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Review

Notch Signaling in the Immune System

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The Notch signaling pathway regulates many aspects of embryonic development, as well as differentiation processes and tissue homeostasis in multiple adult organ systems. Disregulation of Notch signaling is associated with several human disorders, including cancer. In the last decade, it became evident that Notch signaling plays important roles within the hematopoietic and immune systems. Notch plays an essential role in the development of embryonic hematopoietic stem cells and influences multiple lineage decisions of developing lymphoid and myeloid cells. Moreover, recent evidence suggests that Notch is an important modulator of T cell-mediated immune responses. In this review, we discuss Notch signaling in hematopoiesis, lymphocyte development, and function as well as in T cell acute lymphoblastic leukemia.

Overview of Notch Signaling

The Notch signaling cascade is highly conserved and found in organisms as diverse as worms and humans. In 1917, the geneticist Thomas Hunt Morgan and his colleagues described fruit flies with notches at the margins of their wing blades (Morgan, 1917). It turned out that this notched wing phenotype is the result of a partial loss of function of the *Drosophila Notch* gene, which was cloned in the mid eighties (Kidd et al., 1986; Wharton et al., 1985). *Drosophila Notch* encodes an unusual type I transmembrane receptor that is activated by two different membrane-bound ligands called Delta and Serrate. Mammals posses four Notch receptors (Notch1–4) that are bound by five ligands (Delta-like1, 3, and 4 and Jagged 1 and 2) (Figure 1; Bray, 2006). The molecular and biochemical details of Notch signaling have recently been covered by excellent reviews (Gordon et al., 2008; Kopan and Ilagan, 2009). In brief, newly synthesized Notch receptors are proteolytically cleaved in the Golgi (at site S1) during their transport to the cell surface by a furin-like protease. This cleavage generates a heterodimeric receptor consisting of an extracellular subunit (N^{EC}) that is noncovalently linked to a second subunit containing the extracellular heterodimerization domain and the transmembrane domain followed by the cytoplasmic region of the Notch receptor (N^{TM}). The extracellular part of the receptors contains 29–36 epidermal growth factor-like repeats involved in ligand binding, followed by three cysteine-rich LIN12 repeats that prevent ligand-independent activation and a hydrophobic stretch of amino acids mediating heterodimerization between N^{EC} and N^{TM}. The cytoplasmic tail of the receptor harbors multiple conserved elements including nuclear localization signals, as well as protein-protein interaction and transactivation domains.

Notch signaling is initiated by ligand-receptor interaction between neighboring cells, leading to two successive proteolytic cleavages of the receptor. The first is mediated by metalloproteases of the ADAM family, which cleave the receptors 12–13 amino acids external to the transmembrane domain (at site S2). The shedded extracellular domain is endocytosed by the ligand-expressing cell, a process that is dependent on monoubiquitinylation of the cytoplasmic tail of the ligands by E3-ubiquitin ligases of the mind bomb and neuralized family. Ligand binding to N^{EC} presumably induces a conformational change within the Notch receptors to expose the S2 cleavage site for proteolysis. After shedding of the extracellular domain, a second cleavage within the transmembrane domain (at site S3) is mediated by the γ-secretase activity of a multiprotein complex. This liberates the intracellular domain of Notch receptors (NICD), which subsequently traffics to the nucleus and heterodimerizes with the DNA binding transcription factor CSL in order to form a short-lived nuclear transcription complex. The transcription factor CSL is also known as CBF-1 in humans, Suppressor of hairless in *Drosophila*, Lag in *Caenorhabditis elegans*, and RBP-J in the mouse. Once bound to CSL, NICD recruits other coactivators including mastermind proteins (MAML1-3), which in turn recruit the MED8-mediator transcription activation complex in order to induce transcriptional expression of downstream target genes (Figure 2). Members of the Hairy enhancer of split (Hes) or Hairy related (Hey or Hrt) genes have been identified as Notch target genes in many tissues, while other targets are more tissue restricted. Recent studies via genome-wide expression and chromatin immunoprecipitation (ChIP) arrays point to the existence of a large number of genes that can be directly regulated by Notch (Palomero et al., 2006; Weng et al., 2006). The challenge will now be to distinguish the drivers from the passengers among the large number of target genes. Moreover, there is emerging data suggesting that Notch can crosstalk to or cooperate with other signaling pathways (including NF-κB, hypoxia, or TGF-β) and thereby broaden the spectrum of target genes that are influenced by Notch signaling (Poellinger and Lendahl, 2008; Samon et al., 2008).

Notch signaling is regulated at multiple levels. For example, cell type-specific and spatial expression of ligands and Notch receptors can restrict signaling to a certain cell population or context. The ability of Jagged ligands to trigger Notch receptor-mediated signaling is dependent on the glycosylation status of the extracellular domain of Notch. Fringe proteins are glycosyl transferases that add N-Acetylglucosamine to O-fucose residues present within certain epidermal growth factor-like repeats of the receptors (Haines and Irvine, 2003). Notch

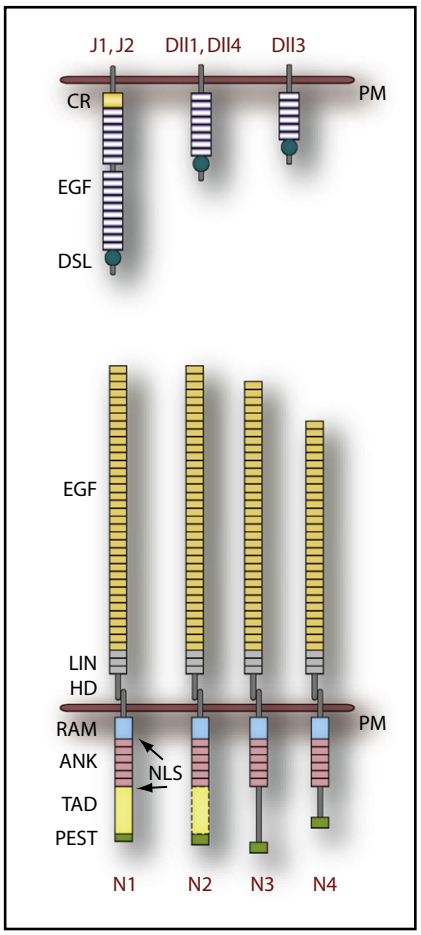

Figure 1. Notch Ligands and Receptors
To date, five conventional Notch ligands are known: Jagged1 (J1), Jagged2 (J2), Delta-like1 (Dll1), Delta-like3 (Dll3), and Delta-like4 (Dll4). A common structural feature of all ligands is an amino-terminal domain called DSL (Delta, Serrate, and Lag-2) involved in receptor binding followed by EGF-like repeats. A cysteine-rich domain (CR) is located downstream of the EGF-like repeats of J1 and J2 close to the plasma membrane (PM). Vertebrates have four Notch receptors (Notch1–Notch4; N1–N4). The extracellular domain of the receptors contains EGF-like repeats (36 in N1 and N2, 34 in N3, and 29 in N4) followed by three cysteine-rich LIN domains that prevent ligand-independent activation and the heterodimerization domain (HD). The cytoplasmic domain contains a RAM domain followed by six ankyrin repeats (ANK) that bind to the CSL transcription factor, two nuclear localization signals (NLS), a transactivation domain (TAD; present in N1 and N2), and a PEST sequence involved in regulating protein stability.

receptors carrying these additional sugar moieties preferentially signal via Delta ligands, while Jagged-mediated Notch signaling is inhibited. Another level of regulation is to ensure that a Notch signal is short lived. Notch receptors carry a PEST domain at the very C terminus that is responsible for rapid turnover of the activated NICD via E3-ubiquitin ligase (including Fbw7)-mediated proteosomal degradation (Figure 2; O'Neil et al., 2007; Thompson et al., 2007).

