Notch-induced myeloid reprogramming in spontaneous pancreatic ductal adenocarcinoma by dual genetic targeting

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RUNNING TITLE: Myeloid-specific Notch modulation in pancreatic cancer

KEYWORDS: Genetically engineered mice, Notch, pancreatic cancer, tumor-associated macrophage, nanostring, Flp, Cre

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ACKNOWLEDGEMENTS
The authors would like to thank M. Schmid-Supprian for helpful discussions and providing recombinant CRE protein; U. Zimber-Strobel for providing R26<sup>LSL-N2IC</sup> mice; H. Nakhai for floxed Rbpj and Ptf1a<sup>Cre</sup> mice; T. Jacks and D.A. Tuveson for Kras<sup>LSL-G12D</sup> mice; A. Berns for floxed p53 mice. We thank N. Bielefeld, R. Hillermann, and S. Schäfers for excellent technical assistance and the International Max Planck Research School for Molecular Life Sciences (IMPRS) for providing educational and financial support to F. Neff.

FUNDING
This work was supported by the European Union’s Seventh Framework Programme for research, technological development and demonstration (FP7/CAM-PaC) under grant agreement n° 602783 (to J.T.S), the German Cancer Consortium (DKTK) (to J.T.S.) and the Deutsche Forschungsgemeinschaft (DFG, SI1549/1-1 to J.T.S.).

CONFLICT OF INTEREST DISCLOSURES
The authors declare no conflict of interest.
ABSTRACT (word limit: 250)

Despite advances in our understanding of the genetics of pancreatic ductal adenocarcinoma (PDAC), the efficacy of therapeutic regimens targeting aberrant signaling pathways remains highly limited. Therapeutic strategies are greatly hampered by the extensive desmoplasia that comprises heterogeneous cell populations. Notch signaling is a contentious pathway exerting opposite roles in tumorigenesis depending on cellular context. Advanced model systems are needed to gain more insights into complex signaling in the multi-layered tumor microenvironment. In this study, we employed a dual recombinase-based in vivo strategy to modulate Notch signaling specifically in myeloid cells to dissect the tumorigenic role of Notch in PDAC stroma. Pancreas-specific KrasG12D activation and loss of Tp53 was induced using a Pdx1-Flp transgene, while Notch signaling was genetically targeted using a myeloid-targeting Lyz2-Cre strain for either activation of Notch2-IC or deletion of Rbpj. Myeloid-specific Notch activation significantly decreased tumor infiltration by protumorigenic M2 macrophages in spontaneous endogenous PDAC, which translated into significant survival benefit. Further characterization revealed upregulated antigen presentation and cytotoxic T effector phenotype upon Notch-induced M2 reduction. This approach is the first proof-of-concept for genetic targeting and reprogramming of myeloid cells in a complex disease model of PDAC and provides evidence for a regulatory role of Notch signaling in intratumoral immune phenotypes.
INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies with no effective therapeutic options for long-term tumor control thus far. Therapeutic targeting of PDAC focusing on deregulated molecular signaling pathways in cancer cells has been largely disappointing. One of the hallmarks of PDAC is the presence of extensive desmoplasia, which comprises heterogeneous cell populations including fibroblasts, immune cells, and extracellular matrix (1, 2). Recent studies have revealed crucial role of stromal components in promoting the induction and progression of tumorigenesis (1, 3-5). Comprehensive studies on differential roles of signaling pathways in regulating tumor cells and stromal components in PDAC will advance our understanding of the complicated pathogenesis.

Notch signaling is a key developmental pathway that is known to regulate cell proliferation, apoptosis, as well as tumorigenesis of various solid tumors including pancreatic cancers (6-8). However, the role of Notch signaling in PDAC still remains highly contentious. Notch receptors and ligands, and their downstream targets have been frequently reported to be overexpressed in PDAC, suggesting a role in PDAC development and progression (7, 9). Activation of Notch1-IC led to progression of preneoplastic lesions in a genetic mouse model (10), while another study showed that genetic ablation of Notch1 in a mouse model of KRAS-induced PDAC resulted in an increase in high-grade pancreatic intraepithelial neoplasia (PanIN) lesions (11). We described an oncogenic role of Notch2 but not Notch1 in regulating PanIN progression and tumor differentiation (12). Notably, phase II clinical trials assessing the use of blocking antibodies against Notch2/3 or the Notch ligand Dll4 in combination with chemotherapy have reported no benefit or even detrimental outcomes (13, 14), therefore casting doubts onto the efficacy of Notch as a therapeutic target in PDAC.

Given the importance of stromal components in tumorigenesis, Notch might exert either oncogenic or tumor-suppressive role depending on cellular context. Indeed, impairment of Notch signaling in epidermis and fibroblasts was shown to result in profound changes in stroma, leading to induction of tumor development (15, 16). Increasing evidence has indicated that the Notch-mediated tumor suppression might be at least partially mediated by regulating inflammation in the tumor microenvironment (15, 17). Ablation of Notch in mice significantly upregulated proinflammatory cytokine expression (18, 19). Intriguingly, deletion or inactivation of Notch signaling induced inflammatory responses, which suppressed tumorigenesis through the anti-tumor function of T cells present in the inflammatory milieu (20, 21). Collectively, these studies demonstrated that Notch
signaling in stromal cells could induce tumor-suppressive effects through mediating inflammation in the tumor microenvironment. However, precise cellular and molecular mechanisms underlying these phenomena still need further examination.

Tumor-associated macrophages (TAM) represent the most abundant immune cell population in the tumor microenvironment, and dysregulated polarization facilitates tumor growth and metastasis of PDAC (22-24). In PDAC patients, increased tumor-promoting M2 TAMs are associated with significantly shorter patient survival (25-28). Recently, Notch signaling was shown to regulate myeloid cell differentiation (29), potentially via transcriptional repression of M2-associated genes by Notch activation (30), and upregulation of M2-associated genes via posttranslational modification of IRAK2(31). However, the relevance of these findings in complex disease models of cancer such as in PDAC has not been addressed so far. Moreover, the role of Notch signaling in myeloid cells in tumor formation and progression has not been investigated.

To study the context and spectrum of immune-tumor interactions, endogenous immunocompetent tumor models are necessary. Genetically engineered mouse models based on pancreas-specific activation of oncogenic KRAS resemble human PDAC in many aspects including formation of an extensive fibro-inflammatory stromal reaction (32). To target non-pancreatic and non-tumoral cell lineages, Schonhuber et al introduced a dual-recombinase approach using a Pdx1-Flp transgene to target KRAs and Tp53 (33), allowing selective Cre-based targeting of the tumor microenvironment. In this study, we investigated the role of Notch signaling in TAM polarization in PDAC development and progression using a dual-recombinase gene targeting strategy as a genetic proof-of-concept approach.

