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SOLICITED REVIEW

Notch signaling at the crossroads of innate and adaptive immunity

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Abstract

Notch signaling is an evolutionarily conserved cell-to-cell signaling pathway that regulates cellular differentiation and function across multiple tissue types and developmental stages. In this review, we discuss our current understanding of Notch signaling in mammalian innate and adaptive immunity. The importance of Notch signaling is pervasive throughout the immune system, as it elicits lineage and context-dependent effects in a wide repertoire of cells. Although regulation of binary cell fate decisions encompasses many of the functions first ascribed to Notch in the immune system, recent advances in the field have refined and expanded our view of the Notch pathway beyond this initial concept. From establishing T cell identity in the thymus to regulating mature T cell function in the periphery, the Notch pathway is an essential, recurring signal for the T cell lineage. Among B cells, Notch signaling is required for the development and maintenance of marginal zone B cells in the spleen. Emerging roles for Notch signaling in innate and innate-like lineages such as classical dendritic cells and innate lymphoid cells are likewise coming into view. Lastly, we speculate on the molecular underpinnings that shape the activity and versatility of the Notch pathway.

KEYWORDS

B cells, dendritic cells, hematopoietic stem cells, innate lymphoid cells, stromal cells, T cells

1 | INTRODUCTION

More than a century ago, John S. Dexter and Thomas Hunt Morgan observed a heritable abnormality in *Drosophila melanogaster* characterized by small indentations at the wing margins and subsequently named the underlying mutated allele "*Notch*" for the irregular wing phenotype.[1,2] By 1983, *Notch* was cloned in *Drosophila*, while orthologs were identified in other organisms shortly thereafter.[3-6] Roughly one decade later, the discovery of a mutated gain-of-function *NOTCH* allele in human T cell acute lymphoblastic leukemia captured the interest of cancer researchers and developmental biologists alike, commencing the modern era of Notch-related investigations in mammalian organisms.[7] Thus, a line of research that started with notched wings gave rise to one of the most extensively studied signaling pathways in biology. In this review, we discuss our current understanding of Notch signaling in the mammalian immune system. Although Notch also has well-established roles in embryonic hematopoiesis, our focus is on Notch's activity in the development, differentiation, and function of adult adaptive and innate immune cells.

Notch signaling is highly conserved across invertebrates and vertebrates. The Notch pathway operates via juxtacrine interactions between signal-sending cells presenting Notch ligands and signal-receiving cells expressing Notch receptors (Fig. 1A). Mammals have 4 Notch family members (Notch1-4) and 5 ligands of the Jagged (Jagged1,2 - orthologs to fly Serrate) and Delta-like families (Dll1,3,4—orthologs to fly Delta). Dll3 likely functions as a natural antagonist, leaving four agonistic mammalian Notch ligands.[8] Notch receptors are transmembrane proteins expressed as heterodimers after cleavage during transport to the cell surface. The Notch extracellular domain contains EGF-like repeats that bind either Jagged or Delta-like ligands. Receptor ligation induces regulated proteolytic cleavage that releases the intracellular domain of Notch (ICN) into the cytoplasm. An ADAM-type metalloprotease mediates the first cleavage at an

Abbreviations: BM, bone marrow; cDC, classical dendritic cell; CLL, chronic lymphocytic leukemia; DC, dendritic cell; Dll, delta-like ligand; dnMAML, dominant negative Mastermind-like; DP, $CD4^+CD8a^+$ "double positive" thymocyte; FoB, follicular B cell; GSI, gamma-secretase inhibitor; HSPC, hematopoietic stem and progenitor cell; ICN, intracellular Notch; iILC2, inflammatory ILC2; ILC, innate lymphoid cell; MAML, mastermind-like family transcriptional coactivator; MZB, marginal zone B cell; nILC2, natural ILC2; pDC, plasmacytoid dendritic cell; Pre-TCR, pre-T cell receptor; T-ALL, T cell acute lymphoblastic leukemia; TCR, T cell receptor; Tfh, T follicular helper cell.

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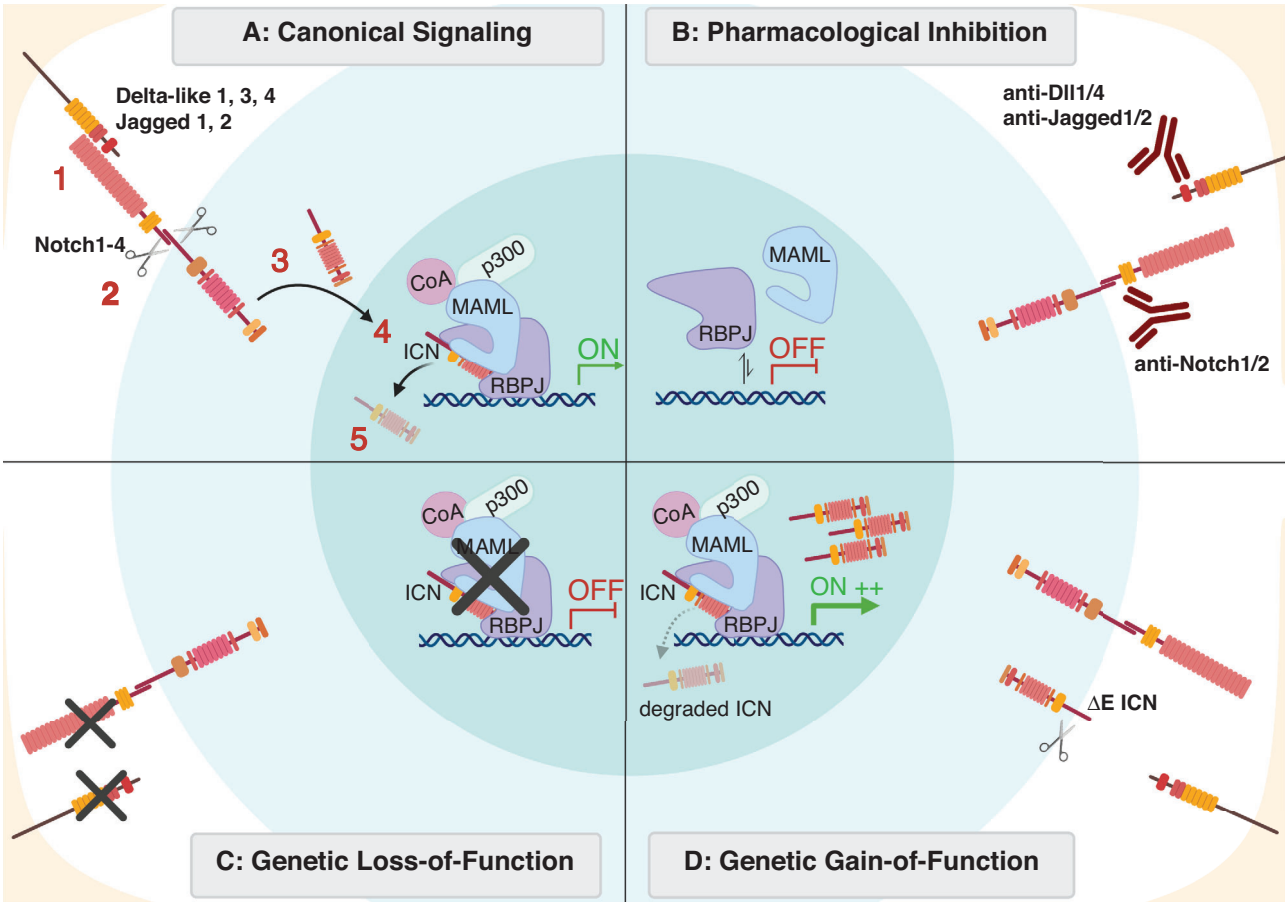

