NOTCH SIGNALING AS A REGULATOR OF TUMOR IMMUNE RESPONSE

EDITED BY: Isabella Screpanti, Antonio Francesco Campese and Barbara A. Osborne

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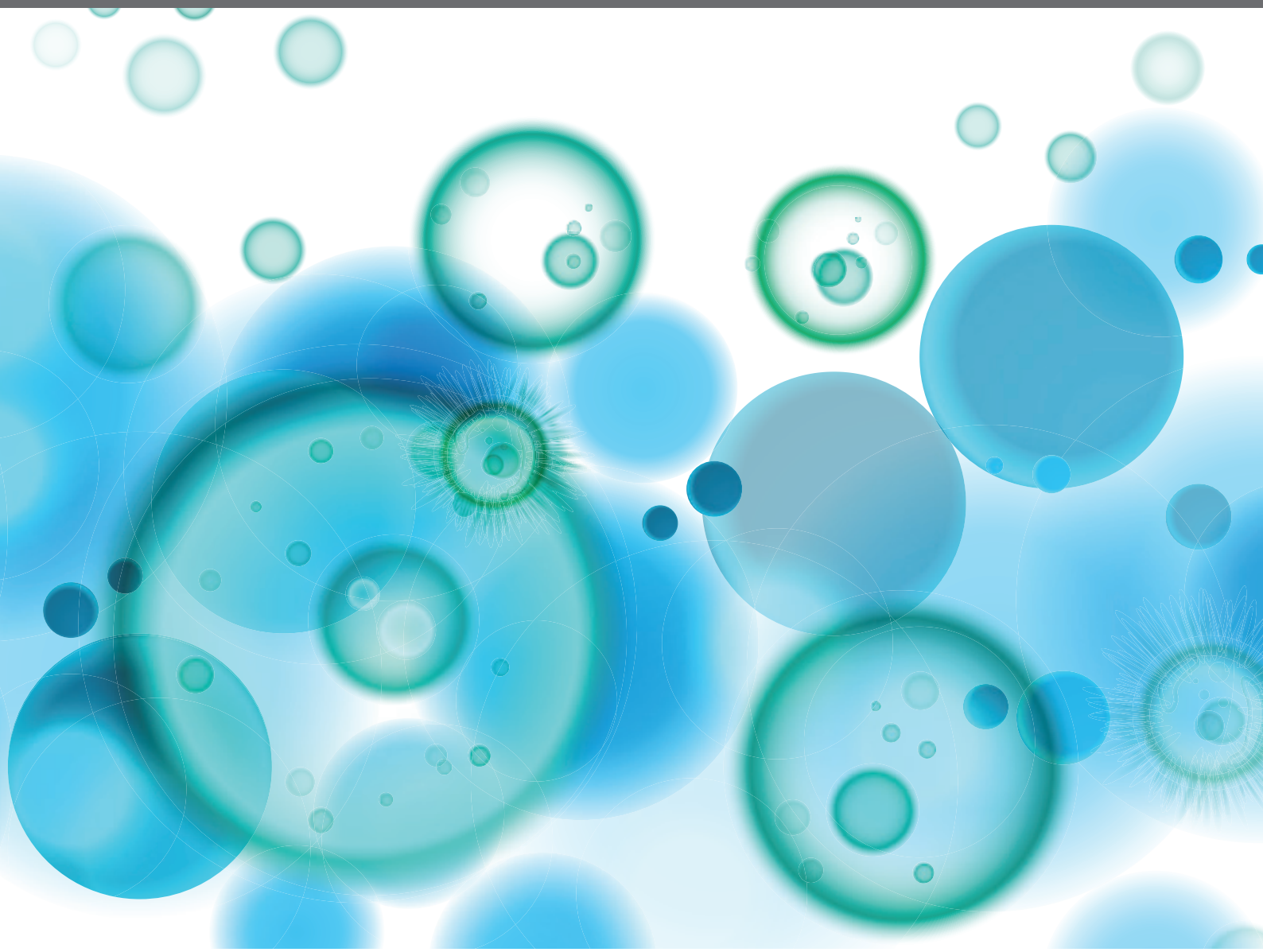

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NOTCH SIGNALING AS A REGULATOR OF TUMOR IMMUNE RESPONSE

Topic Editors:
Isabella Screpanti, Sapienza University of Rome, Italy
Antonio Francesco Campese, Sapienza University of Rome, Italy
Barbara A. Osborne, University of Massachusetts Amherst, United States

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REVIEW
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Regulation of CD8+ T Cells and Antitumor Immunity by Notch Signaling

*Shin-ichi Tsukumo and Koji Yasutomo**

Department of Immunology and Parasitology, Graduate School of Medicine, Tokushima University, Tokushima, Japan

Cancer immunosurveillance is critical for the elimination of neoplastic cells. In addition, recent advances in immunological checkpoint blockade drugs have revealed the importance of the immune system in cancer treatment. As a component of the immune system, CD8+ T cells have important roles in suppressing tumors. CD8+ T cells can kill tumor cells with cytotoxic molecules, such as granzymes and perforin. IFNγ, which is produced by CD8+ T cells, can increase the expression of MHC class I antigens by tumor cells, thereby rendering them better targets for CD8+ T cells. IFNγ also has crucial functions in enhancing the antitumor abilities of other immune cells. Therefore, it has been hypothesized that antitumor immunity could be improved by modulating the activity of CD8+ T cells. The Notch pathway regulates CD8+ T cells in multiple ways. It directly upregulates mRNA expression of granzyme B and perforin, enhances differentiation toward short-lived effector cells, and maintains memory T cells. Intriguingly, CD8+ T cell-specific Notch2 deletion impairs antitumor immunity, whereas the stimulation of the Notch pathway can increase tumor suppression. In this review, we will summarize the roles of the Notch pathway in CD8+ T cells and discuss issues and implications for its use in antitumor immunity.

Keywords: Notch, T cells differentiation, tumor immunity, CD8+ T cells, granzyme B

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Barbara A. Osborne, University of Massachusetts Amherst, United States

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Haidong Dong, Mayo Clinic Minnesota, United States
Amorette Barber, Longwood University, United States

***Correspondence:**
Koji Yasutomo
yasutomo@tokushima-u.ac.jp

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INTRODUCTION

To suppress tumor cell growth, animals use their cell-intrinsic antitumor system, which is regulated by tumor suppressor genes. A second line of defense against tumors includes the immune system itself (1, 2). Acquired immune cells, especially CD8+ T cells, can detect and kill tumors through the latter's expression of abnormal antigens derived from mutated, overexpressed or ectopically expressed molecules. Innate immune cells also have important roles in the antitumor system. For example, NK cells can target tumors by recognizing the expression of MHC class Ib proteins induced by cellular transformation or the lack of MHC class I molecules. Many efforts have been devoted to treating cancer by enhancing immunosurveillance.

Many efforts have been made to enhance antitumor immunity. For example, administration of cytokines, such as type I interferon, IL-2, and IL12, or TLR agonists such as BCG and imiquimod is employed to non-specifically stimulate immune system (3). Vaccine against tumors is also examined to treat them; irradiated tumor cells or selected antigens specifically expressed in tumors are used to increase tumor-specific T cell response (4). In addition, *in vitro* activated and expanded T cells, which can recognize tumors, are adoptively transferred to patients to increase tumor-specific immunity

(5). Notably, recent advances in the development of checkpoint blockade drugs, such as antibodies to PD-1 and CTLA-4, indicate that this field of research is indeed promising (6, 7). To further improve immunotherapy, we need a better understanding of the antitumor immune system.

The Notch pathway is an evolutionarily conserved signaling pathway that regulates various biological systems, including a wide variety of functions of peripheral T cells (8–10). In mammals, the Notch system consists of four receptors (Notch1 to 4) and five ligands (Dll1, 3, 4, and Jagged1, 2). When the receptor is stimulated by the ligand, it is cleaved by an ADAM-family metalloprotease and subsequently the γ-secretase complex, and its cytoplasmic domain is translocated into the nucleus. The cytoplasmic domain then binds to DNA binding protein RBPJκ (encoded by *Rbpj*) and co-activator MAML, leading to transcriptional regulation of specific target genes.

Research into the physiological roles of the Notch pathway in peripheral T cells has mainly focused on $CD4^+$ T cells. The Notch pathway regulates $CD4^+$ T cell differentiation, cytokine production, proliferation, and/or survival, although some of the data among the papers are in disagreement (8, 9). For example, Tanigaki et al. reported that *Rbpj*-deficient CD4 T cells showed decreased Th2 and increased Th1 in *in vivo* and *in vitro* experiments (11). Similarly, Amsen et al. reported Th2 differentiation was dependent on the Notch pathway by using *Notch1/2*-double deficient mice in addition to *Rbpj* (12). On the other hand, Auderset et al. reported that *Notch1* and *2* were required for Th1 differentiation in anti-*Leishmania major* immunity, while *Rbpj*-deficiency did not show any significant effects (13). The causes of these apparent differences have not been resolved. It is possible that the functions of Notch pathway are highly context-dependent in T cells. In this review, we will summarize research into the physiological roles of the Notch pathway in $CD8^+$ T cells and discuss its potentials for antitumor immunity (**Figure 1**).

THE PHYSIOLOGICAL ROLES OF THE NOTCH PATHWAY IN $CD8^+$ T CELLS

To elucidate the roles of Notch in $CD8^+$ T cells, studies have analyzed mice in which the Notch pathway has been knocked out. Maekawa et al. reported that $CD8^+$ T cell-specific (E8I-cre) *Notch2* deletion led to decreased expression of *Gzmb* (encoding granzyme B) and increased sensitivity to *Trypanosoma cruzi* infection (14). This mouse also showed a significant loss of CTL activity against antigen-pulsed cells *in vivo*. They further showed that Notch2 and RBPJκ directly bound to *Gzmb* and *Prf1* (encoding perforin) promoters in combination with the transcription factor CREB and activated their transcription.

Backer et al. described an influenza virus infection model in which T cell-specific (CD4-cre) *Notch1/2*-double KO mice showed almost complete loss of short-lived effector $CD8^+$ T cells (SLECs) that possess the $KLRG1^+CD127^-$ phenotype. On the other hand, the overall ratio of antigen-specific $CD8^+$ T cells to that of $KLRG1^-CD127^+$ memory precursor effector cells (MPECs) was moderately increased (15). They also confirmed this phenotype was present in *Rbpj* KO mice. Then, they analyzed the transcriptome of activated $CD8^+$ T cells, and showed that more than 40% of SLEC-specific genes were decreased in *Notch1/2* KO cells, indicating that the Notch pathway was a critical regulator of SLEC differentiation. In addition, they also found that the Notch pathway was required for the upregulation of CD25 (IL-2Rα chain) and T-bet proteins, both of which are critical regulators of SLEC differentiation. Furthermore, they showed that T-bet overexpression enhanced SLEC differentiation in *Notch1/2* KO $CD8^+$ T cells, while the active form of Notch1 could not do so in *Tbx21* (encoding T-bet) KO cells, suggesting that T-bet is a critical regulator downstream in the Notch pathway.

Similar results were reported by another laboratory. Mathieu et al. used CD8 T cell-specific *Notch1/2* KO mice and showed a reduction of the ratio of SLECs after *Listeria monocytogenes* infection (16). However, they found that the absolute cell number of SLECs was not reduced, and the reduction of the ratio was instead due to an increased number of MPECs and early effector cells (EECs; $KLRG1^-CD127^-$ cells). On the other hand, when they immunized mice with peptide-pulsed dendritic cells (DCs), they found a severe reduction of SLEC cell number, while MPEC cell numbers were not affected. The reason for this difference was not clear, but it might indicate that the roles of the Notch pathway in $CD8^+$ T cells are context-dependent as seen in $CD4^+$ T cells (8). As reported in the paper by Backer et al. above, they found that CD25 protein expression was diminished in *Notch1/2* KO cells. However, the expression of T-bet was not affected. Instead, they found that Eomes, which is a paralog of T-bet, was moderately decreased in *Notch1/2* KO cells. Eomes is reportedly required for MPEC differentiation but not for SLEC (17). Thus, the importance of the Eomes reduction in *Notch1/2* KO cells for SLEC differentiation remains to be investigated.

Instead of KO mice, Maillard and colleagues used the dominant negative form of MAML (DN-MAML)-expressing mice and analyzed its effects on $CD4^+$ and $CD8^+$ T cells in a graft-versus-host disease (GVHD) model (18, 19). They reported that DN-MAML profoundly suppressed GVHD, with reduced production of IFNγ in $CD4^+$ and $CD8^+$ T cells. In contrast to KO mouse experiments, DN-MAML-expressing $CD8^+$ T cells preserved their T-bet and Eomes protein expression. In addition, those cells showed a defect in the activation of Ras/MAPK and NF-κB pathways. Those cells also expressed higher amounts of negative regulators of T cell activation, such as *Dgka*, *Cblb*, and *Pdcd1*, suggesting that these factors might suppress GVHD.

In addition to the genetic approaches described above, γ-secretase inhibitors, blocking antibodies and soluble Notch ligands have been used to investigate the roles of the Notch pathway in $CD8^+$ T cells (20–26). The consensus of these experiments is that the Notch pathway is required for IFNγ production during $CD8^+$ T cell activation. On the other hand, the effect on the cell number after the activation of $CD8^+$ T cells was controversial. Several papers indicated that γ-secretase inhibitors or soluble Notch ligand (Dll4) suppressed proliferation of $CD8^+$ T cells, while their viability was not affected (22–24). Other papers showed that the inhibitors or membrane-bound Notch ligands (Jagged1) did not affect the CD8 T cell number or proliferation after activation (25, 27, 28). In addition, Notch1/2-double KO

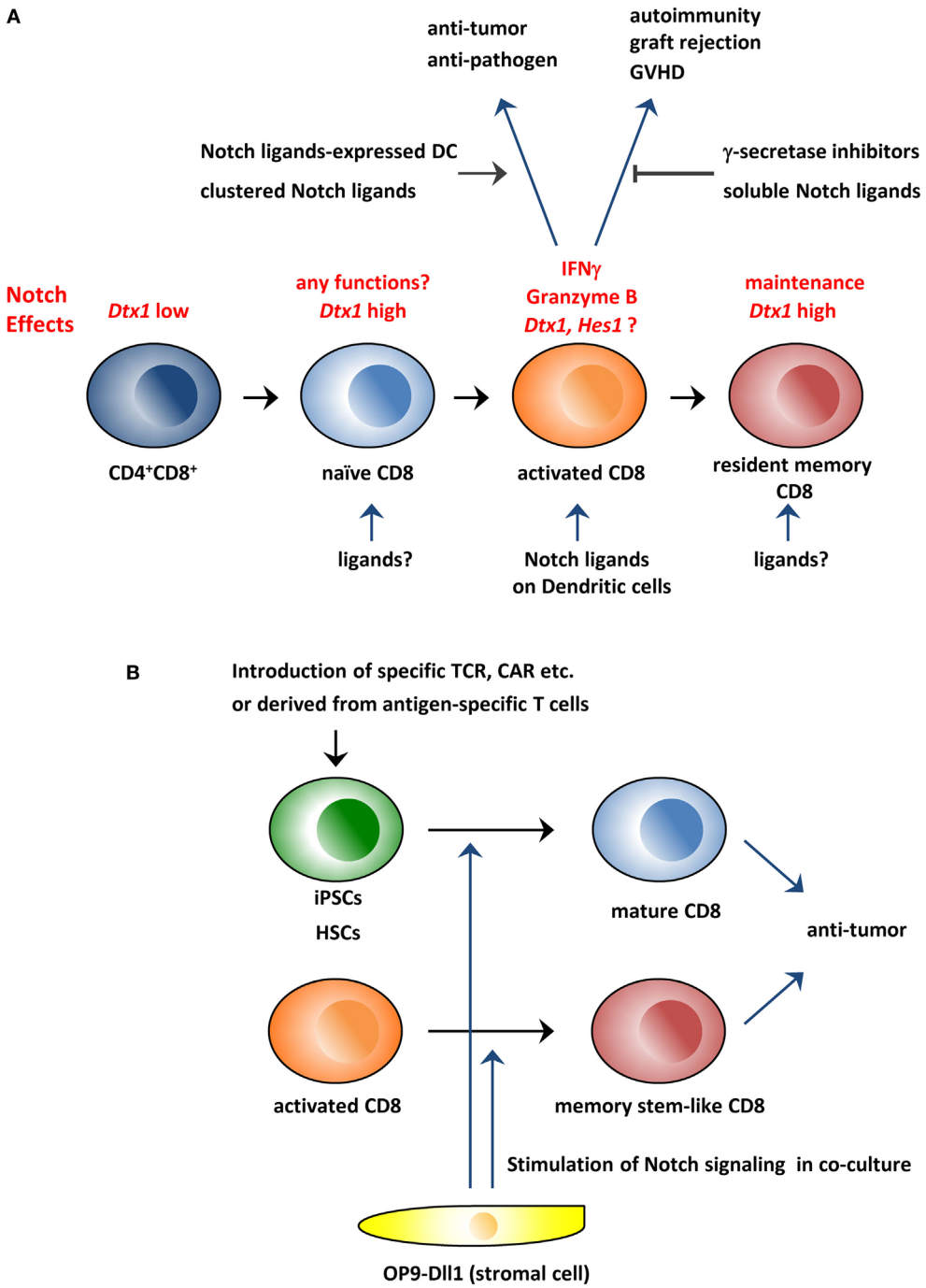

FIGURE 1 | Schematic overview of the roles of the Notch pathway in CD8+ T cells and its application to immunotherapy. **(A)** The Notch pathway is stimulated during CD8+ T cell activation and is required for the production of effector molecules, such as IFNγ and granzyme B. Therefore, the modulation of the Notch pathway could be used to treat various diseases in which CD8+ T cells are involved. In addition, studies indicate that the Notch pathway is active in resting naïve and memory T cells in which the pathway is reportedly needed for the maintenance of these cells. **(B)** Coculture with Dll1-expressing OP9 stromal cells can generate CD8+ T cells from hematopoietic stem cells or iPSCs *in vitro*. In addition, the coculture system can generate memory stem cell-like T cells from activated CD8+ T cells. These *in vitro* generated CD8+ T cells could be superior reagents for antitumor immunity. GVHD, graft-versus-host disease; CAR, chimeric antigen receptor; iPSCs, induced pluripotent stem cells; HSCs, hematopoietic stem cells.

mice showed that the CD8+ T cell number was not affected or even increased when activated *in vivo*, although their differentiation was altered (15, 16). What caused these differences remains elusive. Further examination of the experiment-conditions and the methods of the Notch inhibition should be required in future researches.

Other studies showed that the cell surface expression of Notch1 and 2 was upregulated soon after T cell activation (14, 15, 22, 23, 29, 30). In addition, expression of its ligands (Dll1, Dll4, and/or Jagged1) was also upregulated in activated DCs (15, 21–23, 31). Based on these observations, many researchers have concluded that the Notch pathway is activated early in the process of T cell activation by the ligands on DCs. In fact, it was reported that *Hes1* and/or *Dtx1* (encoding Deltex1), which are well-known targets of the Notch pathway, were upregulated after T cell activation (23, 32). Other papers reported that TCR stimulation caused the cleavage of Notch receptors, indicating that the Notch pathway was activated after T cell activation (20, 33). However, transcriptome analyses clearly show that *Dtx1* is upregulated during the differentiation of $CD4^+CD8^+$ thymocytes to peripheral naïve $CD4^+$ and $CD8^+$ T cells (Immunological genome project[1]; RCAI RefDIC[2]). We confirmed that this upregulation was dependent on *Notch1/2* and *Rbpj* (unpublished data). Unexpectedly, *Dtx1* is moderately downregulated after TCR activation, according to transcriptome data. Subsequently, its expression returns to a high level during the differentiation to memory cells. On the other hand, *Hes1* expression remains low during activation of naïve and activated cells. These results suggest that the Notch pathway is active in resting T cells. The reason why *Hes1* and *Dtx1* were not upregulated during T cell activation remains unclear. The Notch pathway might not be activated under the conditions of T cell activation used in these studies. Alternatively, the epigenetic status of these gene loci or unknown inhibitor(s) might affect their expression during T cell activation.

Interestingly, recent papers support the hypothesis that the Notch pathway is operational in resting $CD4^+$ and $CD8^+$ T cells. Maekawa et al. reported that *Rbpj*-deficient $CD4^+$ T cells normally expanded after antigen stimulation, but could not survive during the contraction phase. They also found that the injection of γ-secretase inhibitor to mice decreased the number of resting memory T cells (34). Hombrink et al. also reported that *Notch1/2*-deficiency or the treatment with γ-secretase inhibitor decreased $CD103^+$ lung-resident memory $CD8^+$ T cells in mice (35). These results suggest that the Notch pathway has important roles not only in activating T cells but also in resting cells.

Although some data disagree, an increasing number of reports have demonstrated that the Notch pathway was required for $CD8^+$ T cell activation and homeostasis. When and how the Notch pathway works remains to be further investigated, but it is very probable that the manipulation of this pathway could be useful in the treatment of diseases in which the immune system is involved.

THE NOTCH PATHWAY IN ANTITUMOR IMMUNE RESPONSES

$CD8^+$ T cells have important roles in antitumor immunity (1, 7), some of which are dependent upon the Notch pathway. Sugimoto et al. reported that CD8-specific deletion of *Notch2*, but not *Notch1*, led to increased tumor size and decreased survival after tumor-inoculation into mice (36). Zhao et al. reported that ovarian cancer imposed glucose restriction on T cells, leading to high expression of microRNAs *miR-101* and *miR26a*, leading to constrained expression *Ezh2*. *Ezh2* is a suppressor of Notch pathway inhibitors *Numb* and *Fbxw7*. As a consequence, the cancer-induced glucose restriction led to the suppression of the Notch pathway. They also showed that downregulation of *Ezh2* elicited poor antitumor immunity, implying that the Notch pathway was important for antitumor immunity (37). Dai et al. found that *1810011o10Rik* (*Tcim*) was upregulated in intratumoral activated $CD8^+$ T cells. They also showed that overexpression of *Tcim* blocked nuclear translocation of the intracellular domain of Notch2 and inhibited the cytotoxic efficacy of $CD8^+$ T cells on hepatocellular carcinoma (38). All of these papers confirm that the Notch pathway in $CD8^+$ T cells has a critical role in antitumor immunity.

Considering these reports, the manipulation of the Notch pathway in T cells could be a good approach to suppress tumors. Several papers pursued the idea in mouse models. Sugimoto et al. showed that injection of agonistic antibody to Notch2 or Dll1-overexpression in DC augmented antitumor immunity (36). Sierra et al. used intracellular Notch1-expressing mice driven by a granzyme B promoter-cre and flox system. They found that such activation of the Notch pathway in $CD8^+$ T cells increased the cytotoxic effects and antitumor response with higher production of IFNγ and granzyme B (39). Thounaojam et al. showed that treatment with the proteasome inhibitor bortezomib caused higher expression of IFNγ in $CD8^+$ T cells in tumor-bearing mice, probably through the upregulation of Notch receptors (40). Biktasova et al. reported that administration of clustered Dll1 enhanced IFNγ-producing $CD8^+$ T cells and suppressed tumor growth (41). These reports reveal that Notch-targeted immune modulation could be promising. However, Notch receptors are broadly expressed in various types of cells, and the modulation of Notch might be highly context-dependent. In addition, Notch receptors are known as proto-oncogenes themselves (42). Therefore, it is possible that the activation of the pathway could exacerbate some types of tumors. Detailed investigations will be needed to examine the possibility of antitumor treatment targeting this pathway.

The therapy by immune checkpoint blockade is recent advance in antitumor immunotherapy (43). The blocking antibodies to PD-1/PD-L1 and CTLA-4 are broadly used to treat melanoma and other types of tumors. Mathieu et al. reported that Notch directly bound to the promoter region of *Pdcd1* (encoding PD-1) gene and upregulated its mRNA expression in activated $CD8^+$ T cells (23). In addition, Yu et al. indicated that γ-secretase inhibitor activated tumor-infiltrating $CD8^+$ T cell probably through the downregulation of PD-1 expression (44). These results indicated that the Notch pathway might also have negative effect during $CD8^+$ T cell activation. Therefore, it is expected that the antitumor therapy by Notch activation would be more efficient in combination with the blocking antibodies to PD-1 and other inhibitory receptors.

[1] http://www.immgen.org/index_content.html.
[2] http://refdic.rcai.riken.jp/welcome.cgi.

GENERATING ANTITUMOR CD8$^+$ T CELLS *IN VITRO* USING THE NOTCH PATHWAY

In addition to efforts to modulate the Notch pathway *in vivo* to enhance antitumor immunity, there have been *in vitro* attempts to create cytotoxic T cells against tumors. CD8$^+$ memory stem cells are reported to have naïve markers, but have self-renewal capacity and can rapidly respond to antigens (45, 46). In addition, they have antitumor capacities exceeding those of central and effector memory T cells (47). Kondo et al. reported that activated CD4$^+$ or CD8$^+$ T cells could be converted to memory stem cell-like cells when cocultured with Dll1-expressing OP9 stromal cells (OP9-Dll1) (48). They also showed that the resultant memory stem cell-like CD4$^+$ and CD8$^+$ T cells had superior antitumor abilities relative to naïve, activated or memory T cells when injected into mice.

In addition to peripheral T cells, the Notch pathway is well known for its role in defining the fate of T cells in early stages of differentiation. By coculturing with Dll1-expressing cells, some types of stem cells can be differentiated to T cells *in vitro* (49). There have been several attempts to create large number of tumor-specific CD8$^+$ T cells through use of this *in vitro* system. Zhao et al. introduced a tumor antigen-specific TCR into human umbilical cord blood-derived hematopoietic cells and generated T cells by coculture with OP9-Dll1 (50). They showed that those T cells could recognize and kill antigen-pulsed antigen-presenting cells. Vizcardo et al. generated induced pluripotent stem cells (iPSCs) from melanoma antigen-specific human cytotoxic T cells and cultured them on OP9-Dll1 cells. They subsequently stimulated the differentiated CD4$^+$CD8$^+$ T cells with anti-CD3 antibody to create CD8$^+$ single positive T cells (51). They found that those CD8$^+$ T cells could respond to the specific melanoma antigen, and had antitumor ability. Themeli et al. introduced a chimeric antigen receptor into iPSCs and generated human T cells targeted against CD19 by using OP9-Dll1 (52). Although the generated T cells showed an innate T cell-like phenotype, those cells had potent antitumor capability specific for CD19-expressing lymphoma cells.

CONCLUSION AND FUTURE DIRECTIONS

Emerging evidence indicates that the Notch pathway has important physiological roles in CD8$^+$ T cell functions, especially in the production of effector molecules. In addition, recent research points out that the Notch pathway probably works in resting T cells to promote homeostasis. On the other hand, the presence of apparently conflicting data suggests that the roles of the Notch pathway might be highly stage and context dependent. Therefore, it is critical to clarify what determines the functions of the Notch pathway under each condition. Comprehensive analyses of Notch signaling by transcriptomic, proteomic, and ChIP-seq analyses would be helpful to elucidate the differences under each condition.

Given the physiological importance of the Notch pathway, it could prove useful in the optimization of antitumor immunotherapy. However, the manipulation of the pathway should be carefully examined because the roles of the pathway could be context-dependent even in peripheral T cells. Furthermore, Notch receptors and ligands are broadly expressed in many tissues, and the manipulation of the pathway could cause unpredicted outcomes.

As well as the administration of cytokines, TLR agonists and immune checkpoint inhibitors, the activation of the Notch pathway induces non-specific activation of immune system, which could lead to autoimmunity or unwanted inflammation. Tumor-specific activation of immune response has been tried by using vaccination against tumor antigens or adoptive transfer of tumor-specific T cells generated or expanded *in vitro*. As described in this minireview, the Notch pathway is an excellent tool to create large amount of CD8$^+$ T cells from iPSCs derived from tumor-specific T cells *in vitro*. In addition, the Notch pathway also can induce memory stem cell-like cells from peripheral T cells. Tuning the culture conditions as well as genetic modification of the cells could be used to create various types of CD8$^+$ T cells for cancer immunotherapy. The best combination of non-specific and specific activation of immune responses should be carefully investigated to fight against tumors in various conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Chemotactic Cues for NOTCH1-Dependent Leukemia

Erich Piovan[1,2], Valeria Tosello[1], Alberto Amadori[1,2] and Paola Zanovello[1,2]**

[1] UOC Immunologia e Diagnostica Molecolare Oncologica, Istituto Oncologico Veneto IOV—IRCCS, Padova, Italy,
[2] Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche, Università di Padova, Padova, Italy

The NOTCH signaling pathway is a conserved signaling cascade that regulates many aspects of development and homeostasis in multiple organ systems. Aberrant activity of this signaling pathway is linked to the initiation and progression of several hematological malignancies, exemplified by T-cell acute lymphoblastic leukemia (T-ALL). Interestingly, frequent non-mutational activation of NOTCH1 signaling has recently been demonstrated in B-cell chronic lymphocytic leukemia (B-CLL), significantly extending the pathogenic significance of this pathway in B-CLL. Leukemia patients often present with high-blood cell counts, diffuse disease with infiltration of the bone marrow, secondary lymphoid organs, and diffusion to the central nervous system (CNS). Chemokines are chemotactic cytokines that regulate migration of cells between tissues and the positioning and interactions of cells within tissue. Homeostatic chemokines and their receptors have been implicated in regulating organ-specific infiltration, but may also directly and indirectly modulate tumor growth. Recently, oncogenic NOTCH1 has been shown to regulate infiltration of leukemic cells into the CNS hijacking the CC-chemokine ligand 19/CC-chemokine receptor 7 chemokine axis. In addition, a crucial role for the homing receptor axis CXC-chemokine ligand 12/CXC-chemokine receptor 4 has been demonstrated in leukemia maintenance and progression. Moreover, the CCL25/CCR9 axis has been implicated in the homing of leukemic cells into the gut, particularly in the presence of phosphatase and tensin homolog tumor suppressor loss. In this review, we summarize the latest developments regarding the role of NOTCH signaling in regulating the chemotactic microenvironmental cues involved in the generation and progression of T-ALL and compare these findings to B-CLL.

Keywords: T-cell acute lymphoblastic leukemia, chemokines, CXC-chemokine receptor 4, stromal-derived factor-1, NOTCH, CXCR7, infiltration

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****Correspondence:***
Erich Piovan
erich.piovan@unipd.it;
Paola Zanovello
paola.zanovello@unipd.it

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INTRODUCTION

The NOTCH signaling cascade is an evolutionarily conserved signaling pathway that in mammals consists of a family of four transmembrane receptors (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) (1) and five ligands of the Delta-Serrate-Lag family [jagged 1 (JAG1), JAG2, delta-like 1 (DLL1), DLL3 and DLL4] (2). This signaling system plays a crucial role in regulating development and tissue homeostasis (3). Given the important role played by NOTCH signaling in regulating key cellular traits such as differentiation, proliferation, and apoptosis, it is perhaps not surprising that deregulation of NOTCH has been implicated in the pathogenesis of a variety of malignancies (4, 5). In this regard, the most firmly established evidence for altered NOTCH signaling in cancer is represented

by activating *NOTCH1* receptor mutations found in over 50–60% of T-cell acute lymphoblastic leukemia (T-ALL) cases (6). In addition, 8–30% of T-ALLs harbor mutations in F-box and WD repeat domain containing 7 (*FBXW7*), a protein that normally promotes NOTCH1 ubiquitination and degradation, which lead to increased NOTCH1 protein stability (7, 8). Moreover, paracrine mechanisms that result in NOTCH1 or NOTCH3 signaling upregulation or rare mutations in *NOTCH3* (9) could contribute to T-ALL. Further, aberrant expression of the NOTCH ligand, DLL4, may contribute to NOTCH1-driven leukemias (10). Thus, the majority of T-ALL cases have hyper-activation of the NOTCH signaling pathway. Interestingly, activating mutations affecting *NOTCH1* are also present in 4–13% of B-cell chronic lymphocytic leukemia (B-CLL) cases (11, 12), and very recently frequent non-mutational NOTCH1 activation in B-CLL has also been reported, irrespective of *NOTCH1* mutational status (13). However, differently from T-ALL, the specific role of NOTCH1 signaling in the pathogenesis of B-CLL remains to be established. T-ALL is an aggressive hematological malignancy arising from the malignant transformation and subsequent clonal expansion of immature T-cell precursors. Clinically, T-ALL patients present with diffuse infiltration of the bone marrow (BM) by immature T-cell blasts, high-blood cell counts (hyperleukocytosis) with extramedullary infiltration of lymph nodes and other organs such as the central nervous system (CNS), and the presence of mediastinal masses (14). T-ALL may arise in the BM from thymus settling progenitors endowed with T-lineage potential or thymus resident T-cell precursor cells. These transformed T lymphoblasts under the influence of oncogenic *NOTCH1* activation and collaborating oncogenes spread infiltrating BM cavities and/or thymus with extensive disease already at time of diagnosis. In addition, leukemic cells invade other tissues such as liver, spleen, lymph nodes, and CNS. B-CLL, on the other hand, is a common hematological malignancy characterized by the clonal expansion of non-functional CD5+ B cells in the BM and lymph nodes (15). The putative normal counterparts of this disease, although debated, are considered naïve and memory B cells (16, 17). Interestingly, B-CLL cells in the lymph node are known to harbor frequent NOTCH1 activation independent of mutations (18) and recent findings have shown that NOTCH1 is physiologically expressed and activated in the cells of origin of B-CLL (13). Additionally, approximately 50% of B-CLL cases without *NOTCH1* mutations express the active form of NOTCH1 ICN1 (intracellular portion of NOTCH1), bringing NOTCH1 signaling to the forefront also in this disease.

Chemokines and their receptors, in particular so-called "homeostatic chemokines" which normally orchestrate leukocyte trafficking and homing during development, have been recently implicated in directing organ-specific metastasis (19, 20). Mechanistic insights on the trafficking of NOTCH-dependent leukemia cells to target organs are still ill-defined, however, recent reports have highlighted the importance of some homing receptors and their ligands (**Figure 1**) such as: (i) CC-chemokine ligand 19 (CCL19)/CC-chemokine receptor 7 (CCR7) (21); (ii) CXC-chemokine ligand 12 (CXCL12)/CXC-chemokine receptor 4 (CXCR4) (22–24); and (iii) CCL25/CCR9 (25). As leukemic relapse remains a major cause of treatment failure in childhood ALL, with leukemic relapses directly linked to the survival of blasts in the BM and/or distant sites such as CNS, the identification of targetable mechanisms behind this phenomenon are of clear impact.

DEREGULATION OF NOTCH1 SIGNALING IN LYMPHOID LEUKEMIAS

NOTCH alterations can be found in a broad spectrum of hematological tumors [reviewed in Ref. (26, 27)]. In particular, NOTCH1 and to a lesser extent also *NOTCH2*, resulted the most frequently mutated. NOTCH1 is well known for its role as a master player in the pathogenesis of T-ALL as demonstrated by the high incidence of mutations in this disease (6). Most of these mutations cluster in the negative regulatory region (NRR), which prevents the extracellular receptor from being cleaved by the Disintegrin and metalloproteinase domain-containing protein 10 in the absence of ligand. These mutations mainly include missense substitutions or short insertions or deletions, which lead to receptor destabilization and ligand-independent activation (28). Other mutations in *NOTCH1* truncate the PEST [proline (P), glutamic acid (E), serine (S), threonine (T)-rich protein sequence] domain through non-sense or frameshift events that lead to premature STOP codons in the C-terminal portion of NOTCH1 and increase half-life of ICN1. In addition, in a significant fraction of T-ALL cases, loss of function mutations or deletions in *FBXW7* gene, an ubiquitin ligase implicated in ICN1 turnover, contribute to activation of NOTCH1 signaling in this malignancy (7, 8). Importantly, in about 20% of T-ALL cases, NOTCH1 signaling results strongly activated by the cooperativity of both mechanisms because of dual mutations affecting the NRR and PEST regions of NOTCH1 or the NRR domain together with the *FBXW7* mutations (6–8). The importance of *NOTCH1* mutations has also been extensively validated in murine mouse models of T-ALL. Forced expression of activated forms of Notch1 in murine hematopoietic progenitors determine T-ALL with a penetrance that depends on the strength of oncogenic Notch1 alleles (29, 30). In addition, numerous T-ALL mouse models showed *Notch1* alterations as significant events in T-ALL progression (31, 32). In the context of NOTCH signaling, a role of Notch3 was also established with transgenic mice expressing ICN3 developing T-ALL with high penetrance, demonstrating a potential role for Notch3 in T-ALL (33). In addition, the human T-ALL cell line TALL1, which has wild-type Notch1 but is sensitive to γ-secretase inhibitors (GSI), carries an NRR mutation in *NOTCH3* gene and shows ICN3 overexpression (9, 34). In T-ALL, the oncogenic function of NOTCH1 has been extensively studied and is linked to its capacity to regulate crucial signaling pathways and genes such as nuclear factor-κB (NF-κB), MYC, IGF-1R, and IL-7R all of which contribute to tumor growth and progression (35–39). NOTCH1 also regulates two families of transcriptional repressors Hes and Hey/Hers which in turn exert several downstream effects of NOTCH1 signaling. In particular, Hes1 sustains the phosphoinositide-3 kinase (PI3K)-AKT pathway and NF-κB activation through the direct suppression of phosphatase and tensin homolog (PTEN) and CYLD, respectively (40, 41). Moreover, Hes1 negatively regulates apoptosis of T-ALL

cells through the repression of the BBC3/Puma pro-apoptotic factor (42). In addition to the consolidated function of NOTCH1 signaling in promoting anabolic processes and growth, NOTCH1 has been found to regulate some chemokine receptors (CCR5, CCR7, and CCR9; see below) thus orchestrating cell migration in specific microenvironments (21, 43).

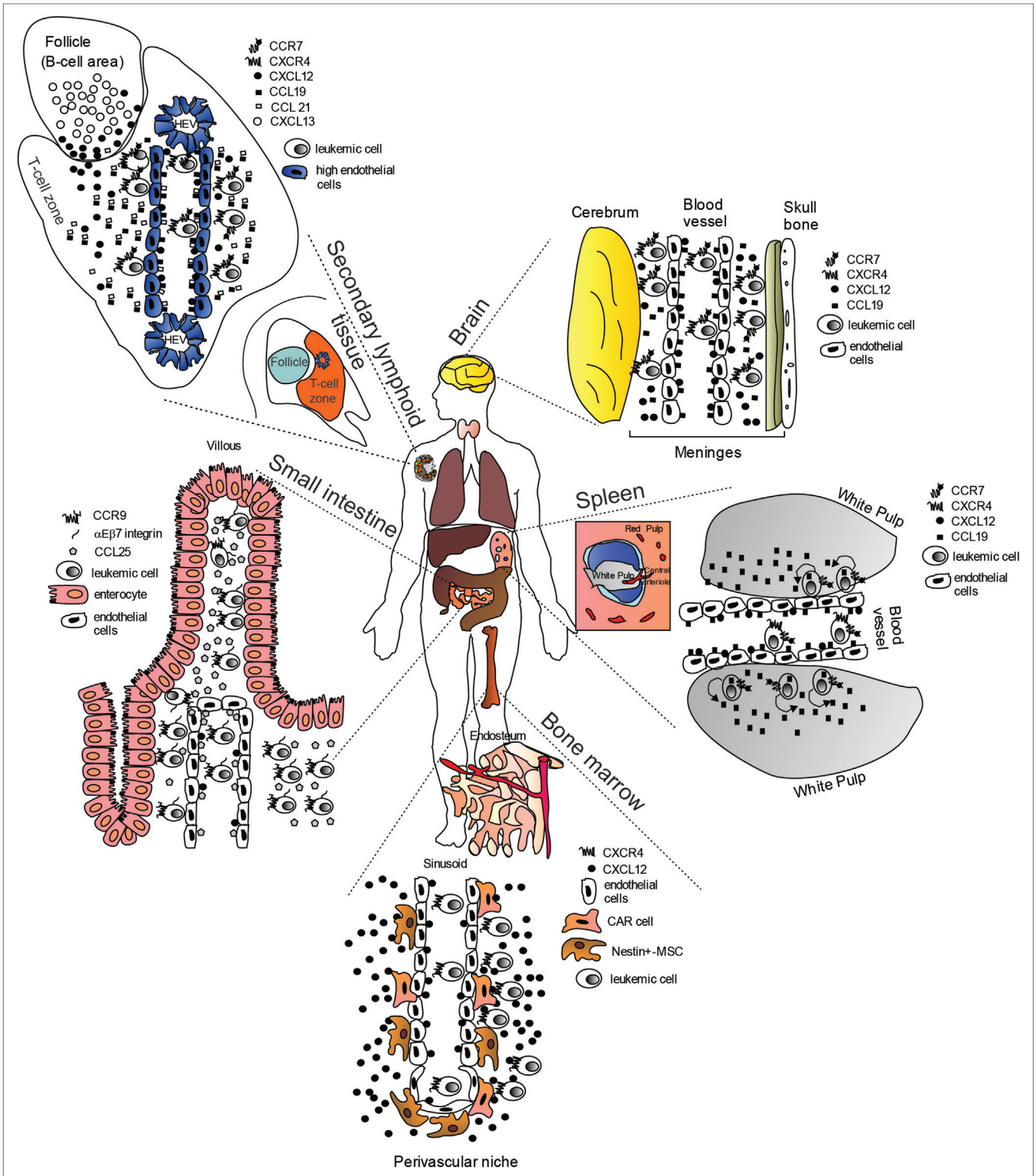

FIGURE 1 | Continued

FIGURE 1 | "Cellular highways" hijacked by leukemic cells implicated in T-cell acute lymphoblastic leukemia dissemination (many of the findings may also apply to B-cell chronic lymphocytic leukemia). Under physiological conditions, homeostatic chemokines control cellular migration by directing cells expressing specific chemokine receptors to appropriate locations expressing their cognate chemokine ligands. These cellular highways are also used by leukemic cells. In the brain, CC-chemokine ligand 19 (CCL19) and CXC-chemokine ligand 12 (CXCL12) recruit CC-chemokine receptor 7 (CCR7)- and CXC-chemokine receptor 4 (CXCR4)-expressing leukemic cells from blood vessels. In the spleen, CCL19 recruits CCR7-expressing leukemic cells from blood vessels possibly in combination with CXCL12. Migrated leukemic cells may then activate an autocrine/paracrine secretion of CCL19. CCR7-expressing leukemic cells together with CD62L (not shown) and CXCR4, gain access to secondary lymphoid organs such as lymph nodes (shown) *via* interactions with CCL19, CCL21, peripheral lymph node vascular addressin (not shown) and CXCL12 presented on high-endothelial venules (HEV). Here, leukemic cells are retained, proliferate, and completely substitute the normal tissue architecture. In the bone marrow (BM), CXCR4-expressing leukemic cells are probably initially recruited to the perivascular niche expressing high levels CXCL12, where a leukemic niche is established. Inhibitors of the CXCL12/CXCR4 interaction release leukemic cells from their BM niche, and allow these cells to enter the blood stream. In the small intestine, CCR9-expressing leukemia cells (together with αEβ7 integrin) are recruited by CCL25, where the presence of phosphoinositide-3 kinase-AKT pathway activation contributes to confer a proliferative advantage to leukemic cells in an otherwise non-supportive microenvironment.

As described above, *NOTCH1* mutations have also been described in B-CLL (11, 12). Mutational activation of NOTCH1 has been found in about 8% of B-CLL at diagnosis and at significantly higher frequency during disease progression toward Richter transformation (about 30%), as well as in chemo-refractory B-CLL (about 20%). Differently from T-ALL, *NOTCH1* mutations clustered uniquely in the PEST domain and the 2-bp frameshift deletion (ΔCT7544–7545, P2515fs) is present in about 80% of cases, making it a potential target for screening and specific targeted therapies. Consistent with the association of *NOTCH1* mutations with clinically aggressive forms of the disease, B-CLL with *NOTCH1* mutations at diagnosis have a poor prognosis similar to B-CLL carrying *TP53* disruption and *NOTCH1* mutations and *TP53* disruption tended to distribute in a mutually exclusive pattern (44). The functional role of *NOTCH1* mutations in B-CLL is not completely understood. A recent study showed that ICN1 is expressed in about 50% of peripheral blood B-CLL cases that present wild-type *NOTCH1*, suggesting that alternative mechanisms are involved in NOTCH1 activation in B-CLL (13). Moreover, independent from the mutational status, $ICN1^+$ cases expressed a *NOTCH1* gene signature and were sensitive to GSI. Notably, NOTCH1 regulated genes included those with a crucial role in the pathogenesis of B-CLL, including CCND3, BCL2, MCL1, BCR signaling pathway genes, and NF-κB pathway members.

CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines are small, secreted cytokines with chemotactic properties that are best known for their capacity to mediate immune cell trafficking and lymphoid tissue homeostasis (45, 46). This subfamily of cytokines which comprise over 48 ligands regulate cell trafficking and positioning by activating 20 seven-transmembrane spanning G-protein-coupled chemokine receptors (GPCR). In addition, chemokines can also bind to non-G-protein-coupled seven-transmembrane spanning receptors called atypical chemokine receptors (ACKR), which due to their incapacity to interact with Gi proteins are supposed to act mainly as decoy receptors, scavenging chemokines to help maintain chemokine gradients in tissues. Chemokines are subdivided into four classes based on the position of the first two cysteine (C) residues at their N-terminal protein sequence: CC-chemokines, CXC-chemokines, XC-chemokines, and CX_3C-chemokines. The chemokine receptor nomenclature is based on the chemokine subclass specificity of the receptor, where L (ligand) is replaced by R (receptor) (47). There is an important degree of promiscuity in the chemokine superfamily, with numerous ligands binding different receptors and vice versa (46). Functionally, chemokines can be divided into "inflammatory" (induced upon inflammation) and "homeostatic" (constitutively expressed in specific tissues or cells) (48). Metaphorically, we can imagine our body as containing "cellular highways" regulated mainly by "homeostatic" chemokines and their receptors through which cells travel to reach specific locations within the body. In this system, chemokines can be envisioned as "traffic directors" responsible for sending cells expressing appropriate chemokine receptors to specific sites. Leukemia cells "hijack" this system to disseminate throughout the body and ensure their survival beyond the primary tumor site (19).

CXCL12/CXCR4–CXCR7 SIGNALING

The stromal cell-derived factor-1 (or CXCL12) initially thought to selectively interact with CXCR4, but now known to signal also through CXCR7 or ACKR3 (49), is widely expressed in numerous tissues and cells, including immature osteoblasts and endothelial cells (EC) within the BM, stromal cells in thymus, lungs, liver, brain, and lymph nodes. CXCR4 is also broadly expressed and is frequently overexpressed in cancer (50). Under homeostasis, the CXCL12/CXCR4 axis is crucial for the homing of hematopoietic progenitor cells (HPC) in the BM and their mobilization into the periphery (51). HPC reside in BM "niches" or specialized areas consisting of diverse cells regulating self-renewal, proliferation, and survival of HPC (52). At least two distinct BM niches have been identified, called "osteoblastic/endosteal" and "vascular" niches. In the hypoxic endosteal niche, osteoblasts lining the endosteum are responsible for HPC retention and quiescence maintenance through the intervention of numerous molecules including granulocyte colony-stimulating factor, bone morphogenetic protein, JAG-1/NOTCH1, Angiopoietin-1/Tie2, and osteopontin signaling (53). The vascular niche, localized at the sinusoidal walls, which includes EC, regulates proliferation, differentiation, and mobilization of HPC by secreting stimulatory and inhibitory soluble factors (54). A third niche, formed by CXCL12-abundant reticular cells (CAR), is located in central

areas of the BM thus surrounding sinusoidal EC. These CAR cells, which comprise reticular Nestin$^+$-mesenchymal stromal cells, as well as leptin receptor positive perivascular stromal cells (55, 56), are essential for the earliest stages of lymphoid development and express high levels of CXCL12, stem cell factor, interleukin-7, Angiopoietin-1, Fms-Related Tyrosine Kinase 3 Ligand, vascular cell adhesion molecule 1, and osteopontin (57–59). These reticular cells promote HPC retention and proliferation.

It is becoming increasingly evident that leukemic cells (and leukemic stem cells) actively interact with the BM microenvironment to promote their proliferation and survival at the expense of normal hematopoiesis (60). Indeed, using a Notch-1-dependent mouse model it has been found that TALL cells suppress normal hematopoiesis through the remodeling of the BM microenvironment by hijacking the proliferative vascular niche and repressing the endosteal/osteoblastic niche (61). The depletion of osteoblasts was due to the aberrant activation of Notch in these cells probably through Hes1-mediated repression of Runx2 transcriptional activity (61). This activation of Notch signaling in osteoblasts (possibly through increased expression of JAG1 or inflammatory cytokines) was associated with a reduced expression of CXCL12 within the stem/perivascular niche. Ultimately, one could envision a feedback loop where leukemic T-cell blasts disrupt homeostatic stem/lymphoid niches leading to compromised hematopoiesis while promoting their own Notch-dependent outgrowth.