MATERIALS AND METHODS
Mouse strains and tumor models
Unless otherwise stated, Ptf1a^{wt/Cre};Kras^{wt/LSL-G12D};p53^{frt/frt} (CKP) was used as tumor model of spontaneous PDAC for phenotypic characterization and quantification of immune cells. For the dual-recombinase system, a myeloid-specific Cre-line (Lyz2^{wt/Cre}) was crossed to R26^{wt/LSL-N2IC} and Rbpj^{fl/fl} mice(34, 35). Lyz2^{wt/Cre};R26^{wt/LSL-N2IC} (Lyz2;N2IC) and Lyz2^{wt/Cre};Rbpj^{fl/fl} (Lyz2;Rbpj) lines were crossed to the Pdx1-Flp;Kras^{wt/FSF-G12D};p53^{frt/frt} (FKP) or Pdx1-Flp;Kras^{wt/FSF-G12D};p53^{frt/frt} (FKP^{het}) model. Details of original and interbred mouse strains are listed in Supplementary Table S1A and B.

Isolation of Bone Marrow-derived Cells and macrophage differentiation
Bone marrow-derived cells were isolated from femur and tibia of mice aged between 6 and
12 weeks. Bone marrow was collected by flushing with RPMI medium (Life Technologies, NY). Red Blood Cells (RBC) were lysed with RBC Lysing Buffer (Sigma-Aldrich GmbH, Germany) for 5 min at RT, washed, and then re-suspended in macrophage medium (RPMI, 15% FBS, 5% horse serum, 1% NEAA, 1% sodium pyruvate) (Life Technologies) containing 50 ng/ml murine M-CSF (PELOBiotech, Germany) for 7 days. The uncoated surface of petri dishes allows attachment of highly adherent cells as macrophages, while floating cells were removed by rinsing with PBS. Macrophage phenotype and purity were assessed based on CD11b and F4/80 expression using flow cytometry.

**Induction of Cre-dependent LoxP site Recombination and polarization in vitro**
BMDM were treated with 1 µM recombinant NLS-His-Tat-NLS-CRE protein(36) in serum-free RPMI medium 1:1 diluted in PBS overnight. Culture supernatant was removed the next day and then changed to complete macrophage medium. To induce M1 polarization, cells were treated with 1 µg/ml LPS (Sigma-Aldrich, St. Louis, MO) for 6 or 24 h; while for M2, cells were treated with 10 ng/ml recombinant murine IL-4 (eBioscience, San Diego, CA) for 72 h.

**Isolation of tumor cells from endogenous PDAC**
Tumors were minced and then digested in 1 mg/ml collagenase type V (Sigma-Aldrich GmbH) for 45 min at 37°C. The cells were filtered through a 100 µm cell strainer. Red blood cells were lysed by RBC lysing buffer for 5 min at RT. Cells were then washed, filtered through 40 µm cell strainer, and subject to subsequent experiments.

**Flow cytometric analysis and cell sorting**
Multi-color flow cytometry experiments were performed using Beckman Coulter Gallios™ flow cytometer (Beckman Coulter, Irving, TX), while cell sorting was performed using BD FACS Aria III (BD Biosciences, San Jose, CA). All samples were incubated with CD16/32 antibody (BD Biosciences) to block unspecific FC receptor-mediated antibody binding prior to antibody incubation. Dead cells were excluded by staining with Fixable Viability Dye 780 (eBioscience, San Diego, CA), LIVE/DEAD® Fixable Yellow stain (Life Technologies), or propidium iodide (PI) (Clontech, Mountain View, CA). For intracellular staining, cells were fixed with 2% PFA/PBS after extracellular staining. Cells were then permeabilized with 0.5% Saponin/PBS prior to antibody incubation. For FOXP3 staining, the Foxp3/Transcription Factor Buffer Set (eBioscience) was applied. After washing, cells were subjected to flow cytometric analysis. The antibody list is shown in Supplementary Table 2. Raw data were analyzed using FlowJo software version 7.5.5 (Tree Star Inc., Ashland, OR).

**Immunohistochemistry**
Immunohistochemical staining was performed using the Dako REAL™ Alkaline Phosphatase or Peroxidase Detection System (Dako, Germany), following manufacturer’s instructions.

Antigen retrieval on formalin-fixed paraffin-embedded (FFPE) sections was performed by heat-induced epitope retrieval using citrate buffer (pH6) for CD11b, hCD2, MRC1, NK1.1, collagen, MHCII, CD11c and CD80; Tris/EDTA (pH9) for CD3, CD4, CD8, Eomes, T-bet and Lag3, and proteinase K treatment for F4/80. After blocking with serum free protein blocking solution (Dako), slides were incubated for primary antibodies for 1 hr at RT, secondary antibody for 30 minutes at RT, and then subjected to Fast Red or DAB chromogen development. The antibody list is shown in Supplementary Table 3. Slides were then counterstained with hematoxylin, dehydrated, and mounted. Stromal content and acinar cells were determined by Movat's pentachrome staining following the manufacturer’s protocol (modified according to Verhoeff, Morphisto GmbH, Germany).

Slides were scanned and digitalized by Zeiss Axio Scanner Z.1 (Carl Zeiss AG, Germany) with 5x and 10x objective magnification. The percentage of positive cells for IHC staining, while the area of collagen, stroma (ground substance/ mucin stained bluish, collagen stained yellowish) and/or acinar cells (deep red) for Movat’s pentachrome staining were quantified by Definiens (Definiens AG, Germany) as the average of 5 representative fields (10X objective magnification) captured from each tissue section of each mouse. For quantification, total number of cells in each field was determined based on nuclei staining (hematoxylin for IHC; DAPI for immunofluorescence staining) detected by the software. The average number of positive cells (percentage in total number of cells in each field) from the 5 representative fields was taken as the percentage of positive cells for each individual mouse. For quantification of Ki67 and cleaves caspase 3, the 5 representative fields were captured in the area of tumor cells as we aimed to show the proliferation and apoptosis of tumor cells upon myeloid-specific Notch modulation. Tumor cells could be distinguished based on their sizes and histology (infiltrating immune cells are largely found in stroma). Besides, area of extensive immune cell infiltration e.g. infiltrating lymph nodes or tertiary lymphoid structures, was avoided.

Immunofluorescence staining

FFPE sections were deparaffinized and then fixed with formaldehyde:methanol (1:10) prior to antigen retrieval by heat-induced epitope retrieval using citrate buffer (pH6) or Tris/EDTA (pH9). Each section was put through several sequential rounds of staining; each includes a protein blocking followed by primary antibody and corresponding secondary horseradish peroxidase-conjugated polymer (Zytomed Systems, Germany or PerkinElmer, MA, USA). Each horseradish peroxidase-conjugated polymer mediated the covalent binding of a different
fluorophore using tyramide signal amplification. The sequential multiplexed staining protocol is shown in Supplementary Table 4. Such covalent reaction was followed by additional antigen retrieval in heated citric buffer (pH6) or Tris/EDTA (pH9) for 20 min to remove antibodies before the next round of staining. After all sequential staining reactions, sections were counterstained with DAPI (Vector lab). Slides were scanned and digitalized by Zeiss Axio Scanner Z.1 (Carl Zeiss AG, Germany) with 10x objective magnification.