Notch in Hematopoietic Stem Cell Development and Homeostasis

The blood system originates from different sites during embryonic development and is generally closely associated with vasculogenesis. The most primitive hematopoietic cells are found within the extraembryonic yolk sac before hematopoiesis shifts to intraembryonic sites including the para-aortic splanchnopleiura and aorta-gonad mesonephros (P-sP and AGM). Later hematopoiesis occurs in the fetal liver before it is finally established in the bone marrow (Godin and Cumano, 2002). The first hematopoietic stem cells capable of long-term repopulation of all blood lineages upon transplantation are found within the AGM region. These cells are generated from a bipotent hemangioblast by budding off from the dorsal aorta of midgestation embryos (de Bruijn et al., 2002). Germline mutant embryos deficient for Notch1 or RBP-J have been shown not to generate intraembryonic HSCs, whereas yolk sac hematopoiesis of these mutant mice was unperturbed (Kumano et al., 2003; Robert-Moreno et al., 2005). These studies led to the suggestion that Notch signaling is important for definitive but not primitive hematopoiesis. However, Notch signaling is also important for arterial cell fate specification in developing blood vessels. Hence, these mutant embryos displayed severe vasculogenic defects characterized by the loss of arterial cell fate (Krebs et al., 2004). Therefore, it was not clear whether the inability to generate intraembryonic HSC is a cell-autonomous defect of hemangioblasts or simply a secondary effect resulting from the absence of arteries. This uncertainty was recently resolved by studies analyzing germline mutant mice for the Jagged ligand family. Jagged1 but not Jagged2 null embryos failed to generate hematopoietic cells in the AGM, without losing the arterial cell fate (Robert-Moreno et al., 2008). Moreover, the same study linked Jagged1-mediated Notch signaling to GATA2 and Runx1 expression, two important transcription factors for hematopoiesis. These observations were important because they were the first studies showing that Notch signaling is directly associated with the generation of hematopoietic cells independently of its role in arterial development. Thus, Notch signaling is indeed essential for definitive hematopoiesis in the developing embryo.

Whether Notch signaling plays a similar role during the generation or maintenance of HSC in the adult bone marrow compartment was debated for several years. Jagged1 was suggested to be part of the HSC stem cell niche, because osteoblast-specific expression of the parathyroid hormone-related protein receptor (PTHRP) resulted in increased numbers of Jagged1-expressing osteoblasts, which correlated with increased numbers of HSCs. This result led to the suggestion that Jagged1-mediated Notch signaling might regulate HSC homeostasis (Calvi et al., 2003). Moreover, multiple gain-of-function studies support a role for Notch in HSC maintenance. Overexpression of N1-ICD or its downstream target gene Hes1 in bone marrow progenitors resulted in increased HSC numbers and/or enhanced self-renewal (Kunisato et al., 2003; Stier et al., 2002). Coculture experiments of murine hematopoietic progenitor cells with immobilized Notch ligands promoted early T cell differentiation and generation of multilog increases in the number of hematopoietic progenitor cells with short-term lymphoid and myeloid repopulating activity (Varnum-Finney et al., 2003). The dose of Notch signaling determines the in vitro process of hematopoietic progenitor cell expansion versus B and/or T cell differentiation. Coculture of hematopoietic progenitor cells in the presence of high densities of Notch ligands increases the propensity to drive

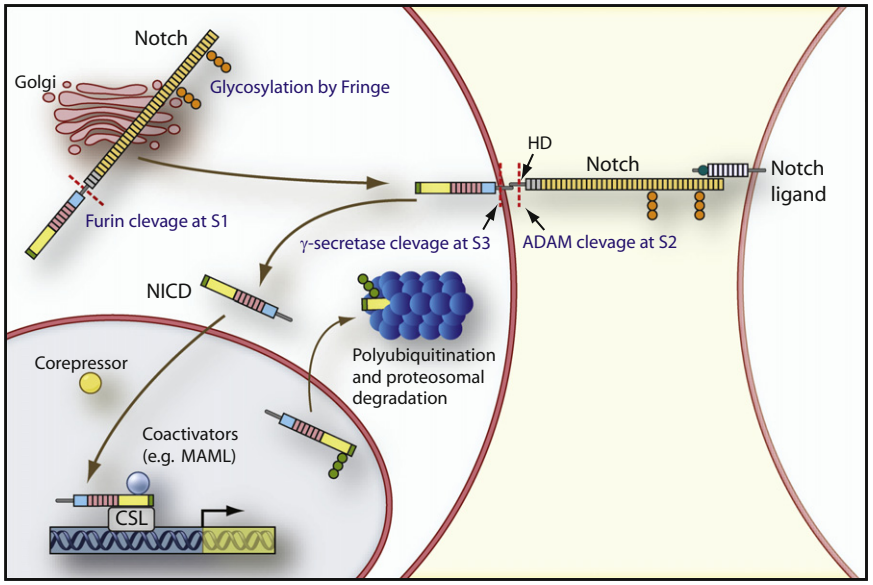

Figure 2. Notch Signaling
Notch proteins are synthesized as single precursor proteins, which are cleaved in the Golgi by a Furin-like convertase at site S1. Cleavage at S1 generates two subunits held together noncovalently by the N- and C-terminal subunits of the heterodimerization domains (HD). EGF-like repeats are glycosylated by Fringe proteins in the Golgi before receptors are transported to the cell surface. Notch signaling is initiated by ligand receptor interaction, which induces a second cleavage at site S2 (close to the transmembrane domain) mediated by ADAM-type metalloproteases followed by a third cleavage at S3 within the transmembrane domain mediated by the γ-secretase activity of a multiprotein complex containing presenilins. This last proteolytic cleavage liberates the cytoplasmic domain of Notch receptors (NICD), which translocate to the nucleus and bind to the transcription factor CSL (CBF1, Suppressor of hairless, and Lag-1), converting it from a transcriptional repressor into a transcriptional activator by recruiting coactivators including mastermind-like proteins (MAML). NICD is polyubiquitinated by E3 ubiquitin ligases (including Fbw7), which marks NICD for proteosomal degradation.

differentiation toward the T cell lineage (Dallas et al., 2005). In particular, the finding that human umbilical cord blood cells (UCB) could also be expanded ex vivo when cocultured with Delta-like1-IgG fusion proteins and that these cells showed a marked increase (approximately 15-fold) in repopulating cell frequency in xenotransplantation assays (Delaney et al., 2005) may be exploited for clinical purposes (Bernstein et al., 2008).

Although it is very encouraging that Notch ligands are currently used to expand murine and human hematopoietic progenitors, there is limited evidence that Notch can be used to expand long-term HSCs. Thus, the question remains whether this is a physiological role of Notch signaling. This has been addressed by analyzing several conditional gene-targeted mice for different components of the Notch pathway. Mice lacking Notch1 or Jagged1 or both did not reveal any defects in HSC maintenance or in the capacity to repopulate the hematopoietic compartment after transplantation (Mancini et al., 2005; Radtke et al., 1999). These results do not exclude the possibility that other Notch receptors or ligands might functionally compensate for the loss of Notch1 and/or Jagged1. Two complementary approaches were recently used to block canonical Notch signaling. The first used a dominant-negative form of the Mastermind-like protein, which inhibits the formation of a functional Notch transactivation complex in HSCs and bone marrow (BM) progenitors, whereas the second inactivated the *Rbp-j* gene within HSCs. These experimental approaches block Notch signaling independently of Notch receptor or ligand usage. Notch signaling-deprived progenitors did not reveal any HSC defects; they showed normal long-term reconstitution even in secondary competitive transplantation assays (Maillard et al., 2008). Taken together, these experiments show that canonical Notch signaling is dispensable for HSC homeostasis in the bone marrow. Moreover, the identification of the proto-oncogene *LRF* (Leukemia/lymphoma Related Factor, encoded by the *Zbtb7a* gene and also known as *Pokemon*) as a negative regulator of Notch signaling in BM progenitors indicates that Notch signaling must be repressed or under very stringent control in HSCs in order to prevent ectopic T cell differentiation in the BM (Maeda et al., 2007). How LRF represses Notch signaling in HSC or progenitor cells is currently unknown.