T-cell lineage cell production relies on the thymic colonization by BM-exported early progenitors (thymus-seeding progenitors) expressing P-selectin glycoprotein ligand-1 and the chemokine receptors CCR7, CCR9, CXCR4, and possibly CCR5 (62, 63). These cells enter the thymus at the cortico-medullary junction where they undergo T-cell development. In the thymus, CXCL12 seems expressed throughout the cortex (64) by cortical thymic epithelial cells and together with the ligands for CCR7 (CCL21/CCL19) and CCR9 (CCL25) (65) contribute to the gradients required for the step-wise migration of immature thymocytes through the cortex toward the medulla. It has been found that chemokine receptor expression is very dynamic during T-cell development, in fact CCR7 is downregulated during double negative (DN) stages such that pre-positive selection double positive (DP) thymocytes are CCR7$^-$, while CD4 and CD8 single positive (SP) thymocytes generated after positive selection re-express CCR7 prior to entering the medulla for tolerance induction (64, 66). On the other hand, DN and DP thymocytes express both CCR9 and CXCR4. The CXCL12/CXCR4 axis seems to have a role beyond acting as a retention signal that maintains DP thymocytes in the cortex, as it critically impacts on the proliferation and survival of DN thymocytes during β-selection acting as a co-stimulator of the pre-T-cell receptor (67). Moreover, CXCL12 may also act as a chemorepellent during the exit of mature SP cells from the thymus into the bloodstream, a process called chemofugetaxis (68). Recently, however, it has been suggested that following positive selection, CXCR4 high CCR9$^+$CD69$^-$ DP cells downregulate CXCR4 to become CXCR4 low CCR9$^+$ CD69$^+$ DP cells and subsequently CD4$^+$ and CD8$^+$ SP cells with very low/undetectable CXCR4 surface expression. Thus, unlike for the DN thymic compartment, CXCR4 expression in DP cells may be dispensable for downstream αβ-T-cell development (64).

CXC-chemokine ligand 12 modulates cancer biology principally through two mechanisms: (i) direct/autocrine effects promoting cancer cell growth, metastasis, and angiogenesis; (ii) by indirect/paracrine effects, including recruitment of CXCR4$^+$ cancer cells to CXCL12-expressing organs (BM, liver, thymus, lymph nodes, brain, among others) or CXCR4-expressing stromal cells to tumor sites (69). CXCR4 is overexpressed in many human cancers (70), with numerous studies demonstrating differential expression patterns (nuclear, cytoplasmic, and membrane) which translated in differences in biological behavior of cancers (71). Thus, membrane and/or cytoplasmic CXCR4 promotes tumor cell proliferation and metastasis, while nuclear CXCR4 is ineffective in explicating these functions. The role for CXCL12/CXCR4 axis in the infiltration of extramedullary sites, which commonly express significant levels of CXCL12 is supported by the correlation between high-surface CXCR4 expression by ALL cells (including T-ALL cells) and infiltration of extramedullary organs such as spleen and liver (72).

Recently, Pitt et al. (22) demonstrated that mouse Notch1-dependent T-ALL cells were directly interacting with CXCL12-producing vascular EC, and that this contact was necessary for leukemia maintenance and progression. In addition, murine and human T-ALL cells were shown to express increased cell-surface CXCR4 compared with mature peripheral T cells. Interestingly, this increased expression was not present at the transcript level, suggesting a non-transcriptional mechanism. Indeed, CXCR4 cell-surface expression, results from a balance between endocytosis, intracellular trafficking, and recycling, as well as gene expression (73, 74). CXCR4 internalization requires phosphorylation of its C-terminus, followed by ubiquitination and subsequent β-arrestin-dependent sorting into early endosomes, which are then processed into late endosomes or multivescicular bodies and further fused with lysosomes, ultimately leading to receptor and ligand degradation. The maturation of endosomes entails a cascade controlled by Rab small GTP-ases (75). CXCR4 internalization also depends on a dileucine motif within the C-terminal tail of CXCR4 (76) and numerous proteins including cortactin (77) and PIM1 (73) have been shown to regulate CXCR4 recycling and cell-surface expression. Remarkably, defects in endocytic trafficking of CXCR4 may contribute to increased surface expression and cancer progression (78). In acute myeloid leukemia (AML), a link has been found between PIM1 kinase activity and the surface expression and function of the CXCR4 receptor, with PIM1 expression levels correlating with CXCR4 surface expression (73). Indeed, PIM1 can phosphorylate serine 339 in the C-terminal domain of the CXCR4 receptor (a site critical for receptor recycling) contributing to high-CXCR4 surface expression and function at least in AML and B-CLL (79) cells. Along these lines, it has recently been shown that calcineurin (a serine/threonine protein phosphatase) previously associated with leukemia initiating cell activity (80), affects CXCR4 cell-surface expression at least partially through increased cortactin expression and thus CXCR4 recycling (23). CXCR4 expression was found to be essential for T-ALL maintenance and progression (22, 23) with loss of CXCL12/CXCR4 signaling leading to reduced Myc expression (a transcription factor directly regulated by NOTCH1) and previously linked to leukemia initiating cell

activity in T-ALL. Surprisingly, although NOTCH1 has been reported to regulate numerous chemokine receptors in T-ALL (CCR5, CCR7, and CCR9; see below) this is not true for CXCR4 (21, 43), suggesting that NOTCH1 activation is not responsible for the increased CXCR4 expression. Differently in B-CLL cells, which also express high levels of surface CXCR4 and where the CXCL12/CXCR4 axis is regarded as a retention signal in tissue niches, CXCR4 has been shown to be a direct NOTCH1 target (13), suggesting a fundamental role of the NOTCH1-CXCR4 axis in the dissemination of B-CLL cells to lymphoid organs. Some of the main consequences on the biological behavior of T-ALL and B-CLL cells determined by NOTCH1 signaling are summarized in **Table 1**.

CXC-chemokine ligand 12 binding to CXCR4 triggers receptor homo- and heterodimerization, often with CXCR7 (a second chemokine receptor for CXCL12; discussed below), depending on the levels of co-expression (88). The binding of CXCL12 to CXCR4 initiates divergent signaling events that result in numerous responses (possibly cell-type specific) such as chemotaxis, cell survival, and/or proliferation, increase in intracellular calcium and gene transcription (**Figure 2**). CXCR4 is a GPCR that uses trimeric G-proteins constituted mainly of a Gαi subunit which inhibits adenyl cyclase activity and to a lesser extent a Gαq subunit which activates phospholipase C-β, which leads to inositol 1,4,5 trisphosphate and diacylglycerol production. Ultimately, these events lead to activation of the transcription factor NF-κB, the tyrosine kinase PYK2, Janus kinase-signal transducer and activator of transcription and PI3K-AKT pathways. The βγ dimer instead is mainly involved in Ras activation of ERK1/2 MAPK and activation of PI3K through direct interaction of the βγ dimer with ion channels. Moreover, following ligand-induced CXCR4 phosphorylation by G-protein receptor kinases the interaction with β-arrestin not only mediates clathrin-dependent endocytosis (see above) but also promotes the activation of MAPKs (p38, ERK1/2) and CXCL12-dependent chemotaxis (89). Recently, CXCR7 has been identified as a second receptor for CXCL12, showing a 10-fold higher affinity for this ligand than CXCR4 (49). This receptor is a member of the ACKR subgroup as it does not activate G-proteins after ligand binding (48). This receptor also binds CXCL11 (known ligand of CXCR3) with low affinity and macrophage migration inhibitory factor (90, 91). CXCR7 has been implicated in cell survival and adhesion (92). CXCR7 can act as a scavenger receptor or decoy receptor that removes CXCL12 from the extracellular milieu. Binding of ligands (CXCL12 or CXCL11) to CXCR7 promotes their internalization (49), ligand trafficking to lysosomes (where ligands are degraded), and CXCR7 recycling back to the cell membrane (93). Such CXCR7-dependent regulation of local CXCL12 availability ultimately leads to reduced CXCL12/CXCR4 signaling. On the other hand, the CXCL12 scavenging function of CXCR7 may positively regulate CXCR4-mediated migration by preventing down-regulation of CXCR4 surface expression and function following the exposure to excessive CXCL12 concentrations. In contrast, in cells with primarily intracellular CXCR7 expression and high-CXCR4 surface expression, CXCR7 blockade was not able to alter CXCR4-mediated phosphorylation of ERK and AKT, suggesting that CXCR7 was not necessary for CXCR4 signaling (94). Emerging evidence suggests that CXCR7 is a fully signaling receptor independent of G proteins and can activate intracellular signaling pathways such as AKT, MAPK, Janus kinase-signal transducer, and activator of transcription 3 either by direct modulation, through a β-arrestin-dependent pathway or after heterodimerization with CXCR4 (95). Thus, the relative expression levels of CXCR4 and CXCR7 could critically influence the cellular response to CXCL12. Recently, CXCR7 expression has been found to be very low in normal BM $CD34^+$ cells compared with high levels of expression of this receptor in malignant ALL cells and cell lines (96, 97). In addition, particularly high levels of CXCR7 transcript were found in the T-ALL subtype. Analysis of the cellular distribution of CXCR7 in T-ALL cell lines disclosed a rather heterogeneous pattern with a sizable fraction being intracellular in Jurkat cells differently from MOLT4 cells. Interestingly, this different cellular distribution did not modify the functional consequences of CXCR7 silencing, as both cell lines exhibited reduced cell migration in the presence of a CXCL12 gradient (97). Notably, through the use of Notch pathway inhibitors, Asters group has identified a subset of Notch-binding sites in leukemia cell genomes that are dynamic, rapidly changing in occupancy when Notch signaling is modulated

TABLE 1 | Functional similarities and differences determined by NOTCH1 in influencing the biological behavior of T-cell acute lymphoblastic leukemia (T-ALL) and B-cell chronic lymphocytic leukemia (B-CLL) cells.

	T-ALL	B-CLL	Reference
Significance of NOTCH1 mutations	Mainly associated with improved therapeutic response and high sensitivity to glucocorticoid therapy	Associated with adverse clinical and biological characteristics (disease progression and chemoresistance)	(44, 81–85)
Effect on CCR7 expression	Transcriptional target (increased expression)	Not known	(21)
Effect on CXCR4 expression	Non-transcriptional increased cell-surface expression[#]	Direct transcriptional target (increased expression[#])	(13, 22)
Effect on CCR5 expression	Indirect transcriptional target (increased expression)	Generally not expressed	(43)
Effect on CXCR7 expression	Direct transcriptional target (increased expression[#])	Not known	(86)
Effect on CCR9 expression	Indirect transcriptional target (increased expression)	Generally not expressed	(43)
Effect on c-MYC expression	Direct transcriptional target (increased expression)	Direct transcriptional target (increased expression)	(13, 36, 37)
Main signaling pathways activated to promote cell growth, proliferation, and survival	NF-κB, c-MYC, and PI3K-AKT-mTOR	NF-κB, c-MYC, and MAPK	(13, 35, 87)

PI3K, phosphatidylinositide 3-kinase; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; AKT, protein kinase B; NF-κB, nuclear factor kappa B subunit; CCR, CC-chemokine receptor; CXCR, CXC-chemokine receptor; [#], to be verified.

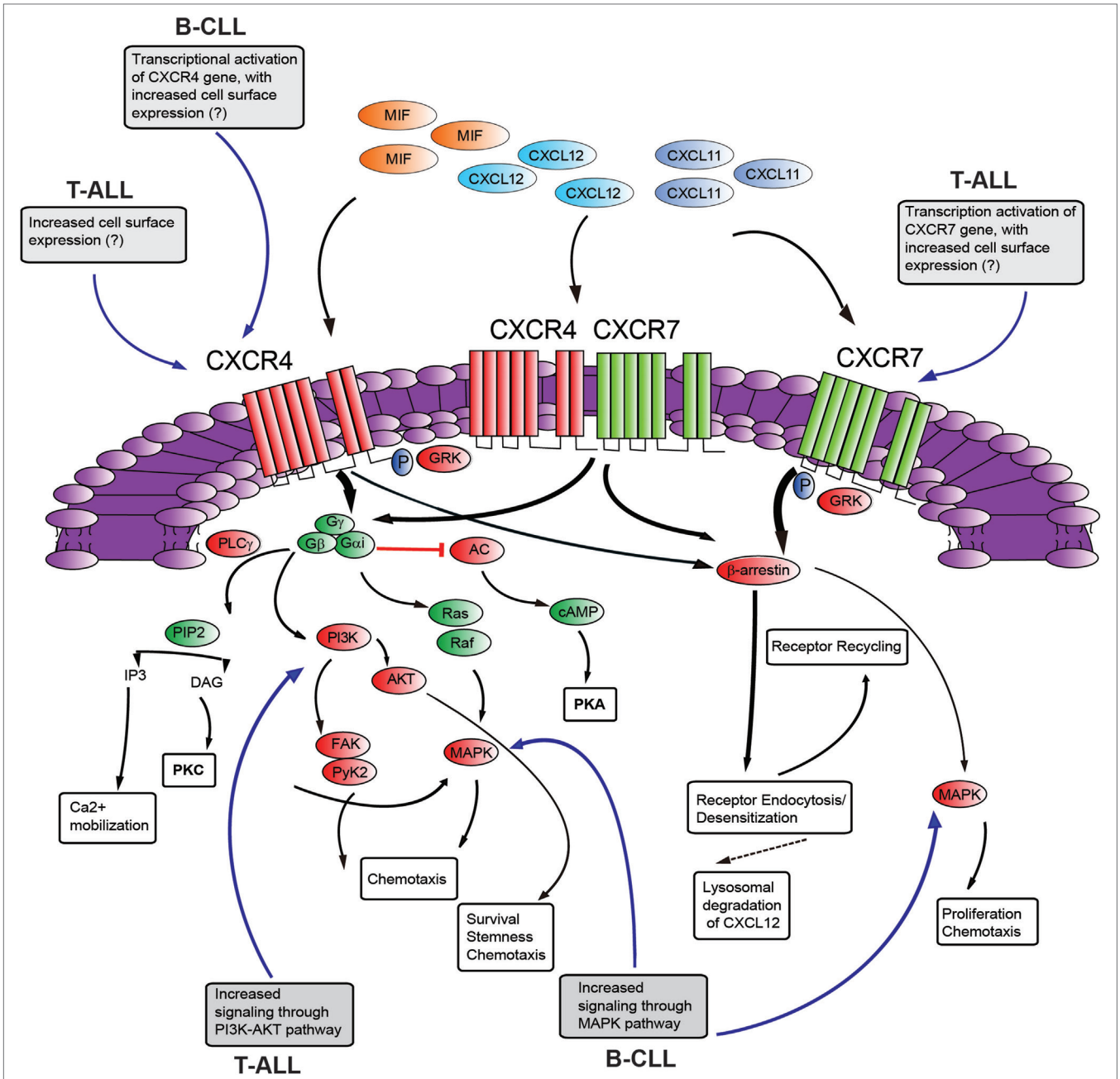

FIGURE 2 | Schematic diagram of putative CXCR4–CXCR7 crosstalk affecting signaling pathways. The influence of NOTCH signaling on the main aspects of this signaling axis is shown in gray boxes [differences between T-cell acute lymphoblastic leukemia (T-ALL) and B-cell chronic lymphocytic leukemia (B-CLL) is presented]. CXCL12 employs two distinct receptors, CXCR4 and CXCR7 which can form homodimers or heterodimers. Additionally, CXCR4 and CXCR7 can act as receptors for macrophage migration inhibitory factor (MIF), while CXCR7 can also bind to CXCL11. Commonly, stimulation of CXCR4 leads to G-protein-coupled chemokine receptors (GPCR) signaling through phosphoinositide-3 kinase (PI3K)/AKT, PLC/IP3, MAPK pathways, and mobilization of $Ca2^{+}$ from intracellular sources. CXCR4/CXCR7 heterodimerization attenuates GPCR signaling, promoting β-arrestin mediated signaling. Activation of CXCR7 triggers β-arrestin mediated signaling. Internalization of the receptors CXCR4 and CXCR7, and subsequent recycling to the cell surface, is also mediated by β-arrestin. Binding of CXCL12 to CXCR7 promotes internalization and scavenging (lysosomal degradation) of CXCL12. AC, adenylyl cyclase; cAMP, cyclic adenosyl monophosphate; PKA, protein kinase A; PLC, phospholipase C; GRK, GPCR kinase; PI3K, phosphatidylinositide 3-kinase; Gα/Gβ/Gγ, heterotrimeric G-protein consisting of subunits α, β, and γ; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-bisphosphate; AKT, protein kinase B; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; Pyk-2, proline rich kinase-2; DAG, diacylglycerol; PKC, protein kinase C. "?", not known; black, pathway activation; red, pathway repression.

(86). These dynamic NOTCH1 sites are highly associated with genes that are directly regulated by Notch and mainly lie in large regulatory switches (termed superenhancers), characterized by exceptionally broad and high levels of H3K27 acetylation (98). The *CXCR7* gene was found to be among these genes with high-dynamic regulatory potential and that are up-regulated following

GSI washout in CUTLL1 cells (86). As the list of genes with highly dynamic regulatory potential are enriched for previously identified putative direct NOTCH1 target genes, it will be interesting to validate CXCR7 as a NOTCH1 direct target as this could add a further layer of complexity to the role played by NOTCH1 in promoting T-ALL retention/dissemination.

CXCL19/CCR7 SIGNALING

This signaling axis is physiologically important for its role in the development of immune responses, as it normally recruits activated dendritic cells and naïve T cells (expressing CCR7) to draining lymph nodes (expressing high levels of the ligands CCL19/CCL21), thus initiating an adaptive immune response (99). In tumors, CCR7 is often overexpressed and its expression mostly correlates with lymph node metastasis (100). Many leukemia and lymphomas also express CCR7, and this may account for their tropism for lymph nodes (especially T-cell zones) (101). Additionally in B-CLL, the interaction between CXCR5 (expressed at high levels in B-CLL, but not T-ALL cells) and its ligand CXCL13 (produced by resident stromal cells) is responsible for recruiting leukemic cells to lymphoid organs and possibly orchestrates the establishment and maintenance of proliferation centers (pseudofollicles) within these tissues (102). T-ALL patients have increased risk of CNS involvement at diagnosis or relapse, with the mechanisms behind this tropism still ill-defined. Possible entry routes for leukemic cells in the CNS include dissemination to the subarachnoid space from the BM of the skull *via* the bridging veins or from the cerebrospinal fluid *via* the choroid plexus; through brain capillaries to the cerebral parenchyma; infiltration of meninges *via* bony lesions of the skull and possibly traumatic lumbar puncture (24, 103). Buonamici et al. (21) showed that CCR7 signaling regulates CNS infiltration of leukemic T cells, using oncogenic Notch1 mouse models. Indeed, gene expression profiling of uncommitted hematopoietic progenitors expressing oncogenic Notch1 (Notch1-IC) showed significant upregulation of *Ccr7*. NOTCH1-dependent regulation of CCR7 was confirmed in T-ALL cell lines and primary T-ALL samples. Furthermore, overexpression of mouse *ccr7* in a T-ALL cell line not expressing CCR7 (DND41) licenses these cells to specifically infiltrate the brain, possibly through interaction with CCL19 expressed on brain EC. Interestingly, using *ccr6*$^{-/-}$, *ccr7*$^{-/-}$, and *cxcr4*$^{-/-}$ fetal liver progenitors transduced with oncogenic Notch1-IC, *cxcr4* rather than *ccr7* was implicated in CNS infiltration by T-ALL cells, in addition to BM engraftment (24). Significantly, in primary T-ALL samples, high CCR7/CXCR4 mRNA levels correlated with increased risk of CNS involvement (104), although only CCR7 expression had an independent predictive impact on CNS status. Taken together, these data suggest that both CXCR4 and CCR7 play a role in the recruitment of leukemic T cells to the CNS.

The spleen is an important organ involved in extramedullary hematopoiesis and is frequently infiltrated in numerous lymphoid malignancies. There is a high incidence of splenomegaly in ALL, especially T-ALL, with the presence of splenomegaly associated with poorer prognosis of leukemia patients (105). Recent findings from Notch1-dependent leukemia models (106), suggest that the higher levels of CCL19 found in the splenic microenvironment compared with BM could be responsible for the initial homing of these leukemic cells to the spleen (given their expression of CCR7), and at the same time the splenic microenvironment could stimulate the expression of CCL19 by T-ALL cells establishing a positive feed-back loop, leading to further recruitment of leukemic cells to the spleen (106).

CCL25/CCR9 SIGNALING

The CCL25/CCR9 chemokine axis normally influences the homing, development, and homeostasis of T cells (107). CCR9 is expressed on the majority of immature DP ($CD4^+CD8^+$) thymocytes, and then is downregulated during their transition to mature SP $CD4^+$ or $CD8^+$ stage (108). Also, approximately half of all γδ TCR^+ thymocytes and peripheral γδ-T cells express functional CCR9 (109). The ligand of CCR9, CCL25, is highly expressed not only by cortical and medullary thymic epithelial cells but also by epithelial cells of the small intestine (108). Intriguingly, a case report of a pediatric T-ALL expressing CCR9 (and CD103 or αEβ7 integrin) at diagnosis, that switched to acute myeloid leukemia at relapse with disease localization to the gut has been reported (110), suggesting a role for CCR9 in the gut tropism of these leukemic cells. Recently, an elegant study found that conditional postnatal knockdown of *Pten* (*shPten*) in the hematopoietic compartment produced a highly disseminated T-ALL with the majority of leukemias harboring activating mutations in the Notch1 PEST domain (25). These *shPten* leukemias expressed high levels of CCR9 and showed marked dissemination to the intestine (and liver). Surprisingly, PTEN reactivation had no effect on tumor growth in the lymph nodes or spleen, while it markedly decreased tumor infiltration into intestine and liver, suggesting that the impact of Pten expression on disease progression is dictated by the anatomical site of leukemic disease. Subsequent experiments to determine how PTEN influences T-ALL homing and survival in the intestine disclosed that reduced PTEN expression (through *Pten* knockdown) sensitized leukemia cells to CCL25-induced Akt phosphorylation leading to their increased migration in transwell assays, and this effect was largely abrogated following PTEN re-expression (25). These findings suggest that leukemic cells with PTEN suppression or loss are facilitated in dissemination to distant sites such as the intestine (if they express CCR9) and amplify weak environmental cues (such as CCL25 signaling) that enable their survival. Consistent with this notion, stimulation of T-ALL cells with CCL25 has been reported to enhance their resistance to TNF-α mediated apoptosis (through the induction of the inhibitor of apoptosis protein Livin) partly through the activation of c-*jun*-NH2-kinase 1 (111). Interestingly, the Notch pathway has been shown to indirectly control the expression levels of CCR9 (and CCR5) in T-ALL cell lines and patient-derived primary leukemia cells, and subsequent biological effects such as cell proliferation and migration (43). It could thus be speculated that PTEN suppression together with NOTCH1 activation (frequently observed in human T-ALL) could cooperate to enhance migration to specific anatomical sites such as the intestine (through the increased expression of selected chemokine receptors such as CCR9) and

confer a proliferative advantage in an otherwise non-supportive microenvironment (CCL25-expressing sites).

CONCLUSION AND PERSPECTIVES

ALL is the most common malignancy in children, with 15% showing markers for the T-lineage (T-ALL). Of these, approximately 20% still die due to disease relapse. Instead in adults, T-ALL represents around 25% of ALL cases, with approximately 50% dying due to disease relapse notwithstanding current combination chemotherapy (112, 113). B-CLL is the most common human leukemia in adults, with patients often presenting an indolent course, surviving for a number of years with relatively mild symptoms (15). In ALL, leukemia relapses have been directly linked to the survival of blasts in organs such as CNS or testes in addition to BM (103). Infiltration of distant organs such as CNS is frequently observed in T-ALL and is an important obstacle for long-term remission. Many genes are implicated in the pathogenesis of T-ALL, including NOTCH, with *NOTCH1* mutations being identified in over half of T-ALL patients (6). Although the mechanisms of normal T-cell homing to lymphoid organs and trans-endothelial migration are relatively well known, the mechanisms exploited by leukemic T cells to gain access to target organs remain elusive. Homeostatic chemokines are considered pivotal molecules in promoting metastasis in solid tumors (19), and may help to account for the non-random metastatic destinations encountered in different neoplasia. In B-CLL, NOTCH1 activation probably reflects the constitutive, dysregulated expression of a physiological signal (13). *NOTCH1* mutations in T-ALL hijack the physiological role of NOTCH signaling during thymocyte development (114) with oncogenic *NOTCH1* alterations expressed in HPC often used as models of human T-ALL to gain mechanistic insights. Mainly through the use of these NOTCH1-dependent leukemias it is emerging that homeostatic chemokines and their receptors are critically involved not only in dictating medullary and extramedullary dissemination but also directly affecting the viability and growth of nascent leukemic niches. Recent studies showing that surface chemokine receptor expression and function may not correlate with mRNA transcript levels and that defects in recycling or endocytic trafficking of chemokine receptors may contribute to cancer progression add a new layer of complexity to the mechanisms acting to fine-tune the functional consequences of chemokine signaling. Thus, future studies evaluating the significance of chemokine receptor expression/signaling will need to go beyond mRNA expression levels, but will also have to take into account receptor phosphorylation, ubiquitination, recycling, and internalization rates. In particular, it may also be worth revisiting the role of CXCL12 biology in T-ALL (and possibly B-CLL) from the CXCR7 perspective. Intriguing are also recent observations that anti-tumor therapies (radiation and chemotherapy, among others) promote a hypoxic environment (19), which through the stabilization of hypoxia-inducible factors can increase the expression of chemokine receptors such as CXCR4 (115); conversely, other chemotherapies can downregulate chemokine receptor expression (19). Thus, some current therapies aimed at killing tumor cells may actually promote a more aggressive phenotype in the surviving cells (116). In B-CLL, the Bruton's tyrosine kinase inhibitor, Ibrutinib, has been shown to determine early lymphocytosis and organomegaly reduction followed by normal cell count restoration, possibly in part due to its effects on CXCR4 expression (117). The effects of contemporary chemotherapy regimens used in T-ALL on chemokine receptor expression remain to be elucidated. Comprehensively, although numerous studies have focused on the role of chemokine receptors and their regulation by NOTCH, much less is known on downstream signals such as integrin activation or actin remodeling dynamics.

Currently, clinically approved targeted therapies to impede organ infiltration in acute leukemia are lacking. Of the chemokine axes that can be targeted, the CXCL12/CXCR4–CXCR7 axis seems most promising in T-ALL, as monotherapy with the selective CXCR4 antagonist, AMD3465, was highly effective in suppressing human disease in a xenograft model (22). However, monotherapy with CXCR4 inhibitors in other malignancies has more modest anti-leukemic effects (including B-CLL), as it mainly sensitized leukemic cells to conventional or targeted therapies through the mobilization of the leukemic cells into the periphery (102, 118, 119). Thus, it is likely that combination therapies comprising chemokine receptors antagonists together with conventional chemotherapeutic agents or specific targeted therapies such as NOTCH1 inhibitors will be required to eradicate the disease and prevent relapse.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Notch Signaling in Macrophages in the Context of Cancer Immunity

Tanapat Palaga[1,2], Wipawee Wongchana[2,3] and Patipark Kueanjinda[2,4]*

[1] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, [2] Center of Excellence in Immunology and Immune-Mediated Diseases, Chulalongkorn University, Bangkok, Thailand, [3] Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, [4] Laboratory for Systems Pharmacology, Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Macrophages play both tumor-suppressing and tumor-promoting roles depending on the microenvironment. Tumor-associated macrophages (TAMs) are often associated with poor prognosis in most, but not all cancer. Understanding how macrophages become TAMs and how TAMs interact with tumor cells and shape the outcome of cancer is one of the key areas of interest in cancer therapy research. Notch signaling is involved in macrophage activation and its effector functions. Notch signaling has been indicated to play roles in the regulation of macrophage activation in pro-inflammatory and wound-healing processes. Recent evidence points to the involvement of canonical Notch signaling in the differentiation of TAMs in a breast cancer model. On the other hand, hyperactivation of Notch signaling specifically in macrophages in tumors mass has been shown to suppress tumor growth in an animal model of cancer. Investigations into how Notch signaling is regulated in TAMs and translates into pro- or anti-tumor functions are still largely in their infancy. Therefore, in this review, we summarize the current understanding of the conflicting roles of Notch signaling in regulating the effector function of macrophages and the involvement of Notch signaling in TAM differentiation and function. Furthermore, how Notch signaling in TAMs affects the tumor microenvironment is reviewed. Finally, the direct or indirect cross-talk among TAMs, tumor cells and other cells in the tumor microenvironment *via* Notch signaling is discussed along with the possibility of its clinical application. Investigations into Notch signaling in macrophages may lead to a more effective way for immune intervention in the treatment of cancer in the future.

Keywords: Notch signaling, macrophages, tumor-associated macrophages, metastasis, tumor immunity

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Rossella Rota, Bambino Gesù Ospedale Pediatrico (IRCCS), Italy
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Masanori Aikawa, Harvard Medical School, United States

****Correspondence:***
Tanapat Palaga
tanapat.p@chula.ac.th

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INTRODUCTION

The biological functions of macrophages are diverse and not only limited to their role as the first line of defense during innate immune response. In addition to their protective role against infections, the known roles of macrophages have expanded in recent years, and their involvement in organ development, tissue homeostasis, and metabolic dysfunctions, such as diabetes and obesity, are increasingly appreciated. Cancer is another area in which macrophages have emerged as a crucial player in the creation of a tumor microenvironment that supports tumor growth and metastasis, in opposition to their traditional role as an innate immune cell, whose function is to eliminate cancer cells (1). Therefore, understanding the signaling pathway(s) governing the development, differentiation, activation, deactivation, proliferation, and cell death of macrophages in the context

of tumorigenesis is expected to reveal novel strategies for targeting cancer growth more effectively.

The critical functions of the evolutionarily well-conserved Notch signaling pathway in myeloid lineage cell development and, in particular, monocyte/macrophage development are well recognized and have been reviewed extensively elsewhere (2, 3). Recent evidence, using state of the art technologies, revealed better defined subsets of circulating monocytes and the uniqueness and the origin of tissue-resident macrophages (TRMs). This new insight reignited the excitement in the field of macrophage biology. In addition, these studies cast new light and controversy over the origin of macrophages found in tumors, called tumor-associated macrophages (TAMs), and the involvement of TAMs in cancer progression and suppression (4, 5). Within tumors of various origins, macrophages have been observed to accumulate in large numbers and exhibit unique combinations of activated phenotypes (6). In general, TAMs in large quantities are associated with poor disease prognosis, partly by promoting tumor growth, dampening immune responses, and inducing angiogenesis and metastasis (7, 8). Together with the recent advances in the understanding of the roles, Notch signaling plays in the activation and regulation of the immune effector functions of macrophages and in TAMs, these observations have led to the conclusion that Notch signaling is one of the candidate pathways to be manipulated to enhance the host anti-tumor response. In this review, we summarize the current knowledge of the involvement of Notch signaling in macrophage activation, with an emphasis on its role(s) in TAMs. We also discuss the cross-talk among macrophages, tumor cells, and other cells associated with the tumor microenvironment and the potential utility and challenges in manipulating Notch signaling in TAMs for tumor suppression in ways that are beneficial to the host.

Notch Signaling in Macrophage Activation and Function

The biological functions of macrophages are multi-faceted depending on the external microenvironment, and some functions may be contradictory or opposing to others. For example, during infection or tissue injury, macrophages sense danger *via* various receptors, actively eliminate the source of danger by phagocytosis and chemical mediators, and trigger inflammation by producing inflammatory cytokines to alert other immune cells. After the elimination phase, wounds are healed mainly by anti-inflammatory wound-healing macrophages (9). The contradictory inflammatory and anti-inflammatory microenvironments are conducive to driving macrophage activation into two opposite functional spectra. The most simplistic view of macrophage effector functions divides activated macrophages into pro-inflammatory macrophages, in which macrophages are activated by pathogen-associated molecular patterns (PAMPs) and/or inflammatory cytokines. In contrast, anti-inflammatory macrophages, activated by IL-4/IL-13, represent a wound-healing and immunosuppressive phenotype (10). However, more detailed characterization and studies in various *in vivo* models have revealed a more complicated view of macrophage effector phenotypes that are often observed in an *in vivo* setting (11). Thus, the narrow concept of pro- vs. anti-inflammatory macrophages may be oversimplified, and the presence of various hybrid phenotypes of macrophages has been described (11). Some of the genes uniquely expressed in pro- or anti-inflammatory macrophages are summarized in **Table 1** (12, 13).

To avoid oversimplification and confusion over macrophage effector phenotypes, this review will adopt the macrophage nomenclatures proposed by Murray et al. to describe specific macrophage subsets based on the stimuli and effector functions described in each referred study (19). In some instances, where the stimuli were not identified, the microenvironments in which macrophages were described will be used.

Initial reports generally found that Notch signaling primarily operates in macrophages that are activated toward inflammatory functions such as in lipopolysaccharide (LPS)-activated macrophages M(LPS) or LPS in combination with IFNγ M(LPS + IFNγ) (15, 20, 21). Subsequent findings in various pathophysiological conditions also indicated the involvement of Notch signaling in activation and effector functions of pro-inflammatory macrophages (3). Notch signaling, therefore, favors inflammatory macrophages, and when the Notch signaling pathway is pharmacologically or genetically blocked, some of the key pro-inflammatory functions are compromised, including the decrease in the production of pro-inflammatory cytokines, such as IL-6, and the reduction in nitric oxide production (15, 22). To this end, Notch signaling is reported to directly or indirectly influence pro-inflammatory effector functions. Notch signaling can directly regulate transcription of some of the inflammation-induced signature genes, such as *il6*, *il12b,* and *nos2* (23–25). Using *Rbpj*-deficient mice, Xu et al. demonstrated that canonical Notch signaling tips the effector phenotypes toward inflammatory ones by directly influencing the transcription of a transcription factor IRF8 (22). In addition, Notch signaling also indirectly regulates pro-inflammatory phenotypes through a cross-talk with other signaling pathways, such as NF-κB and mitogen-activated protein kinases (15, 20). Interestingly, metabolic analysis found that Notch signaling supports inflammatory macrophage phenotypes by reprograming mitochondrial metabolism toward oxidative phosphorylation (25). Abrogating Notch signaling in myeloid lineage cells attenuated inflammation in a mouse model of alcoholic steatohepatitis and reduced the severity of endotoxin-induced hepatitis (25). All evidence, therefore, points to a critical role of Notch signaling in macrophage activation toward pro-inflammatory phenotypes in a canonical Notch signaling-dependent (intracellular Notch and CSL/RBP-Jκ-dependent) manner. The question remains whether inhibition of Notch signaling under an inflammatory microenvironment can switch macrophages toward the opposite phenotype, such as anti-inflammatory functions, or whether a lack of Notch signaling only dampens the inflammatory response without directing the macrophages toward other effector phenotypes.

Is Notch signaling dispensable for other types of macrophage effector functions? In macrophages treated with IL-4/IL-13 M(IL-4/IL-13), which normally induces anti-inflammatory macrophages. Notch signaling was long considered to be irrelevant; however, an indicator that Notch signaling is activated in the form of cleaved Notch1 was observed in this condition, albeit

TABLE 1 | Expression profiles of Notch ligands and receptors and some stage-specific makers in tumor-associated macrophages (TAMs).

Notch receptors/ligands or surface markers related to TAMs	Pro-inflammatory macrophages	Anti-inflammatory macrophages	Differentiation stages of TAMs based on study by Franklin et al. (14)				
			Stage 1	Stage 2	Stage 3	Stage 4	Stage 5 (TAM)
CCR2	+	−	+	+	+	+	+
Ly6C	+	−	+	−	−	−	−
CD11c			−	+	+	+	+
MHCII	+	+	−	−	+	+	+
CD11b	+	+[a]	high	high	high	low	low
Vascular cell adhesion molecule1			−	−	−	−	+[b]
CD38	+	−					
Erg2	−	+[c]					
Notch receptors							
Notch1	+	+		+	+	+	+
Notch2	+[d]	+		+	+	+[e]	+
Notch3	+						
Notch4							
Notch ligands							
Jagged1	+						
Jagged2							
Dll1	+	+					+
Dll3							
Dll4	+	+[g]					+[f]

[a]*Italiani and Boraschi (12) provide reviews on murine blood monocyte subsets based on Ly6C expression and their functions in inflammation and tissue repair.*
[b]*Franklin et al. (14) propose TAM markers found in a breast cancer mouse model.*
[c]*Jablonski et al. (13) propose novel markers of M(LPS + IFNγ) and M(IL-4) (CD38 and Erg2) based on gene expression profiles that can exclusively distinguish M(LPS + IFNγ) from M(IL-4).*
[d]*Palaga et al. (15) report the gene expression profile of LPS-stimulated RAW264.7 macrophages.*
[e]*Ishifune et al. (16) report that Notch receptors are required for CD11c$^+$ CX3CR1$^+$ macrophage (found in the luminal bed of the small intestine) differentiation, thereby suggesting that Notch1 and Notch2, but not Notch3 may be required for TAM differentiation as TAM is also CD11c$^+$.*
[f]*Wang et al. (17) report the Notch gene expression profile in anti-inflammatory-like macrophages isolated from tumors.*
[g]*Bansal et al. (18) report Notch profiles in RAW264.7 M(LPS + IFNγ) and M(IL-4 + IL-13).*

with different kinetics than those reported in M(LPS + IFNγ) (26). More importantly, in macrophages with targeted deletion of *Rbpj*, CSL/RBP-Jκ, possibly through canonical Notch signaling, was found to be required for activation of M(IL-4) or M(chitin), including the expression of the gene signature associated with M(IL-4), such as *Arg1* expression (27). This involvement was independent of STAT6, C/EBPβ, and IRF8. In addition, our observation revealed that Notch signaling functions in macrophages activated by PAMPs in the presence of immune complexes and LPS M(LPS + Ic), which predominantly produce high amounts of IL-10 and low levels of IL-12 to function in dampening the immune response (28, 29). Together, these data indicate the need for re-thinking the roles that Notch signaling plays in macrophage activation. Notch signaling may be involved in various types of macrophage activation in a context-dependent manner. Whether Notch signaling functions as an instructor or a signal amplifier during macrophage activation remains to be determined, but this feature is similar to what has been postulated for the involvement of Notch signaling in the polarization of helper T cells (30).

Notch Receptors and Ligands During Macrophage Activation

Four Notch receptors and five Notch ligands have been identified thus far. Differences in signals sent *via* different combinations of ligand–receptor interactions have long been suspected. For example, two ligands, Dll1 and Dll4, send different signals through the same receptor, Notch1, that are either pulsatile or sustained, thereby inducing different cell fates (31). During macrophage activation, various Notch receptors and ligands have been detected (**Table 1**). All Notch receptors, except for Notch4, are expressed in pro-inflammatory M(LPS) or M(LPS + IFNγ) (15). Notch3 is selectively upregulated in pro-inflammatory macrophages, such as in M(LPS) and M(LDL) (21). Notch1 and Notch2 are required for differentiation of CD11c$^+$ CX3CR1$^+$ macrophage subset in the small intestine (16). Similarly, Jagged1, Dll1, and Dll4 are detected in pro-inflammatory macrophages (18). In M(LPS), Foldi et al. reported that Jagged1 is the ligand responsible for autoamplification of Notch signaling in pro-inflammatory macrophages (32). The importance of the Notch-Dll4 axis in pro-inflammatory macrophages was highlighted in a study using blocking antibodies against Dll4. The results revealed that the anti-Dll4 antibody reduced pro-inflammatory macrophage accumulation in inflammatory lesions and attenuated atherosclerosis and metabolic disease (33). Furthermore, during influenza infection, Dll1 expression on macrophages is crucial for dictating the effective anti-viral responses of CD4 and CD8 T cells (34). Nevertheless, knowledge of the effect of specific combinations of Notch receptors and ligands on macrophage activation is still limited, and requires each receptor and ligand to be specifically blocked to evaluate the relevance of different interaction pairs.

Origins and Functions of TAMs

In solid tumors, TAMs are a dominant cell type in tumor tissues of various origins, often second to the tumor cells themselves (35). This observation leads to the obvious questions of where these TAMs originate and what are their functions in tumors. There are two potential sources of TAMs. TAMs can develop from newly recruited monocytes from circulation or be derived from TRMs. These sources are not mutually exclusive and depend mainly on the tumor type (5). In a breast cancer model, newly recruited monocytes differentiated to become TAMs, while in brain tumors, both blood-derived monocytes and resident microglia cells contributed to the TAM population (14, 36). When TAMs arise from monocytes recruited from circulation, tumor cells need to secrete factor(s) that trigger the migration of monocytes to the tumor sites (**Figure 1**).

Macrophage phenotypes, in general, are considered highly plastic and can change depending on the microenvironment, and this may also be true for the phenotypes of TAMs in the tumor microenvironment (10). In one study, human breast cancer cells skewed TAMs toward an anti-inflammatory phenotype partly by secretion of M-CSF (39). In an *in vivo* model of BALB/c 4T1 mammary carcinoma, the tumor microenvironment condition encouraged monocyte precursors to differentiate into diverse TAM subsets with either pro- or anti-inflammatory phenotypes (40). Furthermore, studies in renal cell carcinoma have shown mixed pro- and anti-inflammatory phenotypes of TAMs (41). These observations indicate that there are variations in TAM phenotype that depend on the type of tumors and that the activation of TAMs is highly complex and context-dependent.

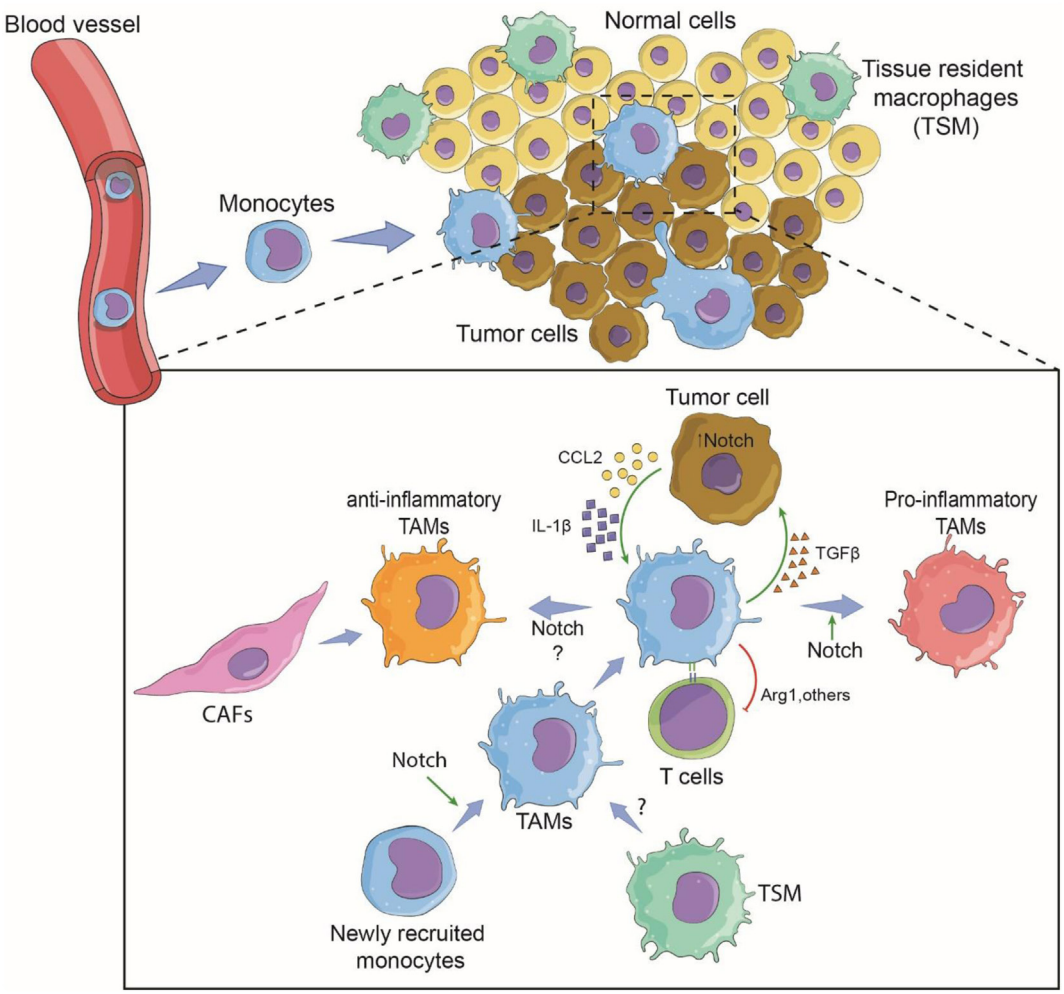

FIGURE 1 | Involvement of Notch signaling during tumor-associated macrophage (TAM) differentiation and tumor growth. Tumor cells recruit monocytes from circulation by secreting chemotactic factors and inflammatory cytokines. Notch signaling may be required for terminally differentiated TAMs. Within the tumor microenvironment, newly recruited monocytes are conditioned to become pro-inflammatory or anti-inflammatory TAMs *via* the cytokine milieu and possibly the canonical Notch signaling (14). Tissue-resident macrophages may also contribute to tumor growth by changing to TAMs. TAMs support tumor growth directly by secreted cytokines and growth factors, and indirectly by affecting T-cell response against the tumor (37). The pro-tumoral function can be Notch signaling dependent or independent. Cancer-associated fibroblasts (CAFs) may also cross-talk with TAMs *via* Notch signaling (38).

Notch Signaling and TAMs

In TAMs, Notch1 and 2 have been detected in breast cancer model, while Dll1 and Dll4 have been detected in a lung cancer model (**Table 1**) (14, 17). Jagged1 expression in a breast cancer cell line was shown to modulate TAM differentiation resulting in anti-inflammatory and IL-10-producing TAMs (42). In human cancer, evidence is still lacking regarding the expression profiles of Notch receptors and ligands in TAMs associated with different types of cancer. Recent study of head and neck head and neck squamous cell carcinoma, increasing Notch1 level is associated with $CD68^+/CD163^+$ TAMs, indirectly suggest the link between Notch signaling and TAMs (43). Knowing the expression profiles of Notch receptors and ligands in TAMs and the importance of the signals that they send will provide better targets for intervention.

Notch Signaling and Migrations of Monocytes and Differentiation Into TAMs

For monocyte-derived TAMs, the presence of TAMs begins with the recruitment of blood monocytes/macrophages to the tumor microenvironment through newly formed blood vessels around the solid tumor (14, 44). Diverse chemokines, i.e., CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), CXCL8 (IL-8), and CXCL12 (SDF1), released by tumor cells induce migration, differentiation, and survival of tumor-infiltrating myeloid cells (45, 46). The chemokine receptor CCR2 has been a subject of intense study as a key molecule of monocyte recruitment into tumors. An *in vitro* study revealed that GM-CSF-induced macrophages M(GC) showed higher CCR2 expression than their M-CSF-induced counterparts M(MC). After CCL2 stimulation, M(GC) exhibited enhanced LPS-mediated IL-10 production, indicating an anti-inflammatory role. These phenomena were confirmed by an *in vivo* study in which *Ccr2*-deficient bone marrow-derived macrophages displayed profiles indicative of inflammatory macrophages (47). In the MMTV-PyMT mammary tumor model, a decrease in the number of TAMs in the tumor site was observed in *Ccr2*-null background animals, suggesting the importance of CCR2/CCL2 signaling in the recruitment of TAMs to tumor sites (14). Further investigation revealed that the deletion of *Rbpj* in macrophages results in loss of CCR2 and TAM markers, suggesting a cross-talk between canonical Notch signaling and the CCR2/CCL2 signaling pathway in TAMs in the tumor microenvironment. One can speculate that in the early phase, monocytes are recruited to the tumor site in a CCR2-dependent manner and perhaps begin to encourage activation toward an inflammatory phenotype, but tumor cells educate these cells by creating a tumor microenvironment that re-directs them toward a tumor-friendly phenotype in a later phase of tumor growth (**Figure 1**). In fact, a gradual increase in M(IL-4)-associated markers such as a high level of CD206 expression and low or no MHC Class II molecule expression has been reported in TAMs in a mouse colon cancer model and in human cancer samples (37). Interestingly, expression of the immune checkpoint receptor, programmed cell death protein 1 (PD1), was significantly increased in $CD206^+$ TAMs compared to the expression in TAMs negative for CD206.