Real-Time quantitative Reverse-Transcription Polymerase Chain Reaction
Real-time quantitative PCR (qPCR) was performed by Roche LightCycler® 480 using LightCycler® 480 SYBR Green I Master Kit (Roche GmbH, Germany). Primers for qRT-PCRs were designed using the NCBI Primer Blast and purchased from Eurofins MWG Operon GmbH, Ebersberg, Germany. The primer list is shown in Supplementary Table 5. The PCR products were designed with a size of~100 bp. All real-time qPCR experiments were run under 58° C annealing condition and amplification was run for 45 cycles. A melting curve was implemented in each experiment to prove single product amplification. Data was analyzed using ΔCt calculations where RPLP0 or cyclophilin A served as housekeeper control for normalization. The amplification efficiency was experimentally determined or assumed as 2 (doubling each cycle). Relative mRNA expression levels compared to housekeeper gene expression (efficiency-ΔCt) were used for visualization.

NanoString nCounter RNA expression analysis
The PanCancer Immune Profiling Panel (NanoString Technologies Inc., Seattle, WA), which includes 730 immune related genes and 40 housekeeping genes, was used in the study. Expression data were normalized and analyzed with the nSolver Advanced Analysis Software 1.1.4 using the PanCancer Immune Profiling Advanced Analysis Module (NanoString Technologies). For background correction, the mean count of negative controls plus two times the standard deviation was subtracted from the counts for each gene. The geNorm algorithm was used to identify the most stable housekeeping genes. The geometric mean of the selected housekeeping genes was used to calculate a normalization factor for each sample. The cell-type specific scores were calculated as mean log2 value of characteristic genes for corresponding immune cell types. Whenever stated, relative score was presented as the relative abundance of certain immune subset with regard to either total infiltrating leukocytes (TIL) or total T cells. The pathway scores were analyzed by the first principal component (PC). For a given pathway, PC analysis scored each sample using a weighted average of its gene expression values.

Statistical data analysis
All statistics were calculated using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).
Two-tailed nonparametric Mann-Whitney test was applied for all analysis, except for survival data by Log-rank (Mantel-Cox) test, and correlation analysis by Spearman’s rank correlation coefficient.

RESULTS

M2-phenotype TAM is the predominant immune subset in PDAC microenvironment

We first studied the dynamics of tumor-stroma interactions at different time points during tumor progression in $Ptf1a^{wt/Cre}$; $Kras^{wt/LSL-G12D};p53^{fl/fl}$ mice (named CKP hereafter; for details refer to table S1), which show a highly aggressive clinical course and display prominent desmoplasia (37). Pancreatic tissue was harvested at different stages: preneoplasia (< 4 weeks of age), early (4-6 weeks) and advanced PDAC (10 weeks). Immunohistochemical (IHC) staining showed that the number of proliferative Ki67+ tumor cells significantly increased from preneoplasia to early PDAC, and then decreased slightly at advanced stage (Supplementary Fig. 1A). A similar trend was observed for apoptotic marker cleaved caspase 3, although the frequency was relatively low (Supplementary Fig. 1A). Tumor histology as revealed by Movat’s pentachrome stain demonstrated that acinar cells significantly decreased, while desmoplasia increased from preneoplasia to advanced stage PDAC (Fig. 1A).

Tumor-infiltrating immune cells are a major component of desmoplasia. We profiled the dynamics of immune subsets along PDAC progression. A significant percentage of CD45+ leukocytes (~20%) was detected in preneoplasia, and increased in early PDAC (~40%), but then dropped to approximately 30% at advanced stage (Fig. 1A). CD3+ T cells demonstrated a similar trend as CD45+ cells, while CD19+ B cells were almost undetectable in preneoplastic lesion, but then continuously increased along PDAC progression (Fig. 1A). However, frequencies of both CD3+ T and CD19+ B cells were relatively low (less than 5 and 1.5%, respectively). On the contrary, a substantial number of CD11b+ myeloid cells was found in preneoplasia (~15%), and increased to ~20% in early and advanced PDAC respectively (Fig. 1A). Further characterization showed that F4/80+ macrophages comprised the major subpopulation of CD11b+ myeloid cells (Fig. 1A). Among F4/80+ macrophages, MRC1+ M2 macrophages represented the dominant subpopulation (Fig. 1A), while iNOS+ M1 macrophages were rarely observed in all stages (< 1.5%, Supplementary Fig. 1A).

Flow cytometric analysis was performed to quantify various immune subpopulations in advanced CKP mice. Firstly, systemic immunoregulatory effects during PDAC progression were assessed by measuring CD3+ T cells, CD19+ B cells and CD11b+ myeloid cells in the spleens of wild type and CKP mice. Spleens of CKP mice showed a significant increase of myeloid cells with a reduction of B cells compared to those of wild type mice (Fig. 1B). However, the number of total and different subsets of T cells was not affected by the presence of pancreatic neoplasms (Fig. 1B).
Next, we characterized the immune landscape in advanced CKP tumors. Approximately 60% of CD45+ leukocytes were positive for the myeloid lineage marker CD11b, while CD3+ T cells and CD19+ B cells accounted for 15% and 20% of leukocytes, respectively (Fig. 1C). Further analysis was performed to comprehensively delineate the composition of tumor infiltrating immune cells (detailed gating strategies as shown in Supplementary Fig. 1C-G). CD4+ and CD8+ T cells contributed to CD3+ population equally (~7% of total tumor-infiltrating leukocytes). Regulatory T cells (Treg), γδ T cells and natural killer (NK) cells accounted for only 1% each (Fig. 1C). Conventional CD43- B2 cells represented 15% of leukocytes, but no CD19+CD5+CD1dhi Breg were found in tumors or spleens. TAMs, characterized by a CD45+CD11b+GR1-/lo/F4/80+ phenotype, were the largest myeloid sub-population (Fig. 1C). Myeloid-derived suppressor cells (MDSC) accounted for 10% of all CD45+ cells, with dominance of granulocytic (G-MDSC) over monocytic MDSCs (M-MDSC). Further characterization on TAMs revealed a clear enrichment of MRC1+ and ARG1+ M2 phenotypes (Fig. 1C).

**Intratumoral T cells do not express markers for effector functions**

Although tumor antigens can elicit T cell-mediated anti-tumor response, T cells are frequently prevented from eradicating tumor cells by various immune-suppressive programs (24, 38). IHC staining showed that CD4+ and CD8+ cells were rarely observed in preneoplastic lesions. Although they increased as tumor progressed, the frequency remained low (less than 1 and 2%, respectively) (Supplementary Fig. 1A). Given the M2 predominated PDAC microenvironment, we hypothesized that the anti-tumor activity of infiltrating T cells might be suppressed. We thus assessed the expression of two critical transcription factors for effector functions of T cells, T-bet and Eomes, as well as the T cell activation marker lymphocyte activation gene-3 (LAG3). T-bet is induced upon naïve T cell priming and is required for production of effector cytokines such as interferon-suppressed. Eomes regulates cytolytic gene program in CTLs, and T-bethiEomeshi cells represent fully differentiated CTL populations(39). IHC staining showed that both transcription factors and LAG3 were found at low expression levels in the tumor infiltrating T cells (Supplementary Fig. 1A), and absent in normal pancreas (Supplementary Fig. 1B).

