Chapter 5
Notch Signaling: A Pivot Regulator of Adaptive and Innate Immunity

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Abstract The coordinated activities of the innate and adaptive arms of the immune system are essential to protect individuals against infectious and neoplastic pathologies and to prevent the development of autoimmune responses. The Notch family of receptors is a highly conserved signaling pathway that controls the development, function, and differentiation of many cell types, including the immune cells. Although the effects of Notch-linked mediators in the innate and adaptive immunity are the focus of an active research field, there are still multiple unknown areas regarding how this cellular signaling pathway plays such a primary role in the regulation of immune responses. In this review, we summarize and discuss the emerging role of Notch in the regulation of adaptive and innate immunity. We postulate that a better understanding of the effects of Notch in immune cells will provide new approaches for therapies in various diseases.

Keywords Cancer · Tumor Immunity · Immune responses · T lymphocytes · Myeloid cells · Immunotherapy · Cytokines · Tolerance · Tumor growth and metastasis

5.1 Introduction

The Notch family of receptors is a highly conserved pathway that controls the development, differentiation, survival, and function of many cell types, including immune cells [1]. Mammals have four Notch receptors (Notch-1 through Notch-4) that are bound by five ligands of the Jagged (Jagged-1 and Jagged-2) and the Delta-like (DLL1, DLL3, and DLL4) families [2]. Binding of the Notch receptors to their

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ligands induces Notch proteolytic processing, including the cleavage through the ADAM metalloprotease and the γ-secretase complexes, thereby leading to the membrane release and nuclear translocation of the Notch intracellular active domain (NICD). Once there, NICD complexes with the recombination signal-binding protein-J (RBP-Jκ, also known as CSL) and the mastermind-like (MAML) coactivator, promoting the canonical induction of multiple transcripts [3]. Moreover, NICD interacts with members of the nuclear factor-κB (NF-κβ), transforming growth factor-β (TGFβ), and hypoxia-induced signaling pathways, inducing noncanonical regulation of various transcripts [4, 5]. These noncanonical signal transduction pathways also occur in the absence of Notch receptor cleavage and through the cross talk between NICD and other signaling mediators [6–8].

The fundamental role of Notch receptors and their corresponding ligands on immune cells was initially established in processes regulating the development and maturation of T cells in the thymus and during marginal zone B (MZB) cell development in the spleen [1]. More recently, Notch signaling has also emerged as a major player in the hematopoietic regulation of various subsets of myeloid cells and a key regulator of lymphocyte function [9]. In this review, we highlight recent advances pertaining to the primary role of Notch signaling in the development and the function of adaptive and innate immune cells. Especial emphasis is placed on the effect of Notch signaling in mature $CD4^+$ and $CD8^+$ T cells and in dendritic cells (DCs).

5.2 Regulation of Lymphocyte Development and Function by Notch Receptors

5.2.1 Notch Regulates the Development of T Cells

Notch signaling is instrumental in the differentiation and maturation of T cells [10]. The development of αβ or γδ T cells in the thymus is initiated after the recruitment of bone marrow-originated common lymphoid progenitors through the bloodstream. Once there, T cell precursors increase the expression of Notch-1 and Notch-3 as they start their differentiation into $CD4^-$ $CD8^-$ T cells and maintain an elevated Notch activity until they reach the double-negative 3 (DN3) stage [11]. Notch-1 and Notch-3 levels then dissipate after the progression of the cells into the β checkpoint selection phase and continue low until the mature T cells are activated in peripheral tissues [12]. As such, deletion of Notch-1 or the Notch canonical partners RBP-Jκ or MAML in bone marrow precursors results in a complete absence of T cells and instead a significant accumulation of ectopic B cells [13, 14]. In contrast, the ectopic expression of Notch-1 intracellular active domain (N1IC) beyond the DN3 phase triggers the development of acute lymphoblastic T cell leukemia (T-ALL) [15]. This effect is physiologically relevant as a high number of the patients with T-ALL carry gain-of-function mutations in Notch-1 or Notch-related genes [16]. Emerging results have also indicated the role of Notch activity in the differentiation of $CD8^+$ T cells [17]. Interestingly, T cell receptor (TCR) tickling by MHC class I is required for the Notch-induced $CD8^+$ T cell development, suggesting the key role of

the interaction between Notch signaling and antigen recognition in CD8⁺ T cells [18]. In addition to the role of Notch receptors in T cell differentiation, recent studies have pointed on the effects of the expression of particular Notch ligands in the thymic epithelial cells as regulators of the T cell commitment. As such, expression of DLL4 or DLL1 in the thymic stroma drives Notch-1 signaling in T cell precursors [19–23]. However, T cell development is unaffected in DLL4 or DLL1 mutant mice, suggesting the potential redundancy of the expression of Notch ligands in the thymic stroma [21]. In addition to the DLL family, Jagged-2, but not Jagged-1, is capable of directing T cell lineage commitment [24]. Therefore, Notch-1 signaling after binding to DLL1, DLL4, or Jagged-2 promotes T cell development in the thymus. It is noteworthy that the high levels of DLL1 and DLL4 inhibited the development of both B cells and myeloid cells, suggesting that the differentiation of each lineage is tightly regulated by Notch signaling in a ligand-specific and a dose-dependent manner [23] (Figs. 5.1 and 5.2).

