Koji Yasutomo *Editor*

Notch Signaling

Immunity and Cancer

Springer

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ISBN 978-981-10-4970-5 ISBN 978-981-10-4971-2 (eBook)
DOI 10.1007/978-981-10-4971-2

Library of Congress Control Number: 2017950490

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

I am very pleased to have had the opportunity to organize this text summarizing the current state of knowledge about Notch. This book is composed of two parts: Notch immunology and cancer biology. Experts in Notch biology summarized their own results, discussed published data, pointed out unresolved issues, and suggested future studies. Indeed, one of the purposes of this book is defining the major questions to be approached as we enter the next era of Notch biology.

Notch signaling uses evolutionarily conserved cellular machinery. Notch signaling and the associated mechanisms were originally defined in the context of cell fate decisions observed in *Drosophila melanogaster*. Thereafter, the fundamental roles of Notch were studied in highly diverse biological processes in many organisms, including mice and humans. The complexity of Notch signaling increased after identification of multiple Notch and Notch ligands in mammals. Plus, each Notch interacts with all of the ligands, although the affinities vary. Furthermore, the interaction is regulated by sugar modulation of Notch receptors. Although those complex fields have been gradually untangled, especially by using genetically modified animals, it remains unclear why mammals have acquired a diverse collection of Notch proteins and ligands.

The role of Notch in the immune systems was first described in 1994. Thereafter, many reports revealed the roles of Notch in various aspects of development, differentiation, and survival of immune cells. As for the relationship between Notch and cancer, one of the interesting discoveries is identification of Notch gene mutations in various types of cancer, including T-cell leukemia. After the discovery of the involvement of Notch in various aspects of immune cells and tumorigenesis, basic researchers as well as pharmaceutical companies became interested in targeting Notch and Notch-related pathways to treat cancer patients. Indeed, there are many ongoing approaches to the modulation of Notch signaling and additional efforts anticipated in clinical trials.

With the explosive accumulation of papers and reviews on Notch in recent years, this is a good time to summarize current understanding about broad aspects of

Notch. This book provides a solid consensus of Notch function as well as insight into unresolved issues of Notch signaling. I hope that readers can raise new questions for further studies after reading this overview.

I would like to thank all authors for their invaluable contributions and the secretarial staff at Tokushima University for help throughout the preparation of this book.

Tokushima, Japan Koji Yasutomo

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Part I
Immunology

Chapter 1
Notch Ligands for Lymphocyte Development

Katsuto Hozumi

Abstract Although Notch signaling is well known to be critical for the specification of cell fate in various developing organs, it has not been fully defined how Notch ligands contribute to triggering through the Notch receptor in those organs, particularly in hematopoietic and lymphoid organs. The timing of the appearance of Notch ligands on the cell surface is thought to be crucial for the triggering between two equivalent progenitors in the lateral inhibition model. By contrast, the features of the Notch-regulating system, in which the Notch ligand functions as an environment factor, can be determined by the cell source of the Notch ligand that is frequently observed in hematopoietic and lymphoid organs. This review focuses on each Notch ligand and its cell source for lymphocyte development; moreover, it emphasizes the characteristics of the bone marrow, thymus, and secondary lymphoid organs based on the Notch system. In particular, the results obtained from the loss-of-function experiments using the defined Cre transgenic mice that are specifically active in the environment are described. In addition, the shared and intrinsic properties, including the structure and function of Notch ligands, are also described. These may be helpful for understanding the physiological significance of Notch ligands and their mediated signaling for the regulation of the lymphoid system.

Keywords Notch ligands • DSL • DOS • Fringe • Bone marrow • Thymus • Secondary lymphoid organs

1.1 Introduction

The Notch system is highly conserved from invertebrates to mammals, and the Notch signaling pathway regulates cell fate specification in many developmental systems (Bray 2006; Kopan and Ilagan 2009). Such signals are transmitted between

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K. Yasutomo (ed.), *Notch Signaling*, DOI 10.1007/978-981-10-4971-2_1

cells in direct contact with one another by the specific binding of Notch with its ligands, which include Delta-like or Jagged family members. These interactions result in the proteolysis of Notch and the movement of the intracellular domain of Notch (NICD) into the nucleus, which is an essential part of the signal transduction process.

The neural development of *Drosophila melanogaster* originally showed that Notch signaling occurs via two equivalent progenitors during embryonic development and leads those progenitors to adopt distinct developmental fates, neurons, and glial cells, to function as signal-sending and signal-receiving cells, respectively, according to the lateral inhibition theory (Greenwald and Rubin 1992; Lewis 1998; Artavanis-Tsakonas et al. 1999). In this case, which cell expresses a Notch ligand (NotchL) and sends the signals to the neighboring cell, which appears to be stochastically regulated, determines its role(s) for signal transduction. This is consistent with findings in the intestine (Vooijs et al. 2011; Sancho et al. 2015), lungs (Morimoto et al. 2012), pancreas (Shih et al. 2012; Nakano et al. 2015), and neurons (Kawaguchi et al. 2013) in mammals. However, for T cell development in the thymus, Dll4 as NotchL is expressed on mature epithelial cells in the thymic environment and induces Notch signaling into immature immigrants expressing Notch1, leading to the T cell lineage (Hozumi et al. 2008; Koch et al. 2008). In this case, NotchL functions as an environmental factor, and Notch-NotchL interactions between cells with distinct origins contribute to the determination of cell fate, which is similarly observed in other lymphoid tissues (below mentioned) or in developing liver tissues (Hofmann et al. 2010). Because almost all immature cells can receive Notch signaling by direct interaction with the environment, in this situation, the cells uniformly develop or contribute to the compartment in the tissue, whereas lateral inhibition allows the cells to differentiate into distinct fates, resulting in a salt-and-pepper pattern (Bray 2006). Thus, which cells express NotchL affects the systematic role of Notch signaling in developing tissues.

The loss-of-function experiments for Notch or its signal transducer, Rbpj (SuH), can effectively reveal the physiological significance of Notch signaling. However, these experiments essentially provide evidence of whether Notch (or Rbpj) is critical and do not provide information regarding when or where Notch signaling contributes to the determination of cell fate. To overcome this problem, both the actual detection of NICD and findings of NotchL are necessary.

The first part of this review describes the functional features of NotchL that trigger Notch signaling; subsequently, the role of NotchL or its mediated Notch signaling for lymphocyte development in the bone marrow, thymus, and other lymphoid tissues is examined. In addition, several subjects, which have remained unclear, are also mentioned.

1.2 General Information of Notch Ligands

1.2.1 Basic Structure

NotchLs are type 1 cell surface proteins that contain two structural shared domains in their extracellular region: DSL (Delta/Serrate/LAG-2) and DOS (Delta and OSM-11-like proteins) located at the 1st and 2nd EGF-like repeats (EGFRs), which are involved in the interaction of Notch receptors (Fig. 1.1a). In addition, Delta-like (Delta-like 1 and Delta-like 4 in mammals; Delta-like 3 is omitted in this review because of its inability to participate in *trans*-activation; Ladi et al. 2005) and Jagged/Serrate (Jag1 and Jag2) family members are Notch ligands that include different EGFRs (8 in Dll and 16 in Jag) or the cysteine-rich domain only found in Jagged/Serrate ligands. While there are no similarities between the two families regarding the intracellular regions, these regions are necessary to trigger the signaling and interchangeable between them (Abe et al. 2010). Thus, Notch signaling is dependent on the shared machinery.

The DSL domain was first identified within fly and worm NotchLs and was shown to be absolutely essential for their function. An X-ray crystallographic analysis revealed that the shared residues, which comprise a DSL motif in the DSL domain, are mapped to the surface, forming the putative Notch binding site (Cordle et al. 2008; Chillakuri et al. 2012; Kershaw et al. 2015). Following the DSL domain, the 1st and 2nd EGFRs display a different secondary structure (DOS domain) from the other EGF-like domains (Komatsu et al. 2008; Pintar et al. 2009), in which the DOS motif is also shared within Dll1, Jag1, and Jag2, but not Dll4 (mammals and zebra fish) (Kopan and Ilagan 2009), and contributes to binding and triggering (Shimizu et al. 1999; Andrawes et al. 2013). As expected, mutations in these domains disrupt the binding activity with Notch and are observed in Alagille syndrome as human Jag1-related disease (Kopan and Ilagan 2009).

The immobilized short fragments of NotchLs, including the N-terminal (module at the N-terminus of Notch ligands, MNNL), DSL, and DOS domains, can sufficiently bind and trigger Notch signaling, suggesting that these fragments can form the binding surface to Notch (Shimizu et al. 1999; Andrawes et al. 2013). The MNNL domain is conserved between the two NotchL families, and missense mutations are found in Alagille syndrome (Chillakuri et al. 2012). Structural and functional analyses have uncovered the structural similarities of the MNNL of Jag1 to the C2 domain observed in protein kinase C or Munc13, which bears phospholipid-binding properties in a calcium-dependent fashion and is necessary for efficient Notch activation (Chillakuri et al. 2012). However, because this ability was not detected in the MNNLs of Dll1 and Dll4, it might function only in Jag1 or Jag family members (Kershaw et al. 2015; Luca et al. 2015). Recently, it was reported that

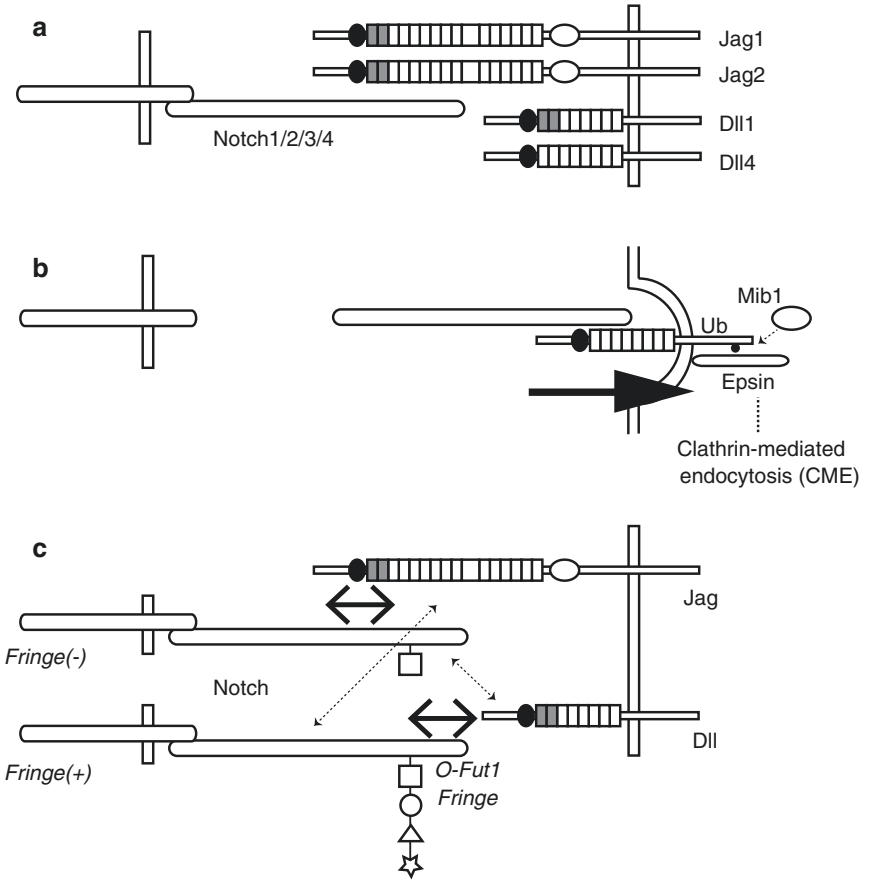

Fig. 1.1 Mammalian Notch ligands. (**a**) Mammalian Notch ligands are composed of Delta-like (Dll) family members, including Dll1 and Dll4, and Jagged (Jag) family members, including Jag1 and Jag2, which share unique characteristics. There are MNNL (module at the N-terminus of Notch ligand), DSL (Delta-Serrate-Lag2, *black circle*), and DOS (Delta and OSM11-like proteins, *gray square*, the first and second EGF-like repeats) regions with 8 (Dll) or 16 (Jag) EGF-like repeats (EGFRs, *square*) as the extracellular domains. Jag members have an additional cysteine-rich domain (*white circle*). Dll4 does not possess the DOS motifs. (**b**) After the binding of Notch ligands with Notch, the Notch ligands are modified with ubiquitin (Ub) by the E3 ubiquitin ligase, Mib1, and subsequently interact with Epsin, resulting in the formation of endocytic vesicles as a step in clathrin-mediated endocytosis (CME). These events produce the mechanical force (*arrow*) that pulls the extracellular domain of Notch, which is necessary for the efficient induction of Notch signaling. (**c**) The EGFR of the Notch extracellular domain is essentially modified O-fucose (*square*) by O-fut1 fucosyltransferase. In the presence of Fringe (Lfng, Mfng, and Rfng in mammals) as β1,3-N-acetylglucosamyltransferase, O-fucosylglycan is extended with GlcNAc (*circle*) and then elongated by other glycosyltransferases to yield a tetrasaccharide (Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc), which is sufficient to enhance receptor binding to Dll (*thick lines* with *arrowheads*) but reduces receptor binding in to Jag members (*thin dotted lines* with *arrowheads*) in vivo and in vitro

in addition to the interaction between Dll4 DSL and Notch1 EGFR 11, the MNNL of Dll4 binds directly to EGFR 12 of Notch1, and some artificial substitutions of amino acids in MNNL can enhance its activity (Luca et al. 2015). Thus, all three domains present in the N-terminal of NotchLs might contribute to Notch activation, although it has not yet been determined whether each one similarly acts in the individual ligand.

1.2.2 Endocytosis of Notch Ligand Is Essential for Signal Induction

A genetic approach in *Drosophila* showed that dynamin (encoded by the *shibire* gene) is required for Notch signaling in both the signal-receiving and the signal-sending cells (Seugnet et al. 1997). Because dynamin plays a critical role in releasing endocytic vesicles, this phenotype first suggested that ligand endocytosis is necessary to trigger Notch signaling in neighboring cells (Weinmaster and Fischer 2011; Musse et al. 2012). This was confirmed in mammalian cells with other essential components, such as clathrin, Epsin (and also Picalm), and actin polymerization, for clathrin-mediated endocytosis (CME) (Fig. 1.1b) (Meloty-Kapella et al. 2012). Endocytosis is thought to be a key process for signal sending in two models: (1) before Notch binding, it recycles NotchL to the cell surface, where the ligand functions well, and (2) after Notch binding, it induces a mechanical pulling force, leading to the dissociation of the Notch extracellular domain (NECD) and the sequential processing of the Notch intracellular fragment by ADAM10 (S2) and γ-secretase (S3) (Musse et al. 2012).

In addition, NotchL requires two distinct E3 ligases, Neuralize (Neu) and Mind bomb (Mib) in Drosophila, Xenopus and zebrafish (Lai et al. 2001; Deblandre et al. 2001; Pavlopoulos et al. 2001; Yeh et al. 2001; Itoh et al. 2003), or only Mib1 in vertebrate (Koo et al. 2005; Koo et al. 2007), for the induction of Notch signaling, which suggests that the ubiquitination of NotchL is a critical event downstream of the Notch/NotchL interaction (Fig. 1.1b). This is consistent with the functional deficit in the ubiquitin-defective or cytoplasm-deficient mutants of NotchL (Itoh et al. 2003; Heuss et al. 2008). The adaptor protein, Epsin, which is a significant component for endocytosis, possesses a ubiquitin-interacting motif (UIM) (Wendland 2002) that is necessary for the transendocytosis of NECD (Meloty-Kapella et al. 2012). The C- and N-terminal regions of Epsin also bind to PtdIns(4,5)P2 and clathrin (Wendland 2002), a major component of vesicle coating, respectively, which suggests that the ubiquitination of a NotchL has a key role in the formation of endocytic vesicles.

Recently, it was revealed that the ubiquitin ligase activity of Mib1 is upregulated by the Notch/NotchL interaction, which precedes the increase in NotchL ubiquitination, suggesting that NotchL endocytosis is stimulated by the interaction-induced Mib1 activity (Okano et al. 2016). The ubiquitination of NotchL is absolutely necessary for

signaling activity but not for binding to Notch, surface expression, and simple endocytosis without the mechanical force to dissociate NECD. Thus, the Mib1-dependent ubiquitination and subsequent endocytosis of NotchL are only required for signal-sending cells to activate the signaling in Notch-bearing cells.

1.2.3 Preferential Interaction with Fringe-Modified Notch

The posttranslational glycosylation of Notch occurs by fringe, which is a β1,3-*N*-acetylglucosaminyltransferase that extends O-fucose glycans attached to EGFRs on the extracellular region. Fringe-producing disaccharides are further elongated by other glycosyltransferases to yield a tetrasaccharide, Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc (Fig. 1.1c) (Moloney et al. 2000; Brückner et al. 2000). These modifications affect the ligand binding potential to NotchL. The fringe-mediated glycosylation of Notch increases the signaling magnitude induced by Delta or Dll family members and inversely decreases that induced by Serrate or Jagged family members (Fortini 2000). In contrast with the upregulation of the signaling based on the increase of the binding affinity with Dll, the downregulation is a result of both the alteration of affinity and the modification of signal transduction with Jag1 (Hicks et al. 2000; Yang et al. 2005). Because the expression of fng family members (Lfng, Mfng, and Rfng in mammals) is substantially detected in hematopoietic cells and lymphocytes (Tsukumo et al. 2006; Visan et al. 2006; Abe et al. 2010), it is generally suggested that Dll members have more critical role(s) than Jag members to trigger Notch signaling in hematopoietic and lymphoid tissues. Exceptionally, fringe activity appears to be low at the $CD4^+CD8^+$ (double-positive, DP) stage in the thymus (Tsukumo et al. 2006). This is physiologically important to maintain early T cell development in the thymus because the overexpression of Lfng converts DP thymocytes into "supercompetitors" with enhanced binding potential of their expressing Notch1 to Dll, which competes for the limited Dll4 on the epithelium with the thymic immigrants and blocks T lymphopoiesis from those (Koch et al. 2001; Visan et al. 2006). This is the first evidence showing that mammalian fringe glycosyltransferase actually modifies the binding affinity of Notch to NotchL and alters the efficiency of the signal induction in vivo.

1.3 Notch Ligand in the Bone Marrow

1.3.1 Jag1-Mediated Notch Signaling for the Maintenance of Hematopoietic Stem Cells

Notch signaling was reported to block the differentiation and maintain the pluripotent state. Based on these findings, it has been speculated that Notch signaling plays a critical role in the maintenance of hematopoietic stem cells (HSCs) in the bone marrow (BM). Parathyroid hormone-induced Jag1 in osteoblasts in the BM was

shown to induce an increase in HSCs through Notch1 activation, which was abrogated by γ-secretase inhibition, suggesting that Jag1 in osteoblasts functions as an environmental factor to regulate HSCs (Calvi et al. 2003). However, the systematic depletion of Jag1, which was observed in polyI-C-treated Mx-Cre, Jag1-floxed mice, did not display any phenotypic changes in HSCs (Mancini et al. 2005), indicating that Jag1-mediated Notch signaling is dispensable for the maintenance of HSCs in the BM. This was consistent with another report using a dominant-negative mutant of MamL1 that attenuated Rbpj/ICN1/Maml1-mediated canonical Notch signaling (Maillard et al. 2008). In contrast, recent report has shown that the deletion of the floxed *Jag1* gene has never been induced by the *Mx-Cre* transgene, which has frequently been used in previous studies; moreover, endothelial-specific Jag1 is necessary for homeostatic and regenerative hematopoiesis from HSCs in VE-cad-CreERT2 Tg mice (Poulos et al. 2013). This was the first evidence demonstrating the physiological significance of Jag1 to support HSCs in a loss-of-function experiment. Interestingly, CAR (Cxcl12-abundant reticular) cells, which were shown to express substantial levels of Jag1, are found at the perivascular region in the BM and may represent another candidate providing a niche for HSCs. Therefore, Jag1-mediated Notch signaling at both the endosteal and vascular or perivascular environment contributes to the maintenance of HSCs, which appears to be transduced via the non-canonical Notch signaling pathway.

1.3.2 Dll4-Induced Weak Notch Signaling Is Present in the BM

A unique phenotype that displayed an accumulation of extrathymic DP T cells and a simultaneous defect of B cell development in the BM was found to have derived from Zbtb7a-deficient hematopoietic stem or progenitors; this phenotype was identical to the phenotype observed with the enforced induction of intracellular Notch1 (ICN1, active form) into hematopoietic progenitor cells (HPCs) (Maeda et al. 2007). These findings suggest that Zbtb7a, a transcriptional repressor that belongs to the POK family, suppresses Notch signaling in the BM and retains the ability to differentiate into the B cell lineage; however, the molecular relationship has remained unclear. Of note, substantial Notch signaling is induced even in the BM, but it is not sufficient for the determination of T cell fate in the presence of Zbtb7a. The role of NotchL in this situation remains unknown. Most likely Dll1 or Dll4 play a role because strong Notch signaling is necessary for the induction of the T cell lineage from HPCs expressing fringe-modified Notch receptors. In addition, Zbtb7a is also necessary for the erythroid-specific repression of Dll4, which prevents HSCs from undergoing T cell differentiation in the BM (Lee et al. 2013).

Recent reports have suggested that HSCs and early lymphoid progenitors reside and are maintained at a distinct niche in the BM (Ding and Morrison 2013). In contrast to HSCs that reside on Cxcl12-expressing perivascular stromal (CAR) cells or endothelial cells (Sugiyama et al. 2006; Greenbaum et al. 2013), early lymphoid

progenitors, including the common lymphoid progenitor (CLP) fraction, mainly exist on osteoblasts that also express Cxcl12 at low levels. Accordingly, B cell development appears in the endostea in a Cxcl12-dependent manner. However, Dll4 is substantially expressed on osteocalcin-producing mature osteoblasts and contributes to the generation of thymus-seeding progenitors in the BM (Yu et al. 2015). Thus, the osteoblast-specific depletion of Dll4 impairs T lymphopoiesis in the thymus. Moreover, the intracellular fragment of Notch1 (NICD) is frequently detected in the CLP fraction compared to HSCs and disappears after the depletion of Dll4 in osteoblasts, suggesting that Dll4-mediated Notch signaling occurs before they arrive at the thymus. However, T cell development is never detected in the BM. Thus, it was speculated that Dll4-mediated Notch signaling induced in CLPs on osteoblasts drives T-lineage competence, but this mechanism is not sufficient for the initiation of the transcriptional program for the T cell lineage. The quantitative or qualitative level of this mechanism should be described.

Significant knowledge about innate lymphoid cells (ILCs) has been revealed (Artis and Spits 2015), but it is not certain whether Notch signaling is necessary for their development. Within that research, only one study clearly demonstrated that canonical Notch signaling is required for the appearance of lung-resident ILC2 through the Tcf1-dependent pathway, which occurs just before the ILCs commit to the ILC lineage (Yang et al. 2013). However, Notch signaling appears to be dispensable for other ILCs or NK cells. Consequently, it is reasonable that Dll4 expressed on osteoblasts in the BM can support the potential of CLP to differentiate into ILC2 cells. Further analysis is required to completely resolve this issue.

1.4 Notch Ligand in the Thymus

1.4.1 The Indispensable Role of Dll4 for the Determination of T Cell Fate

Notch1-floxed mice with the Mx-Cre transgenic allele were used to demonstrate that Notch1 is indispensable for the determination of T cell fate (Radtke et al. 1999). Notch1-null HPCs easily differentiate into the B cell lineage instead of the T cell lineage, even in the thymus, suggesting that Notch1-induced signaling directly promotes T cell development and suppresses B cell development at the branch point. Conversely, the enforced induction of ICN1 into HPCs arrests B cell development and promotes T cell development in the BM (Pui et al. 1999). These studies demonstrate that because environment factors essential for lymphopoiesis are shared within the thymus and the BM, Notch signaling determines which cell fates are induced in the lymphoid condition.

Reconstitution of the environment in the BM was succeeded in the monolayer culture system with BM-derived stromal cells and several cytokines, in which B cell development was completely observed from HPCs. By contrast, it was previously believed that the thymic environment could not be reconstituted in a two-dimensional

(2D) culture system, and no T cells could appear in vitro except in thymic organ culture. The enforced induction of ICN1 into HPCs overcame this difficulty, which led the HPCs to differentiate into the T cell lineage; however, it blocked the cells from entering the B cell lineage, even in the 2D cultures (Hozumi et al. 2003). Similarly, it was shown that Dll1-expressing OP9 stromal cells could enable HPCs to enter the T cell lineage (Schmitt and Zúñiga-Pflücker 2002). These results confirmed the role of Notch signaling at the branch point of T/B cell lineages.

Although Dll1 has the potential to support T lymphopoiesis in vitro, the disruption of the *Dll1* gene in the thymic environment does not perturb that in the thymus, suggesting that another NotchL compensates for this defect (Hozumi et al. 2004). This was consistent with the low expression of Dll1 compared to that of Dll4 in the thymus (Heinzel et al. 2007). In addition, NotchL, which is effective for T lymphopoiesis in the thymus, was estimated to involve a Dll family member because the fringe-modified Notch1 efficiently binds and occupies the ligand as described in Sect. 1.2.3 (Visan et al. 2006). This was validated using Dll4-floxed, FoxN1-Cre mice in which the *Dll4* gene was specifically disrupted in thymic epithelial cells; the obtained results clearly supported the conclusion that Dll4 in the epithelium plays an indispensable role in the determination of T cell fate in the thymus (Fig. 1.2) (Hozumi et al. 2008; Koch et al. 2008).

