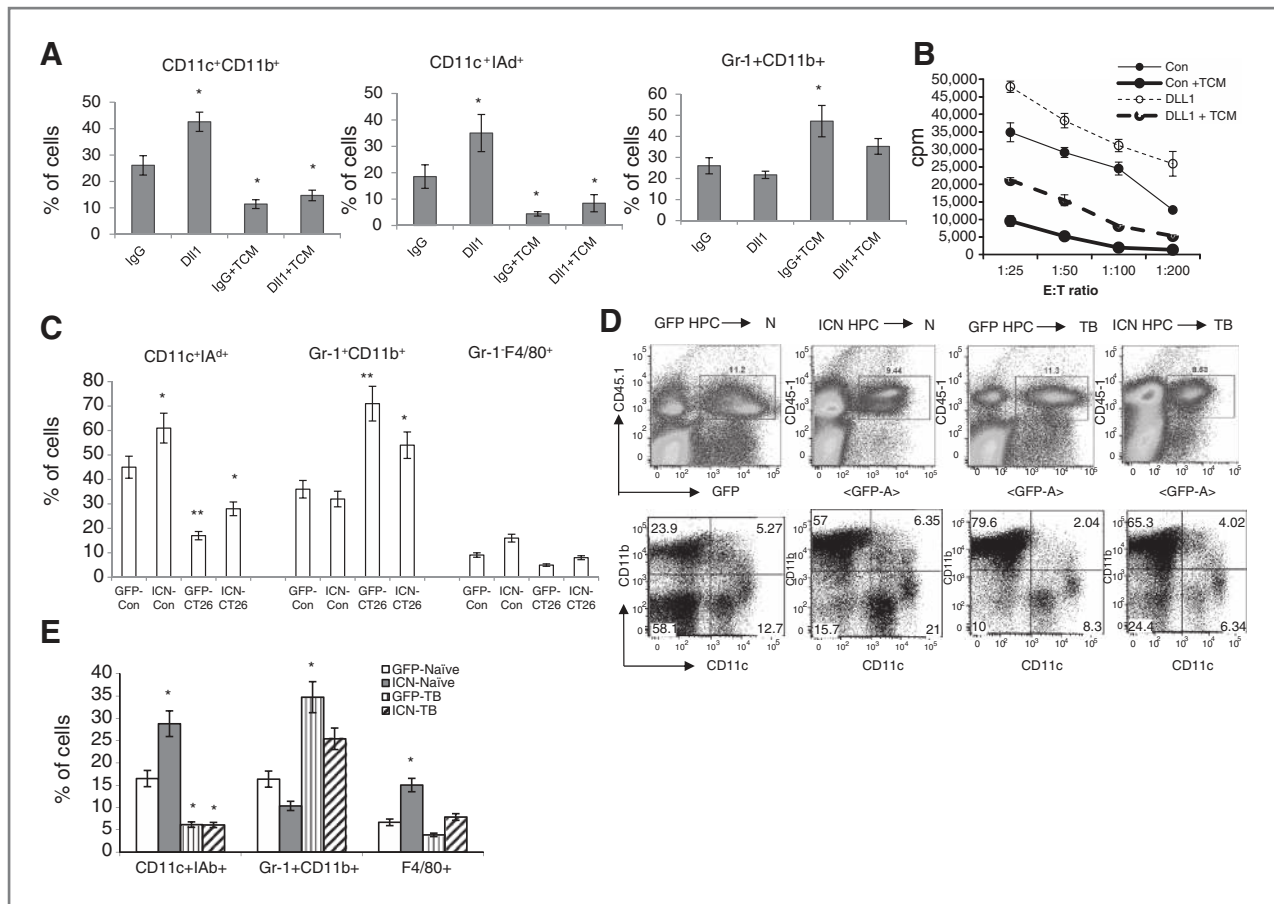
Figure 3. Activation of Notch signaling was not able to rescue inhibited dendritic cell differentiation in TB mice. A and B, differentiation of myeloid cells from HPC in the presence of immobilized Dll1 and TCM. Cells were cultured for 7 days and then phenotype (A) and function (B; allogeneic MLR) was evaluated. Three experiments were performed. C, differentiation of myeloid cells from HPC after transduction with ICN1. HPCs were infected with control (GFP) or ICN1-IRES-GFP (ICN) viruses and cultured with GM-CSF with (TCM) or without (Con) TCM for 7 days. Three experiments were performed. D and E, differentiation of myeloid cells *in vivo*. Lin⁻c-kit⁺ HPCs from CD45.2 naïve bone marrow were infected with control (GFP) or ICN1-IRES-GFP (ICN) viruses and injected intravenously to lethally irradiated CD45.1 naïve (N) or EL4 TB (TB) mice together with CD45.1⁺ bone marrow cells. On day 14, cell phenotype was analyzed within population of GFP⁺CD45.2⁺-transduced donors cells. D, typical example of the analysis. E, cumulative results of three experiments. In all experiments: *, statistically significant difference from control ($P < 0.05$).

improvement in dendritic cell differentiation was seen and changes in the proportion of MDSC were not statistically significant (Fig. 3E). Thus, activation of Notch signaling either by triggering the receptors or by overexpressing ICN1 did not overcome the defect in dendritic cell differentiation observed in TB mice.

Disruption of interaction between CSL and Notch in HPC and myeloid cells in cancer

Formation of Notch/CSL complex is necessary for the activation of the transcription of target genes. To assess physical interaction between Notch and CSL, HPCs were transduced with a retroviral ICN1-myc construct. Myc-specific antibody was used to pull-down ICN1 and membranes were probed with anti-CSL antibody. In HPC, cultured in control medium, the association between ICN and CSL was readily detectable. In contrast, in HPC, cultured in the presence of TCM, it was substantially reduced (Fig. 4A). Culture of HPC with TCM resulted in an increase in serine phosphorylation of

ICN1 (Fig. 4A), whereas no tyrosine phosphorylation was detected (data not shown).