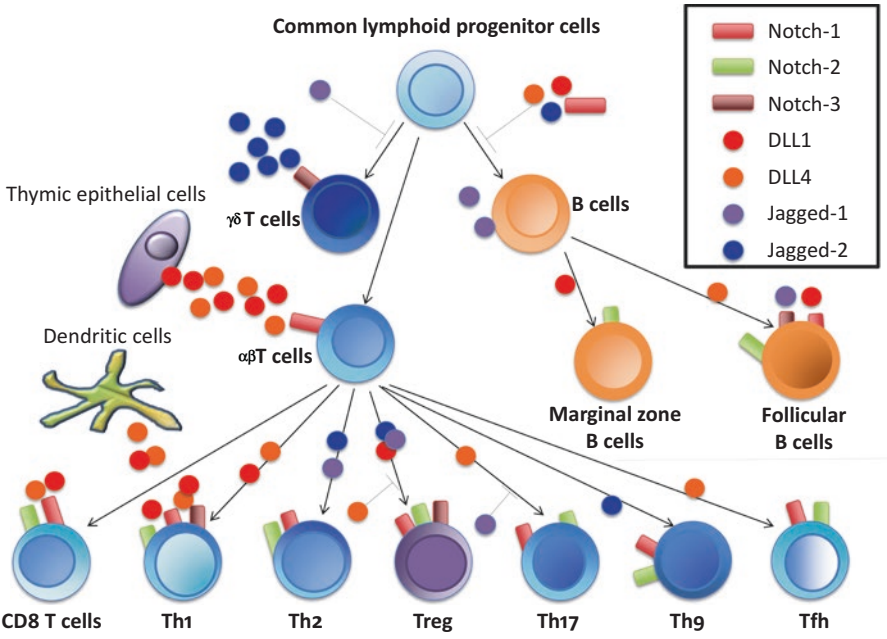

Fig. 5.1 The regulation of lymphocyte differentiation by Notch signaling. Notch-1 and Notch-3 expression and/or DLL1, DLL4, and Jagged-2 stimulation commits common lymphoid progenitor cells to T cell precursor cells [10–14]. After T cell lineage commitment, DLL1 and DLL4 from thymic epithelial cells or APCs induce αβ T cell differentiation including CD8⁺ T cells and Th1 subset [17, 25]. DLL1 is also capable of inducing Treg [32–34]. DLL4 induces Th17 and Tfh but inhibits Treg [35]. Although Jagged-1 is beneficial for Th2 and Treg, this ligand suppresses γδ T cells and Th17 [40]. Jagged-2 elicits the differentiation of γδ T cells, Th2, Treg, and Th9 [28, 29, 34]. After B cell lineage commitment that is suppressed by Notch-1, DLL1, DLL4, and Jagged-2 [50, 51], marginal zone B cells or follicular B cells are induced by DLL1 or DLL1, DLL4, and Jagged-1, respectively [44]

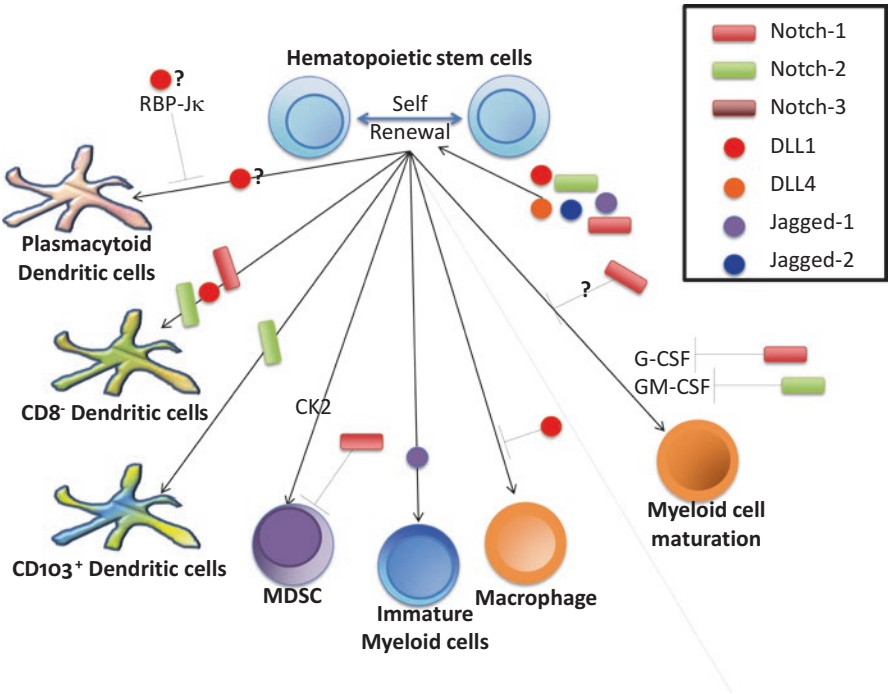

Fig. 5.2 The regulation of myeloid lineage differentiation by Notch signaling. The self-renewal of hematopoietic stem cells is promoted by Notch (Notch-1 and Notch-2) and its ligands (DLL1, DLL4, Jagged-1, and Jagged-2) [96, 100–103]. Immature myeloid cells are maintained via Jagged-1 signaling [96, 100, 101]. G-CSF- or GM-CSF-induced maturation of myeloid cells is inhibited by Notch-1 or Notch-2 signaling, respectively [96, 100]. Macrophage differentiation is suppressed by DLL1 [117]. The canonical Notch pathway inhibits plasmacytoid dendritic cells [108]; however, the effect of DLL1 is context dependent [122, 123]. Notch-2 signaling induces $CD8^-$ and $CD103^+$ dendritic cells [109]. Notch-1 and DLL1 are also inducers of $CD8^-$ dendritic cells [108, 112]. Casein kinase 2 suppresses Notch signaling to maintain the phenotype of MDSCs [113]