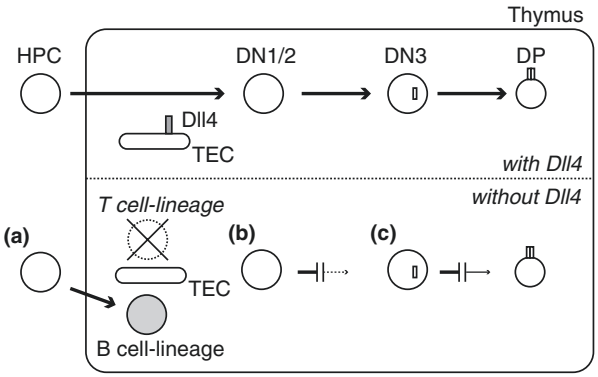

Fig. 1.2 T cell development in the thymus with intact or Dll4-deficient epithelial cells. The thymic immigrants, hematopoietic progenitor cells (HPCs), arrive in the thymus and then receive Notch signaling via Notch1 on the surface. Dll4 on thymic epithelial cells (TECs) binds to Notch1 and triggers the signaling to induce T cell development through the DN1/DN2, DN3, and DP stages. Upon reaching the DN2 stage, thymocytes lose their differentiation potential to other lineages (NK or myeloid cells) and undergo rearrangement of the TCR β gene to produce the TCR β chain. At the DN3 stage, the rearranged TCR β chain (*rectangle*) is composed of pre-TCR with pTα, which is necessary for signal transduction to advance into the DP stage, which expresses TCR αβ (*double rectangle*), referred to as β-selection (with Dll4, the upper layer). Without Dll4 on TECs, HPCs do not differentiate into the T cell lineage; instead, they differentiate into the B cell lineage, even in the thymus (**a**). After, DN1/DN2 cells cannot develop any further without Dll4, resulting in differentiation arrest at an early stage (**b**, the *thin dotted line* with *arrowhead*). In contrast, pre-TCR-bearing DN3 cells, which are competent for further differentiation, efficiently develop in the DP stage without proper proliferation (**c**, *thin line* with *arrowhead*) in the absence of Dll4-expressing TECs (without Dll4, the lower layer)

1.4.2 Dll4 Is Required for Further Developmental Processes

After the migration of HPCs into the thymus, Dll4-mediated Notch signaling is still required for the further differentiation of T cell progenitors. The earliest progenitors (CD44-positive, CD117-high positive ($CD44^{+}CD117^{++}$) and CD4, CD8-double negative (DN) cells (DN1a/b and DN2mt (Porrit et al. 2004; Ikawa et al. 2010)) do not develop into the DP stage in the thymus with Dll4-deficient epithelium (Hirano et al. 2015). Similarly, Notch1- or Rbpj-deficient $CD44^{+}CD25^{+}$ DN cells (DN2t), obtained from Notch1- or Rbpj-floxed mice with the Lck-Cre transgene, do not efficiently undergo rearrangement of the *TCR β* gene and lose the potential for differentiation (Wolfer et al. 2002; Tanigaki et al. 2004). However, the introduction of the exogenous, rearranged *TCR β* gene does not rescue these defects (Maillard et al. 2006; Hirano et al. 2015), suggesting that there is an additional requirement other than the TCR β chain downstream of Notch signaling. These reports concluded that Notch signaling is absolutely necessary for their further development at the earlier stages before DN3, although the downstream target(s) remains to be determined (Fig. 1.2).

By contrast, $CD44^{-}CD25^{+}$ DN cells (DN3) differentiate in the DP stage, although the proliferation is significantly impaired during the differentiation process in a Dll4-deficient thymus (Hirano et al. 2015). This was consistent with previous reports showing that Notch1 and Rbpj are dispensable after the DN3 stage in the thymus (Wolfer et al. 2001; Tanigaki et al. 2004). However, the complete dependency of their differentiation on Notch signaling was suggested from findings observed in monolayer cultures (Ciofani and Zúñiga-Pflücker 2005; Wong et al. 2012; Liu et al. 2010, Kreslavsky et al. 2012). This discrepancy should be explained by the advantage of 3D thymic structures over the 2D cultures. Taken together, these results suggested that Notch signaling induced at the DN3 stage is simply required to maintain the competence of the pre-TCR signaling that triggers differentiation to the DP stage and is not necessary to promote differentiation after the pre-TCR signaling occurs. However, continuous Notch signaling during the DN3/DP transition is essential for efficient proliferation. These requirements of Notch signaling almost correspond to the substantial expression of Notch1 on the cell surface or its intracellular fragment before the DP stage (Fig. 1.2).

1.5 Notch Ligand in Secondary Lymphoid Organs

Although several reports have been published showing that Notch and its mediated signaling play a critical role in the functional regulation of mature T cells, it remained to be fully elucidated which ligand(s) contributes to the triggering of Notch signaling. Moreover, it was also unclear where or when this ligand functions. The following section evaluates studies in which the significance of NotchLs was relatively evident in secondary lymphoid organs (SLOs).

1.5.1 Spleen

The splenic marginal zone (MZ) is a unique site inhabited by a specialized B cell population called MZ B cells; it is exposed to blood flow and links to the capture and follicular delivery of systemic pathogens. Several recent reports have demonstrated that MZ B cells carry and pass antigens to follicular dendritic cells (FDCs) during their shuttling between the MZ and follicles; this process is governed by the Cxcl13/Cxcr5 and sphingosin-1-phospate (S1P)/S1P receptor (S1P1) chemoattractant systems (Cinamon et al. 2008; Arnon et al. 2013) (Fig. 1.3a). The specification of MZ B cell fate is obviously dependent on Dll1/Notch2-mediated signaling in the spleen (Tanigaki et al. 2002; Saito et al. 2003; Hozumi et al. 2004). However, it remains unknown which cells actually express Dll1 that drives MZ B cell development in the spleen. At first, the Dll1 transcript was detected in splenic B cells and DCs, but those were dispensable for the appearance of MZ B cells (Hozumi et al.

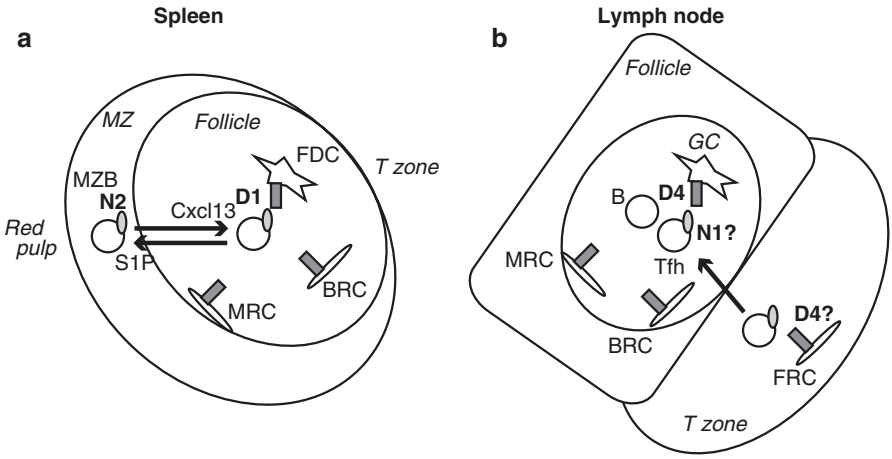

Fig. 1.3 Notch ligands in secondary lymphoid organs. (**a**) The splenic marginal zone (MZ) B cells (MZB) reside in the MZ and entrap systemic pathogens in the blood flow. Continued Notch signaling mediated by Dll1 and Notch2 is necessary to maintain MZ B cells, but Dll1 is detected on follicular dendritic cells (FDCs), B zone reticular cells (BRCs), and marginal reticular cells (MRCs) in the follicle, but not in the MZ of the spleen. To deliver the antigens that were captured in the MZ to the follicle, MZ B cells move to the follicle and pass the antigens to the FDCs; this process is dependent on the Cxcl13 chemokine. After, the MZ B cells return to the MZ; this process is dependent on the S1P chemoattractant (*thick arrows*). During their stay in the follicle, MZ B cells expressing Notch2 (N2) can encounter Dll1 and receive Notch signaling. (**b**) In the draining LNs, antigen-stimulated T cells differentiate into follicular helper T (Tfh) cells expressing Cxcr5 to enter the follicle and cooperate with naïve B cells to initiate the antibody response to T cell-dependent antigens, resulting in germinal center (GC) development. During these processes, Tfh cells must receive Notch signaling, which is mediated by Dll4 (D4), to support the production of antigen-specific high-affinity Abs. Dll4 is mainly observed on FDCs, MRCs, and BRCs in the follicle and is weakly observed on fibroblastic reticular cells (FRCs) in the T cell area (the size of "D4" is smaller than those in follicle), which binds to Notch1 (N1) or Notch2 on activated Tfh cells

2004). In addition, the expression on blood endothelial cells was also negligible (Fasnacht et al. 2014). Finally, the significance of Dll1 on a subset of splenic stromal (CD45$^-$CD31$^-$Podoplanin$^-$) cells expressing a Ccl19-Cre transgene, FDCs, and marginal and B zone reticular cells (MRCs and BRCs) in the follicle for the signal transduction via Notch2 on the immature B cells was revealed (Fasnacht et al. 2014). Thus, MZ B cells can encounter Dll1 and receive Notch signaling in the follicle during their shuttling to maintain their unique characteristics.

Similarly, the differentiation of a proportion of DCs in the spleen is also dependent on Notch2 and Dll1, particularly the Esam$^+$ population, which is critical for priming the CD4$^+$ T cells (Lewis et al. 2011; Fasnacht et al. 2014). As expected, Esam$^+$ DCs disappeared with the absence of Dll1 in the same stromal cells in which MZ B cells disappeared. In consequence, CD4$^+$ T cells did not efficiently divide after antigen stimulation in the spleen without Dll1. These results clearly indicate that Dll1 in the splenic follicle is required for the appearance of some DCs; however, it remains unknown how Esam$^+$ DCs encounter Dll1.

1.5.2 Lymph Node

The T cell-specific gene ablation of *Notch1* and *Notch2* impairs the differentiation of follicular helper T (Tfh) cells in draining lymph nodes (LNs) in mice immunized with T-dependent antigens, resulting in deficient germinal center development and the absence of antigen-specific high-affinity Abs (Auderset et al. 2013). To evaluate the significance of NotchL on blood cells including DCs for this phenomenon, a BM chimera was generated from the NotchL (Dll1, Dll4, Jag1, or Jag2)-deficient donor cells and immunized (Fasnacht et al. 2014). As a result, every NotchL on blood cells was dispensable for the production of T cell-dependent Abs, suggesting that NotchL is critical in the stromal population. Interestingly, Ccl19-Cre$^+$ stromal cells, primarily FDCs (CD45$^-$Podoplanin$^+$CD21/35$^+$CD31$^-$) and MRCs (CD45$^-$Pdn$^+$desmin$^+$) in the B cell zone, substantially expressed Dll4 in LNs after antigen stimulation, which was necessary for the differentiation of Tfh cells and the production of T cell-dependent Abs (Fasnacht et al. 2014). These results suggested that Tfh cells receive Notch signaling after they completely differentiate and arrive to the follicle, which is critical for the maintenance of Tfh cell characteristics. Alternatively, weak expression on fibroblastic reticular cells (FRCs) in the T cell area might occur during their differentiation to Tfh cells. Notably, Dll1 was also detected in LNs after antigen stimulation, which suggests that Dll1 could not compensate for Dll4 deficiency. Interestingly, Dll1 and Dll4 do not share the potential to trigger Notch signaling in LNs. Moreover, it is unknown whether Dll4 similarly functions in the spleen for GC development after the systemic administration of antigens. In that case, the question arises as to whether Dll1 expressed at the splenic follicle can compensate for the role of Dll4. These questions should be investigated to understand the molecular machinery of the preferential combination of Notch/NotchL in detail.

1.5.3 Antigen-Presenting Cells

Notch signaling is believed to upregulate the responsiveness of mature T cells, which is induced by NotchL on DCs as antigen-presenting cells (APCs). In particular, an attractive model has previously shown that Dll and Jag specifically induce Th1 and Th2, respectively (Amsen et al. 2004). Thereafter, it was conceived that Notch signaling positively regulates multiple $CD4^+$ helper T cells (Th1, Th2, Th17, and Tfh) (Maekawa et al. 2003; Tu et al. 2005; Bailis et al. 2013; Auderset et al. 2013) or $CD8^+$ cytotoxic T cells (Maekawa et al. 2008; Backer et al. 2014). However, there has been a lack of evidence for the importance of NotchL on DCs. Further loss-of-function experiments may be necessary to reach a clear conclusion regarding this issue.

Interestingly, Notch signaling was critical for the allogeneic $CD4^+$ T cell response to mediate graft-versus-host disease, and the blockade of both Dll1 and Dll4 or only Dll4 could improve overall survival (Zhang et al. 2011; Tran et al. 2013; Mochizuki et al. 2013). These results indicate the contribution of Dll molecules to generate Notch signaling, but it remains unclear whether the neutralizing Abs block the interaction of Notch/NotchL between T cells and inflammatory DCs (i-DC) that were shown to express Dll4 on the surface (Mochizuki et al. 2013). Moreover, memory $CD4^+$ T cells were maintained by Notch signaling via regulating glucose uptake, which was supported by Dll1 on the $CD11c^+$ DC population in the BM (Maekawa et al. 2015). This phenomenon was impaired, but not completely, in Dll1-floxed mice with the CD11c-driven Cre transgene, suggesting the redundancy of other NotchLs. A recent report indicated the substantial expression of Dll4 on half of the $CD11c^+$ splenocytes, which contributed to the antigen sensitivity of naïve $CD4^+$ T cells (Laky et al. 2015). This defect was only validated in the $CD4^+$ T cell-dependent antitumor response. Because NotchL on blood cells, including DCs, was dispensable for the production of T cell-dependent Abs as described above, NotchL on stromal cells in SLO likely played a critical role in the regulation of other T cell responses.

1.6 Concluding Remarks

Previous studies suggested that Notch signaling is involved in the choice of cell lineages, specifically CD4 helper vs CD8 killer (Robey et al. 1996) and TCR αβ vs γδ T cells (Washburn et al. 1997); these studies first described the possible contribution of the Notch system to the immunological field through gain-of-function experiments. Afterward, it has been revealed that Notch signaling generally supports differentiation and proliferation before the DP stage for T cell development in the thymus. In addition, recent studies have shown that it similarly contributes to the maintenance of functional helper and cytotoxic T cells. Interestingly, the stability of the regulatory T cell lineage appears to be sustained without Notch signaling (Charbonnier et al. 2015), suggesting a critical role of Notch signaling for the

negative regulation of Treg cell function. Because Treg cells are composed of, at least, heterogeneous FoxP3$^+$ subpopulations that share their defined characteristics with each helper T cell lineage (Cretney et al. 2013), Notch signaling may contribute to the regulation of helper and regulatory functions as well as a decision of cell fate in developing states.

However, the physiological significance of Notch signaling should be confirmed in vivo by loss-of-function experiments, particularly for NotchL. Again, these approaches will be able to reveal when or where Notch signaling occurs and regulates the T cell function and provide a better understanding of Notch signaling in lymphoid tissues and in immunological responses.

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Chapter 2
Notch Controls the Differentiation and Function of Cytotoxic CD8 T Cells

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Abstract CD8 positive T cells (CD8 T cells) are immune cells that are crucial in controlling viral infections and the eradication of tumor cells by processes that are primarily dependent on their cytotoxic activities. To exert their effects and due to the consequent strong cytotoxicity, the activation and differentiation of naive CD8 T cells to cytotoxic T lymphocytes (CTLs) are precisely regulated during immune responses. CD8 T cells are primed by antigen-presenting dendritic cells in the presence of permission for activation/differentiation to CTLs. Recent studies have unveiled that Notch signaling gives a license to CD8 T cells to fully activate and become effector cells during priming. In this review, we discuss the recent progresses in the study of the regulation of the activation and function of CD8 T cells by Notch signaling.

Keywords CTL • Cytotoxic molecules • Terminal effector cells (TECs) • Memory precursor cells (MPCs) • CD4/CD8 lineage choice • Tumor immunity • Intracellular infection • Immunosurveillance

2.1 Introduction

CD8 T cells are activated by the interaction of their T cell receptor (TCR) with class I MHC-peptide complexes. As most of all peptides in class I MHC-peptide complexes are derived from intracellular proteins, CD8 T cells mainly sense

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K. Yasutomo (ed.), *Notch Signaling*, DOI 10.1007/978-981-10-4971-2_2

intracellular abnormalities such as viral infections and tumorigenic processes. When CD8 T cells encounter such nonself peptides on class I MHC molecules, they become cytotoxic T lymphocytes in order to eliminate such potentially harmful cells via delivery of cytotoxic molecules, perforin, and granzymes to the target cells (Glimcher et al. 2004; Williams and Bevan 2007). There are several reports showing that Notch signaling might contribute to CD4/CD8 lineage choice during T cell development in the thymus. In contrast, the differentiation and function of peripheral CD8 T cells are completely regulated by Notch signaling in concert with other signaling pathways. It is also expected that intensive investigations focusing on Notch signaling in CTLs will facilitate the development of clinical applications for several disorders.

2.2 Notch Signaling in CD4/CD8 Lineage Choice in Developing T Cells

As Notch signaling generally controls cell lineage and differentiation, many studies have been focused on Notch signaling in CD4/CD8 lineage choice of developing T cells in the thymus. Robey et al. first reported in 1996 that Notch signaling influences the CD4/CD8 lineage decision through studies on NICD transgenic mice (Robey et al. 1996). They showed that an activated form of Notch in thymocytes increased CD8 lineage T cells and decreased CD4 lineage T cells. This increase of CD8 lineage T cells by activated Notch was observed even in the absence of class I MHC. However, the increase of CD8 lineage T cells was not seen when both class I and II MHC were deficient, suggesting that enforced Notch signaling led to differentiation toward CD8 T cell lineage in T cells bearing class II MHC-restricted TCR. Complementary experiments using γ-secretase inhibitors (GSI) reported that low concentrations of the inhibitor or using less potent inhibitors impaired development of CD8 single-positive T cells but not CD4 single-positive T cells in the thymus (Doerfler et al. 2001; Hadland et al. 2001). In 2002, Yasutomo et al. reported that Notch signaling did not appear to be essential for CD4/CD8 fate determination, but was selectively required for CD8 T cell maturation after commitment to CD8 lineage T cells (Yasutomo et al. 2000). In contrary to the in vitro experiments or gain-of-function experiments (Doerfler et al. 2001; Hadland et al. 2001; Fowlkes and Robey 2002), there is no definitive evidence that Notch signaling controls the CD4/CD8 lineage determination of developing T cells in the thymus using the gene-modified mice with loss of function for Notch signaling (Wolfer et al. 2001; Tanigaki et al. 2004). The inconclusive status of Notch signaling involvement in CD4/CD8 lineage choice is partially due to experimental limitations. As Notch signaling is involved in several steps during T cell development, there is currently no suitable gene deletion system that is focused only on the CD4/CD8 lineage decision. Nevertheless, while it is certain that Notch signaling is critical in both the determination of early thymic progenitors to T cell lineage and

the survival of pre-T cells at the β-selection checkpoint (Pui et al. 1999; Radtke et al. 1999; Ciofani and Zuniga-Pflucker 2005), more sophisticated experimental systems are necessary to elucidate the precise contribution of Notch signaling in CD4/CD8 lineage choice.

2.3 Notch Signaling in Controlling T Cell Cytotoxicity

Upon antigen-specific activation of CD8 T cells, these cells acquire the ability to kill the infected cells or tumor. While activation of such processes effectively eliminates the target cells, these processes must be strictly regulated due to their cytotoxic actions. The immune system has several safeguard mechanisms for naive CD8 T cells to become cytotoxic effector cells. To become effector cells, CD8 T cells require co-stimulatory signals such as CD28, IL-2, and other signals in addition to TCR signal (McAdam et al. 1998; Mescher et al. 2007). However, recent studies have shown that such co-stimulatory signals are not sufficient for CD8 T cells to exert effector functions. Barbara Osborne and colleagues were the first to report Notch signaling involvement in peripheral CD8 T cells (Palaga et al. 2003). The investigators examined the effects of Notch inhibitor and downregulation of Notch1 on the proliferation and IFN-γ production of peripheral T cells. They showed that suppression of Notch activity affected the proliferative response and IFN-γ production of both CD4 and CD8 T cells. However, the authors did not address the involvement of Notch signaling in the cytotoxic function of CD8 T cells. Subsequently, our group first reported that Notch signaling was critical for the acquisition of cytotoxic function by CD8 T cells (Maekawa et al. 2008). Of four Notch receptors, Notch2 is expressed on naive CD8 T cells, and Notch1 is induced by TCR stimulation. In the priming of CD8 T cells, mature dendritic cells express Notch ligands to stimulate Notch2 and Notch1 on CD8 T cells. In general, the ligation of Notch with its ligand stimulates release of the Notch intracellular domain (NICD) through the cleavage by γ-secretase, followed by NICD translocation to the nucleus, and consequent formation of a transcriptional complex with RBPj and Mastermind-like (Maml). In the context of CTL differentiation, Notch signaling integrates another signal molecule, phospho-CREB, to activate the transcription of granzyme B, one of the cytotoxic molecules in the cytolytic granules (Fig. 2.1). RBPj-binding elements and cAMP-responsive elements are present in both the human and mouse promoters for granzyme B, suggesting that the transcriptional regulation of granzyme B is fundamentally regulated by Notch signaling. Kuijk et al. reported that Notch controls the generation and function of human effector CD8 T cells (Kuijk et al. 2013). Notch-induced granzyme B expression might be independent of eomesodermin (EOMES), a T-box transcription factor. Cho et al. also reported that Notch1 regulates CTL function by inducing the expression of CTL-related genes such as EOMES, perforin, and granzyme B through direct binding to their promoter regions (Cho et al. 2009). This report and ours suggest that Notch signaling directly controls CTL function independent of EOMES. Natural killer cells (NK cells) are another immune cell type that exerts cytotoxic function similar to

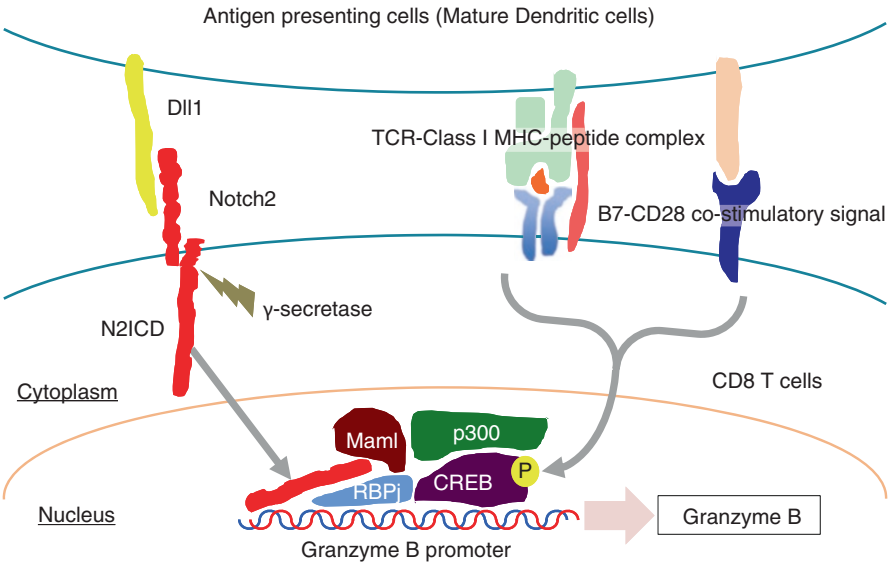

Fig. 2.1 Notch2 integrates signaling by forming a transcription complex with RBPj and phospho-CREB to promote T cell cytotoxicity. Ligation of Notch2 on CD8 T cells with Dll1 on dendritic cells stimulates release of the intracellular domain of Notch2 (N2ICD) to nucleus, followed by NICD translocation to the nucleus, and consequent formation of a transcriptional complex with RBPj and phospho-CREB, to activate the transcription of granzyme B, one of the cytotoxic molecules in the cytolytic granules. RBPj-binding elements and cAMP-responsive elements are present in both the human and mouse promoters for granzyme B

CTLs. Our group reported that the cytotoxic activity of NK cells are also controlled via Notch signaling through interaction of Notch on NK cells with Jagged2 on DCs (Kijima et al. 2008). In contrast, Jagged1, another Jagged family ligand in mammals, has been shown to suppress collagen-induced arthritis by indirectly providing a negative signal in CD8 T cells (Kijima et al. 2009). In CTLs, the Delta-like ligand family mainly induces cytotoxic functions, while NK cells require association with the Jagged ligand family. Generally, the cytotoxic functions in both CTLs and NK cells are mediated by a common mechanism involving Notch signaling, although difference between the two cell types requires further detailed investigations.

2.4 Notch Signaling in the Choice of CD8 T Cells to Effector or Memory

Majority of activated CD8 T cells become terminal effector cells (TECs), whereas a small proportion becomes memory precursor cells (MPCs) (Kaech and Cui 2012). Regulation of this cell fate choice remains to be elucidated. Recently, Derk Amsen and colleagues reported that Notch signaling promotes TEC differentiation but not

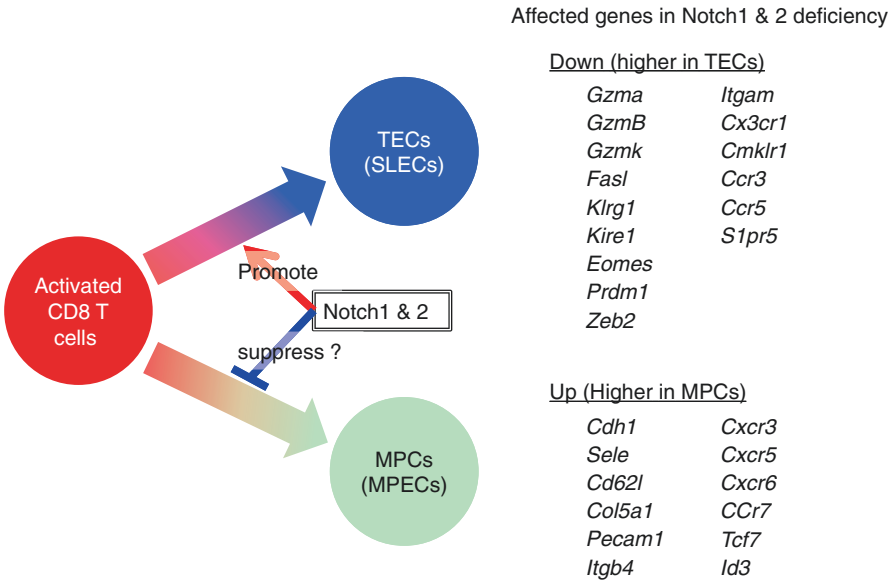

Fig. 2.2 Notch controls activated CD8 T cell fate to become terminal effector cells. Activated CD8 T cells become terminal effector cells (TECs) or memory precursor cells (MPCs). Notch1 and 2 on CD8 T cells are involved in this cell fate choice. Notch signaling promotes TEC differentiation and may suppress MPC differentiation. Several TEC-specific genes are downregulated, and MPC-specific genes are upregulated in Notch1 and 2 deficiency in CD8 T cells

MPC induction (Backer et al. 2014) (Fig. 2.2). Even in the absence of Notch signaling, antigen-specific CD8 T cells could undergo population expansion that was equivalent to WT cells. However, the population of antigen-activated KLRG1^{+}CD127^{-}CD8 T cells (designated as TECs), which express cytotoxic effector molecules, was almost absent in the deficiency of Notch1 and 2 (Notch1-2) in CD8 T cells. In contrast, KLRG1^{-}CD127^{+}CD8 T cells, which are designated as MPCs, increased in parallel under the same conditions. In accordance with these proportional changes in activated CD8 T cells, mice with this T cell-specific Notch1-2 deficiency showed a reduced ability to control an influenza virus infection. The authors further investigated the transcriptional control of TEC/MPC-related genes by Notch signaling. When the global gene expression profile of Notch1-2-deficient effector CD8 T cells was compared with that of WT cells, more than 40% of the TEC-specific transcriptome was lower in Notch1-2-deficient CD8 T cells compared to WT cells. Instead, around 40% of MPC-specific genes showed a higher expression level in Notch1-2-deficient CD8 T cells compared to WT cells. The authors thus concluded that Notch signaling is involved in the commitment of activated CD8 T cells to TECs or MPCs and that activation of Notch signaling commits the activated CD8 T cells to the TEC lineage. In addition, the authors also investigated the relationship of Notch signaling with factors/pathways established to be involved in the control of TEC differentiation. They showed that Akt/mTOR pathway is impaired in activated

Notch1-2-deficient CD8 T cells, resulting in the reduction of the KLRG1^{+} cell population. Conversely, the introduction of active Akt rescues this reduction in Notch1-2-deficient CD8 T cells. Several reports, including ours, showed a strong relation between the Akt/mTOR pathway and Notch signaling, but the precise mechanism underlying the relation is still unknown. Further investigation is required to clarify this issue. Labrecque and colleagues also reported that Notch signaling controls the generation of short-lived effector CD8 T cells (SLECs) but is dispensable for memory precursor effector CD8 T cells (MPECs) (Mathieu et al. 2015). In this study, mature CD8 T cell-specific Notch1-2-deficient mice were used to examine the role of Notch1 and 2 in CD8 T cells in a *Listeria* infection model. In this system using E8I-Cre mice, the influence of gene deficiency is circumvented in CD8 T cell development and mature CD4 T cell function (Maekawa et al. 2008). The authors showed that Notch1-2-deficient CD8 T cells expand more than their wild-type counterparts when activated with Ag. Examination of the activated CD8 T cell phenotype revealed that Notch1-2-deficient CD8 T cells show a twofold reduction in the proportion of SLECs but did not reflect a direct reciprocal increase of MPECs. This reduction of SLECs was due to a defect in the transition from early effector cells (KLRG1^{low} and CD127^{low}) to SLECs in Notch1-2-deficient CD8 T cells. Consequently, the authors concluded that Notch signaling in CD8 T cells was dispensable for the generation of long-lived memory cells despite lower EOMES expression in Notch1-2-deficient effector CD8 T cells. However, maintenance of memory CD4 T cells has been reported to be dependent on Notch signaling (Maekawa et al. 2015); therefore the role of Notch signaling in memory CD8 T cells needs to be examined in further detail.