Since T-bet and Eomes are also implicated in the cytotoxic functions of NK cells, we assessed NK infiltration in PDAC development. IHC analysis showed that NK cells were absent in preneoplastic lesions. However, NK cells were detected in early and late PDAC, albeit at low frequency (<1%) (Supplementary Fig. 1A). Based on the expression patterns of T-bet, Eomes and NK1.1 in consecutive tissue sections, NK cells might also contribute to the expression of T-bet and Eomes in early and late PDAC (Supplementary Fig. 1A). However, since NK cell frequency was relatively low when compared to CD3+ T cells (Fig. 1A), the increased T-bet and Eomes expression should be largely contributed by T cells.
Notch activation counteracts IL-4-induced M2 polarization in vitro

Predominant M2 TAM might be associated with defective T cell responses in PDAC. Therefore, we investigated regulatory mechanisms for M2 polarization. The Notch signaling pathway has been reported to regulate macrophage polarization, in which Notch activation led to M2-associated gene repression (30, 31).

First, we studied the effects of manipulating the Notch pathway using bone marrow-derived macrophages (BMDM). Bone marrow-derived cells (BM) were treated with M-CSF for 8 days, and then confirmed for macrophage phenotype by expression of both CD11b and F4/80 (Supplementary Fig. 2). To test the ability of BMDMs to undergo CRE-mediated recombination, BMDM from R26^{LSL-tdTomato} reporter mice were treated with recombinant NLS-TAT-CRE (40). Approximately 95 % of cells successfully underwent genetic loxP-site recombination as determined by tdTomato fluorescence detected by flow cytometry and fluorescence microscopy (Fig. 2A).

To study the effect of Notch activation in M2 polarization, BMDMs from R26^{LSL-N2IC} mice (34) were pretreated with or without recombinant CRE protein, and subsequently stimulated with IL-4 to induce M2 polarization. In this model, CRE recombinase excises a transcriptional STOP-cassette resulting in constitutive expression of transcriptionally active Notch2-IC and human CD2, a co-expressed reporter molecule (Fig. 2B). CRE-dependent activation of Notch signaling was confirmed by hCD2, as detected by flow cytometry (Fig. 2C). IL-4 strongly induced the expression of M2-associated genes Mrc1, Mgl1 and Arg1, while no significant change was observed for M1-associated genes (Fig. 2D). The IL-4-induced upregulation of Mrc1, Mgl1 and Arg1 expression was significantly abrogated by concomitant Notch activation (Fig. 2D). In addition, effect of Notch activation on MRC1 protein level was validated by flow cytometry (Fig. 2E), supporting the observation that Notch activation counteracts IL-4-induced M2 polarization.

To explore the effect of Notch activation in M1 polarization, BMDMs were stimulated with LPS following treatment with recombinant CRE protein. LPS alone induced an up-regulation of iNos, IL1β, IL6, IL12a, and IL12b genes. However, combination of LPS and CRE did not exert additional effect on M1 gene upregulation or M2 gene reduction (Fig. 2F). The result was validated for protein level of iNOS and MRC1 by flow cytometry (Fig. 2G).

It is noteworthy that IL1β was upregulated by CRE alone treatment (Fig. 2D and F). However, the induced IL1β level by CRE alone is significantly lower when compared to that of CRE plus LPS treatment (Figure 2F). Besides, the expression of other M1-associated genes (e.g. IL-6, IL-12, iNos) induced by CRE alone is generally subtle. Although we cannot exclude the possibility that NLS-TAT-CRE exposure indeed might induce changes in M1- and/or M2-associated genes, the magnitudes of differences induced upon LPS and IL4 treatment are at least several folds greater than CRE alone. Since the induction of M1 and M2-associated
genes is clearly different between N2IC and Rbpj targeted cells, it is deduced that Notch modulation is the dominant factor counteracting IL-4-induced M2 polarization.

**Rbpj knock out blocks LPS-induced M1 polarization in vitro**

Next, we investigated the role of endogenous Notch signaling for M1 or M2 macrophage polarization. Rbpj3/3 construct allows CRE-dependent ablation of canonical Notch signaling (Fig. 2H). BMDMs from Rbpj3/3 mice were treated analogously to those from R26SL-N2IC mice. LPS-induced M1 gene expression was significantly reduced in Rbpj KO macrophages (Fig. 2I). Moreover, LPS-dependent downregulation of Jmjd3, a critical gene for M2 polarization, was retained in Rbpj KO BMDMs (Fig. 2I). Flow cytometric analysis revealed that LPS-induced iNOS expression was also downregulated in Rbpj KO macrophages at protein level (Fig. 2J). However, IL-4-induced M2 polarization in terms of M2C protein expression was not affected by Rbpj ablation (Fig. 2K). These findings suggest that canonical Notch signaling is required for robust M1 polarization.

**Genetic Notch modulation in myeloid cells using in vivo dual recombinase system**

To validate the regulatory role of Notch in M2 polarization in vivo, a myeloid-targeting Cre strain (Ly22/wt;Cre) was crossed to R26wt/LSL-N2IC and Rbpj3/3 mice. Ly22/wt;Cre;R26wt/LSL-N2IC (Ly2;N2IC) and Ly22/wt;Cre; Rbpj3/3 (Ly2;Rbpj) lines were then interbred with Pdx1-Flp;KrasGctF12D;p536fl/+ (FKP) mice (Fig. 3A, B). FKP mice develop and succumb to PDAC highly similar to the CKP model as reported previously (33). Phenotypic characterization of FKP tumors was performed showing comparable histology and biology of tumor cells, as well as immune profile as those of CKP tumors (Supplementary Fig. 3A). Ly2-Cre expression was traced with the R26SL-tdTomato reporter strain to quantify recombination efficiency in myeloid subsets. Approximately 80% of CD11b+ myeloid cells were recombined by Ly2-Cre in bone marrow and spleen (Fig. 3C). Among CD11b- cells, there is less than 10% positivity for tdTomato expression. This population might thus reflect CD11b-CD11c+ dendritic cells that are derived from myeloid cell precursors (41). To confirm myeloid-specific Notch downstream activation in Ly2;N2IC, bone marrow of wild type, Ly2;N2IC, and Ly2;Rbpj mice was isolated and sorted according to CD11b and hCD2 expression for RNA extraction (Fig. 3D). Real time qPCR revealed a strong transcriptional upregulation of the Notch target gene Hes1 in Ly2;N2IC bone marrow, indicating that Notch was Ly2-Cre dependently activated, whereas wild type and Ly2;Rbpj mice both showed low levels of Hes1 expression in CD11b+ bone marrow cells (Fig. 3E). IHC staining for hCD2 and CD11b, as well as immunofluorescence staining for their co-expression pattern in FKP;Ly2;N2IC tumors, further confirmed the selective Notch activation in CD11b+ myeloid cells (Fig. 3F).