Notch in T Cell Development

The essential role of Notch signaling during thymic T cell lineage commitment and maturation is the best-studied function of Notch in hematopoiesis. Via the blood stream, BM progenitors constantly seed the thymus, where they adopt a T cell fate and further differentiate into mature αβ and γδ T cells before emigrating to the periphery. Multiple genetic loss- and gain-of-function studies highlight the importance of Notch1 for T cell lineage commitment. Inducible inactivation of Notch1 or Rbp-j results in a block in T cell development accompanied by the accumulation of ectopic B cells in the thymus (Han et al., 2002; Radtke et al., 1999). These results were initially interpreted to mean that canonical Notch1 signaling instructs a bipotent early thymic progenitor to adopt a T cell as opposed to a B cell fate because no other myeloid or lymphoid lineages were affected. Nevertheless, recent loss of Notch1 function combined with lineage tracing experiments reveal that the inhibitory functions of Notch1 are broader. Notch1 inhibits multiple cell fate potentials of thymus-seeding cells including myeloid and B cells, as well as conventional and plasmacytoid dendritic cell potential (both in a cell-intrinsic and -extrinsic manner) and thereby ensures efficient T cell lineage commitment (Bell and Bhandoola, 2008; Feyerabend et al., 2009; Wada et al., 2008). Similarly, interference with Notch signaling by transgenic expression of Notch modulators (including Fringe, Deltex1, or Nrarp) or dominant-negative forms of the transcriptional coactivator MAML-1 also blocks T cell development concomitant with B lymphopoiesis in the thymus (Izon et al., 2002; Koch et al., 2001; Maillard et al., 2004; Yun and Bevan, 2003). Reciprocal gain-of-function studies involving overexpressing N1-ICD in BM progenitors result in ectopic T cell development at the expense of B cell development

in the BM (Pui et al., 1999). Taken together, these results demonstrate that Notch1 is the key receptor expressed on thymus-seeding cells responsible for T cell lineage commitment.

The question of the ligand(s) required for this process was recently addressed. Historically, Dll1 and somewhat later Dll4 have been favored as potential Notch1 ligands for T cell fate specification based on their capacity to support complete development of mature T cells from BM precursors in vitro (Hozumi et al., 2004; Jaleco et al., 2001; Schmitt and Zúñiga-Pflücker, 2002). Nevertheless, inactivation of Dll4 but not Dll1 in thymic epithelial cells (TECs) resulted in a complete block in T cell development accompanied by ectopic B cell development within the thymus, which phenocopies mice with loss of Notch1 function in BM progenitors (Hozumi et al., 2008; Koch et al., 2008). These results demonstrate an essential interaction between Dll4-expressing TECs and thymus-seeding Notch1-expressing hematopoietic progenitors for T lineage commitment. Previous studies of the thymic epithelium of gene-targeted mice in which T cell development is arrested at early developmental stages showed that the thymocyte progenitors also influence TEC maturation and function. Thus, lymphostromal interactions between developing thymocytes and TECs are bidirectional, a concept known as "thymus crosstalk" (van Ewijk et al., 1994). In this context, a recent report showed that maturation of thymocytes to the $CD4^+CD8^+$ stage induced downregulation of Dll4 on cortical TECs suggesting a negative-feedback loop between developing thymocytes and cortical TECs (Fiorini et al., 2008). This coincides with the maturation and the ability of medullary TECs to mediate positive and negative selection, a trait that is acquired in a thymocyte-dependent manner (Alves et al., 2009). How and whether downregulation of Dll4 on cortical TECs is essential to allow positive and/or negative selection remains an open question.

Once the T cell lineage has been specified, developing thymocytes must choose between the αβ and γδ T cell lineage. γδ T cell development is mostly driven by the successful rearrangement of T cell receptor γ (TCR-γ) and TCR-δ genes and appears to be Notch independent (Ciofani et al., 2006; Wolfer et al., 2002). Interestingly, the helix-loop-helix protein Id3 can induce promotion of the γδ T cell fate as well as rendering γδ T cell maturation independent of Notch signaling (Lauritsen et al., 2009). In contrast, αβ T cell development requires continuous Notch signaling up to the DN3 stage, where cells have to pass a critical checkpoint known as β-selection (Wolfer et al., 2002).

Although it is well established that signaling via the pre-TCR (consisting of productively rearranged TCR-β chain associated with CD3 components and an invariant pTα chain) is essential for β-selection and further thymocyte development, in vitro experiments suggest that successful transition through this checkpoint requires cooperative signaling of both Notch and pre-TCR (Ciofani et al., 2006). This leads to the question of how this functional cooperativity is established at the molecular level and whether Notch and pre-TCR signaling influence each other. Loss-of-function experiments for both Notch signaling and components of the pre-TCR highlight the essential role of each individual signaling pathway during thymocyte development. For example, the consequences of a loss of Notch signaling in vivo (via a dominant-negative MAML-1) in immature thymocytes prior to the β-selection checkpoint cannot be overcome by TCR-β or TCR-αβ transgenes, suggesting that the requirement for early Notch signaling is absolute and independent of the pre-TCR (Maillard et al., 2006). Similarly, RAG2-deficient thymocytes (which lack a pre-TCR because of the inability to rearrange a functional TCR-β chain) cannot progress to the DP stage even if they receive a Notch signal (Allman et al., 2001). Nevertheless, successful transition through β-selection requires the cooperative action of both Notch and the pre-TCR. As thymocytes pass through β-selection, Notch assures survival by regulating glucose metabolism (Ciofani and Zúñiga-Pflücker, 2005). Moreover, transcriptional reporter assays combined with ChIP experiments suggest a direct crosstalk between Notch and pre-TCR because Notch1 and/or Notch3 (which is a Notch1 target gene) can directly activate the transcription of the pTα gene (Bellavia et al., 2007; Reizis and Leder, 2002). Additional, indirect regulation of pTα gene expression by Notch involves Notch3 and Ikaros. Ikaros functions as a transcriptional repressor and recognizes the same DNA binding sites as Rbp-j. Thus, Ikaros and Rbp-j can potentially compete for the same DNA binding site, a process that has been shown to be important during T cell leukemogenesis (Beverly and Capobianco, 2003; Dumortier et al., 2006). Notch3 activation results in the expression of HuD, a RNA binding protein that can trigger the generation of non-DNA binding Ikaros isoforms through alternative splicing. These isoforms competitively block the activity of full-length Ikaros and thereby facilitate the upregulation of pTα directly through Notch-Rbp-j-mediated transcriptional complexes (Bellavia et al., 2007). Thus, Notch1 signaling directly and indirectly participates in the generation of the pre-TCR.

This leads to the question of how Notch1 itself is regulated during thymocyte development. Notch1 (in an autoregulatory loop) together with the transcription factor E2A directly contributes to the progressive increase of Notch1 expression at the earliest stages of thymocyte development, prior to the β-selection stage. Thymocytes that successfully pass β-selection immediately downregulate the expression of Notch1 (Taghon et al., 2006; Yashiro-Ohtani et al., 2009). This process is driven via the pre-TCR-mediated induction of the HLH transcription factor Id3. Id3 is an inhibitor of E-proteins and as such inhibits E2A-dependent activation of Notch1 transcription, leading to a decrease in Notch1 mRNA. Taken together, Notch1-mediated signaling is necessary for assembling a functional pre-TCR and as soon as thymocytes pass β-selection, the pre-TCR ensures the transcriptional repression of Notch1, a mechanism that is presumably essential to avoid the oncogenic properties of Notch signaling and its targets (see below) (Weng et al., 2006). Interestingly, the abrupt downregulation of Notch1 transcription after β-selection is not reflected at the protein level. Surface expression of Notch1 receptor remains at equally high amounts from DN3 stage until the ISP stage and decreases only subsequently in DP thymocytes (Fiorini et al., 2009). These results suggest that the decrease in Notch1 transcription and Notch1 target gene expression after β-selection may occur independently of the regulation of Notch1 surface expression. Thus, it is conceivable that downregulation of Notch1 target genes is not simply the result of absence of Notch signaling resulting from lack of Notch1 surface expression, but may also implicate additional repressive mechanisms. Interestingly, Ikaros is implicated in the negative regulation of the Notch target gene Hes1 in thymocytes that successfully passed β-selection. DN4 thymocytes

thereby lose their capacity to transcribe Hes1 in response to Notch signaling. This event correlates with epigenetic silencing of the Hes1 locus, suggesting that Ikaros might help to shut down Notch target genes once thymocytes passed β-selection (Kleinmann et al., 2008).