As mentioned above, several studies have reported that Notch signaling is associated with the important transcription factors important for CTL differentiation and function, T-bet and EOMES. Amsen's group reported a reduced expression of T-bet in the absence of Notch1-2 in CD8 T cells (Backer et al. 2014). Based on two complementary experiments, they showed that T-bet acts downstream of Notch signaling. T-bet is also important in the upregulation of Notch expression on naive CD8 T cells, indicating that T-bet plays an important role in TEC differentiation by generating a feedback loop with Notch signaling. Meanwhile, Labrecque's group showed that T-bet (Tbx21) expression is reduced at the transcript but not the protein level in Notch1-2-deficient effector CD8 T cells (Mathieu et al. 2015), which suggests that Notch influences SLEC generation via mechanisms independent of T-bet and Blimp-1. This discrepancy in T-bet dependency may be context dependent, where inflammation extent, site, and timing of activation of CD8 T cells may be a factor.

Nonetheless, Notch signaling is likely to preferentially influence activated CD8 T cells to differentiate into effector CTLs. However, how activated CD8 T cells are governed into becoming memory cells is still unknown. Do CD8 T cells activated by Notch ligand negative DCs become memory cells? If so, when, where, and how do such DCs work to stimulate CD8 T cells? Elucidating the answer to these questions is critical to resolving the central problem in immunology.

2.5 Notch Signaling as a Therapeutic Target for Clinical Disorders

CTLs are involved in many clinical disorders, in which CTL modification is thought to be potentially effective, including infection control, tumor rejection, and also transplantation and autoimmune reactions. Notch signaling is considered a potential candidate therapeutic target for the modification of CTL function and/or differentiation. Notch signaling in CD8 T cells is critical to overcome infections in several infection models. *Trypanosoma cruzi* (*T. cruzi*) is an obligate intracellular protozoan parasite, which invades and replicates in several host cell types that do not have inducible antimicrobial activity unlike phagocytic cells. CD8 T cells are the key cells in host immunity needed to eliminate such intracellular parasites in somatic cells. In general, the C57BL/6 mouse strain is relatively resistant to *T. cruzi* infection, whereas mice deficient for Notch2 in peripheral CD8 T cells showed increased susceptibility to the infection with early mortality, indicating that Notch2 in CD8 T cells is indispensable for CTL differentiation and function for infection control (Maekawa et al. 2008). The importance of Notch signaling in CD8 T cells is also shown in an influenza virus infection model (Backer et al. 2014). The authors showed that viral clearance and weight recovery were compromised in mice with Notch1-2 deficiency in CD8 T cells. Moreover, CD8 T cell-specific deletion of Notch1 and 2 genes attenuated immunity to *Listeria monocytogenes* infection, an intracellular bacterium (Mathieu et al. 2015). These reports indicate that Notch signaling, in particular Notch2, in CD8 T cells is crucial for controlling intracellular pathogen infections.

Tumor eradication is a goal in modern medicine that has been the focus of concentrated efforts. One way of tumor control is through enhancement of antitumor immunity. We have shown that Notch signaling is a promising candidate of antitumor immunity enhancement in a tumor-bearing mouse model (Sugimoto et al. 2010). Similar to *T. cruzi* infection in Notch2-deficient mice, these mice but not Notch1-deficient mice have a lower antitumor response to inoculated EG7 cells, indicating that Notch2 is also critical for eradicating tumors. When an agonistic antibody for Notch2 was inoculated into EG7-bearing mice, tumor growth was profoundly suppressed, and mouse survival was prolonged. Another study has also reported that in vivo administration of an agonistic antibody for Notch2 in combination with cytokines such as Flt3L and IL-7 resulted in expansion of antigen-specific CD8 T cells (Haque et al. 2016). These two reports suggest that Notch2 stimulation may be capable of enhancing antitumor immunity in human cancers. Besides using an anti-Notch2 antibody, Biktasova et al. have reportedly developed another agonist for Notch signaling that can evoke strong antitumor immunity (Biktasova et al. 2015). They showed that systemic administration of Dll1-Fc-based multivalent agonist (multivalent Dll1) increased T cell infiltration into tumors and elevated the number of memory CD8 T cells. They also found that the combined treatment of multivalent Dll1 with the EGFR-targeted drug, erlotinib, significantly improved mouse survival without progression by inducing robust tumor-specific T cell immunity. Multivalent Dll1 induced proliferation of human peripheral T cells, but

lacked proliferative or clonogenic effects on lung cancer cells in vitro, suggesting that the target of multivalent Dll1 in vivo might be Notch receptors on T cells.

The rejection of transplanted tissue or organ is dependent on the action of T cell population in the recipient (Priyadharshini et al. 2012). Among T cell populations involved in tissue or organ rejection, effector CD8 T cells largely participate in allograft rejection, while an increase in memory like CD8 T cells is associated with long-term dysfunction of allografted organs (Betjes et al. 2012; Yamada et al. 2012; Yap et al. 2015). Therefore, Notch signaling blockade in CD8 T cells might prevent allograft rejection and be a successful therapy for allogeneic solid tissue transplantation. In the early days of research for Notch signaling in peripheral immunity, one interesting study from Dallman's group has reported that Notch ligation by Dll1 inhibits peripheral immune responses in a CD8 T cell-dependent manner (Wong et al. 2003). They showed that an enforced expression of Dll1 on allogeneic tumor cells induces tolerance to alloantigens with decreased IFN-γ and a concomitant enhancement of IL-10 production in CD8 T cells when these cells are transplanted to a MHC-mismatched recipient. These findings contradict other reports in which Notch signaling in CD8 T cells orchestrates their cytotoxic functions including IFN-γ expression, but this discrepancy might be attributed to differences in Dll1-expressing cells. CD8 T cells usually receive Notch receptor signaling through contact with antigen-presenting cells like DCs during priming, whereby CD8 T cells acquire their cytotoxic functions. Mature DCs in T cell priming highly express co-stimulatory molecules such as CD80 and CD86 that are necessary for full activation and survival of T cells, whereas somatic cells that are usually without co-stimulatory molecule expression or even dendritic cells without CD80 and CD86 expression induce unresponsiveness in T cells with clonal anergy (St. Louis et al. 1993; Gimmi et al. 1993; Fu et al. 1996). It is possible that Notch ligation in interaction of CD8 T cells with Dll1-expressing allogeneic tumor cells without co-stimulation strongly induces T cell unresponsiveness. Graft-versus-host disease (GVHD) induced by donor-derived T cells has still limited allogeneic bone marrow transplantation. Ivan Maillard and colleagues reported that T cell-specific Notch inhibition blocks GVHD by inducing a hyporesponsive program in alloreactive CD4 and CD8 T cells (Sandy et al. 2013). They showed that a dominant negative form of Maml1 (DNMAML) in alloreactive CD8 T cells could block GVHD. These Notch-deprived CD8 T cells preserved their expansion in lymphoid organs of recipients, but profoundly decreased IFN-γ production. They have also shown that DNMAML CD8 T cells maintained their cytotoxic function, suggesting that Notch signaling might differentially regulate each function of CD8 T cells in an immune context-dependent manner.

This paragraph discusses the role of Notch signaling in hyperimmune reactions such as allergy and autoimmunity. Okamoto et al. reported the essential role of Notch signaling in effector memory CD8 T cell-mediated airway hyperresponsiveness (AHR) and airway inflammation (Okamoto et al. 2008). Using a murine asthma model in which adoptive transfer of effector CD8 T cells restores AHR and airway inflammation in CD8-deficient mice, they showed that treatment of effector CD8 T cells with GSI before transfer failed to restore AHR and airway inflammation. They also found that GSI treatment increased the expression of Dll1 on effector CD8 T

cells, leading the authors to speculate that upregulated Dll1 on effector CD8 T cells skewed CD4 T cells from disease-promoting Th2 to disease-ameliorating Th1 phenotype, which could have been another reason for disease attenuation. This study provided evidence for the functional role of Notch signaling in the challenge phase of CD8 T cells in allergic airway disease. Although the expression of several Notch ligands was observed on T cells including CD8 T cells, their function was not totally understood. As mentioned above, amelioration of rheumatoid arthritis was seen in a murine model by injection of plasmid encoding soluble Jagged1, resulting in inhibition of autoreactive CD8 T cell proliferation. Based on these studies, Notch signaling would be a potential target for treating hyperimmune disorders.

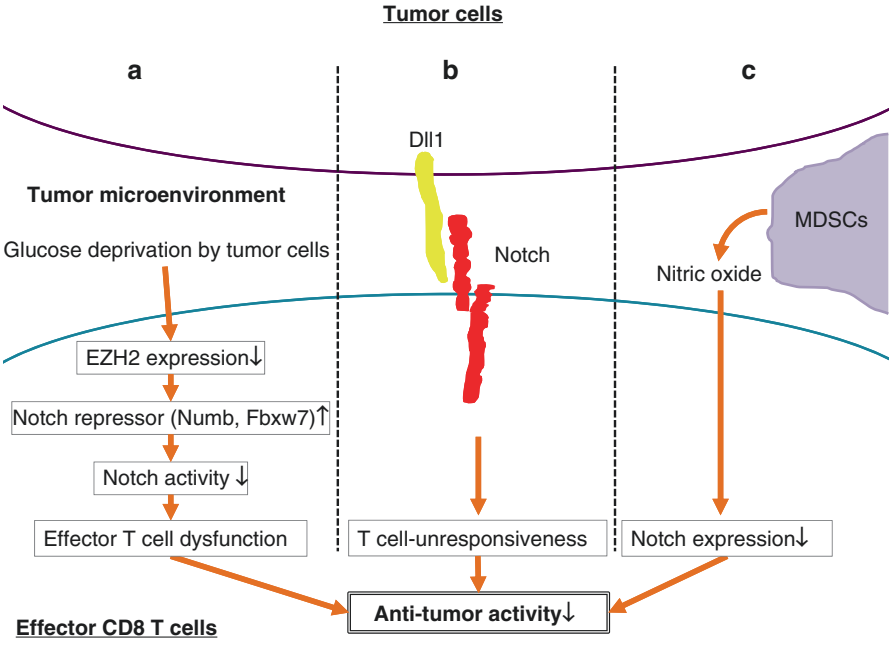

Fig. 2.3 Tumor cells suppress Notch signaling in CD8 T cells to escape immunosurveillance. (**a**) Glucose deprivation by tumor cells in the tumor microenvironment constrains the expression of methyltransferase EZH2 in tumor-infiltrating CD8 T cells, resulting in induction of the repressor genes for Notch, Numb, and Fbxw7. Increased Notch repressors cause Notch signaling to be dampened. Consequently, CTLs lose their polyfunctionality. (**b**) Notch ligation of effector CD8 T cells by Dll1-expressing allogeneic tumor cells mediates alloantigen-specific CD8 T cell unresponsiveness. (**c**) Myeloid-derived suppressor cells (MDSCs) often infiltrate into the tumor. MDSCs prevent expression of full-length and cleaved Notch 1 and h 2 in T cells in a MDSC-derived nitric oxide-dependent manner. Decreased Notch in tumor-infiltrating CD8 T cells impairs IFN-γ production important for antitumor immunity

2.6 Tumor Cells Suppress Notch Signaling in CD8 T Cells to Escape Immunosurveillance

The mechanism of immune evasion for certain tumor cells appears to target Notch signaling in CD8 T cells (Fig. 2.3). Weiping Zou and colleagues reported that ovarian cancers in human and B16 melanoma cells in mouse lung metastasis diminish polyfunctionality of tumor-infiltrating lymphocytes (TILs) including CD4 and CD8 T cells by inhibiting Notch signaling (Zhao et al. 2016). Tumor cells primarily depend on glycolysis for energy production. This Warburg effect of tumor cells consumes a large amount of glucose in the tumor microenvironment, resulting in the dampening of TIL function. This is due to both deprivation of energy source for TILs and inhibition of the CTL regulator, Notch signaling. In the glucose-restricted environment, the expression of methyltransferase EZH2 is constrained in TILs. As EZH2 activates Notch signaling pathway by suppressing the repressor genes for Notch, Numb, and Fbxw7, the decreased expression of EZH2 leads to an increased expression of Notch repressors, causing Notch signaling to be dampened. As Notch signaling is crucial for their cytotoxic functions, CTLs in the tumor microenvironment lose their polyfunctionality to eliminate the tumor cells. Sierra et al. have also reported Notch signaling inhibition in the tumor microenvironment (Sierra et al. 2014). Myeloid-derived suppressor cells (MDSCs) often infiltrate into the tumor to suppress antitumor immunity. The expression of full-length and cleaved Notch 1 and 2 was prevented in T cells cocultured with MDSCs, which consequently led to reduce IFN-γ production. This prevention was dependent on MDSC-derived nitric oxide and could be overcome by an activated form of Notch1. Thus, in addition to the immune checkpoint pathways such as PD-1 and CLTA4, Notch signaling is also a target pathway for immunosurveillance escape by tumor cells. In contrast, one report provided evidence that Notch signaling induces PD-1 expression on CD8 T cells by direct binding of NICD-RBPj complex to PD-1 promoter (Mathieu et al. 2013). If Notch signaling in effector CD8 T cells is dampened in the tumor microenvironment, the downregulation of PD-1 would lead to de-repression of tumor immunity in a similar manner to immune checkpoint blockade. Further investigation will be needed to clarify the precise mechanism regulating this subtle balance between repression and de-repression of tumor immunity by Notch signaling suppression in CD8 T cells. As mentioned, it has been reported that Notch ligation by allogeneic tumor cells with enforced Dll1 expression mediated the unresponsiveness of alloantigen-specific CD8 T cells, indicating that the tumor cells might express Notch ligands to induce tumor-specific CD8 T cell unresponsiveness via stimulation of Notch signaling in CD8 T cells. Indeed, several tumor cells have been reported to express Notch ligands (Hu et al. 2011; Li et al. 2013; Purow et al. 2005). Therefore, in addition to sending the survival signals to tumor cells, Notch ligands on tumor cells may suppress antitumor immunity by inducing Notch signaling-dependent unresponsiveness of tumor-infiltrating CD8 T cells.

2.7 Concluding Remarks

It has been established that Notch signaling in CD8 T cells plays a crucial role in promoting CTL function and differentiation toward short-lived effector cells, while it is unlikely that induction of memory CD8 T cells is Notch dependent. However, it is still unknown whether differences exist in the usage of Notch ligands for regulating the activation, differentiation, and effector functions of CD8 T cells. Moreover, it is also unclear whether Notch signaling is necessary for maintenance and reactivation of memory CD8 T cells. Many questions still exist regarding the biology of CD8 T cells. We believe that these questions can be answered through the investigation of the roles of Notch signaling in CD8 T cells. In this article, we also highlight the potential of Notch signaling in CD8 T cells as a therapeutic target for several diseases. In particular, based on the down-modulation of Notch signaling by tumors, we think that it is important to enhance the activity of CD8 T cells by Notch stimulation and to upregulate the Notch signaling system in tumor-infiltrating CD8 T cells for tumor treatment. As Notch signaling is ubiquitous for many important biological processes, it is important to consider high tropism to the target cells for activation or inhibition of Notch signaling in disease treatment.

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Chapter 3
Notch and Myeloid Cells

Chieko Ishifune and Koji Yasutomo

Abstract The myeloid cell population includes mononuclear phagocytes such as dendritic cells, macrophages, and monocytes. They have roles in phagocytosis, digestion, and presentation of antigens through the use of microbe pattern recognition receptors and secreting effector molecules such as cytokines and chemokines. Notch signaling strictly controls specific subsets in different tissues and developmental stages in both mice and humans. Here, we describe recent reports of Notch regulation in mononuclear phagocytes.

Keywords Dendritic cells • Macrophages • Monocytes • Differentiation • Function • M1 • M2 • Polarization

3.1 Introduction

Monocytes, macrophages, and dendritic cells are derived from macrophage-dendritic cell progenitors (MDP) and categorized as mononuclear phagocytes (Fig. 3.1). In some cases, inflammatory conditions or some tissue macrophages/monocytes became precursors of dendritic cells and macrophages develop from monocytes. It was reported that Notch signaling directly or indirectly controls the development of specific subsets and activation states (M1 or M2 macrophages) (Fig. 3.2). This article describes the function of monocytes, dendritic cells, and macrophages and the role of Notch signaling based on the current literature.

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K. Yasutomo (ed.), *Notch Signaling*, DOI 10.1007/978-981-10-4971-2_3

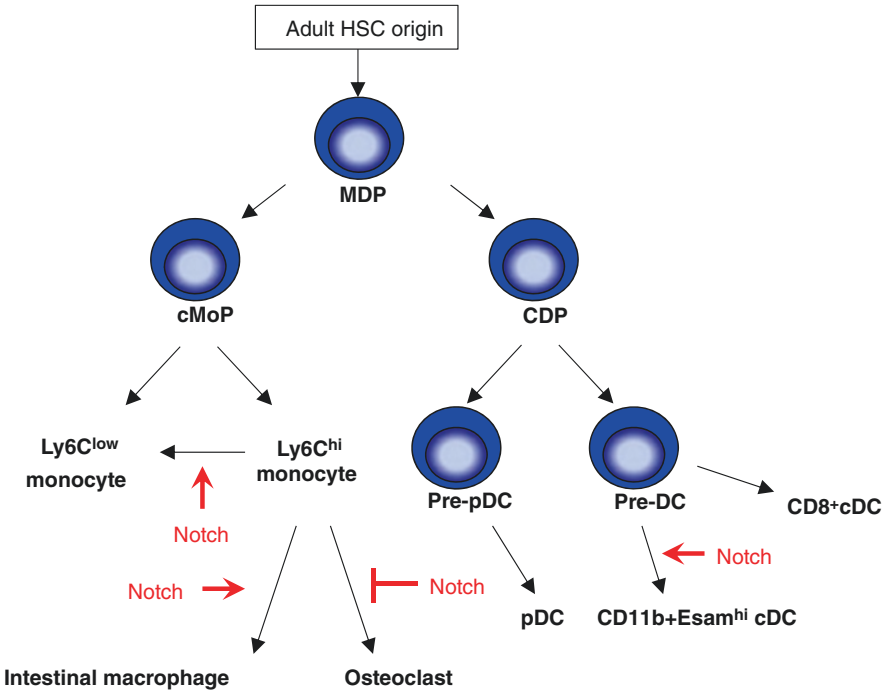

Fig. 3.1 Notch signaling regulates the development of specific subsets of mononuclear phagocytes. Notch promotes the conversion of monocytes from Ly-6C^{hi} to Ly-6C^{low} and the development of intestinal macrophages and CD11b^{+}Esam^{hi} DC. Notch inhibits osteoclast differentiation. *HSC* hematopoietic stem cell, *MDP* macrophage-dendritic progenitor cells progenitor, *CDP* common dendritic precursor, ore-pDC, *pDC* plasmacytoid dendritic cell

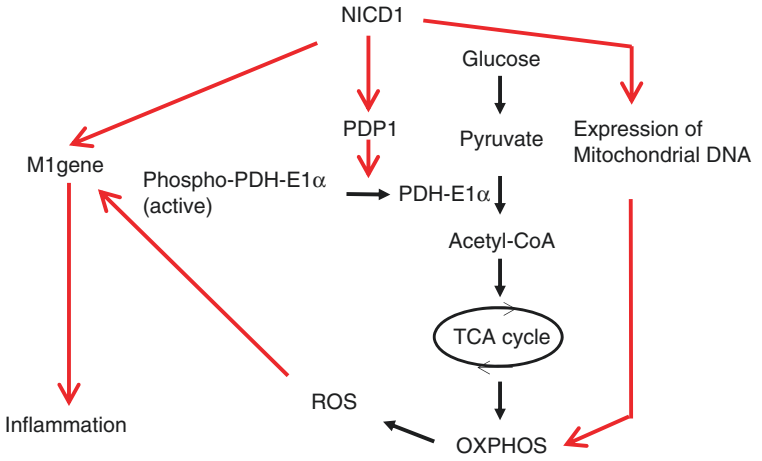

Fig. 3.2 Notch signaling regulates the polarization of M1 macrophage through three mechanisms. NICD1 directly induces M1 gene expression. NICD1 regulates PDP1 expression, which dephosphorylates PDH-E1α and drives the TCA cycle. NICD1 also modulates mitochondrial DNA transcription, promoting oxidative phosphorylation and generation of ROS. ROS also promotes M1 gene expression

3.2 The Ontology of Dendritic Cells and Their Differentiation Is Regulated by Notch Signaling

Mouse dendritic cells (DCs) are divided into three major subsets: classical DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (MoDCs). Each is defined by its specific phenotype, precursor, function, and developmental pathway. Since macrophages and DCs share similar cell surface markers and functions, it is difficult to distinguish DCs and macrophages. Recently, it was suggested that it is possible to identify the cell type by its ontogeny in addition to function and cell surface markers (Guilliams et al. 2014). Both cDCs and pDCs are derived from a common DC precursor (CDP) in an Flt3-dependent manner (Guilliams et al. 2014; Mildner and Jung 2014). CDPs branch further into at least two populations (E2-2$^+$ and Zbtb46$^+$) that become pDCs or cDCs, respectively (Onai et al. 2013; Satpathy et al. 2012; Meredith et al. 2012). It was revealed that E2-2 is an essential transcription factor for the development of pDCs in humans and mice (Cisse et al. 2008). In contrast, not only MoDCs but also monocyte-derived macrophages (MoMF) and some tissue-resident macrophages come from the Ly6C^{hi} monocyte, cells that are derived from a common monocyte precursor (cMoP) in a chemokine receptor, colony-stimulating factor 1 (CSF-1)-dependent manner (Guilliams et al. 2014; Mildner and Jung 2014; Ginhoux and Jung 2014).

The cDCs are identified by their expression of CD11c and MHC class II antigens (Caton et al. 2007). The cDCs can be divided into CD11b$^+$ DCs and CD8α$^+$ DCs (or XCR1$^+$ DCs) (Mildner and Jung 2014; Caton et al. 2007; Schlitzer and Ginhoux 2014; Dalod et al. 2014). In non-lymphoid tissue, CD103$^+$CD11b$^-$ DCs are equivalent to CD8$^+$ DCs in lymphoid tissue and are defined by transcription factors and their expression of chemokine receptor XCR1. The essential transcription factors of CD8α$^+$ DCs and CD103$^+$ CD11b$^-$ DCs are inhibitors of DNA binding 2 (Id2), basic leucine zipper ATF-like 3 transcription factor (Batf3), nuclear factor interleukin 3 regulated (NFIL3), and interferon regulatory factor 8 (IRF8). Defects in each transcription factor lead to severe developmental defects in CD8a$^+$ DC and CD103$^+$ CD11b$^-$ DC (Mildner and Jung 2014).

Most studies show that Notch positively regulates DC development. Delta-like 1 (Dll1) stimuli and GM-CSF promote DC differentiation from bone marrow (BM) cells (Mizutani et al. 2000). Similar effects on DCs were identified in human DC development from monocytes (Ohishi et al. 2001). Dll1 promotes DC development and maturation from human peripheral blood monocytes and CD34$^+$ cell-derived macrophage/DC progenitors. In contrast, macrophage development from those cells was inhibited by Dll1 stimulation. Furthermore, in mice, Notch-related gene modification indicates Notch signaling is important for the development of DCs. DC differentiation in Notch1 antisense transgenic mice was significantly impaired (Cheng et al. 2001; Cheng et al. 2003). Embryonic stem cells from Notch1^{null} mice displayed a reduced ability to develop into DCs (Cheng et al. 2003). As for the ligand, it was reported that Dll1 and Jag1 have opposite effects on DC development (Cheng et al. 2007; Liu et al. 2013). Jagged1 stimulates the accumulation of DC precursors but prevents terminal differentiation of DCs. Dll1 induces generation of DCs. This effect was caused by the expression of a Notch target and a component of

Wnt signaling. Dll1 strongly induces *Hes1* and *Deltex1* (Cheng et al. 2007). The time-dependent expression patterns of *Hes1* and *Deltex1* in vitro are different (Cheng et al. 2007). Furthermore, Dll1 activates Wnt signaling, but Jagged1 suppresses it through inhibition of Frizzled (Liu et al. 2013). It was reported that the Wnt signaling is downstream of the Notch signal to positively regulate DC development (Zhou et al. 2009).

In the spleen, $CD11b^+$ DCs are twice as numerous as $CD8^+$ DC (Caton et al. 2007). $CD11b^+$ DCs are subdivided by the expression of CD4 and endothelial cell-specific adhesion molecule (ESAM) (Mildner and Jung 2014; Lewis et al. 2011). However, it was reported that interferon regulatory factor 4 (IRF4), avian reticuloendotheliosis viral oncogene related B (Relb), and lymphotoxin B receptor (Ltbr) are important for the differentiation of $CD11b^+$ DCs. If any are defective, there may be a partial or tissue-specific reduction of the $CD11b^+$ DC population. Taken together, $CD11b^+$ DCs are likely a heterogeneous population. Notch signaling regulates $CD11b^+$ DCs in the spleen and the lamina propria (LP) of the small intestine (Caton et al. 2007; Lewis et al. 2011).