5.2.2 *Notch Controls the Differentiation of Mature T Cells*

Accumulating evidence indicates the major role of Notch signaling in the differentiation of $CD4^+$ T cells into specific T helper (Th) subsets. As such, activation of Notch-1 and Notch-2 on primed $CD4^+$ T cells by specific Notch ligands on DCs leads to their polarization into different Th subsets [25]. Interestingly, DLL1 and DLL4 promoted the development of Th1 subsets, whereas Jagged-1 and Jagged-2 induced Th2 polarization. Activation of antigen-presenting cells (APCs) with Toll-like receptor (TLR) agonists elicited the expression of Notch ligands Jagged-1, Jagged-2, DLL1, and DLL4 [25]. Expression of STAT3-induced DLL4 upon DC stimulation with LPS mediated the induction of Th1 and Th17 differentiation independent of IL-12 [26, 27]. Additional reports also showed that DLL4-carrying DCs

induced Th1 polarization in an IL-12-dependent manner [28]. The induction of Th1 cells by DLL4 occurred through noncanonical pathways, adding a level of complexity on the effects of DLL/Notch in the promotion of Th subsets. Opposite to the effect induced by DLL1 and DLL4, Jagged-1 and Jagged-2 ligands triggered Th2 differentiation. In fact, Jagged-2 in the tumor microenvironment skewed T cells into Th2 cells, which promoted tumor growth [29, 30]. Moreover, the Th2 differentiation induced by Jagged/Notch signaling was significantly diminished upon deletion of RBP-Jκ, suggesting that canonical Notch activity is required for the Jagged-induced effects [31]. Thus, Th1 polarization was promoted through noncanonical Notch signaling, whereas Th2 induction depended on canonical Notch pathways. Interestingly, the levels of active Notch were similar in T cells primed in the presence of DLL or Jagged ligands, suggesting that unknown mediators are responsible for the opposite effects triggered by these ligands on Th differentiation. Because cytokines are the major determinant of the $CD4^+$ T cell fates and are major targets of Notch signaling [25], current research is exploring the role of Notch-induced cytokines in the polarization of specific Th subsets.

Notch signaling is fundamental for the generation, expansion, and suppressive function of regulatory T cells (Treg). Treg development is increased after overexpression of Notch-1 or Notch-3 in T cells, whereas Treg inhibition is induced upon treatment of T cells with γ-secretase inhibitors (GSI) [32–34]. As ligands, DLL1, Jagged-1, and Jagged-2 play a primary role to support Treg induction. DLL1 maintained the survival of natural Treg and increased Treg conversion by directly upregulating Foxp3 transcription or by cooperatively augmenting TGF-β/Smad3 signaling [34]. Jagged-1 is another ligand that induces Treg by enhancing the signaling through TGF-β/Smad3. Furthermore, $CD4^+$ T cells stimulated in the presence of Jagged-2 became Treg with a high suppressive capacity against autoimmune encephalomyelitis (EAE) [28]. Conversely, DLL4 inhibited the expansion of Treg through downregulation of JAK3 and STAT5 phosphorylation [35]. The mechanisms by which the specific Notch ligands modulate cellular signaling and trigger or inhibit Treg development remain elusive.

Th17 cells are a relatively new subset of $CD4^+$ T cells that play a fundamental role in a variety of autoimmune diseases [36]. Accumulating evidence suggests that Notch signaling regulates the differentiation of Th17 cells [37]. The induction of Th17 differentiation by TLR-activated DCs was abolished by DLL4 neutralization, suggesting that DLL4/Notch signaling is essential for Th17 development [26]. Because RBP-Jκ directly interacted with ROR-γt, the master inducer of Th17 cells, it is likely that DLL4-induced canonical Notch signaling drives Th17 differentiation [38]. Also, the production of IL-6, a well-known inducer of Th17, was decreased in Notch-1 mutant mice, suggesting that Notch signaling can indirectly impact the differentiation of Th17 by regulating IL-6 production [39]. In contrast to the role of DLL4, Jagged-1 expression on DCs had a negative effect in promoting Th17 differentiation [40]. Therefore, although the effect of Notch in Th17 cells remains unclear, it is accepted that the development of Th17 populations depends on the specific binding of Notch receptors to particular ligands on APCs in a microenvironment containing the precise levels of specific cytokines.

Th9 cells were recently identified as a subset of $CD4^+$ T cells with a potent antitumor ability [41]. Polarization of Th9 cells by TGF-β and IL-4 induced the activation of Notch-1, which controlled the Th9 expansion [28]. Moreover, Jagged-2 ligand appears to regulate the Th9 differentiation by Notch, which occurred through the phosphorylation of Smad3 [28]. While Th9 differentiation was suppressed in Notch-1 and Notch-2 knockout mice and after GSI treatment, excessive amounts of IL-4 could compensate the deficiency of Notch signaling and restore Th9 development, suggesting that Notch activity is important only under limiting amounts of IL-4.

Follicular $CD4^+$ T cells (Tfh) are essential for providing B cell help to generate high-affinity antibodies in germinal center by expressing CD40 ligand and by producing IL-4 and IL-21 [42]. The absence of Notch-1 and Notch-2 by gene ablation impaired Tfh differentiation and germinal center formation [43]. By using conditional knockout mice, it has become clear that DLL4 signaling from the stromal cell is primary for the Tfh differentiation [44]. However, the role of Notch in the development and function of Tfh cells remains unclear.

γδ T cells have a restricted TCR repertoire that recognizes phosphoantigens produced by bacteria and parasites. Because endogenous phosphoantigens are also accumulated in tumors, γδ T cells are promising cell types for the target of immunotherapy [45]. The seminal study by Washburn et al. showed that while normal Notch-1 signaling induced αβ T cells, reduced Notch-1 favored the induction of γδ T cell from $CD4^-$ $CD8^-$ progenitor cells [46]. Furthermore, Notch-3 activation by Jagged-2 or constitutive active Notch-3 promoted γδ T cell lineage differentiation [47]. This differentiation was mediated through the inhibition of TCR-β expression, which is necessary for $CD4^+$ and $CD8^+$ T cells. Collectively, the balance between DLL4/Notch-1 and Jagged-2/Notch-3 signaling appears to determine the fate of αβ T cell and γδ T cells.