In basal-like breast cancer, tumor cells secrete both CCL2 and IL-1β in a Notch-dependent manner, and the secreted cytokine/chemokines, in turn, recruit monocytes to the tumor site (48). In this case, canonical Notch signaling directly regulates the expression of CCL2 and IL-1β, leading to the adhesion of monocytes to blood vessel and extravasation to migrate toward tumor tissue. CCL2 can be produced by bone marrow-derived stromal cells or tumor cells, while tumor cells produce IL-1β (49). Once monocytes are recruited, tumor microenvironments train/educate monocytes to differentiate to become TAMs with a pro-tumor phenotype that can function to support tumor growth and metastasis (5). In this breast cancer model, TAMs interact with cancer cells *via* TGFβ to potentiate the expression of Jagged1, one of the Notch ligands (48). The Notch/Jagged1 positive feedback loop amplifies cytokine/chemokine secretion leading to more TAM recruitment. In an animal model of breast cancer using MMTV-PyMT mice, Franklin et al. showed conclusively that TAMs are recruited from blood inflammatory monocytes and exhibit phenotypes and functions that are distinct from mammary TRMs. Importantly, the terminal differentiation of these TAMs from monocytes is CSL/RBP-jκ-dependent, indicating that the canonical Notch signaling pathway plays a vital role in TAM differentiation (14). Therefore, at least for TAMs in this breast cancer model, Notch signaling plays both an extrinsic role, i.e., regulating the production of recruiting factors by tumor cells, and an intrinsic role, i.e., regulating the differentiation of TAMs. Whether TAMs associated with other tumor types also require CSL/RBP-jκ for their differentiation or function is still an open question.

Notch Signaling in Anti-Tumor Responses of TAMs

Forced activation of the Notch receptor in TAMs in a Lewis lung carcinoma cell (LCC) model of cancer was shown to repress tumor-promoting activity by enhancing the anti-tumor phenotype and suppressing the pro-tumor phenotype. The mechanism of anti-tumor activity is reported to be mediated in part by microRNAs (miRNAs) (50). miRNAs are small regulatory non-coding RNAs of 21–22 nt that play important roles in regulating gene expression through post-transcriptional silencing of targets mRNAs. miRNAs play important roles in the activation and effector function of macrophages in TAMs by regulating their target genes and signaling pathway (51). In the LCC model, miR-152a, which is under regulation by Notch signaling, targets factor-inhibiting hypoxia 1 and IRF4, a transcription factor involved in M(IL-4) activation, to enhance the anti-tumor phenotype (52). In addition, another miRNA downstream of Notch signaling, miR-148a-3p, also helps to skew the activation of macrophages toward the anti-tumor phenotype by targeting the PTEN/Akt pathway and activation of the NF-κB pathway (53). This observation is consistent with the role of Notch signaling in favoring anti-tumor macrophage activation, and by forced activation of the Notch signaling pathway, these processes can result in the suppression of tumor growth.

Targeted deletion of *Rbpj* in macrophages resulted in reduced activity of $CD8^+$ T cells by diminishing the cytotoxic activity

against tumor cells in a B16 cell melanoma model (17), suggesting that the cross-talk between TAMs and CTLs is crucial for the anti-tumor immune response, and Notch signaling plays an important role in eliciting the anti-tumor activity of CTL. Moreover, activation of Notch signaling in macrophages was demonstrated to increase the $CD8^+$ T cell population infiltrating the tumor site in the LCC model (50). These data indicate the ability of Notch signaling in TAMs to increase anti-tumor activity directly as pro-inflammatory macrophages or indirectly *via* cytotoxic T cells.

With the use of the opposite approach, manipulating canonical Notch signaling in TAMs in a mouse model of cancer was clearly demonstrated to be able to control tumor growth. Targeted deletion of *Rbpj* in macrophages resulted in anti-inflammatory phenotypes under pro-inflammatory inducers (such as LPS), and these macrophages lost the ability to control tumor growth (17). Therefore, if the Notch signaling pathway is dampened in TAMs, this dampening probably results in TAMs shifting toward an anti-inflammatory-like phenotype and helping tumor growth. One caveat is that this study employed *in vitro*-activated macrophages mixed with a tumor cell line that was administered to mice. Whether switching the Notch signaling on or off in TAMs after differentiation in the tumor influences the anti-tumor immunity remains an open question.

Contradictory to the studies described above, several reports have indicated that activation of Notch signaling supports anti-inflammatory phenotypes of macrophages and possibly favors TAMs (27, 54). A study in breast cancer patients who exhibited resistance to aromatase inhibitor treatment showed higher expression of Jagged1 in the tumor and an increasing density of anti-inflammatory TAM infiltration in breast cancer tissue compared to that in control (42). This study indirectly suggests that Jagged1 on cancer cells may drive TAMs into pro-tumor phenotype by activating Notch signaling in TAMs. These contradictory reports on Notch signaling in TAMs imply that the difference in TAM phenotype possibly depends on the tumor microenvironment and types of tumor, and this need to be taken into consideration. In addition, different Notch ligands may activate Notch signaling in different ways, and this may impact the phenotypes of TAMs.

TAMs, Tumor Angiogenesis, and Notch Signaling

Angiogenesis requires contact between macrophages and endothelial cells together with cytokines and angiogenic molecules. Inflammatory macrophages, including TAMs, are involved in angiogenesis based on the expression of cytokines, such as TNF-α and IL-6, and angiogenic factors, such as vascular endothelial growth factor (VEGF) (5). Because Notch signaling, directly or indirectly, regulates the expression of genes involved in angiogenesis, such as VEGFR and EphrinB2 (55), Notch signaling in TAMs may regulate tumor angiogenesis. In retinal choroidal neovascularization (CNV), the deletion of *Rbpj* in myeloid cells results in the inhibition of the inflammatory response in the retina and choroid after injury. This inhibited inflammatory response is accompanied by suppression of VEGF and TNF-α production and CNV development in the choroid (56). Moreover, Notch1-expressing macrophages interact with two Dll4-expressing sprouts of endothelial cells, leading to the activation of Notch signaling in macrophages. This interaction regulates the function of macrophages during vessel anastomosis in retina angiogenesis (57). Loss of Notch1 in myeloid lineage cells reduces microglia recruitment and results in abnormal angiogenesis (58).

Vascular cell adhesion molecule (VCAM) 1 is highly expressed in TAMs, whereas loss of VCAM1 in macrophages reduces the number of hematopoietic stem cells in the spleen and the inflammation in atherosclerosis due to an inability of macrophages to attach to vascular endothelial cells (59). Although little is known about the role of Notch signaling in the regulation of VCAM1 expression in macrophages, lung endothelial cells express high levels of VCAM1, and increased numbers of TAMs have been observed in lung cancer tissue compared to that in control. Endothelial cells were reported to undergo cellular senescence after implantation of tumor cells expressing Notch ligands (Dll4 and Jagged1), suggesting that VCAM1 expression in endothelial cells is under the regulation by Notch signaling and, together with Notch activation, required for TAM localization (60). VCAM1 expression in endothelial cells is under regulation of the Notch signaling pathway even in the absence of inflammatory cytokines. However, in the presence of IL-1β, VCAM1 expression in endothelial cells is greatly enhanced in a Notch-dependent manner (61). These studies suggest that endothelial VCAM1 is important for the survival of TAMs in the tumor microenvironment. However, this interaction through VCAM1 may be bidirectional because VCAM1 is also highly expressed in TAMs, suggesting that it may play an important role in the survival of endothelial cells as well. Blood vessel endothelial cells have also been found to play a role in TAM differentiation. A recent study demonstrated that Dll1 expressed by endothelial cells lining the blood vessels in mice induced conversion of $Ly6C^{hi}$ to $Ly6C^{lo}$ monocytes in a Notch2-dependent manner (62). This study was the first to demonstrate that the Notch ligand Dll1 in the blood vessel can induce phenotypic changes in monocytes through the Notch2 receptor under steady-state conditions.

The Role of Notch-Dependent TAMs in Supporting Tumor Growth and Immune Suppression

As described above, TAMs can directly support tumor growth by secreting factors, such as TGFβ (48). TAMs also affect the overall anti-tumor immunity mounted by other immune cells, such as T lymphocytes, in tumor sites by dampening the immune functions. Arginase 1, an arginine-degrading enzyme produced by M(IL-4), can suppress CTL activity (63). Recently, anti-inflammatory macrophage-like ($CD206^+$ MHC II^{low} or ^{negative}), but not pro-inflammatory macrophage-like ($CD206^-$ $MHCII^{hi}$) TAMs have been reported to express PD1 in both a mouse model and in human cancers over time with disease progression (37). The so-called immune checkpoint inhibitor is used to block this PD1-PD-L1 interaction and trigger a vigorous host immune response against the tumor. Interestingly, blocking this interaction results in increasing phagocytosis by macrophages and a reduction in tumor growth in mouse models of cancer (37). Although there is no evidence linking Notch signaling and PD1 in TAMs, there

is a report indicating that canonical Notch signaling regulates the expression of PD1 in activated $CD8^+$ T cells (64). Cancer-associated fibroblasts (CAFs) are indicated as accomplices in malignant cancers (38). Because CAFs and TAMs are reported to collaborate *via* cell–cell interaction in promoting tumor progression (65), it is possible that Notch signaling may contribute in the cross-talk between the two cell types. Taken together, these observations suggest that Notch signaling may be involved in regulating this immune suppression mechanism in TAMs *via* an immune checkpoint inhibitor.

Challenges and Potential for Manipulating Notch Signaling in TAMs for Therapy

Notch signaling clearly plays important roles in TAMs, either to promote or suppress tumor growth. Therefore, Notch signaling in TAMs can be a drug target for manipulating host anti-cancer immunity. If Notch signaling in TAMs is pro-tumoral, suppressing it would benefit the host. In contrast, if TAMs require Notch signaling to become more inflammatory anti-tumor macrophages, it needs to be stimulated. Various types of gamma-secretase inhibitor that is a pan-Notch signaling inhibitor are often used to suppress Notch signaling in cancer clinical trials (66). Unfortunately, this inhibitor has off-target effect and is highly toxic if applied systemically. Therefore, designing a method that specifically inhibits Notch signaling in TAMs is desirable. One approach is to use a stapled peptide derived from part of mastermind-like protein that interferes with canonical Notch signaling. If coupled with a TAM-specific delivery system, this peptide could specifically inhibit Notch signaling in TAMs (67, 68). Antibody-based specific antibody blocking has also been investigated for targeting the ligand-binding domain or the negative regulatory region of Notch receptors (69). To activate Notch signaling to favor inflammatory macrophages, an activating antibody that mimics ligand binding may be used. In any case, an intelligent method that targets TAMs is required to minimize the side effects.

Remaining Unresolved Questions and Future Directions

Notch signaling in macrophages clearly affects their biological functions both directly and indirectly. Notch signaling also affects TAMs and functions in monocyte recruitment, tumor-mediated training, and angiogenesis. Notch signaling in TAMs is, therefore, an attractive signal to manipulate to promote anti-tumor immunity. Macrophages have been reported to be epigenetically modified by stimuli that contribute to "trained immunity" and "tolerance," at least *in vitro* (70). If the manipulation of macrophage polarization of TAMs through Notch signaling is to be considered as an alternative for cancer treatment, we must ask whether the epigenetic marks on TAMs imprinted by the tumor microenvironment, created by cancer cells, can be reversed or erased so that TAMs could act to benefit the host.

AUTHOR CONTRIBUTIONS

TP is responsible for designing the article concept and scope, reviewing 50% of the content, and conceptualizing the figure. WW is responsible for reviewing 20% of the content. PK is responsible for reviewing 30% of the content and designing the table and part of the scope of the article.

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REVIEW
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A Review of Notch Processing With New Insights Into Ligand-Independent Notch Signaling in T-Cells

Martin Peter Steinbuck† *and Susan Winandy**

Immunology Training Program, Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA, United States

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Edited by:
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***Correspondence:**
Susan Winandy
swinandy@bu.edu

†**Present address:**
Martin Peter Steinbuck, Vedantra Pharmaceuticals, Inc., Cambridge, MA, United States

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The Notch receptor is an evolutionarily highly conserved transmembrane protein essential to a wide spectrum of cellular systems, and its deregulation has been linked to a vast number of developmental disorders and malignancies. Regulated Notch function is critical for the generation of T-cells, in which abnormal Notch signaling results in leukemia. Notch activation through *trans*-activation of the receptor by one of its ligands expressed on adjacent cells has been well defined. In this canonical ligand-dependent pathway, Notch receptor undergoes conformational changes upon ligand engagement, stimulated by a pulling-force on the extracellular fragment of Notch that results from endocytosis of the receptor-bound ligand into the ligand-expressing cell. These conformational changes in the receptor allow for two consecutive proteolytic cleavage events to occur, which release the intracellular region of the receptor into the cytoplasm. It can then travel to the nucleus, where it induces gene transcription. However, there is accumulating evidence that other pathways may induce Notch signaling. A ligand-independent mechanism of Notch activation has been described in which receptor processing is initiated *via* cell-internal signals. These signals result in the internalization of Notch into endosomal compartments, where chemical changes existing in this microenvironment result in the conformational modifications required for receptor processing. This review will present mechanisms underlying both canonical ligand-dependent and non-canonical ligand-independent Notch activation pathways and discuss the latter in the context of Notch signaling in T-cells.

Keywords: Notch, T-cell, endocytosis, ligand-independent, T-cell receptor, protein kinase C

INTRODUCTION

The Notch receptors are evolutionarily highly conserved transmembrane proteins essential to a wide spectrum of cellular systems (1). Notch signaling is especially important for normal thymic T-cell development (2–5) and remains crucial after the release of T-cells into the periphery (6). Thus, it is not surprising that deregulation of the Notch signal can result in T-cell acute lymphoblastic leukemia (T-ALL) in mice and humans (7, 8).

The earliest lymphocyte progenitors that migrate to the thymus are provided with Notch ligands by the thymic microenvironment, initiating the T-cell program while preventing the B-cell fate (2, 3). It also has been reported that Notch signals are important in subsequent T-cell fate decisions that occur in the thymus [reviewed in Ref. (9)], including αβ vs. γδ (10–14) and CD4 vs. CD8

lineage choices (15, 16). A role for Notch has been described in peripheral T-cells as well, where it has been linked to 1) T-helper (T_H) cell lineage development and cytokine gene expression (6, 17–24), 2) inducible regulatory T-cell development (25), 3) regulatory T-cell survival and function (26–29), 4) differentiation of $CD8^+$ T-cells into terminal effector vs. memory cells (6), and 5) proliferation and survival of T-cells (24, 30–35).

The Notch signaling pathway is unique as Notch is a transcriptional regulator initially expressed as a membrane-bound cell surface receptor. Notch activity is regulated at the level of proteolytic processing of the membrane-bound form to allow release of the active intracellular fragment. In mature T-cells, Notch processing can be triggered *via* two different mechanisms. First, well-defined canonical Notch-ligand-dependent modes have been reported, whereby Notch ligand is expressed on the surface of interacting antigen-presenting cells. However, many groups have reported that Notch can also be activated by T-cell receptor (TCR) complex/CD28 signaling pathways, a much less well-defined process that can occur in the absence of ligand (24, 31–33, 36, 37). Investigation into this unique role of Notch activation is still in its infancy. Our recent report provides arguably the first evidence that ligand-independent Notch activation is required for optimal T-cell proliferation and activation (37). In this review, we will discuss what is known about how Notch processing is regulated, and how these studies, together with our recent report, provide insight into the mechanism underlying this novel activation pathway.

DEREGULATION OF NOTCH FUNCTION IS DANGEROUS TO T-CELLS

Precise regulation of Notch signaling is crucial. Deregulated gain of Notch1 function has been implicated in more than 60% of T-ALL patients, making this an important mutation in leukemogenesis (38). *Notch1* mutations are generally located in two hotspots (**Figure 1B**), which gives insight into how Notch1 function is regulated (38). The most common mutations are located in exons 26 and 27, which code for the heterodimerization domain (HD), a region that is essential in the regulation of Notch activity as discussed in the next section. These mutations destabilize the HD domain and result in loss of autoinhibition (39). Consequently, the receptor is constitutively activated. The second hotspot is located in exon 34, which codes for the PEST [rich in proline (P), glutamic acid (E), serine (S), and threonine (T)] domain. Here, mutations generally cause truncations, most commonly by generating premature stop codons, resulting in deletion of the domain (40). The PEST domain is essential to targeting rapid degradation of the activated Notch protein, and its deletion results in an extended signaling half-life.

STRUCTURE OF NOTCH RECEPTORS AND ITS IMPORTANCE TO REGULATION OF FUNCTION

The Notch family of type-1 transmembrane receptors consists of four protein paralogs (Notch1–4) in humans and mice (**Figure 1A**), with mostly non-redundant functions. T-cells express Notch1, 2, and 3 (41–43). Before integration into the plasma membrane, the Notch receptor is post-translationally cleaved at the S1 site (**Figure 1B**), which is located 70 amino acids (aa) N-terminal of the transmembrane domain. This cleavage occurs inside the *trans*-Golgi network by a furin-like protease, resulting in a heterodimer that is held together by Ca^{2+}-dependent ionic bonds (44, 45). The two polypeptides that constitute the mature membrane-bound form of Notch are called the extracellular domain (ECD) and the transmembrane fragment (TMF). While the ECD is exclusively extracellular, the TMF is comprised of a small 70aa extracellular portion, the transmembrane domain and an intracellular domain.

Starting at the N-terminus, the ECD consists of 29–36 epidermal growth factor (EGF)-like domains, of which some are calcium-binding (cbEGF). cbEGF12 (as counted from the N-terminus) is reported to be the main binding domain involved in receptor–ligand interactions; however, additional EGF sites may contribute to increase binding stability (46, 47).

Following the EGF-like domains is the negative regulatory region (NRR), which encompasses three cysteine-rich Lin12/Notch repeats (LNR) and the fragment-spanning HD domain that results from S1 cleavage, and connects the TMF and ECD polypeptides to form the Notch heterodimer (**Figure 1A**) (44). The NRR is crucial in preventing Notch activation in the absence of the correct signal (48–50). Upon receptor–ligand interaction, conformational changes in the NRR allow access by ADAM proteins to the S2 cleavage site (**Figure 1B**). This site is located 12aa away from the membrane in the extracellular region of the TMF and is usually masked by the LNR (51). In leukemia, mutations in the HD domain either elongate the sequence between the S2 site and the LNR or destabilize the region *via* point mutations, small insertions, or short deletions (**Figure 1B**). These sequence changes prevent the NRR from auto-inhibiting Notch activation, which ultimately leads to unregulated Notch signaling (39).

Within the TMF, C-terminal of the HD domain, is the transmembrane domain. It contains the S3 cleavage site (**Figure 1B**), which is a substrate for regulated intramembrane proteolysis by the γ-secretase complex (γSec) (52). This event will occur only after the rate-limiting S2 cleavage has taken place, making S3 accessible to γSec (53). S3 proteolysis results in the release of the Notch intracellular domain [hereafter referred to as intracellular Notch (ICN)] from the membrane and allows Notch signaling to be initiated.

Canonical Notch ligands of the Delta/Serrate/Lag-2 (DSL) family in humans and mice fall into one of two classes, depending on whether they are a homolog of the *Drosophila* Notch ligand Delta or Serrate (**Figure 1A**). The Delta-like (DLL) proteins include DLL1, DLL3, DLL4, and the Serrate homologs are comprised of Jagged1 (Jag1) and Jag2. Even though functional differences have been ascribed to the four Notch receptors, ligation with either DLL or Jagged family ligands leads to the activation of the same canonical signaling pathway (54). Although Notch can be activated in T-cells through interaction with canonical Notch ligands on adjacent cells (e.g., DLL4, and to a lesser extent DLL1 and Jag2, on dendritic cells, as well as Jag1 on B-cells) (12, 19, 55, 56), it has also been demonstrated by many groups that TCR/

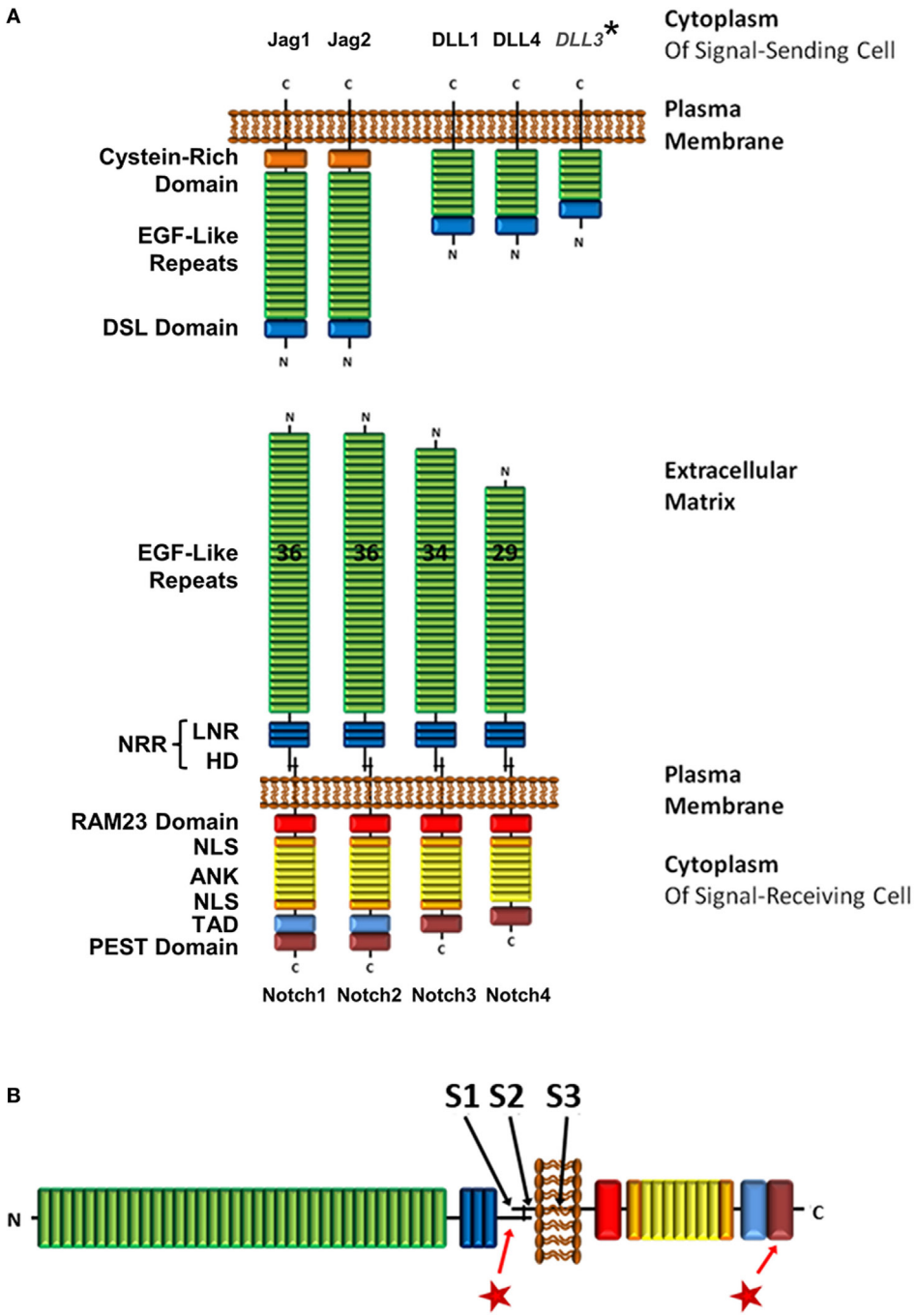

FIGURE 1 | Structure of Notch receptor family and its ligands. **(A)** Structure of Notch receptors on signal-receiving cells and Notch ligands on signal-sending cells. In humans and mice, there are four Notch receptors, and five Notch ligands within two families, the Jagged and the DLL family. *However, DLL3 is expressed exclusively in intracellular compartments. **(B)** Locations of the three cleavage sites on the Notch receptor (S1–3), and mutation hotspot regions in the HD and PEST domains ★. Abbreviations: NRR, negative regulatory region; LNR, cysteine-rich Lin12/Notch repeats; HD, heterodimerization domain; NLS, nuclear localization sequence; TAD, transcriptional activation domain; DSL, Delta/Serrate/Lag-2; Jag, jagged; DLL, delta-like.

CD28 signaling alone is able to initiate Notch cleavage and activation (24, 31–33, 36, 37). In order to understand the mechanism underlying this activation process, a closer look at how Notch receptor is processed *via* canonical ligand-dependent pathways is warranted.

MECHANISMS UNDERLYING LIGAND-INDUCED NOTCH PROCESSING

The mechanism of Notch activation that has been studied most thoroughly is initiated by the canonical ligands described above.

Since Notch activation is auto-inhibited through its NRR domain (49), which masks the S2 site and prevents ADAM-mediated cleavage, conformational changes in the receptor need to take place before Notch can become processed. In the secondary structure of the rod-shaped Notch receptor, the NRR and the ligand-binding EGF domains are spatially far apart from each other. Therefore, it is unlikely that allosteric regulation of the receptor could produce the necessary conformational changes in the heavily folded NRR region to initiate Notch signaling. Using *Drosophila* imaginal disk and retinal cells with defective dynamin function, important for endocytic vesicle formation, it was shown that Notch activation is dependent on ECD dissociation from the receptor and *trans*-endocytosis into the ligand-expressing cells (**Figure 2**) (57). It has been proposed that this pulling force causes conformational changes in the NRR region, which consequently allows S2 cleavage to occur (58), after which the ECD is free to be *trans*-endocytosed. A study in murine C2C12 cells also demonstrated the requirement of Notch ECD *trans*-endocytosis and substantiated that ligand binding alone is insufficient to activate Notch (59). However, this study also provided evidence that ECD dissociation occurs even in the presence of ADAM inhibitors. This suggests that dissociation is not a consequence of S2 site cleavage, but rather that ECD dissociation occurs first, and subsequently allows for S2 site cleavage by ADAMs (59, 60).

Once the S2 site has been exposed, further processing is facilitated by ADAMs (**Figure 2**). In *Drosophila*, ADAM10/Kuz has been shown to activate Notch receptors. This was demonstrated using dominant negative ADAM10 flies (61), ADAM10-deficient flies (62), and RNA interference of ADAM10 (63), all of which inhibited Notch processing and activity. Furthermore, physical contact between Notch and ADAM10 was shown by co-immunoprecipitation (63).

In addition to ADAM10, in mammalian cells, ADAM17/TACE also can process Notch (64, 65). However, ADAM10 and ADAM17 are differentially involved in Notch processing. Using multiple approaches of Notch activation in either ADAM10- or ADAM17-defective cells, it was shown that ADAM10 is absolutely

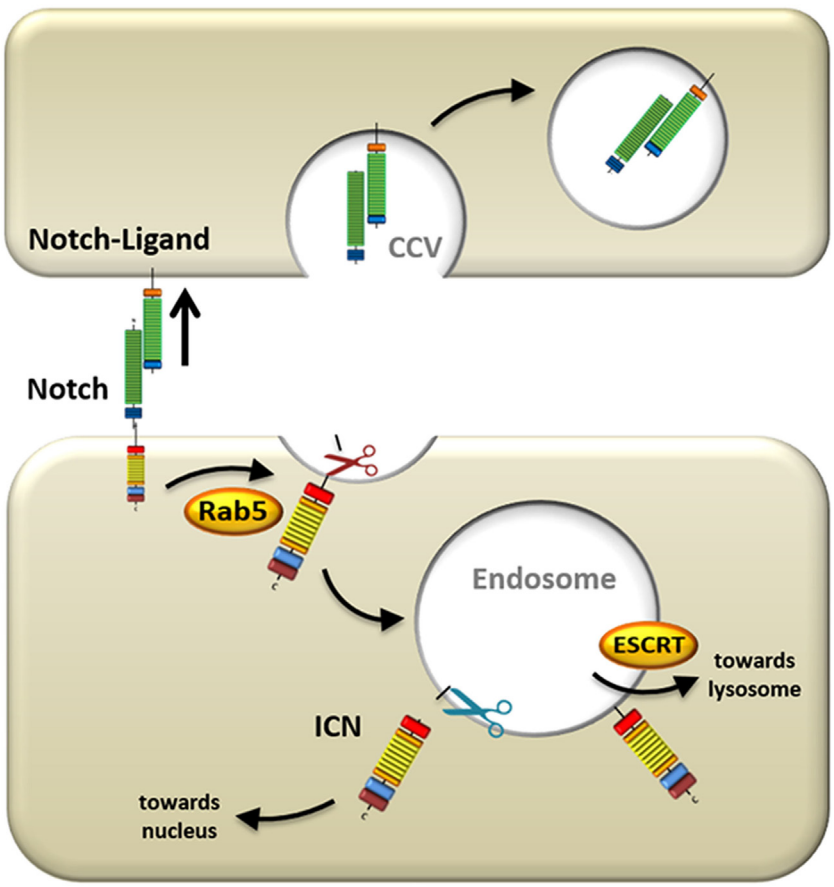

FIGURE 2 | Mechanisms underlying ligand-induced Notch processing. Upon ligand engagement, the signal-sending cell exerts a pulling force on the Notch receptor by internalizing the ligand and Notch extracellular domain. Simultaneously, the residual portion of the Notch receptor is endocytosed into the signal-receiving cell *via* Ras-related protein 5 (Rab5)-positive vesicles, where it is cleaved by ADAMs (red scissors). This first cleavage event primes the Notch receptor for a subsequent cleavage by γ-secretase complex (blue scissors) in the endosome, which releases intracellular Notch (ICN) from the membrane and allows it to transmigrate to the nucleus. Alternatively, the Notch receptor can be escorted by endosomal sorting complexes required for transport proteins (ESCRT) to the lysosome where it is degraded.

required for Notch processing upon ligand engagement in mammalian cells (66). ADAM17 was not able to rescue the ADAM10-deficient phenotype, and dominant negative ADAM17 expression did not inhibit ligand-induced Notch processing. By contrast, ADAM17 was able to process Notch under conditions of ligand-independent activation including EDTA chelation, or when using Notch constructs that harbor a mutated NRR domain, which renders them hyperactive. Thus, it was concluded that ADAM17 is necessary for ligand-independent Notch processing, whereas ADAM10 is responsible for ligand-induced Notch activation. While it has yet to be determined how preferential cleavage by ADAM10 or ADAM17 occurs, it is interesting to speculate that this selective requirement might suggest that the S2 cleavage event occurs in different compartments during ligand-dependent vs. ligand-independent Notch activation.

After S2 processing by the ADAM proteins, the final step in activating Notch and releasing ICN from the membrane is S3 cleavage mediated by γSec (**Figure 2**). This aspartyl protease is an intramembranously cleaving enzyme complex that is comprised of multiple protein subunits including nicastrin, anterior pharynx defective-1, presenilin enhancer-2, and the catalytically active subunit presenilin. γSec substrates generally need to be primed by ectodomain shedding from larger precursor proteins (53), such as is the case in S2-cleavage of Notch, or α-/β-secretase-mediated cleavage of amyloid precursor protein (APP) (67). Shedding of the Notch ECD results in a residual ectodomain of 12aa in length (51) and allows γSec substrate recognition *via* the nicastrin subunit that docks to the new N-terminus of Notch (68).

Although it has been suggested that γSec is present at the plasma membrane as a fully functional and active complex that can cleave APP and Notch (69, 70), γSec also localizes to early and late endosomes (LE) (71), where it processes p75 neurotrophin receptor (72), as well as APP (73). In addition, γSec can be found in lysosomes, where it experiences enhanced activity because of the low pH in the endolysosomal compartment (74). It has even been proposed that in *Drosophila* (75) as well as in mammalian cells (76) Notch/γSec co-localization to the endocytic compartment is absolutely critical to Notch activation. Overall, these data indicate that complete processing of Notch may require internalization of the receptor where it then becomes cleaved and fully activated in the endosome.

In addition to the ligand-induced mechanisms of Notch activation described above, a much less well-understood mechanism of activation has been observed in T-cells, in which TCR/CD28-stimulation is capable of activating Notch (24, 31–33, 36, 37). Notch receptors have been shown through immunofluorescence imaging to associate with the immunological synapse (IS) (33, 77) and, in the absence of Notch ligands, may be activated as bystanders of TCR stimulation. Within the crowded IS, it has been hypothesized that activation could be mediated through undefined mechanical forces acting upon Notch, causing a destabilization of the extracellular region of the receptor. Alternatively, signals downstream of TCR/CD28 may activate the IS-recruited Notch receptor. T-cell stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin leads to robust Notch activation, suggesting that the latter is the case (37).

THE ROLE OF RECEPTOR ENDOCYTOSIS IN NOTCH ACTIVATION

Initially, it was believed that the sole function of endocytosis consisted of terminating plasma membrane signals by either sequestering membrane receptors from ligand binding, or by internalization and degradation of active receptor/ligand complexes [reviewed in Ref. (78)]. But it is becoming increasingly apparent that endocytosis and signaling are intertwined processes that can affect each other reciprocally [reviewed in Ref. (79, 80)]. There are multiple mechanisms of endocytosis, but possibly the most common and best studied form is clathrin-dependent [reviewed in Ref. (81)]. Clathrin is recruited to the plasma membrane by a large variety of adaptor and accessory proteins that in turn adhere to lipid- or protein-binding domains at the membrane (82). These adaptors cause clathrin polymerization into curved lattices called clathrin-coated pits. These pits continue to invaginate with the help of bending-proteins, such as epsin (83), and form clathrin-coated vesicles (CCV) that eventually bud from the internal leaflet of the membrane (**Figure 2**). The budding process is facilitated by dynamin, which is a GTPase that forms a helical polymer around the neck of the CCV (84). Upon dynamin-mediated scission, the fully formed vesicle is released into the cytoplasm where it is uncoated and can then fuse with other membranes (85).

The requirement for endocytosis of Notch ligands during (and even prior to) receptor engagement is well characterized [**Figure 2**; (86)]. However, the need for internalization of Notch itself on the signal-receiving cell as a prerequisite for signal transduction is less well established. Evidence in HeLa cells suggests that Notch endocytosis is not necessary for its activation, but instead promotes attenuation of Notch signal by reducing its expression on the cell surface (87). Notch can indeed be marked for degradation *via* ubiquitination by E3-ligases such as AIP4/Itch (88) or Nedd4 (89). It is then endocytosed with the help of Numb that recruits the AP2-clathrin adaptor-complex (90). But ubiquitination and endocytosis are by no means exclusively linked to the downregulation of Notch signaling.

On the contrary, the majority of data in *Drosophila* support a crucial role for endocytosis in activation of Notch. The first findings implicating endocytosis in Notch activation were studies using dynamin-defective *shibire*-mutants, in which deletion of dynamin in signal-receiving cells disrupted Notch signal induction (91). More recently, detailed studies of Notch localization and activity were conducted, in which successive steps of the endocytic pathway were selectively blocked in *Drosophila* imaginal disks and oocytes (75). Deletion of *shibire* (a dynamin ortholog), Ras-related protein 5 (Rab5; **Figure 2**) and Avalanche (the latter two are proteins that regulate entry of cargo into the early endosome), resulted in Notch accumulation at or just below the plasma membrane and significantly reduced Notch signaling as measured by a *LacZ* reporter assay. However, blocking the endocytic pathway at later stages by deleting "endosomal sorting complexes required for transport proteins (ESCRT)," which control sorting into LE (**Figure 2**), or Fab1 that is important in (pre)-lysosomal compartments,

did not attenuate Notch signaling, but instead elevated it (75). This enhanced signaling may be a result of prolonged retention of Notch in an environment where it can be processed by γSec. In mammalian cells, it was demonstrated that Notch1 is endocytosed during ligand-dependent activation (60). This internalization is dependent on clathrin and dynamin resulting in the presence of Notch receptor in early Rab5-negative endosomal vesicles.

Further evidence placing Notch processing in the endosome comes from experiments in *Drosophila* using defective variants of the vacuolar proton pump V-ATPase (92, 93) and in mammalian cells by pharmacological inhibition of vacuolar H^+ ATPase (94), which prevent the acidification of the endosome. In both cases, Notch activation was attenuated. This, together with data showing that γSec operates optimally in an acidic environment (74), suggests that Notch is preferentially processed in the endosome by γSec (**Figure 2**), and that internalization does play an important role in Notch activation.

In ligand-independent receptor activation, the E3 ubiquitin ligase Deltex is implicated in the regulation of Notch endocytic trafficking in *Drosophila* (**Figure 3**). This was demonstrated with null mutations for Deltex that led to failure of Notch internalization and activation (95), as well as overexpression experiments that strongly enhanced Notch signaling (96). Deltex stabilizes the receptor in the endocytic compartment allowing signal transduction to proceed, as assayed by expression of Notch-reporter genes (97, 98). Deltex also forms a complex with adaptor protein 3 [AP-3; (99)] and "homotypic fusion and vacuole protein sorting" [HOPS; (100)], both of which deliver Notch to the exterior membrane of the multivesicular body (MVB) called the limiting membrane. This localization on the exterior membrane places the intracellular domain of Notch in the cytosol, where, upon its cleavage to form ICN, it is free to translocate to the nucleus and promote activation of its target genes (101). Supporting this model are studies carried out in *Drosophila* examining the role of "Suppressor of Deltex" [Su(dx)], also an E3 ubiquitin ligase, which directly opposes the function of Deltex. Su(dx) facilitates Notch incorporation into the membranes of intraluminal vesicles located inside the MVB, instead of localization to the limiting membrane, and prevents ICN signaling. This results in ICN being spatially sequestered from the cytosol and degraded (101). Interestingly, HOPS and AP-3 are not needed in ligand-dependent processing of Notch indicating that separate pathways underlie ligand-dependent and ligand-independent Notch activation (101).

Overall, it can be concluded that, at least in some forms of Notch activation, endocytosis of the receptor and its shuttling through the endosomal compartment are important factors. In the ligand-independent process, Deltex facilitates the endocytosis of Notch from the plasma membrane and, together with HOPS and AP3, protects it from degradation by targeting it to limiting membranes of the MVB. There, it can be processed by γSec allowing release of ICN into the cytosol.

Whereas, Deltex proteins are not required for thymic T-cell development (102), in which Notch activation is largely dependent on DLL4 (103), we hypothesize that in systems that utilize ligand-independent Notch activation, such as that initiated by TCR/CD28-mediated stimulation, Deltex, as well as many of the other proteins in the endocytic pathway discussed above, will be important players.

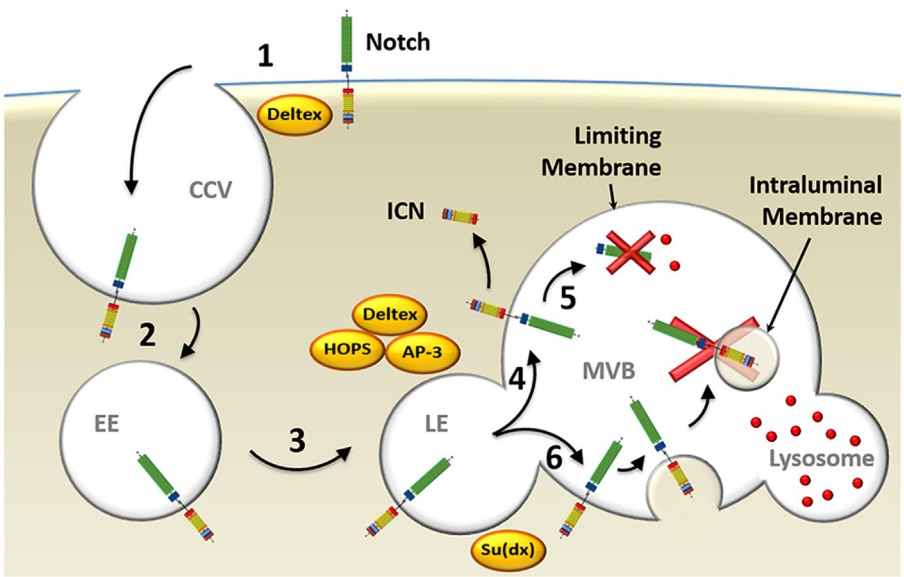

FIGURE 3 | Model of endocytosis in ligand-independent Notch activation. (1) Deltex facilitates ligand-independent Notch receptor internalization into clathrin-coated vesicles (CCV) (2) that fuse with Ras-related protein 5-positive early endosomes (EE). (3) Deltex forms a complex with adaptor protein 3 (AP-3) and homotypic fusion and vacuole protein sorting (HOPS) that directs Notch to Rab7-positive late endosomes (LE) and (4) targets it to the limiting membrane of the multivesicular body (MVB). (5) This protects intracellular Notch (ICN) from lysosomal degradation and allows its release into the cytosol upon γ-secretase complex-mediated processing. (6) Alternatively, Su(dx) can redirect Notch into the intraluminal vesicles, where the Notch receptor will be proteolyzed when the MVB fuses with a lysosome.

ENDOGENOUS PROCESSING OF ENDOCYTOSED RECEPTOR

Canonical ligand-mediated Notch activation relies on conformational changes in the receptor that are induced by the ligand to unmask the protected S2/S3 sites for cleavage by ADAMs and γSec. In ligand-independent Notch activation, these changes must be driven by another method. There are three possible scenarios by which this process might occur within an endosomal microenvironment [**Figure 4**; (104)]. First, lysozymes may proteolyze the Notch-ECD that extends into the intraluminal space of the MVB, thus removing inhibitory sequences that prevent γSec recognition (**Figure 4A**). Whereas this seems to be the simplest answer, it would circumvent any rate-limiting checkpoints of Notch processing (i.e., ADAM-mediated cleavage of S2) and expose Notch to constitutive activation by γSec (105). This would result in Notch signaling that is difficult to regulate. The other two possibilities involve naturally occurring changes in the LE microenvironment. Specifically, adjustments in ion concentrations (106, 107), especially those of Ca^{2+} that are important in NRR and HD stability, and/or the gradual acidification of the endosome could result in (1) destabilization of the NRR and unmasking of the S2 site (**Figure 4B**) or (2) full dissociation of the ECD (**Figure 4C**). Whereas NRR destabilization would mimic a ligand-induced pull, which then still requires ECD removal by ADAM before γSec can recognize Notch, the immediate dissociation of the ECD at the HD domain would leave only a 70aa residual sequence. γSec has been shown to recognize substrates providing the juxtamembrane sequence is <200aa in length (53). However, the longer the sequence, the lower the affinity of γSec to its substrate, suggesting that further processing by ADAM still may be required. Even though ADAM10 and ADAM17 are predominantly expressed at the cell surface—constitutively and upon activation, respectively (108)—intracellular activity of ADAM has been documented (109, 110). Conversely, the acidic optimum for γSec activity may enable it to simply bypass S2 cleavage and directly process the 70aa-stub moiety of Notch (74).

A MODEL OF TCR-ACTIVATED NOTCH SIGNALING

Ligand-independent Notch processing has been described in *Drosophila* cells. However, until recently, whether this method of Notch activation occurs during normal physiological processes in mammalian cells remained murky. We have recently reported that the processing of Notch triggered by TCR/CD28

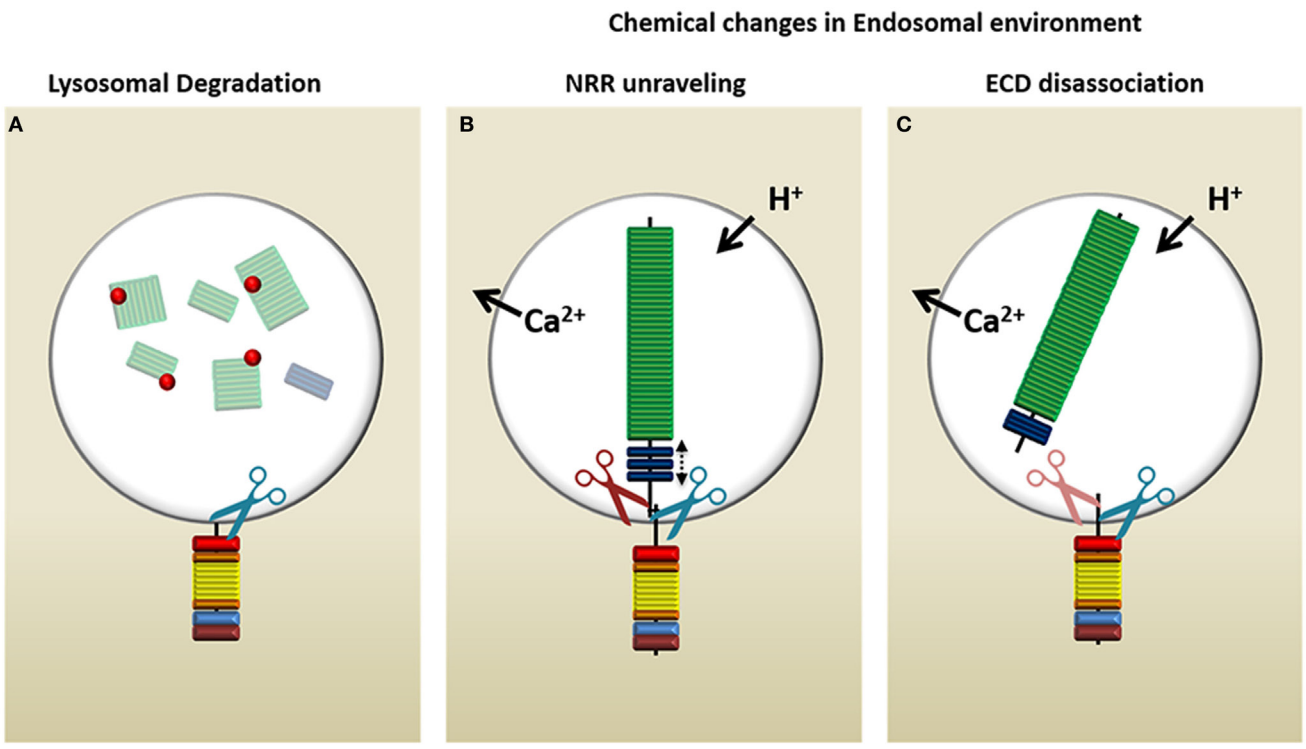

FIGURE 4 | Possible mechanisms of Notch processing in the endosome. Upon delivery of Notch to the multivesicular body, there are three possible ways in which the endocytic environment may prepare the Notch receptor to allow the release of intracellular Notch from the limiting membrane. **(A)** Lysozymes (red dots) present in this compartment may proteolyze the intraluminal region of Notch and therefore allow γ-secretase complex (γSec) cleavage to proceed. **(B)** The change in ion concentrations and decreasing pH may cause the negative regulatory region (NRR) of Notch to unravel, mimicking a ligand-mediated pull, which opens the S2 site for access by ADAM (red scissors), followed by γSec cleavage (blue scissors). **(C)** The lysosomal environment may cause the Notch extracellular domain (ECD) to dissociate entirely, in which case γSec may directly process the 70aa juxtamembrane stub or rely on ADAM proteases (light red because of uncertain requirement) to increase its affinity for the S3 site through processing the juxtamembrane stub to 12aa.