We next addressed the expression of the different Notch receptors. While Notch2 is the target
in R26LSL-N2IC mice, ablation of Rbpj potentially affects other Notch family members. We thus analyzed the constitutive expression of Notch 1-4 in FKP tumors. IHC analysis revealed the expression of Notch 1 and 2, but not Notch 3 and 4 in FKP tumors (Supplementary Fig 3B). Subsequent co-expression analysis by immunofluorescence staining showed that Notch1 expression was largely found in tumor cells, but not in CD11b+ myeloid cells (Supplementary Fig. 3C). Besides, Notch1 expression was similar in either FKP, FKP;Lyz2;Rbpj or FKP;Lyz2;N2IC tumors (Supplementary Fig. 3B and C), suggesting that Notch1 is not expressed in myeloid cells and therefore not subject to myeloid-specific modulation in our system.

Notably, Notch2 was found to be highly co-expressed with CD11b, i.e. in myeloid cells. As shown in Fig. 3G, modulation of Notch2 is a target of the genetic intervention, as an increase in Notch2 was mainly found in CD11b+ cells in FKP;Lyz2;N2IC mice, while it was largely reduced in FKP;Lyz2;Rbpj. In fact, our finding is supported by previous publication that Notch2 is fundamental for myeloid cell differentiation (42, 43).

Importantly, we observed a significant survival benefit in Lyz2;N2IC mice when compared to Lyz2;Rbpj mice in both FKP and FKPhet models with deletion of both or only one Tp53 allele, respectively (Fig. 3H and I), although statistical significance was not reached when compared with FKP controls.

Notch activation reprograms M2 tumor associated macrophages in vivo

We next performed a detailed characterization of tumors from FKP;Lyz2;N2IC, FKP;Lyz2;Rbpj and FKP control mice, and observed that Ki67 expression significantly decreased, while cleaved caspase 3 increased in FKP;Lyz2;N2IC tumors (Fig. 4A), suggesting reduced proliferation and increased apoptosis in tumor cells upon myeloid-targeted Notch activation. Movat’s pentachrome staining showed that stromal content was also reduced in FKP;Lyz2;N2IC group (Fig. 4A). Opposite trend is observed for the above findings in FKP;Lyz2;Rbpj tumors. It is noteworthy that collagen (yellowish stain), which was rarely observed in FKP control tumor (Supplementary Fig. 3A), was increased in FKP;Lyz2;Rbpj tumor, indicating rearrangement of stromal content. IHC staining was performed to confirm the collagen levels in the tumors, and consistently, there were significantly higher levels of collagen in FKP;Lyz2;Rbpj tumor when compared to FKP control and FKP;Lyz2;N2IC group (Supplementary Fig. 4D).

To investigate any alteration in immune landscape upon myeloid-specific Notch modulation, tumors of end-stage FKP;Lyz2;N2IC and FKP;Lyz2;Rbpj mice were characterized for infiltrating leukocyte subsets. Flow cytometric analysis showed a slight increase in CD3+ T cells and CD19+ B cells in FKP;Lyz2;N2IC mice, although statistical significance was not reached (Fig. 4B). CD11b+ myeloid cells remained the predominant population in infiltrating leukocytes in both models. We observed no significant changes in overall MDSC and TAM.
proportions, and TAM comprised the majority of CD11b+ cells (Fig. 4B). However, we found a significant reduction of M2 TAMs (iNOS-MRC1+) in FKP;Lyz2;N2IC tumors when compared to FKP;Lyz2;Rbpj (Fig. 4B). Multiplexed immunofluorescent staining for MDSCs supported the FACS results by showing a slight, but not significant, decrease in G-MDSC in FKP;Lyz2;N2IC mice, while the levels of M-MDSC remained low and there was no change among the three groups (Supplementary Fig. 4E). Besides, IHC staining echoed the findings by demonstrating prominent reduction in MRC1 expression in FKP;Lyz2;N2IC tumors (Fig. 4C). Subsequent multiplexed staining for hCD2, CD11b and MRC1 was performed in FKP;Lyz2;N2IC tumors to assess the M2 phenotype of the CD11b+hCD2+ cells. As shown in Supplementary Fig. 4F, MRC1 expression did not show similar pattern to that of hCD2, suggesting that hCD2+ cells were largely MRC1 negative. The M1 TAM marker iNOS was significantly up-regulated in FKP;Lyz2;N2IC tumors, although the level was relatively low (Fig. 4C). In addition, mice were examined for systemic effect of the Lyz2-Cre-mediated Notch manipulation on immune landscapes in bone marrow, blood and spleen. No significant change was observed among Notch activation (Lyz2;N2IC), blockage (Lyz2;Rbpj), and wild type mice in all myeloid and lymphoid subpopulations (Supplementary Fig. 4A-C).

Association of myeloid-specific Notch modulation with immune profiles in tumor microenvironment

Next, we characterized and compared the immune landscapes of FKP;Lyz2;N2IC (n=7) and FKP;Lyz2;Rbpj (n=9) tumors using the Nanostring PanCancer Immune Panel. The 35 genes most regulated upon Notch modulation are listed in Supplementary Table 6 and plotted in the heatmap (Fig. 5A). Nanostring advanced analysis revealed an increase in infiltrating T cells in FKP;Lyz2;N2IC tumors. Specifically, CD4+ T cells and exhausted CD8+ cells significantly increased in FKP;Lyz2;N2IC tumors (Fig. 5B). Note that the cell scores indicate the relative abundance of immune subsets out of total tumor infiltrating leukocytes (TIL) or total T cells. Consistently, flow cytometric analysis also revealed increased CD3+ (Fig. 4E), CD4+ and CD8+ T cells (Fig. 5C) in FKP;Lyz2;N2IC, although statistical significance was not reached, likely due to small sample size (n=3) (Fig. 5C). No change was observed for the infiltration of other immune cell types including B cells, mast cells, neutrophils (Supplementary Fig. 5E).

Besides, immune pathway analysis by Nanostring showed higher scores for antigen processing, MHC, interferon, senescence, chemokines and receptors, inflammation, and apoptosis pathways in FKP;Lyz2;N2IC tumors (Fig. 5D). This analysis revealed that MHC class II-related genes e.g. Lag3, Ciita, H2-DMb1 and H2-Eb1, comprised the majority of genes that were significantly altered upon Notch modulation in antigen processing and MHC pathways. Indeed, these MHC class II-related genes were all up-regulated for approximately 2-3-fold (log2 scale) in FKP;Lyz2;N2IC tumors (Supplementary Table 6). For chemokines
and receptors pathway, \textit{Ccl24}, a strong chemotactic factor for resting T cells (44), was significantly up-regulated with the greatest fold increase (5-fold in log2 scale) in \textit{FKP;Lyz2;N2IC}. Up-regulation albeit not significant was also observed for other pathways including TNF, NK function and innate immunity (Supplementary Fig. 5F). We next validated findings from Nanostring analysis at protein levels by IHC. Consistent with Nanostring analysis, we observed significantly higher levels of CD3+, CD4+ and CD8+ cells in \textit{FKP;Lyz2;N2IC} tumors (Fig. 5E). In addition, \textit{Eomes} and \textit{Lag3}, markers for both activation and exhaustion of T cells (45, 46), were among the most significantly altered genes in \textit{FKP;Lyz2;N2IC} as revealed by Nanostring analysis (Supplementary Table 6). Their protein levels were also shown to be significantly higher in \textit{FKP;Lyz2;N2IC} (Fig. 5E). To verify whether NK cells also contributed the increase in Eomes expression, we measured infiltrating NK cells in the tumors and found that NK cells were significantly reduced in \textit{FKP;Lyz2;Rbpj} (Supplementary Fig 5A). Therefore, the increase in Eomes in \textit{FKP;Lyz2;N2IC} tumors should be largely T cell-dependent.