NKT cells are unique subset of T cells that recognize lipid antigens presented by the CD1d molecule. There are two subsets of NKT cells, type I (invariant TCR) and type II (diverse TCR) NKT cells, whose function can be both pro-inflammatory and immunoregulatory [48]. Most of the NKT cells are either $CD4^+$ or $CD4^-$ $CD8^-$; however, it remains largely unknown the pathways that drive their development from common lymphoid progenitor cells. Recently, the importance of Notch activity in the differentiation of NKT cells has been proposed. Although deletion of Notch-1 and Notch-2 increased the number of invariant NKT cells in the thymus, these invariant NKT cells were premature ($NK1.1^-$) and sensitive to apoptosis, which thereby resulted in the decreased number of these cells in the periphery [49]. NKT cells in RBP-Jκ mutant mice showed the same increased pro-apoptotic phenotype, suggesting that canonical Notch signaling plays a primary role in NKT cell survival. However, unlike Notch-1- and Notch-2-null NKT cells, RBP-Jκ knockout NKT cells showed comparable thymic development, indicating that noncanonical Notch-1 or Notch-2 signaling is primary for the intra-thymic development and that canonical Notch signaling is essential for the periphery maturation, function, and homeostasis of NKT cells.

Although Notch-1-related activity has been extensively studied during early T cell development, the impact of Notch in the B cell compartment remains poorly understood. Initial reports showed that commitment to early B cell lineage required the inhibition of Notch-1 activity in lymphoid progenitor cells [50, 51]. The increase of B cells in Notch-1-depleted mice was not due to the compensation mechanism by the lack of normal T cell development. Moreover, B cells were increased in DLL4 knockout mice, suggesting that DLL4 is one of the responsible ligands for the inhibition of B cell commitment [22]. Expression of Notch ligands on stromal cells allowed to assess the ability of other Notch ligands in inducing B cell differentiation. In hematopoietic progenitor cells, Jagged-1 stimulation was capable of inducing B cell lineage [24]. On the contrary, DLL1, DLL4, and Jagged-2 suppressed this differentiation. Interestingly, the effect of DLL1 to modulate the fate of B cell differentiation depended on the dose and the density of its expression [52]. More recent results established the role of Notch signaling, especially Notch-2, during the development of specific subsets of B cells in the spleen. Two different populations of B cells accumulate in the spleen, namely, marginal zone B cells (MZB) and follicular B cells [53]. Follicular zone B cells represent the majority of B cells within the spleen and participate in immune responses mediated by T cells. Conversely, MZB cells are a minority of the B lymphocytes in the splenic tissue and regulate antibody responses against lipid antigens, which usually occur in a T cell-independent manner [54]. Although the number of follicular B cells is much higher than MZB, the ability to activate antigen-specific $CD4^+$ T cells of MZB is superior to that of follicular B cells [55]. B cell progenitors from the bone marrow migrate to the spleen and originate MZB cells and follicular B cells through transitional stages T1 and T2. The specification of MZB cells after T2 is highly dependent on Notch-2 signaling induced by DLL1, but not through DLL4 expression [44]. The expression of DLL1 in blood endothelial cells or DCs was dispensable during this process. This differentiation appears to be mediated through canonical pathways, as conditional deletion of RBP-Jκ and MAML resulted in a similar inhibition in the development of MZB as that induced by Notch-2 ablation [56–58]. One of the downstream targets of Notch for MZB induction is the E protein family. Downregulation of E proteins by Notch activation is necessary to drive MZB differentiation [59]. Thus, under physiological conditions, interaction of DLL1 with Notch-2 and further canonical signals induces in transitional B cells to specify MZB cells, as opposed to follicular B cells. Meanwhile, the differentiation of follicular B cells heavily relies on DLL4 on fibroblastic reticular cells [44]. In addition, DLL1 has been shown to enhance the proliferation of follicular B cells after B cell receptor or CD40 stimulation through MAPK activation, suggesting that the Notch ligand required for the differentiation and the proliferation of follicular B cells may be different [60]. For the sake of the survival, Jagged-1 in DCs rendered an anti-apoptotic feature to follicular B cells [61]. Altogether, the results suggest the key role of Notch signaling in B cell development and function.

Innate lymphoid cells (ILCs), a heterogeneous group of lymphocytes that lack T/B cell receptors and that activate in an antigen-independent manner, are emerging as major mediators in the immune responses against infectious agents and tumors

and in the development of tolerance against self-antigens. Similar to $CD4^+$ and $CD8^+$ T cells, ILCs develop from common lymphoid progenitor cells. ILCs are divided into three major subclasses (ILC1s, ILC2s, and ILC3s). ILC1s, ILC2s, and ILC3s consist of Th1-producing T-bet^+ cells, including natural killer (NK) cells, Th2-producing $GATA3^+$ cells, and Th17-producing cells, respectively [62, 63]. While most of the studies focused on the cytokines or transcription factors mediating the induction of ICLs, Notch signaling has been shown to regulate the maturation of several subsets of ILCs, including ILC2s (nuocytes) and ILC3s (lymphoid tissue-inducer (LTi) cells) and IL-22-producing ILCs ($NKp46^+$ ILCs) [64–66]. Nuocytes play an irreplaceable role in anti-helminth and allergic immunity by producing Th2-type cytokines IL-5 and IL-13. DLL1 stimulation in the presence of IL-33 and IL-7 is critical for the induction of nuocytes from progenitor cells [66]. Without nuocyte-skewed cytokines, DLL1 stimulation triggers instead $CD3^+$ T cells. In ILC3s, the necessity of Notch signaling depends on its developmental stage. Notch is prerequisite for the differentiation of common lymphoid progenitor cells to RORγt^- fetal progenitor cells; however, this signaling blocked a successive LTi differentiation [65]. The increased expression of Notch in IL-22^+ $NKp46^+$ ILCs was mediated through the transcription factor aryl hydrocarbon receptor (AHR) and further regulated the expression of the RORγt [64]. Conditional deletion of RBP-Jκ further confirmed that canonical Notch signaling is necessary for the induction of IL-22^+ $NKp46^+$ ILCs. Because unregulated IL-22 can cause an epithelial tumorigenesis [67], Notch inhibition may indirectly suppress tumor development by eliminating IL-22^+ $NKp46^+$ ILCs. Taken together, Notch signaling controls the development and/or expansion of ILC subsets, which also depends on the specific inflammatory modulators present in the microenvironment.