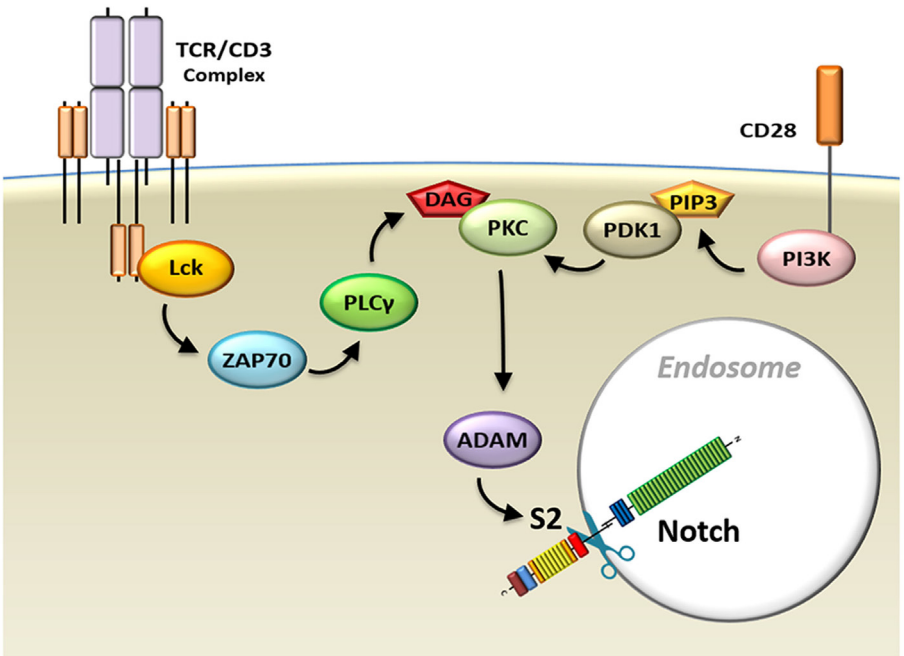

FIGURE 5 | Model schematic diagram of TCR/CD28-induced Notch activation in T-cells. Signals from the TCR–CD3 complex, as well as CD28 co-receptors, activate the Notch cleavage machinery and induce endocytosis of the Notch receptor. Abbreviations: Lck, lymphocyte-specific protein tyrosine kinase; ZAP70, z-chain associated protein kinase 70 kDa; TCR, T-cell receptor.

signaling in T-cells occurs *via* a ligand-independent mechanism (24, 31–33, 36, 37). TCR and CD28 signals cooperate to initiate two processes required for ligand-independent Notch activation (37). The first process consists of internalization of the receptor. Chemical adjustments in the endocytic compartment substitute for the mechanical forces that are generated by conventional Notch receptor ligation, driving conformational changes in the autoinhibitory region of Notch that are required for cleavage. Concurrently, the second process activates the machinery that performs the Notch cleavage events (**Figure 5**).

While it is unknown what pathways trigger internalization of the Notch receptor, Notch processing post-internalization requires signals delivered through both TCR and CD28. These signals cooperate to activate protein kinase C (PKC), which is required for Notch activation. TCR complex cross-linking initiates activation of phospholipase C-gamma, which is required for generation of the membrane anchor diacylglycerol (DAG). DAG, in turn, is a crucial factor in activation of mature PKC. However, in order to be available for activation by DAG, PKC must undergo maturation events that occur downstream of CD28 signal. Specifically, CD28 signals trigger phosphatidylinositide 3-kinase activation, which, through the phosphorylation of PIP_2 (phosphatidylinositol 4,5-bisphosphate), generates another membrane anchor molecule, PIP_3 [phosphatidylinositol (3,4,5)-triphosphate]. The enzyme 3-phosphoinositide-dependent protein kinase-1 (PDK1) is recruited to the IS by PIP_3, where it can now efficiently phosphorylate PKC, priming it for activation by TCR signals (111).

Once PKC has been activated, it induces activity of ADAM10 and ADAM17. It has recently been shown, in agreement with our study, that PKCθ regulates Notch processing downstream of TCR signal and upstream of ADAM activity (112). Cleavage of the Notch receptor at the S2 site by ADAMs is required for Notch activation. However, this proteolytic event cannot occur when the S2 cleavage site of Notch is protected by its NRR autoinhibitory domain. This is why, in the absence of ligand, the internalization of the Notch receptor becomes important. We hypothesize that the gradual acidification of the endocytic environment and efflux of calcium from the endosome (106, 107) result in conformational changes in the Notch receptor. These changes would unravel the NRR and allow activated ADAMs to initiate Notch processing, ultimately resulting in the release of ICN, which can now translocate to the nucleus to activate expression of its target genes.

CONCLUSION

What is the benefit of the TCR/CD28-mediated, ligand-independent mode of Notch activation to T-cells? It may have its origin in the fact that T-cells are part of a fluid system of migrating cells. Generally, Notch receptor initiates cell fate decisions in solid tissues, in which lateral interactions with ligand-expressing cells can be sustained indefinitely. By contrast, T-cells spend some of their life cycle in circulation or percolating through secondary lymphoid organs on the search for cognate antigen (113). Since the Notch pathway does not contain an inherent amplification cascade that would enhance external stimuli—Notch signal input creates a stoichiometric signal output—the one-to-one interaction of ligand on antigen-presenting cells with Notch receptor on T-cells, as well as the short half-life of ICN, may not result

in sufficient Notch signal to robustly activate a full program of Notch target gene activation. TCR/CD28 signaling, which does undergo multiple rounds of signal amplification, may serve to circumvent this deficiency and activate adequate levels of Notch signal when a cognate antigen has been recognized. Since Notch signaling optimizes T-cell responses, such as proliferation and activation, the T-cell is not reliant on additional Notch ligands and can autonomously couple Notch activation to TCR stimulation.

Whether these pathways contribute to ligand-independent Notch activation in leukemia cells is unknown. Our recent report has provided evidence that receptor endocytosis occurs even in Notch-dependent T-leukemic cell lines as a prerequisite step for Notch activation (37). Further studies will be vital to investigate the importance of this pathway to the constitutive Notch activation that drives survival and proliferation of leukemic T-cell as it could be the entryway into development of new therapies.

AUTHOR CONTRIBUTIONS

MS and SW wrote the paper. MS prepared the figures.

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REVIEW
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Notch Signaling in Myeloid Cells as a Regulator of Tumor Immune Responses

*Fokhrul Hossain[1,2], Samarpan Majumder[1,2], Deniz A. Ucar[2], Paulo C. Rodriguez[3], Todd E. Golde[4], Lisa M. Minter[5], Barbara A. Osborne[5] and Lucio Miele[1,2]**

[1] Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA, United States, [2] Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, LA, United States, [3] H. Lee Moffitt Comprehensive Cancer Center, Tampa, FL, United States, [4] Department of Neurosciences, McKnight Brain Institute, University of Florida at Gainesville, Gainesville, FL, United States, [5] Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA, United States

Cancer immunotherapy, which stimulates or augments host immune responses to treat malignancies, is the latest development in the rapidly advancing field of cancer immunology. The basic principles of immunotherapies are either to enhance the functions of specific components of the immune system or to neutralize immune-suppressive signals produced by cancer cells or tumor microenvironment cells. When successful, these approaches translate into long-term survival for patients. However, durable responses are only seen in a subset of patients and so far, only in some cancer types. As for other cancer treatments, resistance to immunotherapy can also develop. Numerous research groups are trying to understand why immunotherapy is effective in some patients but not others and to develop strategies to enhance the effectiveness of immunotherapy. The Notch signaling pathway is involved in many aspects of tumor biology, from angiogenesis to cancer stem cell maintenance to tumor immunity. The role of Notch in the development and modulation of the immune response is complex, involving an intricate crosstalk between antigen-presenting cells, T-cell subpopulations, cancer cells, and other components of the tumor microenvironment. Elegant studies have shown that Notch is a central mediator of tumor-induced T-cell anergy and that activation of Notch1 in CD8 T-cells enhances cancer immunotherapy. Tumor-infiltrating myeloid cells, including myeloid-derived suppressor cells, altered dendritic cells, and tumor-associated macrophages along with regulatory T cells, are major obstacles to the development of successful cancer immunotherapies. In this article, we focus on the roles of Notch signaling in modulating tumor-infiltrating myeloid cells and discuss implications for therapeutic strategies that modulate Notch signaling to enhance cancer immunotherapy.

Keywords: Notch, cancer, immunity, cellular, inflammation, myeloid cells

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****Correspondence:***
Lucio Miele
lmiele@lsuhsc.edu

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INTRODUCTION

Notch signaling, an evolutionarily conserved cell-fate-determination pathway, mediates close contact interactions between neighboring cells. Notch is involved in many aspects of tumor biology, from angiogenesis to cancer stem cells maintenance to tumor immunity (1–3). Mammals have four structurally related Notch receptors (Notch1–4) that bind transmembrane ligands of the Jagged (Jagged-1, Jagged-2) or the Delta-like (DLL1, DLL3, and DLL4) families (2, 4, 5). Binding of

Notch receptors to ligands, or in some cases, ligand-independent receptor activation (6) triggers separation of the extracellular receptor subunit from the transmembrane subunit. The latter undergoes a multistep proteolytic process, which results in the release of a Notch intracellular domain (NICD) (7). NICD translocate into the nucleus and complexes with the CSL (CBF-1/Suppressor of Hairless/LAG-1, also known as RBP-J), and mastermind-like (MAML1-3) coactivator and other proteins to form the Notch transcriptional complex, which regulates the transcription of multiple genes (2, 4, 5, 7). In addition to canonical Notch signaling, several non-canonical (CSL-independent) Notch signals have been described in oncogenesis and inflammation (8–10). Context-dependent Notch signaling regulates many cell fate choices and Notch dysregulation contributes to the development of various malignancies (5). Notch signaling can produce different biological outcomes depending on the timing and the strength of the signals as well as the expression of different ligand/receptor pairs, post-translational modifications, or receptors and specific regulation at both the transcriptional and post-transcriptional level (11, 12). Hyperactivation of Notch has been considered as oncogenic in several cancers including breast cancer and lymphoid malignancies (T-cell acute lymphoblastic leukemia, T-ALL, B-chronic lymphocytic leukemia, and splenic marginal zone lymphoma). On the other hand, loss of function of individual Notch paralogs has revealed tumor-suppressive activities in other malignancies, as reviewed in Ref. (13, 14).

Myeloid cells are essential for the homeostasis of the innate and adaptive immune responses. Myeloid cells [granulocytes, macrophages, and dendritic cells (DCs)] develop from hematopoietic stem cells (HSCs) through sequential differentiation steps under normal physiological conditions. However, multiple soluble factors released by the tumor microenvironment (both tumor cells and tumor-associated stromal cells) perturb the normal myeloid development resulting in the accumulation of myeloid-derived suppressor cells (MDSCs), a heterogeneous group of immature myeloid cells with immune-suppressive properties. In addition, tumor-derived soluble factors induce defects in the differentiation of DCs and accumulation and polarization of tumor-associated macrophages (TAMs), as described in Ref. (15). Although the importance of Notch signaling in myeloid cells differentiation is well understood, the exact nature of Notch effects remains controversial. There is literature supporting a critical role of Notch in the maintenance of progenitor cells to delay the terminal differentiation of myeloid cells, while other data suggest that Notch signaling is required for differentiation of mature myeloid cells, as reviewed in Ref. (16). Overall, it is probably fair to say that the role of Notch signaling in myeloid cell differentiation is context dependent; it depends on the timing of Notch activation and the differentiation stages of myeloid cells.

T-cell-based cancer immunotherapy has shown effectiveness in some highly lethal malignancies and offers a great deal of promise for the treatment of others. Although the Food and Drug Administration (FDA) approved few T cell-based immunotherapy agents and several others are in phase I–II clinical trials, clinical outcomes have not been as universally positive as initially thought. The presence of a tolerogenic microenvironment that blocks the antitumor effector functions of T cells is a major factor limiting the clinical efficacy of T-cell-based immunotherapy (17). Tumor-infiltrating myeloid cells are central components of the tolerogenic tumor microenvironment, along with regulatory T cells (T_{regs}). Recently, Campese et al. described a role of Notch in immunoregulatory cells including T_{reg} in the context of tumor microenvironment (18). In this review, we will discuss the role of Notch signaling in myeloid cells (MDSC, DC, and macrophages) as a modulator of tumor immune response.

NOTCH AND MDSC

Myeloid-derived suppressor cells are major immune response regulators in cancer and other pathological conditions. MDSCs are a heterogeneous population of cells consisting of myeloid progenitor cells and immature myeloid cells that have immune-suppressive functions, as reviewed in Ref. (19). MDSCs adversely modulate the immune response to cancer and also facilitate tumor metastasis and angiogenesis (15, 19, 20). The immune-suppressive function of MDSC is mediated through the expression of arginase1 (ARG1), inducible NOS, formation of peroxynitrite, expression of TGF-β, IL10, and COX2, sequestration of cysteine, and induction of immunosuppressive T_{regs}, among others, as reviewed in Ref. (15, 21). In mice, MDSCs are defined by the co-expression of CD11b and Gr-1 markers and consist of two major subsets, the granulocytic polymorphonuclear (PMN)-MDSC ($CD11b^+Ly6G^+Ly6C^{lo}$) and the M-MDSC ($CD11b^+Ly6G^-Ly6C^{hi}$) (22). However, in humans, the situation appears to be more complex, and several different markers of MDSCs have been described (22).

Although the role of Notch signaling in myelopoiesis remains somewhat controversial, a number of studies have demonstrated that Notch signaling is important for the accumulation of MDSC (18, 23, 24). Transgenic mice that overexpress ADAM10 (responsible for the first proteolytic cleavage of Notch transmembrane subunits) resulted in abrogated B cell development, delayed T cell development in the thymus but systemic expansion of $CD11b^+Gr1^+$ MDSC (25). Gibb et al. (25) suggested that differential cleavage of Notch1 into S2 and S3 products modulated by ADAM10 is important to hematopoietic cell-fate determination. Notch was shown to induce myeloid differentiation of multipotent hemopoietic progenitor cells by upregulating the expression of the transcription factor PU.1, suggesting that Notch signaling functions as an extrinsic regulator of myeloid commitment (26).

Gabrilovich et al. reported that inhibition of Notch signaling in hematopoietic progenitor cells (HPCs), MDSCs, and DCs correlates with abnormal myeloid cell differentiation in cancer (23). The inhibition of Notch signaling in these cells is mediated by NICD phosphorylation by casein kinase 2, which disrupts the interaction between NICD and CSL. Another group (27) also reported that blockade of Notch signaling induced the generation of PMN-MDSC with lower immunosuppressive function, but inhibited the production of mononuclear-MDSC. They also showed that Notch-CSL signals modulate the differentiation process and immunosuppressive functions of MDSC. One possible mechanism whereby Notch signaling could regulate MDSC differentiation is through miR-223. Notch suppresses

miR-223 expression in rheumatoid arthritis macrophages (28). In turn, miR-223 inhibits the differentiation of tumor-induced MDSC (29), regulating their number and immune-suppressive functions (30).

Myeloid-derived suppressor cells within the tumor microenvironment block the effects of adoptive T cell-based immunotherapy (ACT) by inhibiting several T cell functions, including T cell proliferation and the expression of various cytotoxic mediators. The success of ACT depends on differentiation of $CD8^+$ T cells into cytolytic and cytokine-producing effector cells (31). However, limited exposure to MDSCs can paradoxically enhance the effectiveness of ACT. Acquisition of full effector function *in vitro* impairs the antitumor efficacy of $CD8^+$ T cell-based ACT (32). In fact, transfer of activated stem cell memory T cells resulted in higher antitumor responses in mice than effector memory T cells (33). These results suggest that inhibition of $CD8^+$ cell differentiation can enhance the antitumor activity of $CD8^+$ T cells following ACT. Rodriguez et al. (34) reported that transient conditioning of $CD8^+$ T cells with MDSC blocks their differentiations into effector T cells and significantly improves their antitumor activity following ACT. Their results indicated that conditioning of T cells with MDSC induces stress survival pathways through blunted mTOR signaling, which in turn modulated T cell differentiation and ACT efficacy. Thus, short-term conditioning T cells with MDSC could prove beneficial in ACT strategies for cancer immunotherapy.

An elegant study by Peng et al. (35) suggested that the presence of MDSC in tumors is correlated with the presence of cancer stem-like cells (CSCs) and both independently predict poor patient survival. These authors suggested that MDSC-derived IL-6 and nitric oxide (NO) may collaborate to activate STAT3 and Notch signaling and induce breast CSCs. Notch signaling has also been proposed to induce cancer metastasis by promoting the migration of MDSCs. Nakayama et al. reported that F-box protein FBXW7 has tumor-suppressive capacity and inhibits cancer metastasis (36). FBXW7 is an E3 ubiquitin protein ligase involved in the degradation of several oncoproteins including NICD. Deletion of Fbxw7 in murine bone marrow-derived stromal cells resulted in the accumulation of Notch1 and increased expression of CCL2. CCL2 in turn facilitated the recruitment of M-MDSC and macrophages, promoting metastatic tumor growth.

The role of Notch in T cell-mediated cancer immunity has been studied extensively (8, 37). Rodriguez et al. (38) reported that the tumor microenvironment suppresses Notch1 and Notch2 expression in CD8 T cells. Conditional expression of transgenic Notch1 intracellular domain (N1ICD) in activated antigen-specific $CD8^+$ T cells induced cytotoxic responses and caused $CD8^+$ T cells to become resistant to MDSC-mediated tolerogenic effects in tumor-bearing mice (38). MDSC blocked the expression of Notch in T cells *via* NO-dependent mechanisms. The authors suggested that transgenic expression of Notch1 or Notch2 NICD in $CD8^+$ T cells or chimeric antigen receptor T (CAR-T) cells may overcome MDSC-mediated tolerogenic effects and prove therapeutically beneficial. However, the molecular mechanisms whereby MDSC-derived NO inhibits Notch signaling remain unclear.

Recently, the Rodriguez lab in collaboration with the Miele and Osborne labs showed that tumor MDSC, unlike circulating MDSC, upregulate expression of Notch ligand Jagged1, and to a lesser extent, Jagged2. This phenomenon is mediated by NF-κB (39). Treatment with an anti-Jagged1/2-blocking antibody had remarkable therapeutic activity in several mouse models (3LL lung carcinoma and EG-7, an ovalbumin-expressing form of EL-4 lymphoma), which depended upon enhanced CD8 responses (39). In EG-7 tumors, anti-Jagged antibodies enhanced the effect of anti-ovalbumin adoptive T-cell therapy (ACT). Interestingly, anti-Jagged therapy induces the appearance of potentially immune-stimulatory MDSC-like cells (MDSC-LC), which had lower expression of MDSC-suppressive mediators, iNOS and ARG1. It is unclear whether these MDSC-LC derive from the reprogramming of MDSC or from *de novo* differentiation from bone marrow myeloid precursors upon Jagged inhibition. It is also unclear how Jagged blockade produces this effect. It may allow DLL ligands to activate Notch with a different kinetics, or possibly relieve *cis*-inhibition of MDSC Notch receptors by Jagged ligands expressed on the same cells. Further mechanistic investigations are necessary to answer these questions. However, these findings provide a preclinical proof of concept for the use of anti-Jagged1/2 antibodies to reprogram MDSC-mediated T-cell suppression to enhance the efficacy of cancer immunotherapy.

In summary, the Notch pathway can be considered a multifaceted modulator of MDSC biology. Notch signals modulate MDSC activity in different ways, depending on the receptors and ligands involved, microenvironmental clues (e.g., NF-κB activation by inflammatory cytokines), the stages of myeloid cells differentiation, as well as the subpopulation of cells. Targeting Jagged-family Notch ligands to inhibit MDSC is a promising strategy to overcome tumor tolerance.

NOTCH AND DCs

Dendritic cells are professional antigen-presenting cells (APC) that recognize, acquire, process, and present antigens to resting T cells to activate antigen-specific immune responses. The engagement of DC in the induction of immune responses against a myriad of pathogens, tumor cells, and self-antigens is a cornerstone of adaptive immunity (40). DCs include distinct functional subsets including interferon-producing plasmacytoid DCs (pDCs) and classical DCs (myeloid) (41–43). Classical DCs are the dominant subset and differentiate along the myeloid lineage pathway. The mechanisms of differentiation of these two subsets are vastly different, although they converge on some pathways (41–43). Decreased DC function has been suggested as a major cause of the observed defect in cell-mediated immunity in patients with advanced breast cancer (44). DC differentiation from HPCs is controlled both by a network of soluble growth factors and cytokines produced by bone marrow stroma (BMS) and direct cell–cell contact with BMS *via* a complex network of soluble factors and cell-bound molecules. Several studies have implicated Notch signaling in DC differentiation and function (45–47).

There is both consensus and controversy surrounding the extent of Notch involvement in DC differentiation. Several

groups have described a direct role of Notch in promoting DC differentiation. Expression of DLL1 in conjunction with GM-CSF induced differentiation of bone marrow cells to DCs at the expense of other lineages (48). In "emergency myelopoiesis," DLL1 promoted DC differentiation while Jagged1 inhibited it. Both ligands activated Notch, but DLL1 also induced Wnt while Jagged suppressed it by inhibiting the expression of Wnt receptor Frizzled (49). Cheng et al. (50) showed that differentiation of DC was severely compromised in Notch1 antisense mice that have about half the physiological level of Notch1 in HPC. These findings were confirmed in an experimental model of DC differentiation from embryonic stem (ES) cells. Notch1$^{-/-}$ ES are unable to differentiate into DC. In this model, Notch signaling is necessary but not sufficient for DC differentiation (45). On the other hand, Radtke et al. (51) generated *Notch1* conditional knockout mice using the Cre-Lox system and demonstrated that the number of thymic DCs, conventional DCs, and Langerhans cells were normal. Whether other Notch paralogs can compensate for Notch1 deficiency in this model is unclear. Conditional deletion of CSL (RBP-Jκ), which abrogates all canonical Notch signaling in BM cells and DCs resulted in substantial reduction in the presence of conventional DCs in spleens of the knockout mice (52). This decrease affected primarily the CD8$^-$ DC subset in the spleen marginal zone (52). Weijzen et al. (46) demonstrated that peptides from the DSL (Delta-Serrate-LAG1) receptor-binding region of Jagged1 promote the maturation of monocytes into myeloid DC. This effect may be mediated by direct activation of Notch receptors or relief of *cis*-inhibition by endogenous Jagged ligands. Lewis et al. (53) demonstrated that Notch2 is required for the functional differentiation of DCs in the spleen and intestine. De Smedt et al. (54) demonstrated an exquisite dose dependence of Notch signaling in the thymic microenvironment, with different levels of Notch signal intensity biasing cell fate decisions toward NK, B, DC, macrophage, or T cell lineages.

Similar contradictory data exist in the literature with respect to the role of Notch signaling in pDCs. It was reported that Notch signaling *via* DLL1 prevents the differentiation of pDC from early thymocyte precursors by decreasing expression of ETS transcription factor Spi-B. Conversely, Jagged1 did not suppress Spi-B expression. Stromal cells expressing DLL1 blocked pDC development (55). However, in a different study, Notch1$^{-/-}$ bone marrow precursors developed normally into thymic pDC, suggesting that thymocytes and pDC originate from different lineages and that Notch only modulates the thymocyte lineage (56).

There is emerging evidence of crosstalk between Notch and Wnt pathways in the regulation of DC differentiation (57). Inhibition of Notch signaling can lead to accelerated differentiation of HSCs *in vitro* and depletion of HSCs *in vivo* (57). Regulation of Notch signaling by the Wnt pathway also plays a vital role in differentiation of precursors along T or NK differentiation pathways (58). **Table 1** summarizes some of the key findings reported on the role of Notch signaling in the differentiation and function of tumor-associated myeloid cells.

These findings highlight two general features of Notch signaling, namely, its context dependence and dose dependence. Notch signals do not appear to operate as an on/off switch. Rather, in many systems, these signals appear to operate based on an intensity gradient that modulates and is modulated by other pathways. Complete blockade of Notch signals is not always necessary to change cellular phenotypes, and small variations in signal intensity or duration may have major phenotypic consequences. **Figure 1** schematically depicts the current consensus on the role of Notch signaling in the differentiation and function of tumor microenvironment-associated myeloid cells.

TABLE 1 | Notch effects in the differentiation and function of tumor-associated myeloid cells.

Cell population	Observation	Reference
Dendritic cell	Notch signaling induces differentiation	(15, 16, 40)
Hematopoietic progenitor cell (HPC)	Notch signaling promotes NF-κB-dependent differentiation of HPC	(50)
Macrophages	Notch signaling mediators are upregulated in activated macrophages	(77–80)
Macrophages	DLL4-induced Notch signaling mediates inflammatory responses	(76)
Tumor-associated macrophages (TAMs)	Notch signaling modulates the M1 versus M2 macrophages polarization in antitumor immune response. M2-like TAMs have decreased Notch activity	(81)
Myeloid-derived suppressor cell (MDSC)	Notch signaling is important for the accumulation of MDSC	(18, 24)
MDSC	Notch signaling induces multilineage myeloid differentiation	(26)
MDSC	Blockage of Notch signaling promotes MDSC generation	(23, 27)
MDSC	Anti-jagged therapy to reprogram MDSC by relieving Notch inhibition	(39)

Notch signals are involved not only in the maturation of DC but also in their effector functions. DCs express both Notch receptors and ligands as well as toll-like receptors (TLRs) (59). TLRs potently stimulate the expression of Notch ligands in DC (59). TLRs are being increasingly adopted in DC vaccine manufacturing protocols to stimulate DC maturation (60). DCs are composed of subsets that differ in their phenotype, localization, and function. DLL4 + DC promote CD4$^+$ T cell effector response. Blocking DLL4 causes a dramatic reduction of inflammatory T cell responses (60). Gentle et al. (59) demonstrated that DC stimulated concurrently with both Notch and TLR ligands have a distinct cytokine profile and are more pro-inflammatory compared with DCs stimulated with either ligand alone. This effect appears to be mediated by non-canonical Notch signaling (61, 62). Non-canonical Notch signaling regulates various pathways in cancer and immune cells (59). In DC, PI3kinase stimulated by membrane-bound Notch modulates the response to pro-inflammatory signals (59).

In summary, Notch signals play important roles in DC maturation and activity. Canonical and non-canonical Notch signaling are involved. In most cases, Notch activity seems to promote DC maturation and function, but pDC may be an exception. Strategies leading to Notch activation in DC may enhance the effectiveness of DC-based cancer immunotherapy strategies.

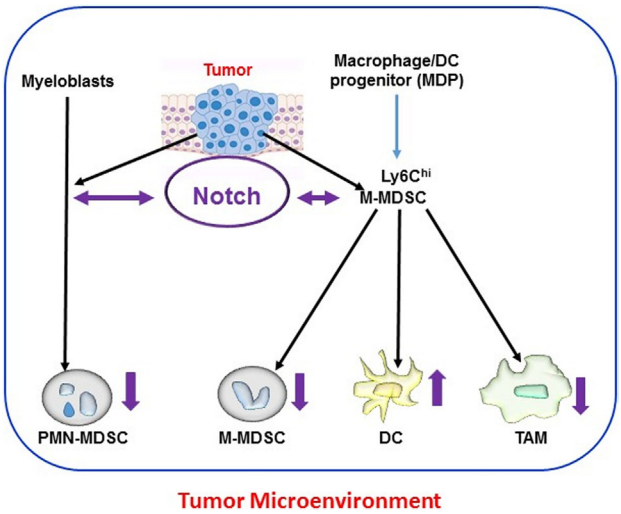

FIGURE 1 | Notch and myeloid cells differentiation within tumor microenvironments. Myeloid cells [polymorphonuclear (PMN) cells, dendritic cells (DCs), and macrophages] derive from hematopoietic stem cells through common myeloid progenitors and the granulocyte-myeloid progenitors lineage. In tumor-bearing hosts, this differentiation process is altered by tumor-derived signals. Expansion of activated PMN-MDSC and M-MDSC occurs from myeloblasts and M-MDSC, respectively, during tumorigenesis. M-MDSC also differentiate into tumor-associated macrophages (TAMs) and DC at the tumor site. Notch signaling mediates bidirectional crosstalk at multiple steps of myeloid cells differentiation in the tumor microenvironment. Differential Notch expression and activity (as indicated by the direction of purple arrows) has been reported in different myeloid populations, with lower Notch expression in myeloid-derived suppressor cell (MDSC) and TAM and higher expression in DC.

NOTCH AND TAMs

Macrophages are a multifunctional and heterogeneous cell population, which can originate from embryonic precursor cells within a tissue or derive from HSCs *via* the myelomonocytic lineage (63). They can function as phagocytes, APC, and modulators of innate and adaptive immune responses, tissue remodeling, and inflammation. Macrophages are phenotypically plastic, and at least in animal models two distinct polarization pathways have been identified: classic activation-M1 macrophages and alternative activation-M2 macrophages (64, 65). M1-macrophages are polarized and activated by interferon-γ and lipopolysaccharide. They are specialized in innate immune responses against intracellular pathogens. TLR receptors such as TLR4 in M1 macrophages trigger the activation of NF-κB, AP-1, and STAT1 and promote the release of pro-inflammatory cytokines such as IL1, TNFα, IL-12, IL-1, IL-6, IFNγ, and chemokines CCL2 and CXCL10 (66). M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and TGF-β. These cells limit tissue damage caused by inflammation and promote tissue repair and remodeling. Their effects on the adaptive immune system are more complex, including activation and inhibition (67). Importantly, the M1 and M2 polarization states are not irreversible. They can be considered phenotypic manifestations of biological plasticity, and intermediate phenotypes are possible. Additional macrophage subpopulations are emerging (68) whose roles in cancer are still unclear.

Tumor-associated macrophages are important components of the tumor microenvironment (69). TAMs tend to acquire an M2-phenotype. Recent studies have shown that TAMs can originate either from resident tissue macrophages or from tumor-infiltrating monocytes (67). Studies in patient samples and animal models reveal that TAMs can promote tumor growth by modulating angiogenesis, remodeling the extracellular matrix, providing a niche for cancer stem cells, as well as directly enhancing invasion and metastasis (70–72). High numbers of TAMs are linked to poor prognosis in cancer and associated with increased angiogenesis, enhanced tumor cell invasion, and suppression of adaptive antitumor immunity (73, 74). In basal-like breast cancer, TAMs are associated with poor clinical outcomes (75).

Notch signals play important roles in the differentiation, polarization, and activation of macrophages. In general, Notch signaling mediators are upregulated in activated macrophages (76–80). Wang et al. reported that Notch signaling modulated the M1 or M2 polarization of macrophages in antitumor immune response (81). M2-like TAMs have decreased Notch activity. Activation of Notch signaling promoted an M1 phenotype, secretion of IL-12, and enhanced tumor immunity. These authors showed that canonical CSL/RBP-J-mediated Notch signaling modulates the M1 versus M2 polarization through SOCS3 (81). Xu et al. showed that Notch1 enhances the M1 polarization of inflammatory macrophages through canonical and mitochondrial signaling, whereby Notch1 NICD induces CSL-mediated expression of mitochondrial genes but also associates with mitochondria and modulates metabolic activity and mitochondrial genome expression (82).

An elegant study by the Reedijk group showed that Notch signaling in tumor cells regulates the expression of pro-inflammatory cytokines, IL1β and CCL2, and induced the recruitment of TAM (83). In addition, these authors found that Notch regulates TGFβ-mediated activation of tumor cells by TAMs, suggesting a paracrine loop between TAMs and cancer cells mediated by Notch signals. These authors found a strong association between Notch activation, IL1β and CCL2 production, macrophages infiltration in basal-like breast cancer (83). Zhang et al. analyzed patient samples of invasive micropapillary carcinoma of the breast and proposed that Jagged1-modulated TAM infiltration is associated with poor prognosis (84). Liu et al. found Jagged1 expression is associated with high stromal M2-like TAM and with reduced disease-free and overall survival in primary breast tumor tissues (85). Interestingly, they also found higher M2-like TAM infiltration in metastatic lesions than in primary tumor of patients with aromatase inhibitor resistant cancers. They concluded that Jagged1 promotes aromatase inhibitor resistance by inducing TAM differentiation in breast cancer patients (85). Tanase et al. proposed that TAM and Notch signaling cooperate in reprogramming the glioma stem cell niche, providing protection and support for glioma stem cells (86). Guo and Gonzalez-Perez described a novel crosstalk between Notch, IL-1, and leptin that induces angiogenesis in breast cancer (87). In their working model, leptin stimulates

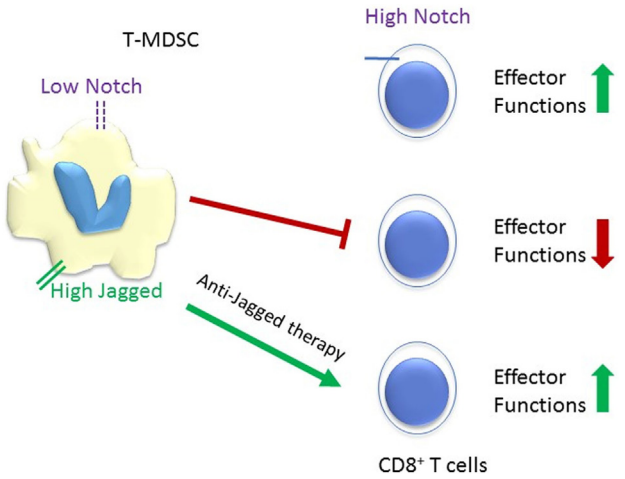

FIGURE 2 | Schematic representation of the reciprocal responses of tumor-infiltrating MDSC (T-MDSC) and $CD8^+$ T cells to Notch signaling. High Notch signaling promotes $CD8^+$ T cells effector functions, while low Notch signaling spurs T-MDSC. Tumor microenvironments upregulate the expression of Notch ligand Jagged on T-MDSC and anti-Jagged therapy overcome tumor-induced T cells tolerance (36). It is unclear whether Jagged expressed in myeloid-derived suppressor cell (MDSC) competes with DLL ligands for Notch1 and Notch2 in CD8 T-cells, or potentially with TCR-induced ligand-independent activation. However, blockade of Jagged1 and 2 in MDSC restores CD8 effector functions.

receptor and ligand expression in breast cancer cells. This phenomenon is dependent on IL-1 signaling. In turn, Notch contributes to the expression of VEGF/VEGFR2 and thus promotes angiogenesis. In this model, IL-1 produced by inflammatory cells such as TAM would enhance leptin-promoted Notch signaling. This crosstalk would be of particular importance in obesity-associated cancers, as leptin is increased in obese patients. Low-grade systemic chronic inflammation in obesity has been proposed to involve M1 macrophages (88). In this case, systemic production of pro-inflammatory cytokines such as IL-1 by M1 macrophages would promote tumor growth at least in part through Notch.

A recent study demonstrated that miR-148a-3p acts downstream of Notch to promote the differentiation of monocytes into macrophages (89). Following Notch activation, miR-148a-3p promoted M1 but inhibited M2 polarization of macrophages. In a transgenic mouse model, conditional overexpression of NICD had no effect of TAM differentiation, but abrogated TAM functions (90). The same study identified miR-125a as a novel downstream mediator of Notch signaling. A miR-125a mimetic increased the phagocytic activity of macrophages and suppressed tumor growth by remodeling tumor microenvironment (90).

In conclusion, Notch signaling participates in the polarization of macrophages and modulates their activity. Furthermore, cytokines produced by macrophages stimulate Notch in cancer cells, and paracrine loops between macrophages and cancer cells can promote tumor survival.

CONCLUDING REMARKS

After decades of preclinical studies with only anecdotal clinical successes, cancer immunotherapy has entered a new phase. Immune checkpoint blockade therapy is one of the most radical innovations in clinical oncology in recent years (91). The FDA approval of CAR-T cell therapy in 2017 was another momentous development (92). However, despite the power of these approaches, there remain plenty of challenges to their clinical application on a large scale. For instance, cancers with low mutational burden are less likely to respond to immunotherapy, perhaps due to their limited antigen repertoire (93). The identification of patients and tumors most likely to respond to immunotherapy through precision medicine approaches is one of the most promising strategies to enhance the impact of cancer immunotherapy. In 2017, in a landmark development, the U.S. FDA granted accelerated approval of an anti-PD-1 antibody to treat patients whose cancers show microsatellite instability or somatic defects in DNA mismatch repair. This was the first FDA approval of an anti-neoplastic agent based not on anatomical cancer location or tumor type but on genomic biomarkers.

Immune suppression by TME myeloid cells is one of the main challenges to the large scale application of cancer immunotherapy. The intricate crosstalk between systemic inflammation, myeloid cells in tumor microenvironment, the cancer cell themselves, and multiple lymphocyte subpopulations modulates tumor immunity. Notch signaling plays multiple roles in this crosstalk (**Figure 2**), and potential therapeutic applications of Notch modulation in immunotherapy have shown significant promise. Among these, the inhibition of MDSC functions by Jagged antibodies and the enhancement of CD8 resistance to MDSC by CD8 T cell-selective Notch activation appear particularly attractive. Another attractive target is DLL4. Tumor-infiltrating myeloid cells activate Dll4/Notch/TGF-β signaling to drive malignant progression (94). A human DLL4 monoclonal antibody by Oncomed Pharmaceuticals is presently in a phase Ib clinical trial in combination with anti PD-1. Combination cancer immunotherapy, particularly targeting the interaction between myeloid cells and T cells in the tumor microenvironment, is a potentially attractive strategy for Notch-targeted drugs and biologics.

AUTHOR CONTRIBUTIONS

FH, SM, and DU wrote different sections of this manuscript. PR, BO, TG, and LMM provided critical input. LM reviewed and edited the draft, and wrote the final version of the manuscript.

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Rotenone Treatment Reveals a Role for Electron Transport Complex I in the Subcellular Localization of Key Transcriptional Regulators During T Helper Cell Differentiation

*Emrah Ilker Ozay[1], Heather L. Sherman[1], Victoria Mello[2], Grace Trombley[3], Adam Lerman[4], Gregory N. Tew[1,2,5], Nagendra Yadava[1,6,7] and Lisa M. Minter[1,2]**

[1] Molecular and Cellular Biology Graduate Program, University of Massachusetts Amherst, Amherst, MA, United States, [2] Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Amherst, MA, United States, [3] Department of Biochemistry and Molecular Biology, University of Massachusetts Amherst, Amherst, MA, United States, [4] Department of Microbiology, University of Massachusetts Amherst, Amherst, MA, United States, [5] Department of Polymer Science and Engineering, University of Massachusetts Amherst, Amherst, MA, United States, [6] Department of Biology, University of Massachusetts Amherst, Amherst, MA, United States, [7] Pioneer Valley Life Sciences Institute, Springfield, MA, United States

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***Correspondence:**
Lisa M. Minter
lminter@vasci.umass.edu

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Recent advances in our understanding of tumor cell mitochondrial metabolism suggest it may be an attractive therapeutic target. Mitochondria are central hubs of metabolism that provide energy during the differentiation and maintenance of immune cell phenotypes. Mitochondrial membranes harbor several enzyme complexes that are involved in the process of oxidative phosphorylation, which takes place during energy production. Data suggest that, among these enzyme complexes, deficiencies in electron transport complex I may differentially affect immune responses and may contribute to the pathophysiology of several immunological conditions. Once activated by T cell receptor signaling, along with co-stimulation through CD28, CD4 T cells utilize mitochondrial energy to differentiate into distinct T helper (Th) subsets. T cell signaling activates Notch1, which is cleaved from the plasma membrane to generate its intracellular form (N1ICD). In the presence of specific cytokines, Notch1 regulates gene transcription related to cell fate to modulate CD4 Th type 1, Th2, Th17, and induced regulatory T cell (iTreg) differentiation. The process of differentiating into any of these subsets requires metabolic energy, provided by the mitochondria. We hypothesized that the requirement for mitochondrial metabolism varies between different Th subsets and may intersect with Notch1 signaling. We used the organic pesticide rotenone, a well-described complex I inhibitor, to assess how compromised mitochondrial integrity impacts CD4 T cell differentiation into Th1, Th2, Th17, and iTreg cells. We also investigated how Notch1 localization and downstream transcriptional capabilities regulation may be altered in each subset following rotenone treatment. Our data suggest that mitochondrial integrity impacts each of these Th subsets differently, through its influence on Notch1 subcellular localization. Our work further supports the notion that altered immune responses can result from complex I inhibition. Therefore, understanding how mitochondrial inhibitors affect immune responses may help to inform therapeutic approaches to cancer treatment.

Keywords: T cell metabolism, T cell differentiation, mitochondria, electron transport complex I, NADH:ubiquinone oxidoreductase, rotenone, Notch1

INTRODUCTION

CD4 T cells can differentiate into effector (Teff) or regulatory [induced regulatory T cell (iTreg)] cells, depending on extracellular cues that they experience at the time they are stimulated (1). During the differentiation process, activated T cells shift their metabolic status and, in this regard, certain metabolic processes have been shown to favor specific T cell programming (1–5). For instance, Teffs usually prefer to use aerobic glycolysis as an energy source, while iTregs are reported to be less glycolytic and primarily utilize fatty acid oxidation (FAO) and oxidative phosphorylation [OXPHOS; (2, 3, 6, 7)]. Studies show that Teffs and iTregs are unable to properly differentiate and function, unless they pass key metabolic checkpoints (8). Reports suggest iTregs fully oxidize the fatty acids and glucose in their mitochondria for ATP production, and using oligomycin to inhibit ATP synthase diminished T cell proliferation and function underscoring the importance of electron transport chain (ETC) activity in T cell activation (3, 9, 10). Given the fact that CD4 T cells display high mitochondrial content and activity, it is not surprising that mitochondrial metabolism may be a critical determinant in Th1, Th2, Th17, and iTreg differentiation (11). Moreover, mitochondrial metabolism has been shown to be essential to T cell plasticity, since inhibiting fatty acid synthesis induces a shift from Th17 toward iTreg differentiation, under Th17-polarizing conditions (1, 8, 10, 12). This inhibition led to decreased nuclear localization of RORγt and reduced binding to the *Il17a* enhancer locus, which ultimately resulted in the Th17-to-iTreg shift (12). Further reports showed the electron transport complex I (ETC-I) inhibitor, rotenone, selectively reduced Foxp3 expression and cytokine production during iTreg differentiation while minimally affecting T-bet and RORγt expression by Th1 and Th17 cells, respectively (13). Of note, rotenone had no effect on Foxp3 expression in fully differentiated iTregs, suggesting OXPHOS is plays a critical role during iTreg differentiation, but not maintenance, programs (13). ETC-I is the largest mitochondrial respiratory chain complex, contributing to ATP synthesis and mitochondrial membrane permeability (14). Rotenone treatment in T cells substantially affects multiple biological functions such as proliferation, cytokine production, and apoptosis (15–17). However, how ETC-I contributes, mechanistically, to T helper (Th) cell differentiation remains unclear.

Notch family proteins are type I transmembrane receptors involved in CD4 Th cell differentiation in response to extracellular polarizing cytokines (18, 19). The intracellular domain of Notch1 (N1ICD) has been shown to regulate T cell differentiation by signaling canonically or non-canonically, and by selectively binding to genes unique to each Th cell subset (18–20). It was shown that Notch1 can regulate the master transcription factors T-Bet, GATA3, RORγt, and Foxp3, as well as their target cytokine genes during Th cell differentiation (20–24). In addition, it has been reported that N1ICD translocates to the mitochondria and can regulate glycolysis, the TCA cycle, and OXPHOS (25, 26). In iTregs, mitochondrial localization of Notch1 was shown to be a critical determinant in fine-tuning differentiation and autophagy responses, thus, linking Notch1 signaling, mitochondrial metabolism, and T cell fate decisions (27).

Cancer cell mitochondrial metabolism may be an attractive therapeutic target, but the impact of mitochondrial inhibitors on immune cell activation and differentiation has not been elucidated. Here, we investigated the relationship between ETC-I activity and Notch1 signaling during Th cell differentiation and report that ETC-I activity influences Notch1 and transcription factor subcellular localization. We found that rotenone treatment increases mitochondrial association of Notch1 in Th2 and iTreg cell subsets and alters nuclear colocalization of Notch1 with Th-specific master transcription factors, especially with RORγt, by reducing Notch1 nuclear residence. Our data suggest that mitochondrial versus nuclear localization of Notch1 may be influenced by ETC-I activity to impact Th cell differentiation.

MATERIALS AND METHODS

Materials

Rotenone ≥95% (Cas No.: 83-79-4) was purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies specific for mouse CD4 APC, CD4 FITC, Notch11 PE, GATA3 APC, and RORγt PE were purchased from eBioscience, Inc. (San Diego, CA, USA) and CD25 PECy7, T-bet APC, T-bet PECy7, and Foxp3 AF488 were purchased from BioLegend (San Diego, CA, USA). Notch1 FITC was purchased from GeneTex, Inc. (Irvine, CA, USA). Unconjugated pyruvate dehydrogenase kinase 1 (PDHK1) (written as PDK1) (Clone: 4A11F5), PDH-E1α (Clone: D-6), and Tubulin AF647 (Clone: A-6) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phospho PDH-E1α (Ser232) was purchased from MilliporeSigma (Burlington, MA, USA). For secondary antibodies, BV510TM donkey anti-rabbit IgG (minimal x-reactivity) (Clone: Poly4064) and BV421TM goat anti-mouse IgG (minimal x-reactivity) (Clone: Poly4053) were purchased from BioLegend. Recombinant IL-2, IFNγ, IL-4, IL-17A, and IL-10 (carrier-free) were purchased from BioLegend. Coating and detection antibodies for IL-2, IFNγ, IL-4, and IL-17A were purchased from BD Biosciences (Billerica, MA, USA) and for IL-10, from BioLegend. For live/dead staining Zombie Violet Fixable Viability Kit was purchased from BioLegend and 7-aminoactinomycin D (7-AAD) staining solution was purchased from BD Biosciences For imaging purposes, MitoTracker Red CMXRos and DRAQ5 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Purified anti-CD3ε (145-2C11) and anti-CD28 (37.51) were purchased from BioLegend. Flow cytometric data were acquired using an LSR II Flow Cytometer, LSRFortessa™ 5 laser (Becton Dickinson, Canaan, CT, USA) and analyzed using DIVA 7.0 software (Becton Dickinson) or FlowJo v10.0 (Tree Star, Ashland, OR, USA). Imaging flow cytometry data were acquired using AMNIS ImageStream X Mark II Imaging Flow Cytometer and analyzed using IDEAS software (EMD Millipore, Billerica, MA, USA).

Animals

All mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts

Amherst. C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Offspring between the ages of 9 and 12 weeks old were used in experiments.

CD4 T Cell Isolation and Rotenone Treatment

Mouse spleens were harvested from C57BL/6J mice and passed through a cell strainer. Red blood cells were lysed in ACK Lysis Buffer, pH 7.2 (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) to obtain bulk splenocytes. Subsequently, primary CD4 T cells were isolated using BD IMag CD4 magnetic particles (Clone GK1.5) (BD Biosciences). CD4 T cells were treated with 20 μM rotenone for 2 h at 37°C prior to stimulating them on anti-CD3ε plus anti-CD28-coated wells.

Cellular Viability Assay

Rotenone-treated primary mouse splenic CD4 T cells were harvested at designated time points and stained with 7-AAD (eBioscience) for 15 min at room temperature followed by centrifugation for 5 min. Cells were resuspended in 0.2% BSA in PBS and analyzed by flow cytometry.

Flow Cytometric Analysis of T Cell Activation

Primary mouse splenic CD4 T cells were pretreated with 20 μM rotenone for 2 h at 37°C prior to stimulating in anti-CD3ε + anti-CD28-coated wells in 5% CO_2 at 37°C. After 24 h of stimulation, cells were harvested and surface-stained with CD4 APC and CD25 PECy7. Cells were then fixed and permeabilized by using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific). They were intracellularly stained for Notch1 PE (Clone: mN1A) and analyzed *via* flow cytometry.

Enzyme-Linked Immunosorbent Assay (ELISA)

Primary mouse splenic CD4 T cell culture supernatants were collected at designated time points and analyzed for cytokine secretion. 96-well Maxisorp plates were coated overnight at 4°C with the appropriate capture antibody (anti-mouse IFNγ, anti-mouse IL-2, anti-IL-4, anti-IL-17A, and anti-IL-10; BD Biosciences). Non-specific protein binding was prevented by blocking wells with 5% BSA in PBS for 3 h at room temperature. Culture supernatants and standards (recombinant IFNγ and IL-2, carrier-free) were diluted appropriately and added to wells. The plate was incubated overnight at 4°C, with continuous rocking. Biotinylated detection antibodies were added to wells followed by TMB substrate reagents (BD Biosciences) at a 1:1 ratio. Color development was monitored, and the reaction was terminated by the addition of stop solution (2N H_2SO_4). Absorbance was read at 450 nm using a microplate reader. Cytokine concentrations were determined relative to the standard curves generated.

In Vitro Mouse T Cell Differentiation Into Th1, Th2, and Th17 Subsets

Primary mouse splenic CD4 T cells were pretreated with 20 μM rotenone for 2 h at 37°C and then they were resuspended in complete RPMI-1640 media (10% fetal bovine serum, 100 U/mL penicillin–streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, β-mercaptoethanol). For polarizations into Th1, Th2, and Th17 subsets, specific cytokine cocktails were added into each cell suspension [Th1: 10 μg/mL anti-IL-4 (BioLegend) + 1 ng/mL recombinant IL-12 (eBioscience, Inc.), Th2: 10 μg/mL anti-IFNγ (BD Biosciences) + recombinant IL-4 (eBioscience, Inc.), Th17: 10 μg/mL anti-IL-4 (BioLegend) + 10 μg/mL anti-IFNγ (BD Biosciences) + 20 ng/mL recombinant IL-6 (BioLegend) + 5 ng/mL TGF-β (BioLegend)]. The cells were incubated for 24, 48, 72, and 96 h for further analysis.