FIGURE 1 **Overview of Notch signaling**. (**A**) Signal-receiving cells express Notch receptors (Notch1–4) and juxtacrine signal-sending cells express agonistic Notch ligands (Jagged 1/2 or Delta-like ligand 1/4). Ligand-receptor interaction **(1)** triggers successive proteolytic cleavages of the Notch receptor **(2)**, first extracellularly by the ADAM10 metalloprotease followed by intramembrane cleavage by the gamma-secretase/presenilin complex. Cleavage releases intracellular Notch (ICN) into the cytoplasm **(3)** where it translocates to the nucleus to form a transcriptional activation complex with the DNA-binding transcription factor RBP-J and a member of the Mastermind-like (MAML) family **(4)**, which in turn recruits additional coactivators (CoA) and enhances gene transcription. Once the transcriptional complex has formed, MAML recruits kinases to phosphorylate ICN, ultimately leading to E3 ligase-dependent degradation and complex destabilization **(5)**. (**B**) Methods of pharmacological Notch inhibition include neutralizing monoclonal antibodies against Notch receptors or ligands. Gamma-secretase inhibitors (not shown) are often used for pan-Notch inhibition, which prevents ICN cleavage and formation of a functional ICN-RBPJ-MAML complex. (**C**) Genetic loss-of-function models include conditional inactivation of Notch ligand or receptor genes, *Rbpj* inactivation, and inhibition of the Notch transcriptional activation complex via expression of a dominant negative MAML (dnMAML). (**D**) Genetic gain-of-function models primarily rely on overexpression of constitutively active ICN. However, models utilizing a gamma-secretase sensitive membrane-tethered ICN (ΔE ICN) are also available and have the advantage of permitting signal withdrawal or titration with GSI when needed. Figure created with BioRender.com

extracellular site, followed by intramembrane proteolysis by the gamma-secretase/presenilin complex that releases ICN to migrate to the nucleus. Once in the nucleus, ICN interacts with the transcription factor RBP-J and a Mastermind-like family transcriptional coactivator (MAML1-3). The ICN/RBP-J/MAML complex recruits a larger transcriptional activation complex to induce target gene transcription. Once the transcription complex has been formed, ICN is rapidly degraded. Together with the lack of signal amplification and of complex signal transduction steps in the cytoplasm, these features render the Notch pathway exquisitely sensitive to precise regulation influenced by dose, duration, and context of the signals. Finally, Notch receptors may also signal independently of RBP-J and MAML in selected circumstances, however we will confine our discussion to "canonical" Notch signaling mediated by RBP-J and MAML, as it underlies the most established functions of Notch in immune cells.[9-11]

Notch signaling can be manipulated in vitro and in vivo by several methods (Fig. 1). Pan-Notch inhibition can be achieved using pharmacological agents such as gamma-secretase inhibitors (GSI), which prevent ICN release. More selective blocking strategies include neutralizing mAbs against individual Notch receptors or ligands (Fig. 1B). Genetic Notch loss-of-function models include conditional inactivation of Notch ligand and receptor genes, *Rbpj*, or inhibition of the Notch transcriptional activation complex via expression of a dominant negative MAML (dnMAML; Fig. 1C). Lastly, genetic gain-of-function models primarily rely on overexpression of constitutively active ICN; however, more subtle approaches exist, including expression of

weaker gain-of-function alleles as well as membrane-tethered forms of constitutively active Notch that still rely on gamma-secretase activity for ICN release, thus allowing for Notch titration in combination with GSI (Fig. 1D).[12-15] Although these refined gain-of-function models have mostly been used in cell lines and cancer studies thus far, they could offer a more nuanced way to deploy Notch gain-of-function strategies in in vivo models.

Despite Notch's simple stoichiometry, its functional consequences are pleiotropic and context-dependent. As with many transcriptional regulators, the number of potential Notch/RBP-J binding sites across the genome greatly exceeds the number of genes that Notch regulates within a given cell.[16] Specificity in part derives from the chromatin state, which controls genomic access and helps contextualize Notch's transcriptional effects. Within a particular epigenetic landscape, the location of binding includes sites proximal to gene promoters as well as distal regulatory regions. Past work in Notch-driven T cell acute lymphoblastic leukemia (T-ALL) showed that many dynamic Notch effects occur at distal enhancers.[17] Moreover, studies comparing Notch-dependent B cell lymphomas and T cell acute lymphoblastic leukemia (T-ALL) suggest that Notch works primarily through lineage specific-enhancers.[18] For example, Notch-mediated up-regulation of Myc expression occurs via different enhancer sites in B cell lymphomas and T-ALL. It is likely that access to these sites requires lineage-specific factors. Evidence is accumulating that elements of this regulation are evolutionarily conserved, as the Notch transcriptional complex associates with a lineage-specific cofactor Runx—or the *Drosophila* orthologue, Lozenge—at enhancer sites in both T-ALL cells and *Drosophila* blood cell equivalents.[17,19] Similarly, Runx1 is essential to promote T cell fate in response to Notch activity during mouse T cell development.[20,21] Whether this paradigm holds true in other mammalian contexts outside of cancer, such as in mature T cells or dendritic cells, remains to be established. Altogether, delineating which Notch binding sites are functionally relevant, and how they contribute to transcriptional changes, is challenging. Although recurrent Notch targets have been identified, including members of the Hairy/Enhancer-of-split (*Hes*) and Hairy-related (*Hey*) families, the diverse outcomes of Notch activation are likely mediated by lineage-specific cooperating factors and accessible Notch targets determined by a preconditioned epigenome (Fig. 2). As scientists uncover more roles for Notch in the immune system, it will be increasingly important to delve into the molecular mechanisms underpinning Notch's transcriptional effects in each cell type.