Next, we validated the markers for antigen presentation as revealed by Nanostring analysis. IHC staining for CD11c, MHCII and CD80 in \textit{FKP;Lyz2;Rbpj}, \textit{FKP;Lyz2;N2IC} and \textit{FKP} control tumors were performed as their genes, CD11c (\textit{itgax}), CD80, MHCII (e.g. \textit{ciita}, \textit{H2-DMb1}, \textit{H2-Eb1}), were among the most significantly upregulated genes upon Notch modulation (Fig. 5A and Supplementary Table 6). Consistent with Nanostring analysis, IHC staining showed increase in CD11c+, MHCII+ and CD80+ cells in \textit{N2IC} targeted mice, while a reduction in \textit{FKP;Lyz2;Rbpj} group is observed (Supplementary Fig. 5A). By multiplexed immunofluorescent staining of CD11c with the human CD2 reporter protein in \textit{FKP;Lyz2;N2IC} showed that CD11c+ cells are mostly, if not all, hCD2 negative (Supplementary Fig. 5B). Subsequent co-staining of CD11b, CD11c, MHCII and CD80 in \textit{FKP;Lyz2;N2IC} showed that upregulation of MHCII and CD80 are largely expressed by CD11c+ cells (Supplementary Fig. 5C). The presence of hCD2+CD11c+ cells might be due to the fact that some CD11c+ cells are derived from myeloid cell precursors, as reported by Clausen \textit{et al} (41). Regarding the cytokine profile, we observed an increase in the M1-associated cytokines IFN\textgamma, IL12p70 and TNF\alpha, while the M2-associated cytokines CXCL1 and TGF\beta were decreased in \textit{FKP;Lyz2;N2IC} mice (Supplementary Fig. 5D). It is noteworthy that serum TGF\beta levels were also decreased in the \textit{FKP;Lyz2;N2IC} group, suggesting a systemic change in this cytokine. However, no statistical significance was reached in the above cytokine profiling due to the small sample size.

DISCUSSION

One of the distinctive features of PDAC is that the malignant epithelial cells often account for only a minority of tumor mass, while the desmoplastic stroma and other non-tumor cells
constitute up to 80% (47). Currently most targeting approaches focus on aberrant signaling of tumor cells, while the effect on other cellular compartments remain elusive given the lack of comprehensive model systems. As such, targeting of Notch signaling has been disappointing clinically in PDAC despite supportive preclinical evidence. However, in more complex disease models, the role of Notch signaling has been controversial in PDAC, exerting both pro- and anti-tumorigenic effects (48).

In this study, we used a genetic approach to modulate Notch signaling specifically in myeloid cells and characterized the consequent effects on the immune landscape in spontaneous endogenous PDAC in immunocompetent mice. In line with previous findings, tumor-promoting M2 TAMs were shown to predominate the tumor infiltrating leukocytes (49, 50). A significant amount of M2 TAMs was observed in preneoplastic lesion, and the level increased along tumor progression, suggesting that M2 TAMs participate not only in PDAC progression, but also in tumor formation (51-53). Here, we demonstrated a tumor-suppressive role of Notch signaling in myeloid cells in PDAC. Upon Notch activation in myeloid cells, M2 TAMs were significantly reduced, while antigen presentation and cytotoxic T cell activity were restored, and importantly, survival of mice with spontaneous PDAC was significantly improved. Our findings therefore may help explain the limited efficacy of targeting Notch signaling in PDAC as previously reported (13, 14).

Earlier studies reporting the re-polarization of macrophages in experimental PDAC demonstrated beneficial outcome when M2 polarization was antagonized either genetically or by low-dose irradiation(54, 55). These studies, however, were conducted either by tumor transplantation or transgenic mouse models, which do not recapitulate seminal features of human PDAC as faithfully as spontaneous Kras<sup>G12D</sup>-driven PDAC mouse models do. We thus employed a novel dual-recombinase system to genetically induce both Kras<sup>G12D</sup>-driven pancreatic tumorigenesis and myeloid-specific modulation of Notch signaling. In our Lyz2-CRE model, Notch signaling was genetically targeted in myeloid cell-specific manner. It is noteworthy that although TAMs represent the majority (~50-60%) of intratumoral myeloid cells in our PDAC model, MDSCs also account for a significant proportion (~20-30%). Indeed, recent studies have shown that Notch signaling can regulate MDSC differentiation. Blockage of Notch signaling promoted the differentiation and expansion of G-MDSCs both in vitro and in vivo(29, 56). Our finding of a reduction in G-MDSCs upon Notch activation, though not statistically significant, would be consistent with this effect (Fig. 4E). Thus, we cannot rule out the possibility that Notch-induced MDSC differentiation also contributed to the observed alterations in the tumor microenvironment. Further investigation will be required to further dissect the roles of MDSCs and TAMs in modulating immunosuppression.
Analysis of immune landscape of FKP;Lyz2;N2IC and FKP;Lyz2;Rbpj tumors by Nanostring showed that Notch-induced M2 reduction was significantly associated with gene expression signatures of increased antigen processing and presentation, infiltrating T cells, and interferon pathway. Intriguingly, a strong expression of exhausted T cell signature was observed in FKP;Lyz2;N2IC mice, in which Eomes, Lag3 and PD1 were expressed at high levels. Although described as exhaustion markers for T cells, they are in many ways considered as T cell activation markers. Upregulation of these markers may suggest that these cells have undergone priming and were consequently activated. One key feature of T cell exhaustion is the continuous exposure to antigen rather than acutely terminated or intermittent exposure. Here, we observed that upon M2 reduction, antigen processing and presentation was significantly enhanced, which might at least partially explain the restored T cell activation and potentially the survival benefit of FKP;Lyz2;N2IC mice. This is supported by the strong correlation between the mRNA levels of Eomes, Lag3 and PD1, with antigen processing/presentation-related genes that are highly up-regulated upon Notch activation in myeloid cells.