In Vitro Mouse iTreg Differentiation

Primary mouse bulk splenocytes were incubated with CD4 T cell enrichment antibody cocktail (BD Biosciences) for 15 min on ice, then incubated with Streptavidin Particles Plus—DM (BD Biosciences) for 30 min on a rotator at 4°C. The negative fractions from magnetic separation were collected in a conical tube. Later, biotin-conjugated anti-CD25 (2.5 μg) was added into the negative fractions and incubated on ice for 30 min. Subsequently, they were incubated with Streptavidin Particles Plus—DM (BD Biosciences) for 30 min on a rotator at 4°C and separated magnetically to obtain $CD4^+$ $CD25^-$ T cells in the negative fraction. The cells were resuspended in complete RPMI-1640. IL-2 (135 U/mL) and TGF-β (20 ng/mL) were added into the cell suspension to polarize them toward iTregs. The cells were incubated for 24, 48, 72, and 96 h for further analysis.

Imaging Flow Cytometry

Each Th subset was harvested at the indicated time points following polarization. Mitochondria were visualized using 300 nM MitoTracker Red CMXRos and nuclei using DRAQ5 (1:1,000). Notch1 FITC was used to stain the cells intracellularly. Later, 1,000 cells were imaged, and fluorescent intensities were quantified using an AMNIS ImageStream X Mark II Imaging Flow Cytometer at 60× magnification. To determine nuclear localization of desired proteins, the nuclear localization wizard was applied in the IDEAS software upon masking the nuclear area (Intensity mask: Ch05-DRAQ5 staining). For mitochondrial localization of Notch1, the colocalization wizard was used to determine the bright detail similarity between MitoTracker Red signal and Notch1 FITC signal. A similar protocol was followed to assess localization of Th-specific master transcription factors in the mitochondria and nuclei of imaged cells. To visualize Th17-polarized cells treated with rotenone or dichloroacetate (DCA), primary mouse splenic CD4 T cells were pretreated with 20 μM rotenone for 2 h or 1 mM DCA (left in the cell suspension throughout the experiment) at 37°C and then they were resuspended in complete RPMI-1640 media (10% fetal bovine serum, 100 U/mL penicillin–streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, β-mercaptoethanol). Later, cells were polarized into Th17 subset by adding 10 μg/mL of anti-IL-4 (BioLegend) + 10 μg/mL of anti-IFNγ (BD Biosciences) + 20 ng/mL of recombinant IL-6 (BioLegend) + 5 ng/mL of TGF-β (BioLegend) and cultured for 72 h on anti-CD3ε + anti-CD28-coated wells. At 72 h, cells were harvested and stained for mitochondria with

MitoTracker Red CMXRos, cytosol with Tubulin AF647, and nuclei with propidium iodide for imaging flow cytometry *via* AMNIS ImageStream X Mark II Imaging Flow Cytometer at 60× magnification. Th17-polarized cells were gated as RORγt-positive cells and analyzed for localizations for PDHK1 (followed by secondary BV510 anti-rabbit IgG), total PDH-E1α (followed by secondary BV510 anti-rabbit IgG), pPDH-E1α (Ser232) (followed by secondary BV421 anti-mouse IgG), RORγt PE, and Notch1 FITC. The data were analyzed *via* IDEAS software.

Statistical Analysis

Data are the mean ± SEM; all experiments were repeated at least two or three times. Unpaired, two-tailed Student's *t*-test and two-way ANOVA with post-Bonferroni test were applied for statistical comparison by using GraphPad Prism 5 software. p Values of ≤ 0.05 were considered significant.

RESULTS

Rotenone Treatment Reduces T Cell Activation Upon Anti-CD3 Plus Anti-CD28 Stimulation

Mitochondrial metabolism has been previously implicated in antigen-specific T cell activation *in vivo*, through the production of reactive oxygen species by ETC-III (9). To investigate whether ETC-I also functions to mediate CD4 T cell activation, we pretreated primary mouse splenic CD4 T cells with the ETC-I inhibitor, rotenone (20 μM), for 2 h at 37°C, then stimulated cells on anti-CD3ε- plus anti-CD28-coated plates for 24 h. Upon stimulation, the high affinity IL-2 receptor, CD25, is upregulated on CD4 T cells through a Notch1-dependent mechanism (28, 29). Using conventional flow cytometry, which quantifies total protein, but not its subcellular localization, we evaluated CD25 expression following T cell stimulation and found that, compared with cells incubated only with vehicle control, DMSO, significantly fewer cells upregulated CD25 following rotenone treatment, and the cells that did express CD25 showed markedly reduced levels of protein expression (**Figures 1A–C**). We and others have shown that stimulating CD4 T cells *in vitro* generates the cleaved, signaling competent, intracellular domain of Notch1, N1ICD (20, 30). Therefore, we measured the percentage of cells that expressed N1ICD, as well as the total N1ICD they expressed on a per cell basis. Consistent with CD25 results, we noted reduced percentages of CD4 T cells that expressed N1ICD, following rotenone treatment (**Figure 1D**), but interestingly, the overall expression of N1ICD did not appear to be significantly different between rotenone- and DMSO-treated cells (**Figure 1E**). However, when we analyzed the culture supernatants from cells left untreated or treated with rotenone, we noted that secretion of IFNγ and IL-2, in the culture supernatants was reduced (**Figures 1F,G**). Thus, rotenone treatment reduces CD4 T cell activation potential upon anti-CD3ε plus anti-CD28 stimulation as measured by CD25 expression and secretion of IFNγ and IL-2, although the cellular levels of N1ICD did not appear to differ between cells treated with rotenone or DMSO.

Rotenone Treatment Alters the Kinetics of Th17 and iTreg Cell Differentiation

Studies have highlighted how metabolic pathways are selectively utilized during Th cell differentiation (31, 32). Teff cells rely on glucose as their main energy source, while iTregs will oxidize fatty acids to produce the energy they need. Rotenone acts to block NADH → NAD, a process important both in glycolytic and OXPHOS pathways. Therefore, we sought to further investigate whether rotenone treatment would alter the differentiation potential of Th cells. We performed polarization assays to differentiate Th1, Th2, Th17, and iTreg cells *in vitro*. CD4 T cells were isolated from mouse spleens and left untreated or treated with rotenone (20 μM) for 2 h at 37°C. Cells were stimulated with anti-CD3ε and anti-CD28 in the presence of specific combinations of antibodies and cytokines to promote their differentiation toward distinct Th cell lineages. We harvested polarized cells 24, 48, 72, and 96 h after plating and used standard ELISA techniques to analyze the secretion kinetics of their signature cytokines: Th1:IFNγ, Th2:IL-4, Th17:IL-17A, and iTreg:IL-10. Overall, rotenone treatment did not affect cellular viability over their time course (Figures S1A–D in Supplementary Material). Interestingly, we observed that rotenone affected the cytokine secretion of distinct Th subsets, but with different kinetics. We did not detect significant difference in IFNγ secretion by Th1-polarized cells or in IL-4 secretion by Th2-polarized cells over time, regardless of whether the cells were polarized in the absence or presence of rotenone (**Figures 2A,B**). However, we found IL-17A levels were greatly diminished by 72 h in Th17-polarized cells exposed to rotenone treatment (**Figure 2C**). IL-10 secretion by iTregs was also significantly lower in rotenone-treated cells, compared with those cultured in DMSO. However, we noted that the kinetics of reduced IL-10 production were different from Th17-polarized cells, with the maximum differences in effects on IL-10 production occurring 48 h after stimulation (**Figure 2D**). Altogether, these findings suggest that rotenone treatment uniquely modifies the kinetics of cytokine production in Th cells during *in vitro* polarization, with Th17 and iTreg cells being particularly responsive to ETC-1 inhibition.

Notch1 Expression and Cellular Localization in Th Cells Is Differentially Affected by Rotenone Treatment

Earlier studies showed Notch1 can regulate IL-17A production in Th17 cells, as well the differentiation of iTreg cells induced *in vitro* (19, 23). Therefore, we asked whether the kinetics of Notch1 expression in each Th cell subset also varied in Th cells in response to rotenone treatment. Using conventional flow cytometry, which quantifies total protein, but not its subcellular localization, we determined the percent of cells that stained positively for Notch1, as well as the total level of Notch1 expressed, in Th1-, Th2-, Th17-, and iTreg-polarized cells. Notch1 expression was significantly diminished in Th17 and iTreg cells, 72 and 48 h after polarization, respectively (**Figures 3A–D**), displaying response kinetics that overlapped with those of the IL-17A and IL-10 cytokine expression. These data suggest that rotenone may be affecting cytokine secretion in Th17 and iTreg cells through

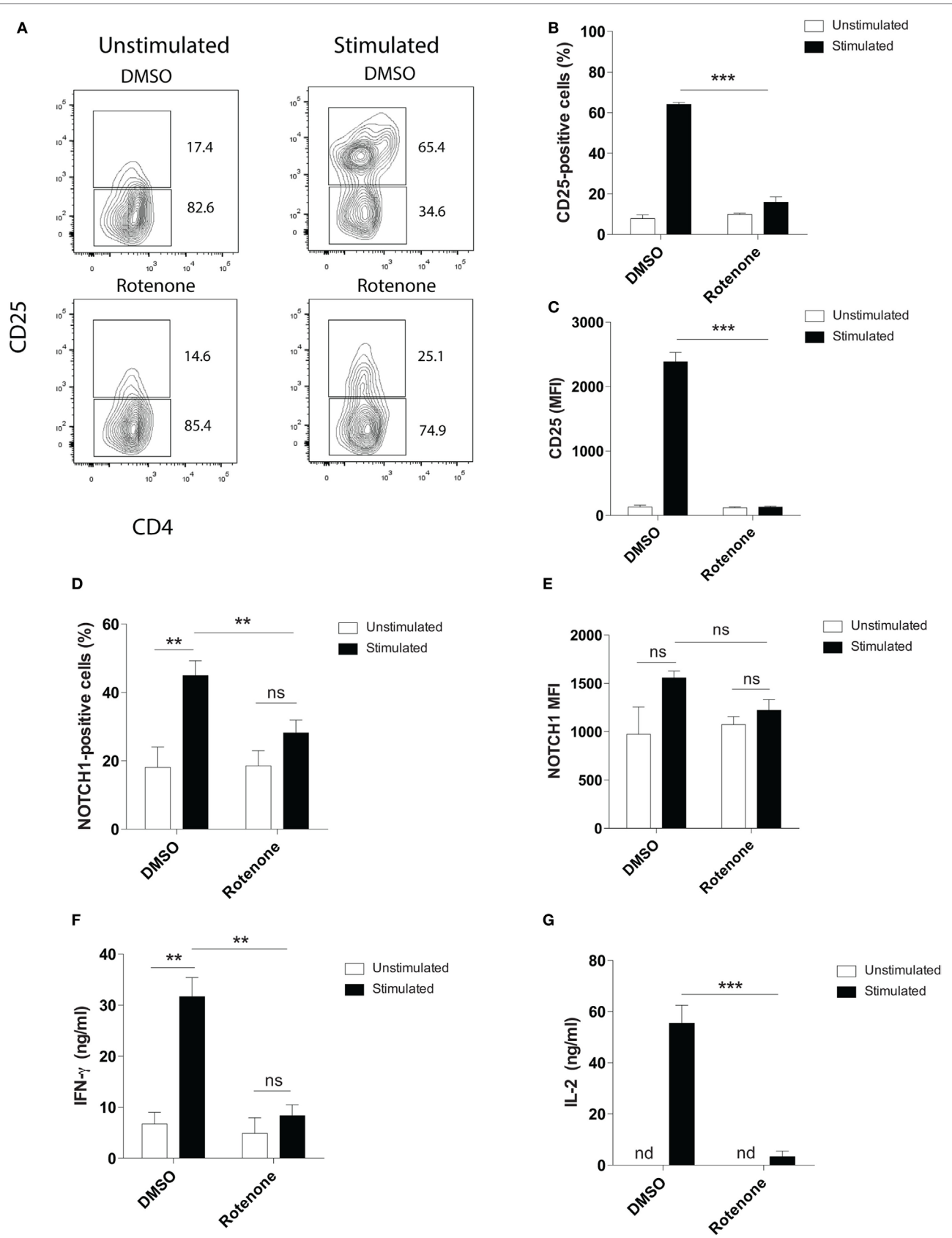

FIGURE 1 | Continued

FIGURE 1 | Rotenone treatment reduces T cell activation upon anti-CD3ε plus anti-CD28 stimulation. Mouse splenic CD4 T cells were left untreated or treated with 20 μM rotenone for 2 h, then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24 h. CD25 and Notch1 levels were measured as read-outs of T cell activation *via* flow cytometry. **(A)** Representative contour plot showing CD25-expressing cells left unstimulated or stimulated in the presence or absence of rotenone for 24 h. The **(B)** percent CD25-positive, and **(C)** median fluorescence intensity (MFI) of CD25, as well as the **(D)** percent Notch1-positive **(E)** MFI of Notch1 on CD4 T cells, cultured as described above. At the end of 24 h of stimulation, culture supernatants were collected, and standard enzyme-linked immunosorbent assay techniques were used to quantify secreted **(F)** IFNγ and **(G)** IL-2. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; calculated using an unpaired, two-tailed Student's *t*-test.

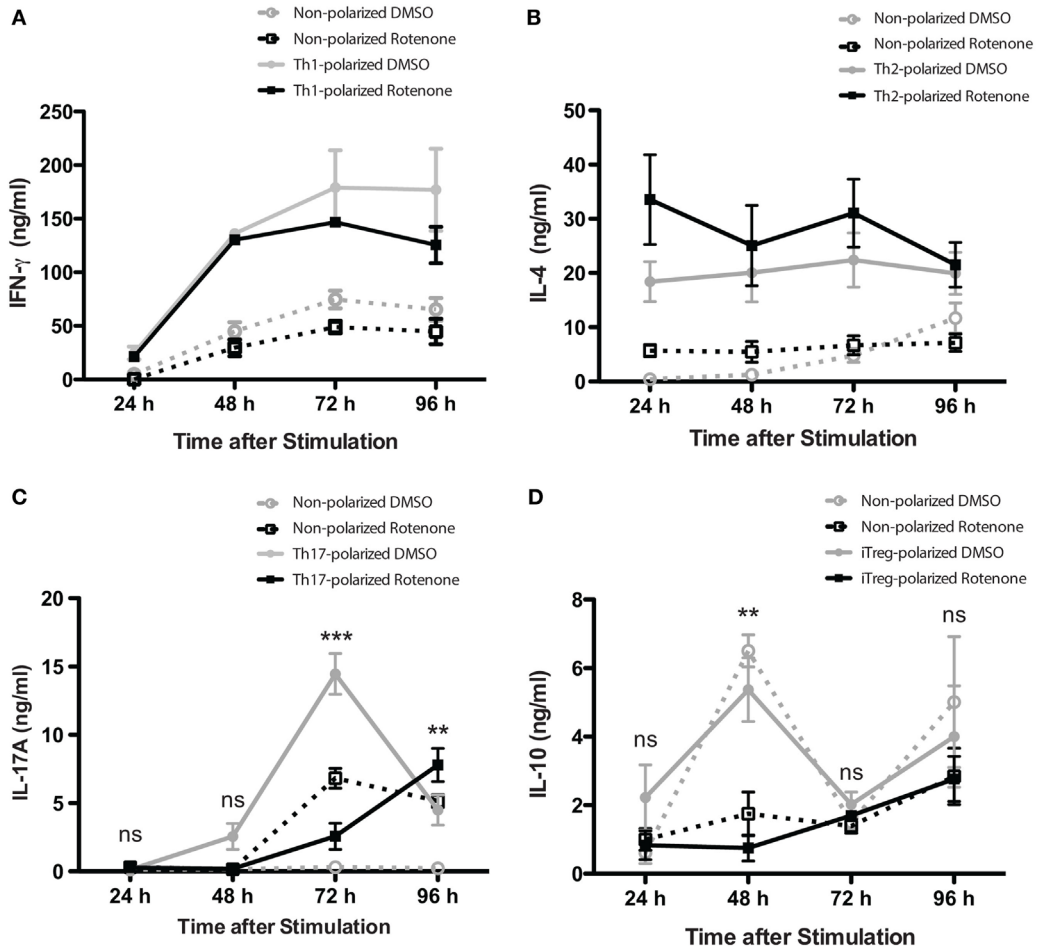

FIGURE 2 | Rotenone treatment alters the kinetics of Th17 and induced regulatory T cell (iTreg) cell differentiation. Mouse splenic CD4 T cells and $CD4^{+}CD25^{-}$ T cells were used for T helper (Th) cell and iTreg differentiation, respectively. Cells were left untreated or treated with 20 μM rotenone for 2 h and then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24, 48, 72, and 96 h in the presence of specific Th cell polarization conditions. At the indicated time points, cell supernatants were collected, and signature cytokines were quantified using standard enzyme-linked immunosorbent assay techniques for **(A)** IFNγ by Th1-polarized cells, **(B)** IL-4 by Th2-polarized cells, **(C)** IL-17A by Th17-polarized cells, and **(D)** IL-10 by iTreg-polarized cells. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; calculated using two-way ANOVA with post-Bonferroni test applied.

its effects on Notch1. Consistent with this conclusion, we did not find any significant differences in Notch1 expression in Th1 or Th2 cells over time (Figures S2A–D in Supplementary Material).

To further understand the effects of complex I inhibition on Th cell differentiation, we measured the mitochondrial mass in Th cells following rotenone treatment. We also asked whether rotenone treatment influenced the subcellular localization of Notch, by assessing its distribution across mitochondrial, cytosolic, and nuclear compartments. When we measured mitochondrial mass using CMXRos dye, we noted that rotenone treatment significantly reduced the mitochondrial mass in Th1, Th2 (Figures S3A,B in Supplementary Material), and Th17 cells (**Figure 3E**). By contrast, the mitochondrial mass in iTreg cells was notably increased (**Figure 3F**). We next quantified the frequency of Th cells that show mitochondrial association of Notch1 using imaging flow cytometry, which can distinguish the subcellular localization of a specific protein of interest. We detected low levels of mitochondrial Notch1

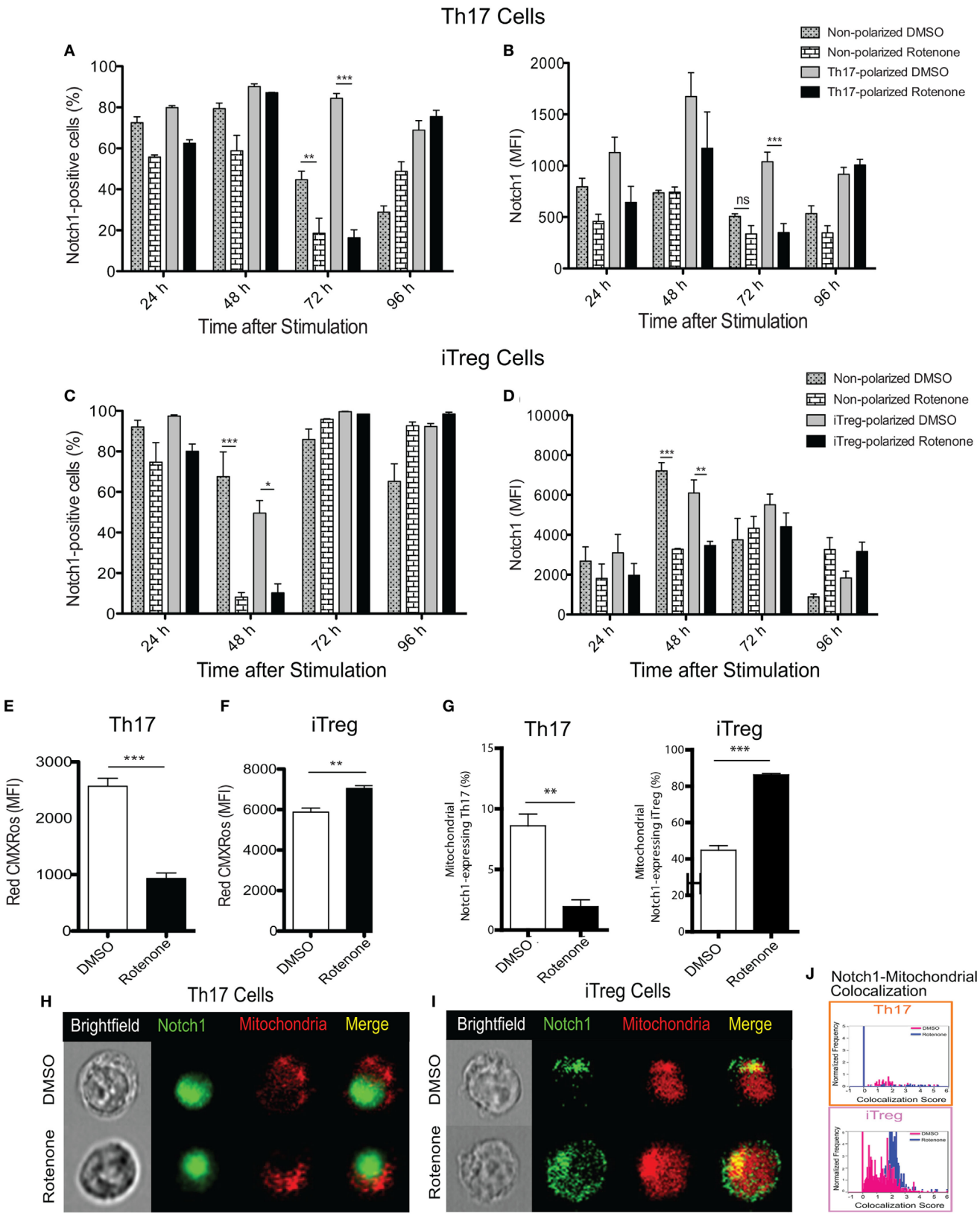

FIGURE 3 | Continued

FIGURE 3 | Notch1 expression and cellular localization in T helper (Th) cells is differentially affected by rotenone treatment. Notch1 levels were measured in Th cells, using flow cytometric approaches. Cells were left untreated or treated with 20 μM rotenone for 2 h and then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24, 48, 72, and 96 h in the presence of specific Th cell polarization conditions. At the indicated timepoints, cells were harvested to determine the **(A)** percent Notch1-positive and **(B)** Notch1 median fluorescence intensity (MFI) in Th17-polarized cells, and the **(C)** percent Notch1-positive, and **(D)** Notch1 MFI in induced regulatory T cell (iTreg)-polarized cells. We visualized mitochondria within the cells using Mitotracker Red CMXRos and imaging flow cytometry. Mitochondrial mass was determined based on the MFI of Red CMXRos for DMSO control and rotenone-treated cells under **(E)** Th17-polarizing conditions 72 h after stimulation and **(F)** iTreg-polarizing conditions 48 h after stimulation. **(G)** We calculated the percent of Th17 (left panel) and iTreg (right panel) cells which showed mitochondrial Notch1 localization. Representative cell images showing Notch1 and mitochondrial colocalization in **(H)** Th17 and **(I)** iTreg cells, differentiated in the presence and absence of rotenone. **(J)** We used the AMNIS Colocalization Wizard to calculate Th17 and iTreg cell frequency histograms that show Notch1 colocalized with mitochondria, together with their corresponding colocalization scores. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; calculated using two-way ANOVA with post-Bonferroni test applied or an unpaired, two-tailed Student's *t*-test.

in DMSO-treated Th1, Th2, and Th17 cells (Figures S3C–E in Supplementary Material). Although rotenone treatment did not alter mitochondrial Notch1 colocalization score in Th1 or Th2 cells, the frequency of cells that expressed mitochondrial Notch1 increased with rotenone treatment, and to a much greater extent in Th2 cells (Figures S3C–E in Supplementary Material). We observed almost no mitochondrial-associated Notch1 in Th17 cells, regardless of whether they were differentiated in the presence or absence of rotenone (**Figures 3G,H,J**). iTregs, however, showed a substantial amount of mitochondrial Notch1 when they were differentiated in the presence of DMSO, and this increased further with rotenone treatment, as determined by the higher colocalization score assigned by the AMNIS Colocalization Wizard algorithm (**Figures 3G,I,J**). Finally, we applied the AMNIS intensity mask for nucleus, mitochondria, and cytosol to calculate the percent of Notch1 protein residing in these different compartments and determined whether complex I deficiency altered this distribution. We noted slight increases in nuclear Notch1 in rotenone-treated Th1 and iTreg cells, marked decreases in cytosolic Notch1 in Th2 and iTregs, with concomitant increases in mitochondrial Notch1. Most striking was a near complete loss of nuclear Notch1 in Th17 cells, with redistribution to the cytosol (Figure S3E in Supplementary Material). These results suggest that Notch1 localization may be related to cytokine signaling during Th17 and iTreg differentiation and can be altered by ETC-I activity.

Rotenone Treatment Does Not Affect the Expression of Th Cell Master Transcriptional Regulators

Signature Th cytokines are regulated by their master transcription factors. Therefore, we asked whether rotenone also affects the levels of these transcription factors expressed over time. We first assessed levels of RORγt and Foxp3, since changes in Notch1 expression correlated with IL-17A and IL-10 levels during Th17 and iTreg differentiation, respectively. Surprisingly, we did not detect differences in total in RORγt in Th17 cells (**Figures 4A,B**) or Foxp3 levels in iTreg cells (**Figures 4C,D**), as measured by flow cytometry, regardless of whether cells were pretreated with rotenone. We did note modest decreases in T-bet levels in Th1-polarized cells (Figures S4A,B in Supplementary Material), but there was no significant effect on GATA3 expression in Th2-polarized cells (Figures S4C,D in Supplementary Material). These results showed that the levels of each transcription factor were not substantially changed after rotenone treatment. However, we found that rotenone treatment decreased the percent of iTregs, characterized as $CD4^{+}CD25^{+}Foxp3^{+}$ cells, at 48 and 72 h upon iTreg differentiation (**Figures 4E,F**) suggesting that regulatory T cells rely on their mitochondrial function during the differentiation process.

Th17 Cells Lose Expression of Nuclear RORγt Following Rotenone Treatment

One means by which protein activity may be regulated is through selective localization within subcellular compartments. Since we did not detect differences in the total levels of master transcriptional regulators following ETC-I inhibition, we asked whether treating cells with rotenone affected their cellular distribution. We stained differentiated Th cells with antibodies specific for their master transcription factor, together with the nuclear marker, DRAQ5. We then determine the nuclear localization of each of these transcription factors using imaging flow cytometry. We also determined the percent distribution of each transcription factor within and outside of the nucleus by applying the AMNIS nuclear masking wizard. T-bet in Th1 cells and GATA3 in Th2 cells were localized exclusively to the nucleus in DMSO-treated, polarized cells, with only minimal redistribution to the cytosol following rotenone treatment (Figures S5A–D in Supplementary Material). Rotenone treatment had the greatest effect on RORγt localization in Th17 cells, completely abrogating RORγt nuclear localization and concomitantly redirecting it entirely to the cytosol (**Figures 5A,B**). We expected to see differences in Foxp3 nuclear localization as well, since rotenone treatment had such robust effects on the percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ cells generated during iTreg differentiation. Interestingly, we found Foxp3 to be similarly distributed between the nucleus and the cytosol, both in DMSO- and in rotenone-treated iTregs (**Figures 5C,D**). Reports are increasingly linking Notch1 function to Th17–iTreg differentiation axis (33–37); therefore, we also assessed Notch1 localization in both these cell types. We detected nuclear Notch1 in a high percentage of Th17 cells, and the percentage of Th17 cells showing nuclear Notch1 was decreased significantly, following rotenone treatment (**Figure 5E**). In complementary fashion, fewer than 10% of iTregs expressed nuclear Notch1, but in rotenone-treated iTregs, nuclear Notch1 could be detected in nearly 40% of cells (**Figure 5F**). Altogether, these results suggest that, although inhibiting ETC-1 with rotenone negatively impacts Th17 and iTreg differentiation *in vitro*, it likely does so through different mechanisms. However, nuclear residence of Notch1 may be necessary for a sustained Th17 differentiation program.

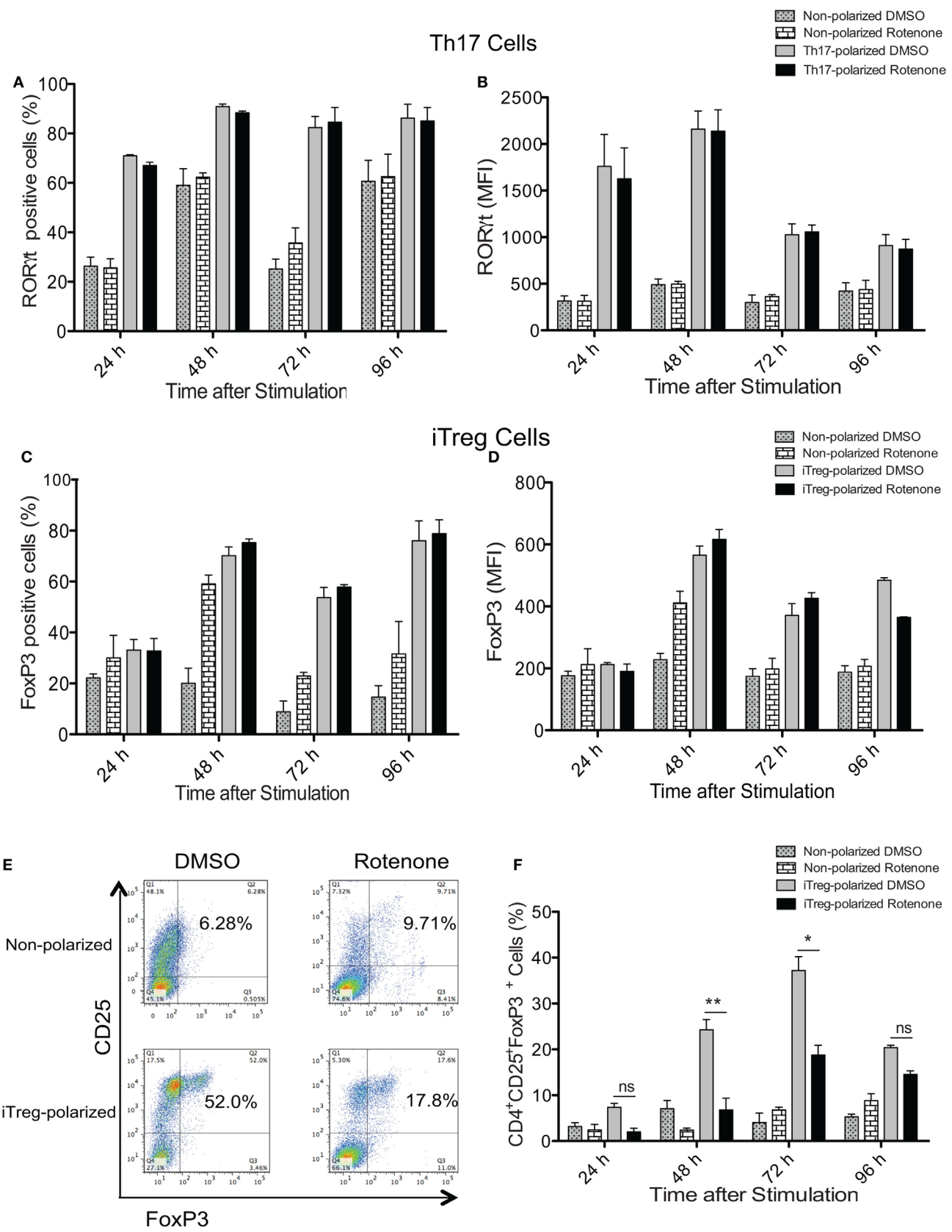

FIGURE 4 | Continued

FIGURE 4 | Rotenone treatment does not affect the expression of T helper (Th) cell master transcriptional regulators. CD4 T cells were left untreated or treated with 20 μM rotenone for 2 h and then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24, 48, 72, and 96 h in the presence of specific Th17- or induced regulatory T cell (iTreg)-specific polarization conditions. At the indicated timepoints, cells were harvested, and we determined the **(A)** percent RORγt-positive and **(B)** median fluorescence intensity (MFI) of RORγt expressed in Th17-polarized cells, and the **(C)** percent Foxp3-positive and **(D)** MFI of Foxp3 expressed in iTreg-polarized cells. **(E)** Representative dot plot and **(F)** collated data showing percentages of $CD4^+CD25^+Foxp3^+$ iTregs following differentiation in the absence or presence of rotenone. Data represent the mean ± SEM of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; calculated using two-way ANOVA with post-Bonferroni test applied.

Rotenone Treatment Inhibits Mitochondrial Localization of PDHK1 and Promotes RORγt and Notch1 Colocalization With Its Phosphorylated Substrate, pPDH-E1α, in Th17-Polarized Cells

In the presence of the appropriate cytokine environment, Notch signaling can serve to reinforce Th cell fate decisions, including differentiation into Th17 and iTreg cells (19, 23, 24, 38, 39). Since we observed significantly reduced levels of nuclear Notch1 in Th17 and iTreg-polarized cells, we asked what effects rotenone imparts on Notch1 nuclear localization and its colocalization with master transcription factors. We stained differentiated Th cells with antibodies specific for their signature master transcriptional regulator, together with Notch1, and the nuclear marker DRAQ5, and analyzed colocalization using imaging flow cytometry. Rotenone treatment significantly diminished colocalization of Notch1 with T-bet, GATA3, and RORγt, while Notch1–Foxp3 nuclear colocalization increased in rotenone-treated iTregs (Figures S6A–D in Supplementary Material). Furthermore, data suggest Th17 cells rely heavily on glycolysis, whereas iTregs primarily utilize OXPHOS and FAO, to meet their respective energy needs. Specifically, Gerriets and Kishton identified PDHK1 as a selective regulator of glycolytic and oxidative metabolism by inhibiting PDH (13). PDH inhibition suppressed glucose oxidation and shifted metabolic processes to lactate production and glycolysis (13). The authors found that inhibiting PDHK1 specifically weakened Th17 cells while benefiting iTregs. To further explore how ETC-I integrity affects PDHK1, we generated Th17 CD4 T cells, *in vitro*, in the presence of rotenone or DCA, an inhibitor of PDHK1. DMSO was used as a vehicle control. Cells were stained with a mitochondrial dye, together with antibodies specific for Notch1, RORγt, PDHK1, and its substrate, PDH-E1α, then visualized using imaging flow cytometry. We observed that, in Th17 cells, PDHK1 is localized primarily to the mitochondria (**Figure 6A**), as is its non-phosphorylated substrate, PDH-E1α (**Figure 6B**). However, treating cells either with rotenone or DCA diminishes mitochondrial-associated PDHK1 and PDH-E1α, and redistributes PDH-E1α to the cytosol (**Figures 6A,B**; Figure S7A–C in Supplementary Material). We next asked whether Notch1 or RORγt associated with the phosphorylated form of PDH-E1α, pPDH-E1α (Ser232), in Th17 cells. We observed increases both of cytosolic and mitochondrial Notch1-pPDH-E1α colocalization when Th17 cells were polarized in the presence of rotenone or DCA (**Figure 6C**; Figure S7D in Supplementary Material). Unexpectedly, when we assessed colocalization of RORγt and pPDH-E1α, we found increased colocalization in Th17 cells only after rotenone treatment (**Figure 6D**; Figure S7E in Supplementary Material). In addition, colocalization of total PDH-E1α and RORγt did not seem to change in either of the treatments (Figures S7F,G in Supplementary Material), suggesting that, as nuclear RORγt is lost, it may be redistributed and sequestered selectively by phosphorylated form of PDH-E1α [pPDH-E1α (Ser232)] outside of the nucleus in rotenone-treated Th17 cells. These data suggested to us that PDHK1 and, more specifically, its substrate, PDH-E1α, may act as a "rheostat" to regulate Th17–Treg cell fate decisions. Therefore, we examined the effects of rotenone and DCA treatment, both, on the expression of *foxp3* and *rorgt* in Th17 cells. Consistent with this prediction, following treatment with either inhibitor, *foxp3* expression was significantly increased in Th17 cells. However, only in rotenone-treated Th17 cells did we observe decreased *rorgt* expression, suggesting ETC-I integrity is also important for sustained *rorgt* transcription (**Figure 6E**).

Altogether, we show that using the ETC complex I inhibitor, rotenone, disrupts Th17 and iTreg differentiation, *in vitro*. Furthermore, we demonstrate that, in Th17 cells, rotenone acts to abrogate RORγt nuclear localization and subsequently its colocalization with Notch1 in the nucleus, resulting in diminished IL-17A cytokine production. We conclude from our data, that rotenone decreases the expression of Notch1, the percentage of cells that express nuclear Notch1, and the percentage of Th17 cells that express mitochondrial Notch1, compared with DMSO-treated counterparts, which strongly suggests Notch1 has an integral function in Th17 cell differentiation. We also noted decreased nuclear colocalization of Notch1-RORγt in rotenone-treated Th17 cells, as RORγt is redistributed almost solely to non-nuclear compartments following rotenone treatment, which further suggests that nuclear Notch1 and nuclear RORγt may co-regulate Th17 cell fate. Non-nuclear Notch1 and RORγt, both, associated with pPDH-E1α in rotenone-treated Th17 cells, leading to increased *foxp3* transcription in these cells (Figure S8 in Supplementary Material). These data suggest that the Notch1-RORγt signaling pathway intersects with key regulators of T cell metabolism to influence Th17–iTreg cell fate potential.

DISCUSSION

Here, we report the effects of inhibiting mitochondrial function on CD4 T cell activation and differentiation. Treating murine splenic CD4 T cells with rotenone, a known ETC-I inhibitor, attenuated T cell activation potential upon anti-CD3ε and anti-CD28 stimulation. This decreased activation was accompanied

by reduced secretion of proinflammatory cytokines such as IFNγ and IL-2. We also examined how inhibiting ETC-I function impacts CD4 T cell differentiation into four major Th subsets and showed treatment with rotenone primarily affected Th17 and iTreg differentiation while exhibiting milder effects on Th1 and Th2 cells.

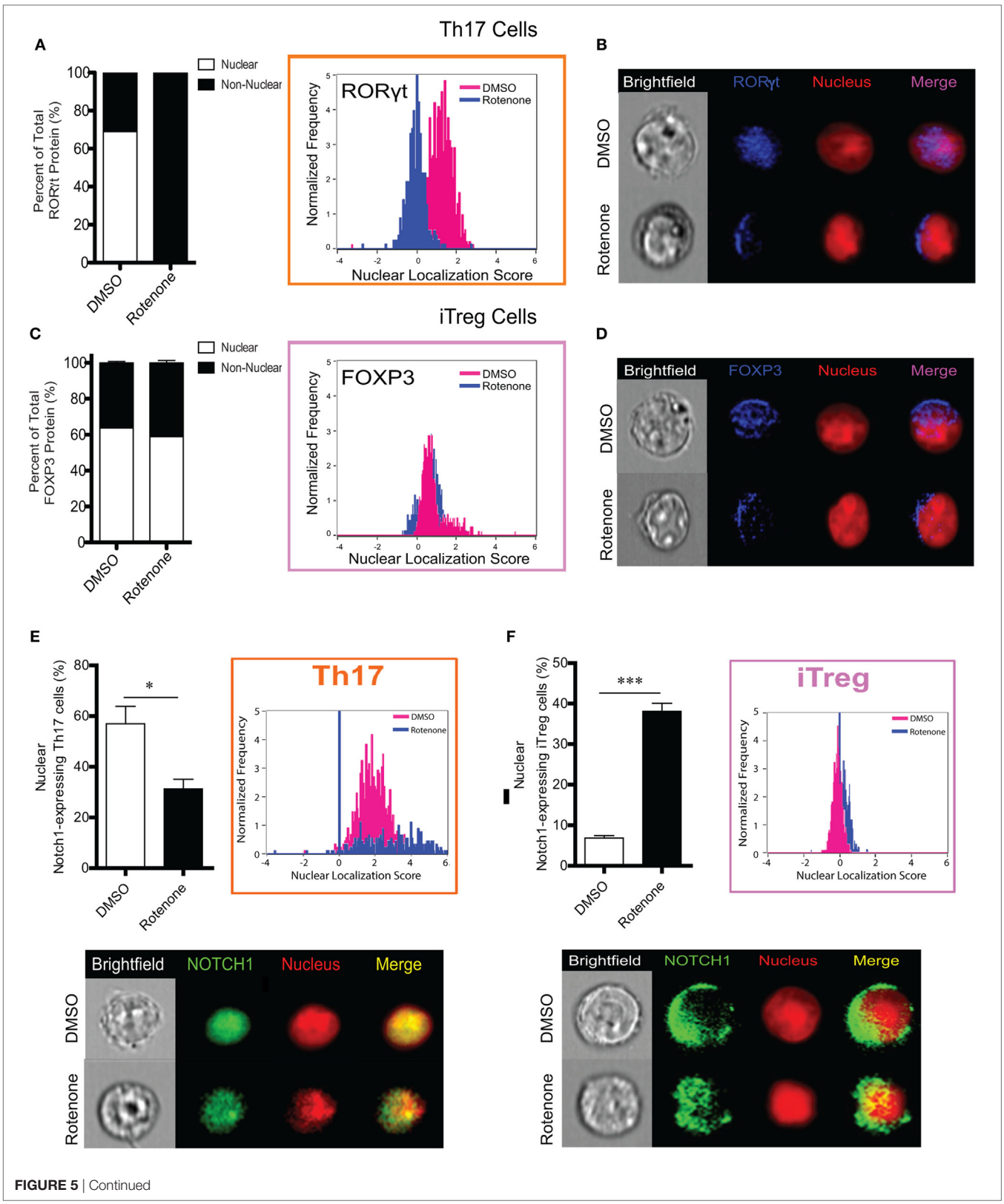

FIGURE 5 | Continued

FIGURE 5 | T helper (Th)17 cells lose expression of nuclear RORγt following rotenone treatment. CD4 T cells were left untreated or treated with 20 μM rotenone for 2 h and then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 24, 48, 72, and 96 h in the presence of specific Th17- or induced regulatory T cell (iTreg)-specific polarization conditions. For each master transcriptional regulator, we determined the total, nuclear, and non-nuclear median fluorescence intensity (MFI), using an AMNIS Imaging Flow Cytometer. nuclear localization scores were determined by an algorithm that calculates the probability of the protein of interest resides within the nucleus. The higher the nuclear localization score, the greater the probability of the protein of interest is in the nucleus. Scores above "1" are considered to be positive for nuclear expression. Nuclear and non-nuclear MFI were calculated using AMNIS IDEAS Software and applying the nuclear mask. Percent total nuclear and non-nuclear transcription factor proteins were calculated for cells differentiated in the absence or presence of rotenone as follows: [nuclear MFI]/[Total MFI] × 100, or [non-nuclear MFI]/[Total MFI] × 100, respectively. For cells differentiated without or with rotenone, **(A)** percent distribution of total RORγt protein and RORγt nuclear localization score in Th17-polarized cells. **(B)** Representative images showing nuclear localization of RORγt. **(C)** Percent distribution of total Foxp3 protein and Foxp3 nuclear localization score in iTreg-polarized cells. **(D)** Representative images for nuclear localization of Foxp3 in iTreg cells. For cells differentiated without or with rotenone. **(E)** Percent of nuclear Notch1-expressing Th17 cells and their representative corresponding Nuclear Localization Scores and representative images of Notch1 nuclear localization in Th17 cells after 72 h of differentiation without or with rotenone in Th17 cells 72 h after differentiation, and **(F)** percent of nuclear Notch1-expressing iTreg cells and their representative corresponding Nuclear Localization Scores and representative images of Notch1 nuclear localization in iTreg cells after 48 h of differentiation without or with rotenone. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; calculated using two-way ANOVA with post-Bonferroni test applied or an unpaired, two-tailed Student's *t*-test.

Notch1 localization in Th cell subsets is a critical determinant in their differentiation processes and previous studies have linked Notch signaling to cell survival, based on its mitochondrial versus nuclear association (40, 41). In this study, in iTregs Notch1 localized to the mitochondria, whereas Th17 cells exhibited high levels of nuclear Notch1. Surprisingly, we found Notch1 selectively colocalized with master transcription factors in the nucleus. For instance, RORγt and Notch1 predominantly colocalized in the nucleus and this was abrogated upon rotenone treatment. On the other hand, we did not observe Foxp3 and Notch1 colocalization, although rotenone treatment did reduce the percent of iTregs differentiated in culture. Thus, although rotenone treatment affected both Th17 and iTreg differentiation, through its inhibition of ETC-I function, it is likely that mitochondrial involvement in regulating Th17 and iTreg differentiation is different and distinct in these subsets.

Evidence shows that metabolic reprogramming plays an important role in T cell activation, differentiation, and function (42, 43). As the central hubs of cellular energy production, mitochondria are key regulators of cell metabolism, survival, and signal transduction (44–46). Certain subsets of Th cells rely on OXPHOS to meet their energy demands which, in turn, is directed by a group of proteins found on the outer mitochondrial membrane (47). ATP produced *via* OXPHOS is mediated by the four protein complexes within the ETC, through the creation of a proton gradient. After T cell activation, CD4 T cells undergo a switch from a resting to a proliferating metabolic state, with substantial increases in glucose uptake and in rates of glycolysis reported. This increase in glycolysis is dependent upon CD28-mediated signaling (14). Our results show that the failure to upregulate CD25 and produce IFNγ and IL-2 suggest that rotenone-treated cells do not undergo full TCR-mediated activation. In addition to determining T cell activation, mitochondrial function impacts CD4 T cell differentiation. We measured signature cytokine production in four main Th subsets and found that rotenone treatment reduced Th17 and iTreg cytokine secretion, but not that of Th1 or Th2 cells. One explanation for the differences in sensitivity to ETC-I inhibition could be that Th subsets have intrinsically distinct metabolic characteristics and requirements, especially during the early stages of Th cell commitment.

Data in the literature suggest Th1, Th2, and Th17 cells rely heavily on glycolysis, whereas, iTregs primarily utilize OXPHOS and FAO to meet their respective energy needs. Our findings demonstrate that ETC-I integrity may factor prominently in Th17 and iTreg cell differentiation, and that Notch1 and RORγt do, indeed, converge at the level of cell metabolism in Th17 cells. Gerriets and Kishton identified PDHK1 as a selective regulator of glycolytic and oxidative metabolism by inhibiting PDH (13). PDH inhibition suppresses glucose oxidation and shifts metabolic processes to lactate production and glycolysis (48). Our data are consistent with their findings that inhibiting PDHK1 shifts Th17 cells toward an iTreg phenotype, since we observed increased *foxp3* expression in Th17 cells treated either with rotenone or the PDHK1 inhibitor, DCA. We further add the critical mechanistic observation that PDHK1 activity is linked to ETC-I function during Th17 differentiation, by altering the cellular distribution of pPDH-E1α, as well as its association with Notch1 and RORγt.