2 | ADULT HEMATOPOIESIS AND LYMPHOPOIESIS

2.1 | Hematopoietic stem and progenitor cells

Early work in *Drosophila* and *C. elegans* described the ability of Notch to promote self-renewal and inhibit progenitor differentiation, laying the groundwork for a potential role in mammalian stem cell function.[22-28] The discovery that the human Notch ortholog is expressed in $CD34^+$ bone marrow (BM) hematopoietic stem and progenitor cells (HSPCs) then led to a cascade of studies implicating Notch signaling in HSPC maintenance, myelopoiesis, and regulation of megakaryocyte/erythroid cell development.[29-36]

Mechanistically, many early reports relied on gain-of-function experiments and assumed that Notch receptors in HSPC directly interacted with ligands expressed by supportive niche cells.[30,37,38] Constitutive activation of Notch in conjunction with hematopoietic cytokines promoted HSPC survival and expansion.[33] Similarly, exposure to immobilized Dll4, Dll1, or Jagged1 supported HSPC survival and multilineage reconstitution upon transplantation.[34-36] Both endothelial and osteoblastic cells were reported to express Notch ligands and promote ex vivo HSPC maintenance, suggesting that direct ligand/receptor interactions were a critical component of the HSPC niche.[30,38] Moreover, conditional *Jagged1* deletion in BM endothelial cells led to a decrease in HSPCs, as well as impaired recovery after sublethal irradiation.[37] These data were interpreted as evidence of Notch's importance in maintaining HSPCs within their niche in the BM.

Beyond supporting HSPC maintenance and self-renewal, other studies implicated Notch in regulating myelopoiesis. Mouse models with conditional inactivation of Notch signaling displayed an accumulation of granulocyte-monocyte progenitors and development of chronic myelomonocytic leukemia-like disease.[31] Additional reports suggested that Notch inhibits granulopoiesis while promoting megakaryocyte/erythrocyte development, while signaling through Notch2 was mapped to steady-state and stress erythropoiesis.[32] In addition, mouse HSPC co-cultured with OP9-Dll1 stromal cells induced megakaryocyte differentiation, and ICN4 overexpression rescued inhibition of megakaryopoiesis when OP9-Dll1 co-cultures were treated with gamma-secretase inhibitors (GSI).[39] However, in contrast to mouse HSPC studies, experiments with human HSPC indicated that Notch signaling through Dll4 axis inhibits, rather than promotes, megakaryocyte development.[40] Despite species differences, these data overall suggested roles for Notch signaling in regulating non-lymphoid hematopoietic lineages, in addition to its role in HSPCs.

Although gain-of-function and in vitro studies identified multiple effects of Notch signaling, genetic models inhibiting all routes of Notch transcriptional activation in HSPC showed canonical Notch signaling to be dispensable for HSPC maintenance and myelo-erythropoiesis under homeostatic and stress conditions in vivo.[41-43] Transplantation of Notch-deficient HSPC via *Rbpj* inactivation or expression of a dnMAML pan-Notch inhibitor did not reveal marked defects in reconstitution or lineage potential. Several considerations may help to reconcile these conflicting bodies of data. First, a majority of the gain-of-function experiments exposed HSPC to supraphysiological levels of Notch activation. Rather, HSPCs experience low levels of in situ Notch signaling in the BM, as evidenced by low Notch target expression.[41,44] Instead, sustained levels of intense Notch signaling drive ectopic T cell development that can eventually progress to T-ALL.[45-47] Second, it is often assumed that the only functionally significant Notch signals for hematopoiesis are mediated between niche cells and HSPC. However, it is possible that non-cell-autonomous signals regulate HSPC function indirectly. In support of this concept, transplantation of wild-type BM

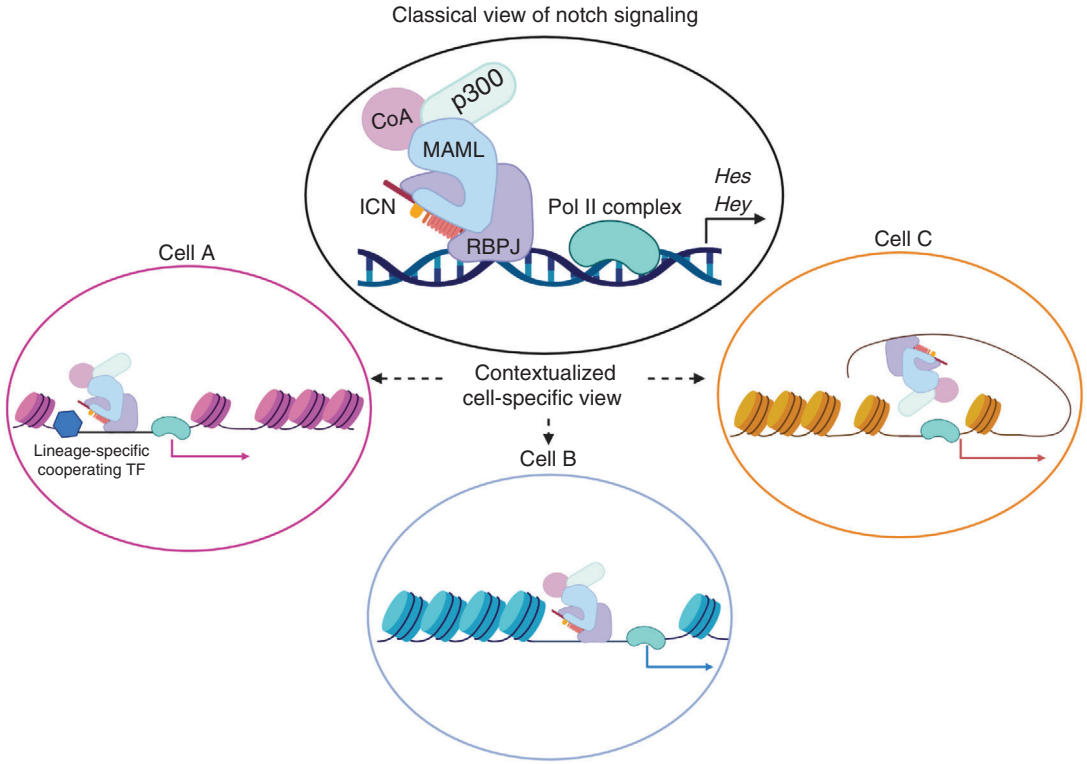

FIGURE 2 **Cell-specific regulation of Notch-mediated transcriptional activation.** Classical Notch transcriptional targets include members of the Hairy/Enhancer-of-split (*Hes*) and Hairy-related (*Hey*) families. However, the lineage and context-dependent consequences of Notch activation are likely mediated by cell-specific cooperating factors (Cell A) as well as a preconditioned epigenetic landscape that defines target accessibility (Cell A vs. Cell B). Moreover, the ICN/RBPJ/MAML complex can regulate transcription via binding either proximally to gene promoters or to distal enhancer sites, adding to the versatility of Notch as a transcriptional regulator (Cell C). Figure created with BioRender.com

cells into mice lacking *Rbpj* in radioresistant host cells led to altered hematopoiesis and eventually to myeloproliferative disease.[48] Similarly, Notch signaling may be critical for niche cell maintenance. Indeed, Notch signaling between BM endothelial cells appeared necessary for niche regeneration and timely hematopoietic recovery after BM injury.[49] Thus, while Notch signaling may regulate the BM microenvironment, its cell-autonomous role in HSPCs remains debated.