Notch in Marginal Zone B Cell Development

A second well-characterized role for Notch signaling in the lymphoid system involves the specification of marginal zone (MZ) versus follicular B cell fate in the spleen. Mature splenic B cells are comprised of two principal subsets, follicular B cells and MZ B cells (Pillai and Cariappa, 2009). Follicular B cells, which are the most abundant subset, are recirculating cells that home to B cell follicles and participate in T cell-dependent immune responses to protein antigens. In contrast, MZB cells are not recirculating and localize in the outer region of the splenic white pulp between the marginal sinus and the red pulp. MZB cells provide an important line of defense against blood-borne pathogens by mounting T cell-independent antibody responses. In addition, MZB cells express high amounts of CD1d, which allows them to capture lipid antigens from the circulation and present them to CD1d-restricted Vα14 invariant natural killer T cells. In some respects, MZB cells can thus be considered to be an "innate-like" population because they exhibit a constitutively activated phenotype similar to NK cells, NKT cells, and γδ T cells.

Both MZB cells and follicular B cells in the spleen are derived from B lineage progenitors in the BM. During development, immature B cells that productively rearrange heavy- and light-chain immunoglobulin genes express a B cell receptor (BCR) at the cell surface. Similar to T cells, immature B cells that express strongly self-reactive BCR undergo clonal deletion or receptor editing. Further B cell maturation proceeds through transient transitional stages (T1 and T2), ultimately leading to the differentiation of mature follicular or MZB cells in the spleen. The specification of splenic follicular versus MZB cell fate from immature T2 B cells is determined by several factors and has been reviewed in detail recently (Pillai and Cariappa, 2009). In this section, we will concentrate on Notch signaling, which has a critical and nonredundant role in specifying MZB cell fate.

It is now widely accepted that MZB cell fate specification in the spleen depends upon nonredundant interaction between Notch2 and Dll1. Thus mice conditionally deficient in either Notch2 or Dll1 have greatly reduced numbers of MZB cells (Hozumi et al., 2004; Saito et al., 2003). Further evidence supporting a requirement for Notch signaling in MZB cell development comes from the analysis of mice deficient in other components of the Notch signaling pathway such as Rbp-j (Tanigaki et al., 2002) or MAML1 (Oyama et al., 2007; Wu et al., 2007), which also fail to generate MZB cells. In reciprocal experiments, deletion of MINT (a negative regulator of Notch signaling) led to an increase in splenic MZB cells (Kuroda et al., 2003). Collectively these loss-of-function experiments provide compelling evidence that the strength of signaling via Notch2:Dll1 interactions controls the rate of development of MZB cells.

Although it has been known for some time that Dll1 is the relevant ligand of Notch2 in MZB cell development, the identity of Dll1-expressing cells in the spleen remained elusive. Nonhematopoietic cells (Hozumi et al., 2004), and in particular endothelial cells located in the red pulp and MZ of the spleen (Tan et al., 2009), selectively express Dll1. It seems likely that endocytosis of Dll1 by these ligand-expressing endothelial cells may be required for efficient signaling via Notch2 on MZB cells or their precursors, because deletion of Mindbomb 1 (Mib1), an E3 ligase known to regulate Dll1 endocytosis, phenocopies conditional Notch2 and Dll1 mutant mice (Song et al., 2008).

Another modulator of Notch signaling that plays an important role in MZB cell development is the Fringe family of glycosyltransferases. As discussed earlier, Fringe can enhance interactions of Notch receptors and Dll ligands by adding N-acetyl glucosamine to O-linked fucose residues on Notch. Two members of the Fringe family (Lunatic fringe and Manic fringe) function cooperatively to strengthen the presumably weak interaction between Notch2 on MZB cells (or their precursors) and Dll1-expressing endothelial cells in splenic niches, thereby promoting development of the MZB cell lineage (Tan et al., 2009). According to this scenario, MZB cell homeostasis depends upon Fringe-regulated competition between Notch2-expressing precursor cells for access to Dll1 ligands.

Notch in Other Hematopoietic Lineages

Although much attention has been focused on the controversial role of Notch signaling in HSC homeostasis and differentiation, Notch may also play a role in other cell fate decisions within the hematopoietic system. Thus megakaryocyte development is enhanced in vitro when BM precursors are cocultured with Dll1-expressing OP9 stromal cells (Mercher et al., 2008). In reciprocal experiments, inhibition of canonical Notch signaling in vivo by a dominant-negative MAML1 decreased megakaryocyte numbers, but (somewhat surprisingly) did not affect platelet counts, even after challenge with 5-Fluorouracil (5-FU) (Mercher et al., 2008). Further studies will be needed to identify the physiologically relevant Notch receptors and ligands implicated in the development of the megakaryocyte lineage in vivo.

Dendritic cells (DCs) appear to be another hematopoietic lineage that is influenced by Notch signaling during development. Several in vitro systems have demonstrated that Dll1-mediated Notch signaling promotes the development of either plasmacytoid or conventional DCs at the expense of other lineages such as T cells or macrophages (Ohishi et al., 2001; Olivier et al., 2006). Moreover, differentiation of ES cells or BM progenitors into DCs in vitro was shown to depend upon Notch signaling via downstream activation of the Wnt pathway (Zhou et al., 2009). More compellingly, loss of function of canonical Notch signaling via Rbp-j inactivation specifically in the DC lineage led to a selective impairment of the splenic $CD8^-$ DC subset (Caton et al., 2007). Intriguingly, $CD8^-$ DCs in the spleen were found adjacent to unidentified Dll1-expressing cells in the marginal zone, raising the possibility that $CD8^-$ DCs may compete with MZB cells for Notch signals during development (see previous section). In a genetic fate mapping model, inactivation of Notch1 in early intrathymic precursors revealed their potential to develop into DCs in the thymus (Feyerabend et al., 2009). Taken together, these experiments indicate that at least some aspects of DC development may depend upon Notch signaling.

Notch in Peripheral T Cell Differentiation and Function

Once T cells leave the thymus, they migrate to the periphery, where they orchestrate immunity against different pathogens.