There is a growing body of literature that identifies a Notch1-regulated "switch" between Th17 and iTreg cells (33–37). Our findings are consistent with a function for nuclear Notch1, together with nuclear RORγt, in promoting Th17 differentiation and suggest that defects in ETC-I function alter Notch1, as well as RORγt, nuclear localization leading to reduced cytokine production. How mitochondrial function and protein localization are linked is not entirely clear. In macrophages, Notch1 has been shown to be recruited to the promoters of nitric oxide synthase 2 (*Nos2*) and pyruvate dehydrogenase phosphatase 1 (*Pdp1*) genes to mediate cell activation and mitochondrial glucose oxidation, respectively. PDP1 functions in opposition to PDHK1, suggesting that PDH activation may promote iTreg differentiation. Moreover, Notch1 signaling coordinates both components of OXPHOS: TCA cycle and ETC (25). For instance, one report demonstrated that, in response to Notch1 activation, the mitochondrial proteome is altered, thus impacting both the TCA and ETC in mitochondria (49). Hyperactivation of Notch1 signaling accelerates glycolysis by activating the phosphatidylinositol 3-kinase/AKT pathway, whereas hypoactivation of Notch1 signaling attenuated mitochondrial activity and induced glycolysis. However, only when Notch1 had been hyperactivated could cells switch back to OXPHOS (26). At this stage, then, Notch1 localization to

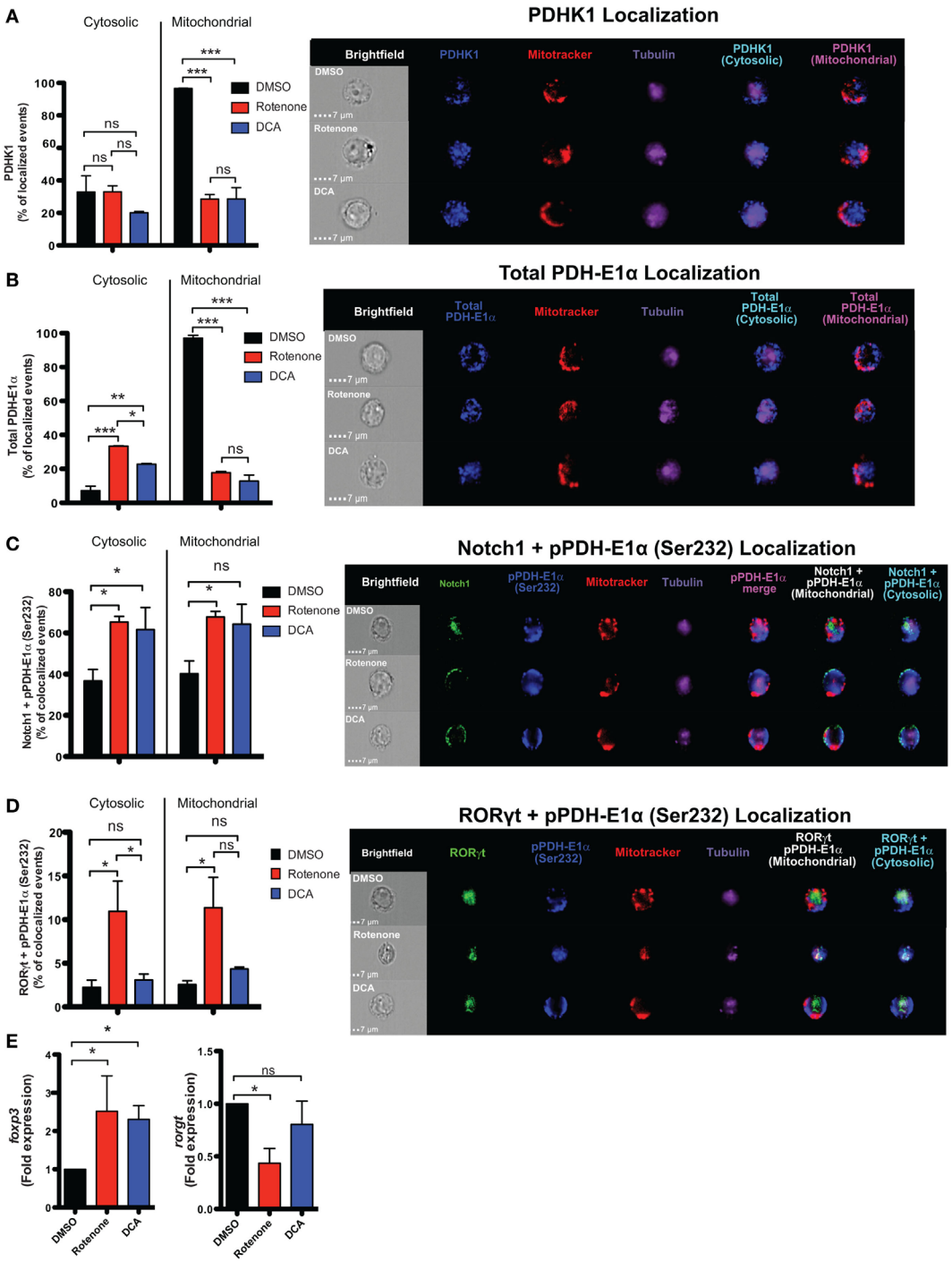

FIGURE 6 | Rotenone treatment inhibits mitochondrial localization of pyruvate dehydrogenase kinase 1 (PDHK1) and promotes RORγt and Notch1 colocalization with its phosphorylated substrate, pPDH-E1α, in Th17-polarized cells. CD4 T cells were treated either with 20 μM rotenone for 2 h or 1 mM dichloroacetate (DCA) (left in the cell suspension throughout) and then stimulated with plate-bound anti-CD3ε plus anti-CD28 for 72 h in the presence of Th17 polarization conditions. After 72 h, samples were split and half of the cells were stained for PDHK1, total PDH-E1α, pPDH-E1α (Ser232), RORγt, and Notch1 to determine their localization in cytosol (Tubulin AF647 staining) and mitochondria (Mitotracker CMXRos). Data were acquired *via* AMNIS ImageStream X Mark Imaging Flow Cytometer. Quantification of percent of cytosolic or mitochondrial **(A)** PDHK1-localizing cells and **(B)** total PDH-E1α-localizing cells along with corresponding representative images are shown. For colocalization, percent of **(C)** Notch1 + pPDH-E1α (Ser232)-colocalizing and **(D)** RORγt + pPDH-E1α (Ser232) Th17-polarized cells for each treatment condition and representative images are shown. Total RNA was collected from the other half of treated cells and qPCR was performed for measuring **(E)** *foxp3* and *rorgt* expressions upon rotenone treatment (DCA treatment was used as a control which triggers *foxp3* expression). Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ calculated using an unpaired, two-tailed Student's *t*-test.

mitochondria may be essential to modulating specific metabolic pathways and, thus, intersect with distinct Th cell differentiation programs.

Our data show that Notch1 can colocalize with RORγt in the nucleus and nuclear localization of RORγt, but not of Foxp3, is abrogated upon rotenone treatment. Although we did not find any difference in Notch1-Foxp3 colocalization in the nucleus, mitochondrial Notch1 localization was significantly increased in rotenone-treated iTregs. These data may support the notion that Notch1 in mitochondria can negatively impact iTreg differentiation and act independently of Foxp3 regulation. We detected negligible amounts of mitochondrial Notch1 or RORγt in Th17 cells differentiated in DMSO or in rotenone, suggesting these proteins are not resident in the mitochondria in Th17 cells. However, a pool of mitochondrial-associated STAT3 has been shown to associate within and enhance the functions of the ETC-I (50). A recent report has identified an IL-6-STAT3-dependent pathway that maintains mitochondrial membrane potential, keeps intracellular Ca^{++} stores high, and is necessary in the late stages of Th17 differentiation (51). It is interesting to speculate that the decreased mitochondrial mass, observed in rotenone-treated Th17 cells, may also impact this pool of STAT3 to further negatively regulate Th17 polarization, although this awaits further exploration.

Cancer cells require intact mitochondrial activity during tumor progression (52), identifying mitochondrial complex I as an attractive therapeutic target. Altogether, we have demonstrated that using rotenone to inhibit electron complex I function decreases CD4 T cell activation and impacts Th17 and iTreg differentiation *in vitro*, through its effects on the cellular distribution of Notch1 and RORγt, as well as on key components of cellular metabolism, PDHK1, and its substrate, PDH-E1α. Our results implicate mitochondrial function as a critical contributing factor in the proper differentiation of Th17 and iTreg cells and may pave the way for further examination of mitochondrial deficiency as a regulator of immune dysfunction, especially in the context of tumor therapy to target mitochondrial metabolism.

AUTHOR CONTRIBUTIONS

EO, HS, VM, GT, and AL acquired and analyzed data. GNT, NY, and LM provided scientific input. LM conceived of the experimental plan and wrote the manuscript together with EO.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01284/full#supplementary-material.

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The Notch Signaling Pathway Is Balancing Type 1 Innate Lymphoid Cell Immune Functions

*Thibaut Perchet[1,2,3], Maxime Petit[1,2,3], Elena-Gaia Banchi[1,2,3], Sylvain Meunier[1,2,3], Ana Cumano[1,2,3] and Rachel Golub[1,2,3]**

[1] Unit for Lymphopoiesis, Department of Immunology, Pasteur Institute, Paris, France, [2] INSERM U1223, Paris, France, [3] Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, Paris, France

The Notch pathway is one of the canonical signaling pathways implicated in the development of various solid tumors. During carcinogenesis, the Notch pathway dysregulation induces tumor expression of Notch receptor ligands participating to escape the immune surveillance. The Notch pathway conditions both the development and the functional regulation of lymphoid subsets. Its importance on T cell subset polarization has been documented contrary to its action on innate lymphoid cells (ILC). We aim to analyze the effect of the Notch pathway on type 1 ILC polarization and functions after disruption of the RBPJk-dependent Notch signaling cascade. Indeed, type 1 ILC comprises conventional NK (cNK) cells and type 1 helper innate lymphoid cells (ILC1) that share Notch-related functional characteristics such as the IFNg secretion downstream of T-bet expression. cNK cells have strong antitumor properties. However, data are controversial concerning ILC1 functions during carcinogenesis with models showing antitumoral capacities and others reporting ILC1 inability to control tumor growth. Using various mouse models of Notch signaling pathway depletion, we analyze the effects of its absence on type 1 ILC differentiation and cytotoxic functions. We also provide clues into its role in the maintenance of immune homeostasis in tissues. We show that modulating the Notch pathway is not only acting on tumor-specific T cell activity but also on ILC immune subset functions. Hence, our study uncovers the intrinsic Notch signaling pathway in ILC1/cNK populations and their response in case of abnormal Notch ligand expression. This study help evaluating the possible side effects mediated by immune cells different from T cells, in case of multivalent forms of the Notch receptor ligand delta 1 treatments. In definitive, it should help determining the best novel combination of therapeutic strategies in case of solid tumors.

Keywords: Notch, innate lymphoid cells, liver, cancer, inflammation, transcription factors, cytotoxicity, molecular biology techniques

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***Correspondence:**
Rachel Golub
rachel.golub@pasteur.fr

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INTRODUCTION

Type 1 innate lymphoid cells (ILC) are defined by the capacity to secrete IFNg and comprise at least two distinct subsets, type 1 helper innate lymphoid cells (ILC1) that are the tissue-resident counterparts of the circulating conventional NK (cNK) cells found in blood and in numerous tissues. ILC1 have been identified in liver, gut, salivary glands, skin, peritoneum, spleen, and uterus (1). cNK and ILC1 both express the receptors NKp46 and NK1.1 that distinguishes them from other ILC subsets.

ILC1 express markers of tissue residency with an immature CD49a$^+$CD49b$^-$ phenotype in the liver. cNK and ILC1s could be discriminated by the identity of T-box transcription factors they expressed. The T-box protein in T cells, T-bet, is encoded by the *Tbx21* gene involved in IFNg production. Another T-box transcription factor eomesodermin (Eomes) shares homology with T-bet. Mature cNK cells are T-bet$^+$ Eomes$^+$ and T-bet upregulation is induced during ILC differentiation in the liver. Studies in Eomes reporter mice showed that despite their immature phenotype, T-bet$^+$ hepatic ILC1 are not precursors of Eomes$^+$ cNK cells (2) and ILC1 and cNK lineages diverge early in ontogeny (3). There is limited information on the mechanisms inducing or repressing T-box transcription factors in different organs. Because *Tbx21* is a known potential target of the Notch canonical pathway, we investigated the role of the Notch signaling pathway on the differentiation and function of ILC1 and cNK cells residing in enterohepatic sites. The Notch pathway is highly conserved and regulates several aspects of development and differentiation (4, 5). Vertebrates express four different Notch receptors (Notch 1–4), which can engage five known ligands (Delta-like 1, 3, 4, Jagged 1, and 2) (6). The co-factor RBP-Jk and mastermind-like (MAML) mediate the signaling cascade of the canonical Notch pathway. Upon recognition of Notch ligands and after serial proteolytic cleavages, the Notch intracellular domain translocates to the nucleus where it binds to the transcription factor CSL/RBP-Jk, recruiting co-activator members of the MAML family, and enabling transcription of target genes (6, 7). Notch receptors are expressed in the hematopoietic cells with a well-known role of Notch1 signaling in regulating T versus B cell fate decisions (8). *In vitro* studies have shown that multipotent progenitors differentiate into T/cNK progenitors *via* the Notch1/Dll1 or Dll4 interaction; however, at later stages, Notch1 favor the T cell potential to the expense of cNK cells (9–11). It has been proposed that the Notch ligand Jagged2 promotes the development of cNK from murine hematopoietic progenitors (12). At later stages, Notch signaling has been implicated in the upregulation of KIR molecules (13) and in human peripheral cNK cells it increases IFNg secretion (14). cNK cells participate to immune surveillance of tumors and viral infection (15, 16). They are important cytotoxic players by the release of granules containing both perforin and granzyme B (GzmB) and by the production of inflammatory cytokines, such as IFNg and TNFa.

There is, however, limited information for a role of Notch pathway in ILC1 development and function. Single-cell transcriptional analyses of hepatic ILC1 and cNK showed that more than half of both cell types express Notch receptors. Moreover, gene expression analysis indicated a possible implication of the Notch signaling pathway on the heterogeneity of these populations. We therefore analyzed ILC1 in mice where the canonical Notch pathway is abrogated in all lymphoid lineages using a conditional knockout of RBP-Jk in cells expressing IL-7Ra, a receptor upregulated at the common lymphoid progenitor stage (11, 17). We found that both cNK and ILC1 are altered in the absence of the Notch signaling pathway. Hepatic and circulating ILC1 from Il7r^{Cre} Rbpj$^{F/F}$ mice showed decreased expression of CD49a and the ratio of cNK versus ILC1 were affected possibly due to a deregulation of proliferation/survival.

Considering that the Notch pathway activates Th1 type responses, we expected that the lack of Notch signaling would reduce inflammatory responses in type 1 ILC. Instead, we found that RBPJ deficiency enhanced the inflammatory and cytotoxic functions of the type 1 ILC subsets present in the enterohepatic region. Notably, we showed that T-bet was inhibited in RBPJ-deficient cells resulting in the upregulation of the complementary Eomes and of an inflammatory gene signature. Finally, we showed that RBPJ deficiency also increased the control of tumor proliferation at the early time-points due to the recruitment of highly inflammatory cNK cells. We conclude that Notch signaling, in type 1 ILC cells, prevents the over-expression of pro-inflammatory cytokines through the regulation of T-bet and Eomes expression.

MATERIALS AND METHODS

Mice

IL7r$^{+/+}$, IL7r$^{Cre/+}$ Rbpj$^{F/+}$, IL7r$^{Cre/+}$ Rbpj$^{F/F}$, Vav$^{Cre/+}$ Rbpj$^{F/+}$, Vav$^{Cre/+}$ Rbpj$^{F/F}$, IL7r$^{Cre/+}$ Notch2$^{F/+}$, and IL7r$^{Cre/+}$ Notch2$^{F/F}$ mice were bred in the animal facilities at Pasteur Institute, Paris. Mice were bred in accordance with Pasteur Institute guidelines in compliance with European animal welfare regulations, and all animal studies were approved by Pasteur Institute Safety Committee in accordance with French and European guidelines.

Cell Preparation

Bone marrow (BM), thymic lobes, spleens, and lamina propria lymphocytes (LPL) were harvested, dissociated, and resuspended in Hanks' balanced salt solution (HBSS) supplemented with 1% fetal calf serum (FCS; Gibco). To isolate LPL, the small bowel was flushed with phosphate-buffered saline (PBS), and the conjunctive tissue and Peyer's patches were carefully removed. The intestine was opened and cut into 1-cm pieces. To eliminate epithelial cells and intraepithelial lymphocytes, these fragments were incubated at 37°C in 50 ml of RPMI 1640 (Gibco) containing 10% FCS and 10 mM Hepes buffer under strong agitation for 30 min, which was followed by vortex treatment for 4 min. For LPL isolation, the remaining fragments were incubated in identical medium to which was added type VIII collagenase (0.5 mg/ml; Sigma-Aldrich) and were shaken for 30 min at 37°C. To complete digestion, the suspension was repeatedly passed through a 10-ml syringe for 5 min and then filtered through a 40-mm cell strainer (BD Biosciences) and collected by centrifugation. The cell pellet was resuspended in 44% Percoll (GE Healthcare), laid over 67% Percoll, and centrifuged at 600 *g* for 20 min at 20°C. Cells at the interface were collected, washed in HBSS containing 1% FCS, and recovered. Livers were harvested, dissociated, and resuspended in RPMI 1640 supplemented with 2% FCS. Cells were collected by centrifugation and resuspended in 44% Percoll. After centrifugation at 600 *g* for 20 min at 20°C, the cell pellet was washed in HBSS containing 1% FCS. Blood and portal vein blood (PVB) were harvested using a 1-ml syringe (BD Plastipak) and laid on Ficoll Paque Plus (GE Healthcare). After centrifugation at 600 *g* for 20 min at 20°C, the cell at the interface were washed in HBSS containing 1% FCS and recovered. Tumors were harvested and then washed with PBS, then separated into different tubes. The

tumors were resuspended in 2 ml of thermolysin (Liberase™, Roche Diagnostics, Mannheim, Germany) at 0.13 U/ml (concentrations as recommended by the manufacturer). Incubations were performed for 30 min at 37°C. Following incubation, the digestate was crushed and passed through a 70-µm filter and washed with RPMI supplemented with 10% FCS. Samples were centrifuged at 370 *g* for 7 min and resuspended in HBSS containing 1% FCS.

Flow Cytometry

Flow cytometry data were acquired with a LSRFortessa flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Tree Star). Dead cells were eliminated by exclusion with propidium iodide. Cells were stained intracellularly after permeabilization and fixation with True Nuclear Transcription Factor Buffer Set (BioLegend). Cells were purified with a FACSAria III (Becton Dickinson) and recovered in tubes or in 96-well quantitative PCR (qPCR) plates for gene expression analysis.

Antibodies

All antibodies were from BD Biosciences, eBioscience, BioLegend, Cell Signaling Technology, or R&D Systems. Antibodies were biotinylated or conjugated to fluorochromes (fluorescein isothiocyanate, phycoerythrin, PECy5, PerCPCy5.5, PECy7, allophycocyanin, Alexa Fluor 647, APCCy7, Pacific Blue, BV421, eFluor450, V500, BV605, BV655, BV700, and BV786) and were specific for the following mouse antigens: Ly76 (TER119), Gr-1 (RB6-8C5), CD3e (145-2C11), CD19 (6D5), NK1.1 (PK136), IL-7Ra (A7R34), CD8 (53-6.7), TCRb (H57-597), TCRd (GL3), CD4 (GK1.5), Thy1.2 (53-2.1), NKp46 (29A1.4), IFNg (XMG1.2), CD27 (LG.3A10), CD45.2 (104), CD49a (HMa1), CD49b (DX5), Eomes (Dan11mag), TNFa (MP6-XT22), PD1 (29F.1A12), CD226 (10E5), Mac1 (M1/70), and GzmB (GB12).

RT-qPCR Analysis

Cells were sorted in Buffer RLT (Qiagen) containing 2-mercaptoethanol (Sigma-Aldrich) and were frozen at −80°C. RNA was obtained with an RNeasy Micro Kit (Qiagen), and complementary DNA (cDNA) was obtained with the PrimeScript RT Reagent Kit (Takara). A 7300 Real-Time PCR System (Applied Biosystems) and TaqMan technology (Applied Biosystems) or SYBR Green Technology (Qiagen) were used for qRT-PCR analysis. A bilateral unpaired Student's *t*-test was used for statistical analysis. The following primers were from SABiosciences: Ifng (Mm_01168134_m1), Eomes (Mm_01351984_m1), Tnfa (Mm_00443258_m1), GzmB (Mm_00442837_m1), Hprt (Mm_00446968_m1), and Actb (Mm_02619580_g1).

Tumor Injection

Cancer Hepa 1.6 cells were cultured in Opti-MEM with GlutaMAX (Gibco) containing 10% FCS (Gibco), 1% penicillin–streptomycin (Gibco), and 60 mM 2-mercaptoethanol (Sigma-Aldrich) and were maintained in a 37°C incubator (Thermo Scientific) with 5% CO_2. Cells were harvested, washed, and resuspended in PBS. 3×10^6 cells were injected subcutaneously in 150 µl of PBS. Mice were monitored every day and tumor growth was measured every 2/3 days.

T Cell Transfer

CD3 cells were isolated using magnetic microbead (Miltneyi Biotech, Bergisch Gladbach, Germany) from spleen of B6 wild-type mice. 3×10^6 purified CD3 positive cells were injected in 150 µl of PBS in host mice 3 days before Hepa1.6 injection.

Cytotoxicity Assay

Freshly isolated splenic cNK cells (Lin^- $CD45^+$ $CD4^-$ $NKp46^+$ $NK1.1^+$) were sorted to high purity (>98%) and used as effectors. Killing of the cNK-sensitive YAC-1 (European Collection of Cell Cultures) target cells was assessed using a fixable viability dye. Percentage specific of killing was calculated as: 100 × (experimental release − spontaneous release)/(total release − spontaneous release).

Single Cell Multiplex RT-qPCR

Cells were sorted in 96-well qPCR plates in 9 µl of a CellsDirect One-Step quantitative RT-PCR Kit (Life Technologies), containing mixtures of diluted primers (0.05× final concentration). Preamplified cDNA was obtained after reverse transcription (15 min at 40°C, 15 min at 50°C and 15 min at 60°C), and preamplification (22 cycles: 15 s at 95°C and 4 min at 60°C), and diluted 1:5 in TE pH8 Buffer (Ambion). Sample mix was as follows: diluted cDNA (2.9 µl), Sample Loading Reagent (0.29 µl; Fluidigm), TaqMan Universal PCR Master Mix (3.3 µl; Applied Biosystem), or Solaris quantitative PCR Low ROX Master Mix (3.3 µl; GE Dharmacon). The assay mix was as follows: Assay Loading Reagent (2.5 µl; Fluidigm) and TaqMan (2.5 µl; Applied Biosystem). A 48.48 dynamic array integrated fluidic circuit (IFC; Fluidigm) was primed with control line fluid, and the chip was loaded with assays (TaqMan) and samples using an HX IFC controller (Fluidigm). The experiments were run on a Biomark (Fluidigm) for amplification and detection (2 min at 50°C, 10 min for TaqMan reagents or 15 min for Solaris reagents at 95°C, 40 cycles: 15 s at 95°C and 60 s at 60°C).

Bioinformatic Analyses

For visualization, the dimensionality of the datasets was further reduced using the "Barnes-hut" approximate version of t-SNE. This was implemented using the Rtsne function from the Rtsne R package using 800 iterations and a perplexity setting that varied from 10 to 30 depending on the size of the dataset. PhenoGraph takes as input a matrix of N single-cell measurements and partitions them into subpopulations by clustering a graph that represents their phenotypic similarity. PhenoGraph builds this graph in two steps. First, it finds the k nearest neighbors for each cell (using Euclidean distance), resulting in N sets of k-neighborhoods. Second, it operates on these sets to build a weighted graph such that the weight between nodes scales with the number of neighbors they share. The Louvain community detection method is then used to find a partition of the graph that maximizes modularity. Given a dataset of N d-dimensional vectors, M distinct classes, and a vector providing the class labels for the first L samples, the PhenoGraph classifier assigns labels to the remaining N_L unlabeled vectors. First, a graph is constructed as described above. The classification problem then corresponds to the probability that a random walk originating

at unlabeled node x will first reach a labeled node from each of the M classes. This defines an M-dimensional probability distribution for each node x that records its affinity for each class.

Statistical Analysis

Statistical analysis was performed with the Student's *t*-test or two-way analysis of variance. The analysis was performed using Prism Software (GraphPad). Statistical significance is represented as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Four Distinct Populations of Type 1 ILC Are Defined in the Liver

Mutually exclusive expression of CD49a and CD49b separates the NKp46$^+$ NK1.1$^+$ population into ILC1 and cNK cells in the murine liver (**Figure 1A**). Purified single ILC1 and cNK cells were subjected to multiplex transcriptional analysis, as described (18). We analyzed 48 transcripts known or supposed to be expressed in type 1 ILC with some of them also being possible Notch pathway

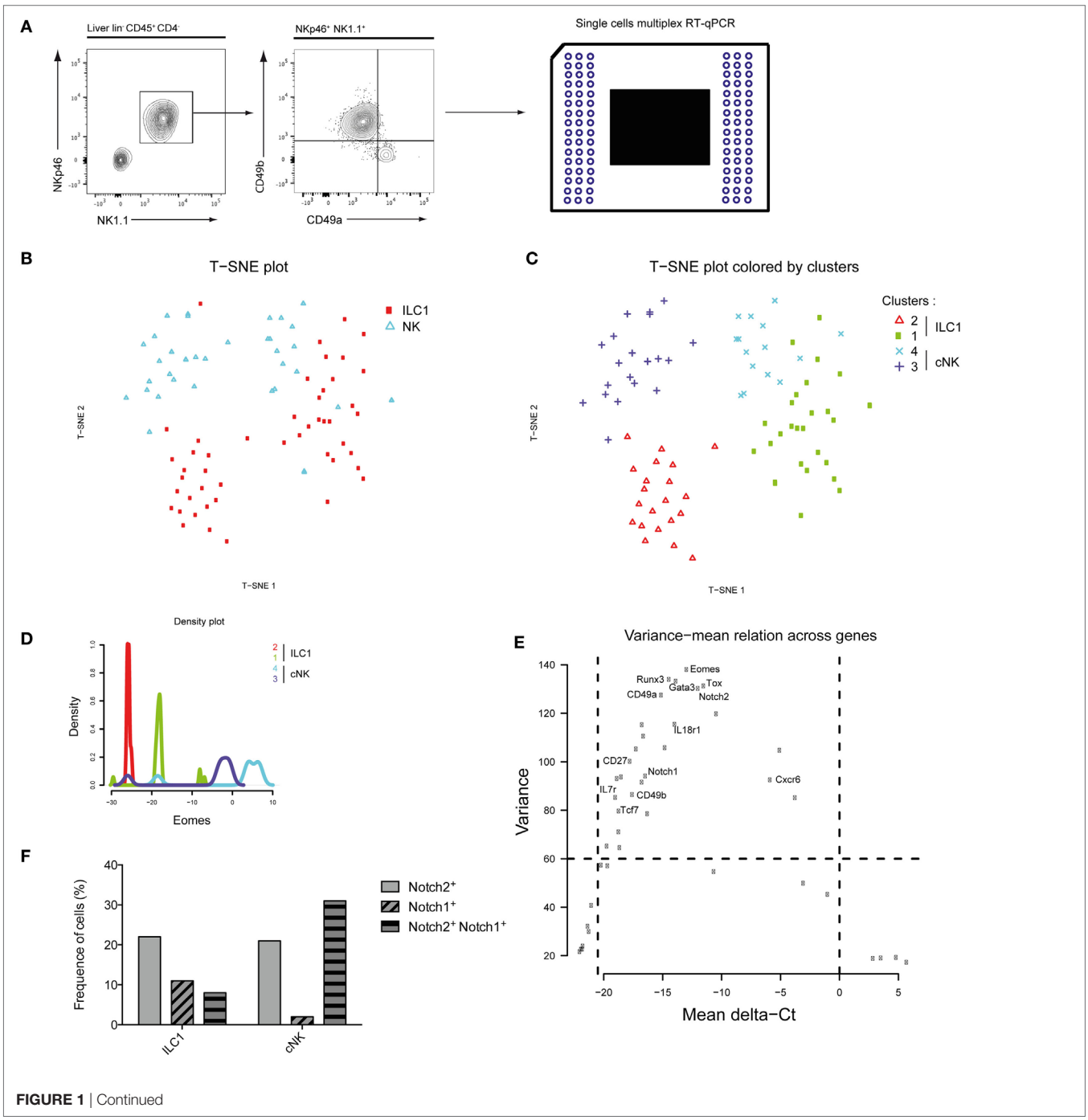


FIGURE 1 | Continued

FIGURE 1 | Group 1 innate lymphoid cell is composed of four distinct populations in the liver at homeostasis. **(A)** Single cells were sorted for transcriptomic analyses using the Biomark technology. Hepatic lineage negative CD45-positive cells (lin$^-$ CD45$^+$) were separated into type 1 helper innate lymphoid cells (ILC1) (CD4$^-$ NKp46$^+$ NK1.1$^+$ CD49a$^+$ CD49b$^-$) and conventional NK (cNK) cells (CD4$^-$ NKp46$^+$ NK1.1$^+$ CD49a$^-$ CD49b$^+$). **(B)** Application of t-SNE to single cells using 48 genes expression separates 48 ILC1 and 48 cNK into two distinct subsets. **(C)** Phenograph algorithm clustering performed and plotted on t-SNE graph, showing four different clusters among hepatic ILC1 and cNK. **(D)** Density plot of *Eomes* gene expression show four distinct types of expression among phenograph clusters of hepatic ILC1 and cNK. **(E)** The variance mean of delta Ct across expressed genes allows the selection of genes that are differentially expressed (variance > 60) with a significant expression (dCt from −20.5 to 0). **(F)** Frequency of hepatic ILC1 and cNK expressing Notch1 and/or Notch2. **(G)** Heatmap with unsupervised hierarchical clusters of gene selected in panel **(D)**.

targets. A t-SNE analysis of the data set indicated that both ILC1 and cNK cells were subdivided into two populations (**Figure 1B**), populations 1 and 2 for ILC1 and populations 3 and 4 for cNK (**Figure 1C**). Surprisingly, population 1 of ILC1 clustered closer to the cNK population 4 rather than to its ILC1 counterpart. Inversely, population 3 of cNK clustered closer to ILC1 population 2 (**Figure 1C**). We ascertained that cNK express high levels of Eomes transcripts and that ILC1 subsets had low expression levels (**Figure 1D**). Unexpectedly, Eomes gene expression is found as highly variable and different levels are observed for these four populations (**Figure 1E**). We then restricted our analysis to the genes that were the most variable (>60 of variance) and significantly expressed (mean values selection between 0 and −20) (**Figure 1E**). With *Eomes, Gata3, Tox, Notch2, Runx3*, and *Itga1* were among the most variable transcripts (**Figure 1E**). Notch receptors were expressed more frequently in cNK cells than in ILC1 (**Figure 1F**). Notch2$^+$ cells were more frequent than Notch1$^+$ cells (**Figure 1F**) and cells expressing both Notch1 and Notch2 transcripts represented 9% of ILC1 and 30% of cNK. It is interesting to notice that population 1 of Eomes^{lo} ILC1 was enriched in Notch expressing cells compared with Eomes$^-$ ILC1 (population 2). An unsupervised hierarchical cluster was constructed based on this restricted list of genes. The segregation of ILC1 and cNK into four different subsets was consistent with the t-SNE analysis (**Figure 1G**). The genes could be separated into two cluster signatures. The first comprises genes directly related to the Notch pathway (*Notch1, Gata3, Ahr, Tcf7, IL7ra*, and *c-myc*) and genes that define the identity of ILC1 versus cNK (CD49a, *Cd27*, and *Il7ra*). In the second cluster, *Notch2* together with *Cxcr6, Eomes*, CD49b, and *Il18r1* define the signature 2 and also cNK identity. Correlation heatmaps confirmed that most of genes among each signature are correlated (Figure S1A in Supplementary Material). A good correlation is shown between most genes of signature 1 with a strong correlated core for Tcf7, CD27, Rora, Klr5, Ahr, and Il7r (Figures S1A,B in Supplementary Material). A good correlation is described between Eomes, IL18r1, Tsc22d3, CD49b, and Tle4 for the signature 2 (Figure S1C in Supplementary Material). The data suggest that the Notch signaling pathway could play a role in the specification of the subsets of ILC1 and cNK in the liver.

RBPJ-Deficient Type 1 ILC Have Different Characteristics

We analyzed type 1 ILC (Lin$^-$ CD45$^+$ NK1.1$^+$ NKp46$^+$ cells) in Il7r^{Cre} Rbpj$^{F/F}$ mice to define the role of the canonical Notch signaling pathway in the maturation of this population (11). CD49a and CD49b expression that distinguishes hepatic ILC1 from cNK cells was tested in the spleen, BM, and thymus of Notch-competent and Notch-deficient mice (**Figure 2A**). In Notch-deficient mice, CD49a levels were decreased in most ILC1 while CD49b levels remained unchanged. Interestingly, a CD49a^{lo} CD49b$^-$ population appears in the circulation especially in the PVB (**Figure 2A**). Notch-deficient hepatic ILC1 showed decreased levels of CD49a (**Figure 2B**) and increased frequencies and absolute numbers (**Figure 2C**).

We then analyzed mice where the Notch signaling pathway was defective in hematopoietic cells (Vav^{Cre} Rbpj$^{F/F}$) or downstream of Notch2 (IL7r^{Cre} Notch2$^{F/F}$) (Figure S2A in Supplementary Material). In Notch2-deficient liver ILC (IL7r^{Cre} Notch2$^{F/F}$), CD49a and CD49b expression on type 1 ILC was unchanged contrasting with the decreased levels of CD49a in RBPJ-deficient ILC irrespective of whether deletion of RBPJ occurred in IL7r or in Vav-expressing cells (Figure S2B in Supplementary Material). Interestingly, while RBPJ deletion resulted in an increased frequency of ILC1, the Notch2 deletion induced an opposite effect with a decreased frequency of ILC1 suggesting non-redundant roles of Notch1 and Notch2 (Figure S2C in Supplementary Material) that was not due to differences in proliferation (Figure S3 in Supplementary Material). The analyses of Thy1 expression showed an increase from 10 to 50% in Notch-deficient cNK and to virtually 100% in Notch-deficient ILC1. Mac-1 expression among cNK showed a significant decrease (**Figures 2D,E**).

We have previously shown that type 1 ILC (Lin$^-$ NKp46$^+$ NK1.1$^+$ cells) in the intestinal lamina propria (LP) were not affected by RBPJ deletion (17). However, the changes in the expression levels of CD49a and CD49b described above led us to reevaluate the representation of the subsets of Notch-deficient type 1 ILC1 in the LP. In LP, CD49a and CD49b could not strictly separate ILC1 from cNK cells as numerous cells express both markers (**Figure 3A**). We designed a panel of surface markers that allowed the enrichment of NKp46$^+$ NK1.1$^+$ cells into Eomes$^-$ versus Eomes$^+$ subsets. Cells separated as CD226$^+$ CD49b$^-$ Mac1$^-$ are enriched for Eomes$^-$ ILC1 and CD226$^-$ CD49b$^+$ Mac1$^+$ cells are enriched into Eomes$^+$ cNK cells (**Figure 3A**). We found that Notch-deficient CD226$^+$ CD49b$^-$ Mac1$^-$ ILC1 comprises 15% of Eomes$^+$ cells whereas the enriched cNK subset is exclusively composed of Eomes$^+$ cells (**Figures 3A,B**). Similar to liver type 1 ILC all subsets in the LP have increased Thy1 levels (**Figure 3B**). Consistent with our previous observations, the absolute numbers of ILC1/cNK remained unchanged in RBPJ-deficient compared with control mice (**Figure 3C**). Altogether, these results indicated that the Notch signaling pathway was modulating several properties of the type 1 ILC including expression of transcription factors, integrins, and the capacity to circulate.

Type 1 ILC Functions Are Altered in RBPJ-Deficient Cells

We then assessed the functional properties of RBPJ-deficient hepatic ILC1 and cNK cells. We found that, after activation with PMA-ionomycin, TNFa and IFNg secretion by RBPJ-deficient

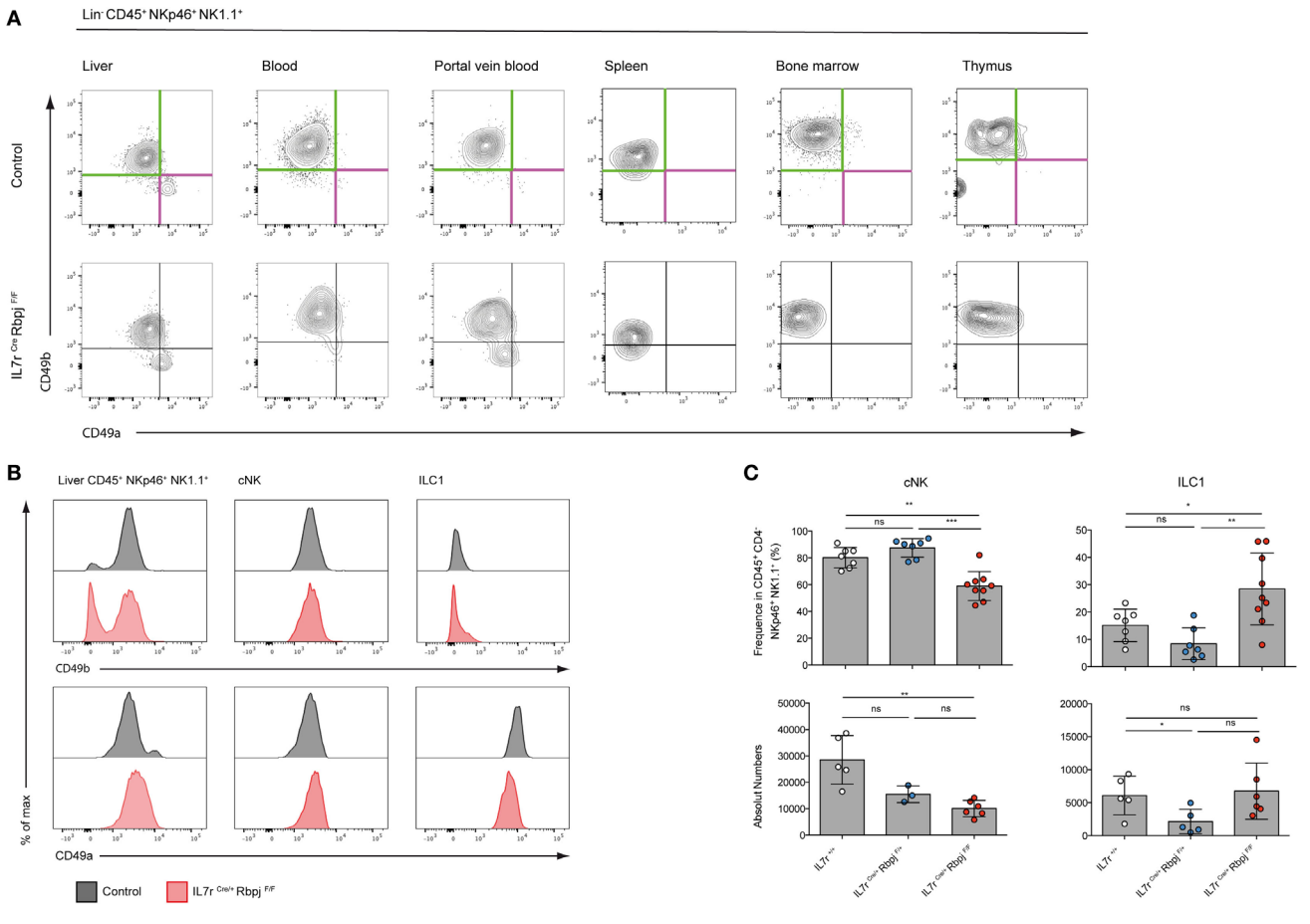


FIGURE 2 | Continued

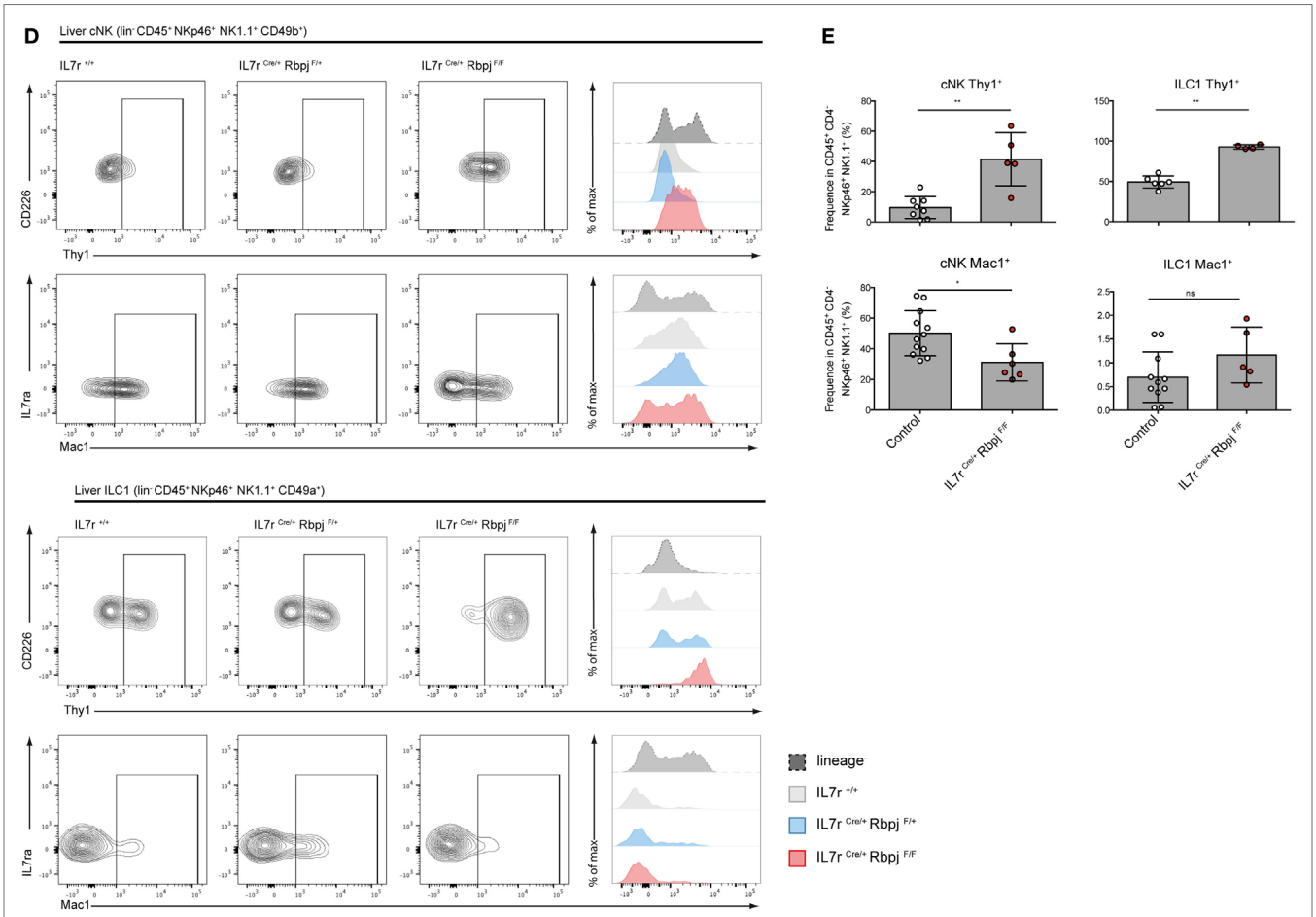


FIGURE 2 | Altered phenotype of NKp46$^+$ NK1.1$^+$ cells in IL7r^{Cre} Rbpj$^{F/F}$ mice. **(A)** Expression of CD49a (purple) and CD49b (green) in lin$^-$ CD45$^+$ NKp46$^+$ NK1.1$^+$ cells from the liver, blood, portal vein blood, spleen, bone marrow, and thymus of control IL7r^{Cre} Rbpj$^{F/+}$ mice (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ mice (bottom panel). **(B)** Surface expression of CD49b (top panel) and CD49a (bottom panel) on total liver lin$^-$ CD45$^+$ NKp46$^+$ NK1.1$^+$ cells, hepatic conventional NK (cNK) and type 1 helper innate lymphoid cells (ILC1) in control IL7r^{Cre} Rbpj$^{F/+}$ mice (gray) and IL7r^{Cre} Rbpj$^{F/F}$ (red). **(C)** Frequency and absolute numbers of hepatic cNK and ILC1 in IL7r$^{+/+}$ (white), IL7r^{Cre} Rbpj$^{F/+}$ (blue), and IL7r^{Cre} Rbpj$^{F/F}$ (red) mice. **(D)** Surface expression of Thy1 and Mac1 on hepatic cNK (top panel) and ILC1 (bottom panel) in IL7r$^{+/+}$ (gray), IL7r^{Cre} Rbpj$^{F/+}$ (blue), and IL7r^{Cre} Rbpj$^{F/F}$ (red) mice. Lineage-negative cells were used as control for histograms (dashed gray). **(E)** Frequency of hepatic cNK and ILC1 expression of Thy1 (top panel) and Mac1 (bottom panel) in control IL7r^{Cre} Rbpj$^{F/+}$ mice (white) and IL7r^{Cre} Rbpj$^{F/F}$ (red).

ILC1 and cNK cells were more strongly increased compared with control cells (**Figure 4A**). TNFa was also more expressed by ILC1 than cNK, whereas IFNg was produced by most hepatic ILC1 and cNK, after Notch depletion. GzmB production by ILC1 remained unchanged while it was produced by few cNK Mac1$^+$, in absence of the Notch signaling pathway (**Figure 4A**). Similar to those from LP, hepatic RBPJ-deficient ILC1 comprise a fraction of Eomes$^+$ cells, whereas a subset of Eomes$^-$ Mac1$^-$CD49b$^+$ cells becomes more prominent among cNK cells. These differences in Eomes expression were also apparent in qRT-PCR (**Figure 4B**). Overall, the mRNA expression for *Tnfa, Gzmb*, and *Ifng* genes confirmed the increase of protein levels and high production of IFNg (**Figure 4B**). Similar experiments done in hepatic ILr7^{Cre} Notch2 $^{F/F}$ mice showed consistent increase of TNFa and IFNg production by Notch 2-deficient type 1 ILC (Figure S4 in Supplementary Material). RBPJ-deficient splenic NKp46$^+$NK1.1$^+$ cells showed significantly increased lytic abilities on YAC-1 mouse lymphoma cells (**Figure 4C**) that correlated with an increase frequency of cells capable to produce TNFa and IFNg (**Figure 4D**). To assess the *in vivo* functions of Notch-deficient type 1 ILC in inflammatory conditions, we used a model of liver damage with inflammation, immune infiltration, and fibrosis (19) induced by methionine-choline deficient (MCD) diet. Under MCD diet, RBPJ-deficient mice showed no differences in frequency of TNFa$^+$ and IFNg$^+$ cells (**Figure 4E**), in weight loss, and in the ratio of liver size versus body weight (**Figure 4F**). The levels of the circulating transaminase aspartate aminotransferase were also not different after 24 days of MCD diet (**Figure 4F**).

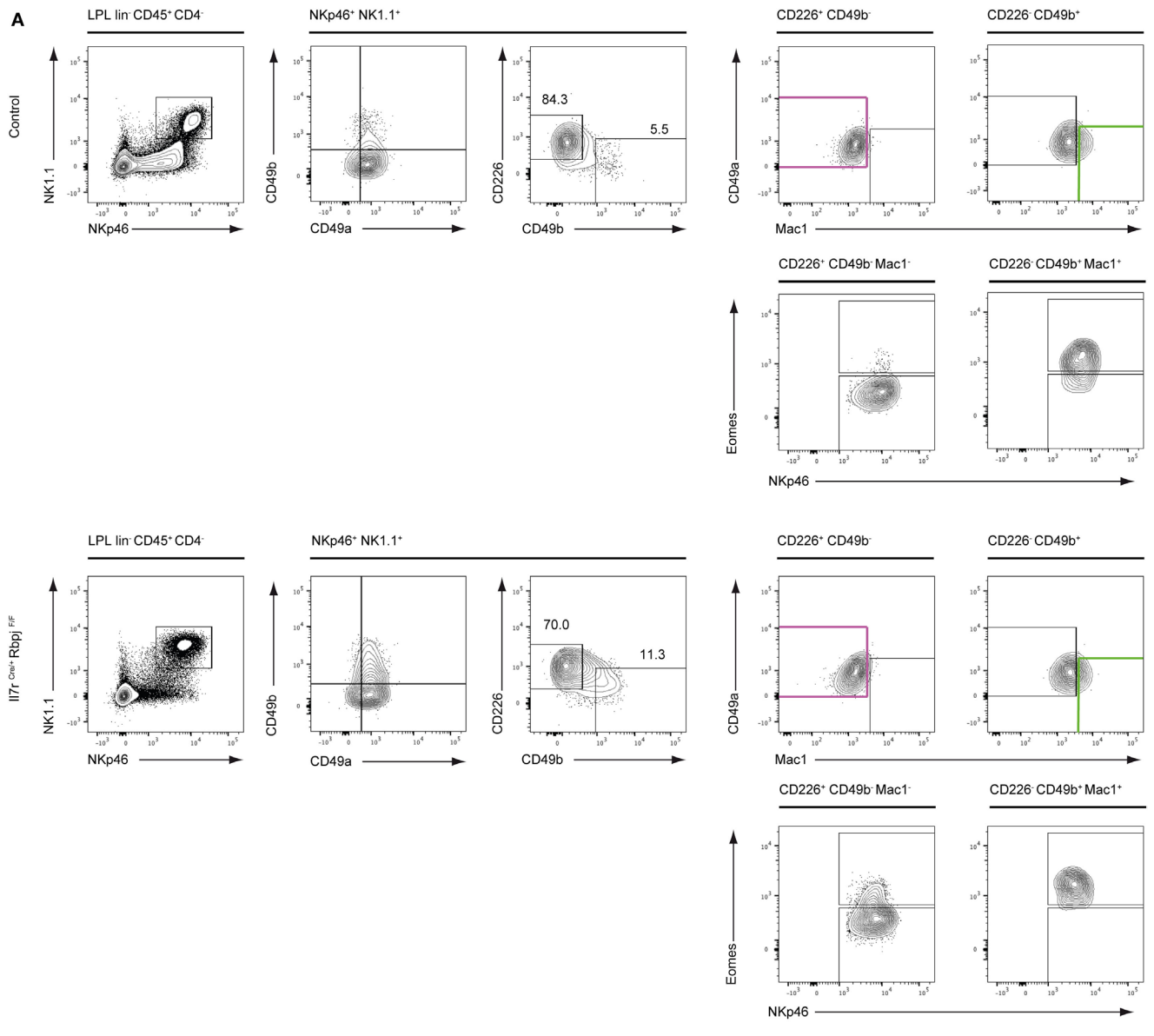
Taken together, these experiments indicated that RBPJ-deficient type 1 ILC had increased levels of inflammatory cytokines and increased cytotoxic activity that are not modified by a liver inflammatory inducing diet.