2.2 | T cell development

Notch signaling is essential to establish T lineage identity in lymphocyte progenitors that emigrate from the BM to the thymus. Although not the focus of this review, others have already contributed in-depth discussion of the transcriptional networks sequentially regulating T lineage specification, commitment, and development—all of which are contingent upon initial Notch signals.[50]

Unraveling Notch's critical role in T cell development began with the identification of a Notch activating mutation in human T-ALL with a rare t(7;9) chromosomal translocation.[7] The ortholog to the *Drosophila* Notch receptor was found to be highly expressed in human and mouse lymphoid tissues, suggesting a potential role in lymphocyte development and/or differentiation.[7,45] Mice receiving BM transplantation with cells transduced to express ICN1 displayed thymus-independent production of T cells and suppressed B cell development in the BM.[46] Conversely, mice with an inducible *Notch1* inactivation in hematopoietic cells developed a hypoplastic thymus with markedly reduced T cells and a concomitant accumulation of intrathymic B cells.[51] Further in vitro and in vivo studies revealed that early T cell development was supported by signals mediated through the Notch1 receptors in T lineage progenitors and Dll4 Notch ligands in thymic epithelial cells.[52-54] Although both Dll1 and Dll4 can support T lineage development in vitro, the essential endogenous ligand expressed by thymic cortical epithelial cells is Dll4.[52,53,55]

Together, these complementary gain- and loss-of-function studies indicate that Notch signaling is necessary and sufficient to support T lineage development. The accumulation of B cells in loss-of-function experiments and suppression of B cell development in gain-of-function experiments was interpreted as evidence for a cell-intrinsic fate switch in the absence of Notch. Thus, analogous to reports in *Drosophila*, mammalian Notch appeared to regulate a binary cell fate decision, enforcing T lineage at the expense of B cell development. The earlier identification of lymphoid-restricted BM progenitors fortified the concept that Notch promoted 1 of 2 diametrically opposed fates in bipotent progenitors.[56] However, direct experimental evidence supporting cell-autonomous T-to-B conversion in the absence of Notch was lacking. A closer examination of thymic B cells observed in Notch1-deficient mice revealed that a large fraction of these cells arose from rare Notch1-sufficient progenitors. Thus, an alternative explanation was that most

intrathymic B cells were not linked to an intrinsic *Notch1* deficiency, but a thymus lacking abundant Notch1-sufficient progenitors was permissive to B cell development. Additionally, early T cell progenitors were found to have dendritic cell and myeloid cell potential, further undermining a simple binary model of Notch controlling T versus B cell fates.[57,58]

The requirement for Notch signaling evolves through successive stages of T cell development. In the earliest T lineage progenitors, Notch kickstarts expression of gene networks essential for T cell identity.[59,60] Multipotent lymphoid progenitors co-cultured with OP9 stromal cells that constitutively express Dll4 potently up-regulate expression of the transcription factor *Tcf7*, a master regulator of T cell fate.[61,62] *Tcf7* expression is directly up-regulated by Notch activation, as supported by chromatin immunoprecipitation showing that Notch and RBP-J bind within the *Tcf7* locus. Importantly, thymocytes continuously remodel their epigenetic landscape and become conducive to the induction of specific Notch-regulated targets, such as the oncogene *Myc*, at later stages. For example, *MYC* contains a long range enhancer controlled by NOTCH1 in human T-ALL.[63] Genetic deletion of this conserved regulatory sequence in mice led to impaired T cell development and markedly reduced *Myc* expression in pre-T cells, but without impacting earlier steps of Notch-driven T lineage development.

Beyond lineage commitment, Notch remains an essential pro-survival signal through the beta-selection checkpoint, a critical stage during T cell development when immature thymocytes are selected for productive rearrangement of the *Tcrb* locus. T lineage committed progenitors require Notch activation to provide essential survival signals via the PI(3)K-Akt pathway, independent of signals downstream of the pre-TCR.[64] In the absence of Notch1, developing thymocytes committed to the T lineage undergo apoptosis. Conditional inactivation of *Notch1*, *Rbpj*, or inhibition of Notch signaling by dnMAML arrests T cell development prior to beta-selection, when developing T cells are exposed to high levels of signal.[65-67] After clearance of the beta-selection checkpoint, Notch1 expression is rapidly down-regulated downstream of pre-TCR activation, and Notch ultimately becomes dispensable for positive and negative selection.[67-69]

Interestingly, a growing body of evidence supports a prethymic role for Notch signaling in early T cell development. *Dll4* inactivation in mesenchymal progenitor cells was reported to decrease BM lymphoid progenitors and impair thymopoiesis.[70] Similarly, endothelial *Dll4* inactivation was linked to decreased lymphoid progenitors and enhanced myelopoiesis.[71] Moreover, mice transplanted with *Notch1* hypomorphic hematopoietic stem cells displayed a cell-autonomous defect in T cell production that was associated with a decrease in BM lymphoid progenitors.[49] Most recently, a new mouse model allowing for temporal and hematopoietic-specific control of *Rbpj* expression and Notch responsiveness supported these findings, suggesting that lymphoid precursors first experience Notch signaling in the BM.[72] Nevertheless, Notch ligand expression and signaling intensity is tightly regulated to prevent extrathymic T cell development during steady-state conditions. Overt overexpression and constitutive activation of Notch signaling in BM progenitors leads to T-ALL.[45,46] Additionally, loss of repression of Dll4 in erythroblasts induces in situ differentiation of BM progenitors into T lineage cells.[44] Thus, Notch signals are delivered at different locations, and spatial regulation of signaling intensity is critical for efficient T cell production without excessive extrathymic T cell development.

In sum, Notch signaling does not occur as a single, inductive pulse in progenitors, but rather is sustained over the course of multiple developmental stages. An initial Notch signal is essential for converting multipotent precursors into T lineage-restricted progenitors that display differential receptivity to continuous Notch activation. This progressive transformation—a complex interplay between Notch, other master transcriptional regulators, and an evolving epigenetic landscape—is fundamental to propagate cells through developmental phases and allow for successful maturation. Understanding the stage-specific kinetics and intensity of Notch signals is critical to explain how a seemingly simple signal transduction pathway can have diverse effects, even within a single step-wise process such as T cell development.