The murine *Rbpj* gene codes for recombining binding protein suppressor of hairless, which interacts with Notch. In CD11c-*Cre*-specific $Rbpj^{f/f}$ mice, the ratio of splenic $CD11b^+$ cells to $CD8^+$ DCs changed from 2:1 to 1:1 (Caton et al. 2007). $CD11b^+$ cells express the Notch signal target genes *Hes1* and *Dtx1* at higher levels than do $CD8^+$ DCs. The expression of these genes by $CD11b^+$ DCs was reduced in CD11c-*Cre* $Rbpj^{f/f}$ mice. Although the progenitors of $CD11b^+$ DCs are normal, the expression of annexin V and uptake of BrdU label by $CD11b^+$ DCs in Rbpj-deficient mice are elevated. Those data indicate that Notch signaling regulates the survival of $CD11b^+$ DCs. $Esam^{hi}$ $CD11b^+$ DCs are selectively reduced in Rbpj-deficient mice (Caton et al. 2007). Furthermore, Caton et al. demonstrated that $CD11b^+$ DCs are located close to the marginal zone and express Dll1. Sekine et al. assessed whether maintenance of $CD11b^+$ DCs was controlled by Dll1 (Sekine et al. 2009). A single injection of anti-Dll1 monoclonal antibody was ineffective at changing the number of $CD11b^+$ DCs, whereas a combination of anti-Dll1 and a second antibody against another ligand (Dll4, Jagged1, Jagged2) reduced the number of $CD11b^+$DCs by half. Taken together, Dll1 and other ligands synergistically regulate the size of the $CD11b^+$ DC population. As for the receptor, $CD11b^+$ DCs express low levels of *Notch1*, but a substantial level of *Notch2* and *Notch4* at mRNA and protein levels (Caton et al. 2007; Sekine et al. 2009). Splenic $CD11b^+$ DCs were reduced in CD11c-*Cre* $Notch2^{f/f}$ mice. Moreover, CD11c-*Cre* mediated overexpression in DN-Maml1 mice but not in CD11c-*Cre* $Notch1^{f/f}$ mice (Lewis et al. 2011). Those results suggest that Notch2 is a major receptor for regulation of $CD11b^+$ DCs. These data are consistent with the report that Notch1 is not essential for the development of DCs (Radtke et al. 2000).

Notch2 also regulates the development of $CD11b^+$ DCs in non-lymphoid tissue. For example, Notch2 is indispensable for the development of DCs in the skin, lung, and liver. CD11c-cre-mediated deletion of Notch2 contributes to selective deletion of $CD103^+CD11b^+$ DCs that are $CD8^{neg}$ in the LP of the small intestine, and they are important for inducing Th17 in the LP (Lewis et al. 2011). The reduction of

$CD103^+CD11b^+$ DCs in LP DCs results in impairment of Th17 cells. In Lewis' report (Lewis et al. 2011), there are some discrepancies between phenotypes in CD11c-specific *Notch2* and *Rbpj*-deficient mice. *Notch2* deletion from CD11c cells leads to a reduction of splenic $CD11b^+$ DCs similar to *Rbpj* and also leads to impaired numbers of $CD103^+CD11b^+$ LP cDCs but not in *Rbpj*-deficient mice. This contradiction is caused by rapid turnover, timing of CD11c-*Cre* recombination, and/or protein perdurance rather than noncanonical Rbpj-independent Notch signaling (Lewis et al. 2011).

Mouse pDCs are defined by the expression of CD11c, BST2, Siglec-H, and B220. Human pDCs are characteristic by the expression of CD123, CD303, and CD304. Mouse and human pDCs produce type I interferon via TLR7 and 9, which is important for viral infection. The master regulator of pDC development in mice and humans is E2-2 (Onai et al. 2013; Cisse et al. 2008). In Caton's study, mouse pDCs were slightly increased in CD11c-*Cre* $Rbpj^{f/f}$ mice (Caton et al. 2007). Another report used bone marrow chimeric mice reconstituted by Notch1-deleted BM cells by Mx1-Cre. The data suggested that Notch1 was not important for pDC development (Radtke et al. 2000). Collectively, Notch signaling has a stimulatory rather than inhibitory role for mouse pDC regulation. In contrast, Notch signaling in human pDCs is controversial. Additionally, there are few in vitro studies. One paper indicated that $CD34^+CD1a^-$ human thymic precursor cells differentiated to functional $BDCA2^+CD123^{hi}$ pDCs. Those cells produced IFN-α after stimulation of TLR9 by CpG-ODN and HSV-1 during coculture with the OP9 stromal cell line, IL-7, and Flt3L (Dontje et al. 2006). This pDC development was blocked by coculture with OP9 that expressed Dll1 but not Jag1. Inhibition by Dll1 was blocked by γ-secretase inhibitor. Dll1 promotes expression of T cell lineage-specific factor Gata3 on pDCs but reduces expression of Spib, which regulates pDC development and Hes1. In another report, $CD34^+$ cells from umbilical cord blood develop $BDCA-2^+$, $CD123^+$, $CD4^+$, $CD11c^+$, and pDCs, which express TLR9, pre-Tα mRNAs, and IFN-α (Olivier et al. 2006).

Genetic blockage of nicastrin (a component of γ-secretase signaling) or treatment with anti-Dll4 antibody induced accumulation of thymic DCs in the cortex (Billiard et al. 2012). This thymic DC is derived from DN1 stage ex vivo and developed in Flt3-independent manner with upregulation of *PU.1*, *Irf-4*, *Irf-8*, and *Csf-1* (Billiard et al. 2012). Although Notch1 deficiency causes severe defects in T cell development, Notch1-deficient thymic tissue can still develop DCs (Radtke et al. 2000). From this report, it appears that Notch1 is not essential for generation of thymic DCs. We have previously reported that Thy1-expressing thymic cDCs ($Thy1^+$DCs) could differentiate in the presence of Dll1-stimulated BM cells or OP9 stromal cells in the presence of GM-CSF (Ishifune et al. 2011). In vitro generated $Thy1^+$ DCs express the Notch target genes *Hes1* and *Dtx1* at high levels, but not *E2-2* or *Spib*, which are pDC-specific transcription factors. The cells had a phenotype similar to in vitro-induced $Thy1^+$ DCs that were mainly localized in the thymus, but a few were found in lymph nodes and the spleen. In the thymus, $CD8^+$ DCs constitute a major proportion of cDCs. When we classified thymic DCs for Thy1 expression, 70% of $Thy1^{neg}$ DCs were $CD8^+$ DCs and the rest were $CD11b^+$ DCs.

Furthermore, 70% of thymic Thy1$^+$ DCs were CD8a$^+$CD11b^{neg}, data that indicate that Thy1$^+$ DCs were heterogeneous and mainly identified as CD8$^+$ DCs. Thy1$^+$ DCs have an expression pattern of co-stimulatory molecules and clonal deletion activity similar to those of Thy1^{neg} DC but not pDCs. However, the number of thymic Thy1$^+$ DCs was normal in CD11c-*Cre Rbpj*$^{f/f}$ mice, but reduced in BM chimeric mice reconstituted by Cre-transfected BM cells from *Rbpj*$^{f/f}$ mice. These data suggested that Notch signaling plays crucial roles in the differentiation of thymic Thy1$^+$ DCs after acquiring CD11c$^+$ expression. Thymic Thy1$^+$ DCs express Notch2 and 3 but not Notch1. Notch1 may not regulate the development of Thy1$^+$ DCs.

3.3 DC Function

Notch signaling regulates the maturation state of DCs (Wang et al. 2009; Weijzen et al. 2002). *Rbpj*-deficient monocyte-derived DCs stimulated by LPS show few dendrites, low expression of MHC class II antigens, or CXCR4; in addition they have low antigen-presenting ability and motility. Promoters of CXCR4 are activated by Rbpj. Overexpression of CXCR4 could rescue that phenotype. Notch signaling regulated the maturation of DCs through regulating CXCR4. Furthermore, Jagged1 induces maturation of human DCs (Weijzen et al. 2002). Jagged1-stimulated DCs from human peripheral blood upregulated the expression of maturation markers (MHC class II, CD80, and CD86) and IL-12. DCs' intrinsic Notch signaling is important for tumor immunity (Feng et al. 2010).

3.4 Monocyte Development

There are two monocyte subsets in mice: Ly6C^{hi} monocytes (Ly6C^{hi}CX3CR1^{low}CD11b$^+$CD11c^{neg}) and Ly6C^{low} monocytes (Ly6C^{low}CX3CR1^{hi}CD11b$^+$CD11c$^+$). The origin of Ly6C^{low} monocytes is poorly understood. Ly6C^{hi} monocytes give rise to Ly6C^{low} monocytes in transfer experiments (Varol et al. 2007; Yona et al. 2013). Both monocyte subsets express Notch1 and Notch2 receptors (Gamrekelashvili et al. 2016). Lyz2-*Cre* could delete the floxed gene in Ly6C^{hi} monocytes but not in Ly6C^{low} monocytes. Lyz2-*Cre Notch2*$^{f/f}$ mice but not Lyz2-*Cre Notch1*$^{f/f}$ mice had reduced numbers of Ly6C^{hi} monocytes with an atypical phenotype in the BM, spleen, and peripheral blood, including those with upregulated MHC class II and CCR2 in addition to downregulated CD11c and CD43 without changes in cell death. Reduced number of Ly6C^{low} monocytes in Lyz2-*Cre Notch1*$^{f/f}$ mice was caused by conversion from Ly6C^{hi} monocytes to Ly6C^{low} monocytes. In contrast, CD11c-*Cre* deleted the floxed gene in Ly6C^{low} monocytes but not in the Ly6C^{hi} monocytes. CD11c-*Cre Notch2*$^{f/f}$ mice also have reduced numbers of Ly6C^{low} monocytes. Notch2 regulates monocyte conversion, generation, and maintenance. As Ly6C^{low} monocytes are localized near the population of endothelial cell that

generate vascular niches in the BM and spleen, endothelial deletion of Dll1 but not Dll4 specifically reduces Ly6C^{low} monocyte numbers. In human CD14^{+} monocytes, Notch1 and Notch2 are highly expressed. Furthermore, a truncated extracellular form of Dll1 induced the apoptosis of monocytes in the presence of M-CSF but not GM-CSF (Ohishi et al. 2000).

3.5 Macrophage Differentiation

Tissue-restricted macrophages are defined and named by anatomical localization. For example, macrophages in the liver, brain, and lung are termed Kupffer cells, microglia, and alveolar macrophages. Recently, it was reported that each tissue-resident macrophage has a different origin, and some of them develop in the fetal stage (Sheng et al. 2015; Ginhoux et al. 2010).

Microglia cells belong to the glia cell group, along with astrocytes and oligodendrocytes. However, the microglia are the only glia cell derived from mesoderm (Sheng et al. 2015; Ginhoux et al. 2010). Microglia are tissue-resident macrophages and have characteristic development that is derived from yolk sac macrophages at the embryonic stage. Notch1, Dll1, and Jagged1 proteins are expressed by rat primary microglia. Stimulation of the MMGT12 microglia cell line by LPS, IFN-γ, or TNF-α upregulates *Notch1* mRNA and downregulates *Hes1* mRNA. Inhibition of *Notch1* mRNA by *Notch1* siRNA induces higher expression of IL-6 and IL-1β.

Since Langerhans cells (LC) come from embryonic precursors in the AGM or fetal liver, the LC are tissue-resident macrophages rather than DCs (Guilliams et al. 2014; Mildner and Jung 2014; Sheng et al. 2015). LC development is normal in BM chimeric mice reconstituted by *Notch1*-deficient BM cells (Radtke et al. 2000). In this context, BM cells were not suitable for assessing whether Notch signaling regulates LC development (Radtke et al. 2000).

CX_3CR1^{hi} LP cells in the intestine were first described as DCs and thought to transfer antigens from the intestinal lumen across the epithelial cell tight junctions (Niess et al. 2005). Recently, CX_3CR1^{hi} LP cells are considered as intestinal tissue-resident macrophage that are continuously replaced from monocyte in a CSF1-dependent manner (Sheng et al. 2015). Because CX_3CR1^{hi} LP cells have poor antigen-presenting and trafficking capacity compared to cDCs (Schulz et al. 2009), CX_3CR1^{hi} LP cells rarely induce excessive immune responses to microbiota (Diehl et al. 2013). Normally, CX_3CR1^{hi} LP cells express F4/80, CD11b, CD68, MHC class II, and CD11c. We found that CX_3CR1^{hi} LP cells show an unusual CD11c^{low} phenotype in CD11c-*Cre* Rbpj$^{f/f}$ mice (Ishifune et al. 2014). The unusual CX_3CR1^{hi}CD11c^{low} LP cells had a large cytosol. Furthermore, the expression of the co-stimulatory molecule CD86 (but not CD40 or CD80) was upregulated, and the ability to uptake intestinal luminal antigens was elevated. It was reported that CX_3CR1^{hi} LP cells produce IL-10, which maintains intestinal inducible Tregs. Unusual CX_3CR1^{hi}CD11c^{low} LP cells in *Rbpj*-deficient mice can produce IL-10 similar to CX_3CR1^{hi} LP cells. CX_3CR1^{hi}CD11c^{low} LP cells and CX_3CR1^{hi} LP cells

do not change CD11c expression during cultivation. Thus, $CX_3CR1^{hi}CD11c^{low}$ LP cells and CX_3CR1^{hi} LP cells belong to different lineages. Notch controls the cell fate of CX_3CR1^{hi} LP cells.

Bone homeostasis maintains the balance between osteoblasts and osteoclasts, the multinucleated cells responsible for bone resorption and bone formation, respectively. In contrast to osteoblasts derived from mesenchymal progenitors, osteoclasts are a class of tissue-resident macrophages derived from the monocyte lineage. RANKL stimulation of osteoclast precursors is important for their differentiation. RANKL induces the expression of the key transcription factor NFATc1 (Takayanagi et al. 2002; Asagiri et al. 2005). Notch signaling components are expressed by osteoclasts. RANKL-stimulated BM cells express Notch2, Dll3, Jag1, and Hes-1 (Fukushima et al. 2008). Other reports suggested that BM-derived osteoclasts express *Dll1*, *Jagged1*, *Jagged2*, *Notch1*, and *Notch2* mRNAs (Yamada et al. 2003). BM-derived osteoclasts express Notch2 at the protein level (Jin et al. 2016). In fact, all Notch ligands and receptors are expressed at different times by BM-derived osteoclasts (Ashley et al. 2015). Many reports suggested that Notch is crucial for the differentiation, activation, or function of osteoclasts. Fukushima et al. suggested that Notch2 promoted RANKL-dependent osteoclastogenesis. RANKL- and M-CSF-induced osteoclast differentiation from BM cells was inhibited by γ-secretase inhibitor (GSI) treatment or by silencing *Notch2* mRNA with shNotch2 (Fukushima et al. 2008). Conversely, stimulation by the active form of Notch2 or Jagged1 can block the GSI-mediated reduction of osteoclast differentiation. Notch signaling promotes osteoclast differentiation by positively regulating *NFATc1* promoter activity (Fukushima et al. 2008). Another paper showed that the GSI suppressed RANKL- plus M-CSF-induced osteoclast differentiation from BM cells and their resorption activity (Jin et al. 2016). The expression of NFATc1 and the phosphorylation of PYK2 were both inhibited by GSI treatment. Intracellular domain of Notch2 has a greater ability than that of Notch1to rescue reduced PYK2 phosphorylation and the anti-resorptive effect caused by DBZ (Jin et al. 2016). Notch signaling promotes osteoclast differentiation by RANKL-prestimulated precursors in contrast to the inhibitory role in noncommitted precursors (Ashley et al. 2015).

In vitro studies generally have difficulty in showing the real roles of Notch signaling in the development of osteoclasts. Most reports indicated that Notch has inhibitory effects on osteoclasts (Yamada et al. 2003). FLAG-fused human Dll1 (Dll1-FL) inhibited osteoclast development from the BM, spleen, and peritoneal macrophages in the presence of RANKL and M-CSF. Furthermore, Dll1-FL reduced the expression of c-Fms (the receptor for M-CSF) by BM cells. Notch also affects stromal cells' support of osteoclast differentiation. The ST2 stroma cell line expressed Notch1-3, Dll1, and Jagged1. Overexpression of the active form of Notch1 enhanced the expression of RANKL and the decoy receptor OPG in contrast to reduced M-CSF expression. Notch signaling might regulate osteoclasts as well as stromal cells. Further studies using genetically modified animals support the notion that Notch functions in the generation of osteoclasts (Bai et al. 2008). M-CSF and RANKL stimulation of BM cells from *Lyz2-Cre Notch1*$^{f/f}$*2*$^{f/f}$ *Notch3*$^{-/-}$ (*Notch1-3* $^{OC-/-}$) mice

increased the number and cell size of osteoclasts and promoted resorptive activity compared to control mice in vitro. BM precursors from *Notch1-3* $^{OC-/-}$ mice upregulate c-Fms and their proliferation is enhanced. Furthermore, in vivo experiments showed that injection of *Notch1-3* $^{OC-/-}$mice with RANKL increased osteoclast numbers and the level of the resorption marker, CTx. In contrast, stimulation by overexpression of intracellular domain of Notch1 and Jagged1 inhibited osteoclastogenesis in vitro. The Notch components responsible for inhibiting osteoclast differentiation and function are Notch1, Notch3, and Jagged1. Osteoclastogenesis is induced by inflammatory cytokines such as TNF-α, IL-1β, IL-17, and TLR ligand in synergy with RANKL, which is associated with diseases including rheumatoid arthritis, psoriatic arthritis, and periodontitis. Notch signaling also suppresses osteoclastogenesis associated with TNF-α-mediated inflammatory conditions (Zhao et al. 2012). TNF-α-induced osteoclastogenesis in vivo and in vitro was enhanced in *Lyz2-Cre Rbpj*$^{f/f}$ mice (Zhao et al. 2012). Consistent with past reports, RANKL-induced osteoclast differentiation of BM cells from *Lyz2-Cre Rbpj*$^{f/f}$ mice was modestly increased. However, TNF-α-induced differentiation was significantly increased compared to control BM cells in vitro.

It is believed that Rbpj negatively regulates NFATc1 expression through two mechanisms. The first mechanism is that TNF-α-induced Rbpj inhibits the expression of c-Fos, which positively regulates NFATc1. The second mechanism is that Rbpj induced by TNF-α attenuates IRF-8 which is a negative regulator of NFATc1 through suppression of Blimp1 (Zhao et al. 2012). The inhibitory role of Notch signaling in osteoclastogenesis is supported by other molecules. For example, Tak1 inhibits NUMB-like (NUMBL), which is important for degradation of Notch (Swarnkar et al. 2015). Further study demonstrated that Rbpj negatively controls PLCγ2, and this modulates intracellular calcium concentrations through suppressing TGF-β signaling, thereby upregulating osteoclastogenic genes such as *NFATc1*, *BLIMP1*, and *c-Fos* (Li et al. 2014). As several papers suggested that Notch signaling regulates osteoblast development from mesenchymal precursor, it is possible that Notch indirectly regulates osteoclastogenesis through control of osteoblastogenesis (Zanotti et al. 2011; Engin et al. 2008; Hilton et al. 2008).

3.6 Macrophage Polarization

Environmental conditions can change the activation state of macrophages toward M1 inflammatory macrophages or alternatively M2 macrophages (Galvan-Pena and O'Neill 2014). LPS and/or IFN-γ induce M1 macrophages that produce IL-12, iNOS, and IL-6. Activated M1 macrophages are important for inflammation and host defense. In contrast, M2 macrophages are important for wound healing and tissue repair. They are activated by the parasitic infection and the associated produced cytokines, including IL-4 and IL-13. M2 macrophages express Arg-1, which is important for producing urea, polyamines, and ornithine for wound healing (Corraliza et al. 1995; Munder et al. 1998). M1 and M2 macrophages possess different metabolic

programs such as glycolysis and fatty acid oxidization, respectively (Galvan-Pena and O'Neill 2014). Several papers indicated that Notch signaling regulates the polarization to the M1 macrophage and produces M1-related cytokine (Espinosa et al. 2010; Wongchana and Palaga 2012; Xu et al. 2012, 2015; He et al. 2015; Bansal et al. 2015; Boonyatecha et al. 2012). Bone marrow-derived macrophages (BMDMs) stimulated by LPS plus IFN-γ produce M1-type cytokines (IL-6 and IL-12), and the production is regulated by Notch signaling (Wongchana and Palaga 2012; Boonyatecha et al. 2012). Treatment of BMDM with GSI inhibits the production of IL-12p70, which is constituted by Il12p40 and p35. This inhibition reduces the nuclear translocation of c-Rel and Erk1/2 independent of *Irf5*, which is a master regulator of *Il12p40* in macrophages (Boonyatecha et al. 2012). Overexpression of the active form of Notch1 increases the level of *Il12p40* mRNA. However, in the presence of GSI, addition of TNF-α partially rescued *Il12p40* expression by macrophages. Thus, Notch controls *Il12p40* directly via c-Rel and indirectly via TNF-α (Boonyatecha et al. 2012). M1-polarization stimuli (LPS plus IFN-γ) lead to the binding of Notch1-IC to the IL-6 promoter region near the NK-κB binding site in mouse BM-derived macrophage (BMDM), which subsequently controls the production of IL-6 in mouse BMDM stimulated by LPS plus IFN-γ (Wongchana and Palaga 2012). Other reports showed that BMDM (from *Lyz2-Cre Rbpj*$^{f/f}$ mice) that were stimulated by LPS plus IFN-γ reduced TNF-α, IL-1β, and IL-6 at mRNA and protein levels (Dou et al. 2016). Another paper indicated that Notch regulated transcription factor IRF8, which controls M1 polarization induced by IFN-γ. Expression of M1 macrophage-related genes, *Il12a*, *Il12b*, and *Nos2*, was reduced in LPS-stimulated BMDM of *Lyz2-Cre Rbpj*$^{f/f}$, *Lyz2-Cre Adam10*$^{f/f}$ mice, and *Notch1*$^{f/f}$ mice compared with that of wild-type mice (Xu et al. 2012). This phenomenon is caused by the delayed synthesis of IRF8 protein. The expression of IRF8 was regulated by the TLR4 signaling. Rbpj controls synthesis and degradation of *IRAK2*, which is proximal to TLR4 signaling, without affecting *IRAK2* mRNA expression. Reduced amounts of IRAK2 protein reduced downstream signaling such as phosphorylation of MNK1 kinase and cap-binding protein eIF4E, a translation initiation factor. Because the reconstitution of IRF8 in Rbpj-deficient BMDM did not recover *Nos2* expression, the data suggest that Rbpj regulates not only IRF8 but also other factors. Taken together, it is possible that Notch signaling regulates M1 macrophage polarization by modulating IRF8 expression through control of the TLR4-IRAK2-MNK1-eIF4E signal pathway.

Notch can also drive liver macrophages and macrophage cell lines to the M1 type either directly or indirectly by controlling the expression of M1-related genes (Xu et al. 2015). For direct regulation, Notch modulates transcription of *Nos2* and *Pdp1* (pyruvate dehydrogenase (PDH) phosphatase (PDP) catalytic subunit (1)). Intracellular domain of Notch binds to promoter region of *Nos2* and *Pdp1* with Rbpj and controls their expression. Pdp1 is the phosphatase that catalyzes the α-subunit of PDH. Dephosphorylation of PDH by PDP1 activates the protein that synthesizes acetyl CoA from pyruvate and drives the TCA cycle. There are two other mechanisms by which Notch upregulates the generation of mitochondrial reactive oxygen species (mtROS) that induce M1-related gene expression. The first is achieved by

stimulating NF-κB and hypoxia-inducible factor-1α (Hif-1α). mtROS are produced when Notch enhances glucose oxidization by increasing glucose flux into the TCA cycle combined with PDP1 upregulation (Fig. 3.2). In the second mechanism, Notch modulates mitochondrial oxidative phosphorylation by driving the expression of mitochondrial genes, including NADH dehydrogenase, cytochrome b, cytochrome c oxidase, and ATP synthases (Fig. 3.2). This process increases ATP production through electron transport chain complexes.

Notch-mediated M1-macrophage regulation and monocyte recruitment are relevant to liver inflammation in the mouse model of alcoholic steatohepatitis (ASH), which is induced by a high fat diet and alcohol (OF + Alc). Liver macrophages from OF + Alc mice show elevated expression of M1 macrophage-related genes (*Nos2*, *Tnfa*, and *Il1b*; *Notch1 and Hes1*) compared to untreated mice. Furthermore, *Lyz2-Cre Notch1*$^{f/f}$ mice show reduced migration of blood monocytes into the liver and lower macrophage numbers in addition to reduced Nos2 expression and ROS production. However, the number of tissue-resident Kupffer cells and their expression level of M1-/M2-related genes and M2-related gene expression in monocytes and macrophages were normal in *Lyz2-Cre Notch1*$^{f/f}$ mice. The survival rate of OF + Alc mice with induced liver inflammation was ameliorated in the *Lyz2-Cre Notch1*$^{f/f}$ strain. Thus, Notch-mediated gene regulation and M1 polarization of metabolic programs are important in the ASH model.

Sustained excessive inflammation sometimes results in fibrosis. It was reported that Notch signaling exacerbates carbon tetrachloride (CCl_4)-induced fibrosis in the mouse model (He et al. 2015; Bansal et al. 2015). The expression of Notch1-3, Dll1, Dll4, Jag1, and Hes1 was elevated in the mouse model of fibrosis induction (Bansal et al. 2015). Treatment with the γ-secretase inhibitor avagacestat inhibited fibroblast-driven M1 polarization of macrophages, which reduced CCl_4-induced liver fibrosis (Bansal et al. 2015). Another report suggested that profibrotic factors such as platelet-derived growth factor (PDGF)-B and TGF-β1 produced from hepatic stellate cells in addition to infiltration of immune cells (macrophages and neutrophils) and the production of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokines (CCL2 and CXCL1) in the liver were significantly reduced in *Lyz2-Cre Rbpj*$^{f/f}$ mice (He et al. 2015). A past study indicated that deubiquitinase cylindromatosis (*CYLD*), which negatively regulates NF-κB and MAPK, is a target of Notch signaling in T cell leukemia (Espinosa et al. 2010). Notch modulates fibrosis controlled by NF-κB and MAPK through regulation of *CYLD*, which protects hepatocytes from injury and fibrosis. Patients suffering hepatic fibrosis have a more severe fibrosis and tend to exhibit high Notch activity that correlates with lower levels of *CYLD* expression (He et al. 2015). As for the ligand, Dll4 is important for M1 polarization. In a coculture system, Dll4 supplied from endothelial cells induced M1 polarization of human monocytes from PBMC in vitro (Pabois et al. 2016). Furthermore, thioglycollate-induced macrophages upregulate M1-related cytokines (IL-6, IL-1β, and TNF-α) and VEGF that display angiogenetic potential (Camelo et al. 2012).

There are some reports indicating that Notch can either negatively or positively regulate M2 macrophage polarization (Zhang et al. 2015; Zhao et al. 2016; Franklin et al. 2014; Wang et al. 2010; Zhang et al. 2010; Brifault et al. 2015; Foldi et al.