5.2.3 Effects of Notch Signaling in the Function of $CD4^+$ and $CD8^+$ T Cells

The role of Notch signaling in the modulation of $CD4^+$ T cell function is well established [68–70]. Treatment of activated mature $CD4^+$ T cells with GSI impaired their activation [71], proliferation [72, 73], and survival [74], suggesting the importance of Notch signaling in $CD4^+$ T cells. The $CD4^+$ T cell survival effects induced by Notch appear to be mediated through the promotion of glycolysis and occurred in a Notch canonical-dependent manner [75]. Furthermore, inhibition of DLL1 and DLL4 impaired the function of activated $CD4^+$ T cells, indicating the major role of these ligands in the function of $CD4^+$ T cells [76]. Accordingly, DLL4 stimulation increased the sensitivity of $CD4^+$ T cells to antigens by upregulating the PI3K/mTOR/GLUT1 signaling cascade [77]. As stated above, ligation of Notch to DLL1 or DLL4 ligands promoted Th1 responses, whereas their engagement to Jagged-1 or Jagged-2 induced the development of Th2 and Treg populations [31, 68, 78, 79]. Furthermore, Treg development was induced by DLL4 blockade, which resulted in

the attenuation of EAE [35]. In contrast to DLL4, DLL1 not only induced Th1 but also promoted Treg differentiation. DLL1 increased the suppressive function of Treg, which was significantly inhibited upon Notch-1 blockade [33, 34]. DLL1-induced Treg cells express CD39 expression, which is a key enzyme for ATP/AMP conversion, suggesting that Notch signaling in Treg might indirectly suppress immune cells via production of adenosine. Interestingly, the development and function of Treg were significantly increased upon overexpression of Notch-1 or Notch-3 [32, 33]. Notch-3 induction in thymocytes expanded Treg that were fully competent to suppress the proliferation of bystander cells [32]. Although it is not clear if Notch-3 is solely important for the expansion of Treg, it is evident that Notch-3 acts as a pro-Treg receptor. Besides the benefit of DLL1 and Notch-3 signaling in Treg induction, the overall role of Notch-1 in Treg remains controversial [80]. The function of Th17, a prominent mediator of a variety of autoimmune diseases [36], is regulated by Notch. GSI or DLL4 blockade alleviates inflammation in asthma or EAE models by suppressing IL-17 [35, 81]. In line with this, Notch signaling supported the survival of Th17 cells by upregulating anti-apoptotic gene Bcl2 partly via HIF-1 [82]. Also, DLL3 significantly increased the number of pathogenic Th17 in collagen-induced arthritis model [83].

Although naïve $CD8^+$ T cells do not express significant levels of Notch receptors [30], they still require Notch signaling to be fully activated. Indeed, GSI treatment decreased proliferation [73], survival [74], cytokine production [73, 84], and cytotoxicity [84] of $CD8^+$ T cells. Expression of Notch-1 and Notch-2 in $CD8^+$ T cells was induced upon anti-CD3/CD28 activation and relied on mTOR and T-bet signaling [30, 85]. The role of Notch-1 and Notch-2 in the function of $CD8^+$ T cells has been demonstrated by the impaired lytic capacity found in $CD8^+$ T cells from Notch-1- and Notch-2-null mice or after the blocking of Notch-1 [30, 84, 86]. Notch signaling promoted cytotoxic activity in $CD8^+$ T cells through the induction of the effector molecules granzyme B, IFNγ, and perforin, which were upregulated through canonical signals or through the binding of NICD to Eomes or NF-κB [84, 87]. Interestingly, the induction of short-lived effector $CD8^+$ T cells (SLECs, $CD127^{low}$ $KLRG1^{high}$) was inhibited in mice lacking Notch-1 or Notch-2 after DC vaccination [85, 87]. Instead, early effector $CD8^+$ T cells ($CD127^{low}$ $KLRG1^{low}$) were increased in these mutant mice, suggesting that Notch-1 and Notch-2 are important for the conversion of early effector cells to short-lived effector cells [87]. Although Notch is dispensable for the CD8 memory generation [87], $N1IC^+$ $CD8^+$ T cells possessed a central memory phenotype ($CD44^+$ $CD62L^+$ $CD122^+$ $CD127^+$) and displayed an elevated cytotoxicity and antitumor activity after adoptive cellular transfer into tumor-bearing mice [30]. Thus, Notch signaling in $CD8^+$ T cells could represent an important immunotherapy target for cancer. With regard to Notch ligands, DLL1 overexpression increased the antitumor activity of antigen-specific $CD8^+$ T cells [88]. Indeed, Notch-2 activation on $CD8^+$ T cells by DLL1 on DCs results in a high production of granzyme B [86]. In contrast to the role of DLL1, Jagged-1 expression suppressed collagen-induced arthritis by providing negative signals in $CD8^+$ T cells [89].