3 | ADAPTIVE IMMUNE CELLS

3.1 | Mature T cells

3.1.1 | T cell maturation

Given Notch's prominent role in cell fate decisions, many research groups hypothesized that Notch might regulate T cell development and maturation beyond establishing T lineage identity. Several reports suggested a role for Notch signaling in $\alpha\beta$ versus $\gamma\delta$ as well as CD4 versus CD8 lineage decision.[73-78] Early work assessing the contribution of *Notch1* heterozygous BM cells to the $\alpha\beta$ versus $\gamma\delta$ T cell lineages upon transplantation into *Rag1*-deficient hosts suggested that Notch may favor the $\alpha\beta$ over the $\gamma\delta$ T cell lineage.[75] Similar reports showed that Lck-Cre-driven *Rbpj* inactivation, but not CD4-Cre-driven inactivation, resulted in enhanced $\gamma\delta$ T cell production.[66] These 2 transgenic models differ in the timing of *Rbpj* deletion: Lck proximal promoter-driven Cre expression initiates *Rbpj* inactivation earlier than the CD4-Cre model. However, Lck-Cre-induced dnMAML expression, which also inhibits all routes of canonical Notch signaling, did not affect generation of $\gamma\delta$ T cells.[67] While both in vivo loss-of-function studies are equally valid, the discrepancy may be due to minor differences in the timing or efficiency of Cre-mediated Notch inactivation. Single cell clonal assays of T lineage progenitors refined our understanding of the stage-dependent impact of Notch signaling on $\gamma\delta$ T cell generation, demonstrating that Notch signaling is required at earlier stages of development, but dispensable later on.[68,74] Notch signaling also plays an important role in generating human $\gamma\delta$ T cells. However, unlike in mice, intense levels of Notch signal provided to early precursors inhibited, rather than promoted, $\alpha\beta$ T cell production.[79] These data highlight an important point: Notch signaling has persisted throughout evolution as a powerful mode of cellular communication, yet dynamic regulation allows for divergent functional consequences within a cellular process and even across species.

Early reports explored a connection between Notch activation and the CD4 versus CD8 lineage decision. Constitutive ICN1 expression in

immature thymocytes resulted in increased numbers of either CD8$^+$ or both CD4$^+$ and CD8$^+$ thymocytes.[76,78] Moreover, ICN1 overexpression in T cell lines resulted in increased resistance to glucocorticoid-induced cell death, suggesting a role for Notch in promoting cell survival during DP maturation.[77] While gain-of-function studies supported a role for Notch at later stages of T cell development, in vivo Notch inactivation at the CD4$^+$CD8$^+$ double positive (DP) stage challenged these reports and showed no effect on the CD4/CD8 lineage decision, as well as subsequent maturation, survival, and emigration of thymocytes.[65,67] In this case, gain-of-function studies proved to be of limited physiological relevance because DP thymocytes experience low Notch signaling intensity, much like BM HSPCs. Thus, it is critical to evaluate both stage-specific gain and loss-of-function experiments, with a contextualized understanding of the intensity of in vivo Notch signaling.

3.1.2 | Effector T cell function

The importance for Notch signaling in T cells extends beyond development and influences T cell effector differentiation and function. Although Notch activity precipitously declines after the DP stage in the thymus, mature naïve CD4$^+$ and CD8$^+$ T cells express Notch1 and Notch2 receptors in the periphery and up-regulate their expression upon T cell receptor (TCR) stimulation. Both ex vivo overexpression and in vivo loss-of-function studies implicate Notch signaling in a variety of immune contexts, including host defense, autoimmunity, and alloimmunity. Encapsulating the breadth of work on Notch in mature T cells is beyond the scope of this review, although this has been discussed in detail recently.[80] Here, we focus on key findings that shaped our current view of Notch's effects on mature T cell function.

Early overexpression in vitro work suggested that Notch activation in mature T cells had a tolerogenic effect. CD4$^+$ T cells exposed to APCs that constitutively expressed Jagged1 acquired Ag tolerance, leading to decreased proliferation and IFN-γ production.[81-83] Although these data supported a suppressive role for Notch signaling, they conflicted with subsequent reports illustrating that Notch ligand expression is up-regulated among APCs in response to inflammatory stimuli.[84-86] In vivo loss-of-function studies corroborated earlier ex vivo work that supported a pro-inflammatory role for Notch. Mice with mature CD4$^+$ T cells lacking Notch1 and Notch2 receptors were more susceptible to *Leishmania major* infection, demonstrating higher parasite burden and lower levels of CD4$^+$ T cell IFN-γ secretion.[87] Similarly, expression of dnMAML in CD4$^+$ T cells led to impaired *Cryptococcus neoformans* clearance and higher fungal burdens.[88,89] Moreover, inactivation of *Notch1/2* in CD4 T cells blunted expression of S1PR1 and subsequent lymph node egress during a house dust mite challenge, suggesting that Notch's role in promoting a Th2 inflammatory response may extend beyond mediating cytokine expression.[90] Taken together, these studies support the notion that Notch signaling was critical to promote an inflammatory response to host pathogens. Much like CD4$^+$ T cells, mature cytotoxic CD8$^+$ T cells also depend on Notch signaling during host defense. TCR stimulation up-regulates surface Notch1/2 expression in CD8$^+$ T cells, and genetic inactivation of *Notch1* and *Notch2* weakened the CD8$^+$ T cell cytotoxic response to several pathogens, including *Trypanosoma cruzi*, *Listeria monocytogenes*, and influenza.[91-93] Collectively, these data argue for a proinflammatory role of Notch among both CD4$^+$ and CD8$^+$ T cells during infection.

Alloimmune and autoimmune models likewise support a proinflammatory role for Notch signaling. Numerous lines of evidence from models of rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis highlight Notch's proinflammatory functions and suggest that its inhibition may have therapeutic benefit.[94-100] Furthermore, Notch is critical to regulate alloreactive T cell function during solid organ transplant rejection and graft-versus-host disease after allogeneic bone marrow transplantation, as reviewed in detail elsewhere.[80,101] Understanding the cell types involved, the associated receptor-ligand pairs, and the downstream consequences of Notch activation across multiple cell types is essential to pinpoint critical cellular interactions that lead to excessive inflammation, immune dysregulation, and disease.

In sum, Notch signaling is emerging as a pro-inflammatory pathway in T cells in vivo, but it remains debated whether Notch activation promotes specific T effector responses. Early models proposed that individual Notch ligands could specify patterns of CD4$^+$ T cell effector differentiation. Overexpression of Dll4 in APCs led to increased Th1 differentiation ex vivo, whereas Jagged1 led to Th2 polarization.[85,86] Th1 polarizing conditions increased *Dll4* expression in APCs, whereas Th2 conditions up-regulated expression of *Jagged* ligands. Dll1 appeared to drive Th1 commitment ex vivo and aided in control of intracellular pathogens such as *Leishmania major*.[102] Notch blockade via GSI suppressed Th1 differentiation in vivo.[95] In contrast, mouse models of pan-Notch inhibition using dnMAML expression in CD4$^+$ T cells showed that Notch blockade impaired Th2 cytokine secretion and defense against the helminth *Trichuris muris*, suggesting that Notch promoted Th2 differentiation.[103,104] Beyond Th1 and Th2 cells, Notch was also reported to enhance Th9 responses, promote follicular T helper (Tfh) differentiation, and suppress Treg differentiation.[105-107]

Taken together, the simple paradigm of Notch regulating binary cell fate decisions cannot collectively account for these data. More recent models inspired by additional experimentation may help clarify and synthesize otherwise conflicting studies. Indeed, Notch-deprived T cells expressing dnMAML could initiate a Th2 program following *Trichuris muris* infection, but were unable to sustain the appropriate immune response.[108] In vitro stimulation of naïve CD4$^+$ T cells with/without GSI showed that expression of essential gene targets in both Th1 and Th2 conditions decreased, but could be restored upon recovery from Notch blockade. Furthermore, chromatin immunoprecipitation assays suggested that Notch bound a variety of effector lineage-determining transcription factor loci, such as *Gata3*, *Tbx21*, and *Rorc*, independently of cytokine signals.[108] Whether or not Notch occupancy at these loci is functionally relevant and corresponds to dynamic changes in transcription remains an open question. Nonetheless, Notch may function as a general signal-amplifier and orchestrate multiple effector programs by sensitizing cells to environmental cues. Further support for this model comes from evidence indicating that in vivo deletion of *Dll4* in APC resulted in a global decrease in T cell

activation.[109] The authors proposed that Notch signaling bolsters co-activation of CD4+ T cells downstream of CD28, suggesting that Notch functions as a regulator of co-stimulation through mechanisms that remain to be identified.

