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Effects of Notch signaling on regulation of myeloid cell differentiation in cancer

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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 (DC) 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 down-regulation 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.

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 (DC), and accumulation and polarization of tumor associated macrophages (1). Although some specific proteins factors such as STAT3, CEBP β , PIR-B, NF-kB, 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 (BM) is regulated by complex network of cytokines and by the direct physical interaction between hematopoietic progenitor/stem cells (HPC) and stromal cells (reviewed in (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 comprised of noncovalently associated extracellular (ECN), transmembrane (TMN), and intracellular (ICN) subunits. The intracellular region contains a RAM (RBPjk association module) domain that binds the transcriptional repressor CSL (CBF-1, RBP-J), a series of cdc10/

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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, two major Notch ligand families, Delta (Dll, Dll1-4) and Jagged (Jag, Jag1-4), have been described. Binding of the extracellular 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 co-activators, it interacts with CSL. The binding of ICN with CSL displaces co-repressor 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- κ B 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 (rev. in (18)). It appears 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 DC differentiation (19–26). Notch signaling was shown to be also important for macrophage localization and for interactions of endothelial cells during sprouting angiogenesis (27). Notch was also implicated in accumulation of immature myeloid cells 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 down-regulation of Notch signaling plays a major role in abnormal myeloid cell differentiation in cancer. Further, this inhibition could be caused by the disruption of physical interaction between Notch and CSL and implicated up-regulation of casein kinase 2 (CK2) activity in that disruption.

Material and Methods

Mice and tumor models

6–8-wk-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 s.c. 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 FACS Aria cell sorter (BD Biosciences). PMNs were collected after centrifugation on 63/72% 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 (BM) 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 >92% in all samples. In some experiments, Gr-1⁺CD11b⁺ cells were sorted using FACS Aria cell sorter (BD Biosciences).

Casein kinase 2 α and Notch 1 constructs and transfection

Casein kinase 2 α plasmid pZW6 was obtained from Addgene. pcDNA3.1-ICN1-Myc plasmid was kindly provided by Dr. Osborne, University of Massachusetts. Preparation of retroviruses expressing CK2, ICN1-Myc, and mutant constructs is described in supplementary data. All retroviruses were generated by co-transfection of the constructs with pCL-Eco into the packaging cell line 293T using Geneporter-2 (Genlantis, San Diego, CA). The retroviral supernatants were harvested 48 hr post-transfection and used for infection of cells for 4 hr at 32°C in the presence of 4 μ g/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 hr at 37°C, and then infection was repeated.

Cell cultures on Notch ligands and differentiation of DC

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 MiniMACS columns with more than 93% purity. Culture of HPC on Dll1 immobilization on plastic was performed as described previously (29). DCs were generated from enriched BM HPCs for 7 days with 20 ng/ml GM-CSF and tumor conditioned media (TCM, 30% v/v). For DC activation, LPS 1 μ g/ml was added 24 hr prior to 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.

DC 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 FACS Aria. 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, 10⁶ cells were injected i.v. into lethally irradiated (9.5 Gy) naïve or EL4 TB CD45.1 congenic mice with 0.5 $\times$ 10⁶ 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 (MLR)

Splenocytes from naïve or TB Balb/c mice were irradiated at 20 Gy and mixed in triplicates with 2 $\times$ 10⁵ T cells from naïve C57BL/6 mice at different ratios. [³H]-thymidine (1 μ Ci) was added 18 hr prior to cell harvesting. Radioactivity was counted on a liquid scintillation counter (Packard Instrument, Meriden, CT).

TBCA Treatment *in vivo* and *in vitro*

CT26 TB mice were treated with TBCA (1.7 mg in 200 μ l 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 BM were cultured in the presence TCM. TBCA (0.6 - 2.5 μ M) was added and medium was changed every day.

Western blot assay, immunoprecipitation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and western blotting was performed as described previously (29). Immunoprecipitation was carried out using 800 μ g–1.2 mg of whole cell lysates. EMSA was performed as previously described (31) and details are provided in supplementary data.

Small interfering RNA transfection

The cells were mixed with 100 nM Silencer® Select Pre-Designed siRNA (*Csnk2a1*) specific to sequences encoding CK2 α 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 24–48 hr, the cells were collected and *Hes 1* expression was analyzed by quantitative RT-PCR.

Real-time qPCR

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 delta CT. 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 hr after infection in duplicates on a Lumat LB 9501 luminometer (Berthold, Germany) and normalized to protein concentration.