2016; Chen et al. 2015). Compared to controls, *Rbpj*-deficient macrophages stimulated by M1-inducing LPS have a higher expression of *Jmjd3*, a crucial molecule for M2 polarization (Xu et al. 2012). Notch signaling is important for M2 polarization under some conditions. Metformin, a drug for type 2 diabetes and cancer, promotes the in vitro expression of M2-related genes (*Arg1*, *Il4*, and *Il10*) and Notch components (receptor, ligand, and target genes) in RAW264.7 cells (Chen et al. 2015). When *Lyz2-Cre Rbpj*$^{f/f}$ mice were injected intraperitoneally with chitin (an *N*-acetyl-β-D-glucosamine polysaccharide and a major component of helminthes, fungi, and arthropods), the peritoneal macrophages had lower expression of M2-related genes (*Arg1*, *Mrc1*) without a change of *Marco* or *Il12p40* expression compared to control mice (Foldi et al. 2016). In *Lyz2-Cre Rbpj*$^{f/f}$ mice, chitin-mediated recruitment of eosinophils was reduced. However, unstimulated BMDM from *Lyz2-Cre Rbpj*$^{f/f}$ mice showed reduced expression of M2-related *Arg1* mRNA at baseline. When IL-4 stimulated BMDM from *Lyz2-Cre Rbpj*$^{f/f}$ mice in vitro, *Arg1* expression was promoted to a level similar to BMDM derived from control mice. Those data suggest that Rbpj controls the maintenance of *Arg1* expression. Although a previous study suggested that Stat6 activation, which is a downstream signal for IL-4, is important for *Arg1* expression (Chawla et al. 2011) and that IRF8 regulation by Rbpj is crucial for M1 polarization (Xu et al. 2012), expression of pSTAT6 and IRF8 is intact in IL-4-stimulated Rbpj-deficient BMDM. Under conditions of chitin stimulation, Rbpj controls M2 polarization through *Arg1* expression independent of STAT6 signaling. Moreover, M2 macrophages play crucial roles in the pathogenesis of proliferative vitreoretinopathy (PVR), as they infiltrate the fibrous membrane in a mouse PVR model (Zhang et al. 2015). Treatment with a γ-secretase inhibitor reduced the infiltration of M2 macrophages and their expression of M2 marker Arg1.

Tumor-associated macrophages (TAMs) are important for tumor initiation, growth, invasion, and metastasis. They have features similar to M2 macrophages and produce IL-10 and TGF-β. However, there are some discrepancies between gain of function and loss of function in experimental models (Zhao et al. 2016; Franklin et al. 2014; Wang et al. 2010). With regard to loss of function studies, TAMs were investigated in CD11c-*Cre Rbpj*$^{f/f}$ mice using a myeloid-dominant MMTV-PyMT (PyMT) mammary tumor model (Franklin et al. 2014). TAMs and conventional M2 macrophages possess different features. TAMs express CD206 and VCAM1 but not M2 markers such as YM1, FIZZ1, and MRC1. The number of MHCII^{hi}CD11c^{hi} macrophages and their Ly6C^{neg}MHCII$^{neg/low}$ precursors were unchanged in CD11c-*Cre Rbpj*$^{f/f}$ PyMT mice, and TAMs lost the expression of VCAM1 and their tumor-promoting activity. In the PyMT tumor model, Notch signaling positively regulated the differentiation of TAMs from monocytes in a CCR2-dependent manner. Increasing the number of TAMs by tumor growth was associated with upregulation of PD-1 in contrast to downregulation of granzyme B on CTL in PyMT mice. PD-1^{+} CTLs were decreased in CD11c-*Cre Rbpj*$^{f/f}$ PyMT mice. Although there was no evidence that macrophages directly induce the expression of PD-1 in CTL, it is possible that Notch-Rbpj signaling induces the tolerance to tumors by inducing TAM.

In contrast, another report used a *Lyz2-Cre*-mediated Notch overexpression system and tumor model induced by Lewis lung carcinoma (LLC) cells. In this gain of

function model, the system obtained the opposite results, i.e., Notch prevented TAM function but not differentiation via miR-125a induction of M1 macrophage polarization (Zhao et al. 2016). In *Lyz2*-Cre-driven Notch intracellular domain overexpressing (NIC^{cA}) mice, LLC tumor growth was markedly delayed compared to control mice. The study reported increasing $CD8^+$ T cell numbers in the LLC tumor in contrast to decreasing myeloid-derived suppressor cells (MDSCs). However, the number of TAMs was comparable between NIC^{cA} and control mice. TAMs from NIC^{cA} mice lost several M2 features (MR and *Arg1*) and gained M1 features (*Nos2* and *Il12*). In the LLC tumor model, TAMs from *Lyz2-Cre*-driven NIC-overexpressing mice highly expressed *miR-152a* compared to control mice. pri-*miR-125a* is located in the first intron of the gene for sperm acrosome-associated protein 6A (*Spaca6a*) along with a putative enhancer element containing a Rbpj-binding site. In another study, BMDMs were treated with a lentiviral vector to overexpress *miR-152a.* The resultant cells were transferred into an LLC tumor, and the transfectants significantly delayed tumor growth (similar to NIC^{cA} mice) with decreasing MDSCs and increasing $CD8^+$ T cells in the tumor. Notch directly controls the expression of miR-152a in BMDM. BMDM transfected with *miR152a* showed enhanced phagocytosis, increased M1 markers (*Nos2*, *Il12*, *Tnfa*), and iNOS production relative to the control BMDM. In contrast, the M2 marker *Mrc1*, which encodes a mannose receptor, was not increased. The targets of *miR-152a* are the 3′-UTR of hypoxia-inducible factor 1, alpha subunit (*Hif1an*), *IRF4*, and YY1-binding protein (*Rybp*). *miR-152a* suppresses *Hif1an* and inhibits HIF-1α activity, leading to M1 polarization through its control of glycolysis and iNOS production. In addition, *miR-152a* negatively regulates IRF4 to induce *Mrc1* expression upregulating PU.1. Furthermore, *miR-152a* positively controls its own expression through downregulation of *Rybp.* Rybp binds to and subsequently suppresses the transcription factor YY1 (*Yy1*), which binds to *pri-miR-152a.* The latter reports indicated that Notch suppresses the tumor-inducing TAM M1 phenotype rather than the M2 phenotype. The data suggesting that Notch plays opposite roles in TAM regulation were possibly caused by the use of different tumor cells and/or Cre-mediated gene deletions. The NIC overexpression model might activate canonical as well as noncanonical Notch signaling. Furthermore, Lyz2-*Cre* controls neutrophils in addition to macrophages and monocytes.

Mouse tumor models involving macrophage transfer show results similar to those described above in which Notch plays a key role for indirectly inhibiting TAMs by promoting M1 macrophage polarization (Wang et al. 2010). Mice inoculated with B16 or LLC tumors in addition to LPS-primed BMDM from Mx-*Cre* $Rbpj^{f/f}$ mice exhibited increased tumor weights and volumes. BMDMs differentiated in culture on OP9 stromal cells expressing Dl1 promoted the M1 phenotype after LPS stimulation as shown by production of IL-12 and expression of *Nos2*. In contrast, the BMDMs decreased the M2 phenotype after IL-4 stimulation, as shown by IL-10 production and expression of *Mrc1*, *Ym1* mRNAs. GSI-treated BMDMs or BMDMs from Mx-*Cre* $Rbpj^{f/f}$ mice predominantly display the M1 phenotype rather than the M2 phenotype. This M1 polarization by Notch is linked to *SOCS3* upregulation. LPS stimulation induces SOCS3 expression in BMDM and RAW264.7 cells, whereas GSI treatment prevents LPS-induced *SOCS3* expression.

Notch drives M2 macrophage polarization in mice that had systemic lupus erythematosus (SLE) or stroke (Zhang et al. 2010; Brifault et al. 2015). It was reported that transplantation of mice with stem cells producing pituitary adenylate cyclase-activating polypeptide (PACAP) improved functional recovery after ischemia without reducing lesion size (Brifault et al. 2015). Phenotypic analysis of PACAP-treated ischemic mice indicated that microglia had a neuroprotective M2 phenotype in which *Il10* and *Ym1* were elevated and *Tnfa* mRNA was reduced. These changes were related to downregulation of *Rbpj*, supporting the association between M2 microglia and Notch signaling. M2 macrophages can be further divided into four subpopulations: M2a, M2b, M2c, and M2d (Colin et al. 2014). M2b macrophages produce IL-1-β, IL-10, IL-6, TNF-α, and IL-12 when stimulated by combined immune complexes, TLR ligands, and IL-1R ligands. In an SLE model, disease is induced by immunizing BALB/c female mice with activated lymphocyte-derived DNA (ALD-DNA). In this setting, F4/80^{+} macrophages accumulated in renal tissue and displayed an M2b phenotype, with an elevated expression of *Il10*, *Tnfa*, *Il1b*, *Il6*, *Mcp1*, and *Nos2* (Zhang et al. 2010). However, renal macrophages only express *Il12*, *Tgfb1*, *Il1ra*, and *Arg1* at low levels. Those expression patterns exhibited features of M2b macrophages. The exposure to ALD-DNA appeared to induce Notch signaling, as the ALD-DNA-treated RAW264.7 cells have increased *Notch1* mRNA and elevated expression of Notch target genes (*Hes1* and *Hey1*), but not other receptors. BMDMs and RAW264.7 cells stimulated with ALD-DNA showed elevated expression of MHC class II, CD80, and CD86 and expressed high levels of TNF-α, IL-1β, IL-6, IL-10, and MCP-1, but not IL-12. In contrast to that, DAPT-treated RAW264.7 cells decreased the production of TNF-α, IL-6, IL-12, IL-10, and MCP-1. The symptoms of SLE, such as increased auto antibody, urine protein production, and glomerulonephritis, were ameliorated by GSI treatment that prevented Notch signaling. Renal macrophages in DAPT-treated mice showed reduced expression of Notch1 and M2b macrophage-related gene expression. Additionally, Notch inhibition by DAPT partially reduced already established SLE symptoms through decreasing urine protein production and reducing glomerulonephritis although anti-dsDNA and autoantibody levels were not affected. Notch promotes M2b polarization in ALD-DNA-treated macrophages, thereby controlling P13K/Akt-ERK1/2 and P38 MAPK pathways. Those finding suggests that Notch might be a target for prevention and therapy of SLE. One paper indicated that adenylate cyclase-activating polypeptide (PACAP) induced neuroprotective effects after intra-cerebroventricular injection. A different study showed that Notch signaling regulated both M1 and M2 macrophages. Macrophages are important for choroidal neovascularization (CNV). In Lyz2-*Cre Rbpj*$^{f/f}$ mice, laser-induced formation of retinal CNV lesions was inhibited with reduced macrophage infiltration and M1 and M2 polarization (Dou et al. 2016). M2 reduction was greater than that of M1.

3.7 Cross Talk with TLR Signaling

According to the RefDIC database, mouse BMDMs and peritoneal macrophages express high levels of *Notch1*, *Notch2*, and *Jagged1* at the mRNA level. Past papers suggested that the RAW264.7 macrophage cell line and peritoneal macrophages

express *Notch1* and *Notch2* mRNAs, but rarely express *Notch3* or *Notch4* (Zhang et al. 2012). With regard to ligands, the RAW264.7 cell line and peritoneal macrophages express mRNAs for *Dll1*, *Dll3*, *Dll4*, as well as *Jagged 1 and Jagged 2 (Jag1, 2)* (Zhang et al. 2012). Furthermore, others reported that *Jagged 1 and Jagged 2* and *Dll1* were expressed by peritoneal and splenic F4/80$^+$ macrophages (Yamaguchi et al. 2002). Human primary macrophages express *Dll4* and *Notch3* at mRNA and protein levels (Fung et al. 2007). Several reports indicated that there are relationships between TLR and Notch signaling. There are many reports that suggest that TLR signaling upregulates the Notch receptor, ligand, and/or target genes. One paper demonstrated the effects of stimulating the macrophage cell line RAW264.7 or BMDMs with one of the following: LPS (a TLR4 ligand), poly I:C (a TLR3 ligand), Pam_3Cys (a TLR2 ligand), or CpG (a TLR9 ligand). The exposure upregulated Notch1 expression and Notch targets *Hes1* and *Deltex1*, which modulate the production of IL-6, TNF-α, and IL-10 (Palaga et al. 2008). Others reported that LPS-stimulated RAW264.7 cells upregulated *Jagged1* and *Notch1* at mRNA and protein levels (Monsalve et al. 2006). Expression of Jagged1 by LPS-simulated RAW264.7 cells depends on JNK activation (Tsao et al. 2011). In a mouse sepsis model, DAPT plus LPS treatment induced RAW264.7 cells and macrophages to reduce their production of IL-1β and IL-6, which is important for survival from life-threatening sepsis (Tsao et al. 2011). Murine peritoneal macrophages elevate the expression of Notch1 after exposure to *Mycobacterium bovis* or *Bacillus* Calmette-Guerin (BCG)-derived TLR2 and LPS-induced TLR4 signaling (Narayana and Balaji 2008; Monsalve et al. 2009; Palaga et al. 2013). Murine BMDMs and human monocyte-derived macrophages stimulated by soluble egg antigen (SEA) from the helminth *Schistosoma mansoni* upregulate Jagged1 through TLR2 or TLR4/MD2 signaling in an ERK-dependent manner (Goh et al. 2009). Furthermore LPS-stimulated primary human macrophages highly express *Dll4* at mRNA and protein levels (Fung et al. 2007). TLR2 and TLR4 induce the expression of Notch ligands Jagged1, Dll1, and Dll4 on human primary macrophages and mouse macrophages. The increase of Jagged1 was greater than that of the other two ligands. The mechanism by which TLR2 and 4 upregulate Jagged1 on macrophages is partially dependent on NF-κB and MAPK (Foldi et al. 2010). In addition, upregulation of Jagged1 is Notch1 and 2-Rbpj dependent. Thus, TLR and Notch pathways cooperate to upregulate the expression of Jagged1 leading to amplification of Notch signaling, which contributes to IL-6 production.

It has also been shown that there is a pathological link between NO, Notch, and TLR2. When murine macrophage is activated by treatment with *M. bovis* BCG-TLR2 signaling, iNOS modulates the upregulation of Notch1 and its targeted metalloproteinase-9 (*Mmp9*) and *Hes1* (Kapoor et al. 2010). Interestingly, NOS is also essential for the recruitment of Rbpj to the Rbpj-binding site in the promoter region of *Mmp*. It is possible that Notch1 activation is related to infection because human tuberculosis patients tend to have high expression of Notch1, Hes1, and/or Mmp9 (Kapoor et al. 2010). From a different perspective, treatment of mice with docosahexaenoic acid (DHA, an omega-3 long-chain fatty acid) inhibits the LPS-induced elevated expression of Notch1 and Jagged1 on macrophages in the presence of O_2 (Ali et al. 2016). On the other hand, it has been suggested that TLR-mediated signaling directly induces Notch target genes in human macrophages without requiring the expression of a Notch receptor or ligand, suggesting there is cross talk between TLR and Notch signaling (Hu et al. 2008).

Macrophages stimulated with LPS, Pam_3Cys, or R848 (TLR7 and TLR8 ligands) upregulate Notch target genes *Hes1* and *Hey1*, which subsequently induce the production of IL-6, TNF, and IL-12 (Hu et al. 2008). Notch target genes induced by TLR are Notch signal dependent because treatment with a γ-secretase inhibitor and knockdown or the absence of Rbpj inhibits the expression of *Hes1* and *Hey1*. Production of IL-6 is directly regulated by binding of Rbpj to the promoter of the IL-6 gene. Furthermore, canonical IKK- and MAPK-dependent TLR signaling is also required for TLR-mediated Notch target gene expression. TLR might induce *Hes1* expression through IKK- and MAPK-induced phosphorylation of serine 10 of histone H3 at the *Hes1* locus. In some reports, IFN-γ repressed TLR-induced gene expression (Hu et al. 2006, 2007). IFN-γ also suppressed primary transcription of *Hes1* and *Hey1* by suppressing the recruitment of RNA polymerase II to their promoters (Hu et al. 2008). Furthermore, TLR-induced *Hes1* and *Hey1* are important for negative feedback. BMDMs from BM chimeric mice reconstituted with *Heyl1*^{null} or *Hes1*^{null} BM reduced the production of IL-6, IL-12, and IL-27 in response to Pam_3Cys. What is the mechanism for inhibiting *Hes1* and *Hey1* gene expression? It is known that the *Il6* promoter contains E-box and N-box sequences to which Hey1 binds and suppresses promoter activity. This suggests that Hey1 function as a transcriptional repressor to inhibit cytokine coding genes. These data demonstrated that a combination of Notch signaling with TLR and IFN-γ regulates macrophage function. However, most papers have suggested that Notch signaling promotes LPS-induced inflammatory responses and that Notch signaling suppresses production of IL-6, TNF-α, and IL-10 through Erk1/2 inactivation and NF-kB transcription by using the NICD overexpression system (Zhang et al. 2012). This discrepancy is likely caused by different sources of macrophages, cell lines, or BMDMs.

3.8 Macrophage Function

Human $CD14^+$ monocytes and macrophages from RA patients express high levels of *miR-223* compared to healthy individuals. *miR-223* links the lower expression of *Notch3* and its target *Hey1*. Notch3-modulated Hey1 negatively regulates *miR-223* (Ogando et al. 2016). *miR-223* promotes production of IL-1β, IL-6, and TNF-α through inhibition of the aryl hydrocarbon receptor (Arnt), which induces further *Notch3* expression. Thus, Notch might be a negative regulator of RA pathogenesis.

Notch signaling regulates the cell cycle to keep cells in the G1 phase. It also promotes cell survival in LPS-stimulated RAW264.7 cells by controlling the phosphorylation of Akt, which is partially linked to G protein signaling (RGS19) (Sangphech et al. 2014). Notch signaling also regulates macrophage cell death (Palaga et al. 2013). In addition to upregulation of Notch1 in macrophages by *M. bovis* BCG described above, tuberculin purified protein derivative (PPD) upregulates Notch1 expression on BMDMs. Notch1 upregulation is associated with upregulation of the antiapoptotic gene *Mcl-1* and slightly increasing annexin V^+ macrophages. GSI or si*Notch1* treatment inhibits the expression of *Notch1* and

Mcl-1. In the *Mcl-1* promoter region, there are conserved Rbp-j binding regions in human and mouse genes. Chip analysis indicates an association of Notch1 with the *Mcl-1* promoter.

Mice that are transplanted with macrophages from Mx1-*Cre Notch1*$^{f/f}$ mice showed lower clinical score of EAE that mice with those from Mx1-*Cre Rbpj*$^{f/f}$ mice (Wongchana et al. 2015). Furthermore, macrophages from Mx1-*Cre Notch1*$^{f/f}$ mice suppress production of IL-17A from T cells in the spleen. Because of this phenomenon, macrophages from Mx1-Cre Notch1$^{f/f}$ mice had less ability to produce IL-6 and express CD80 molecules. However, it remains unclear whether Notch regulation in macrophages is related to CNS infiltration enhanced by Th17, which is important for the pathogenesis of EAE. During retinal angiogenesis, Dll4 and Notch1 on endothelial cells are important for angiogenic sprouting. Intrinsic Notch1 in retinal macrophages is important for the localization of those cells during retinal angiogenesis (Outtz et al. 2011).

3.9 Conclusions

The literature suggests that Notch signaling is required for the differentiation and activation of monocytes, macrophages, and dendritic cells. Recent studies show that tissue-resident macrophages have a specific developmental origin and that there are appropriate markers to identify those monocytes, macrophages, or dendritic cells. Future studies of ligand distribution, genetically modified mice based on ontology or the analysis of organizations, and subjects that have not yet been studied will enable us to clarify the roles of Notch signaling.

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Chapter 4
Taking CD4 T Cells Up a Notch

Christina Helbig and Derk Amsen

Abstract CD4 T cells orchestrate immune protection against pathogens from different classes. For this, naïve CD4 T cells must be activated, proliferated, and differentiated into various lineages that are each dedicated to fighting specific types of pathogens. Furthermore, some CD4 T cells must differentiate into and persist as memory cells for long-term protection against recall infection. The highly conserved Notch signaling pathway, known for its many functions in cell fate decisions, has been implicated as a powerful regulator in all of these processes. How this ostensibly simple pathway controls such diverse cellular programs remains incompletely understood. We here review the, sometimes seemingly contradictory, findings regarding the role of Notch signaling in CD4 T cell activation, differentiation, memory formation, and persistence. Consensus is starting to emerge that Notch acts via induction of basic metabolic programs as well as by activation of CD4 T cell lineage-specific genes. Outlining both unifying principles involved in Notch-mediated T cell fate decisions and context-specific differences may lead the way to successful therapeutic exploitation of this pathway in immunity.

Keywords CD4 T cell subsets • Notch • Differentiation • Activation

4.1 Introduction

Different CD4 T cell subsets exist, each dedicated to a specific group of tasks (Basu et al. 2013; Zhu et al. 2010). These lineages can be classified according to a matrix in which the first dimension classifies CD4 T cell subsets according to the types of microorganisms against which they react, while the second dimension distinguishes subsets based on the types of cells they control (Fig. 4.1).

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K. Yasutomo (ed.), *Notch Signaling*, DOI 10.1007/978-981-10-4971-2_4

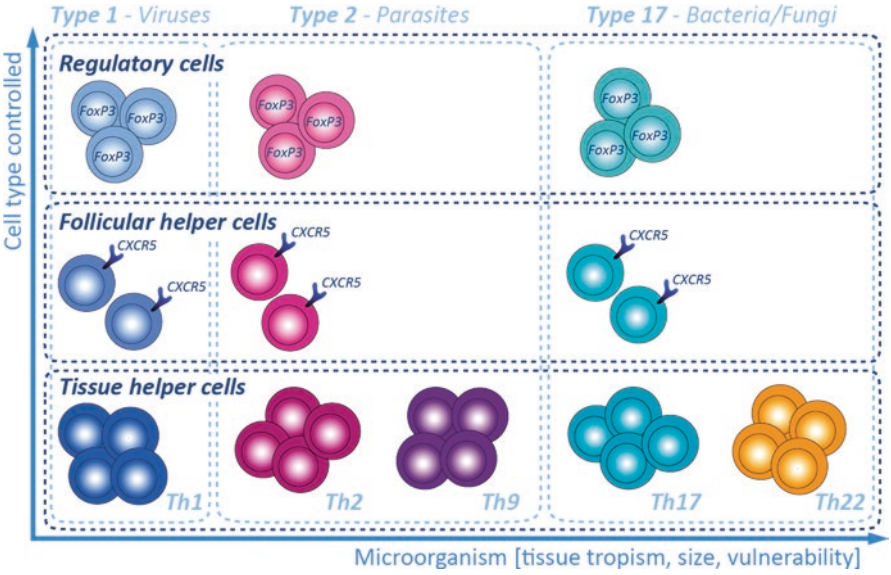

Fig. 4.1 Two-dimensional classification of CD4 T cells. Displayed are the known CD4 T cell subsets in a matrix defined by the microorganisms against which the CD4 T cells react (x-axis) and the cells controlled by these CD4 T cells (y-axis). Types of microorganisms are indicated on top of the graph

The first dimension is that differences between microorganisms in tissue tropism, size, and vulnerabilities necessitate different types of immune effector mechanisms. The CD4 T cell lineages each produce their own cocktail of cytokines and express distinct combinations of chemokine receptors. These attributes allow them to home to the correct environment and mobilize effector responses suited to fight the infectious agent encountered (Abbas et al. 1996). For instance, Th1 cells produce IFNγ to stimulate phagocytic capacity by macrophages and are important for defense against intracellular bacteria (Abbas et al. 1996). T helper 2 cells produce IL4, IL5, and IL13 and thereby induce effector mechanisms such as mucus production by enterocytes and activation of eosinophilic granulocytes (Abbas et al. 1996). Other lineages include Th9 cells (which produce IL9 and orchestrate responses against parasites), IL17-producing Th17 cells (for protection against fungi and pathogenic intestinal bacteria), and IL22-producing Th22 cells, also thought to be responsible for defense against such bacteria by inducing epithelial cell-mediated production of antimicrobial peptides (Basu et al. 2013).

The second dimension is that the classical Th1, Th2, and Th17 cells mostly activate innate effector mechanisms and exert effector functions in the tissues (Abbas et al. 1996). We propose to classify these as *tissue* T helper cells. A second lineage along this axis, known as follicular helper T cells (Tfh), provides signals that promote Immunoglobulin isotype class switching and affinity maturation by B cells. These cells again exhibit different first dimension properties, depending on the type of microorganisms to which they respond (Fig. 4.1). Thus, Tfh cells produce the

Th1 cytokine IFNγ, which promotes isotype switching toward IgG2a, and the Th2 cytokines IL4 and IL5, which drive switching to IgG1 and IgE (and IgG4 in human B cells) or IgA, respectively (Crotty 2014). Although less well studied, a role for IL17 in isotype class switching has also been described, promoting recombination to IgG2a and IgG3 (Mitsdoerffer et al. 2010; Peters et al. 2011; Keerthivasan et al. 2011). Differential production of these cytokines may reflect an ability of a single type of Tfh cell to modify its cytokine production. However, it is also possible that distinct Tfh1, Tfh2, and Tfh17 lineages exist.

Finally, a third type of CD4 T cell along the second axis is the regulatory T cell (Treg). These cells suppress other immune cells and are critical for maintenance of immune tolerance and tissue homeostasis (Campbell 2015). Experiments with Treg-specific knockout alleles for transcription factors have shown that the suppressive capacity of Tregs is divided into modules along the first dimension of this matrix (Josefowicz et al. 2012). Thus, when Tregs lack expression of T-bet, the major Th1-associated transcription factor, Th1-mediated pathology develops (Xiong et al. 2016). Likewise, when IRF4 or Gata3 (two Th2-associated transcription factors) or the Th17 factor STAT3 are eliminated specifically in Tregs, Th2- and Th17-mediated pathology ensues, respectively (Josefowicz et al. 2012). In contrast to tissue T helper cells, which are relatively committed to their lineage, regulatory T cells appear to switch between these properties depending on the cytokine milieu (Yu et al. 2015).

Fine antigen specificity is a defining feature of adaptive immunity. For robust T cell responses that match antigen specificity with microbe-specific functional properties, naïve T cells (with the right antigen receptors) must expand their numbers and acquire effector properties by differentiation in response to signals that provide information about the nature of the infection. Another hallmark of adaptive immunity is preservation of antigen-specific immune memory, which requires differentiation of memory T cells as well as their long-term persistence (Harty and Badovinac 2008).

All processes described above are controlled by signals from antigen-presenting cells (APC) and stromal cells. The traditional view is that activation and differentiation of naïve CD4 T cells depend on three signals derived from APC (Kapsenberg 2003). The major APC involved is the dendritic cell, which phagocytoses microorganisms in tissues and carries these to lymphoid organs, where naïve T cells can be activated. The first signal delivered by APC to naïve T cells derives from engagement of the TCR by an antigen-MHC complex. A second, or costimulatory signal, can be generated, among others, by binding of CD80/86 on activated APC to CD28 on the T cell (Kapsenberg 2003). Expression of costimulatory ligands on APC occurs in response to recognition of characteristic microbial structures (pathogen-associated molecular patterns or PAMPs) by germ line-encoded pattern recognition receptors, such as Toll-like receptors (TLR) (Iwasaki and Medzhitov 2015). Such recognition signifies the presence of microbial danger and therefore justifies the generation of a T cell response. Because different classes of microorganisms express different characteristic molecules, PRR are also important in determining the direction of the T cell response. Guided by the molecular patterns detected, APC generate a third type of signal to direct differentiation of the naïve cells into the appropriate

lineages. This third signal can derive from cytokines (de Jong et al. 2002; Reis e Sousa 2004) or from membrane-bound molecules like Notch ligands (Amsen et al. 2004; Sauma et al. 2011) on the APC. Multiple signals may be integrated to jointly compose a third signal. It is conceivable that not all of these are delivered simultaneously during the first T cell interaction with the APC and evidence is starting to emerge that some of the requisite signals may be provided by stromal cells.