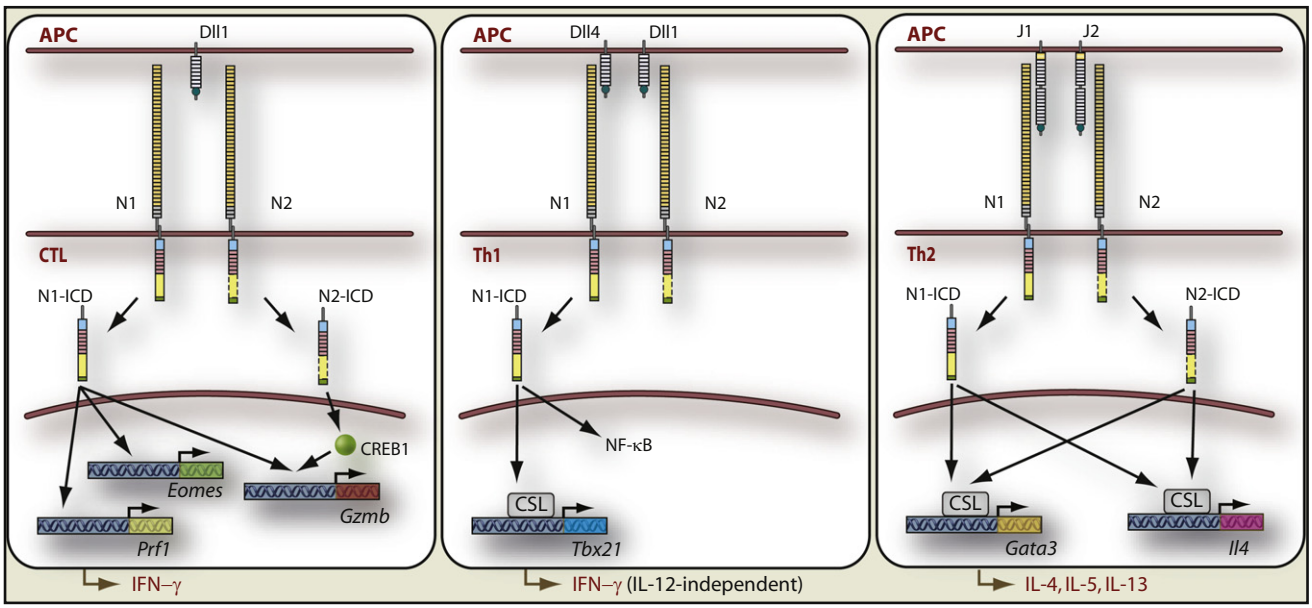

Figure 3. Consensus on the Role of Notch in Peripheral T Cell Differentiation

After encounter with pathogens, Notch ligands are upregulated on antigen-presenting cells (APCs). Activation of naive $CD8^+$ T cells requires binding of Dll1 on APCs to N1 or N2 receptors. After receptor:ligand interaction N1-ICD is liberated and moves to the nucleus where it binds to *Eomes*, *Prf1*, and *Gzmb* promoters to activate functional $CD8^+$ T cells producing IFN-γ. Alternatively, N2-ICD interacts with CREB1 to bind the Gzmb promoter. Naive $CD4^+$ T cells are generally induced to become Th1 or Th2 cells. Th1 cell-inducing microbes upregulate Dll ligands on APCs. Then, N1-ICD can directly promote Th1 cell differentiation by binding to CSL on the *Tbx21* promoter or by activating the NF-κB pathway, eventually leading to IFN-γ production. After exposure to Th2 cell stimuli, APCs upregulate ligands of the Jagged family. N1-ICD and N2-ICD induce Th2 cell differentiation by forming complexes with CSL, either on the upstream promoter of *Gata3* or on a 3′ enhancer of the *Il4* gene, ultimately leading to the production of IL-4, IL-5, and IL-13.

After receiving appropriate signals, naive T cells become activated and exert their function. For example, cytotoxic $CD8^+$ T cells are recruited against viruses and kill infected cells by the production of interferon-γ (IFN-γ) and cytotoxic molecules including granzyme B and perforin. Pathogens such as bacteria, helminths, fungi, and also viruses are recognized by antigen-presenting cells (APCs), which then activate $CD4^+$ T helper (Th) cells. These cells drive adaptive immunity and induce specific responses against these microbes. Although first classified as either Th1 or Th2 cells according to their cytokine profiles, it appears that many more discrete Th cell subsets exist, exemplified by the newly characterized Th17 cells. Th1 cells produce IFN-γ and are involved in the fight against intracellular pathogens, whereas Th2 cells secrete interleukin-4 (IL-4), IL-5, and IL-13 and provide immunity to helminths. Moreover, some extracellular bacteria and fungi are cleared by the presence of Th17 cells that produce IL-17 and recruit neutrophils to the site of infection (Weaver et al., 2007). Once an immune response has been initiated, it has to be dampened and/or switched off again in order to avoid exaggerated immune responses that could cause autoimmunity or allergies. Notch signaling has been associated with all of the above-mentioned functions. The multitude of different disease models used has contributed to a complex picture of possible Notch functions in peripheral immune responses. A potential role for Notch signaling in peripheral T cells linked Notch receptor expression to T cell activation, proliferation, and cytokine production. TCR activation of peripheral T cells in vitro led to upregulated expression of Notch1, which correlated with increased proliferation. γ-secretase inhibitor (GSI)-mediated inhibition of Notch signaling in peripheral $CD4^+$ and $CD8^+$ T cells resulted in decreased T cell proliferation and reduced IFN-γ production (Palaga et al., 2003). A second study showed that forced NICD expression can induce a positive feedback loop by increasing IL-2 production and the expression of the IL-2 receptor α-subunit on T cells in vitro (Adler et al., 2003). Notch signaling is also associated with the differentiation of naive $CD8^+$ T cells to cytotoxic T lymphocytes (CTLs), a process that is in part mediated by the upregulation of the transcriptional regulator eomesodermin (Eomes), which regulates the expression of perforin and granzyme B (Cho et al., 2009). Interestingly, this study showed that Notch1 regulates the expression of all three genes that encode Eomes, perforin, and granzyme B through direct binding to the corresponding promoter. Moreover, CTLs in mice expressing a Notch1 antisense transgene showed decreased expression of Eomes, perforin, and granzyme B, and GSI-mediated inhibition of Notch signaling attenuated CTL function (Cho et al., 2009). Similarly, Notch2 was also reported to regulate granzyme B expression. N2-ICD was shown to interact with the transcription factor CREB1 and together to bind to the granzyme B promoter. Notch2-deficient $CD8^+$ T cells produced lower amounts of granzyme B after stimulation with Dll1-expressing DCs in vitro compared to control animals (Figure 3; Maekawa et al., 2008).

Taken together, these experiments suggest that Notch signaling can participate in regulating genes necessary for CTL differentiation and function; however, whether Notch signaling is required in vivo for mounting an efficient CTL response against a viral infection has not been investigated.

Notch in Th1 Cells

Another function that was assigned to Notch is the regulation of Th cell differentiation. The differentiation of naive $CD4^+$ T cells to different Th cell subsets is regulated, first by TCR and costimulatory signals and then by cytokines present in the environment. In this context, IL-12 produced by APCs drives naive $CD4^+$ T cells to differentiate into Th1 cells, whereas IL-4 present in the milieu allows Th2 cell polarization. Although certain aspects of how Notch regulates Th cell differentiation are controversial, the consensus is that Notch is essential for Th2 cell differentiation but can also influence a Th1 cell response.

The evidence that Notch signaling is important for Th1 cell induction is mostly derived from experiments implicating Notch ligands or from studies with pharmacological Notch inhibitors. APCs encountering pathogens that promote their ability to mount a Th1 cell response show an upregulation of the Notch ligands Dll1 and/or Dll4 (Amsen et al., 2004; Skokos and Nussenzweig, 2007; Sun et al., 2008). Moreover, ectopic expression of Dll ligands on DCs promoted Th1 and inhibited Th2 cell differentiation (Amsen et al., 2004; Krawczyk et al., 2008; Maekawa et al., 2003). Th1 cell induction by forced Dll expression required the presence of the Th1 cell transcription factor T-bet, which was dispensable for Dll-mediated inhibition of Th2 cells (Krawczyk et al., 2008). In addition, Dll ligands were suggested to inhibit Th2 cell differentiation by interfering with IL-4 receptor signaling (Krawczyk et al., 2008). Skokos and colleagues investigated the dependence of Dll4-mediated signaling within different splenic DC subsets for their capacity to induce a Th1 cell response after LPS exposure. $CD8^-$ DCs upregulated Dll4 in response to LPS but did not express IL-12. In contrast, $CD8^+$ DCs expressed IL-12 but not Dll4. Inhibition of Notch signaling (with a Dll4-Fc fusion protein) of $CD8^+$ DCs expectedly did not affect Th1 cytokine production. In contrast, inhibition of $CD8^-$ DCs led to a pronounced reduction of IFN-γ production. Taken together, these results suggest that Dll4-mediated Notch signaling might be involved in the generation of IL-12-independent Th1 cell responses (Skokos and Nussenzweig, 2007).