Casein kinase 2 activity

CK2 activity of whole cell lysates was measured using Millipore Assay Kit after precipitation of 750 μ g of protein with CK2 α 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

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

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

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

Expression of Notch target genes *hes1* and *hes5* was dramatically reduced in all three populations of cells isolated from TB mice as compared to the cells from naïve mice (Fig. 1A, B). In recent years two subsets of MDSC were identified (1): CD11b⁺Ly6C^{hi}Ly6G⁻ monocytic MDSC (M-MDSC) representing ~10% of all MDSC and CD11b⁺Ly6C^{lo}Ly6G⁺ polymorphonuclear MDSC (PMN-MDSC) representing ~ 90% of these cells (Fig. S1C). We compared the expression of Notch target genes in these populations sorted from BM of TB mice with monocytes and PMN with the same phenotype sorted from BM 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 cancer patients 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 kidney cancer patients.

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 (two major Notch receptors in HPC), and CSL in nuclear extracts from BM 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 BM HPC and spleen derived DCs. Expression of *notch1* in MDSC from TB mice was even higher than in IMC from control mice (Fig. 2B, C). Similar results were obtained in experiments *in vitro* where BM 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 down-regulation of the receptors, their cleavage and nuclear translocation, or the amount of CSL.

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

We hypothesized that if decreased expression of Notch ligands was responsible for down-regulation of signaling, then Notch signaling and DC 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 up-regulation 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 DC 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 DC differentiation. However, Dll1 was not able to overcome the inhibitory effect of TCM on DC differentiation (Fig. 3A and Fig S2). The presence of DCs was tested functionally using allogeneic MLR, the hallmark of DC 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 DC 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 DCs (Fig. 3C). However, the overexpression of ICN1 was not able to overcome the defect in DC 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 BM of naïve CD45.2⁺ mice, infected with retrovirus expressing ICN1, and then injected i.v. 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 DCs but higher proportion of MDSC than HPC transferred to control mice (Fig. 3D, E). Expression of ICN1 in HPC transferred to naïve recipients resulted in up-regulation of DCs and of macrophages' differentiation. However, in TB mice no improvement in DC 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 DC 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 construct with mutations in serine residues in RAM domain of ICN1. Mutations in S₁₇₇₉ and S₁₈₅₆ were not sufficient to abrogate interaction between ICN1 and CSL (Fig. 4B). However, after mutations in S₁₉₀₁ and T₁₉₈₉ 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.

Caseine kinase 2 regulated Notch activity in myeloid cells in cancer

What could induce Notch phosphorylation in myeloid cells in cancer? Serine/threonine caseine kinase 2 (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 up-regulation 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). Co-transfection 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 BM HPC were infected with control and CK2 retroviruses and cultured for 5 days with GM-CSF. Overexpression of CK2 in HPC reduced DC differentiation by half, whereas the proportion of Gr-1⁺CD11b⁺ cells increased more than two-fold (Fig. 5C). Thus, up-regulation of CK2 in HPC blocked Notch signaling, inhibited DC differentiation, and caused accumulation of IMC.

Next, we tested the possibility that down-regulation of CK2 might improve Notch signaling in myeloid cells from TB mice. MDSCs were isolated from EL4 TB mice, transfected with either control or CK2 α 2 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-tetrabromophenyl)acrylic acid, TBCA] (34). Treatment with TBCA did not affect CK2 activity in myeloid cells cultured without TCM, but at concentrations 1.2–2.5 μ M 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 phosphorylation of ICN caused by TCM and restored interaction between ICN and CSL (Fig. 6B).

To test the effect of CK2 inhibitor on DC differentiation, TBCA was added to the culture of HPC with TCM. Addition of TBCA to the cultures abolished negative effect of TCM on DC 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 two experimental models: CT26 and EG-7 (EL-4 tumor cells expressing chicken ovalbumin). *In vitro* testing in MTT assay revealed lack of TCDA toxicity in both cell lines at concentration as high as 10 μ M (**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 i.p. injections of TBCA and cells were evaluated two 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 BM and spleens (Fig. 7B). This was associated with significant increase in the expression of *hes1* in these cells (Fig. 7C). Treatment of mice with TBCA resulted in significant increase in the proportion and absolute number of DCs in spleens (Fig. 7D, E) or LN (Fig. 7D, F). Splenocytes from TBCA treated mice demonstrated substantially higher stimulatory activity in allogeneic MLR, reflecting the presence of higher proportion of DCs (Fig. 7G). Thus, inhibition of CK2 activity resulted in improved Notch signaling and DC 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 two 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 cancer patients as compared to their control counterparts. Since PMN-MDSC Since 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 down-regulation of all Notch ligands in BM of TB mice. However, to our surprise, this decrease was not responsible for the down-regulation 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 (33), mutations in S₁₉₀₁ and T₁₈₉₈ 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 G0/G1, G1/S, and G2/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). On the other hand, 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 up-regulation 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 DC differentiation, which mimics the conditions observed in TB hosts. Down-regulation 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 IC50 values 0.11 μ M, 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 μ M, 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 to 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 since 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 towards PMN-MDSC and cells that are not able to progress towards PMN-MDSC (46).