The regulation of Notch signaling in T cells has emerged as a novel mechanism of tumor to escape from immunosurveillance. Myeloid-derived suppressor cells

(MDSC) suppressed the expression of full-length and cleaved Notch-1 and Notch-2 in $CD8^+$ T cells in a nitric oxide-dependent manner, suggesting that the tumor microenvironment blocks Notch signaling in $CD8^+$ T cells as a strategy to evade protective immunity [30]. Higher levels of VEGF from tumor or stromal cells would be another determinant of Notch inhibition in T cells by inhibiting the expression of DLL1 and DLL4 in the bone marrow microenvironment [88]. A recent report suggests that Notch signaling is controlled by epigenetic regulation in $CD8^+$ T cells. Enhancer of zeste homolog 2 (EZH2) stimulated Notch by the methylation of Notch repressor Numb in activated $CD8^+$ T cells [90]. $EZH2^+$ $CD8^+$ T cells were capable of producing multiple cytokines and had an anti-apoptotic feature. In ovarian cancer patients, high accumulation of $EZH2^+$ $CD8^+$ T cells correlated with good prognosis demonstrating that Notch signaling endows $CD8^+$ T cell with high antitumor activity. Notably, tumor cells dampened EZH2 expression in $CD8^+$ T cells by consuming glucose [90]. Several approaches have been reported to activate CD8 Notch signaling in the tumor microenvironment. As well as N1IC overexpression, DLL1-Fc fusion complex induced central memory $CD8^+$ T cells, which had an increased antitumor activity [30, 91]. The decreased Notch signaling in $CD8^+$ T cells of tumor-bearing mice was counteracted by a proteasome inhibitor bortezomib [92]. Collectively, decreased expression of Notch in $CD8^+$ T cells represents a key element in tumor-induced tolerance, and the restoration of Notch signaling in $CD8^+$ T cells could be a possible strategy to overcome immunosuppression in the tumors.

In addition to $CD4^+$ and $CD8^+$ T cells, the role of Notch in regulating function of γδ T cells and NKT cells has been described. Phosphoantigen stimulation by bromohydrin pyrophosphate (BrHPP) increases the expression of Notch-1 in γδ T cells. Accordingly, the γδ T cell survival, cytokine production, and cytotoxicity against tumor cells heavily rely on Notch-1 signaling [93]. The production of IL-4 by NKT cells is regulated by conserved noncoding sequences (CNS)-2 through canonical Notch signaling [94]. Moreover, Notch-1 and Notch-2 are crucial for the IFN-γ and IL-4 production in NKT cells [49]. The cytokine production of NKT cells is suppressed in RBP-Jκ knockout mice, indicating that canonical Notch signaling is indeed important for the function of NKT cells. These results suggest that the effects of Notch activity go further than those induced on classic $CD4^+$ and $CD8^+$ T cell subsets.

5.3 Notch Signaling Controls the Development and Function by Various Subsets of Myeloid Cells

5.3.1 Notch Regulates the Development of Myeloid Subsets

Myeloid cells regulate adaptive immunity through their ability to acquire and present antigens and through the expression and release of inflammatory mediators. Myeloid subsets in peripheral tissues are represented by monocytes, granulocytes, macrophages, and DCs. Additional subgroups that expand under inflammation are

MDSC, tolerogenic DCs, and suppressive plasmacytoid DCs (pDCs). Most of the myeloid populations are originated from common myeloid progenitors; however, pDCs are derived from common lymphoid progenitors. Initial reports provided compelling evidence supporting the key role of Notch in the function and differentiation of myeloid cells [95–98]. However, the exact nature of Notch-related signaling in specific subsets of myeloid cells remains unclear. Previous reports showed the role of Notch in the maintenance of myeloid progenitor cells and blockage of their terminal differentiation [99–101]; whereas others indicated the effect of Notch for the final differentiation of mature myeloid cells [16]. These differences can be explained by the stage of myeloid cell differentiation when Notch is activated, the specific Notch receptor or ligand triggering the signaling, and the inflammatory milieu present under Notch-signaling conditions.

In support of the effect of Notch as a mediator for maintaining the pool of myeloid precursors, a delayed hematopoietic cell differentiation in response to G-CSF and GM-CSF and promotion of hematopoietic precursor self-renewal were observed after specific activation of Notch-1 or Notch-2 [96, 100] or through interaction with Jagged-1 [102], Jagged-2 [101], DLL1 [103], or DLL4 [104]. Accordingly, the inhibition or deletion of Notch-1 triggered the spontaneous maturation of erythroid and myeloid precursors [105]. On the other hand, Notch activity has been shown to be required for the differentiation of mature myeloid cells. Ectopic expression of Notch-1, or its active forms, promoted the differentiation of hematopoietic progenitors and into myeloid cell [98, 106]. Furthermore, inactivation of Notch signaling by targeting γ-secretase member Nicastrin resulted in an aberrant accumulation of granulocyte/monocyte progenitors in the peripheral blood, spleen, and liver [107]. Despite the potential role of Notch signaling in the homeostatic expansion of myeloid cells, myeloid cell lineages are normal in mice deficient for Notch-1 [13] and RBP-Jκ [108], indicating that the interaction of Notch signaling and the maturation of myeloid cells are a complex process that is context dependent.