Analysis of the structure of Notch suggested a number of serine residues in RAM domain, phosphorylation of which could affect binding of ICN1 with CSL. For instance, phosphorylation of serine 1901 and threonine 1898 were implicated in decreased formation of a Notch–Maml1–CSL ternary complex on DNA (33). To clarify the possible role of the specific serine residues in ICN/CSL interaction, we made ICN1 several constructs with mutations in serine residues in RAM domain of ICN1. Mutations in S_{1779} and S_{1856} were not sufficient to abrogate interaction between ICN1 and CSL (Fig. 4B). However, after mutations in S_{1901} and T_{1898}, ICN1 construct lost binding to CSL in MDSC (Fig. 4C) and was associated with substantially reduced level of serine phosphorylation in the presence of TCM (Fig. 4C). These data suggested that serine phosphorylation of ICN1 in cancer may block its binding to CSL and thus prevents the transcription of target genes.
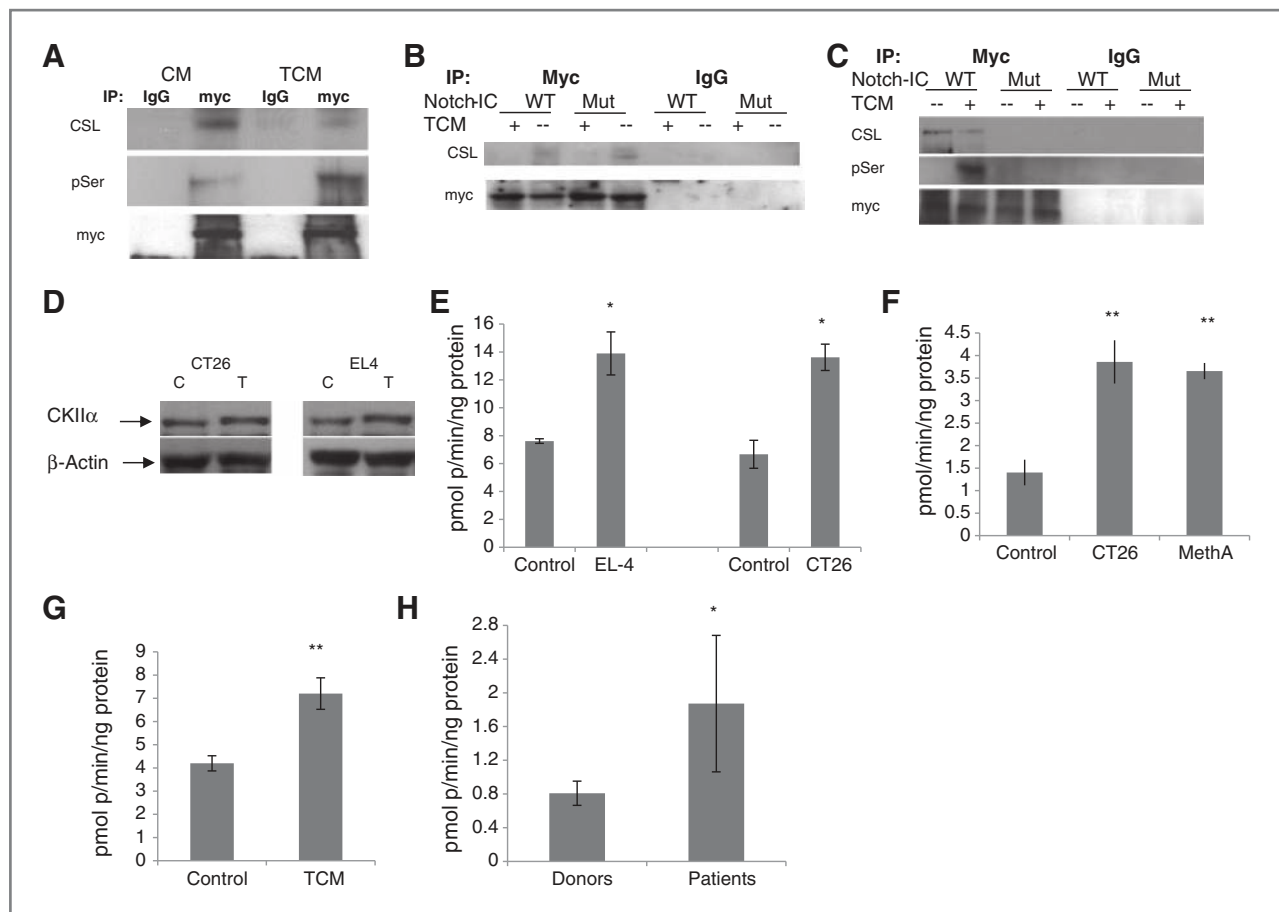
Figure 4. Disruption of interaction between CSL and Notch and CK2 activity in myeloid cells. A–C, HPCs were transduced with ICN1-Myc virus. Coprecipitation and serine phosphorylation of ICN1 (A), ICN-Myc virus with two mutations (B), or four mutations (C) as described in Materials and Methods. Cells were cultured with control media or TCM. On day 4, cells were collected and whole cell lysates were used for immunoprecipitation with Myc antibody or control immunoglobulin G (IgG) and then probed with CSL antibody or phosphor-serine antibody as indicated. Each experiment was performed at least twice. D, the amount of CK2α in Gr-1^{+} cells from bone marrow of naïve or TB mice. E and F, CK2 activity in HPC (E) or Gr-1^{+} cells (F) from bone marrow of naïve or TB mice. Cumulative results of four performed experiments are shown. G, CK2 activity in Gr-1^{+} cells generated from HPCs after 5-day culture with control or TCM-containing media. Cumulative results of three performed experiments are shown. H, CK2 activity in human CD11b^{+} CD15^{+} cells isolated from blood of renal cell carcinoma patients and healthy donors ($n = 3$). In all experiments, statistically significant difference from control: *, $P < 0.05$; **, $P < 0.01$.