Experiments *in vivo* demonstrated that CK2 inhibitor significantly increased differentiation of DCs 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 down regulation. We implicated up-regulation 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Down-regulation of Notch signaling in myeloid cells

A, B. Expression of *hes1* (**A**) and *hes5* (**B**) in CD34⁺ BM cells, Gr-1⁺ or CD11c⁺ splenocytes isolated from naïve or TB mice by qPCR. Cumulative results of 4 experiments are shown. * - statistically significant differences between groups (p<0.05). **C.** Expression of *hes1* and *hes5* in BM M-MDSC and PMN-MDSC from EL-4 TB mice and BM Mon and PMN from naïve mice (n=3, * - statistically significant differences between groups (p<0.05)). **D.** CSL EMSA assay 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 BM 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 cells carcinoma and healthy donors. Four samples were analyzed by qPCR. 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.

Figure 2. The role of Notch receptors and ligands in down regulation of Notch signaling

A. Nuclear translocation of Notch1 and Notch2. Nuclear proteins were analyzed by Western blot in HPCs from naïve or TB mice. Two experiments with the same results were performed. **B, C.** Expression of *Notch1* (**B**) and *Notch2* (**C**) in indicated cells isolated from naïve or CT26 TB mice and assessed by qPCR. Each experiment was performed in triplicate and includes 3 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 3 mice. **E.** Notch ligands in BM of naïve and CT26 TB mice. Two experiments with the same results were performed. **F.** Expression of *hes1* evaluated by qPCR in HPCs cultured on immobilized Dll1 with and without TCM. Each experiment was performed in triplicates. 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 ($p < 0.05$).

Figure 3. Activation of Notch signaling was not able to rescue inhibited DC differentiation in TB mice

A, 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, E.** Differentiation of myeloid cells *in vivo*. Lin⁻c-kit⁺ HPCs from CD45.2 naïve BM were infected with control (GFP) or ICN1-IRES-GFP (ICN) viruses and injected i.v. to lethally irradiated CD45.1 naïve (N) or EL4 TB (TB) mice together with CD45.1⁺ BM 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).

Figure 4. Disruption of interaction between CSL and Notch and CK2 activity in myeloid cells
A–C. HPCs were transduced with ICN1-Myc virus. Co-precipitation and serine phosphorylation of ICN1 (**A**), ICN-Myc virus with 2 mutations (**B**) or 4 mutations (**C**) as described in methods. Cells were cultured with control media (CM) or TCM. On day 4, cells were collected and whole cell lysates were used for immunoprecipitation with Myc antibody or control 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 BM of naïve or TB mice. **E, F.** CK2 activity in HPC (**E**) or Gr-1⁺ cells (**F**) from BM 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: * - $p < 0.05$; ** - $p < 0.01$.

Figure 5. CK2 regulates Notch activity and inhibits DC differentiation

A, B. 293T cells were co-transfected 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 hr later by Western blotting. Two experiments with the same results were performed. **C.** Phenotypes of DC 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, E**). *Hes1* expression in MDSC transfected with CK2α siRNA. **D** - confirmation of down-regulation 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 24h later (**D**). ** - statistically significant difference from control ($p < 0.01$).

Figure 6. Improvement of Notch signaling and DC differentiation by CK2 inhibitor

A, B. MDSC from spleen of TB mice were cultured for 48 hr with control or TCM containing medium in the presence of indicated concentrations of TBICA. CK2 activity (**A**) and serine phosphorylation of ICN (**B**) were assessed. **C.** HPC were cultured for 7 days with GM-CSF on immobilized DII1 or control IgG in medium alone or in the presence of TCM. TBICA (2.5 μ M) (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 DII1 or control IgG in medium alone or in the presence of TCM. TBICA (5 μ M) (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^{hi}Ly6G⁺ cells; M-MDSC – Ly6C^{hi}Ly6G⁻ cells. Two experiments with the same results are shown.

Figure 7. Effect of CK2 inhibition on DC differentiation *in vivo*

CT26 TB mice were treated with TBCA (1.7 mg in 200 μ l DMSO/water (1:4), i.p. daily) or vehicle alone (Con) for 6 days. Cells were collected on day 7. Each experiment include 4 mice. **A**. Tumor size in treated mice; **B**. CK2 activity of MDSC isolated from BM and spleens; **C**. Expression of *hes1* mRNA in the same cells; **D**. Proportion of myeloid cells in spleens and lymph nodes. **E**, **F**. Absolute number of cells in spleens (**E**) and lymph nodes (**F**). **G**. Allogeneic MPR of splenocytes from treated mice. T cells from C57BL/6 mice were used as responder cells. In all experiments * indicated $p < 0.05$ from control. **H**. EG-7 tumors were established s.c. 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.