Impact of Notch disruption on myeloid development

The Notch pathway is a conserved signaling network that regulates many cellular processes including renewal of stem cells, differentiation of multiple cell lineages, proliferation and apoptosis. Notch signaling involves the binding of Notch ligands to Notch receptors followed by proteolytic cleavage events, translocation of intracellular Notch (ICN) to the nucleus and regulation of target genes via the interaction of transcription factor CSL/RBPJ and the MAML family of transcriptional co-activators. The Notch pathway is involved in lymphoid development and recurrent activating mutations in NOTCH1 contribute to T lymphoblastic leukemias. Whether Notch signaling is actively involved in the regulation of myeloid development and myeloid leukemogenesis is less clear due to conflicting reports. In this study, we abrogated canonical Notch signaling throughout the hematopoietic system to evaluate the role of Notch in myelopoiesis and to conclusively determine if inhibition of Notch signaling can contribute to aberrant myelopoiesis and lead to the development of a myeloid neoplasm.

To abrogate Notch signaling, we utilized the well-studied conditional DN-MAML1-GFP mouse model previously shown to block canonical Notch signaling via inhibition of Notch receptors 1–4. We disrupted Notch signaling throughout the hematopoietic system, including myeloid stem and progenitor cells, by intercrossing DN-MAML1-GFP mice with Vav-cre mice. The DN-MAML1-GFP is a fusion protein; thus evaluation of GFP levels by flow cytometry was used to track cells expressing DN-MAML1. As expected, doubly heterozygous mice demonstrated GFP expression in most of the cells in the bone marrow and to a lesser extent in the spleen and thymus (Figure 1A; Supplementary Figure 1). Some mice demonstrated a notable reduction of DN-MAML1 expressing cells only in the thymus, suggesting there is strong selective pressure in T cells to not express DN-MAML1.

To validate our mouse model, we first determined if canonical Notch signaling is decreased in mice doubly heterozygous for DN-MAML1-GFP and Vav-cre (DNMf/−Vav−/+). The development of marginal zone (MZ) B-cells in the spleen relies on Notch 2 signaling and Notch blockade results in the reduction of the MZ B-cell pool in murine spleens. As shown in Figures 1B and C, a significant reduction in the percentage of MZ B-cells was observed in DNMf/−Vav−/+ mice compared to controls at 6 months. Second, we confirmed that these mice exhibited the expected abnormalities in thymocyte development, a Notch1 process. We found a significant increase in the double negative (DN) population within the GFP+ fraction of thymocytes taken from DNMf/−Vav−/+ mice compared to controls (Figure 1D). Further evaluation of the DN thymocyte populations in DNMf/−Vav−/+ mice showed a significant increase in the frequency of the more immature DN1/2 cells, a decrease in DN3/4 cells (Figure 1E) and an increase in B220+ B cells (Figure 1F). Taken together, these data provide confirmatory evidence that our in vivo model is sufficient to inhibit canonical Notch signaling over time.

Earlier studies suggest that loss of Notch signaling can impair megakaryopoiesis leading to a decrease in megakaryocyte–erythroid progenitors (MEPs) at the expense of an increase in granulocyte–monocyte progenitors (GMPs). To determine whether loss of Notch signaling in our model affects the myeloid progenitor pool, we analyzed stem and progenitor compartments of control and DNMf/−Vav−/+ mice at 6 months and 15–18 months. Analyses of DNMf/−Vav−/+ mice at 15–18 months revealed a trend toward an increase in myeloid progenitors (Figure 2A), a significant increase in GMPs (Figure 2B) and a significant decrease in CMPs (Figure 2B). There was, however, no significant difference in the LSK (Figure 2A) or the CD150+CD48−LT-HSC populations (data not shown). A similar trend was observed in the spleens of these mice along with an increase in CD11b+ cells (Supplementary Figures 2A–E). This significant increase in GMPs was observed as early as 6 months (Supplementary Figure 3). Finally, bone marrow cells from mice expressing DN-MAML1-GFP produced fewer myeloid colonies in methylcellulose at 6 months (Supplementary Figure 4) compared to age-matched controls. The collective data suggest that loss of Notch signaling contributes to a decrease in CMPs and a mild but stable expansion of the GMP and myeloid compartments.