Altogether, Notch signaling is integral to a T cell's existence. From specification to commitment, maturation, and effector functions, Notch is an indispensable communication pathway for T cells to interact with their environment. As such, a more nuanced, complex model defining the dynamics of Notch regulation and function is necessary. In particular, Notch's role as a regulator of the transcriptome and epigenome in mature T cells remains poorly understood. Compared to what we know about Notch in other contexts such as immature T cells and T cell leukemia, this is an important gap of knowledge and an exciting avenue of study. Importantly, context-specific epigenetic mechanisms likely underlie Notch's multidimensional role and are key to understanding its ability to synchronize multiple inputs and coordinate immune responses.

3.2 | Mature B cells

The importance of Notch signaling in B cells appears restricted mostly to particular subsets of B lymphocytes, such as marginal zone B (MZB) cells.[110] Unlike other recirculating mature B lymphocytes that reside in B cell follicles, MZB cells are retained in the blood-rich marginal sinus of the spleen at the red pulp interface. MZB cells rapidly generate plasma cells in response to blood-borne pathogens and are critical for protective responses to encapsulated bacteria.[111] After an initial report pinpointing a role for *Rbpj*, other groups described non-redundant roles for the Dll1 ligand, Notch2 receptor, and Maml1 coactivator in MZB cell homeostasis.[110,112-114] Together, these studies demonstrated that Dll1/Notch2 interactions are essential for MZB cells. Interestingly, Notch's effects are highly sensitive to gene dosage as reductions in MZB cells were reported upon loss of only 1 *Dll1*, *Notch2*, or *Maml1* allele. More recently, a viable hypomorphic missense mutation of *Ncstn*, the gene encoding for the nicastrin protein of the gamma-secretase complex, led to defects in intestinal homeostasis, MZB cell development, and T cell-independent Ab responses.[115] Thymocyte development was unaffected, suggesting that Notch2 processing for MZB cells was more sensitive to the mutation compared to Notch1 processing. Thus, MZB cells appear uniquely sensitive to and reliant on robust Notch2/Dll1 activation.

Mechanistically, Notch was proposed to control a binary cell fate decision whereby immature transitional B cells choose between follicular B (FoB) and MZB cell fates.[110,116] At the time, this concept satisfied prevailing models influenced both by findings in lower organisms and in other hematopoietic contexts (e.g., the "T/B lineage decision" that was proposed to underlie the effects of Notch signaling in early lymphoid development). However, this model is not consistent with more recent work showing that continuous Notch signals are required for the maintenance of MZB cells, nor with the capacity of FoB cells to yield MZB cells in lymphopenic environments.[117-119] Interestingly, MZB cells rely on Dll1 Notch ligands expressed by non-endothelial stromal cells lineage traced with a *Ccl19-Cre* transgene, a source that was also recently shown to regulate T cell function in secondary lymphoid organs.[120,121]

Beyond MZB cells, less is known so far about roles of Notch signaling in other B cell subsets. Ex vivo, Notch signaling can cooperate with critical signals delivered through the B cell receptor or CD40, and this cooperativity has been hypothesized to play a role in the B cell immune dysfunction that underlies aspects of chronic graft-versus-host disease.[122,123] Genetic evidence also suggests that Notch1-mediated signals can regulate plasma cell differentiation, although limited studies have been performed.[124] Importantly, activating *NOTCH1* and *NOTCH2* mutations have been reported in human B cell lymphomas, including chronic lymphocytic leukemia lymphoma (CLL) and marginal zone lymphomas.[125-129] In these tumors, *NOTCH1/2* mutations truncate the C-terminal PEST domain, which increases the half-life of cleaved intracellular NOTCH. Moreover, the gain-of-function outcomes of these mutations remain ligand-dependent, and Notch activation can also occur via unmutated Notch receptors/ligands in >50% of CLL.[130,131] In some cases, active Notch was detected only within lymph nodes and abruptly lost in disease areas outside of the capsule.[130] Thus, human Notch-driven B cell lymphomas appear to thrive on stroma-derived signals driven by Notch ligands, likely hijacking functions of the Notch pathway that operate in normal B cell homeostasis and function. However, the overall functional impact of Notch signaling remains to be fully explored, especially considering the limited availability of in vivo Notch-dependent B cell lymphoma models. Moreover, mechanistic information thus far has been limited to few cell lines harboring rare, potent Notch-activating events that do not mimic the more prevalent scenarios of non-mutational activation or weak ligand-dependent Notch gain-of-function alleles commonly observed in disease.

4 | INNATE AND INNATE-LIKE CELLS

4.1 | Dendritic cells

Innate cell types, such as dendritic cells, also depend on Notch signaling for their in situ terminal differentiation and maintenance. Classical dendritic cells (cDC) are specialized APCs that link innate and adaptive immunity by recognizing pathogens via pattern recognition receptors and by recruiting other immune cells to orchestrate an Ag-specific response. Mouse cDC are composed of two main subsets, cDC1 and cDC2. cDC1 are capable of Ag cross-presentation to CD8+ T cells and can be identified through expression of CD8$\alpha\alpha$ and CD103. cDC2 are characterized by expression of CD11b and mostly present exogenous Ags to CD4+ T cells.[132]

cDC and plasmacytoid dendritic cells (pDC) derive from progenitors that emigrate from the BM to undergo differentiation in peripheral lymphoid tissues. Both transcription factors and tissue-specific signals are required for terminal cDC differentiation. For instance, IRF8 and BATF3 are required for cDC1 differentiation in humans and mice.[132] Signaling through the Dll1-Notch2 axis appears necessary for differentiation of selected splenic and intestinal cDCs. Targeted inactivation of *Rbpj* or *Notch2* in DCs led to decreased frequency and numbers of

$CD8^{-}CD11b^{+}$ cDC2, as well as a modest decrease in $CD8\alpha\alpha^{+}$ cDC1 in the spleen.[133,134] Refined analyses identified a near absence of a particular subpopulation of cDC2 defined by expression of endothelial selective-adhesion molecule (Esam).[134]

Loss of Notch-dependent cDCs is specific to the spleen and to a population of intestinal $CD11b^{+}CD103^{+}$ DCs that reside in the lamina propria.[133,134] The localized defects suggest that Notch signaling plays a tissue-specific role in splenic and intestinal DC differentiation, both known sites of Notch activity. In fact, *Notch2* deletion disrupted characteristic clusters of $CD11b^{+}$ cDC2 in the marginal zone and bridging channels of the spleen, leaving a disorganized scattering of cDC throughout the T cell zone and red pulp.[135] Other studies revealed that *Dll1* inactivation in *Ccl19-Cre*$^{+}$ fibroblastic stromal cells resulted in loss of $Esam^{+}$ cDC2, as well as reduction in cDC1 and other cDC2 subsets.[120] Thus, the Dll1/Notch2 axis is critical for proper differentiation and localization of $Esam^{+}$ cDC2 within a particular splenic niche. However, the nature and source of Notch ligands in the intestine remain to be discovered.