In addition to T cell infiltration and activation, another interesting impact of myeloid-specific Notch modulation is antigen presentation. Nanostring analysis and subsequent IHC staining demonstrated augmented antigen presentation in FKP;Lyz2;N2IC tumors. Upregulated MHCII and CD80 were predominantly expressed by CD11c+ dendritic cells, which are mostly hCD2 negative. One possible explanation for increased CD11c+ dendritic cells may be an altered cytokine and chemokine profile in the tumor microenvironment. Although not statistical significant, cytokine profiling revealed increased cytotoxic cytokines IFNγ, IL12p70 and TNFα, and decreased tumor promoting cytokines CXCL1 and TGFβ in FKP;Lyz2;N2IC tumors (Supplementary Fig. 5D). Besides, a systemic change in TGFβ was observable in FKP;Lyz2;N2IC mice, as illustrated by decreased serum TGFβ level. It is notable that TGFβ is crucial for inducing M2 phenotype and inhibiting dendritic cell maturation and activation. A reduction in TGFβ might at least partially explain the increased CD11c+ dendritic cells with higher MHCII and CD80 expression levels in FKP;Lyz2;N2IC cohort. In this regard, it can be speculated that the application of Notch inhibitors in clinical settings might lead to suppressed antigen presentation, which potentially compromise the efficacy of other immunotherapies. However, further investigation is needed to comprehensively characterize the impact of the Notch-induced myeloid polarization on the complex antigen presentation machinery.

The present study may serve as proof-of-concept for genetic targeting of myeloid subsets in complex models of immunocompetent endogenous PDAC. The dual recombinase-based approach offers a platform to assess their contribution in PDAC progression and immune-based approaches. Our findings on the anti-tumorigenic role of Notch signaling
specifically in myeloid cells call for attention on the need to dissect the differential roles of signaling pathways in different cellular components within the tumor microenvironment. The dual-recombinase system is a useful model system for dissecting the complex network among tumor and stromal components to validate non-tumor targeting strategies in PDAC, a disease in high need for better treatment approaches.
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FIGURE LEGENDS

Fig. 1. M2 macrophages predominated tumor infiltrating leukocytes in CKP tumors. (A) Representative Movat, CD45, CD19, CD3, CD11b, F4/80 and MRC1 staining of different tumor stages: preneoplasia (< 4 weeks), early (4-6 weeks) and advanced (> 8 weeks) PDAC in CKP mice (n≥5 per group). Magnification: 25X, 200X. Scale bars represent 100 µm. Right panel shows the percentage of positive cells or area (for Movat’s pentachrome staining only) as the average of 5 fields from each mouse (10X objective magnification). Mean ± SD is shown. (B) Flow cytometric analysis on the immune phenotype of spleens from wild type or end-stage CKP mice. Percentage of viable CD45+ cells were pre-gated for T cells (CD3+), B cells (CD19+), and myeloid cells (CD11b+) quantification. Percentage of CD4+ and CD8+ cells in total CD3+ T cells. Naïve: CD44loCD62Lhi, EM (effector memory): CD44hiCD62Llo, CM (central memory): CD44hiCD62Lhi in total CD4+ and CD8+ T cells were quantified. WT: n=6; CKP: n=6. (C) PDAC tumors of end-stage CKP mice were digested into desegregated cells and analyzed by flow cytometry. Live CD45+ cells were gated as viable leukocytes for subsequent immune subpopulation characterization: T cells (CD3+), B cells (CD19+), and myeloid cells (CD11b+); CD4+ T (CD3+CD4+CD8-), CD8+ T (CD3+CD4-CD8+), Treg (CD3+CD4+Foxp3+CD25+), γδT (CD3+γδTCR+), B1 (CD19+CD43+) and B2 (CD19+CD43-), NK (NK1.1+), M-MDSCs (CD11b+F4/80-Gr1-/Ly6Cch), G-MDSCs (CD11b+F4/80-Gr1hi) and TAM (CD11b+F4/80+Gr1+). TAMs were further dissected into M1 (iNOS+) and M2 (MRC1+, ARG1+). n=12, except for Treg and NK (n=5), γδT (n=3), M-MDSC (n=7). Mean ± SD are shown.

*: p<0.05; **: p<0.005.

Fig. 2. Notch modulation regulates macrophage polarization in vitro. (A) BMDM isolated from R26LSL-tdTomato reporter mice treated with 1 µM recombinant NLS-TAT-CRE overnight and analyzed for tdTomato fluorescence on day 4 by flow cytometry or observed under microscope. Scale bars represent 100 µm. (B) Simplified description of genetic R26LSL-N2IC construct. (C) In vitro LoxP recombination assessed by detecting hCD2 on N2IC BMDM. (D, E) BMDM from R26LSL-N2IC stimulated with 10 ng/ml IL-4 for 72 hrs with or without previous CRE treatment. (D) Relative expression levels of M1 and M2 marker genes determined by qRT-PCR (n=6). Mean ± SD is shown. (E) Representative flow cytometric analysis showing MRC1 level on CD11b+F4/80+ cells upon CRE and IL-4 treatment. (F, G) BMDMs from R26LSL-N2IC stimulated with 1 µg/ml LPS for 6 hrs or (G) overnight with or without previous CRE treatment. (F) Relative mRNA expression levels of M1 and M2 marker genes determined by qRT-PCR. n=6, except for +CRE-LPS: n=3. (G) Flow cytometry analysis showing percentage of iNOS+ and MRC1+ cells in F4/80+ BMDM. n=4. Mean ± SD is shown. (H) Simplified description of conditional
Rbpj\(^{fl/fl}\) construct. (I-K) BMDMs from Rbpj\(^{fl/fl}\) mice stimulated with 1 \(\mu\)g/ml LPS for (I) 6 hrs or (J) overnight, or (K) with IL-4 for 72 hrs with or without previous CRE treatment. (I) Relative mRNA expression levels of M1 and M2 marker genes determined by qRT-PCR (n=6). Mean + SD is shown. (J) Flow cytometry analysis showing percentage of iNOS+ and MRC1+ cells in F4/80+ BMDM (n=4). Mean + SD is shown. (K) Representative histogram showing relative fluorescence intensity of MRC1 on CD11b+F4/80+ cells upon CRE and IL-4.

*: p<0.05, **: p<0.005.

Fig. 3 Lyz2-Cre directs genetic Notch activation to myeloid cells.