Previous results from the disruption of Nicastrin showing marked expansion of myeloid cells prompted speculation that Notch functions as a tumor suppressor. To determine if canonical Notch signaling can function as a tumor suppressor in myelopoiesis, a tumor watch was established (n = 19 DNMf/−Vav−/+; n = 15 controls). The mice were aged to 15–18 months and there was no evidence of a highly penetrant myeloid disease with loss of canonical Notch signaling as shown by no significant difference in survival (Figure 2C), immunophenotype of the peripheral blood (Figure 2D) and white blood cell count between the two groups (Figure 2E). At the end point, the collective DN-MAML1 cohort also did not have splenomegaly (Figure 2F). During the course of the experiment, two mice died in the DNMf/−Vav−/+ group near the end of the study; one had an enlarged spleen (0.510 g) but viable cells could not be obtained and the other died abruptly without splenomegaly. A single mouse in the control group also died abruptly without splenomegaly. These mice continued to show >90% GFP expression at 12 months and decreased MZ B-cells at 18 months (Supplementary Figures 5A–C), demonstrating that there was continued abrogation of Notch signaling. The above data suggest that although there is an expansion of GMPs and myeloid cells in mice that lack canonical Notch signaling, these changes are not sufficient to produce a myeloid neoplasm. It is possible that loss of Notch signaling in myeloid cells is insufficient for the development of myeloid neoplasms, but may cooperate with other events such as genes frequently mutated in AML. To test this hypothesis, we retrovirally expressed FLT3ITD (MSCV-Ires-mCherry) in control and DNMf/−Vav−/+ lineage negative bone marrow cells followed by transplantation into syngeneic recipients (Figure 2G; Supplementary Figure 6A). By ~150 days, all control-FLT3ITD mice died, while there was an observed delay in disease progression and subsequent death of DNMf/−Vav−/+ FLT3ITD mice (Figure 2H). There was a significant reduction in the spleen weights (Figure 2I) and frequency of mCherry+ cells in the spleen of DNMf/−Vav−/+ FLT3ITD mice (Figure 2J). Immunophenotyping of spleenocytes showed no significant differences within the myeloid lineage (Figure 2K). Further, in vitro methylcellulose experiments performed using Lin-marrow cells from both groups transduced with FLT3ITD mCherry construct showed no advantage in growth of DNMf/−Vav−/+ FLT3ITD compared to control-FLT3ITD cells (Supplementary Figure 6B). Overall, these data suggest that loss of Notch signaling does not cooperate in vivo with FLT3ITD to induce a myeloid disease.
The results from our study demonstrate that prolonged loss of Notch signaling in vivo does not lead to a highly penetrant myeloid neoplasm. We found that expression of DN-MAML1-GFP in bone marrow cells, including hematopoietic stem and progenitor cells (HSPCs), resulted in a relative increase in GMPs that was stable over time and an increase in myeloid cells in the spleen. Despite these observations, there were no overt clinical features consistent with the development of a myeloid neoplasm. Our results are consistent with other studies that evaluated the impact of Notch inhibition on normal hematopoiesis via expression of DN-MAML1 or conditional ablation of the Rbpj gene. Additionally, our results regarding the impact of Notch blockade on leukemia growth using the FLT3 ITD model is congruent with our previous data, which demonstrated that inhibition of Notch signaling, via retroviral expression of DN-MAML1 or treatment with gamma secretase inhibitors, can inhibit leukemia growth. Furthermore, inactivating mutations in the Notch pathway are uncommon in primary myeloid neoplasms. These studies are in contrast to the results from disruption of Nicastrin, which led to a marked expansion of myeloid cells and a fully penetrant myeloid neoplasm at 5 months, prompting speculation that Notch functions as a tumor suppressor in myeloid cells.

These differences are likely multifactorial and include cell context, the varying contribution of intrinsic and extrinsic signaling and dosage of pathway modulation; altogether highlighting the complexity of the Notch pathway. To achieve loss of Notch1-4 signaling in vivo, we utilized the dominant-negative mastermind-like transgene in which the ICN transcriptional co-activator MAML1 is truncated and fused to GFP preventing the formation of the ternary complex that is required for the transcriptional activation of Notch target genes. This approach should yield a more specific disruption of canonical Notch signaling than deleting Nicastrin, which has other substrates besides Notch. Finally, we avoided the Mx-1 cre system, and relied on the more hematopoietic-specific Vav-cre model. Not only is the Mx-1-Cre transgene active in mesenchymal stem cells in the bone marrow stroma, but it requires an interferon response for induction and it’s well known that interferon signaling can alter stem cell function and induce proliferation of myeloid cells. Collectively, these features may lead to cell extrinsic effects on myeloid development and leukemogenesis.

Altogether, our results demonstrate that loss of Notch signaling in vivo is insufficient for the development of myeloid neoplasms. This lack of a strong phenotype observed after Notch blockade on normal hematopoiesis is encouraging for clinical studies evaluating the impact of different Notch pathway inhibitors on both solid and liquid tumors. Furthermore, our results highlight the importance of using selective in vivo mouse models of Notch perturbation for future evaluation of the underlying mechanisms of Notch signaling in myeloid malignancies.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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