The identity of the Notch receptors that mediate Dll-induced Th1 cell differentiation is currently unclear. Two reports showed that retroviral expression of either N1-ICD or N3-ICD led to the generation of Th1 cells with a mechanism that was independent of IL-12 (Maekawa et al., 2003; Minter et al., 2005). However, genetic inactivation of Notch1 did not affect Th1 cell differentiation (Tacchini-Cottier et al., 2004), suggesting that the development of Th1 cells may require Notch3 rather than Notch1-mediated signaling. Consistent with this possibility is the finding that the Th1 cell response in an experimental autoimmune encephalomyelitis (EAE) model (murine model of multiple sclerosis) was inhibited after Notch3, but not Notch1, blockade. This correlated with the downregulation of protein kinase C theta (PKCθ), which plays an important role in the regulation of Th1 cell differentiation (Jurynczyk et al., 2008). Finally, GSI-mediated inhibition of Notch signaling suppressed the Th1 cell immune response in vitro and in vivo. Moreover, the same study showed that exogenous N1-ICD and Rbp-j were able to form transcriptional complexes on the *Tbx21* promoter (of the T-bet gene), indirectly suggesting that Notch might transcriptionally regulate T-bet expression (Figure 3; Minter et al., 2005).

Notch may induce the promotion of Th1 cells by interacting with the NF-κB pathway. Complexes of Notch1 and NF-κB1 (p50) or N1-ICD and RelA (p65) could be immunoprecipitated on the *Ifng* promoter, in regions lacking Rbp-j binding sites (Shin et al., 2006). This raises the possibility that N1-ICD can contribute to Th1 cell differentiation in a Rbp-j-independent manner (Figure 3).

Although a role for Notch in the promotion of the Th1 cell lineage is well documented, genetic evidence for the absolute requirement of Notch signaling is lacking. Indeed, inactivation of Rbp-j or both Notch1 and Notch2 (N1N2) or T cell-specific expression of dominant-negative MAML1 had no effects on Th1 cell differentiation (Amsen et al., 2004, 2007; Tu et al., 2005), suggesting that Notch signaling is not essential for Th1 cell differentiation.

Notch in Th2 Cells

The cell fate decision between Th1 and Th2 cells may depend on the ligand used to activate Notch signaling. Expression of Jagged ligands, but not Dll, on the surface of APCs was shown to induce Th2 cell differentiation in vitro (Amsen et al., 2004). Jagged2 is induced on DCs after stimulation with multiple Th2 cell-inducing products such as Schistosoma egg antigen (SEA), dust-mite extracts, or Prostaglandin E2 (Amsen et al., 2004; Krawczyk et al., 2008; Worsley et al., 2008). However, although capable of driving Th2 cell differentiation in vitro, Jagged2 seems to be dispensable in vivo for Th2 cells (Krawczyk et al., 2008; Worsley et al., 2008). In contrast, expression of Jagged1 on DCs might be involved in the initial phase of Th cell differentiation by inducing IL-4 production in a model of airway hyperresponsiveness (Okamoto et al., 2009). Taken together, these data implicate Jagged ligands in the development of Th2 cell immune responses. How Dll- and Jagged-mediated Notch signaling differ on the molecular level to drive Th1 or Th2 cell differentiation is currently unknown.

Genetic loss-of-function experiments showed that Notch signaling is dispensable for Th1 cell differentiation, and additionally they were instrumental to demonstrate that Notch is essential for the development of Th2 cell immune responses in physiological settings such as parasite infection (Amsen et al., 2004, 2007). For example, mice in which Notch signaling was inhibited in T cells through dominant-negative MAML could not mount a protective Th2 cell response against the intestinal parasite *Trichuris muris* in vivo (Tu et al., 2005). Moreover, mice deficient for Rbp-j or N1N2 in T cells and immunized with Th2 cell antigens or alum adjuvants were incapable of mounting a Th2 cell immune response in vivo. However, addition of exogenous IL-4 in vitro restored the ability of these T cells to generate a Th2 cell cytokine profile (Amsen et al., 2004, 2007). In another set of experiments, GSI treatment after ovalbumin immunization in an asthma model led to a decrease in Th2 cell cytokine production and inhibition of NF-κB signaling (Kang et al., 2009).

Although Th1 cell immune responses can be induced in an Rbp-j-independent manner, Th2 cell differentiation is Rbp-j dependent (Amsen et al., 2004). It is therefore not surprising that *Il4* and *Gata3* were identified as two direct Notch target genes. The importance of the Rbp-j containing hypersensitive site 5 (HS5) within a 3′ enhancer of the *Il4* gene was clearly demonstrated (Amsen et al., 2004). In $CD4^+$ T cells, Notch1 and Rbp-j could be immunoprecipitated onto the HS5 site of

the *Il4* enhancer (Fang et al., 2007). Further experiments showed that Notch-mediated activation of HS5 within the 3′ enhancer is indeed important for IL-4 expression (Amsen et al., 2004; Tanaka et al., 2006). These findings suggest that IL-4 is downstream of Notch signaling, which may explain why Th2 cells could be generated from Rbp-j-deficient or N1N2-deficient $CD4^+$ T cells in vitro by adding exogenous IL-4 (Amsen et al., 2007; Tu et al., 2005). Efficient Th2 cell immune responses are usually induced after IL-4R signaling, which leads to STAT6 activation and subsequent increased transcription of *Gata3*, which is an important transcription factor for Th2 cell polarization (Ouyang et al., 2000). Two recent reports showed elegantly that Notch could directly regulate expression of the *Gata3* gene. Notch-Rbp-j transcription complexes were found to bind to the most upstream *Gata3* promoter and thereby regulate its expression (Figure 3; Amsen et al., 2007; Fang et al., 2007). Taken together, these data provide two molecular mechanisms by which Notch-Rbp-j signaling can promote Th2 cell differentiation. The first consists in driving transcription of the *Gata3* gene. Increased GATA3 protein engages in a positive feedback loop and then drives its own expression (Fang et al., 2007). Moreover, GATA3 may help to render the *Il4* enhancer accessible to Notch, which then results in increased *Il4* gene expression and protein levels. IL-4 can then bind to the IL-4R receptor and thereby further drive Th2 cell polarization.

Notch in Th17 and Regulatory T Cells

In addition to the well-described Th1 and Th2 cell subsets, Th17 cells were recently identified and classified as a proinflammatory subset distinct from Th1 cells (Korn et al., 2009). Dll4-expressing DCs, when activated with Toll-like receptor (TLR) ligands or *Mycobacterium* antigens, can promote the generation of Th17 cells through activation of the Th17 cell-specific transcription factor RORγt (Ito et al., 2009; Mukherjee et al., 2009). In EAE, pathogenic Th1 and Th17 cells develop in the central nervous system, causing autoimmunity. GSI-mediated inhibition of Notch signaling in this disease model resulted in reduced Th1 and Th17 cytokines (Jurynczyk et al., 2008; Minter et al., 2005). In addition, specific antibodies against Dll1, which attenuated EAE, have the opposite effects to antibodies against Jagged1 which exacerbated EAE (Elyaman et al., 2007). These results suggest that Dll ligands on DCs seem to be involved in the promotion of pathogenic Th1 and Th17 cells, whereas Jagged ligands might suppress autoimmunity.

Suppression of autoimmunity could also at least in part be regulated by the influence of Notch signaling on regulatory T (Treg) cells. Treg cells have an important role in negatively regulating hyperactive T cells that are induced during strong immune responses in peripheral tissues. Several reports show that the presence of Notch ligands (mostly of the Jagged family) can enhance Treg cell differentiation and function in vitro (Vigouroux et al., 2003). For example, exposure of Treg cells to Jagged2-expressing hematopoietic progenitor cells resulted in Treg cell proliferation and prevented the development of diabetes in an experimental autoimmune disease model in mice (Kared et al., 2006). Similar effects have been observed in mice overexpressing N3-ICD (Anastasi et al., 2003; Campese et al., 2009). It is unlikely that Notch signaling in vivo is required for the development of Treg cells because none of the genetic Notch loss-of-function mutant mice reported the lack of Treg cells. Nevertheless, a recent report suggests that Notch signaling might cooperate with TGF-β signaling components (P-Smad3) to activate FoxP3 expression (Samon et al., 2008). FoxP3 is an essential transcription factor regulating Treg cell development and function. GSI-mediated inhibition of Notch signaling inhibited TGF-β-induced Foxp3 expression as well as the suppressive activity of Treg cells in vitro. In vivo administration of GSI also resulted in reduced Foxp3 transcription. Moreover, these mice developed symptoms of an autoimmune hepatitis, a disease that was previously shown to result from deregulated TGF-β signaling and Treg cells (Samon et al., 2008). Collectively, these results indicate that Notch signaling facilitates the action of TGF-β to sustain Foxp3 expression in Treg cells in order to maintain their immune-suppressive function.