In this chapter, we will focus on the contribution of Notch to activation, survival, and differentiation of T cells.

4.2 The Notch Pathway in a Nutshell

As the Notch signaling pathway is discussed elsewhere in this book, we will here provide only a brief overview. The four mammalian Notch receptors (Notch1–4) are expressed as heterodimers at the cell surface with an extracellular ligand binding domain and a non-covalently associated transmembrane polypeptide (Kopan and Ilagan 2009). Naïve CD4 T cells express low levels of Notch1 and 2. After T cell activation, expression of both these receptors increases, and also Notch 3 starts to be expressed (Auderset et al. 2012; Maekawa et al. 2008; Koyanagi et al. 2012; Amsen et al. 2015). There are five canonical, membrane-bound ligands for Notch. These belong to two families, called Jagged (Jagged1, Jagged2) and delta-like ligand (DLL1, DLL3, and DLL4). This diversity is conserved from fruit flies to mammals, suggesting functional (but poorly understood) importance. All ligands can bind to all Notch receptors and activate the same biochemical signaling pathway, involving cleavage by a transmembrane protease complex known as γ-secretase (Kopan and Ilagan 2009). Pharmacological inhibitors of this enzyme (GSI) effectively prevent activation of Notch (Kopan and Ilagan 2009). After cleavage, the intracellular domain of Notch (NICD) dissociates from the plasma membrane and migrates to the nucleus. The major Notch effector is the DNA-binding protein RBPJκ (also known as CSL). Together with proteins of the Mastermind-like family (MAML), these proteins assemble a transcriptional activator complex (Kopan and Ilagan 2009; Kovall 2007).

The RBPJκ protein associates with transcriptional repressor proteins that are displaced by NICD binding. This has led to the concept that RBPJκ would actively suppress target genes of the pathway in the absence of Notch signaling (Bray 2006). Evidence for this concept indeed exists in Drosophila (Kopan and Ilagan 2009). Somewhat confusingly, studies in mammalian cells showed that RBPJκ is recruited to most target sites only after complex formation with NICD (dynamic sites), seemingly at odds with a major role for RBPJκ in transcriptional repression (Castel et al. 2013). Nonetheless, a modest number of static sites are occupied by RBPJκ regardless of the presence or absence of NICD and thus may be subjected to active repression (Castel et al. 2013).

The Notch pathway is notoriously sensitive to signal strength. Furthermore, the outcome of Notch activation is influenced by the activity of other signaling pathways.

This at least partially depends on physical interaction of NICD with effector proteins such as Smad3, CREB, HIF1α, Nur77, and Zmiz1 (Maekawa et al. 2008; Mukherjee et al. 2011; Wang et al. 2011; Jehn et al. 1999). As these interacting proteins possess DNA-binding domains themselves, it is conceivable that these partnerships affect target preference, perhaps allowing binding to low-affinity sites that would not be effectively occupied by either of the partners individually.

Hes family transcriptional repressors are the prototypical effectors of Notch throughout the metazoan kingdom. However, Notch also has many cell type-specific target genes (Bray 2006). Notch operates in signaling networks together with some prominent other pathways, including NFκB and mTOR. Some of these may involve as yet poorly characterized noncanonical (RBPJκ-independent and/or ligand-independent) Notch signaling (Ayaz and Osborne 2014).

4.3 T Helper Cell Activation, Survival, and Memory

During initial activation, signaling by TCR and CD28 leads to secretion of IL2 and cell cycle entry. This process requires metabolic changes to meet the demands of proliferation and effector function, which require large amounts of energy as well as building blocks to generate membranes, proteins, and nucleotides (MacIver et al. 2013). T cells therefore strongly increase their uptake of nutrients and switch their energy metabolism from one based on oxidative phosphorylation in mitochondria to an aerobic glycolytic program. This latter pathway only partially degrades glucose and therefore yields less energy than oxidative phosphorylation, but helps preserve building blocks for anabolic purposes (MacIver et al. 2013). An important pathway-regulating nutrient uptake and the glycolytic switch involve the mTOR protein (Chi 2012). This is a serine/threonine kinase that functions in two separate complexes, known as TORC1 and TORC2. Of these, TORC1 is especially important for the metabolic changes required for efficient activation of T cells (Chi 2012). This complex responds to signals from TCR and CD28 as well as to growth factor receptors, such as the IL2 receptor. Among the many processes controlled by TORC1 are induction of nutrient receptors on the cell surface and translation of proteins with critical roles in cell cycle progression (Chi 2012).

Recent findings have established a role for Notch signaling in activation of CD4 T cells. Laky et al. showed that DLL4-induced Notch signaling allowed efficient activation of CD4 T cells at low doses of antigen, which increased the magnitude of primary responses (Laky et al. 2015). They showed that DLL4 is expressed on a subset of splenic myeloid dendritic cells (DCs) and genetic ablation of the *Dll4* gene in these cells markedly diminished their ability to activate naïve CD4 T cells. Expression of DLL4 is induced on DCs in response to TLR ligation (Amsen et al. 2004; Napolitani et al. 2005). Upregulation of this Notch ligand may therefore represent part of a code that allows activated DCs to license activation of naïve T cells. Consistent with this possibility, Notch signaling was found to complement costimulation by CD28 rather than substitute for it (Laky et al. 2015).

Activation of Notch led CD4 T cells to express higher amounts of nutrient transporters (for uptake of glucose, glutamine, amino acids, and iron). Consequently, such cells developed increased biomass compared to CD4 T cells activated without the benefit of a Notch-activating signal (Laky et al. 2015). Notch may boost metabolic reprogramming by promoting activity of TORC1, as downstream mediators of this complex were stimulated by activation of Notch in CD4 T cells (Laky et al. 2015). Notch has been linked to TORC1 in several other contexts, including during T cell development in the thymus and during effector differentiation of CD8 T cells (Wong et al. 2012; Backer et al. 2014). A direct mechanism to explain how Notch controls TORC1 activity has not definitively been established. A difficulty here is that, while TORC1 promotes expression of nutrient transporters on the cell surface, its own activity is in turn heavily dependent on the presence of nutrients (Chi 2012). It can therefore not be excluded that the stimulatory effect of Notch on TORC1 in CD4 T cells is a consequence rather than a cause of the elevated surface expression of nutrient receptors. However, more direct mechanisms are also possible. One of these involves regulation of PTEN. This phosphatase antagonizes activity of PI3 kinase, a major activator TORC1 (Chi 2012). At least in immature T cells, the Notch target Hes1 represses expression of the *Pten* gene, thereby enhancing PI3K activity (Wong et al. 2012). Notch may however also promote TORC1 by inducing expression of CD25, thereby sensitizing T cells to IL2 (Amsen et al. 2015).

Apart from activating the mTOR pathway, a recent study showed that Notch promotes protein O-GlcNAcylation, a modification that requires constant influx of glucose and glutamine (Swamy et al. 2016). Interestingly, one of the proteins modified by O-GlcNAcylation is Myc, a factor that is critical for cell cycle progression (Swamy et al. 2016), suggesting that it may function as a sensor for nutrient sufficiency downstream of Notch.

After initial activation, survival signals are required to prevent premature termination of the response. This is reflected, for instance, in the abortive responses generated by immunogens in the absence of adjuvant, where initial expansion is followed by apoptosis of the activated CD4 T cell clones (Jenkins et al. 2001). Notch can provide such survival signals (Helbig et al. 2012). Some of this function may depend on maintenance of the appropriate metabolic program. However, Notch also couples to survival pathways in a more direct way. Using the classical RBPJκ-dependent pathway, it enhances expression of antiapoptotic Bcl2 family members as well as of inhibitors of death receptor signaling (e.g., CD95) such as Faim3 (Helbig et al. 2012). In addition, Notch may regulate survival by direct physical interaction with apoptosis mediators such as XIAP, Bax, and Nur77 (Jehn et al. 1999; Liu et al. 2007; Sade et al. 2004).

Despite being a potent activator of survival pathways in CD4 T cells, Notch is not always required for that function (Laky et al. 2015), suggesting the existence of other signals that can similarly protect activated CD4 T cells from cell death. Whether Notch is required may depend on the nature and dose of the immunogen/pathogen. Furthermore, Notch controls survival of memory CD4 T cells (Maekawa et al. 2015). Memory CD4 T cells persist poorly in mice lacking expression of RBPJk, and short-term treatment of mice with GSI resulted in rapid loss of already

established memory CD4 T cells. This was attributed to a requirement for Notch in maintaining cell surface expression of the Glut1 glucose transporter in a manner that is apparently independent of TORC1 (Maekawa et al. 2015). Maintenance of CD4 memory T cells depended on the expression of DLL1 by DCs, and a population of DLL1 expressing bone marrow DCs was identified that may be responsible for providing this survival signal (Maekawa et al. 2015). Importantly, treatment of mice with GSI could prevent development of pathology by adoptively transferred encephalitogenic memory CD4 T cells (Maekawa et al. 2015), suggesting that this pathway may hold therapeutic potential for treatment of established autoimmune disease.

4.4 T Helper Cell Differentiation and Notch

Notch is most famous for its role in directing cellular differentiation processes throughout the metazoan kingdom. It is no surprise, therefore, that Notch is heavily involved in differentiation of CD4 T helper cells into effector cells. Although early studies indicated that Notch imparted direction to this differentiation process, more recent insights favor a more general role for Notch in acquisition of effector competence.

4.4.1 Th1 Cell Differentiation

Differentiation of Th1 cells is initiated by TCR signaling, but potently enhanced by IL12 and IFNγ, cytokines produced by innate immune cells upon bacterial or viral encounter (Zhu et al. 2010; Bonelli et al. 2014). The IL12 receptor cascade involves activation of the STAT4 transcription factor, which activates expression of target genes, including *IL12rb2*, *Ifng*, and *Tbx21* (encoding the lineage-defining transcription factor T-bet) (Bonelli et al. 2014). Binding of IFNγ to its receptor leads to activation of STAT1 and interferon regulatory factor 1 (IRF1) (Stark and Darnell 2012). The latter factor enhances expression of *IL12rb1* (Kano et al. 2008). STAT1, like STAT4, directly induces expression of *Tbx21*. T-bet, in turn, promotes expression of *IL12r*β2 and *Ifng* (Zhu et al. 2010; Bonelli et al. 2014).

Even though IL12 promotes Th1 cell differentiation, Th1 cell responses can be generated in IL12-deficient mice (Jankovic et al. 2002), among others by Notch. Expression of DLL family Notch ligands is induced on DCs by stimuli that promote Th1 cell differentiation (Amsen et al. 2004; Sauma et al. 2011; Brombacher et al. 1999; Napolitani et al. 2005; Skokos and Nussenzweig 2007; Sun et al. 2008; Debarry et al. 2007), and DLL1-Fc fusion proteins induced Th1 cell differentiation in vitro (Maekawa et al. 2003) and in vivo (Maekawa et al. 2003; Elyaman et al. 2007; Okamoto et al. 2008). However, IL12-induced Th1 cell responses were not altered by deletion of Notch1, Notch2, RBPJ, or the γ-secretase components presenilins 1 and 2 (Amsen et al. 2007, 2004; Ong et al. 2008). It was therefore hypothesized that

Notch might specifically regulate IL12-independent Th1 responses. In support of this, Th1 differentiation induced by CD8α⁻ DCs expressing DLL4, but not IL12, could be inhibited using blocking DLL4-Fc (Skokos and Nussenzweig 2007). Confusingly, however, one study reported that GSI did block the ability of IL12 to induce Th1 differentiation in vitro after all (Minter et al. 2005). This was initially attributed to the existence of other targets of γ-secretase than Notch (Beel and Sanders 2008; Wolfe and Kopan 2004). However, a more recent study made a strong case that Notch does in fact contribute to IL12-induced Th1 cell differentiation, but only when IL12 levels are low (Bailis et al. 2013). Correspondingly, Th1 responses and resistance to *Leishmania major*, a potent inducer of IL12, were unaffected in mice expressing a dominant-negative MAML (DN MAML) transgene (Tu et al. 2005). On the other hand, DN MAML did reduce Th1 cell responses in a model for graft versus host disease after allogeneic T cell transplantation, which probably does not elicit strong production of IL12 (Roderick et al. 2013; Tran et al. 2013; Zhang et al. 2011).

A two-pronged mechanism has been described to explain the role of Notch in Th1 cell differentiation. Notch signaling can induce expression of the lineage-defining transcription factor T-bet, presumably via an RBPJk-binding element in the promoter of the *Tbx21* gene (Minter et al. 2005; Bailis et al. 2013). Furthermore, Notch is recruited to RBPJk-binding sites in an enhancer of the *Ifng* gene (Bailis et al. 2013). Interestingly, this recruitment occurred regardless of the presence of polarizing cytokines. This suggests that Notch does not so much provide direction to the response but rather serves as an enhancer of a fate dictated by other truly polarizing signals.

4.4.2 Th2 and Th9 Cell Differentiation

Differentiation of Th2 cells can be efficiently elicited in vitro by TCR activation in the presence of IL4. Binding of IL4 to its receptor activates STAT6, which activates Th2-specific genes, including the gene that encodes the lineage-defining transcription factor Gata3. Gata3 enables expression of the *Il4*, *Il5*, and *Il13* genes and silences Th1 genes (Zhu et al. 2010). Despite the potent ability of IL4 to induce Th2 cell differentiation in vitro, strong IL4-independent Th2 differentiation occurs in vivo in the context of parasitic infections (King et al. 2008; Jankovic et al. 2000).

Microbial products (such as *Schistosoma mansoni* egg antigen (SEA) or *Vibrio cholerae* toxin), allergens (house dust mite extract), and pro-inflammatory mediators (prostaglandin E2) induce expression of Jagged2 on DCs (Amsen et al. 2004; Krishnamoorthy et al. 2008; Krawczyk et al. 2008). All these stimuli also promote the ability of DC to induce Th2 cell differentiation. Ectopic expression and genetic knockdown of Jagged, respectively, promoted and reduced Th2 cell differentiation in vitro in some (Liotta et al. 2008), but not in other studies (Ong et al. 2008; Krawczyk et al. 2008). It is presently not clear, therefore, whether Jagged truly has a role in T helper cell differentiation. Nonetheless, an important role for Notch signaling itself in Th2 cell differentiation is strongly supported by genetic experiments both in vitro and in vivo (Amsen et al. 2015).

Ectopic expression of the intracellular domain of each of the four Notch receptors (NICD1–4) promoted differentiation of Th2 cells (Amsen et al. 2004, 2007; Tu et al. 2005). Deficiency for Notch1 and Notch2, or for RBPJk, as well as expression of dominant-negative MAML1, resulted in loss of Th2 cell responses to parasite antigens or to antigens adsorbed to the adjuvant alum (Amsen et al. 2007; Tu et al. 2005). Th2 cell responses were also reduced by GSI in a model for asthma (Okamoto et al. 2009, 2012). Remarkably, one study showed that Notch may not necessarily regulate the entire Th2 cell differentiation program. In this study, deletion of *Notch1* and *Notch2* in CD4 T cells in mice infected with *L. mexicana* abrogated production of IL4, but not IL5 or IL13 (Fasnacht et al. 2014). In contrast, all three Th2 cell cytokines were inhibited by T cell-specific expression of a DN MAML transgene in response to the gastrointestinal parasite *Trichuris muris* (Tu et al. 2005). The requirement for Notch may thus be greatest for IL4 production. Part of the Th2 program can, however, apparently be regulated by Notch-independent pathways. Indeed, such a modular program would allow for much more flexible tailoring of the response to the demands of the infection.

Like Th1 differentiation, the requirement for Notch in Th2 cell differentiation in vitro can be overcome by high amounts of cytokine (IL4) (Amsen et al. 2007; Ong et al. 2008; Tu et al. 2005). One explanation might be that IL4 is genetically downstream of Notch. As the *Il4* gene is a direct target for Notch, addition of recombinant IL4 obviates the requirement for Notch signaling. Notch binds to a 3′ enhancer of the *Il4* gene, called HS5/CNS2. Responsiveness of this enhancer to Notch was documented by genetic experiments in mice, in which the entire enhancer was deleted or the RBPJκ binding elements were mutated (Amsen et al. 2004; Harada et al. 2012). Notch likely also transactivates the *Il4* gene in Tfh cells (see below) and NKT cells via HS5/CNS2 (SJ et al. 2015; Tanaka et al. 2006).

The *Gata3* gene is also directly transactivated by Notch-RBPJκ (Amsen et al. 2007; Fang et al. 2007) via the upstream of its two promoters. As with Th1 cell differentiation, Notch therefore seems to rely on a two-pronged mechanism to induce Th2 cell differentiation. It promotes expression of the lineage-defining transcription factor Gata3 and enhances expression of the major Th2 cell product IL4, which can further bolster Th2 cell differentiation in an auto/paracrine manner.

Notch was recently also implicated in differentiation of Th9 cells, which are related to Th2 cells and secrete IL9 and IL10 (Elyaman et al. 2012). Th9 cells also function in host defense against parasitic helminth infections (Noelle and Nowak 2010). They can derive from naïve CD4 T cells in the presence of IL4 and TGFβ but may also be generated from already developed Th2 cells. Th9 cells use similar transcription factors as Th2 cells, including STAT6, Gata3, Irf4, Batf, PU.1, and STAT5. The cytokines IL1β, IL6, IL21, and type I interferons enhance Th9 differentiation (Kaplan et al. 2015), whereas IL25 and IL2 promote IL9 secretion (Kaplan et al. 2015).

Induction of Th9 cells by the combination of IL4 and TGFβ is diminished by deficiency for Notch1 and Notch2 or for RBPJκ. Together with TGFβ, Jagged2 can induce differentiation of Th9 cells and functionally replace the role of IL4 in this process. Differentiation of Th9 cells is one example where interaction of Notch with another pathway (in this case TGFβ) modifies response output. In this case, this

interaction depends on physical interaction between NICD and the TGFβ responsive transcription factor Smad3, which together transactivate the *Il9* promoter (Elyaman et al. 2012).

4.4.3 Th17 and Th22 Cell Differentiation

Th17 cells produce the namesake IL17a and IL17f cytokines, as well as IL21 and IL22, and are involved in immunity against fungi and extracellular bacteria. TGFβ, IL6, IL1β, IL21, and IL23 have all been implicated in differentiation of Th17 cells and may control different stages in this process. Signaling induced by IL6, IL21, and IL23 activates STAT3, which induces expression of the lineage-specific transcription factors Rorγt and Rorα (Basu et al. 2013).

Expression of DLL4 on DCs correlates with their competence to induce Th17 cell differentiation (Meng et al. 2016) and DLL4 enhanced the generation of Th17 cells in the presence of the skewing cytokines IL6 and TGFβ (Mukherjee et al. 2009). NICD is recruited to the *Il17* and *Rorγt* genes, suggesting that Notch may directly transactivate key Th17 genes (Mukherjee et al. 2009; Bailis et al. 2013). Inhibition of Notch signaling with GSI or by siRNA against Notch1 during in vitro Th17 cultures resulted in a reduction of IL17a and Il17f production, but only when applied early during differentiation. As was the case for Th1 and Th2 cell differentiation, inhibition of Notch only effectively prevented Th17 cell differentiation when suboptimal concentrations of inducing cytokines were used (Bailis et al. 2013).

In vivo, inhibition of Notch signaling reduced production of IL17 and the progression of Th17-mediated disease in a mouse model for multiple sclerosis (EAE) (Keerthivasan et al. 2011; Eixarch et al. 2013). Finally, treatment with GSI alleviated acute airway inflammation of allergic asthma in mice, which was also attributed to downregulating Th17 cell responses (Zhang et al. 2015).

IL22 is one of the cytokines produced by Th17 cells, but this cytokine can also be produced by Th22 cells, which are akin to Th17 cells, but do not produce IL17 family cytokines (Basu et al. 2013). IL22 elicits innate antimicrobial responses from epithelia and promotes wound healing. Th22 cells differentiate from naïve CD4 T cells in the presence of IL6 and TNFα and depend on the transcription factor AHR. Notch signaling can induce IL22 production via a transcriptional mechanism involving RBPJκ, which led to the production of an as yet unidentified endogenous stimulator of AHR signaling (Alam et al. 2010).

4.4.4 Tfh Cell Differentiation

Tfh cells support B cell maturation, class switching, and affinity maturation in the germinal centers (King and Sprent 2012). They are characterized by expression of the surface molecules PD1 and ICOS as well as the chemokine receptor CXCR5.

Expression of CXCR5 allows Tfh cells to migrate into B cell areas in response to CXCL13. Naïve CD4 T cells can differentiate into Tfh cells in the presence of IL6 and IL21. Differentiation of Tfh cells is a stepwise process marked by intermediate expression of PD1, ICOS, and CXCR5 in early Tfh cells and high expression of these markers in fully functional Tfh cells. Characteristic for Tfh cells is expression of the transcription factors Bcl6 and STAT3. Cytokines can be produced by Tfh cells which include IL21, a cytokine known to promote B cell responses (Leonard and Wan 2016), as well as IL4 and IFNγ, consistent with the role of these cytokines in isotype class switching (Crotty 2014). Expression of CD40L is an important attribute of Tfh cells to control B cell expansion, survival, and class switching in germinal centers.

The discussion whether Tfh cells represent a distinct lineage is ongoing. The fact that many markers expressed on Tfh cells are also expressed on activated CD4 T cells makes the analysis difficult. Furthermore, Tfh cells can be generated from Th2 cells in response to helminth antigens (Glatman Zaretsky et al. 2009) and Th1 cells go through a Tfh-like transition (Nakayamada et al. 2011).

Th2-dependent antibody isotypes are strongly diminished when the Notch pathway is genetically disabled in T cells (Amsen et al. 2007; Tu et al. 2005; Bailis et al. 2013). In contrast, titers of unswitched IgM are actually sometimes even elevated in mice with such deficiencies, suggesting an accumulation of B cells unable to class switch (Amsen et al. 2007). This finding suggested a role for Notch in Tfh development and/or function. Indeed, the number of fully mature Tfh cells elicited by different immunization regimens was strongly reduced by T cell-specific deficiency for Notch1 and 2 (Auderset et al. 2013). Although a small number of Tfh cells did still develop in these mice, these produced reduced IL21 and expressed low levels of the lineage-defining transcription factors Bcl6 and cMaf (Auderset et al. 2013). The findings of this study suggested that Notch controls generation of (especially late stage) Tfh cells in general. It should be noted, however, that production of some class-switched antibody isotypes (notably IgG2a/c) was not reduced, or even enhanced, when Notch signaling in T cells was disabled (Amsen et al. 2007; Bailis et al. 2013; Tu et al. 2005). Moreover, production of Th2-dependent antibody isotypes can be restored in these mice by neutralization of IFNγ (Bailis et al. 2013). Finally, help by Tfh cells is required for affinity maturation, and high-affinity neutralizing antibodies were made by mice lacking Notch1 and 2 in T cells after infection with influenza virus (Backer et al. 2014). These findings all argue against a general requirement for Notch in Tfh generation and instead support a more specific role specifically in class switching to the type 2-dependent isotypes IgG1 and IgE. A specific role for Notch in type 2 antibody isotype switching may be explained, at least partially, by the fact that the Notch responsive HS5/CNS2 enhancer of the *Il4* gene (Amsen et al. 2004; Tanaka et al. 2006) is essential for IL4 expression by Tfh cells (Harada et al. 2012; Vijayanand et al. 2012). However, the dramatic defect in Tfh cells in the study by Auderset et al. suggests that the role of Notch may be broader (Auderset et al. 2013). Relevant may be that the immunization models used in this latter study all heavily favor type 2 immunity. It is attractive, therefore, to invoke the existence of a type 2 Tfh cell, whose differentiation would be strongly dependent on Notch.

4.5 Tolerogenic T Cells

Several subsets of CD4 T cells serve to maintain immunological tolerance to self-antigens as well as antigens derived from commensal and food. The most prominent among these are FoxP3$^+$ CD25$^+$ Tregs. Under some conditions, even traditional Th1 cells can assume tolerogenic properties by producing IL-10, and Notch was identified as one of the signals that can elicit such protective IL10 production (Kassner et al. 2010; Neumann et al. 2015). The suppressive mechanisms used by FoxP3$^+$ Treg cells are diverse and involve production of suppressive cytokines, competition for growth factors, and removal of costimulatory ligands from antigen-presenting cells (Josefowicz et al. 2012). Two types of Tregs exist: those that develop from immature T cells in the thymus (thymic Tregs) and others that develop in the periphery from naïve CD4 T cells (peripheral Treg). This latter cell type can be induced in vitro by activating naïve CD4 T cells in the presence of TGFβ, in which case these cells are referred to as induced Treg (iTreg) (Josefowicz et al. 2012).

The role of Notch in Tregs is currently confusing. In vitro generation of iTreg in the presence of TGFβ was less efficient when Notch signaling was disabled by GSI or by genetic deletion (Samon et al. 2008; Marcel and Sarin 2016), although this was not confirmed in another study (Charbonnier et al. 2015). In several studies, stimulation with Jagged elicited or promoted expansion of T cells with suppressive capacity (Samon et al. 2008; Cahill et al. 2015; Gopisetty et al. 2013; Kared et al. 2006; Vigouroux et al. 2003; Lin et al. 2015), perhaps explained by induction of IL9 production by Notch, which promotes Treg expansion (Yvon et al. 2003). DLL1-Notch signaling enhanced the in vitro conversion of human memory cells into Treg cells (Mota et al. 2014). Finally, noncanonical Notch signaling was implicated in Treg resistance to growth factor deprivation by promoting protective autophagy (Marcel and Sarin 2016).

Surprisingly, however, when expression of Notch or RBPJk was deleted specifically in Tregs, these cells exhibited enhanced, rather than reduced, regulatory function and survival in vivo (Charbonnier et al. 2015). Vice versa, expression of NICD1 in Treg cells diminished their regulatory capacity and converted them into Th1-like effector cells (Charbonnier et al. 2015), suggesting that Notch antagonizes the Treg program.

These results seem to fit with a systems biological view of Notch as a pathway involved in promoting T cell effector responses by boosting T cell activation and effector function on the one hand and eliminating opposition to such responses by Tregs. Despite the appeal of such a coherent view, it must be mentioned that a recent study found that increased Treg-specific deletion of Notch1 did result in pathology (Marcel and Sarin 2016), in stark contrast to the study mentioned above (Charbonnier et al. 2015). At this stage, the jury is therefore still out regarding the role of Notch in Tregs. It does seem safe, however, to conclude that a potential positive role for Notch in Tregs is modest at best, considering that mice with T cell-specific deletions in the Notch pathway (Notch1, Notch2, RBPJ) do not present with overt pathology or even elevated activation of conventional T cells (Amsen et al. 2007) (CH, DA unpublished observations).