CK2 regulated Notch activity in myeloid cells in cancer

What could induce Notch phosphorylation in myeloid cells in cancer? Serine/threonine CK2 previously was implicated in regulation of Notch activity under physiological conditions (33). We evaluated the presence of CK2 protein in Gr-1^{+}CD11b^{+} myeloid cells and found that the amount of catalytic α domain of CK2 protein was similar between control and TB mice (Fig. 4D). However, when kinase activity of CK2 was measured, it was significantly higher in HPC (Fig. 4E) or MDSC (Fig. 4F) than in cells from control mice. Gr-1^{+}CD11b^{+} myeloid cells generated from HPC in the presence of TCM had higher CK2 activity than Gr-1^{+}CD11b^{+} cells generated in the control medium (Fig. 4G). In humans, we observed similarly that CD14^{−}CD11b^{+}CD15^{+} MDSC isolated from blood of patients with renal cancer had significantly higher levels of CK2 activity than neutrophils with the same phenotype from healthy donors (Fig. 4H).

These results suggest that upregulation of CK2 activity may play a role in regulation of Notch activity in cancer. To test this possibility directly, 293T cells were transfected with the plasmid encoding catalytic α subunit of CK2 gene (csnk2a1; Fig. 5A). Cotransfection of CK2 plasmid with ICN1-myc plasmid caused phosphorylation of ICN1 and inhibited interaction of ICN1 with CSL (Fig. 5B). Csnk2a1 expression vector was cloned to a retroviral construct expressing GFP. Enriched bone marrow HPC were infected with control and CK2 retroviruses and cultured for 5 days with GM-CSF. Overexpression of CK2 in HPC reduced dendritic cell differentiation by half, whereas the proportion of Gr-1^{+}CD11b^{+} cells increased more than 2-fold (Fig. 5C). Thus, upregulation of CK2 in HPC blocked Notch signaling, inhibited dendritic cell differentiation, and caused accumulation of IMC.

Next, we tested the possibility that downregulation of CK2 might improve Notch signaling in myeloid cells from TB mice.
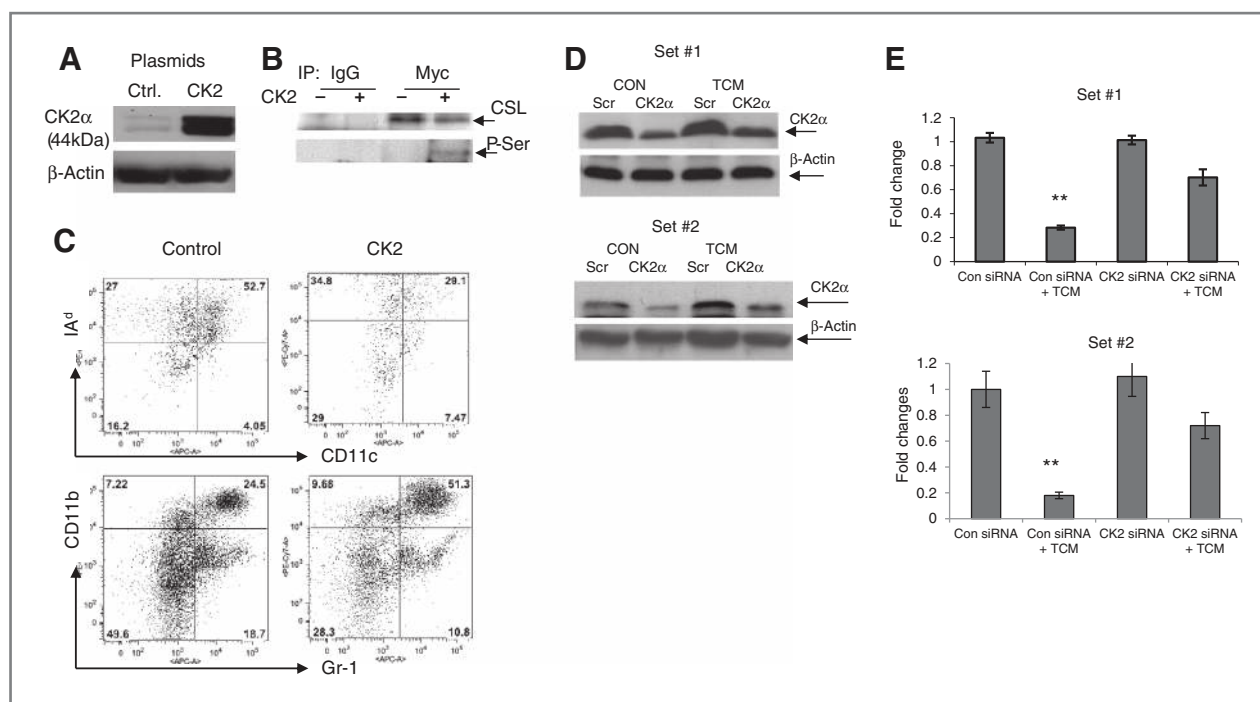
Figure 5. CK2 regulates Notch activity and inhibits dendritic cell differentiation. A and B, 293T cells were cotransfected with CK2 plasmid pZW6 and pcDNA3.1-ICN-Myc plasmid. Expression of CK2α (A) and ICN1 serine phosphorylation (B) after immunoprecipitation with myc antibody was measured 48 hours later by Western blotting. Two experiments with the same results were performed. C, phenotypes of dendritic cell differentiated from HPC transduced with CK2. Cells were analyzed on day 7 after infection with control or CK2 retroviruses and GFP$^+$ cells were evaluated. Two experiments with the same results were performed. D and E, *Hes1* expression in MDSC transfected with CK2α siRNA. D, confirmation of downregulation of CK2 expression using two different sets of siRNA. E, *Hes1* expression. Cumulative results of three experiments are shown. MDSC were isolated from spleen of TB mice, transfected with scrambled or CKIIα siRNA, *Hes1* expression was evaluated 24 hours later (E). **, statistically significant difference from control ($P < 0.01$).