Thus, Notch signaling has been linked to many aspects of peripheral immune responses. For many of these functions, Notch is not absolutely required. However, Notch signaling appears to be critical in fine-tuning immune responses. There is clearly more work needed to understand the full spectrum of Notch functions in the periphery.

Notch in T Cell Leukemia

Jeff Sklar and colleagues identified the first human homolog of the *Drosophila Notch* gene in the late 1980s (Reynolds et al., 1987) and beginning of the 1990s (Ellisen et al., 1991) by cloning and sequencing of a chromosomal translocation within a small number of T cell lymphoblastic leukemia patient samples. In this original study, 4 out of 40 screened T cell leukemia patient samples carried a t(7;9)(q34;q34.3) chromosomal translocation, which juxtaposes the C-terminal region of EGF repeat 34 of the human *NOTCH1* gene to the TCR-β enhancer. This translocation results in the expression of an N-terminal truncated, dominant active, and ligand-independent human NOTCH1 receptor, which was named TAN1 for translocation-associated Notch homolog (Ellisen et al., 1991). The proof that TAN1 is indeed causative for disease development was shown by murine BM reconstitution experiments. Mice transplanted with BM progenitors expressing TAN1 developed T cell neoplasms as early as 2 weeks after BM transplantation (Pear et al., 1996). Other truncated Notch isoforms, including Notch2 and Notch3, were subsequently shown to induce T cell leukemias when expressed in BM progenitors or immature thymocytes (Bellavia et al., 2000; Rohn et al., 1996). Although the association of Notch and T cell leukemia has been widely demonstrated in mouse models, the rare frequency (<1%) of the t(7;9) translocation in human T cell acute lymphoblastic leukemia (T-ALL) patients questioned the clinical importance of these findings. This changed dramatically when Aster and colleagues analyzed 96 pediatric primary T-ALL tumors and thereby found that 55% of the samples had at least one mutation in the HD or the PEST domain within the *NOTCH1* gene, with approximately 20% of tumors having a mutation in both domains (Weng et al., 2004). Whereas mutations within the HD domain render Notch1 susceptible to ligand-independent S2 cleavage (Malecki et al., 2006), mutations and/or deletions within the PEST domain stabilize the intracellular Notch1 protein (Chiang et al., 2006), because essential recognition sequences for ubiquitin ligases that ensure a rapid turnover of the protein get lost. Fbw7 (F-box and WD repeat domain

containing 7) is an E3 ubiquitin ligase that is important in this latter context. Fbw7 has multiple targets including c-Myc and cyclin E, but it also physically binds to the C terminus of NICD and targets it for proteosomal degradation (O'Neil et al., 2007; Thompson et al., 2007). More importantly, the loss-of-function Fbw7 mutation was indentified in T-ALL cell lines as well as in a substantial number (20%) of T-ALL patients. Most of the Fbw7 mutations were identified in patients undergoing disease relapse, suggesting that these mutations conferred a selective advantage to tumor cells in order to render them resistant to the treatment. A causative role for Fbw7 in mouse T-ALL was confirmed by using conditional gene targeted mice for Fbw7. These mice exhibited increased Notch protein expression in thymocytes and developed T cell leukemia over time (Onoyama et al., 2007). These results suggest that the Fbw7-mediated regulation of protein stability of Notch is important during T-ALL development.

Although it is well documented that expression of NICD in BM progenitors causes a rapid induction of T-ALL, the impact of the different NOTCH1 mutations for disease development was less clear and has only recently been addressed. Pear, Aster, and colleagues tested different human gain-of-function NOTCH1 alleles for their ability to drive ectopic T cell development and to induce leukemia when expressed in murine BM progenitors. NOTCH1 mutant alleles that are more commonly found in T-ALL patients and induce a relatively weak Notch signaling activity (read out via transcriptional reporter assays) were able to induce ectopic T cell development but failed to initiate T cell leukemia. Only uncommon gain-of-function mutations, which correlated with strong downstream signaling, were able to drive both ectopic T cell development and T cell leukemia. These results question the importance of the more frequently observed *NOTCH1* mutations for the development of T cell leukemia. However, the weak gain-of-function *NOTCH1* alleles accelerated the onset of leukemia when tested in combination with a constitutively active K-Ras oncogene and importantly gave rise to tumors that were sensitive to inhibition of Notch signaling (Chiang et al., 2008). These data show that the induction of T cell leukemia is dependent on the signaling strength of NOTCH1 and that the signaling strength of the more common *NOTCH1* mutant alleles are insufficient to induce T-ALL on their own. Thus, *NOTCH1* mutations appear to be additional events that can cooperate with oncogenic hits and thereby influence tumor development and onset. Importantly, these tumors seem to remain "addicted" to NOTCH signaling (Chiang et al., 2008), which justifies further exploration of Notch inhibitors for treating T cell leukemia.

One important question that gained a lot of attention in the past years is the identification of target genes and signaling pathways that are regulated by or cooperate with mutant *NOTCH1* alleles in T-ALL. Gene expression arrays performed by several laboratories identified c-Myc as an important Notch target gene. c-Myc is rapidly downregulated in murine and human T-ALL cell lines upon GSI-mediated inhibition of Notch. c-Myc expression was also directly correlated to NICD expression and ChIP analysis revealed c-Myc to be a direct target of NOTCH1 (Weng et al., 2006). Ferrando and colleagues extended these findings and came to similar conclusions by performing "ChIP on ChIP" analysis and thereby showed that c-Myc and NICD share common targets, regulating cell growth, metabolism, and proliferation. They proposed a feed-forward loop through which NOTCH1 and c-Myc reinforce the expression of genes required for growth of leukemic cells (Figure 4; Palomero et al., 2006).

Aberrant Notch signaling is directly linked to the regulation of cell cycle proteins. Protein amounts of the cell cycle-dependent kinase inhibitor $p27^{Kip1}$ increased upon Notch inhibition. Notch signaling induces the transcriptional expression of the F-box protein Skp2, which is part of the E3-ligase complex that degrades $p27^{Kip1}$ and $p21^{Cip1}$, leading to enhanced G1-S transition (Sarmento et al., 2005). In mouse models, $p27^{Kip1}$ deficiency itself was shown to contribute to T-ALL development (Kang-Decker et al., 2004). Another cell cycle protein involved in Notch-induced T-ALL is cyclin D3. Mice lacking cyclin D3 show greatly reduced susceptibility to Notch-induced leukemogenesis, suggesting that cyclin D3 might be an essential cell cycle protein through which Notch mediates its oncogenic effects (Sicinska et al., 2003). With a tetracycline-inducible mouse model for Notch-induced T cell leukemia, Notch was shown to suppress p53 through repression of the ARF-mdm2-p53 surveillance network (Beverly et al., 2005). Attenuation of Notch signaling led to increased p53 expression and to tumor regression by inducing apoptosis (Beverly et al., 2005). Thus, Notch-mediated suppression of p53 appears to be another important event for T-ALL development (Figure 4).

Protein microarray screens identified the mTOR pathway as being positively regulated by Notch in T-ALL cells. Treatment of T cell leukemia cell lines with GSI induced hypophosphorylation of multiple signaling proteins within the mTOR pathway, a phenomenon that could be rescued by expressing NICD. Expression of a dominant-negative MAML-1 also mimicked the GSI effects, suggesting that activation of the mTOR pathway involves transcriptional activation of Notch target genes. How Notch activates the mTOR pathway is not fully understood. Interestingly, the effects of GSI on mTOR were rescued by expression of c-Myc and because c-Myc is a direct target gene of Notch, it is possible that mTOR is activated via c-Myc (Chan et al., 2007). An alternative possibility, as suggested in flies and in human T-ALL cell lines, is that the Notch target gene *Hes1* can negatively regulate the expression of PTEN (Palomero et al., 2007). PTEN is a tumor suppressor that counteracts the PI3-kinase activity and thereby negatively regulates the Akt pathway and its downstream target mTOR (Figure 4). In this context, loss of PTEN was suggested to induce resistance to NOTCH1 inhibition in T cell leukemia (Palomero et al., 2007). This hypothesis was challenged by showing that primary murine T cell leukemias remained dependent on Notch signaling despite the loss of PTEN. Moreover, investigations of 13 primary human T-ALL samples did not show any correlation between the PTEN status and resistance to Notch inhibition. However, loss of PTEN-accelerated disease onset in a murine T-ALL model suggested that Notch1 activation and loss of PTEN may collaborate in leukemia induction (Medyouf et al., 2009).