Loss of these DC subpopulations has functional consequences. Notch2-deficient mice immunized with sheep red blood cells had reduced splenic Tfh differentiation and germinal center formation.[136] In the intestine, Notch2-deficient mice had reduced IL-17-producing T cells.[134] Additionally, mice deficient in lamina propria Notch2-dependent DC exhibited decreased survival after challenge with *C. rodentium*, a mouse model pathogen of attaching-and-effacing intestinal bacteria. These DCs are a critical source of IL-23, which is essential for activation of innate lymphoid cells (ILC) to produce IL-22 and provide protection against enteropathogens.[135]

Altogether, a picture for how Notch contributes to DC biology is coming into focus, although much remains to be learned. Cumulative data suggest that Notch plays distinct roles in DC differentiation and survival, much like it does in T cells. Early studies using a DC-specific Cre recombinase to inactivate Notch signaling showed a modest decrease in splenic cDC1 as well as a dramatic loss of cDC2.[134] Notch ablation in BM HSPCs using a Vav1-Cre recombinase similarly led to a reduction of $CD8\alpha^{+}$ cDC1 and $CD11b^{+}$ cDC2, suggesting that early loss of Notch signals impaired splenic DC development.[135] Moreover, primary BM cells cultured on OP9-Dll1 stroma exhibited more efficient generation and phenotypic resolution of cDC1 and cDC2 compared to cells cultured without Notch ligands on OP9 stroma alone. Human cord blood progenitors also produced more cDC1 when exposed to human DLL1.[137] These data support the concept that Notch acts as a critical regulator of both cDC1 and cDC2 terminal differentiation from an upstream precursor. However, the defect in cDC1 was modest compared to the complete absence of $Esam^{+}$ cDC2. Reconciling this discrepancy may come in part from assessing Notch signaling activity in mature cDC. Only mature cDC2 express high levels of Notch2 receptor and target genes such as *Hes1* and *Dtx1*.[133,134] Moreover, Notch-deprived cDC2 demonstrate increased apoptosis and inappropriate localization in the spleen. In contrast, cDC1 express few or no classical Notch target genes, indicating that active Notch signal may not be critical after terminal differentiation. As a possible explanation, it has been proposed that Notch2 signaling may stay active in differentiated $Esam^{+}$ cDC2 that remain in contact with Dll1-expressing marginal zone and bridging channel stroma.[138] Thus, Notch2/Dll1 signals might be required for cDC2 survival, maintenance, and positioning with abrogation of the signal leading to apoptosis, loss, and mislocalization of residual cDC2. In contrast, Notch2/Dll1 signals may be turned "off" in mature cDC1, which exhibit little transcriptional Notch activity and thus might migrate through the spleen with a lessened Notch dependence.

Overall, it will be exciting to elucidate the mechanisms underlying Notch's dual roles in DCs. Notch's ability to transcriptionally promote cDC1 and cDC2 lineage programs is reminiscent of its potential role as a signal amplifier in mature T cells.[108,109,138] As the DC transcriptional and epigenetic landscape evolves from pre-DC precursors to differentiated cDC1 and cDC2, Notch's potential binding partners and range of targets likely shifts. Notch's target genes in developing and mature cDC remain unknown. Interestingly, recent work investigating the regulation of *Irf8* expression in cDC1 showed that 2 distinct enhancer regions are required at different stages of early cDC1 development.[139] One enhancer region is required during cDC1 development and maintained in mature cDC1. However, another region is only transiently accessibly during cDC1 specification. It would be interesting to know whether Notch can bind and/or regulate either region and if occupancy has consequences on *Irf8* expression and cDC1 specification. Lastly, it will be interesting to explore if cDC1 negatively regulate Notch signaling after terminal differentiation in order to become independent of Notch for survival. Altogether, Notch's target genes in developing and mature cDC remain unknown, and their impact on overall differentiation versus specific DC functions remain to be established.

4.2 | Innate lymphoid cells

Innate lymphoid cells (ILCs) are a diverse class of immune cells that lack Ag receptors, but otherwise functionally and transcriptionally parallel effector T cells.[140] ILCs can be categorized as "cytotoxic" ILCs, for example, NK cells, and "helper" ILCs, including ILC1, ILC2, and ILC3 cells that produce effector cytokines in response to environmental stimuli as part of type 1, type 2, or type 3 immune responses. Given the transcriptional and functional similarities between ILC and T cells, Notch activity is a logical candidate for investigation. Although data on the importance of Notch exists, rigorous in vivo loss-of-function studies that specifically target Notch signaling in ILC subsets are only starting to emerge. This is in part due to the difficulty of specific in vivo targeting of ILC. Thus, most data so far relied on in vitro co-cultures with Notch ligands or *Il7ra*-driven Cre recombinase that targets deletion to the early lymphoid progenitors from which ILC, T, and B cells all derive. A more recent genetic model that targets ILC2s has just emerged, as illustrated using a complex scheme to inactivate *Icos* in ILC2, but not $CD4^{+}$ T cells.[141] Such models will be critical to further refine our understanding of Notch signaling in ILC biology.

Fate mapping reveals that, like T and B cells, ILCs originate from BM common lymphoid progenitors.[142-144] Much less is known about cellular and molecular cues supporting early ILC development in the BM. Direct assessment of Notch's requirement for ILC precursors

using in vivo loss-of-function models has yet to be reported. However, evidence suggests that Notch promotes ILC lineage multipotentiality among early precursors. Single cell in vitro co-cultures of mouse and human ILC progenitors with OP9-Dll4 or OP9-Dll1 generate more colonies with mixed lineage potential compared to OP9 stroma.[145-147] Although some groups showed reduced NK cell generation and increased ILC2 production when ILC progenitors were exposed to Notch ligands, the overall effects of Notch signaling were to maintain or promote multi-lineage output.[145] These studies suggest that Notch is not required for early ILC precursors to mature, survive, or differentiate, as they can do so on OP9 stroma alone. Interestingly, transfer of lymphoid precursors from OP9-Dll1 to OP9 within 3–6 days of co-culture halted ILC2 development.[148] Furthermore, transplantation of BM HSPCs expressing the dnMAML pan-Notch inhibitor led to severely reduced frequency and numbers of lung ILC2.[149] Co-transduction with a Tcf7 retrovirus partially restored the defect, suggesting that Notch may be upstream of *Tcf7* expression in ILCs, as in T cell development.[61,62,149] Altogether, these data suggest that Notch signaling may regulate early ILC2 development.