5.3.2 Notch Activity in the Development of DCs

In recent years, multiple studies based on the pharmacological inhibition of Notch activity, the overexpression of Notch receptors or ligands, and the conditional deletion of Notch receptors in DCs or hematopoietic precursors have provided conclusive evidence that Notch signaling plays an important role in the development and function of DCs. As such, conditional deletion of RBP-Jκ or Notch-2 in DCs reduced the expansion of specific DC subsets in the spleen, but not in other lymphoid tissues [108, 109]. In addition, differentiation of DCs was inhibited in mice lacking Notch-1 in hematopoietic precursors [110], as well as in embryonic stem (ES) cells mutant for Notch-1 [111]. Similar results were obtained after the conditional deletion of Notch canonical member RBP-Jκ in bone marrow cells, which induced a substantial reduction in the presence of splenic DCs, specifically the

$CD8^-$ DCs present in the marginal zone [108]. The mechanism maintaining $CD8^-$ DC expansion appears to be mediated through DLL1 [112]. Surprisingly, there was also an increased in the accumulation of pDCs in RBP-Jκ null mice, suggesting that Notch signaling controls the homeostasis of $CD8^-$ DCs and inhibits the expansion of pDCs in the spleen. In addition to splenic DCs, a subset of $CD103^+$ DCs located in the lamina propria of the intestine is strongly reduced in the absence of Notch-2 [109]. Thus, the final commitment to DC differentiation during myelopoiesis is regulated by the Notch receptor and ligand expressed through the overall microenvironment components.

In addition to the effect of Notch receptors and ligands on the differentiation of DCs, recent studies suggested the posttranslational modification of Notch in immature myeloid cells. As such, phosphorylation of Notch by casein kinase 2 (CK2) was suggested as a major mediator of the expansion of MDSC under chronic inflammation and a key inhibitor of DC differentiation. Silencing of CK2 restored Notch signaling and enabled the maturation of MDSC into DCs [113]. Furthermore, current research aims to determine the Notch ligand responsible for controlling splenic DC development. Initial studies suggested a potential distinct effect of DLL1 and Jagged-1 as mediators of Notch signaling in DCs. Incubation of bone marrow precursors with fibroblasts expressing DLL1 induced DC differentiation, whereas Jagged-1-carrying fibroblasts promoted the accumulation of immature myeloid cells [114]. The mechanisms mediating this opposite effect were mediated through the activation or inhibition of Wnt pathways by DLL1 and Jagged-1, respectively [115, 116]. Interestingly, DLL1-induced activation of Notch blocked differentiation of monocytes into macrophages but also enabled their differentiation into DCs [117]. *In addition to the interaction of Notch and Wnt pathway in DCs, there is evidence that Notch partners with* NF-κB to control the differentiation of myeloid cells. In fact, a significant decrease in the NF-κB signaling and expression is observed in bone marrow precursors from Notch-deficient mice, which was restored after reconstitution of Notch signaling [110, 118, 119]. The regulation of NF-κB by Notch is mediated by a transcriptional induction of NF-κB members and through the direct interaction of Notch active forms and NF-κB subunits [120, 121]. Recent research also established the interaction of DLL4, Notch, and NF-κB in the function of DCs [26].

pDCs are phenotypically and functionally a distinct subset of DCs. Several reports described opposite effects of Notch receptors and ligand DLL1 on the differentiation of pDCs [122, 123]. Initial studies showed that DLL1 enhanced the numbers of pDCs by promoting their differentiation rather than proliferation [123]. Conversely, stromal cells expressing DLL1 drove differentiation of thymic progenitor cells to T cells and blocked pDC development [123]. Furthermore, deletion of Notch-1 in bone marrow populations did not affect the development of pDCs in vivo [124, 125]. Opposite results were found in RBP-Jκ-deficient mice, which showed increased numbers of pDCs [108], suggesting that Notch signaling may play distinct roles in the development of pDCs. This could be explained by the redundancy in the function of Notch in different environments. In fact, an example that the

effects induced by Notch signaling are highly complex and context dependent is suggested by studies showing that deletion of Notch-1 in the thymus favors the development of DCs [124, 126], without affecting pDCs.

5.3.3 *Signaling Through Notch Modulates the Activity of Myeloid Cells*

In recent years, an active research field has suggested the importance of the Notch signaling in the activation of myeloid cells and the subsequent effect on T cell responses. RBP-Jκ-lacking DCs had a defect in the activation, maturation, and antigen presentation in response to LPS [127]. Further studies showed that loss of RBP-Jκ in DCs impaired their ability to contain tumor growth [128]. These results demonstrated the role of canonical Notch signaling in the function of mature DCs. Additional studies have shown the important role of Jagged-1 and DLL4 in the modulation of DC-related inflammation. Activation of DCs through Jagged-1 induced the production of IL-10 and promoted the development of Treg [129]. Moreover, DLL4-dependent Notch activation in DCs triggered Th1 and Th17 responses via NF-κB activity [26, 130]. However, DLL4 also induced IL-10 production from DCs that had an ability to attenuate airway hyperresponsiveness [131]. Thus, signaling through Notch in mature DCs may result in tolerogenic or immunogenic environments, which depend on the context and the strength of the Notch activation. In fact, a switch in the Notch ligands has been observed in myeloid populations as they approach the tolerogenic tumor microenvironment [132].

Activation of Notch in myeloid populations has been reported to be mediated through TLRs and various cytokines. TLRs are widely expressed in macrophages and DCs and allow them to rapidly respond to pathogen infections. Signaling through TLR ligands leads to the induction of Notch receptors and Jagged-1, Jagged-2, DLL1, and DLL4 [25, 133, 134]. Induction of Notch receptors by TLR ligands induces synergistic effects through unknown mediators that enable the inflammatory capacity of myeloid cells. Furthermore, inflammatory cytokines such as TNF and IL-1β induce the expression of Notch-1 and Notch-4, leading to the activation of inflammatory mediators [135–138]. A common potential mediator for the induction of Notch signaling by TLRs and cytokines is the activation of NF-κB [139]. Interestingly, IFN-γ signaling blocks the induction of Notch-induced genes through unknown mechanisms adding into the complexity of Notch induction in DCs under inflammation [139].