4.6 Concluding Remarks

Notch has now been implicated in the generation and function of every major CD4 T cell subset as well as in general T cell activation and survival. It seems likely, therefore, that this signaling pathway does not so much function to impart direction on the response but rather serves as a general promoter of T cell responses. The induction of Notch ligands by pattern recognition receptors is consistent with the idea that activation of Notch is a licensing signal that marks the presence of microbial danger, justifying progression of T cell responses. Although Notch is not universally required for all (aspects of) CD4 T cell responses under all conditions, the role for Notch is often more pronounced in vivo than in traditionally used in vitro differentiation systems. One explanation for this discrepancy is the finding that high concentrations of skewing cytokines, used to obtain robust differentiation in experiments in vitro, can override the requirement for Notch. How Notch functions in so many different processes is still not completely understood but may depend on context-dependent accessibility of promoter and enhancer regions as well as on physical interactions with signaling effectors from other signaling pathways. Proof of principle exists that Notch can be targeted to mitigate CD4 T cell-dependent immune pathology, for instance, in graft versus host disease, asthma, and autoimmune disorders. Clinical exploitation may, however, be complicated by the many critical roles of Notch in other cell types. Identification of the molecular mechanisms that determine specificity of the response to Notch may ultimately hold the key to unlock the therapeutic potential of Notch in immune pathology.

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Part II
Cancer

Chapter 5
NOTCH in Malignant Lymphoma

Shigeru Chiba

Abstract Next-generation sequencing has provided knowledge about frequent somatic mutations in *NOTCH1* and *NOTCH2* genes in malignant lymphomas, represented by mature B-cell neoplasms including chronic lymphocytic leukemia. While physiologic roles of NOTCH signaling through NOTCH2 have been amply understood in splenic marginal zone B-cell development, functional significance of NOTCH1 expression in various mature B cells has been elusive, though well-understood in developing thymocytes. The discovery of frequent *NOTCH1* mutations in several mature B-cell malignancies, however, reminds us of the importance of NOTCH signaling in mature B cells, by means of reverse genetics.

Keywords NOTCH • NOTCH1 • NOTCH2 • Lymphoma • Leukemia • B-cell receptor • Mutation

5.1 Introduction

A quarter century has passed since the first discovery of disease-associated abnormalities in NOTCH signaling, i.e., truncated NOTCH1 expression in T-cell acute lymphoblastic leukemia (T-ALL) due to a rare somatic chromosomal translocation t(9;11) (Ellisen et al. 1991). For over a decade since then, several seminal findings were aligned in a well-ordered manner. These included (1) truncated NOTCH1 transduced signaling independent of ligand binding (Jarriault et al. 1995); (2) hematopoietic stem/progenitor cells expressing truncated forms of NOTCH1 induced a T-ALL-like disease when transplanted into mice (Pear et al. 1996); (3) deficiency of *Notch1* gene in mice caused a defect in thymocyte development, and in its compensation, B-cells expanded in the thymus (Radtke et al. 1999); and (4) *NOTCH1* gene was somatically mutated and thus activated in approximately a half the human

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K. Yasutomo (ed.), *Notch Signaling*, DOI 10.1007/978-981-10-4971-2_5

T-ALL patients (Weng et al. 2004). In contrast, *Notch2* gene disruption in the blood cell compartment resulted in the defect in splenic marginal zone B cells (SMZB) in mice (Saito et al. 2003).

These early findings led to the conclusions, with a contrast, as follows: physiologic signaling through NOTCH1 is important for normal T-cell specification and development, while suppressing B-cell developmental pathway; acceleration of NOTCH1 signaling causes T-ALL; and NOTCH2 is specifically important for development of SMZB (Chiba 2006). Such a scenario, however, was complicated by the discovery of frequent *NOTCH1* gene mutations in a variety of mature B-cell neoplasms, after the introduction of the second-generation sequencing (NGS) technology.

Neoplasms of mature B lymphocytes can be roughly divided into three major categories: indolent types of lymphomas/leukemias, aggressive types of lymphomas, and plasma cell neoplasms. Indolent types of lymphomas/leukemias, comprising relatively small-sized tumor cells, include marginal zone lymphoma (MZL), mantle cell lymphoma (MCL), follicular lymphoma (FL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), etc. Aggressive types of lymphomas, comprising medium- to large-sized tumor cells, are represented by diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). Plasma cell neoplasms are mostly represented by multiple myeloma. Each disease entity, particularly that in the indolent lymphomas/leukemias, has distinct biological, pathological, and clinical features. In contrast, DLBCL, the most common diagnosis accounting for 30–40% of mature B-cell lymphomas, contains heterogeneous groups such as transformed indolent lymphomas/leukemias, in addition to de novo diseases. Anyway, the origin of tumor cells of each disease is thought to correspond to a certain B-cell subset in the immune compartment.

As described above, understanding of significance of NOTCH signaling for development/activation of each mature B-cell subset has been largely limited to that in SMZB. Genetic studies of human neoplasms should provide us, by means of reverse genetics, with tales of NOTCH biology that have not been clarified by mouse studies.

5.2 Genetic Aberrations of *NOTCH* Genes in Mature B-Cell Neoplasms: Prologue

As mentioned, it had been established that signaling through Notch2 is important for physiologic development of mouse SMLB (Hozumi et al. 2004; Kuroda et al. 2003; Saito et al. 2003). In the years of 2008–2009, 7 *NOTCH2* mutations were reported in 2 out of 41 MZL (5%) (28 splenic MZL and 13 nodal or extranodal MALT-type MZL) (Trøen et al. 2008) and 5 out of 63 DLBCL (8%) (Lee et al. 2009). Five of the seven mutations predicted to produce NOTCH2 with the truncation of the PEST domain. Such truncated NOTCH2 is presumed to have a prolonged half-life and thus enhanced signaling, considering the similarity with the

case of NOTCH1 (McGill and McGlade 2003; Oberg et al. 2001; Weng et al. 2004). The others were missense mutations at the PEST domain and the heterodimerization domain, predicting (Weng et al. 2004) or demonstrating (Lee et al. 2009) enhancement of NOTCH signaling. Thus, although the precise origin of SMZL tumor cells was elusive, it was discussed that, in contrast to physiologic importance of NOTCH2 signaling in SMZB, its enhancement in SMZB due to *NOTCH2* mutations causes SMZL.

5.3 *NOTCH1* Mutations in Chronic Lymphocytic Leukemia

Such a hypothesis, however, was obscured by the discovery of frequent somatic *NOTCH1* mutations causing truncation of the PEST domain in CLL (Fabbri et al. 2011; Puente et al. 2011; Wang et al. 2011). CLL is characterized by CD5/CD23 expression in tumor cells, and in Caucasian, by the greatest frequency in all kinds of leukemias (Swerdlow et al. 2008). The origin of tumor cells is postulated to be antigen-experienced B cells (Chiorazzi et al. 2005). Because of the highly selected usage of immunoglobulin heavy chain variable (*IGHV*) gene, it is argued that a limited set of autoantigens is responsible for cell growth (Swerdlow et al. 2008). At the later phase of the disease, if not at the initial presentation, most of CLL patients experience lymphadenopathy, the pathologic findings of which are identical to small lymphocytic lymphoma, and CLL is often referred to as CLL/SLL. *NOTCH1* mutations were found in approximately 10% of CLL at diagnosis (Table 5.1), while 20% in chemotherapy-refractory cases of CLL (Fabbri and Dalla-Favera 2016; Zent and Burack 2014). The *NOTCH1* mutations were associated with a poorer prognosis in a number of retrospective analyses, the results confirmed in several clinical trial settings (Zent and Burack 2014). CLL, having an indolent nature at the beginning, is known to occasionally develop into DLBCL, an aggressive form of neoplasms designated Richter transformation, and *NOTCH1* mutations were found to be more frequent in this type of transformed tumors (30–40%) (Fabbri et al. 2011; Rossi et al. 2012a).

According to extensive genetics studies, CLL is extremely heterogeneous in nature; while a large number of genes are identified to be mutated, no single genes are mutated at frequencies over 10–15%, and only a handful number of genes are recurrently mutated at 10–15% (Fabbri and Dalla-Favera 2016; Quesada et al. 2013) (Fig. 5.1). Among them, *NOTCH1* mutations (Fabbri et al. 2011; Puente et al. 2011) are the most frequent. The other recurrently mutated genes are categorized into several biological mechanisms and signaling pathways such as RNA splicing (represented by *SF3B1*), DNA damage (*ATM* and *TP53*), B-cell receptor (BCR)-NF-kB-Toll-like receptor (TLR)/B-cell activation (*PAX5* and *MYD88*), cell cycle/apoptosis (*BRAF*), etc. (Table 5.1; Fig. 5.2). The mutations in *ATM* and *TP53* predict the worst prognosis, followed by those in *NOTCH1* and *SF3B1*. In the vast majority of cases having *NOTCH1* mutations are found in a mutually exclusive manner with either *TP53* or *SF3B1* mutations and are abstracted as a risk factor independent of *TP53* or *SF3B1*

Table 5.1 Mutations in *NOTCH* genes and NOTCH signaling pathway genes in mature B-cell neoplasms

Disease	NOTCH pathway genes	Frequencies	Biological relevance/ characteristics	Other frequently mutated genes	References
CLL[*1]	*NOTCH1*[*8] *FBXW7* *SPEN*	10% (all cohort) 20% (chemo-r[*9]) 30–40% (Richer[*10]) 24–40% (+12[*11])	Unknown	*SF3B1*[*8] *PAX5* *MYD88* *ATM* *TP53*	Fabbri and Dalla-Favera (2016) and Zent and Burack (2014) Fabbri et al. (2011) and Rossi et al. (2012a)
SMZL[*2]	*NOTCH2*[*8] *NOTCH1 SPEN* *DTX1* *MAML1/MAML2*	10–25% 30–40%	Development of SMZB	*KLF2*[*8]	Kiel et al. (2012), Martínez et al. (2014), Parry et al. (2015), and Rossi et al. (2012b)
MCL[*3]	*NOTCH1* or *NOTCH2*	10–12%	Unknown	*ATM*[*8] *CCND1*[*8] *TP53*[*8]	Kridel et al. (2012) and Beà et al. (2013)
DLBCL[*4]	*NOTCH2* *NOTCH1* *DTX1*	6–8% 7–8% 12%	Transformation from MZL?	*MLL2*[*8] *CREBBP*[*8] *B2M*[*8]	Lee et al. (2009), de Miranda et al. (2014), and Rossi et al. (2013)
FL[*5]	*NOTCH1* *or* *NOTCH2*	6.3%	(+) in 7/7 female, 6/7 ExN[*12], 5/7 splenic[*13]; 1/7 t(14;18)	*MLL2*[*8] *CREBBP TNFRSF14*	Karube et al. (2014)
NMZL[*6]	*NOTCH2*, NOTCH pathway genes	20% 40%	Unknown	*MLL2*[*8] *PTPRD*[*14]	Spina et al. (2016)
HCV-DLBCL[*7]	*NOTCH2*	20%	Relevance to SMZL	*TNFAIP3*[*8]	Arcaini et al. (2015)

*1, chronic lymphocytic leukemia/small lymphocytic lymphoma; *2, splenic marginal zone lymphoma; 3, mantle cell lymphoma; *4, diffuse large B-cell lymphoma; *5, follicular lymphoma; *6, nodal marginal zone lymphoma; *7, hepatitis C virus-associated DLBCL; *8, most frequently mutated gene(s) in the entity; *9, chemotherapy-resistant cohort; *10, cohort of Richter transformation; *11, cohort with trisomy 12; *12, extranodal involvement; *13, splenic involvement; *14, unique to or enriched in the entity

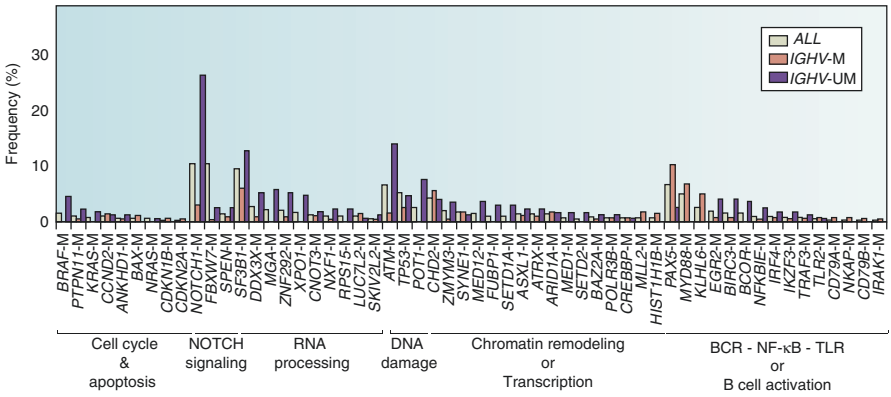

Fig. 5.1 Genetic landscape of chronic lymphocytic leukemia (CLL). Genes are grouped according to the biological program or pathway. *All* all cases, *IGHV-M* immunoglobulin heavy chain variable region-mutated, *IGHV-UM* IGHV-unmutated. Modified from Fabbri and Dalla-Favera 2016 with permission

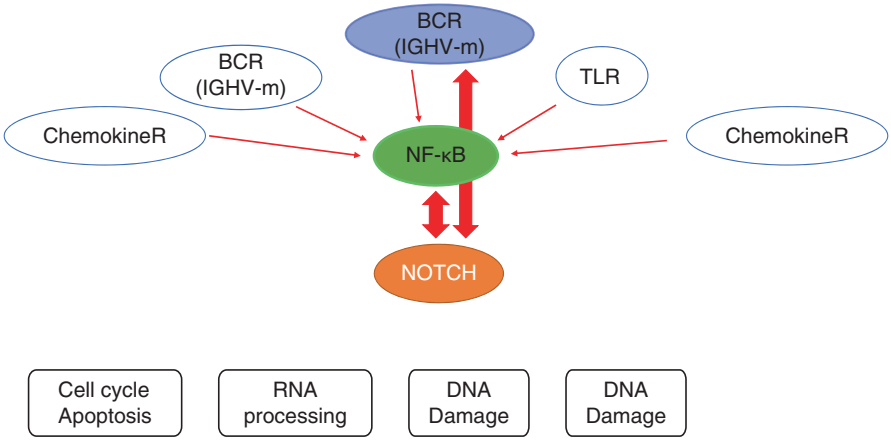

Fig. 5.2 Possible interactions with biological programs and pathways whose component genes are frequently mutated in mature B-cell neoplasms

mutations. These lines of evidence imply that CLL could be subclassified due to distinct pathway abnormalities, without a major overlap.

Four types of chromosomal abnormalities are frequent in CLL: deletions of 13q (present in approximately 50%), 11q (15–20%), 17p (5–10%), and trisomy 12 (20%) (Swerdlow et al. 2008). *NOTCH1* mutations are the most common in CLL having trisomy 12 (24–40%) (Balatti et al. 2012; Del Giudice et al. 2012; Villamor et al. 2013), while the biological significance of this finding is elusive.

In CLL, *IG* gene is hyper-mutated (40–50%) or unmutated (50–60%), and many gene mutations are associated preferentially with either *IG* hyper-mutated or

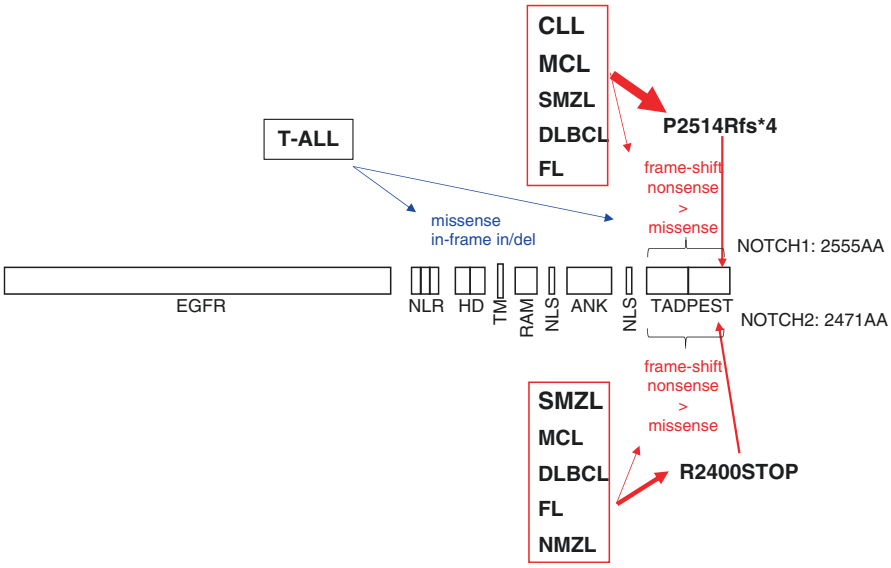

Fig. 5.3 Distribution of mutations within *NOTCH1* and *NOTCH2* genes. *NOTCH1* is mutated in approximately 50% of T-cell acute lymphoblastic leukemia (T-ALL) patients; the majority are missense or in-frame in/del mutations at the heterodimerization domains (HD) or frameshift or nonsense mutations at the transactivation domain (TAD) or PEST domain (PEST). In contrast, mutations are found in both *NOTCH1* and *NOTCH2* genes in mature B-cell neoplasms, and accumulated at TAD/PEST domains, whereas mutations are very rare at HD. *NOTCH1* mutations are frequent in chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) and less frequent in splenic marginal zone lymphoma (SMZL), diffuse large B-cell lymphoma (DLBCL), and follicular lymphoma (FL). *NOTCH2* mutations are most frequent in SMZL and very rare in CLL. Unlike TAD/PEST domain mutations in T-ALL, *NOTCH1* and *NOTCH2* mutations have a hotspot at c.7541_7542CTdel, resulting in p.P2514Rfs*4, and c.7198C>T, resulting in R2400STOP, respectively. Particularly, approximately 80% of *NOTCH1* mutations in CLL are accumulated at c.7541_7542CTdel (p.P2541Rfs*4)

unmutated cases (Fabbri and Dalla-Favera 2016). Signaling from hyper-mutated and unmutated BCR could be different in multiple aspects because of the differences in the affinity to antigens and the usage of IG repertoires. This could, at least in part, influence the CLL pathophysiology. Each chromosomal and genetic abnormality is linked to the *IG* mutational status; *NOTCH1* mutations are identified in both types of CLL, but much more frequent in unmutated cases (Fabbri and Dalla-Favera 2016). It is yet to be elucidated whether mutated NOTCH1 has a role in association with signaling from unmutated IG-based BCR.

Interestingly, *NOTCH1* mutations in CLL have a hotspot; approximately 80% of mutations are the frameshift mutation at a single position in the PEST domain, c.7541-7542delCT (p.P2514Rfs*4) (Puente et al. 2011; Weissmann et al. 2013; Zent and Burack 2014) (Fig. 5.3). The consequence of this mutation is the PEST domain truncation, basically the same as other frameshift mutations. It is unknown why this specific mutation is commonly accumulated in CLL. Nevertheless, this hotspot mutation

provides a diagnostic value. Because the frequencies of *NOTCH1* mutation alleles are low in a relatively large proportion of CLL patients, increased detection sensitivity improved the discovery of *NOTCH1* mutations by the use of an allele-specific primer-based PCR method (Sportoletti et al. 2014). According to this report, the frequencies of *NOTCH1* mutations may be substantially higher than described so far.

Furthermore, mutations in the *NOTCH1* were recurrently found in CLL at 3′ UTR, resulting in increased stability of mRNA, emphasizing the importance of enhanced NOTCH signaling in CLL even without carrying PEST domain-truncated NOTCH1 expression (Puente et al. 2015).

5.4 Mutations of *NOTCH2* and Other NOTCH Pathway Genes in Splenic Marginal Zone Lymphoma

Splenic MZL (SMZL) is a mature B-cell neoplasm comprising less than 2% of lymphoid neoplasms, mainly infiltrating splenic white pulp. The tumor cells are negative for both CD5 and CD23 and often circulate in the peripheral blood (Swerdlow et al. 2008). Other than the spleen, the bone marrow and the liver are often involved, but the peripheral lymph nodes except for the splenic hilar nodes are not typically involved. *IG* somatic hypermutation is present in 50% of cases.

As described in the "prologue" section, a previous work identified only 1 *NOTCH2* mutation in 28 SMZL cases (Trøen et al. 2008). Nevertheless, gain-of-function mutations of *NOTCH2* were found to be among the most frequent genetic lesions, accounting for 10–25% of the in SMZL cases by NGS (Kiel et al. 2012; Martínez et al. 2014; Parry et al. 2015; Rossi et al. 2012b), together with *KLF2* mutations (Clipson et al. 2015) (Table 5.1; Fig. 5.2). Mutations in other NOTCH pathway-related genes such as *NOTCH1*, *SPEN*, *DTX1*, and *MAML1/MAML2* were also identified, and 30–40% of SMZL have mutations in *NOTCH2* or one of the NOTCH pathway genes (Rossi et al. 2012b). "SMZL" was named by pathologists based on morphologic findings of lymphoma samples, but the origin of the tumor cells of SMZL has been still unclear. This is because the identity of the human analogous subset of mouse SMZB is obscure (Benitez et al. 2014; Weill et al. 2009), in contrast to the fact that the mouse version has been extensively characterized. The discovery of highly frequent *NOTCH2* as well as other NOTCH pathway gene mutations in SMZL may be a clue to locating the origin of SMZL tumor cells to the analogue of mouse SMZB.

Most of the *NOTCH2* mutations identified in SMZL cause the PEST domain truncation, as seen in *NOTCH1* mutations in CLL. There is a hotspot mutation in *NOTCH2* in SMZL, a missense mutation, c.7198C>T (p.R2400X), similar to the hotspot *NOTCH1* mutation c.7541-7542delCT in CLL (Fig. 5.3). Notably, the c.7198C>T mutation was also identified in 3 of 5 *NOTCH2* mutations previously described in DLBCL (Lee et al. 2009). Again, the reason of the selection of this hotspot is unknown.

Recurrent mutations found in SMZL other than NOTCH signaling genes included those in the NF-κB signaling pathway, which is also important for marginal zone development (Clipson et al. 2015; Martínez et al. 2014; Rossi et al. 2012b)

5.5 *NOTCH1* and *NOTCH2* Mutations in Mantle Cell Lymphoma

MCL accounts for approximately 3–10% of mature B-cell lymphomas, characterized by the cyclin D1 expression. The tumor cells also express CD5 in most of the cases. The postulated normal counterpart is naïve pre-germinal center B cell at the inner mantle zone. The expression of cyclin D1 is caused by the t(11;14)(q13;q32) chromosomal translocation, at the break point of which *CCND1* expression is positively regulated by the *IGH* enhancer (Swerdlow et al. 2008).

Mutations in cell cycle/apoptosis and DNA damage-response genes, such as *ATM*, *CCND1*, and *TP53*, have been reported. The transcriptome (Kridel et al. 2012) and whole genome/exome (Beà et al. 2013) sequencing confirmed that mutations in those genes were indeed the most frequent. In addition, by these NGS studies, mutations in *NOTCH1* or *NOTCH2* were discovered in 10–12% of MCL cases. In the cohort of 172 MCL, *NOTCH1* and *NOTCH2* mutations were found in 8 and 9 cases in a mutually exclusive manner, with only one exception. Both mutations were significantly more frequent in blastic/pleomorphic MCL, being known to have poorer survival. Indeed, mutations in *NOTCH1* or *NOTCH2* conferred a shorter survival (Beà et al. 2013).

The vast majority of mutations in both *NOTCH1* and *NOTCH2* cause truncation of the PEST domain. The *NOTCH1* mutational hotspot found in CLL, c.7541_7542CTdel (p.P2514Rfs*4) also serves in MCL; 8 of 16 *NOTCH1* mutations occurred at this location (Kridel et al. 2012). Similarly, *NOTCH2* mutational hotspot found in SMZL, c.7198C>T (p.R2400X), also serves in MCL; 4 of 9 *NOTCH2* mutations occurred at this location (Beà et al. 2013; Kridel et al. 2012) (Fig. 5.3).

5.6 NOTCH Pathway Gene Mutations in Diffuse Large B-Cell Lymphoma

As described, the discovery of *NOTCH2* mutations in a fraction of DLBCL was one of the initial findings of NOTCH pathway gene mutations in mature B-cell neoplasms (Lee et al. 2009). In accordance with this, *NOTCH2* mutations were found in 6–8% of DLBCL in the subsequent works (de Miranda et al. 2014; Rossi et al. 2013). In addition to *NOTCH2*, mutations were also found in *NOTCH1* at 7–8% of DLBCL (de Miranda et al. 2014). Intriguingly, the latter paper reported more frequent (16/136; 12%) mutations in *DTX1*, one of the NOTCH signaling molecules. Physiologically, N-terminal domains of DTX1 directly interact with the NICD and repress NOTCH signaling by impeding the recruitment of transcriptional coactivators (Izon et al.

2002; Matsuno et al. 1998). At least some DTX1 mutants lose this repressive function, allowing enhanced NOTCH signaling (de Miranda et al. 2014).

5.7 NOTCH Pathway Gene Mutations in Other Mature B-Cell Neoplasms

NOTCH pathway gene mutations have been found in mature B-cell neoplasms other than the mutations described above, as those among major gene mutations. These include FL (Karube et al. 2014), nodal MZL (NMZL) (Spina et al. 2016), and hepatitis C virus (HCV)-associated DLBCL (Arcaini et al. 2015). Both NMZL and HCV-associated DLBCL demonstrate the mutational profiles very similar to SMZL.

In mice, SMZB have been characterized as innate-like B cells responding to blood-borne antigens in a T-cell-independent manner, and this subset of B cells had been considered to be unique to the spleen. Just recently, the nodal marginal zone B cells in mice were firstly described (Palm et al. 2016). On the other hand, NMZL is named mostly by the morphological observations similar to the case of SMZL and distributions of tumors. Nevertheless, genetic similarities between SMZL and NMZL may provide us with triangular connections in marginal zone B cells in the spleen and lymph nodes and those in mouse and humans.

HCV-associated lymphomas are mainly diagnosed as SMZL or DLBCL, and HCV-associated DLBCL usually shows splenomegaly. Therefore, the origin of HCV-associated lymphomas is speculated as splenic marginal zone B cells, and it appears reasonable that HCV-associated DLBCL shows the similar mutational profiles with SMZL.

FL is the second most common type of lymphoma accounting for approximately 15% of cases. This type of lymphoma is characterized by a somatic t(14;18) translocation, which causes deregulated expression of BCL2 by the *IGH* gene enhancer. BCL2 is strictly repressed in the normal germinal center cells and maintains unexpressed condition in the reactive follicular hyperplasia. Thus, the BCL2 expression in the germinal center is among the hallmarks of FL. It is remarkable therefore that only one of seven FL cases having *NOTCH1* or *NOTCH2* mutations show t(14;18), together with the marked female dominance; all of the seven were female (Table 5.1). *NOTCH1*- or *NOTCH2*-mutated FL is likely to fall into a distinct entity.