MDSCs were isolated from EL4 TB mice, transfected with either control or CK2α2 siRNA, and cultured for 24 hours in either complete medium or with TCM. Two different sets of siRNA, targeting different parts of the sequence were used (Fig. 5D). We observed that MDSC transfected with control siRNA cultured with TCM had significantly ($P < 0.01$) lower *hes1* expression than cells cultured in complete medium. The treatment with the CK2 siRNA did not affect *hes1* expression in cells cultured in complete medium but substantially reduced negative effect of TCM of *hes1* expression (Fig. 5E). Thus, these data indicated that CK2 negatively regulated Notch activity in myeloid cells in cancer.

Inhibition of CK2 improves Notch signaling and differentiation of myeloid cells in cancer

We asked whether pharmacological inhibition of CK2 may increase Notch signaling in myeloid cells and consequently restore their differentiation in cancer. We used the selective CK2 inhibitor, tetrabromocinnamic acid [(E)-3-(2,3,4,5-tetra-bromophenyl)acrylic acid, TBCA; ref. 34). Treatment with TBCA did not affect CK2 activity in myeloid cells cultured without TCM, but at concentrations 1.2 to 2.5 μmol/L it abrogated increased activity of CK2 in MDSC (Fig. 6A). At those concentrations, no effect on cell viability was seen (data not shown). Inhibition of CK2 abrogated serine phosphoryla-

tion of ICN caused by TCM and restored interaction between ICN and CSL (Fig. 6B).

To test the effect of CK2 inhibitor on dendritic cell differentiation, TBCA was added to the culture of HPC with TCM. Addition of TBCA to the cultures abolished negative effect of TCM on dendritic cell differentiation (Fig. 6C). TCM caused expansion of PMN-MDSC from HPC, which was not corrected by Dll1 (Fig. 6D). However, TBCA in combination with Dll1 substantially reduced the effect of TCM on PMN-MDSC expansion (Fig. 6D).

We tested the possible effect of *in vivo* treatment with the CK2 inhibitor on 2 experimental models: CT26 and EG-7 (EL-4 tumor cells expressing chicken ovalbumin). *In vitro* testing in MTT assay revealed lack of TBCA toxicity in both cell lines at concentration as high as 10 μmol/L (data not shown). First, we tested mice with established, large CT-26 tumors (>1 cm in diameter) with short (6 days) treatment protocol. Mice were treated with intraperitoneal (i.p.) injections of TBCA and cells were evaluated 2 days after the finish of the treatment. One week of TBCA treatment did not significantly affect the size of established large tumors (Fig. 7A). Mice did not display any gross signs of toxicity (changes in body weight, posture, or behavior). TBCA caused significant decrease in CK2 activity in myeloid cells, both in bone marrow and spleens (Fig. 7B). This was associated with significant increase in the expression of
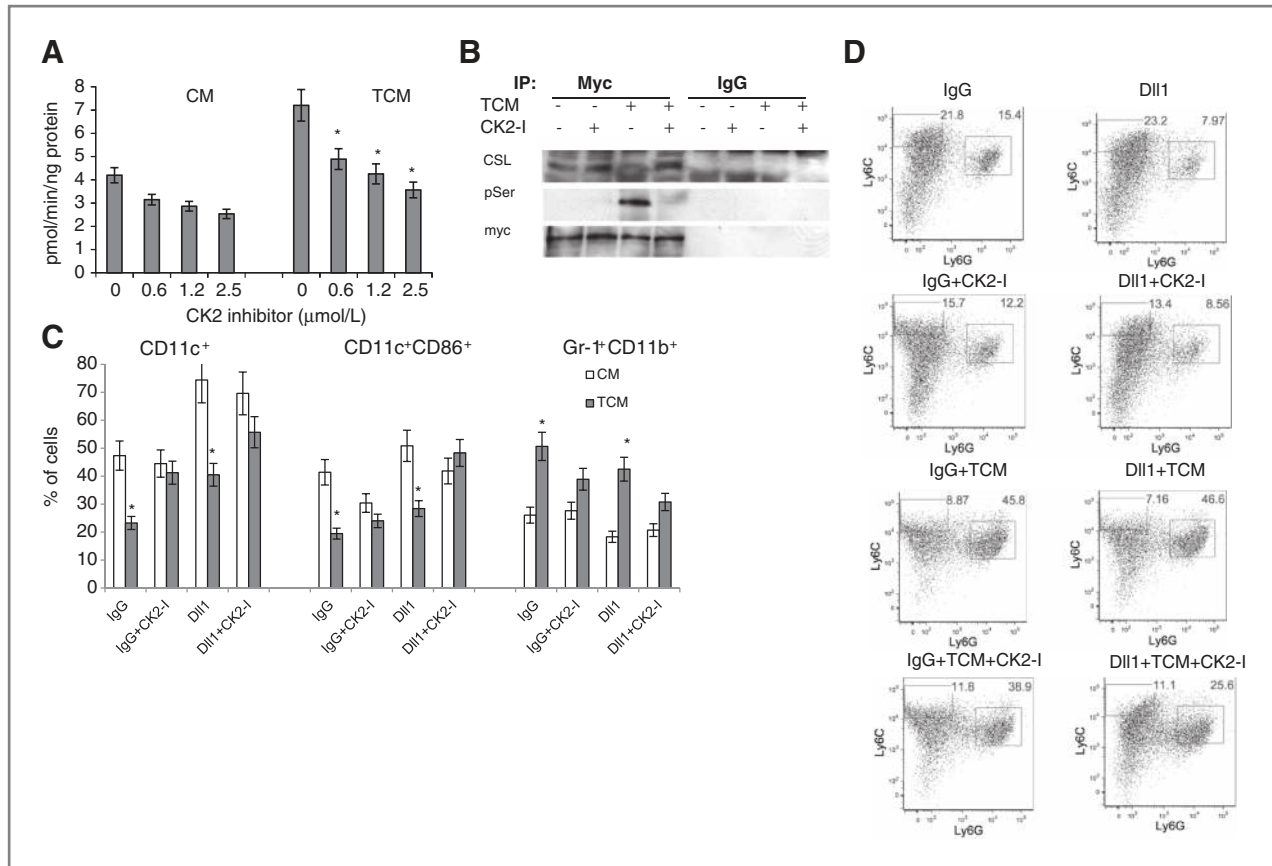
Figure 6. Improvement of Notch signaling and dendritic cell differentiation by CK2 inhibitor. A and B, MDSC from spleen of TB mice were cultured for 48 hours with control or TCM containing medium in the presence of indicated concentrations of TBCA. CK2 activity (A) and serine phosphorylation of ICN (B) were assessed. C, HPC were cultured for 7 days with GM-CSF on immobilized Dll1 or control IgG in medium alone or in the presence of TCM. TBCA (2.5 μmol/L; CK2-I) was added at the start of the culture. Medium was replaced every other day. Cell phenotype was analyzed by flow cytometry. Results of three experiments are shown. *, statistically significant difference from control (*P* < 0.01). D, HPC were cultured for 6 days with GM-CSF on immobilized Dll1 or control IgG in medium alone or in the presence of TCM. TBCA (5 μmol/L; CK2-I) was added at the start of the culture. Medium was replaced every other day. The phenotype of cells is shown. PMN-MDSC, Ly6C^{lo}Ly6G$^+$ cells; M-MDSC, Ly6C^{hi}Ly6G$^-$ cells. Two experiments with the same results are shown.