The molecular mechanisms by which Notch regulates DC activity remain unclear. Most of the effects triggered by Notch signaling in myeloid cells are mediated through activation of NF-κB. The mechanisms by which NF-κB regulates the function of myeloid cells include cooperation with Notch transcriptional activity [140], release of the inhibitor of NF-κB (IκB) that binds to Notch targets [141], and

chromatin modification of Notch target genes [141]. Moreover, Notch signaling may activate the signal transducer and activator of transcription 3 (STAT3), which then interacts with specific NF-κB subunits [142, 143]. Another group of signaling molecules implicated in mediating Notch effects is the mitogen-activated protein kinases (MAPKs) [144].

Although there are overlapping effects of Notch signaling in DCs and macrophages (TLR and Notch interaction), recent studies have delineated unique features for canonical Notch signaling during macrophage activation. Treatment of macrophages with GSI decreased the production of IL-6, iNOS, and TNFα after activation with LPS, which correlated with a lower activity of NF-κB [145]. Similar results were obtained after deletion of RBP-Jκ in macrophages [144]. Conversely, production of IL-6 and TNFα was decreased, and IL-10 was increased in macrophages carrying the active forms of Notch-1 or Notch-2 [146], suggesting a potential effect of Notch in the promotion of tolerogenic macrophages. Accordingly, depletion of RBP-Jκ in tumor-associated macrophages (TAMs) restored the infiltration of $CD8^+$ T cells into tumors [147]. These results also support the potential context-dependent effects of Notch in macrophages.

5.4 Notch Signaling in Regulation of T Cell Polarization by APCs

Although Notch activity is a clear regulator of inflammation, the interaction of Notch on DCs and T cell function remains poorly understood. Unstimulated DCs express low levels of DLL and Jagged ligands; however, TLR activation upregulates the expression of both Notch and its ligands. As mentioned above, expression of DLL1 and DLL4 on DCs appears to favor Th1-type responses, whereas Jagged-1 and Jagged-2 induce Th2-type responses [25, 148]. In agreement with this, blockade of DLL4 in RSV-infected mice reduced Th1 cell polarization and promoted Th2 responses [149]. In contrast, activation of Notch through Jagged-1 promoted the development of Th2 responses that protected mice against autoimmune encephalitis [150]. In addition, silencing of Jagged-1 in immature human DCs prevented their ability to induce Th2 polarization [151]. These results provide a strong indication that polarization of $CD4^+$ T cells indeed depends on activation of Notch signaling. Although this aspect remains highly controversial, strong evidence supports the notion that DCs direct Th2 polarization via Jagged and Th1 polarization via DLL. In addition to DCs, Notch signaling in macrophages also affects CD4 differentiation. Activated macrophages derived from Notch-1 knockout mice produced less IL-6 and have low costimulatory molecule CD80 expression that results in less induction of Th17 [39]. Collectively, the interaction between innate and acquired immunity is dynamically impacted by Notch signaling.

5.5 Future Perspectives

Despite the aforementioned fact that Notch is important for both innate and acquired immunity, the question arises whether Notch signaling is a simple ligand/receptor pathway that plays an identical role regardless of the cell types. A recent finding partially answers this question by depleting Zmiz1, a cofactor of Notch-1 signaling. Surprisingly, Zmiz1 was required for T cell development but was dispensable for Notch-dependent intestinal homeostasis or myeloid suppression [152]. Although further investigations are necessary, this result offers a novel insight that cofactors would be a cell-specific determinant of the downstream signaling of Notch in addition to the type of ligands and receptors. Also, the influence of Notch pathway in tumor or stroma cells over acquired antitumor immunity is particularly interesting. The induction of N1IC in cancer cells reduced SERPINE1 expression and inhibited granzyme H-mediated cytotoxicity, indicating that Notch activity in the malignant cells enables escape from immune surveillance [153]. Since Notch is widely accepted as an oncogene and GSI (RO4929097) has passed a phase 1 study with a manageable safety profile [154], Notch activation or inhibition could not be a simple solution for cancer therapy. Although a substantial number of studies used GSIs as Notch inhibitors, gamma secretase is not specific for Notch, and cleaves over 95 different substrates including CD44. Because γ-secretase also cleaves CD44, a marker for cancer stem cells and activated T cells [155], the results obtained from using GSI must be interpreted with caution.

There are several regulators other than GSI that have potentials to regulate Notch signaling in immune cells. Because ADAM10 is necessary for the ligand-induced Notch-1 activation and ADAM17 is required for the ligand-independent Notch-1 signaling [156], the comparison between ADAM10 inhibitors and GSIs would be useful to differentiate ligand-dependent or -independent Notch-1 signaling. Jagged-1-mimic peptide (17 amino acids) would be a cost-effective alternative for Notch stimulation [157]. Valproic acid and suberoylanilide hydroxamic acid (SBHA), both of which have been identified as histone deacetylase inhibitors [158, 159], activate Notch-1 signaling and have a potential to suppress tumor proliferation. In addition, a dietary polyphenol resveratrol activates Notch-2 and suppresses carcinoid cell growth [160]. Interestingly, treatment of tumor-bearing mice with agonistic antibodies against Notch-2 or DLL1 or DLL4-Fc fusion proteins led to antitumor responses [88, 161, 162], suggesting the potential therapeutic effect of promoting Notch signaling in cancer. Although these therapeutic approaches were systemic and did not specifically target immune cells, further understandings of Notch signaling in innate and acquired immunity will enable us to pave the way for developing powerful strategies to treat cancer and autoimmune diseases.

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