Another signaling pathway that becomes activated in response to the expression of NICD or human T-ALL *NOTCH1* mutations in hematopoietic progenitors is the NF-κB cascade. NICD expression could induce the nuclear localization of NF-κB, which resulted in the expression of multiple NF-κB targets

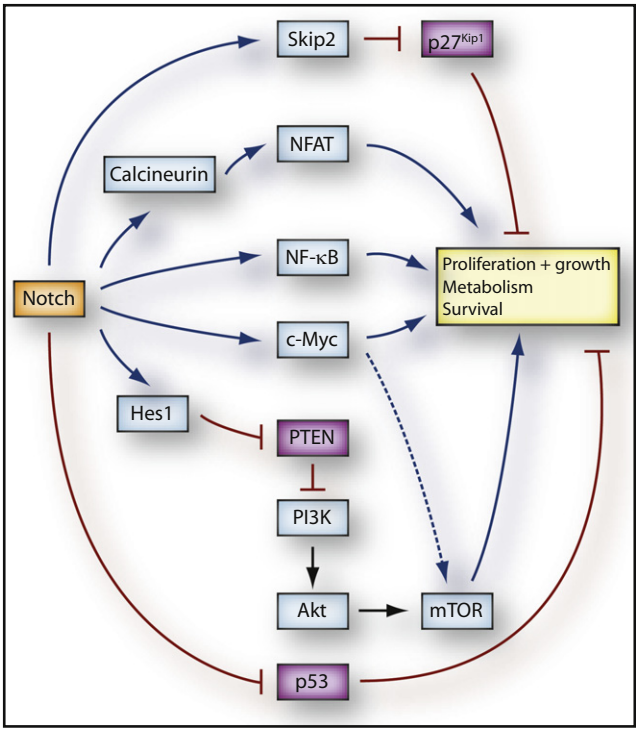

Figure 4. Molecules and Signaling Pathways Downstream of Notch Mediated T-ALL Induction

Arrows indicate positive and bars negative regulatory interactions contributing to the development and/or maintenance of T-ALL. Growth-promoting molecules or pathways are highlighted in blue, whereas growth-inhibitory molecules are shown in purple. Details of the molecules involved are described in the text. NFAT, nuclear factor-activated T cells; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositol 3-kinase; mTOR, mammalian target of rapamycin.

(including Bcl-2A1, NF-κB2, and ICAM1). Importantly, attenuation of NF-κB signaling resulted in the suppression of T cell leukemia both in vitro and in vivo (Vilimas et al., 2007). Finally, the NFAT cascade was also shown to be activated in response to aberrant Notch signaling. Notch activates calcineurin, which is a calcium-activated phosphatase that is important for the activation and translocation of NFAT factors from the cytoplasm to the nucleus. Calcineurin inhibitors such as Cyclosporin A (CsA) or FK506 induced cell death of T-ALL cells and resulted in tumor regression as well as in substantial prolonged survival of leukemic mice (Figure 4; Medyouf et al., 2007). In this context, it is interesting to note that c-*myc*, NF-κB, and NFAT are all signaling pathways that also get activated when immature thymocytes transit through β-selection.

Although the details of how Notch regulates and intersects with all these different signaling pathways are not fully understood, they highlight important connections and open possibilities to treat T-ALL from different angles. To date, the most frequently used strategy to block Notch signaling that is also currently explored in clinical trials is to use small molecule inhibitors of the γ-secretase complex (Seiffert et al., 2000). These molecules block the S3 cleavage and thereby inhibit the liberation of NICD (Seiffert et al., 2000). However, γ-secretase inhibitors are not NOTCH specific nor are they selective for individual Notch receptors; they block signaling of all receptors. Administration of γ-secretase inhibitors to mice or T-ALL patients results in unwanted side effects including dose-dependent gastrointestinal toxicity (Milano et al., 2004). Alternative strategies such as designing blocking antibodies for specific Notch receptors and/or ligands are currently being developed by several groups and pharmaceutical companies. A strategy for inhibiting Notch signaling was successfully explored by Bradner and colleagues, who aimed at inhibiting the NOTCH transcription complex. The design of the new inhibitors is based on the X-ray structure of the CSL-NICD-dnMAML complex (Nam et al., 2006). The dnMAML protein forms a long α helix that is located in a groove formed by the CSL-NICD complex. Bradner and colleagues designed and tested small α-helical peptides (hydocarbon-stapled α-helical peptides) for their ability to mimic this α helix and then bind to and thereby block the intracellular protein-protein interaction. Treatment of leukemic cells with such a peptide resulted in the suppression of the Notch-activated transcriptome. Most importantly, the peptide inhibited the proliferation of leukemic cells in vitro as well as in a NOTCH1-driven T-ALL mouse model without causing gut toxicity (Moellering et al., 2009). Whether these α-helical peptides are specific inhibitors of the NOTCH1 transcription complex remains to be investigated. Nevertheless, this is a very encouraging study showing that transcription factor complexes can indeed be targeted (Moellering et al., 2009).

Conclusions

Over the last decade, Notch signaling was shown to regulate multiple cell fate decision and differentiation processes during development and function within the hematopoietic system. Most, if not all, of these functions seem to be mediated through canonical (Rbp-j-mediated) Notch signaling. Notch is essential for the development of definitive hematopoiesis during embryogenesis, whereas under physiological conditions it is dispensable for the maintenance or homeostasis of adult HSCs. Furthermore, it is absolutely necessary for T cell lineage commitment and early stages of thymocyte as well as for MZB cell development. All these developmental aspects of Notch function are mediated by nonredundant Notch receptor ligand pairs (Jagged1:Notch1 for embryonic HSCs; Dll4:Notch1 for thymocyte development, and Dll1:Notch2 for MZB cell development). In more recent years, investigators found increasing evidence that Notch also plays important roles during T cell-mediated immune responses, in particular for the regulation of T helper cell differentiation. Genetic loss-of-function experiments point to an essential role of Notch signaling in Th2 cell differentiation. Nevertheless, Notch seems also to influence Th1 cell differentiation. Th1 and Th2 cell differentiation may depend on the ligand used to activate Notch. The question of ligand receptor specificity is currently unclear and will need to be answered in the future. Moreover, it is not known how Jagged- and Dll-expressing APCs differ in their ability to induce Notch signaling. Is this a question of signaling strength and how does this translate into differential target gene expression followed by Th cell differentiation?

The disease outcome of several patient-relevant experimental murine autoimmune models can be influenced by interfering with Notch signaling, suggesting that inhibitors or activators of Notch might be used to treat inflammatory and/or autoimmune

diseases. In this context, it is important to note that many functions of Notch are conserved between mice and men. Nevertheless, the effects of interfering with Notch signaling in human systems remain to be systematically investigated.

Although the oncogenic properties of Notch signaling for T-ALL were discovered approximately 20 years ago, research in this field gained increasing interest only after the finding that gain-of-function *NOTCH1* mutations are the most frequent genetic alterations found in T-ALL. The predominant role of Notch signaling in T-ALL cells is to drive a gene expression program maintaining growth, high metabolism, and survival. Thus, the Notch pathway became an attractive therapeutic target and multiple tools (e.g., γ-secretase inhibitors, neutralizing antibodies against Dll4 or NOTCH1) that interfere with Notch signaling are currently being developed and tested in various murine cancer models or even in clinical trials. In the future, the same tools might also be exploited to treat immunological disorders.

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