hes1 in these cells (Fig. 7C). Treatment of mice with TBCA resulted in significant increase in the proportion and absolute number of dendritic cells in spleens (Fig. 7D and E) or LN (Fig. 7D and F). Splenocytes from TBCA-treated mice demonstrated substantially higher stimulatory activity in allogeneic MLR, reflecting the presence of higher proportion of dendritic cells (Fig. 7G). Thus, inhibition of CK2 activity resulted in improved Notch signaling and dendritic cell differentiation in TB mice. To assess whether these changes in myeloid cells can result in antitumor effect, we used immunogenic EG-7 tumor and longer treatment. Mice with palpable tumors were treated for 2 weeks with TBCA. No signs of toxicity were evident. TBCA caused significant decrease in the tumor growth (*P* = 0.01; Fig. 7H).

Discussion

In this study, we observed dramatic reduction of Notch signaling in HPC and myeloid cells from TB mice and patients with cancer as compared with their control counterparts. Because PMN-MDSC decreased expression of Notch ligands

in TB hosts was previously described (35), this could explain the observed defect in Notch signaling. We have confirmed substantial downregulation of all Notch ligands in bone marrow of TB mice. However, to our surprise, this decrease was not responsible for the downregulation of Notch signaling. Neither Dll1, a potent activator of Notch signaling, nor overexpression of ICN1 were able to restore Notch signaling or myeloid cell differentiation in HPC isolated from TB mice or cultured in the presence of tumor-derived factors. This observation, together with the fact that the amount of cleaved ICN1 translocated to the nuclei was not changed, indicated that the defect in Notch signaling in cancer was downstream of the receptor.