(A) Mouse crossing strategy. Pdx1-Flp;Kras\(^{G12D}\);p53\(^{frt/frt}\) (FKP) mice crossed to Lyz2;Rbpj or Lyz2;N2IC strains to generate FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC mice respectively. (B) Oncogenic Kras\(^{G12D}\)-driven pancreatic tumorigenesis (FKP) and myeloid-specific manipulation of Notch signaling were concomitantly genetically induced. (C) The R26\(^{LSL-tdTomato}\) reporter mouse line was used to visualize Lyz2-Cre expression and recombination activity in vivo. tdTomato+ fractions in CD11b+ and CD11c- cells isolated from bone marrow (BM) and spleen (SP) were quantified by flow cytometry (n=3). Mean + SD is shown. (D) Bone marrow derived cells of wild type, Lyz2;N2IC, and Lyz2;Rbpj mice was sorted based on hCD2 and CD11b expression, and mRNA was extracted from the sorted populations (red gates). Lower gate: CD11b+hCD2-, upper gate: CD11b+hCD2+. (E) Specific Hes1 expression in sorted CD11b+hCD2- and CD11b+hCD2+ cells was assessed by qRT-PCR. (F) Left panel: IHC staining for CD11b, hCD2 and isotype control on the tumor of FKP;Lyz2;N2IC tumor. Magnification: 100X, 200X. Scale bars represent 50 \(\mu\)m. Right panel: Immunofluorescence staining for co-expression of CD11b (green) and hCD2 (red) on the tumor of FKP;Lyz2;N2IC tumor. DAPI (blue) indicates nuclei. Magnification: 25X, 200X. Scale bars represent 100 \(\mu\)m. (G) Immunofluorescence staining for co-expression of CD11b (green) and Notch2 (red) on the tumor of FKP, FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC tumors. DAPI (blue) indicates nuclei. Magnification: 25X, 200X. Scale bars represent 100 \(\mu\)m. (H, I) Kaplan-Meier plot showing the survival of FKP;Lyz2;Rbpj, FKP;Lyz2;N2IC, and FKP control mice in (H) FKP and (I) FKP\(^{het}\) models. (H) Median survival: FKP;Lyz2;Rbpj 67 d (n=24), FKP;Lyz2;N2IC 78 d (n=11), FKP 70 d (n=19). Log-rank test: FKP;Lyz2;Rbpj vs FKP;Lyz2;N2IC: \(p=0.014\); FKP;Lyz2;Rbpj vs FKP: \(p=0.482\); FKP;Lyz2;N2IC vs FKP: \(p=0.095\). (I) Median survival: FKP\(^{het}\);Lyz2;Rbpj 147 d (n=17), FKP\(^{het}\);Lyz2;N2IC 186.5 d (n=8), FKP\(^{het}\) 172 d (n=24). Log-rank test: FKP;Lyz2;Rbpj vs FKP;Lyz2;N2IC: \(p=0.013\); FKP;Lyz2;Rbpj vs FKP: \(p=0.184\); FKP;Lyz2;N2IC vs FKP: \(p=0.317\). *: p<0.05 when comparing between groups denoted by horizontal bars.

Fig. 4. Notch signaling antagonizes M2 polarization in PDAC TAMs
Movat's pentachrome staining, IHC staining of Ki67 and cleaved caspase 3 in FKP; FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC tumors (n≥5 per group). Magnification: 25X, 200X. Scale bars represent 100 µm. Lower panel shows the percentage of positive cells or area (for Movat’s pentachrome staining only) as the average of 5 fields from each mouse (10X objective magnification). Mean + SD is shown. 

Tumors of end-stage FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC mice were digested into desegregated cells and analyzed by flow cytometry. Live CD45+ cells were gated as viable leukocytes for subsequent immune subpopulation characterization: T cells (CD3+), B cells (CD19+) and myeloid cells (CD11b+); M-MDSCs (CD11b+F4/80-/hiGR1-/loLy6Chi), G-MDSCs (CD11b+F4/80-GR1hi) and TAM (CD11b+F4/80+GR1-/lo). TAMs were further dissected into subpopulations based on iNOS and MRC1 expression. Mean + SD were shown.

IHC staining for CD11b, F4/80, MRC1, and iNOS in FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC tumors (n≥5 per group). Magnification: 25X, 200X. Scale bars represent 100 µm. Lower panel showed the percentage of positive cells as the average of 5 fields (n≥5 per group) (10X objective magnification). Mean + SD is shown.*: p<0.05; **: p<0.01.

**Fig. 5.** Association of myeloid-specific Notch modulation with immune landscapes in tumor microenvironment

(A, B, D) Profile of immune-related expression signatures in FKP;Lyz2;Rbpj (n=9) and FKP;Lyz2;N2IC (n=7) tumors determined by the NanoString PanCancer Immune Profiling Panel. (A) Heat map with hierarchical clustering for genes with at least 2-fold change up or down with p<0.05 as cut-off. Significantly up-regulation is shown in red and down-regulation in green. (B) The cell-type specific scores of T cells and exhausted CD8+ T cells (relative to total tumor infiltrating cells), and CD4+ T cells (relative to total CD3+ T cell), were calculated by PanCancer Immune Profiling Advanced Analysis as described in the Material and Methods section. (C) Flow cytometric analysis showing the percentage of CD4+ T cells (CD3+CD4+CD8-) and CD8+ T cells (CD3+CD4-CD8+) of total leukocytes (CD45+) in FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC tumors (n=3 per group). (D) Pathway scores of antigen processing, MHC, interferon (IFN), senescence, chemokines and receptors, inflammation, and apoptosis were calculated by PanCancer Immune Profiling Advanced Analysis as described in the Material and Methods section. (E) IHC staining of CD3, CD4, CD8, Eomes and LAG3 in FKP;Lyz2;Rbpj and FKP;Lyz2;N2IC tumors (n≥5 per group). Magnification: 25X, 200X. Scale bars represent 100 µm. Right panel showed the percentage of positive cells as the average of 5 fields (n≥5 per group) (10X objective magnification). Mean + SD is shown.*: p<0.05; **: p<0.005; ***: p<0.001.
Fig. 2

A. -CRE, +CRE

B. R26-CAG

C. -CRE, +CRE

D. M1 genes
   -CRE - IL-4
   +CRE - IL-4
   -CRE + IL-4
   +CRE + IL-4

E. MRC1

F. M1 genes
   -CRE - LPS
   +CRE - LPS
   -CRE + LPS
   +CRE + LPS

G. MRC1+
   NOS+  

H. Rbpj

I. M1 genes
   -CRE - LPS
   -CRE + LPS
   +CRE + LPS

J. MRC1+
   NOS+  

K. MRC1
   -CRE - IL-4
   -CRE + IL-4
   -CRE + IL-4

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Fig. 4

A

Movat

Ki67

Cleaved Casp3

FKP-Lyz2;Rbpj

FKP-Lyz2;N2IC

Stroma (% of area)

Ki67 (%)

Cleaved Casp3 (%)

FKP

FKP-Lyz2;Rbpj

FKP-Lyz2;N2IC

B

% of CD45+ cells

% of CD11b+ cells

% of M-MDSC

% of G-MDSC

% of TAM

% of iNOS-MRC1+

% of iNOS-MRC1−

% of iNOS+MRC1−

% of iNOS−MRC1−

FKP-Lyz2;Rbpj

FKP-Lyz2;N2IC

C

CD11b

F4/80

MRC1

iNOS

FKP-Lyz2;Rbpj

FKP-Lyz2;N2IC

CD11b+ (%)

F4/80+ (%)

MRC1+ (%)

iNOS+ (%)
Notch-induced myeloid reprogramming in spontaneous pancreatic ductal adenocarcinoma by dual genetic targeting


*Cancer Res* Published OnlineFirst May 29, 2018.

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