ILC precursors eventually leave the BM to seed the periphery where they complete maturation. Notch may also play a role in the activation of mature ILC2. The lineage stability and flexibility of ILCs remain unclear and mounting evidence suggests that there is plasticity among ILC lineages. For instance, ILC2 possess the ability to up-regulate expression of transcription factors (e.g., *Rorc*) and cytokines associated with the ILC3 lineage. Natural ILC2 (nILC2) respond to IL-33 and produce type 2 cytokines such as IL-13; however, inflammatory ILC2 (iILC2) up-regulate *Rorc*, can respond to IL-25, and coproduce both IL-13 and IL-17. Compared to nILC2, iILC3 express higher levels of *Dtx1*, suggesting increased Notch activity.[150] Treatment of mice with GSI or systemic anti-Notch1/2 Abs reduced lung iILC2 in mice treated with IL-25 or inoculated with a nematode infection, and impaired co-production of IL-13 and IL-17. The effects of Notch blockade were cell-intrinsic, given that ex vivo co-culture of nILC2 on OP9-Dll1 increased co-production of type 2 and type 3 cytokines compared to OP9 stroma. Future studies dissecting the cell-intrinsic role of Notch in ILCs as well as the source and regulation of Notch ligands are warranted.

Recent in vivo loss-of-function data are beginning to uncover potential tissue-specific requirements for ILC3 and helper ILC1 populations. A role for Notch signaling has been reported previously for NK cell development and function; however, the focus here is on recent data highlighting Notch's importance for liver ILC1 populations.[151-153] *Rbpj* inactivation in lymphoid progenitors using an *Il7ra*-driven Cre recombinase showed an increase in liver, but not lamina propria, ILC1.[151] The authors reported alterations in surface phenotype, which makes distinguishing NK and helper ILC1 populations difficult, but transcriptional analyses supported the phenotypic findings. Interestingly, the increase in ILC1 was not recapitulated in Notch2-deficient ILCs, suggesting nonredundant roles for the Notch receptors. While Notch-deficient ILC1 and NK in the lamina propria remained unaltered, ILC3 subsets were reduced.[154] Both *Vav-Cre* and *Il7ra-Cre* models of *Notch2* or *Rbpj* inactivation showed marked reduction in the frequency and number of lamina propria Nkp46$^+$ ILC3.[154,155] Indeed, ILC progenitors cultured on OP9-Dll4 can give rise to RORγt$^+$ ILC3, an effect that is inhibited by treatment with GSI.[154,156] Some propose that other ILC3 subsets, such as NKp46- ILC3 are precursors to the NKp46$^+$ ILC3s, and that Notch is involved in the in situ differentiation of NKp46$^+$ ILC3 from NKp46$^-$ ILC3; however, future studies are necessary to determine the lineage relationships of these populations.[154,157,158]

In summary, the anatomic site(s) of ILC maturation and cues regulating terminal differentiation are only just beginning to unravel. Elucidating Notch's role in these processes is paramount. Similar to cDC2, certain ILC3 appear dependent on Notch signals specifically in the lamina propria. A better understanding of peripheral ILC niches is needed. In fact, some strides have been made in understanding the ILC2 lung niche. Although ILC2 localize with neighboring dendritic cells and Th2 cells, quantitative imaging studies pinpoint a close association with adventitial stromal cells.[159] It will be interesting to investigate these stromal cells further to understand if Notch ligand up-regulation and signaling plays a part in ILC2 activation—and perhaps a role in the generation of inflammatory ILCs.[150] Moreover, mature ILC are not static and can migrate to lymphoid tissues.[160,161] Thus, a systematic investigation of ligand regulation and ILC exposure to these ligands—from the BM to the lymphoid and non-lymphoid periphery—will be important to clarify mechanisms of ILC development and activation.

Furthermore, how the ILC fate is molecularly distinct from the T cell fate remains unresolved. ILC and T cells share remarkably analogous biological functions as well as transcriptional and epigenetic profiles.[162,163] Many transcription factors critical for T lineage specification and commitment are also vital at early stages of ILC development.[145,164-166] Notch's relevance to both lineages positions it as a unique candidate that could in part underlie such differences. Perhaps Notch signaling aids in imprinting either the similarities or differences between these distinct lineages during development from a common progenitor. The nature and intensity of signaling, as well as integration with other cues, could alter Notch's effects and help impart essential distinctions between the ILC and T cell lineages.

5 | CONCLUDING REMARKS

From *C. elegans* and *Drosophila* to mammals, Notch signaling has an extensive evolutionary history. Notch's phylogeny speaks to its potency as a mode of communication—a direct translation of an environmental signal to a cellular transcriptional response. However, a panoptic view of Notch in the immune system challenges us to reconsider its role as a simple regulator of binary cell fate decisions. Indeed, Notch signaling may have been propagated through evolution because of its versatility in converting environmental cues into responses that are lineage and context-dependent.

To better understand Notch's pleotropic nature, we need to define the microanatomical niches where Notch ligands are restricted and regulated. In certain contexts, such as thymocyte development, we have a clear view of Notch ligand expression—namely, the transcription factor critical to thymic epithelial development and maintenance, *Foxn1*, is required for *Dll4* expression in cortical thymic epithelial

cells.[167] In secondary lymphoid organs, specialized fibroblastic reticular cells were recently reported to function as the critical source of Delta-like Notch ligands.[120,121] Other contexts, such as the BM hematopoietic stem cell niche, remain controversial. For yet other situations, such as cDC and ILC differentiation, the cellular source(s) and nature of Notch ligands remain completely unknown. Additionally, Notch signaling within the niche cell compartment per se may directly regulate stromal cell biology and thus subsequently affect both the innate and adaptive immune response. Given the role for Notch signaling during bone marrow endothelial cell regeneration, and the non-cell autonomous myeloproliferative disease following *Rbpj* inactivation in non-hematopoietic compartments, it is likely that Notch signaling between stromal niche cells also has functional consequences on innate and adaptive immune cells.[48,49]

New efforts are also needed to resolve the precise mechanisms underlying Notch as a transcription factor. Rigorous analyses in models of T-ALL have forged a path to identify direct Notch transcriptional targets.[16,17] As technologies to dissect genomic and epigenetic landscapes become more accessible for low cell numbers, we will soon be able to pursue similar studies in other contexts, such as mature T cell function during an immune response. Altogether, research on Notch signaling in the immune system keeps unraveling versatile functions for this ancient pathway, with a profound impact on both innate and adaptive immunity. Deepening our understanding of Notch signaling regulation will provide new insights into key aspects of immunobiology and may identify potential Notch-based therapeutic opportunities for patients with cancers and immune-mediated disorders.

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DISCLOSURE

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