Formation of ternary complex is required for activation of canonical Notch signaling. Although this complex includes several different components, the central element of that complex is the interaction between ICN and CSL. We found that this physical interaction between ICN1 and CSL was disrupted in myeloid cells from TB mice. This disruption was associated with serine phosphorylation of ICN1. Consistent with recent observation made under physiological conditions
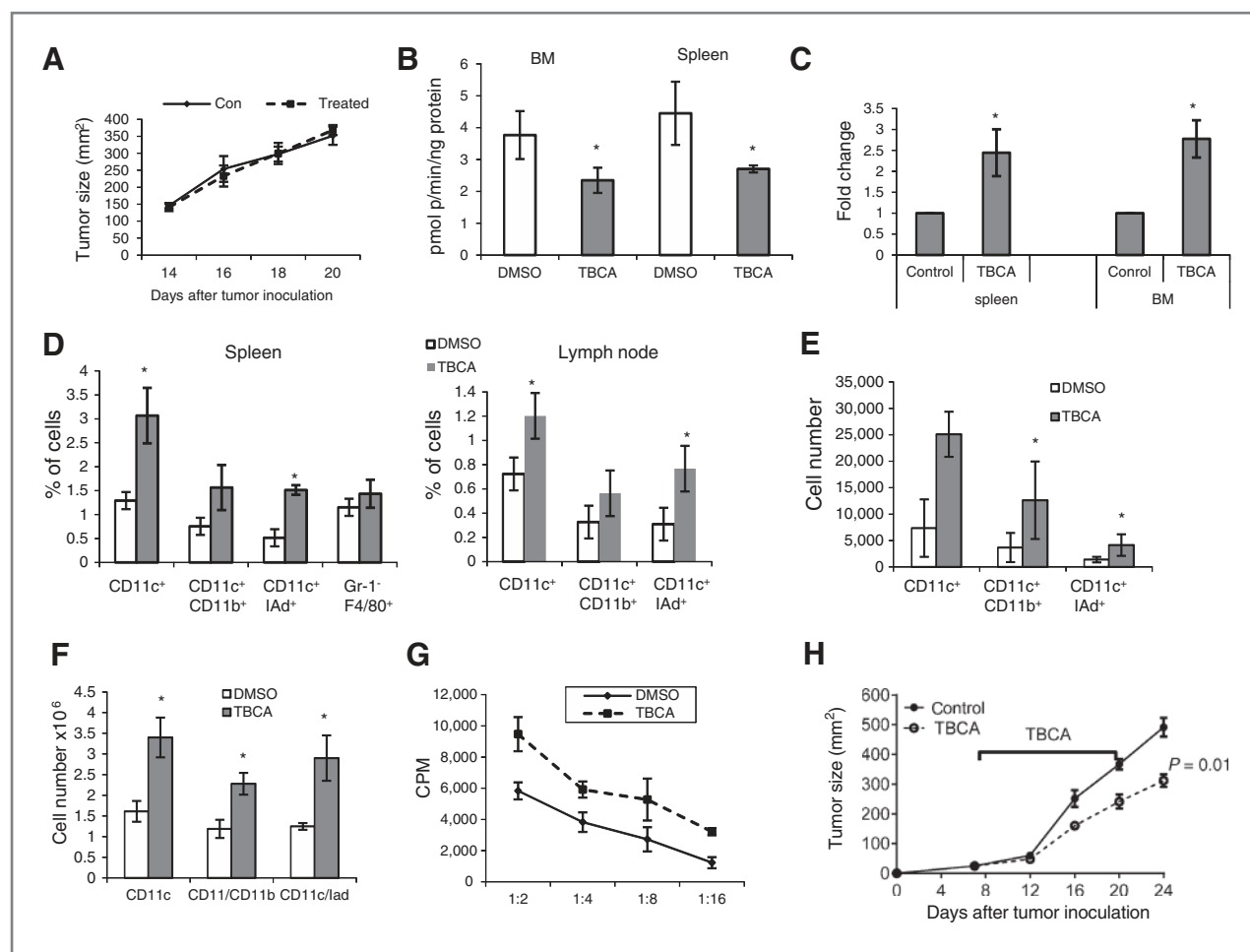
Figure 7. Effect of CK2 inhibition on dendritic cell differentiation *in vivo*. CT26 TB mice were treated with TBCA [1.7 mg in 200 µL DMSO/water (1:4), i.p. daily] or vehicle alone (Con) for 6 days. Cells were collected on day 7. Each experiment includes four mice. A, tumor size in treated mice; B, CK2 activity of MDSC isolated from bone marrow and spleens; C, expression of *hes1* mRNA in the same cells; D, proportion of myeloid cells in spleens and lymph nodes. E and F, absolute number of cells in spleens (E) and lymph nodes (F). G, allogeneic MLR of splenocytes from treated mice. T cells from C57BL/6 mice were used as responder cells. In all experiments, *, $P < 0.05$ from control. H, EG-7 tumors were established subcutaneously by injecting 0.5×10^6 cells. TBCA treatment started on day 7 after tumor inoculation and was continued for 14 days. Tumor size was measured. Each group included 3 mice.

(33), mutations in S_{1901} and T_{1898} completely abrogated physical interaction between ICN1 and CSL in MDSC. Taken together, these data suggested that inhibition of Notch signaling in HPC and myeloid cells in cancer could be the result of increased serine phosphorylation of RAM domain of Notch and disruption of formation of ternary complex. Our data implicated CK2 as the kinase possibly responsible for this phenomenon.

Serine/threonine kinase CK2 is ubiquitously expressed in a variety of cell types and tissues, and is considered to be constitutively active. CK2 is involved in every stage of cell cycle, by phosphorylating proteins required for G_0–G_1, G_1–S, and G_2–M transitions. CK2 may act as a general regulator of all cellular transcription and apoptosis (36). The function of CK2 in normal hematopoiesis is largely unknown. However, there are some indications that CK2 may play an important role in this process. For instance, it has been shown that CK2 activate Wnt and hedgehog signaling pathways (37, 38). However, CK2

negatively regulate PTEN activity (39) and activate Akt (40). In addition, CK2 stimulates activity of several transcription factors involved in hematopoiesis: c-Myb (41), PU.1 (42), and Ets-family transcription factors Sp1 and Sp3 (43). Although CK2 is constitutively active, its overexpression was documented in the number of solid and hematologic malignancies (44, 45). Our data indicate that although the amount of this enzyme was not changed in myeloid cells from TB mice, its activity was substantially higher than in the corresponding control leukocytes. Our experiments demonstrated CK2 upregulation was caused by tumor-derived factors, although the nature of these factors needs to be elucidated. Overexpression of catalytic domain of CK2 was sufficient to disrupt interaction between Notch and CSL and, most importantly, to block dendritic cell differentiation, which mimics the conditions observed in TB hosts. Downregulation of CK2 in MDSC improved Notch signaling. These data support the role of CK2 in negative regulation of Notch signaling and myeloid cell differentiation

in cancer. From this perspective, pharmacologic inhibition of CK2 may have value in an immunotherapeutic anticancer approach.