ILC1 and cNK Have Variations in Gene Expression After Abrogation of the Notch Signaling Pathway

To understand the role of Notch signaling pathway in ILC1 and cNK cells, we performed multiplex quantitative transcriptional analysis of 41 immune genes in these different populations from various organs. We used small numbers of cells (25 cells per subset) to reduce the averaging generated by population-level studies. We sorted the type 1 ILC populations of Notch-competent (Ctrl) and -deficient (Flox) mice in distinct organs according to the strategy outlined in **Figure 5A**.

We only analyzed samples expressing all three "housekeeping" genes and did an unsupervised hierarchical clustering analysis of the transcriptional profiles from 79 samples (**Figure 5B**). The population identity is indicated in the first line with a color code for cNK, ILC1 versus BM subsets of preNKP, iNK, and mNK cells. The genotype of the subset analyzed is indicated in the second line with a color code for Notch-competent (Ctrl-Blue) and Notch-deficient (Flox-Red) subsets and the third line indicates the tissue of origin.

The separation between the RBPJ-competent and -deficient samples was evident in most samples. Only, some splenic samples, exclusively composed of mature cNK cells, showed similar distribution of RBPJ-competent and -deficient subsets and clustered together with RBPJ-competent blood and RBPJ-deficient liver cNK samples. This indicates that RBPJ-deficient cNK cells from liver resemble cNK splenic subsets, insensitive to Notch inactivation. It also suggests that the canonical Notch pathway maintains an identity of cNK cells in liver, LP, mesenteric lymph



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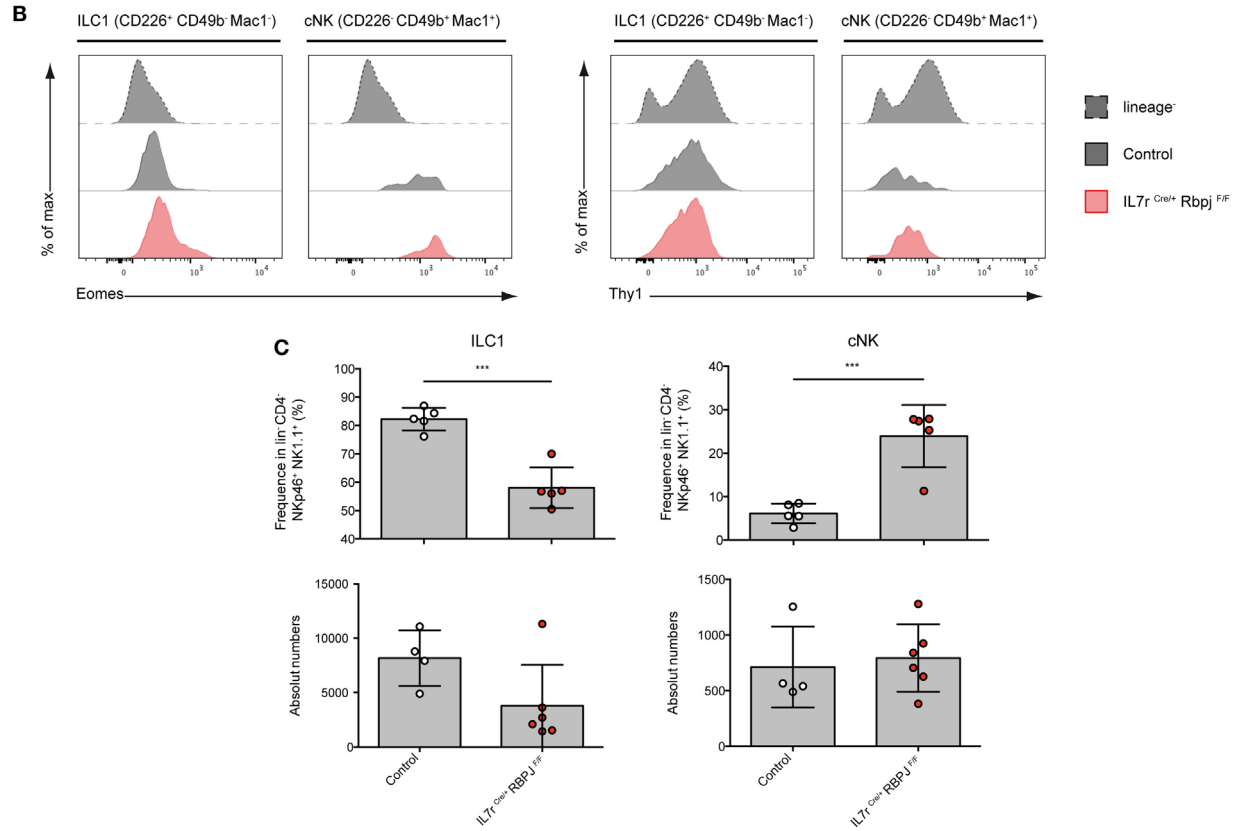


FIGURE 3 | Conventional NK (cNK) cells are increased in the lamina propria lymphocytes (LPL) of IL7r^{Cre} Rbpj$^{F/F}$ mice. **(A)** Flow cytometry of control IL7r^{Cre} Rbpj$^{F/+}$ mice (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ LPL (bottom panel). Repartition of type 1 helper innate lymphoid cells (ILC1) (lin$^-$ CD45$^+$ CD4$^-$ NKp46$^+$ NK1.1$^+$ CD226$^+$ CD49b$^-$ CD49a$^+$ Mac1$^-$, in purple) and NK (lin$^-$ CD45$^+$ CD4$^-$ NKp46$^+$ NK1.1$^+$ CD49b$^+$ CD226$^-$ CD49a$^{-/+}$ Mac1$^+$, in green) in lamina propria (LP). **(B)** Eomesodermin (Eomes) and Thy1 expression in LP ILC1 and cNK in control IL7r^{Cre} Rbpj$^{F/+}$ mice (gray) and IL7r^{Cre} Rbpj$^{F/F}$ (red). Lineage-negative cells were used as control for expression (dashed gray). **(C)** Frequency and absolute numbers of LP ILC1 and cNK in control IL7r^{Cre} Rbpj$^{F/+}$ mice (white) and IL7r^{Cre} Rbpj$^{F/F}$ (red).

node (mLN), and PVB different from that found in the spleen and circulation.

Two subsets of BM iNK clustered with their Notch-deficient counterparts. Because all type 1 ILC and their precursors express *Tcf7* and these two samples express low levels of *Tcf7* and *Cd27*, we concluded that they contained few NK precursors. We decided not to eliminate them from the analysis, as they did not alter its global architecture of the hierarchy.

As discussed above, while surface expression of CD49a and CD49b separated hepatic ILC1 from cNK their separation by transcriptional profiling is less stringent. Indeed, even if most ILC1 subsets clustered together and separated from cNK, a few samples of hepatic ILC1 are interspersed with cNK cells. Notch-competent hepatic ILC1 subsets clustered with intestinal ILC1 and BM precursors (preNKp + iNK), whereas Notch-deficient hepatic ILC1 cluster together and were in the vicinity of mLN and intestinal LP cNK populations.

Our analysis allowed the separation of genes that varied with the activity of the Notch pathway. Genes in cluster 2 were upregulated and conversely genes in cluster 3 were dowregulated, in most Notch-deficient samples. We observe that interfering with the canonical Notch pathway in cNK cells of the enterohepatic axis led to the upregulation of *Eomes, Ly6c, Sell, Il18r1, Notch2, Tcf7, Cx3cr1, Cir1*, and *Cxcr6*. Nearly all were also upregulated in ILC1 subsets with the exception for *Sell* that is never found in this population. Despite being generally increased, *IL18r1* and *Tcf7* were undetectable in few ILC1 Notch-deficient samples illustrating a variable expression in this subset.

A group of genes related to ILC1 signature (*Il21r*, *Lnpp4b*, and *Tnfsf10*) were also upregulated in most Notch-deficient ILC1. *Itga1* was maintained in most hepatic RBPJ-deficient ILC1, although the BM and LPL cNK subsets silenced *Itga1* after RBPJ depletion. *Tnfsf10* expression was also downregulated after RBPJ depletion in cNK subsets. Other genes such as *IL12rb1*, *IL2ra*, and *Notch1* displayed increased expression after Notch depletion.

With the exception of *Tcf7* that was upregulated, most other known direct targets of the canonical Notch pathway comprising *Dtx1, Dtx3, Dtx3l, Zbtb16, Bcl2, Bik*, and *Tbx21* were silenced in Notch-deficient cells.

Genes implicated in apoptosis (*Bcl2l1*, *Crebbp*, and *Bcl2*) were down-modulated, whereas *Bcl2l11* showed increased expression in Notch-deficient cells.

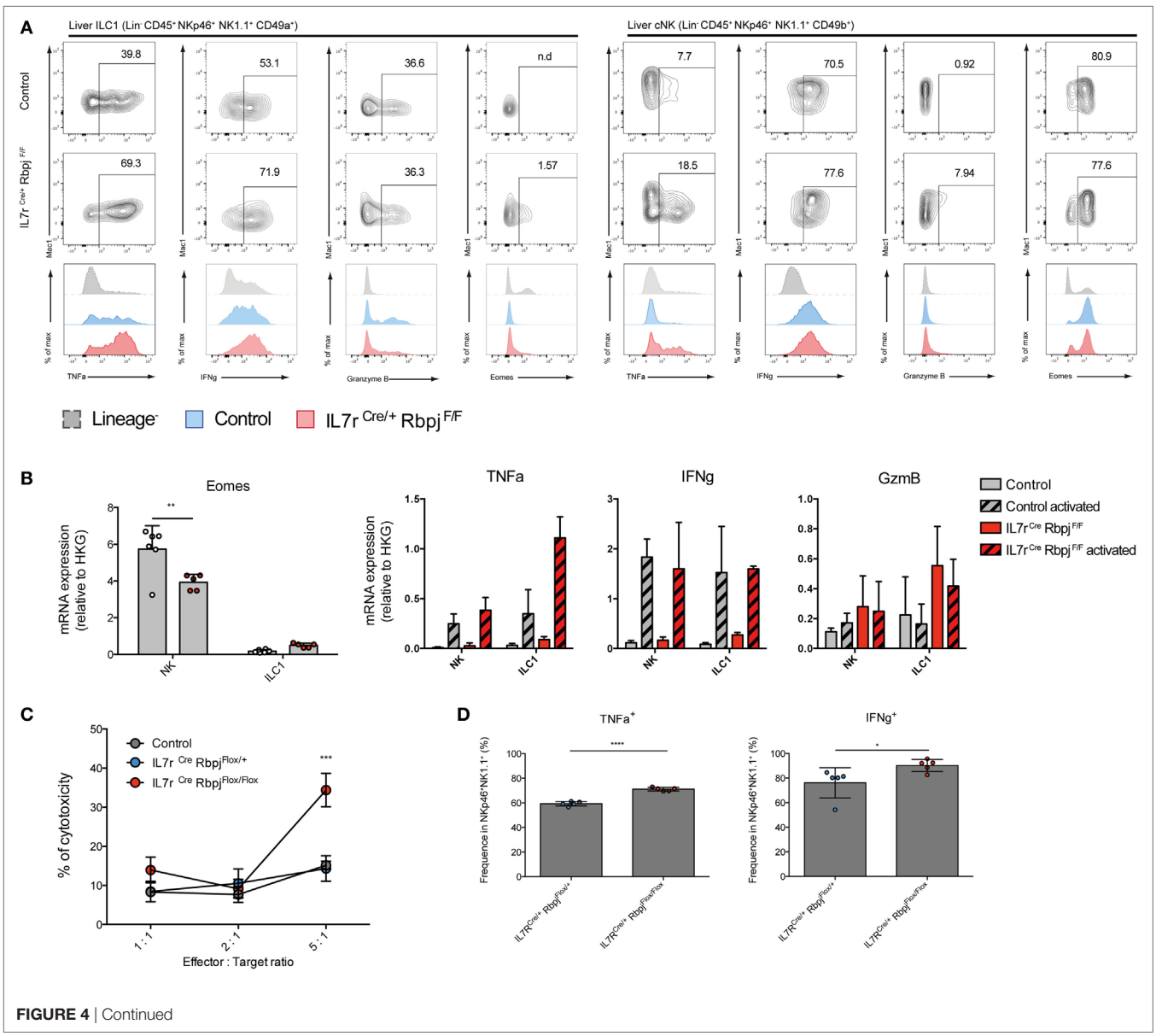
Maml2, a co-activator of the Notch pathway, is decreased, whereas *Cir* a RBPJ co-repressor is upregulated, in RBPJ-deficient

cells. Splenic and circulating cNK subsets that were scattered within Notch-competent and -deficient genotypes did not express *Dtx1, Dtx3, Dtx3l, Crebbp, Bcl2, Zbtb16*, and *Tbx21*, thus appearing Notch independent. These subsets also did not express *Il21r, Tnf, Tnfsf10, Lnpp4b*, and *Tgfr2*.

To better visualize the genes that are co-modified by inactivation of the Notch pathway, we built a heatmap (Figure S5 in Supplementary Material) that clusters together genes that vary in a similar manner comparing RBPJ-proficient and -deficient cells. Gene enrichment analysis of the clusters thus obtained allowed identifying some hallmark for different pathways. *Il2ra*, *Eomes*, *IL18r1*, *Gzmb*, and *Sell* that are co-regulated belong to the inflammatory response genes and to those that are stimulated by Stat5 in response to IL2 stimulation. It is interesting to notice that these genes are related to *Notch2*, *Tcf7*, *Ly6c*, and the chemokine receptors *Cx3cr1* and *Cxcr6*.

A few genes at the bottom of the heatmap (*Tnfsf10, Bcl2*, and *Bcl2l1*) (Figure S5 in Supplementary Material) are also associated to the Stat5/IL2 pathway. Moreover, they correlated with genes implicated in the Notch signaling (*Notch1, Maml2, Crebbp, Dtx3*, and *Dtx3l*) and with Notch target genes (*Tbx21, Zbtb16*). *Itga2* correlated with *Notch1, Dtx3, Dtx3l*, and *Tbx21*, whereas *Itga1* correlated to *Dtx1*, *IL21r*, and *Ctbp2*.

We have previously observed after stimulation of RBPJ-deficient ILC1 subsets, a marked upregulation of *Tnfa* transcripts with similar levels for *Infa* and *Gzmb* (**Figure 4B**). However, in homeostatic conditions, *Tnfa, Ifng*, and *Gm-csf* transcripts were decreased in opposition to an increase for those coding *Gzmb*. Finally, we found that genes differentially regulated by the Notch pathway in type 1 ILC had a highly conserved binding site motif for NFAT and FOXO4. This suggests interactions between NFAT/FOXO4 and the Notch signaling pathway in regulating immune processes in these cells.



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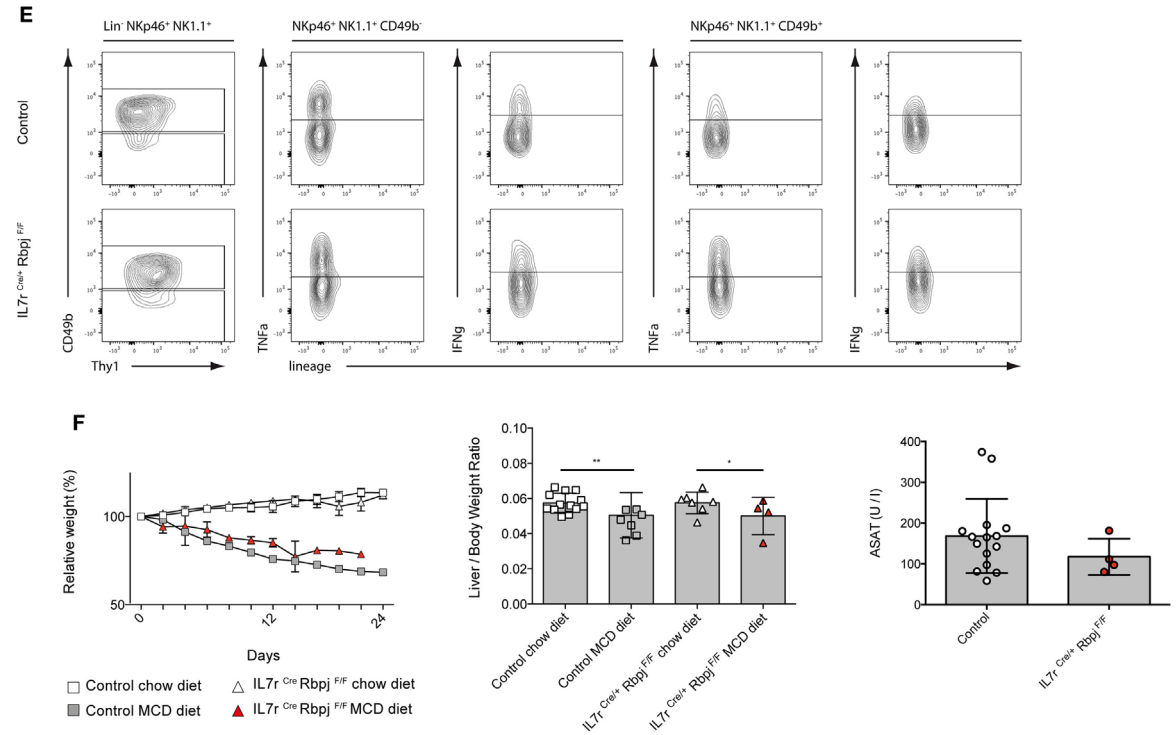

FIGURE 4 | Altered cytokine profile of NKp46$^+$ NK1.1$^+$ cells in IL7r^{Cre} Rbpj$^{F/F}$ mice. **(A)** Expression of TNFa, IFNg, granzyme B, and eomesodermin (Eomes) in hepatic type 1 helper innate lymphoid cells (ILC1) (left panel) and conventional NK (cNK) (right panel) of control IL7r^{Cre} Rbpj$^{F/+}$ mice (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ mice (middle panel). Levels of expression were compared (bottom panel) between control IL7r^{Cre} Rbpj$^{F/+}$ mice (blue) and IL7r^{Cre} Rbpj$^{F/F}$ mice (red). Lineage-negative cells were used as control for surface expression (dashed gray). **(B)** Expression of *Eomes*, *Tnfa*, *Ifng*, and *Gzmb* mRNA in control IL7r^{Cre} Rbpj$^{F/+}$ mice (gray) and IL7r^{Cre} Rbpj$^{F/F}$ mice (red). For *Tnfa*, *Ifng*, and *Gzmb*, mRNA expression was also tested on activated cells (dashed histograms). **(C)** Splenic cNK cells killing capacity in control C57BL/6J (gray), IL7r^{Cre} Rbpj$^{F/+}$ (blue), and IL7r^{Cre} Rbpj$^{F/F}$ (red) mice. **(D)** Frequency of cNK expressing TNFa and IFNg during cytotoxicity assay. **(E)** Cytokine profile of hepatic ILC1 and NK cells in control IL7r^{Cre} Rbpj$^{F/+}$ (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ mice (bottom panel) after methionine-choline deficient (MCD) diet. **(F)** Relative weight and liver/body weight ratio in control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/+}$ mice (square) after chow (white) and MCD diet (gray) and in IL7r^{Cre} Rbpj$^{F/F}$ mice (triangles) after chow (white) and MCD diet (red). Aspartate aminotransferase (ASAT) levels of control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/+}$ (white) and IL7r^{Cre} Rbpj$^{F/F}$ mice (red) after MCD diet.

RBPJ Deficiency Increases Type 1 ILC Control at the Initial Stages of Hepatic Tumor Development

To assess the effect of RBPJ deficiency on hepatic type 1 ILC function, we chose a model of hepatocellular carcinoma by injection a Hepa1–6 mouse liver cancer cell line. It was shown that cNK and T cells are important in the control of the tumors *via* IFNg and lytic granules (20). Three weeks after subcutaneous injection of Hepa1–6 cells, the area of the tumor was significantly increased in RBPJ-deficient mice compared with control mice (**Figure 6A**). Moreover, at 21 days post-transplantation, RBPJ-deficient mice showed no sign of tumor rejection while 50% of control mice were tumor free (**Figure 6B**). The analysis of the tumor-infiltrating cells indicated that CD49a$^+$ CD49b$^+$ cNK subset was only found in RBPJ-deficient animals (**Figure 6C**) that were more efficient than the conventional cNK subset in secreting GzmB and TNFa (**Figure 6C**). However, since RBPJ-deficient animals contain defective T cell subsets, they are not able to efficiently eliminate the tumors despite more cytotoxic cNK populations. Therefore, the tumor area difference between RBPJ-competent and -deficient mice starts around day 14 post-injection (**Figure 6A**). Consistent with a role of T cells in tumor rejection, we found infiltrated CD4$^+$ and CD8$^+$ T cells 14 days after injection in C57Bl6 mice (Figure S6 in Supplementary Material). T cells transferred resulted in the control of RBPJ-deficient mice tumor growth similar to that in Notch-competent mice (**Figures 6D,E**). Tumor-infiltrating cells were analyzed revealing a lower frequency of T cells and conversely a higher frequency of type 1 ILC in RBPJ-deficient mice compared with controls (**Figure 6E**). We showed that even if T cells secreting GzmB, TNFa, and IFNg were more numerous the cytotoxicity produced by cNK populations against the tumor was higher in RBPJ-deficient mice, even after T cell transfer (**Figure 6E**). Because RBPJ-deficient controlled better than RBPJ-competent mice the expansion of the tumor at early time points (**Figure 6A**), we analyzed the type 1 ILC composition and functions 5 days after tumor injection, a time point at which no infiltrating T cells could be detected. In RBPJ-deficient mice, the CD49a$^+$ subset was still absent but the intratumoral cNK cells were more prone to release GzmB, TNFa, and IFNg (**Figure 6F**). We propose that the canonical Notch signaling pathway is involved in the downregulation of cytotoxic capacities of specific cNK cell

A

Population name	ILC1	NK	NK	ILC1	ILC1	mNK/NK$^+$	NK	NK	NK	mNK/NK$^+$	iNK	iNK	preNKP
Sort strategy (Lin-)	CD226+ CD49a+ Mac1-	CD49b+ Mac1+ CD90-	CD49b+ Mac1+ CD90+	CD90-	CD90+	CD27+	CD27+ Mac1+	CD90-	CD90+	Mac1+	NKp46- CD49b- NK1.1+	NKp46- CD49b+ NK1.1+	NKp46- NK1.1- CD27+ CD127+
Blood	×	×	×	×	×	×	×	✓	✓	×	×	×	×
BM	×	×	×	×	×	✓	×	×	×	✓	✓	✓	✓
Liver	×	×	×	✓	✓	×	×	✓	✓	×	×	×	×
LP	✓	✓	✓	×	×	×	×	×	×	×	×	×	×
mLN	×	×	×	×	×	✓	×	×	×	✓	×	×	×
PVB	×	×	×	×	×	×	×	✓	✓	×	×	×	×
Spleen	×	×	×	×	×	✓	✓	×	×	✓	×	×	×

B

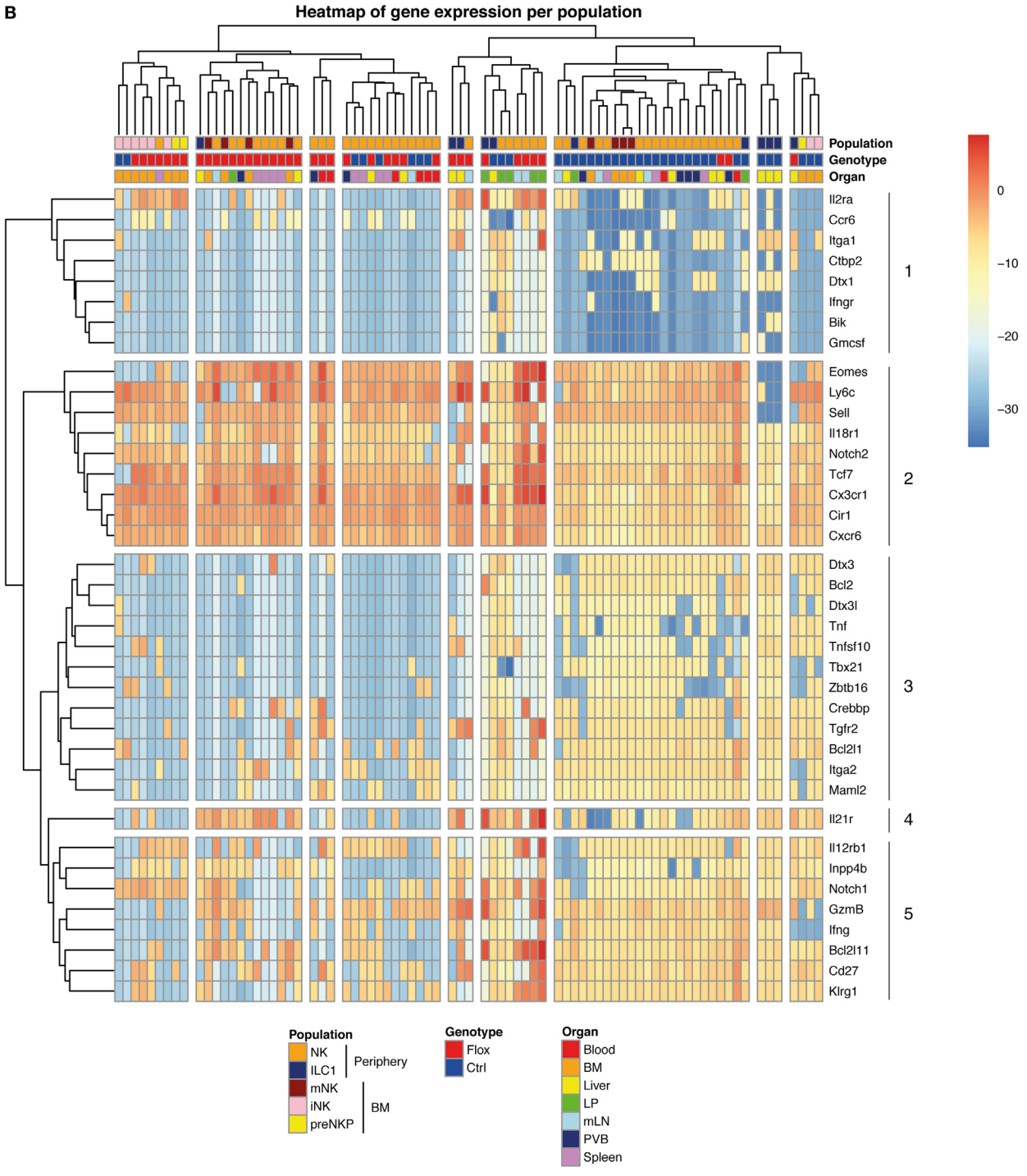

FIGURE 5 | Molecular signature heterogeneity of group 1 innate lymphoid cell (ILC) in IL7r^{Cre} Rbpj$^{F/+}$ and IL7r^{Cre} Rbpj$^{F/F}$ mice. **(A)** Sorting strategy of group 1 ILC cells in the different tissues **(B)** Heatmap of genes expression in group 1 ILC of IL7r^{Cre} Rbpj$^{F/+}$ and IL7r^{Cre} Rbpj$^{F/F}$ mice in blood, bone marrow (BM), liver, lamina propria (LP), mesenteric lymph nodes (mLN), portal vein blood (PVB), and spleen. Cluster of cells and genes were obtained using hierarchical clustering.

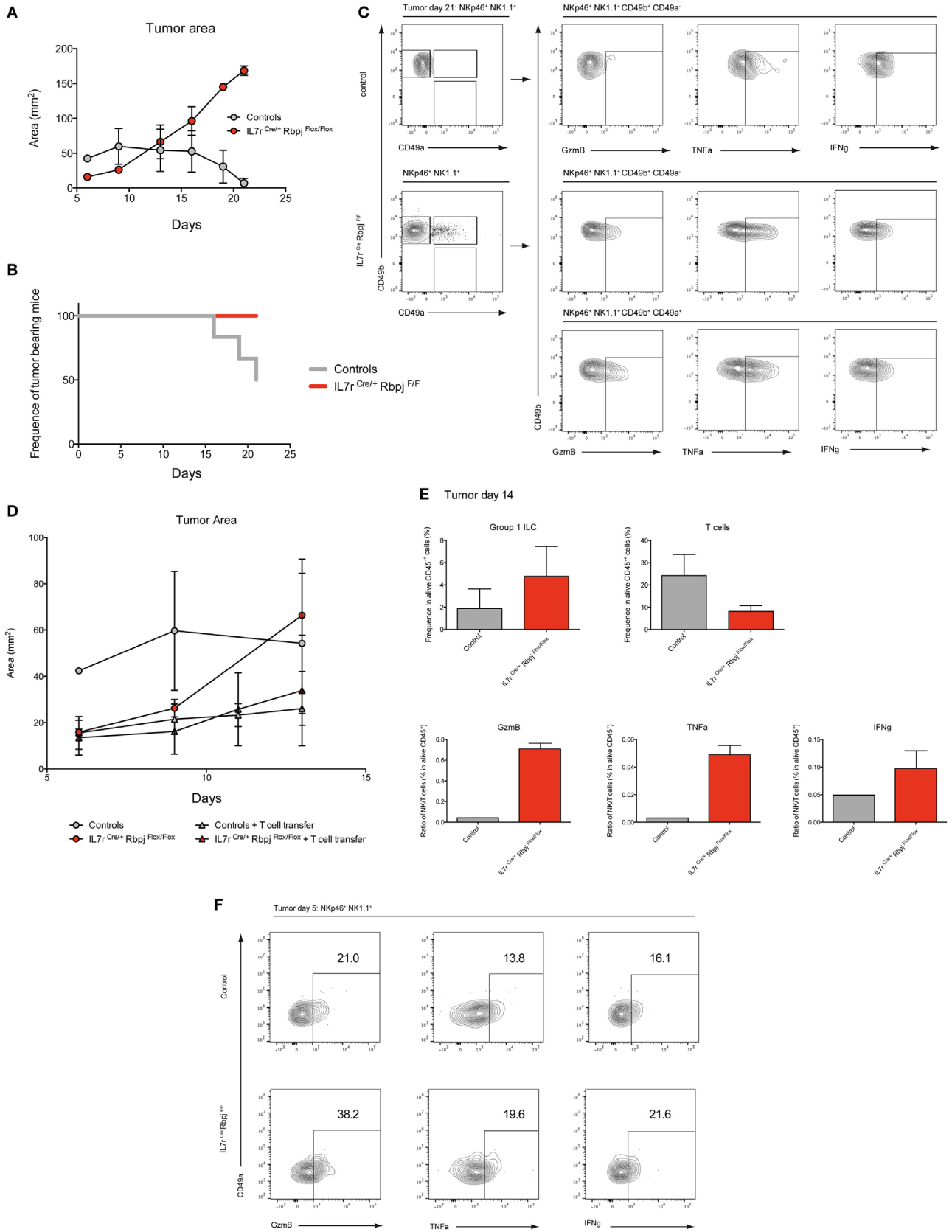

FIGURE 6 | Continued

FIGURE 6 | Notch signaling participates to early antitumoral activity. **(A)** Tumor area after subcutaneous injection of 3×10^6 Hepa1.6 cells in control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ (gray) and IL7r^{Cre} Rbpj$^{F/F}$ mice (red). **(B)** Frequency of mice with a tumor after subcutaneous injection of 3×10^6 Hepa1.6 cells in control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ (gray) and IL7r^{Cre} Rbpj$^{F/F}$ mice (red). 50% of control mice rejected the tumor after 21 days. **(C)** Group 1 innate lymphoid cell (ILC) tumor infiltrates in control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ mice (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ mice (bottom panel) 21 days after Hepa1.6 cells injection. **(D)** Tumor area after subcutaneous injection of 3×10^6 Hepa1.6 cells in control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ (gray dot), IL7r^{Cre} Rbpj$^{F/F}$ mice (red dot), control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ mice with T cell transfer (gray triangle) and IL7r^{Cre} Rbpj$^{F/F}$ mice with T cell transfer (red triangle). **(E)** Frequency of group 1 ILC and CD3-positive cells in tumor of control IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ (gray), IL7r^{Cre} Rbpj$^{F/F}$ mice (red) with T cell transfer 14 days after Hepa1.6 cells injection. Ratio of group 1 ILC on T cell expressing granzyme B (GzmB), TNFa, and IFNg. **(F)** Expression of GzmB, TNFa, and IFNg in group 1 ILC tumor infiltrates of IL7r^{Cre} Rbpj$^{F/+}$, IL7r$^{+/+}$ Rbpj$^{F/+}$, and IL7r$^{+/+}$ Rbpj$^{F/F}$ control (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ mice (bottom panel) 5 days after Hepa1.6 cells injection.

subsets and also in the control of CD49a expression levels on recruited type 1 ILC populations.

DISCUSSION

In the liver, cNK and ILC1 are more heterogeneous than initially thought. Contrary to CD49b$^+$ cNK, resident hepatic ILC1 have been defined as Eomes$^-$ CD49a$^+$ CD49b$^-$ T-bet dependent (2, 21–24). We distinguished here two subsets of ILC1 and of cNK based on the expression of a limited set of transcripts. Surprisingly, Eomes transcripts were found expressed in one of the hepatic ILC1 subgroup and they were differentially expressed among cNK subsets. Differences in tissue ILC1 populations have been attributed to the environment, particularly in glands where ILC1 express both Eomes and T-bet (25). This made us consider the possibility that Eomes levels may be actively suppressed in hepatic ILC1. Since Eomes expression is repressed in T-bet$^+$ hepatic ILC1 (2), and that T-bet is a possible target of the Notch pathway, we investigated whether the Notch pathway acts on hepatic type 1 ILC.

Using single-cell transcriptomic analyses, we confirmed that nearly half of hepatic ILC1 and cNK cells expressed Notch receptors. We found that expression is heterogeneous as cells could either be Notch1$^+$, Notch2$^+$, or both. Hence, we suspected that the Notch signaling pathway could also play a role on different characteristics of type 1 ILC from other organs. Therefore, by selecting genes belonging to type 1 ILC developmental program and to the Notch signaling pathway, we designed a comparative transcriptomic study on numerous small populations of type 1 ILC subsets from diverse tissues. We showed that Notch pathway is actively operating in populations from most tissues, except for spleen where a substantial amount of cells was found to be Notch insensitive.

We found that RBPJ deficiency was associated with the reduction of the *Tbx21* gene expression in half hepatic ILC1 validating our hypothesis of the Notch pathway implication on hepatic subset specification and functions. We assume that T-bet might also be activated by other signaling pathways because the other half of ILC1 that are not expressing Notch receptors should have a path to upregulate T-bet. We observed identical changes of ILC1 and cNK transcriptional program in other organs. In a previous study, we reported that T-bet levels were maintained in type ILC1 in the intestinal LP (17). However, in our previous study, the Notch sensitive population was diluted among Notch insensitive cells masking the effect of the Notch signaling depletion. Therefore, to overcome this limitation, we used only 25 cells per population in this comparative transcriptomic assay. In the absence of the Notch signaling pathway, we also detected an increase of Eomes expression in ILC1 and cNK subsets both at transcriptional and protein levels. The presence of a new immature Eomes$^-$ cNK population in the liver diluted the Eomes transcript levels from the global population as observed in **Figure 4**. The expression of Eomes is probably due to the decrease of T-bet, as previously observed in T-bet-deficient cNK cells (2, 26). Moreover, T-bet$^-$ cNK cells fail to express Mac1 and maintain their CD27 levels (27, 28). Consistently, we observed a decrease of Mac1 expression in liver cNK cells and the presence of CD27$^+$ Mac1$^-$ immature subset. The presence of these immature subsets might be linked to the decrease of T-bet expression in type 1 ILC. It has been hypothesized that hepatic ILC1 depends on T-bet for their development, although no direct evidence has been provided. In our RBPJ-deficient model, it is possible that all Notch-dependent ILC1 subsets are absent because they are not able to differentiate *in situ*. This immature subset could therefore represent accumulating ILC1 precursors that could not progress to the T-bet$^+$ stage. On other hand, peripheral immature subsets could also represent accumulating immature cNK cells. It has been shown that environment could induce conversion between ILC1 and cNK (25, 29, 30). Our study illustrates a probable hepatic ILC1 differentiation into cNK where the absence of the Notch pathway leads to the induction of Eomes *via* the downregulation of its regulator T-bet. Consistent with this hypothesis, in T-bet-deficient mice, precursors in the BM show an increase of the immature CD27$^+$ and a decrease of the mature Mac1$^+$ populations (Figure S7 in Supplementary Material). Clustering of BM RBPJ-competent and -deficient subsets confirmed a role for Notch signaling in the early development of type 1 ILC. Even if T-bet levels are actively maintained low in BM cNK precursors, T-bet is expressed at the immature CD27$^+$ Mac1$^-$ stage. T-bet has been shown to control S1P5 expression which participates to cNK trafficking (31, 32). As in T-bet-deficient mice, we also found more cNK cells in the BM (Figure S7 in Supplementary Material) of RBPJ-deficient mice while their ratio were decreased to the expense of ILC1 in the periphery (32, 33). We proved that the increase of ILC1 was not due to excessive proliferation and propose that the Notch induced decrease of T-bet results in a reduced exit from the BM.

Notch1 and Notch2 were correlated to different set of genes in our study suggesting that Notch1 was related to CD49a expression, IL21r, IL12rb1. Modifications in integrin expression are observed mainly in ILC1 with a clear decrease of surface CD49a. CD49a is not changed in Notch2-deficient mice arguing that modulation of CD49a levels is a specific Notch1 related feature.

Hence, different Notch receptors could have different repercussions on the resulting subset especially if they are expressed at different frequencies. To consider whether Notch signaling could directly act on CD49a expression, we looked for potential binding sites for RBPJ in the promoter region of the itga1 gene (Figure S8 in Supplementary Material). Typical RBPJ-binding sequence (Figure S8A in Supplementary Material) was searched by screening the itga1 promoter region for a minimum of 5-mer motif. Three potential-binding sites were found suggesting a possible direct action of the Notch signaling pathway on the level of CD49a expression (Figure S8B in Supplementary Material). Due to the important proportion of type 1 ILC expressing Notch2 in the periphery and the superposition of the effects driven by RBPJ and Notch2 deficiency on enhanced effector functions, we suggest that Notch2 could be the main player in cell activation. Notch2 has been proposed as a central receptor for peripheral T cell maturation (34, 35). However, Notch1 could also control Eomes, perforin, and GzmB (36). Among others, Notch2 gene expression is correlated with the upregulation of Eomes, GzmB Cx3cr1, Ly6c, Il18r1, and IL2ra expression that constitute a hallmark of activated mature cNK cells. Increased expression of CX3CR1 was also described in T-bet-deficient mice (26) and is a marker of circulating peripheral cNK cells (37). Increased of Th1 cytokine receptors coupled with the increase of GzmB correspond to the phenotype of peripheral-activated cytotoxic cells. In our RBPJ-deficient model, type 1 ILC also increases their capacity to release IFNg and TNFa. The increase of Ly6C by RBPJ-deficient cNK cells is reminiscent of cells previously designated as peripheral resting inert mNK cells that could produce an effective and strong response in case of reactivation by cytokine stimuli (38). We suggest that maturation to the Ly6C^{hi} stage is linked to modification of T-bet/Eomes quantities driven by a deficiency in Notch signaling.

It was shown that Notch signaling impacts Th1 differentiation and cytotoxicity (34, 35, 39, 40). We found the opposite effect in type 1 ILC where the absence of the Notch signaling induces maturation of peripheral subsets toward a more "activated" state with enhanced cytotoxic functions. Nonetheless, our results are in agreement with other studies showing the maintenance of the Th1 response in Notch1/Notch2, RBPBJ-deficient, and MAML1 dominant-negative mice (41, 42).

Type 1 helper innate lymphoid cells and cNK cells are developmentally and functionally related and it has been suggested that cells can interconvert in certain conditions such as in a tumoral environment (25). In addition, dysregulations of the Notch signaling were described in diverse types of cancer where an oncogenic or tumor suppressive role depended on tissue type and particular microenvironments (43). In hepatocellular carcinoma, the implication of Notch signaling is currently under intense investigation (44).

To test antitumor ability of Notch-deficient cNK subsets, we used an *in vivo* hepatocellular carcinoma model. Hepa1–6 tumors grew slower at early phase when transplanted into Notch-deficient mice than into Notch-competent littermates thanks to an increase of intratumoral type 1 ILC frequency in RBPJ-deficient conditions. Moreover, deficient Notch signaling pathway leads to the presence of new CD49a^{+} cNK cells with a higher ability to release cytotoxic and inflammatory signals. Since in Notch-deficient mice, T cell subsets are reduced, this model is not ideal to compare later stages of tumor progression or regression. The progression of tumor area and analyses of intratumoral immune content in control animals allowed us to determine that tumors start to regress 2 weeks after hepatocellular carcinoma injections due to intratumoral effector T cells. The tumor regression is fast with already half of control littermates that have totally eradicated the tumor 3 weeks after injection. Hence, we decided to transfer T cells to both Notch-deficient and littermate controls to compare the tumor growth and analyze their immune compartment. As previously observed for early phase, RBPJ-deficient conditions allowed a better control of tumor growth until 10 days. To recover enough immune cells from the tumor, we did not extend our analysis over 2 weeks and observed that even after T cell transfer, type 1 ILC were more frequent and more cytotoxic in RBPJ-deficient animals. We concluded that inhibition of the Notch signaling pathway is beneficial for the early control of tumor growth by type 1 ILC. Other studies have shown that depending on Notch receptor and ligands identity, antagonistic effects could be found on tumor progression (45). Our study adds a stone by dissecting the regulation of important cytotoxic subsets implicated in the tumor immunosurveillance. Collectively, our data suggest that cNK cells unable to signal *via* the Notch pathway are more critical effector cells to restrain early carcinoma growth. These cells displayed features that resemble ILC1 but also mature reactivated cNK secreting higher amounts of cytotoxic and inflammatory cytokines. Tumor immunosurveillance studies using T-bet-deficient mice demonstrated that T-bet is essentially required at late stages of the immune response but is not crucial in primary tumors (26, 46, 47). Nonetheless, forced expression of Eomes in cNK cells was shown to decrease tumor growth and enhance survival (48). Hence, we propose that the Notch pathway in mature peripheral type 1 ILC represents a modulator of the inflammatory response. This is achieved by the regulation of T-bet versus Eomes expression and by regulating expression of pro/anti-apoptotic molecules, as observed on our comparative transcriptomic analyses. The Notch signaling pathway is also implicated in the regulation of the cNK cell-mediated tumor immunosurveillance. Hence, the tumoral environment could also temper the immune response *via* the regulation of Notch ligand expression.

Finally, we propose that the Notch signaling pathway as one of the extrinsic signals that control the intrinsic T-bet/Eomes balance. This pathway is implicated at multiple levels since T-bet/Eomes ratio are so important for cytotoxic lymphocyte differentiation and functions (49). Like TGF-β signaling that directs differentiation of salivary gland ILC1 through suppression of Eomes (25), we propose that the Notch signaling pathway participates to reduce Eomes levels in both cNK and ILC1, with a strong effect on hepatic ILC1. The spatio-temporal regulation of Notch receptor expression is participating to this equilibrium and enhances the complexity of the global picture.

ETHICS STATEMENT

Mice were bred in accordance with Pasteur Institute guidelines in compliance with European animal welfare regulations, and

all animal studies were approved by Pasteur Institute Safety Committee in accordance with French and European guidelines.

AUTHOR CONTRIBUTIONS

TP, SM, MP, and E-GB performed the experiments. RG, TP, and SM designed the experiments. RG, TP, AC, MP, SM, and E-GB analyzed the data. RG supervised the experiments and wrote the manuscript with the contribution of AC and TP.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01252/full#supplementary-material.

FIGURE S1 | **(A)** Correlation heatmap of gene expression from transcripts of **Figure 1**. **G** using Spearman method. **(B)** Correlation heatmap of gene expression from transcripts of signature 1 from **Figure 1**. **G** using Spearman method. **(C)** Correlation heatmap of gene expression from transcripts of signature 2 from **Figure 1**. **G** using Spearman method. Levels of correlation are shown from blue (low level) to red (high level).

FIGURE S2 | **(A)** Flow cytometry of hepatic group 1 innate lymphoid cell in IL7r^{Cre} Notch2$^{F/F}$, Vav^{Cre} Rbpj$^{F/F}$ mice and their respective controls. **(B)** Mean fluorescence intensity of CD49a and CD49b in hepatic conventional NK (cNK) and type 1 helper innate lymphoid cells (ILC1) in control (white) and Vav^{Cre} Rbpj$^{F/F}$ (red) mice. **(C)** Frequency of hepatic ILC1 and cNK in IL7r^{Cre} Notch2$^{F/F}$, Vav^{Cre} Rbpj$^{F/F}$ mice, and their respective controls.

FIGURE S3 | **(A)** Frequency of hepatic type 1 helper innate lymphoid cells (ILC1) and conventional NK (cNK) in G0 (white), G1 (gray), and S/G2/M (black) phase. **(B)** T cells in the thymus were used as control of cell cycle.

FIGURE S4 | Expression of TNFa, IFNg, and granzyme B in hepatic type 1 helper innate lymphoid cells (ILC1) (left panel) and conventional NK (cNK) (right panel) of control IL7r^{Cre} Notch2$^{F/+}$ mice (top panel) and IL7r^{Cre} Notch2$^{F/F}$ mice (middle panel). Levels of expression were compared (bottom panel) between control IL7r^{Cre} Notch2$^{F/+}$ mice (blue) and IL7r^{Cre} Notch2$^{F/F}$ mice (red), Lineage-negative cells were used as control for expression (dashed black).

FIGURE S5 | Correlation heatmap of gene expression using Spearman method. Levels of correlation are shown from blue (low level) to red (high level).

FIGURE S6 | **(A)** Flow cytometry of T cell infiltrate from tumor at day 14. Intracellular granzyme B (GzmB), TNFa, and IFNg expression of T cells. **(B)** Frequency of T cells infiltrate in alive CD45$^+$ cells and frequency of T cells expressing GzmB, TNFa, and IFNg in alive CD45$^+$ cells.

FIGURE S7 | NK cells and NK progenitors (NKP) repartition in bone marrow (BM). **(A)** Flow cytometry of NKP (NKp46$^-$ NK1.1$^+$ CD49b$^{+/-}$), and NK cells (NKp46$^+$ NK1.1$^+$) in BM of control IL7r^{Cre} Rbpj$^{F/+}$ (top panel) and IL7r^{Cre} Rbpj$^{F/F}$ mice (bottom panel). **(B)** Frequency of NKP (NKp46$^-$ NK1.1$^-$ CD49b$^{+/-}$) and NK cells (NKp46$^+$ NK1.1$^+$) in BM of control IL7r^{Cre} Rbpj$^{F/+}$ (white) and IL7r^{Cre} Rbpj$^{F/F}$ mice (red). NKP were divided based on CD49b expression and NK cells were divided based on CD27 and Mac1 expression.

FIGURE S8 | **(A)** Consensus sequence for RBPJ-binding sites to promoter regions. **(B)** Location of 5-mer motifs for potential RBPJ binding sites along the itga1 (CD49a) promoter region. Different motifs are represented in different colors.