5.8 Perspectives and Speculations

5.8.1 Physiologic NOTCH Signaling in Mature B Cells.

The physiologic role of NOTCH signaling in mature B cells has been best understood in murine SMZB development, and this signaling has been understood to be transmitted through NOTCH2. In contrast, despite the fact that NOTCH1 is expressed on various B cells, significance of this expression has not been addressed

well. Although involvement of NOTCH1 signaling for B-cell differentiation into antibody-secreting cells was reported in an in vitro study (Santos et al. 2007), definitive proof is lacking.

It might be possible that NOTCH signaling plays a significant role in the physiologic development of various subsets of B cells other than marginal zone B cells, such as mantle zone B cells, germinal center/follicular B cells, and antigen-experienced B cells. This could be proven by analyzing mice with deleted *Notch1* or *Notch2* in specific compartment of mouse B cells. Should this be proven, abnormal enhancement of the normal developmental signals might lead to neoplastic transformation of each subset of mature B cells, as is in SMZB and possibly in nodal marginal zone B cells.

5.8.2 NOTCH Signaling in B-Cell Activation

Irrespectively of whether NOTCH signaling is physiologically involved, enhancement of NOTCH signaling may accelerate B-cell activity through BCR, chemokine receptors, or Toll-like receptors (Fig. 5.2). Particularly, modulation of *IG*-unmutated BCR may be an important mechanism how enhanced NOTCH signaling is involved in neoplastic transformation. MCL and CLL might be explained by this scenario. Downstream of BCR, NOTCH pathway could cross talk with NF-κB signaling, given that the interactions between NOTCH and NF-κB signaling pathways have been reported in different contexts (Espinosa et al. 2003; Guan et al. 1996), as well as in CLL (Xu et al. 2015). Throughout the different categories of mature B-cell malignancies, mutations are frequent in the NF-κB pathway genes, as well as in the BCR pathway genes and the NOTCH pathway genes.

Epstein-Barr virus (EBV) infection is well known to be associated with B-cell growth as well as establishment of several B-cell lymphomas, such as BL and a fraction of DLBCL (Swerdlow et al. 2008). While the EBV genes actively involved in each condition are variable, EBV nuclear antigen 2 (EBNA2), known to be a growth driver, uses NOTCH pathway by binding to CBF1/Rbp-J (Thorley-Lawson and Allday 2008). Although this information implies, albeit indirectly, involvement of NOTCH signaling in B-cell growth, EBNA2 binding to CBF1/Rbp-j is reported to activate a distinct set of target genes from that by NOTCH signaling (Kohlhof et al. 2009). Thus, it is still elusive whether EBV infection fills the missing link between NOTCH signaling and lymphomagenesis.

5.8.3 NOTCH Signaling and Epigenetic Regulation

Upon activation, the intracellular NOTCH1 (ICN1) is reported to bind to histone deacetylases. According to this report, this molecular association negatively regulates expression of CD20, resulting in downregulation of its cell surface presentation (Pozzo et al. 2016). This might explain, albeit partially, association between *NOTCH1* mutation and drug resistance.

5.8.4 NOTCH Signaling and Tumor Microenvironment

Recently, NOTCH signaling was shown to be actually activated in the tumor cells in the lymph nodes of CLL patients carrying *NOTCH1* mutations (Arruga et al. 2014). The authors also demonstrated that NOTCH signaling was turned off when the cells were transferred into an in vitro condition. This indicates that ligand stimulation is indeed provided within the tumor microenvironment. This might convey an answer to the question: why the vast majority of *NOTCH1* as well as *NOTCH2* mutations in mature B-cell neoplasms cause PEST domain truncation, rather than ligand-independent activation due to mutations at the heterodimerization domains, which are also frequent in T-ALL (Weng et al. 2004). In malignant lymphoma, ligand-independent activation might not be important because of abundant ligand density in the tumor microenvironment. Rather than that, prolonged half-life of intracellular NOTCH after the ligand-dependent NOTCH cleavage might be specifically important. As such, the consideration in the context of tumor cell-environmental cell interaction could be a key to better understanding NOTCH signaling in mature B-cell neoplasms.

5.8.5 Implications in Clinical Settings

Mutations in NOTCH pathway genes found in myeloid malignancies cause reduction of the signaling activity (Klinakis et al. 2011), corresponding to another genetic evidence showing tumor-suppressive role of NOTCH signaling (Kato et al. 2015). A similar tumor-suppressive role of NOTCH signaling was demonstrated in B-lineage cells through in vitro experiments. However, genetic evidence in human diseases clearly indicates that abnormally enhanced NOTCH signaling plays tumor-promoting roles in mature B-cell neoplasms. Thus, clinical application of NOTCH signaling inhibitors may be considered in this category of cancers.

In prospective and retrospective clinical observations, mutations in *NOTCH1* and *NOTCH2* are both associated with poor prognosis in most of the mature B-cell malignancies. Biological significance is to be clarified in the future.

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Chapter 6
The Two Faces of Notch in Solid Cancers

Craig S. Nowell and Freddy Radtke

Abstract Aberrant Notch signalling is associated with a variety of solid tumours. Therefore, understanding the role Notch signalling plays during the development and progression of cancer is an area of considerable interest, and a deeper knowledge of its influence on cellular processes will potentially lead to improvements in both the prevention and treatment of cancer.

Keywords Cancer stem cells • Differentiation • Inflammation • Tumour stroma • SCC • ECM

Interestingly, Notch can act as an oncogene or tumour suppressor depending on the tissue context (Koch and Radtke 2007) (Fig. 6.1). Thus, some cancers display increased Notch signalling activity and are dependent on Notch for growth and malignant progression. Conversely, in other cancers, inactivation of Notch signalling is essential for carcinogenesis, indicating that Notch can function as an important tumour suppressor. In the following sections, the supporting evidence for both oncogenic and tumour suppressive roles of Notch will be discussed, as will the mechanisms by which Notch signalling influences carcinogenesis.

6.1 Cancers Associated with Active Notch Signalling

Historically, the evidence supporting an oncogenic role for Notch signalling has been provided predominantly by the study of the haematological malignancy T-cell acute lymphoblastic leukaemia (T-ALL). In this disease, activating mutations in

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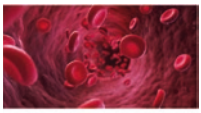
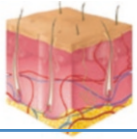
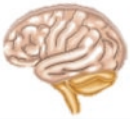
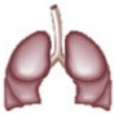
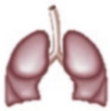
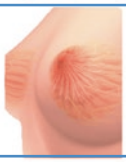
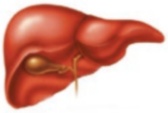
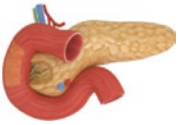
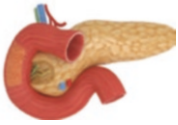
Oncogenic functions of Notch in	Ref.	Tumor suppressive function of Notch in	Ref.
	Koch and Radtke 2011		Nicolas et al. 2003 Pickering et al. 2014 Wang et al. 2011
	Gao et al. 2007 Kanamori et al. 2007 Purow et al. 2005 Zhao et al. 2010 Zhung et al. 2008 Gaetani et al. 2010 Fan et al. 2004		Alcolea et al. 2014 Gao et al. 2014 Song et al. 2014
	Zheng et al. 2013		George et al. 2015
	Sansone, P. et al, 2006 Shipitsin et al. 2007 Reedijk et al. 2005 Hu et al. 2006 Pece et al. 2006 Ayyanun et al. 2006		Viatour et al. 2011
	Miyamoto et al. 2003 Mazur et al. 2010 Plentz et al. 2009 Maniati et al. 2011 Cook et al. 2012 De La et al. 2008		Hanlon et al. 2010

Fig. 6.1 Schematic representation of tissues in which Notch signalling is oncogenic and/or tumour suppressive. *Left side* of the panel represents major human tissues in which an oncogenic role for Notch has been described, whereas the *right side* shows tissues where Notch exerts tumour-suppressive activities. References related to oncogenic and tumour-suppressive functions in a given tissue are indicated

Notch1 are present in 50–60% of cases, and there is considerable functional data indicating that Notch plays a crucial role in driving the development and growth of T-ALL (Koch and Radtke 2011a, b). However, numerous studies also suggest that Notch acts as an oncogene in a variety of solid tumours (Galluzzo and Bocchetta 2011; Reedijk 2012; Teodorczyk and Schmidt 2014).

For example, increased expression of Notch pathway components has been observed in human gliomas, which are a group of primary brain tumours derived from the glial tissue of the central nervous system. In these malignancies, increased expression of Notch receptors, Notch ligands and downstream targets such as Hey-1, is associated with increasing tumour grade (Somasundaram et al. 2005; Phillips et al. 2006; Xu et al. 2009, 2010; Hulleman et al. 2009). Furthermore, inhibition of Notch1 in glioma cell lines induces cell cycle arrest,

while constitutive activation of Notch signalling results in increase proliferation (Gao et al. 2007; Kanamori et al. 2007; Purow et al. 2005; Zhao et al. 2010, 2008). Inhibition of Notch1 or Dll1 in in vivo models also results in delayed tumour growth (Xu et al. 2010; Purow et al. 2005), and high expression of Hey-1 in human gliomas is associated with a poor prognosis (Hulleman et al. 2009; Gaetani et al. 2010).

Notch may also function as an oncogene in medulloblastoma, which is a brain tumour derived from neuronal precursor cells in the cerebellar cortex and is distinct from the gliomas discussed above. In this example, the expression of Notch2 and the target gene Hes-1 is upregulated in medulloblastoma and can promote proliferation when overexpressed (Xu et al. 2009; Fan et al. 2004).

Increased Notch signalling activity is also linked to the development and progression of breast cancer. High expression levels of Notch1, Notch3 and Jag1 are observed in many cases of breast cancer and are associated with a poor prognosis (Sansone et al. 2007; Shipitsin et al. 2007; Reedijk et al. 2005). Consistent with this, overexpression of Notch1 and Notch3 in mice promotes mammary tumour development (Sansone et al. 2007; Hu et al. 2006). Furthermore, loss of numb expression, which is a negative regulator of Notch activity, is frequently observed in primary human breast cancers (Pece et al. 2004). Notch signalling can also cooperate with other signalling cascades, such as Wnt, to promote the transformation of human primary mammary epithelial cells (Ayyanan et al. 2006) further supporting the hypothesis that overactive Notch signalling is oncogenic in this tissue.

Pancreatic cancer has also been linked to increased Notch signalling. Notch target genes are frequently expressed in PDAC cells, suggesting that Notch activity is associated with development and progression of the disease (Miyamoto et al. 2003). Perhaps more significantly, several studies demonstrate that inhibition of Notch signalling, either by genetic ablation of Notch2 or by administering gamma-secretase inhibitors, can prevent or reduce PDAC following activation of oncogenic k-ras (Mazur et al. 2010; Plentz et al. 2009). Notch signalling has also been shown to cooperate with Nf-KB during k-ras-driven murine PDAC development (Maniati et al. 2011), and pharmacological inhibition of Notch signalling can sensitize PDAC to chemotherapeutic drugs by disrupting the tumour vasculature (Cook et al. 2012) In addition, a synergistic role for Notch during k-ras-mediated carcinogenesis in the pancreas has been reported (De La et al. 2008). However, in direct contrast to these studies, genetic ablation of Notch1 in a mouse model of k-ras-induced PDAC resulted in an increase in high-grade PanIN lesions (Hanlon et al. 2010) suggesting that Notch1 exerts a tumour suppressive function. In addition, the genetic status of members of the Notch pathway in pancreatic ductal adenocarcinoma (PDAC) remains to be resolved. Thus, further work is needed to definitively establish the role of Notch signalling during pancreatic carcinogenesis, although at present, the balance of the evidence supports an oncogenic function.

Mouse models also suggest an oncogenic role for Notch signalling in non-small-cell lung cancer (Zheng et al. 2013). In this example, tumour-propagating cells express high levels of components of the Notch cascade, and Notch3 appears to be essential for their capacity to initiate tumour development. However, it should be noted that in small-cell lung cancer, which is a distinct disease, Notch is thought to be a tumour suppressor (see below).

6.2 Cancers Associated with Loss of Notch Signalling

The strongest evidence of a tumour-suppressive function for Notch signalling is provided by the analysis of squamous cell carcinomas (SCC) that occur in stratified epithelial tissues such as the skin. Initial studies found that genetic ablation of Notch1 in the murine epidermis substantially increased the susceptibility to chemical-induced carcinogenesis (Nicolas et al. 2003). Subsequently, analysis of other SCC types using mouse models also indicated a tumour-suppressive function for Notch signalling. For example, urothelium-specific deletion of the Notch transcriptional effector RBPJk or presenelins, which is essential for Notch receptor activation, results in accelerated development of bladder SCC following chemical carcinogenesis (Maraver et al. 2015). Furthermore, ablation of Notch signalling in this model is strongly associated with the predomination of highly invasive SCC.

In the mouse oesophagus, genetic inhibition of Notch signalling in epithelial progenitor cells promotes the expansion of preneoplastic clones carrying carcinogenic mutations, thus establishing a field from which oesophageal SCC can develop (Alcolea et al. 2014). This therefore indicates that loss of Notch signalling in the oesophagus is likely to be an early event during tumorigenesis, similar to the findings from analysis of cutaneous SCC.

Validation of the results obtained from mouse models has now been made possible with the advent of next-generation sequencing technology, which has enabled the mutational landscape in several types of human SCC to be determined robustly from clinical specimens. This has revealed that loss-of-function mutations in Notch family members are among the most recurrent mutations in a variety of SCC, including head and neck SCC (Agrawal et al. 2011; Stransky et al. 2011), cutaneous SCC (Pickering et al. 2014; South et al. 2014; Wang et al. 2011), bladder SCC(Rampias et al. 2014) and oesophageal SCC (Gao et al. 2014; Song et al. 2014). The mutations identified to date are predominantly found in the Notch receptors, particularly Notch1, and include missense mutations in critical functional regions, nonsense mutations that result in truncated proteins lacking the C-terminal transactivation domain, mutations in splice sites that result in truncation or deletion and frameshift insertion/deletions (indel) (Agrawal et al. 2011; Stransky et al. 2011; South et al. 2014; Gao et al. 2014). In addition, a clinical trial of semagacestat, a γ-secretase inhibitor evaluated for the treatment of Alzheimer's disease, reported an increased risk of skin cancer in patients who received the drug,

providing further evidence that Notch signalling performs an antitumour function in humans (Extance 2010).

In addition to SCC, there is also evidence that Notch suppresses tumour development in other solid malignancies, including small-cell lung cancer (George et al. 2015), some types of brain cancer (Giachino et al. 2015) and liver cancer (Viatour et al. 2011)

6.3 Mechanisms Underlying Notch-Mediated Oncogenesis or Tumour Suppression

6.3.1 Regulation of Stem Cells

The capacity for Notch to operate as an oncogene or tumour suppressor in particular tissues is in part a consequence of its role in regulating stem and progenitor cells (Koch et al. 2013; Wilson and Radtke 2006). Advances in our understanding of cancer biology in recent years have revealed that aberrations in stem and/or progenitor cells are often essential steps during carcinogenesis, and considerable evidence supports the so-called 'cancer stem cell' hypothesis, which posits that the growth of tumours is driven by distinct populations of malignant cells that share many traits with normal stem cells, such as self-renewal, drug resistance and the capacity to repopulate all cell types within the tumour (Clevers 2011; Visvader and Lindeman 2012; Visvader 2011). Importantly, Notch signalling plays critical and diverse roles in regulating stem cell function in many tissues, including processes such as self-renewal, proliferation and differentiation (Wilson and Radtke 2006). Thus, abnormal Notch signalling activity can have a profound effect on stem cell compartments and as a consequence lead to carcinogenesis.

Notch-mediated oncogenesis frequently occurs in tissues where Notch functions to maintain or expand the stem and/or progenitor cell compartment (Fig. 6.2a). In the central nervous system, Notch plays an important role in the maintenance of neural stem cells (Yoon and Gaiano 2005). Consistent with this, cancer stem cells isolated from brain tumours frequently exhibit high expression of Notch family members (Lee et al. 2006; Fan et al. 2006; Gunther et al. 2008). Furthermore, in vitro studies indicate that high levels of Notch are associated with the maintenance of an undifferentiated phenotype in neurosphere cultures derived from brain tumour cancer stem cells, which also correlates with tumorigenicity and malignant traits such as invasiveness (Gunther et al. 2008).

Similar observations have been made with respect to the mammary gland. In this example, the propagation of mammosphere cultures, which is derived exclusively from mammary stem cells, was found to require Notch signalling activity (Dontu et al. 2004) indicating that maintenance of the mammary stem cell compartment is indeed Notch dependent. In addition, constitutive activation of Notch in subpopulations of progenitor cells in murine mammary glands resulted in tumour development

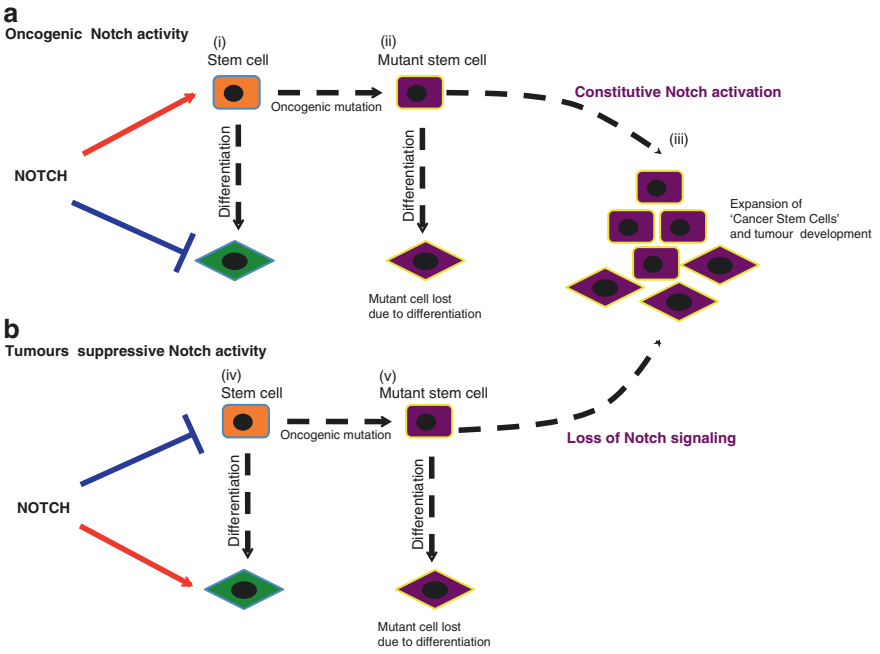


Fig. 6.2 Notch-mediated stem cell regulation and carcinogenesis. (**a**) Oncogenic Notch signalling can occur in tissues in which Notch functions to maintain stem cells and/or prevent their differentiation. In such cases, high Notch activity is normally restricted to the stem cell compartment and is down-regulated as cells differentiate (i). Stem cells that acquire potentially oncogenic mutations are therefore lost as they down-regulate Notch and initiate terminal differentiation (ii). However, if Notch signalling in stem cells remains active, for example, by activating mutations, mutant stem cells expand and can function as 'cancer stem cells' and drive tumour growth (iii). (**b**) Conversely, in tissues where Notch promotes differentiation, it functions as a powerful tumour suppressor by imposing terminal differentiation of mutant stem cells (iv), thus extinguishing clones that may initiate cancer development (v)

(Bouras et al. 2008). These examples highlight the link between the oncogenic function of Notch in specific tissues and its role in stem cell maintenance.

In contrast, in tissues where Notch functions as a tumour suppressor, active Notch signalling is strongly associated with cell cycle exit and the promotion of differentiation, thus extinguishing stem and/or progenitor cells that acquire oncogenic mutations (Fig. 6.2b).

The most prominent example of this is the epidermis. In this tissue, Notch activity is confined to the differentiating cells in the suprabasal layers and is absent in the proliferative stem/progenitor cells of the basal layer (Blanpain and Fuchs 2009; Nowell and Radtke 2013). Ablation of Notch signalling in the murine epidermis results in perturbed differentiation (Yamamoto et al. 2003), while activation induces commitment to differentiation (Blanpain et al. 2006). Furthermore, in vitro experiments show that Notch plays a functional role in promoting cell cycle exit and differentiation of epidermal stem/progenitor cells (Okuyama et al. 2004; Rangarajan

et al. 2001). At a molecular level, several studies indicate that Notch regulates factors that control the proliferation of epidermal stem/progenitor cells, such as p63 (Nguyen et al. 2006; Senoo et al. 2007), p21/CDKN1A(Rangarajan et al. 2001) and AP-1 (Eferl and Wagner 2003; Guinea-Viniegra et al. 2012; Murthy et al. 2012; Nowell et al. 2016) while also promoting differentiation via the induction of cascades such as retinoic acid signalling (Collins and Watt 2008). Consistent with the tumour suppressor activity of Notch being linked to its pro-differentiation function, cutaneous SCC that carry loss-of-function mutations in Notch family members express high levels of stem cell-associated factors, such as p63, and exhibit reduced expression of gene signatures associated with differentiation (Parsa et al. 1999; Rocco et al. 2006). Notch may perform a similar function in other stratified epithelia. For example, inhibition of Notch signalling in the murine oesophageal epithelium results in the expansion of undifferentiated progenitors, thus increasing the pool of cells that have the capacity to form tumours following the acquisition of oncogenic mutations (Alcolea et al. 2014).

6.3.2 Regulation of Inflammation

Recent developments in cancer biology have revealed that inflammatory cells perform important functions during tumour initiation, development and progression, and they thus constitute an important component of the tumour stroma (Grivennikov et al. 2010). Intriguingly, several studies have now shown that an important role of Notch signalling in stratified epithelial tissues is to attenuate inflammatory responses (Nowell et al. 2016; Demehri et al. 2008, 2010). Given that Notch is generally a tumour suppressor in stratified epithelia, a key element of the antitumour function of Notch may be related to its ability to negatively regulate the inflammatory response (Fig. 6.3).

Ablation of Notch signalling in the murine epidermis induces chronic inflammation, the severity of which is dependent on the degree of Notch signalling impairment. Ablation of Notch1 alone results in significant up-regulation of pro-inflammatory cytokine expression, and additional deletion of Notch2 causes a much more pronounced inflammatory response resembling atopic dermatitis (Demehri et al. 2008, 2010). Intriguingly, the inflammatory response induced following complete inactivation of Notch signalling actually prevents carcinogenesis due to the anti-tumorigenic function of T cells present in the inflammatory milieu (Demehri et al. 2012; Di Piazza et al. 2012). However, abrogation of T-cell-mediated immunity in this setting leads to rapid tumour development that is dependent on myeloid inflammatory cells present in the inflamed dermis. These studies demonstrate that loss of Notch signalling in the epidermis can induce pro- and anti-tumorigenic inflammation depending on the degree to which Notch signalling is impaired. Further investigations are needed to establish the precise cellular and molecular factors that underpin these observations. However, the outgrowth of tumours in the Notch-deficient epidermis is dependent on high levels of β-catenin

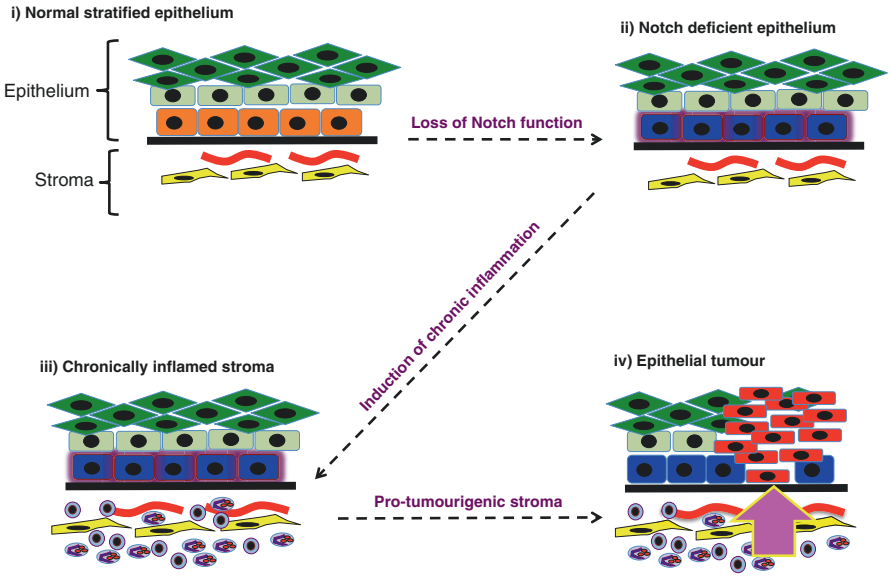


Fig. 6.3 Notch-mediated regulation of inflammation and carcinogenesis. A key function of Notch in many stratified epithelial tissues is to attenuate the inflammatory response and maintain normal tissue architecture (i). Thus, upon loss of Notch signalling in epithelia such as the epidermis, a chronic inflammatory response can be initiated in the underlying stroma (ii) and (iii). This can subsequently promote tumour development by eliciting a variety of responses in the epithelium (iv)

signalling, and pro-tumorigenic myeloid cells that accumulate following ablation of Notch signalling express high levels of Wnt ligands (Di Piazza et al. 2012) suggesting that induction of the Wnt/β-catenin cascade by inflammatory cells is an important mechanism by which loss of Notch signalling promotes carcinogenesis. Other experimental models also support a link between Notch, inflammation and Wnt/β--catenin signalling. For example, ablation of Notch1 in the corneal epithelium results in severe chronic inflammation on the ocular surface that induces squamous cell metaplasia in a β-catenin-dependent manner (Nowell et al. 2016). In this example, the induction of β-catenin signalling is due to inflammation-induced ECM deposition in the corneal stroma, which subsequently induces β-catenin signalling in epithelial cells through mechanotransduction. Although not directly related to carcinogenesis, this study highlights how loss of Notch signalling can induce Wnt/β-catenin signalling, which is frequently pro-tumorigenic, via the induction of inflammation and stromal remodelling. Thus, in stratified epithelial tissues such as the epidermis, negative regulation of inflammation is likely to be a key mechanism by which Notch signalling mediates tumour suppression.

In light of the evidence obtained from the study of the epidermis, it will be important to address if Notch signalling has a similar influence on inflammation in other tissues and whether or not this is relevant with respect to carcinogenesis. Furthermore, delineating how Notch signalling controls the inflammatory response will potentially identify therapeutic targets that can ameliorate the effects of Notch

loss of function and so can potentially be used as anticancer therapeutic agents. In this respect, Notch signalling has been shown to interact with several factors that play an important role in regulating the inflammatory response, including Nf-KB(Espinosa et al. 2010) and AP-1 (Guinea-Viniegra et al. 2012; Murthy et al. 2012; Nowell et al. 2016) although detailed mechanisms remain to be resolved.

6.4 Concluding Remarks

It is clear that Notch signalling has an important impact on the development of many solid cancers, whether as an oncogene or tumour suppressor. In addition, continued advances in our understanding of the role of Notch signalling during development, homeostasis and disease have revealed that the mechanisms by which Notch influences carcinogenesis are diverse and include cell autonomous and non-cell autonomous effects. Therefore, the development of therapeutic strategies that aim to manipulate the Notch cascade directly or the downstream consequences it elicits will potentially lead to improvements in the prevention and treatment of cancer.

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