Our initial data supported this possibility. We used selective CK2 inhibitor TBCA. It was described to inhibit CK2 at IC_{50} values 0.11 μmol/L, without having any comparable effect on a panel of 28 protein kinases (34). In our experiments, TBCA had little effect of CK2 activity in control myeloid cells but at concentration 0.6 μmol/L, TBCA significantly attenuated the increased activity in myeloid cells from TB mice. Similarly, TBCA did not have effect on Notch signaling in control myeloid cells, but restored inhibited Notch signaling in the cells from TB hosts. These results may suggest that TBCA could target only elevated levels of CK2 activity.

We observed interesting dichotomy in changes in Notch signaling between PMN-MDSC and M-MDSC. Notch signaling was substantially reduced in PMN-MDSC as compared with PMN and CK2 inhibition markedly reduced TCM inducible expansion of these cells. In contrast, no differences in Notch signaling were observed between M-MDSC and Mon and CK2 inhibition had no effect on the presence of M-MDSC during incubation with TCM. These results are consistent with our observations made in the total population of Gr-1$^+$CD11b$^+$ MDSC because PMN-MDSC represented more than 90% of these cells. The exact mechanism of this phenomenon is not clear. However, given recent findings suggesting that M-MDSC could be a major precursors of PMN-MDSC in tumor-bearing hosts (46), this may suggest that M-MDSC in TB mice are transition cells where changes in Notch signaling are difficult to detect. As we demonstrated earlier, the population of M-MDSC is heterogenic and consist of the cells with high potential to differentiate toward PMN-MDSC and cells that are not able to progress toward PMN-MDSC (46).

Experiments *in vivo* demonstrated that CK2 inhibitor significantly increased differentiation of dendritic cells and inhibited MDSC expansion in TB mice without displaying signs of toxicity. Thus, this study is a first report describing the mechanism that negatively regulates Notch signaling in myeloid cells in cancer and functional consequences of that downregulation. We implicated upregulation of CK2 in this process. In the future, a more detailed analysis of toxicity and the effect of the CK2 inhibitor on other immune cells will be necessary to establish its potential therapeutic utility. However, our findings here suggest that this approach can be of interest to improve regulation of myeloid cell function in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Cheng, V. Kumar, D. Gabrilovich
Development of methodology: P. Cheng, V. Kumar, H. Liu, D. Gabrilovich
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Cheng, V. Kumar, H. Liu, J.-I. Youn, M. Fishman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Cheng, V. Kumar, H. Liu, S. Sherman, D. Gabrilovich
Writing, review, and/or revision of the manuscript: P. Cheng, H. Liu, J.-I. Youn, M. Fishman, D. Gabrilovich
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Sherman
Study supervision: D. Gabrilovich

References

1. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 2012;12:253–68.
2. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPβ transcription factor. Immunity 2010;32:790–802.
3. Nefedova Y, Huang M, Kusmartsev S, Bhattacharya R, Cheng P, Salup R, et al. Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. J Immunol 2004;172:464–74.
4. Nefedova Y, Nagaraj S, Rosenbauer A, Muro-Cacho C, Sebti SM, Gabrilovich DI. Regulation of dendritic cell differentiation and antitumor immune response in cancer by pharmacologic-selective inhibition of the janus-activated kinase 2/signal transducers and activators of transcription 3 pathway. Cancer Res 2005;65:9525–35.
5. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 2005;11:1314–21.
6. Ma G, Pan PY, Eisenstein S, Divino CM, Lowell CA, Takai T, et al. Paired immunoglobin-like receptor-B regulates the suppressive function and fate of myeloid-derived suppressor cells. Immunity 2011;34:385–95.
7. Martino A, Badell E, Abadie V, Balloy V, Chignard M, Mistou MY, et al. Mycobacterium bovis bacillus Calmette-Guerin vaccination mobilizes innate myeloid-derived suppressor cells restraining *in vivo* T cell priming via IL-1R-dependent nitric oxide production. J Immunol 2010; 184:2038–47.
8. Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, et al. Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells. Am J Pathol 2010;176:2490–9.
9. Zhang Y, Liu Q, Zhang M, Yu Y, Liu X, Cao X. Fas signal promotes lung cancer growth by recruiting myeloid-derived suppressor cells via cancer cell-derived PGE2. J Immunol 2009;182:3801–8.
10. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res 2007;67:4507–13.
11. Donkor MK, Lahue E, Hoke TA, Shafer LR, Coskun U, Solheim JC, et al. Mammary tumor heterogeneity in the expansion of myeloid-derived suppressor cells. Int Immunopharmacol 2009;9:937–48.
12. Eruslanov E, Daurkin I, Ortiz J, Vieweg J, Kusmartsev S. Pivotal advance: tumor-mediated induction of myeloid-derived suppressor cells and M2-polarized macrophages by altering intracellular PGE2 catabolism in myeloid cells. J Leukoc Biol 2010;88:839–48.
13. Despars G, O'Neill HC. A role for niches in the development of a multiplicity of dendritic cell subsets. Exp Hematol 2004;32:235–43.
14. Osborne BA, Minter LM. Notch signalling during peripheral T-cell activation and differentiation. Nat Rev Immunol 2007;7:64–75.
15. Miele L. Notch signaling. Clin Cancer Res 2006;12:1074–9.

16. Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. Dev Cell 2009;16:633–47.
17. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009;137:216–33.
18. Cheng P, Zhou J, Gabrilovich D. Regulation of dendritic cell differentiation and function by Notch and Wnt pathways. Immunol Rev 2010;234:105–19.
19. Cheng P, Zlobin A, Volgina V, Gottipati S, Osborne B, Miele L, et al. Notch-1 regulates NF-κB activity in hematopoietic progenitor cells. J Immunol 2001;167:4458–67.
20. Cheng F, Wang HW, Cuenca A, Huang M, Ghansah T, Brayer J, et al. A critical role for Stat3 signaling in immune tolerance. Immunity 2003;19:425–36.
21. Yamamura K, Ohishi K, Katayama N, Kato K, Shibasaki T, Sugimoto Y, et al. Notch ligand Delta-1 differentially modulates the effects of gp130 activation on interleukin-6 receptor α-positive and -negative human hematopoietic progenitors. Cancer Sci 2007;98:1597–603.
22. Ohishi K, Varnum-Finney B, Serda RE, Anasetti C, Bernstein ID. The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells. Blood 2001;98:1402–7.
23. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell 2004;117:515–26.
24. Caton ML, Smith-Raska MR, Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8− dendritic cells in the spleen. J Exp Med 2007; 204:1653–64.
25. Lewis Kanako L, Caton Michele L, Bogunovic M, Greter M, Grajkowska Lucja T, Ng D, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. Immunity 2011;35:780–91.
26. Sekine C, Moriyama Y, Koyanagi A, Koyama N, Ogata H, Okumura K, et al. Differential regulation of splenic CD8− dendritic cells and marginal zone B cells by Notch ligands. Int Immunol 2009;21: 295–301.
27. Outtz HH, Tattersall IW, Kofler NM, Steinbach N, Kitajewski J. Notch1 controls macrophage recruitment and Notch signaling is activated at sites of endothelial cell anastomosis during retinal angiogenesis in mice. Blood 2011;118:3436–9.
28. Gibb DR, Saleem SJ, Kang DJ, Subler MA, Conrad DH. ADAM10 overexpression shifts lympho- and myelopoiesis by dysregulating site 2/site 3 cleavage products of Notch. J Immunol 2011;186:4244–52.
29. Cheng P, Nefedova Y, Corzo CA, Gabrilovich DI. Regulation of dendritic cell differentiation by bone marrow stroma via different Notch ligands. Blood 2007;109:507–15.
30. Cheng P, Corzo CA, Luetteke N, Yu B, Nagaraj S, Bui MM, et al. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. J Exp Med 2008;205:2235–49.
31. Cheng P, Nefedova Y, Miele L, Osborne BA, Gabrilovich D. Notch signaling is necessary but not sufficient for differentiation of dendritic cells. Blood 2003;102:3980–8.
32. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162–74.
33. Ranganathan P, Vasquez-Del Carpio R, Kaplan FM, Wang H, Gupta A, VanWye JD, et al. Hierarchical phosphorylation within the ankyrin repeat domain defines a phosphoregulatory loop that regulates Notch transcriptional activity. J Biol Chem 2011;286:28844–57.
34. Pagano MA, Poletto G, Di Maira G, Cozza G, Ruzzene M, Sarno S, et al. Tetrabromocinnamic acid (TBCA) and related compounds represent a new class of specific protein kinase CK2 inhibitors. Chembiochem 2007;8:129–39.
35. Huang Y, Lin L, Shanker A, Malhotra A, Yang L, Dikov MM, et al. Resuscitating cancer immunosurveillance: selective stimulation of DLL1-Notch signaling in T cells rescues T-cell function and inhibits tumor growth. Cancer Res 2011;71:6122–31.
36. St-Denis NA, Litchfield DW. Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. Cell Mol Life Sci 2009;66:1817–29.
37. Seldin DC, Landesman-Bollag E, Farago M, Currier N, Lou D, Dominguez I. CK2 as a positive regulator of Wnt signalling and tumourigenesis. Mol Cell Biochem 2005;274:63–7.
38. Jin Z, Mei W, Strack S, Jia J, Yang J. The antagonistic action of B56-containing protein phosphatase 2As and casein kinase 2 controls the phosphorylation and Gli turnover function of Daz interacting protein 1. J Biol Chem 2011;286:36171–9.
39. Al-Khouri AM, Ma Y, Togo SH, Williams S, Mustelin T. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3β. J Biol Chem 2005;280:35195–202.
40. Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F, et al. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death Differ 2005;12:668–77.
41. Cures A, House C, Kanei-Ishii C, Kemp B, Ramsay RG. Constitutive c-Myb amino-terminal phosphorylation and DNA binding activity uncoupled during entry and passage through the cell cycle. Oncogene 2001;20:1784–92.
42. Lodie TA, Reiner M, Coniglio S, Viglianti G, Fenton MJ. Both PU.1 and nuclear factor-κB mediate lipopolysaccharide-induced HIV-1 long terminal repeat transcription in macrophages. J Immunol 1998;161: 268–76.
43. Tan NY, Khachigian LM. Sp1 phosphorylation and its regulation of gene transcription. Mol Cell Biol 2009;29:2483–8.
44. Trembley JH, Wang G, Unger G, Slaton J, Ahmed K. Protein kinase CK2 in health and disease: CK2: a key player in cancer biology. Cell Mol Life Sci 2009;66:1858–67.
45. Piazza F, Manni S, Ruzzene M, Pinna LA, Gurrieri C, Semenzato G. Protein kinase CK2 in hematologic malignancies: reliance on a pivotal cell survival regulator by oncogenic signaling pathways. Leukemia 2012;26:1174–9.
46. Youn JI, Kumar V, Collazo M, Nefedova Y, Condamine T, Cheng P, et al. Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. Nat Immunol 2013;14: 211–20.