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REVIEW
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NOTCH1 Aberrations in Chronic Lymphocytic Leukemia

*Emanuela Rosati[1], Stefano Baldoni[2†], Filomena De Falco[3†], Beatrice Del Papa[3†], Erica Dorillo[3], Chiara Rompietti[3], Elisa Albi[3], Franca Falzetti[3], Mauro Di Ianni[4,5] and Paolo Sportoletti[3]**

[1] Department of Experimental Medicine, Biosciences and Medical Embryology Section, University of Perugia, Perugia, Italy, [2] Department of Life, Hematology Section, Health and Environmental Sciences, University of L'Aquila, Perugia, Italy, [3] Institute of Hematology-Centro di Ricerche Emato-Oncologiche (CREO), University of Perugia, Perugia, Italy, [4] Department of Medicine and Aging Sciences, University of Chieti Pescara, Chieti, Italy, [5] Department of Hematology, Transfusion Medicine and Biotechnologies, Ospedale Civile, Pescara, Italy*

Chronic lymphocytic leukemia (CLL) is an incurable B-cell neoplasm characterized by highly variable clinical outcomes. In recent years, genomic and molecular studies revealed a remarkable heterogeneity in CLL, which mirrored the clinical diversity of this disease. These studies profoundly enhanced our understanding of leukemia cell biology and led to the identification of new biomarkers with potential prognostic and therapeutic significance. Accumulating evidence indicates a key role of deregulated NOTCH1 signaling and *NOTCH1* mutations in CLL. This review highlights recent discoveries that improve our understanding of the pathophysiological NOTCH1 signaling in CLL and the clinical impact of *NOTCH1* mutations in retrospective and prospective trials. In addition, we discuss the rationale for a therapeutic strategy aiming at inhibiting NOTCH1 signaling in CLL, along with an overview on the currently available NOTCH1-directed approaches.

Keywords: NOTCH1, chronic lymphocytic leukemia, prognostic biomarker, targeted therapy, gene mutation

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****Correspondence:***
Paolo Sportoletti
sportolp@gmail.com

[†]These authors have contributed equally to this work.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by a clonal expansion of mature CD5+ CD23+ B-lymphocytes that accumulate in the bone marrow and infiltrate lymphoid tissues such as the spleen and lymph nodes (1). CLL, the most common leukemia in the Western world, is a heterogeneous disease and remains incurable in virtually all cases. CLL predominates in the elderly, and the incidence of the disease increases exponentially with age (2). Thus, the number of CLL patients is expected to rise in the future, given the increase in the aging population, bringing to light new clinical challenges and public health issues. Patients with CLL show a tremendously variable clinical course ranging from excellent prognosis with no treatment to short-term survival, despite early initiation of therapy (3). Genomic and molecular characterization of CLL has largely explained the heterogeneous clinical course of this disease, improving the prognostic risk stratification (4). Features predicting CLL outcome include somatic mutations of the immunoglobulin heavy chain variable (*IGHV*) genes, expression of CD38 and ZAP-70 surrogate markers, identification of chromosomal abnormalities (deletions of chromosome 13q, 17p, and 11q, and trisomy 12), and recurrent mutations in *TP53*, *NOTCH1*, and *SF3B1* genes (5).

NOTCH1 has emerged as the most commonly mutated gene in CLL at diagnosis, and its frequency rises with disease progression (6–8). *NOTCH1* mutations are associated with poor outcomes and result in more difficulties to treat CLL (9–12). Mutated CLL shows a biologically active form of NOTCH1, though NOTCH1-dependent transcriptional responses have also been described in CLL cases lacking the mutation (13). Therefore, *NOTCH1* represents a new key cancer gene in CLL whose genetic and pathway alterations are likely to represent a novel oncogenic process in this disease.

In this review, we discuss the impact of NOTCH1 aberrations on the pathogenesis, prognosis, and therapeutic strategies in CLL, based on available literature.

NOTCH1 PROTEIN STRUCTURE AND PATHWAY

NOTCH1 is a single pass transmembrane heterodimeric receptor. It is synthesized as a single precursor that undergoes a proteolytic cleavage by a furin-like convertase in the Golgi apparatus. The mature receptor expressed on the cell surface is composed of an N-terminal extracellular subunit (NOTCH1-EC) and a C-terminal transmembrane and intracellular subunit (NOTCH1-TMIC), held together by non-covalent interactions. The NOTCH1-EC contains a series of epidermal growth factor-like repeats, involved in ligand binding, and three LIN-12/NOTCH repeats that stabilize the heterodimerization domain (HD), preventing ligand-independent activation of the receptor. The NOTCH1-TMIC consists of a transmembrane region followed by different cytoplasmic domains that form the NOTCH1 intracellular domain (ICD) (NOTCH1-ICD). NOTCH1-ICD includes an RBPJ-associated molecule domain, a series of ankyrin (ANK) repeats, flanked by nuclear localization signals, a transactivation domain (TAD), and a C-terminal PEST domain, a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T), which regulates stability and proteasomal degradation of active NOTCH1-ICD (14, 15) (**Figure 1A**). NOTCH1 signaling is triggered when a ligand, from the SERRATE/JAGGED or DELTA families, expressed on an adjacent cell, binds the receptor.

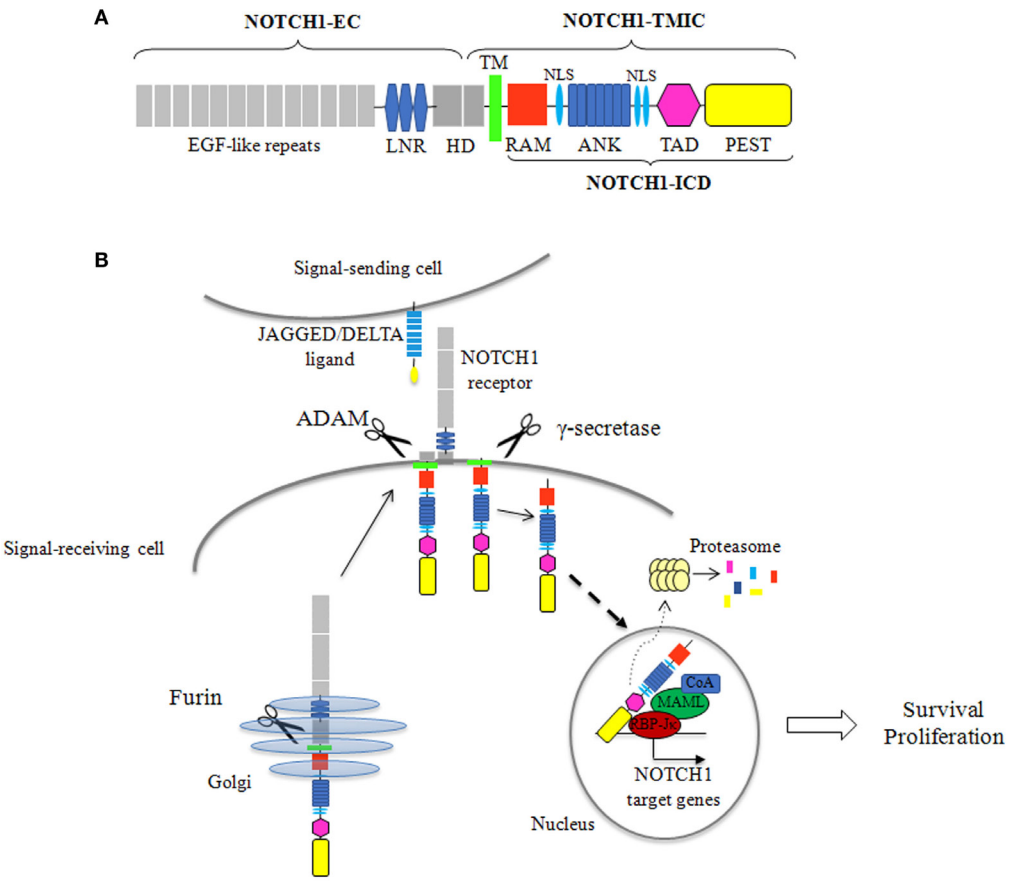

FIGURE 1 | NOTCH1 protein structure and signaling activation. **(A)** The mature NOTCH1 receptor is a heterodimer composed of an extracellular subunit (NOTCH1-EC) and a transmembrane and intracellular subunit (NOTCH1-TMIC). The NOTCH1-EC includes epidermal growth factor (EGF)-like repeats, involved in ligand binding, three LIN-12/NOTCH repeats (LNR), which prevent receptor activation in the absence of ligands, and the heterodimerization domain (HD) involved in non-covalent interactions between the NOTCH1-EC and NOTCH1-TMIC. The NOTCH1-TMIC consists of the transmembrane domain (TM) and the intracellular domain (ICD) (NOTCH1-ICD). NOTCH1-ICD comprises an RBPJ-associated molecule (RAM) domain, seven ankyrin (ANK) repeats, nuclear localization signals (NLS), a transactivation domain (TAD), and a PEST domain, which is involved in proteasomal degradation of active NOTCH1-ICD. **(B)** Newly synthesized NOTCH1 precursor is cleaved by a furin-like convertase (Furin) in the Golgi apparatus to generate the mature receptor. NOTCH1 signaling initiates when a JAGGED or DELTA ligand expressed on a signal sending cell interacts with NOTCH1 on a signal receiving cell. This interaction triggers two sequential cleavages of NOTCH1: the first, by an a disintegrin and metalloproteinase (ADAM) metalloproteinase, generates the substrate for the second cleavage by γ-secretase, which releases the active NOTCH1-ICD. NOTCH1-ICD translocates to the nucleus where it forms a transcriptional activation complex by interacting with the transcription factor CSL/RBP-Jk, mastermind-like proteins, and others coactivators (CoA), leading to the expression of NOTCH1 target genes. In physiological conditions, NOTCH1 signal attenuation is mediated by ubiquitination and proteasomal degradation of NOTCH1-ICD.

This interaction starts two successive proteolytic cleavages: an extracellular juxtamembrane cleavage, by a disintegrin and metalloproteinase that occurs in the HD and generates the substrate for the intramembrane cleavage, by γ-secretase complex, resulting in the release of the active NOTCH1-ICD which translocates to the nucleus. In the nucleus, NOTCH1-ICD forms a transcription complex with the transcription factor RBP-Jk, mastermind-like (MAML) proteins and other coactivators, switching on the expression of NOTCH1 target genes (15). The signal is terminated through the ubiquitination of degron sites on the PEST domain, followed by proteasome-dependent degradation of the active NOTCH1-ICD (**Figure 1B**).

HISTORY OF NOTCH1 SIGNALING AND EXAMINATION OF GENE ALTERATIONS IN CLL

Initially, NOTCH1 was considered essential to direct T-cell lineage commitment at the expense of B-cell development (16), and its oncogenic potential has been demonstrated in T-cell leukemia (17, 18). In a pioneering study, we demonstrated that CLL cells expressed high levels of NOTCH1 receptor together with its ligands JAGGED1 and JAGGED2. NOTCH1 was constitutively activated in CLL cells and contributed to their survival and resistance to apoptosis (19). Based on these observations, an initial *NOTCH1* gene defect in relation to CLL was reported in 2009, when our group identified a frameshift deletion of *NOTCH1* gene in a percentage of unselected CLL patients (6). Soon after, we determined the significant prognostic implication of *NOTCH1* mutation in a pivotal retrospective single-institution cohort study (9).

The advent of next-generation sequencing (NGS) technology confirmed the presence of stabilizing mutations of *NOTCH1* in several independent CLL groups (8, 20, 21). Fabbri et al. were the first to identify the link between *NOTCH1* mutations and chemotherapy refractoriness and disease progression to large cell lymphoma (7). Other publications further confirmed the impact of *NOTCH1* mutations on poor clinical outcome and delineated their association with other genetic markers (i.e., unmutated *IGHV* genes and trisomy 12) (22–24). Based on this observation, *NOTCH1* mutations have been integrated in the hierarchical cytogenetic prognostic stratification model devised by Döhner et al. that is still of major clinical relevance (5, 25). Nowadays, the recommendation for the assessment of *NOTCH1* mutations has not been introduced into general practice but is often performed in clinical trials. The analysis of prospective series yielded conflicting results with retrospective studies on the prognostic potential of NOTCH1 in CLL (12, 26). Genetic and molecular findings were paralleled by a number of biological studies aimed to define the leukemogenic role of *NOTCH1* mutations. Results of these studies will be discussed in detail in this review. More recently, the identification of non-mutational activation of NOTCH1 in CLL (13) implied that NOTCH1-ICD levels might represent a prognostic biomarker to refine the mutation/cytogenetic hierarchical model of risk stratification. Furthermore, these findings implicated a much broader role of NOTCH1 in CLL pathogenesis and raised the question about the mechanisms leading to the signaling activation.

NOTCH1 MUTATIONAL STATUS IN CLL

Types of *NOTCH1* Mutations in CLL

NOTCH1 genetic alterations have been described in different human malignancies, including hematopoietic and solid tumors (27). Chromosomal rearrangements (28) and mutations (29) of the *NOTCH1* gene were initially described in T-cell acute lymphoblastic leukemia (T-ALL), which displayed aberrant activation of NOTCH1 signaling in over 60% of the cases. In T-ALL, mutations occur more frequently on the HD domain, resulting in the activation of the receptor independent of ligand binding (29, 30). Unlike T-ALL, the most common *NOTCH1* mutation in CLL affects the C-terminal PEST domain causing prolonged half-life of the cleaved protein (**Figure 2**). This mutation, accounting for approximately 80% of cases (**Table 1**; **Figure 3**) consists in a 2-bp (CT) frameshift deletion (c.7541_7542delCT) localized in the exon 34 that generates a premature stop codon causing truncations of the C-terminal PEST domain (P2514fs*4) (6). The truncated protein lacks the F-box and WD repeat domain-containing 7 (FBXW7) and WSSSSP degron domains required to target activated NOTCH1 for proteasomal degradation (Table S1 in Supplementary Material). This leads to increased NOTCH1-ICD stability and aberrantly prolongs its activation, indicating that mutations may contribute to enhance NOTCH1 signaling in CLL. The reasons for which *NOTCH1* mutations affect two different domains in CLL and T-ALL samples remain unclear. We can hypothesize the existence of distinctive genetic contexts that probably depend on highly conserved mechanisms. Alternatively, the domain targeted by mutation may depend on either a distinct epigenetic regulation in T versus B cells or on a selective pressure from microenvironmental conditions.

NGS studies in CLL revealed several mutations other than the hotspot dinucleotide deletion targeting the function of the C-terminal PEST domain (8, 21, 31–34) (**Table 1**; **Figure 3**). These genetic alterations include frameshift mutations or truncations that affect different nucleotides in exon 34 and occur with lower frequency than that of the canonical delCT mutation (12.5%). The majority of these mutations lead to the disruption of the Cdc phosphodegron domain targeted by FBXW7 and the following WSSSSP sequence. Rare non-delCT mutations include Q2519* and W2520* substitutions (35, 36) that result in the removal of the WSSSSP sequence alone. Similar alterations have been shown to be leukemogenic in T-ALL, suggesting a specific function in CLL. In addition, PEST domain mutants include a small number of missense mutations (34), some of which are reported in the single-nucleotide polymorphism database, suggesting their limited role in CLL. The TAD and ANK domains are targets of frameshift non-sense mutations in 1.9 and 0.4% of CLL, and generally determine a premature stop codon.

In 2015, Puente et al. reported recurrent mutations in the 3′ untranslated region (3′-UTR) of the *NOTCH1* gene of previously untreated CLL or monoclonal B-cell lymphocytosis (MBL)

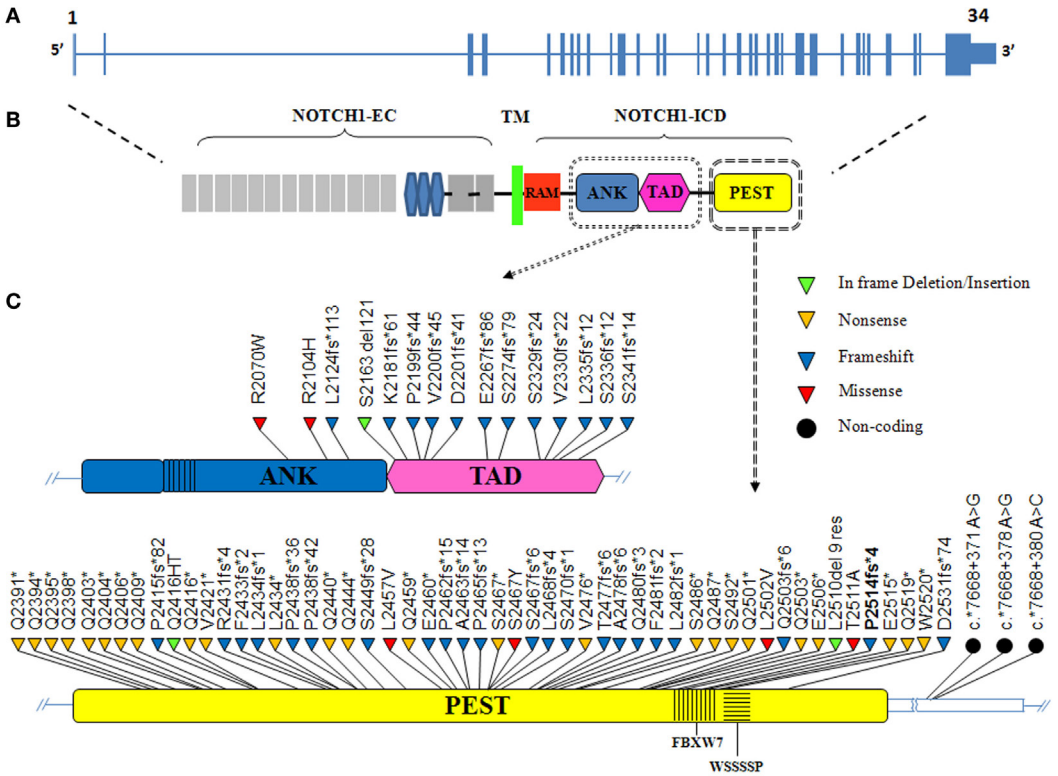

FIGURE 2 | The distribution of *NOTCH1* mutations in chronic lymphocytic leukemia (CLL). **(A)** Schematic diagram of the human *NOTCH1* gene consisting of 34 exons (vertical bars) and spanning 50 kb. **(B)** The NOTCH1 protein includes an extracellular domain (EC) and an intracellular domain (ICD) linked by a transmembrane portion (TM). The NOTCH1-ICD consists of RBPJ-associated molecule (RAM) and ankyrin (ANK) domains involved in CSL/RBP-Jk binding, a transactivation domain (TAD) and a PEST (polypeptide enriched in proline, glutamate, serine and threonine) domain, which is a hotspot for mutation in CLL. **(C)** The ANK/TAD (upper panel) and PEST (lower panel) domains are magnified, and the color-coded shapes indicate the position of the in frame deletion/insertion (green), non-sense (yellow), frameshift (blue), missense (red), and non-coding (black dot) mutations found in CLL. The F-box and WD repeat domain-containing 7 (FBXW7) and WSSSSP degron are indicated within the PEST domain.

cases (32). The presence of these mutations was confirmed at low frequency (2–4%) in heterogeneous CLL cohorts of retrospective studies and clinical trials (33, 37). The most common target of 3′-UTR mutations is the non-coding region of the exon 34 at position 7668+371A>G, 7668+378A>G, and less frequently, 7668+380A>C (74.5, 21.3, and 4.2%, respectively). Each of these non-coding variants creates a new splice acceptor site that favors an alternative splicing event with a cryptic donor site located in the coding exon 34. As a result, most of the coding bases of the PEST sequence are removed, resulting in an increased NOTCH1 protein stability as in the case of delCT mutation. In most cases, 3′-UTR mutations are mutually exclusive with other *NOTCH1* somatic variants, supporting the analysis of exon 34 non-coding region to identify additional patients with pathogenic *NOTCH1* mutations. Preliminary evidence showed that patients with different *NOTCH1* mutations display constitutive levels of cleaved protein whose size is consistent with the type of mutations (34). This observation suggests potential differences in biological and prognostic impacts on CLL that need to be further analyzed. Besides mutations in the *NOTCH1* gene itself, several NOTCH1 pathway regulatory genes such as *FBXW7*, mediator complex subunit 12 (*MED12*), and spen family transcriptional repressor (*SPEN*) were also identified as mutated with low frequency in CLL. *FBXW7* and *MED12* loss-of-function mutations preventing proteasomal degradation of NOTCH1 were present in 2–5% CLL (20, 38). SPEN is a co-repressor of RBPJ and a putative negative regulator of NOTCH1 signaling. Inactivating mutations of the *SPEN* gene were detected in approximately 1% of CLL cases (39).

In addition to NOTCH1, CLL cells also express the NOTCH2 receptor which is constitutively activated (19). Despite NOTCH2 signaling appears to have a role in CLL cell survival similar to that of NOTCH1 (19, 40, 41), *NOTCH2* mutations have not been detected in CLL (42, 43). By contrast, *NOTCH2* mutations have been found in other non-Hodgkin B-cell lymphoma subtypes, such as splenic marginal zone lymphoma (SMZL) and diffuse large B-cell lymphoma (DLBCL). In DLBCL, *NOTCH2* mutations affect approximately 8% of patients with some cases having increased copies of the mutated *NOTCH2* allele (44). In SMZL, *NOTCH2* mutations represent the most recurrent genetic lesion accounting for approximately 20–25% of cases (42, 45, 46). Most of identified mutations were frameshift or non-sense mutations affecting PEST domain and resulting in protein truncation and increased NOTCH2 activation (45). Remarkably, NOTCH2

TABLE 1 | Frequency of genetic variants of *NOTCH1* mutations across different chronic lymphocytic leukemia studies.

No. patients	Mutation	Domain	%
646	P2514fs*4	PEST	78.78
35	c.*7668+371 A>G	UTR	4.2
14	Q2444*	PEST	1.7
10	L2482fs*1	PEST	1.2
10	c.*7668+378 A>G	UTR	1.2
6	Q2503*	PEST	0.73
5	Q2394*	PEST	0.61
4	Q2440*	PEST	0.48
4	Q2501*	PEST	0.48
3	S2336fs*12	TAD	0.36
3	S2341fs*14	TAD	0.36
3	Q2404*	PEST	0.36
3	A2463fs*14	PEST	0.36
3	F2481fs*2	PEST	0.36
3	Q2519*	PEST	0.36
2	Q2403*	PEST	0.24
2	Q2406*	PEST	0.24
2	Q2409*	PEST	0.24
2	P2415fs*82	PEST	0.24
2	Q2416HT	PEST	0.24
2	Q2416*	PEST	0.24
2	V2421*	PEST	0.24
2	Q2459*	PEST	0.24
2	S2470fs*1	PEST	0.24
2	V2476*	PEST	0.24
2	A2478fs*6	PEST	0.24
2	c.*7668+380 A>C	UTR	0.24
1	R2070W	ANK	0.12
1	R2104H	ANK	0.12
1	L2124fs*113	ANK	0.12
1	S2163del121	TAD	0.12
1	K2181fs*61	TAD	0.12
1	P2199fs*44	TAD	0.12
1	V2200fs*45	TAD	0.12
1	D2201fs*41	TAD	0.12
1	E2267fs*86	TAD	0.12
1	S2274fs*79	TAD	0.12
1	S2329fs*24	TAD	0.12
1	V2330fs*22	TAD	0.12
1	L2335fs*12	TAD	0.12
1	Q2391*	PEST	0.12
1	Q2395*	PEST	0.12
1	Q2398*	PEST	0.12
1	R2431 fs*4	PEST	0.12
1	F2433fs*2	PEST	0.12
1	L2434fs*1	PEST	0.12
1	L2434*	PEST	0.12
1	P2438fs*36	PEST	0.12
1	P2438fs*42	PEST	0.12
1	S2449fs*28	PEST	0.12
1	L2457V	PEST	0.12
1	E2460*	PEST	0.12
1	P2462fs*15	PEST	0.12
1	P2465fs*13	PEST	0.12
1	S2467*	PEST	0.12
1	S2467Y	PEST	0.12
1	S2467fs*6	PEST	0.12
1	L2468fs*4	PEST	0.12
1	T2477fs*6	PEST	0.12
1	Q2480fs*3	PEST	0.12
1	S2486*	PEST	0.12
1	Q2487*	PEST	0.12
1	S2492*	PEST	0.12
1	L2502V	PEST	0.12
1	Q2503fs*6	PEST	0.12
1	E2506*	PEST	0.12
1	L2510 del9 res	PEST	0.12
1	T2511A	PEST	0.12
1	E2515*	PEST	0.12
1	W2520*	PEST	0.12
1	D2531fs*74	PEST	0.12

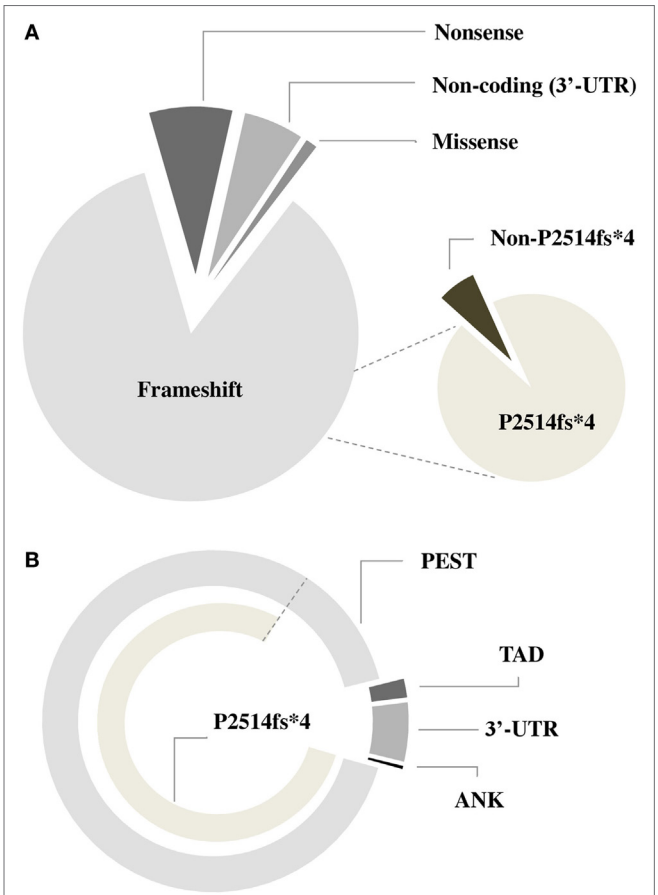

FIGURE 3 | Frequency of different types of *NOTCH1* mutations in chronic lymphocytic leukemia. Pie charts describing the incidence of *NOTCH1* mutations according to the type of genetic variant **(A)** and domains **(B)** involved.

signaling has been shown to play a key role in marginal zone B-cell development in the spleen, and to be dispensable for the development of other B-cell lineages (47, 48).

Based on these observations, one can hypothesize that the selective requirement of NOTCH2 signals for the development of normal splenic marginal zone B cells provides a functional basis for the involvement of *NOTCH2* mutations in SMZL and not in other B malignancies, such as CLL, that derive from other cell types. In agreement with this hypothesis, the selective pressure to acquire *NOTCH1* mutations in CLL presumably reflects a special context-dependent role for NOTCH1 activation in

normal naïve and memory B cells (13), which are considered the cells of origin of CLL (49, 50). The involvement of NOTCH1 alterations in CLL leukemogenesis will be detailed in a specific section of this review.

Frequency of *NOTCH1* Mutations in CLL

The prevalence of *NOTCH1* mutations in CLL varies greatly across studies, depending on differences between cohorts relative to time from diagnosis, stage of the diseases analyzed (**Figure 4**), and other genetic alterations enriched in the study. The frequency of mutated cases is between 6 and 12% at initial diagnosis of CLL, whereas only approximately 3% of patients with MBL harbor a mutation (51, 52). The average mutation rate increased to approximately 15–20% when considering only patients with fludarabine-refractory CLL (7). *NOTCH1* lesions are much more prevalent after disease progression to Richter transformation relative to newly diagnosed CLL, with 30% patients harboring mutations, mostly the classical delCT (7, 53). A high mutation rate has been described in independent cohorts of CLL cases with trisomy 12 (23, 54). Specifically, mutations were predominant in CLL with isolated trisomy 12, and less common in cases associated with additional chromosomal aberrations (55). Balatti et al. reported a mutation frequency of 41.9% in aggressive trisomy 12 cases, suggesting a critical role of NOTCH1 activation in this CLL subgroup (22). In addition, *NOTCH1* mutations frequently occur with deletions of the long arm of chromosome 14 (56), and are inversely correlated with chromosome 11 deletions (23). *NOTCH1* lesions are considerably associated with flow cytometry-based markers of poor prognosis including ZAP-70, CD38, and CD49d (26, 57–59). Analyses on larger number of patients documented a high frequency of unmutated *IGHV* genes in both coding and non-coding *NOTCH1*-mutated CLL cases (60). Evidence indicates that *NOTCH1* mutations can occur in the context of other genetic variables. The concurrent presence of *NOTCH1* and *TP53* mutations has been described in 1.2–2.6% of CLL patients (12, 21), and single cell analysis revealed that gene defects preferentially affected the same leukemic cells (61). Additional studies observed a high co-occurrence of mutations in *MGA*, *BCOR* (32), and *XPO1* genes (62) with those in *NOTCH1*. Interestingly, *NOTCH1* mutations were found to be mutually exclusive with *SF3B1*, *BIRC3*, and *MYD88* mutations (63). The patterns of co-occurrence and mutual exclusivity between *NOTCH1* mutations and other genetic features in CLL are shown in **Figure 5**.

The molecular method used to identify *NOTCH1* mutations represents one important factor that influences observed mutation rates (**Table 2**). Earlier studies used standard Sanger sequencing to detect delCT variants in the exon 34 region, therefore mutations with allelic frequencies below 20% were not detected (6, 9, 64). Similarly, early NGS techniques discarded variants below the sensitivity of Sanger sequencing, allowing the identification of clonal mutation in 4–12% CLL patients (7, 8). In many subsequent NGS-based analyses, deep sequencing allowed to sequence a genomic region even thousands of times and to detect rare subclonal mutations (≤1%). This type of approach permitted to reveal a mutation frequency of the *NOTCH1* gene between 12 and 25.5% (33, 34, 65). The presence of recurrent delCT mutations was investigated by high-sensitivity polymerase chain reaction (PCR)-based methods (i.e., allele-specific and droplet digital PCR) to identify the *NOTCH1* mutant in up to half of CLL patients (66–68). These techniques help uncover subclonal mutations and may be used to identify patients in need of close clinical follow-up or for prospective minimal residual disease studies.

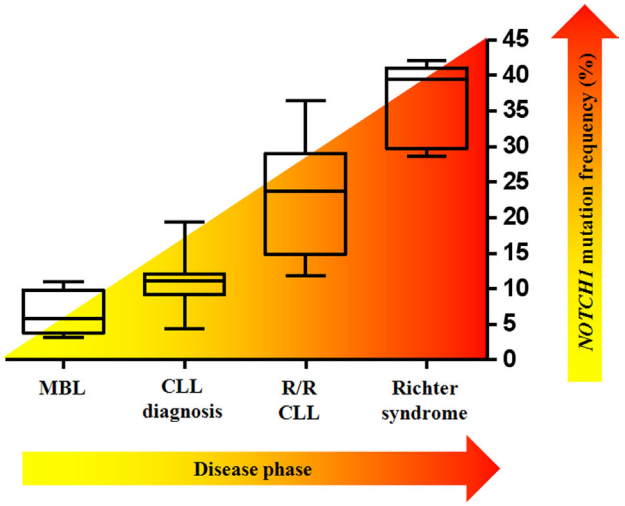

FIGURE 4 | *NOTCH1* mutation frequency varies according to the clinical time point of the disease. Box–whisker plots indicate the prevalence of mutated NOTCH1 in monoclonal B-cell lymphocytosis (MBL), in chronic lymphocytic leukemia (CLL) at diagnosis, in relapsed/refractory (R/R) CLL, and in Richter syndrome.

PROGNOSTIC AND PREDICTIVE IMPACT OF *NOTCH1* MUTATIONS IN CLL

NOTCH1 mutations have been strongly associated with clinical outcomes, making them ideal prognostic biomarkers for an accurate risk-adapted stratification of CLL patients. The following paragraphs provide a comprehensive review of the most relevant findings on the role of *NOTCH1* in CLL prognosis.

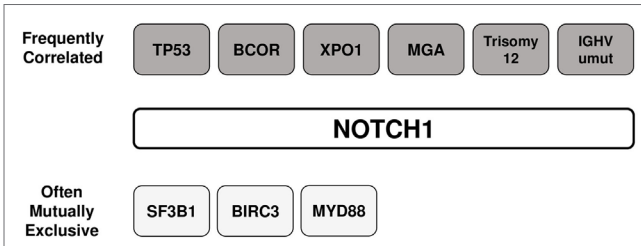

FIGURE 5 | Association between *NOTCH1* mutations and other genetic features in chronic lymphocytic leukemia. The patterns of co-occurrence and mutual exclusivity between *NOTCH1* mutations and other genetic features are shown.

TABLE 2 | Comparison between different molecular methods used to identify *NOTCH1* mutations.

Approach	Target region	Limit of detection (%)	Frequency (%)		Reference
			Clonal	Subclonal included	
Sanger	Exons 26, 27, and 34	10	4.6–12.1	N.A.	Di Ianni et al. (6), Quesada et al. (31), and Villamor et al. (58)
WGS/WES	All genome and exome	10	8.3–12.2	N.A.	Puente et al. (8) and Fabbri et al. (7)
ARMS-PCR	7541_7542delCT	10	11.0	N.A.	Rossi et al. (10)
Pyrosequencing	Exons 25, 28, and 34	2.5	13.0	17.0	Kluk et al. (69)
NGS (454)	Exons 33 and 34	2	10.5	13.2	Weissmann et al. (21) and Jeromin et al. (36)
AS-PCR	7541_7542delCT	0.1	10.2	20.1	Sportoletti et al. (66)
NGS ultra-deep targeted	Exons 26, 27, and 34 and 3′-UTR	0.3–1	11.8–14.3	18.6–25.5	Nadeu et al. (33), Pozzo et al. (70), and D'Agaro et al. (34)
ddPCR	7541_7542delCT	0.03	18.1	53.4	Minervini et al. (68)
Fragment Analysis	7541_7542delCT	5	8.4	8.4	Campregher et al. (67)
Real-time PCR	7541_7542delCT	10	10.1	N.A.	Bilous et al. (71)
HRM	7541_7542delCT	1	6.02	6.02	Xu et al. (72)

WGS, whole-genome sequencing; WES, whole-exome sequencing; ARMS-PCR, amplification refractory mutation system PCR; NGS, next-generation sequencing; NGS (454), next-generation sequencing (Roche 454 sequencing system); AS-PCR, allele-specific PCR; ddPCR, droplet digital PCR; HRM, high-resolution melting; N.A., not applicable.

NOTCH1 Mutations in Retrospective CLL Studies

Several retrospective analyses demonstrated that patients harboring *NOTCH1* mutations have altered survival statistics and treatment outcomes compared with patients with the wild-type *NOTCH1*. In 2010, we first demonstrated that the presence of a 2-bp frameshift deletion (c.7541_7542delCT) localized in the exon 34 of *NOTCH1* reduced time from diagnosis to initial treatment in a small cohort of 133 CLL patients (9). Thereafter, two unbiased whole-exome studies of large European CLL cohorts also identified *NOTCH1* mutations in different functional domains (7, 8) associated with a significantly shorter overall survival (OS). Additional analysis of numerous independent cohorts of patients confirmed the adverse clinical outcome of *NOTCH1*-mutated CLL in univariate analysis (21, 36, 43, 58, 66, 73–75). Reasons for being allocated to a high-risk category could be explained by a strong correlation of *NOTCH1* mutations with other markers of poor prognosis, including unmutated *IGHV*, high ZAP-70 expression, and trisomy 12. Conflicting data about the independent prognostic effect of the *NOTCH1* mutant in CLL have been reported. According to Rossi et al., multivariate analysis identified *NOTCH1* mutation as an independent predictor of shorter survival, similar to that observed with *TP53* abnormalities (10). By contrast, *NOTCH1* mutations did not retain independent significance as a predictor of time-to-first treatment in one of the largest series of general practice CLL patients (76).

NOTCH1 Mutations in Clinical Prospective CLL Trials

The role of *NOTCH1* has been further refined shifting from the investigation of heterogeneous cohorts of retrospective studies to clinical trials with well-characterized CLL patients. The LRF CLL4 study was the first to validate the importance of *NOTCH1* gene mutations in the context of a prospective trial. In this context, *NOTCH1* was a prognostic biomarker of OS identifying patients with intermediate survival rather than the poor survival associated with *TP53*-deleted or -mutated individuals (26). Conversely, *NOTCH1* was not confirmed to be an independent prognostic factor for progression-free survival (PFS) in both the CLL4 and CLL8 studies (12, 26), employing either less intensive therapies or fludarabine-based CLL treatments. Similar information emerged from the analysis of the Gruppo Italiano Malattie Ematologiche dell'Adulto LLC0405 protocol that used the association of fludarabine and alemtuzumab in high-risk CLL (57), while *NOTCH1* mutation was unexpectedly identified as an independent favorable marker for PFS in fludarabine-refractory patients treated with alemtuzumab in the CLL2H trial of the German CLL Study Group (77). Allogeneic stem cell transplantation (Allo-SCT) offers the only potentially curative treatment option for patients with poor-risk disease. In the CLL3X trial, the use of reduced-intensity Allo-SCT provided long-term disease control in CLL patients independent of the presence of different adverse prognosticators including *NOTCH1*, *SF3B1*, and *TP53* mutations (78).

NOTCH1 Mutations and New Targeted CLL Treatments

Besides NOTCH1, several other signaling pathways and molecules, including the B-cell receptor (BCR)-associated kinases, Bruton tyrosine kinase (BTK) and PI3K, and the antiapoptotic protein BCL-2, are altered in CLL contributing to disease pathogenesis and representing new therapeutic targets. Indeed, the advent of agents inhibiting these key CLL players has dramatically changed treatment algorithms of high-risk CLL and downscaled the role of Allo-SCT as the most effective treatment for this disease. Clinically relevant CLL targeted drugs include inhibitors of the BTK (Ibrutinib) and PI3Kδ (Idelalisib) pathways and the antagonist of the antiapoptotic protein BCL-2 (Venetoclax). The results of randomized clinical trials demonstrated impressive activity of Ibrutinib as single agent for the treatment of relapsed/refractory disease (79) and del 17p CLL patients (80). The drug is well tolerated in the vast majority of patients although there are some common side effects, including an increased rate of

clinically significant bleeding and atrial fibrillation, that have to be managed to optimize outcome (81). Its efficacy in relapsed patients as well as its tolerability has led to its increased use in previously untreated patients, especially in those with poor prognostic markers and/or the elderly. A recent report on a 5-year experience showed that Ibrutinib is associated with a high overall response rate of 89% with complete response rates increasing over time to 29% in treatment-naïve patients and 10% in relapsed/refractory patients (82).

Based on a randomized clinical trial, Idelalisib in combination with rituximab appeared to benefit pre-treated patients with CLL and showed equivalent activity in patients with and without abnormalities of the TP53 pathway (83). Recent evidence demonstrated the lack of adverse prognostic impact of complex karyotype on Idelalisib-treated patients (84) Despite efficacy of Idelalisib in CLL, adverse effects are common and often limit treatment.

Venetoclax is the first BCL-2 inhibitor to enter routine clinical practice. In a phase I study, Venetoclax induced durable responses in 79% of patients with relapsed/refractory CLL, including complete remissions in 20% of patients (85). The antileukemic effects of Venetoclax occurred rapidly, with high response rates, independent of negative prognostic indices. Reductions in tumor burden were consistent and deep in most patients, with the depth of response increasing over time. In the setting of clinical trials, Venetoclax is undergoing testing for use in treatment-naive patients and in combination with other new agents.

The marked benefit of CLL drugs targeting the BCR signaling or BCL-2 has been investigated in the contest of NOTCH1 alterations. In particular, the presence of a *NOTCH1* mutation did not negatively affect the efficacy of Ibrutinib on disease progression outcomes in the extended follow-up from the RESONATE study of relapsed/refractory CLL (86). On the other hand, Idelalisib showed a shorter duration of response in *NOTCH1*-mutated CLL compared with unmutated patients in a phase I trial (87). Recently, the association of *NOTCH1* mutation and low BAX/BCL-2 ratio showed synergistic prognostic properties in patients treated with Ibrutinib, identifying a CLL subset with reduced OS and PFS (88). These data support the rationale to improve the efficacy of Ibrutinib in *NOTCH1*-mutated CLL by using the BCL-2 inhibitor Venetoclax.

In addition, innovative CLL therapies include novel immunotherapeutic regimens using new anti-CD20 antibodies, immune checkpoint inhibitors, and adoptive immunotherapy using modified T lymphocytes (89). To date, only intravenous obinutuzumab, a novel antibody that targets CD20, in combination with chlorambucil, has entered clinical practice as a first-line treatment for patients with CLL (90). The predictive role of *NOTCH1* mutation in the context of immunotherapy will be discussed in a specific section of this review.

Role of *NOTCH1* Mutations in New Integrated CLL Scoring Systems

Collectively, published data support mild negative effects of *NOTCH1* mutations in the prognosis of CLL, and such effects have been incorporated into novel prognostic scoring systems. Rossi et al. combined the genetic status of *NOTCH1* and other CLL mutations to the cytogenetic profile (5). The accuracy of survival prediction is significantly improved by including *NOTCH1* in the cytogenetic hierarchical model (25), leading to reclassification of approximately 20% of low-risk patients into higher-risk classes. According to this integrated model, *NOTCH1*-mutated patients belong to an intermediate-risk group that accounts for approximately 15–20% of newly diagnosed CLL and shows a 10-year survival of 37%. In particular, the detection of clonal *NOTCH1* mutations has allowed to refine the conventional fluorescence *in situ* hybridization-based prognostic stratification of trisomy 12 CLL patients (23, 91).

Clinical Impact of Subclonal *NOTCH1* Mutations in CLL

Clinical information on *NOTCH1* mutations is mainly restricted to lesions represented in more than 10% leukemic cells, the limit of detection for Sanger sequencing. The high sensitivity of PCR methods and ultra-deep NGS allowed the accurate detection of low allele frequency somatic mutations in CLL. Recently, the presence of few *TP53* clones has been associated with poor CLL outcome (92). However, the prognostic impact of *NOTCH1*-mutated subclones is still controversial. We demonstrated that the presence of subclonal *NOTCH1* mutations from early phases of the disease affected patient survival, providing a proof-of-principle that very few leukemia subclones detected at diagnosis are important drivers of the subsequent disease course (66). Ultra-deep NGS revealed subclonal *NOTCH1* mutations that predicted shorter time-to-first treatment irrespective of *IGHV* mutational status (33). Conversely, this approach failed to find statistically significant differences in OS between patients harboring small subclonal mutations of *NOTCH1* and wild-type patients in two independent cohorts with similar numbers of patients (33, 93). These data have been confirmed in a larger cohort of 1,000 patients, where the presence of subclonal *NOTCH1* mutations influenced time to the first treatment but not OS of CLL patients (34). These data suggest the need for additional studies with large uniformly treated datasets to determine whether an allele frequency cutoff is necessary when evaluating these mutations in relation to clinical outcome.

Clinical Impact of Non-delCT *NOTCH1* Mutations and Concurrent Genetic Abnormalities

Screening of *NOTCH1* mutations in CLL identified several genetic alterations outside the canonical region involved in delCT, but little is known about the association between different types of *NOTCH1* mutations and clinical outcome. These mutations are localized both in the coding sequence and 3′-UTR of exon 34, resulting in impaired protein degradation (32). Regardless of the mutation type, all *NOTCH1*-mutated cases retain their adverse prognostic impact on time-to-first treatment in a large retrospective analysis (34). In univariate analysis, patients with 3′-UTR mutations behaved similarly to patients with coding mutations in *NOTCH1* in terms of OS (32). Conversely, the independent impact of non-coding mutations on OS was either non-significant or not reliably assessed because of the small number of cases (60). The distinctive prognostic impacts of different

NOTCH1 mutations can be attributed to their capacity to generate NOTCH1-cleaved proteins with different sizes, suggesting unique leukemogenic effects that need further investigation. One hypothesis is that the presence of a mutated NOTCH1 protein with a specific configuration may correlate with a more sustained NOTCH1 signaling deregulation. As outlined elsewhere in this review, mutations in *NOTCH1* primarily result in premature protein truncation that prevents protein degradation. In this respect, different frameshift mutations may lead to the deletion of distinct portions of NOTCH1 degrons, with a potential impact on the inactivating phosphorylation of the protein. We might not exclude that mutated NOTCH1 proteins acquire additional functions contributing to stabilize the constitutive activation of the signaling.

NOTCH1 Mutation as a Predictive Factor

Recent reports provided evidence of an association between *NOTCH1* mutations and lack of benefit of CD20 antibody therapies, suggesting that NOTCH1 could have predictive potential. In the CLL8 trial, patients carrying *NOTCH1* mutations did not benefit of the inclusion of rituximab to standard fludarabine and cyclophosphamide (FC) chemotherapy, displaying a significantly lower PFS than that of *NOTCH1* wild-type patients and comparable to those treated with the FC protocol (12). These data were further confirmed by the analysis of homogeneously prospective CLL series treated with chemoimmunotherapy followed by a rituximab-based consolidation (94). Ofatumumab and obinutuzumab represent new generations of anti-CD20 monoclonal antibodies that have been developed for potential benefits over rituximab. *NOTCH1*-mutated patients randomized to ofatumumab in the RESONATE trial fared significantly worse than their non-mutated counterparts (86). In addition, *NOTCH1* gene mutation appears to predict reduced efficacy of ofatumumab in relapsed/refractory CLL, according to data from the phase III COMPLEMENT 2 trial. Conversely, mutational status of *NOTCH1* gene did not affect the B-cell depletion efficacy of obinutuzumab against CLL *ex vivo*. These data suggest a potential improved clinical outcome, although direct comparison between *in vitro* and *in vivo* data should be considered with caution. More recently, Estenfelder et al. described the predictive power of the *NOTCH1* gene mutation in 689 patients enrolled in the CLL11 protocol. In this study, obinutuzumab in addition to chlorambucil improved PFS compared with what was observed in patients treated with rituximab and chlorambucil in the subgroups with mutated *NOTCH1* (95). The application of all these findings to clinical practice is not yet fully defined, as these data need confirmation in independent cohorts before being applied in routine practice.

ROLE OF NOTCH1 ALTERATIONS IN CLL LEUKEMOGENESIS

NOTCH1 Alterations in the Transformation Process of Mature B Cells Into CLL Cells

Chronic lymphocytic leukemia has traditionally been considered a malignancy originating from oncogenic transformation of a mature antigen-experienced B cell (96) with a gene expression profiling related to normal CD27+ memory B cells (50, 97). The discovery that CLL cells could be distinguished by the presence or absence of *IGHV* gene mutations led to postulate that antigenic stimulation of naïve B cells might proceed either through a T cell-dependent reaction occurring in the germinal center (GC) and leading to CLL with mutated *IGHV* genes, or in a T cell- and GC-independent manner leading to CLL with unmutated *IGHV* genes (98). Recent studies revealed that *IGHV*-mutated CLL was derived from a previously unrecognized CD5+ CD27+ post-GC memory B cell subset (49), whereas *IGHV*-unmutated CLL was generated from pre-GC CD5+ CD27− B cells that were derived from naïve B cells, a separate lineage of B cell precursor or GC-independent memory B cells (4).

A growing body of evidence indicated that a major driver of CLL pathogenesis was the BCR signaling (99–101), which provided a survival and proliferative advantage to CLL cells, leading to malignant clone selection (98). Given that CLL cells often carry stereotyped BCR, it is likely that a role in the leukemic clone selection is played by recognition of common epitopes or classes of structurally similar epitopes of autoantigens or microbial antigens (102–104). Even normal neighboring cells within the proliferation centers of peripheral lymphoid tissues may favor cell growth or prevent apoptosis of the leukemic clone by providing essential cell–cell and cell–soluble factor interactions (105, 106). Interestingly, it has been shown that stromal cells within lymph nodes expressed the NOTCH1 ligand JAGGED1 that induced NOTCH1 activation in CLL cells (107), suggesting that an aberrant NOTCH1 activity might contribute to CLL development. In line with this hypothesis, the evidence that NOTCH1 is activated in normal naïve, and memory B cells together with the finding that NOTCH1 regulates genes involved in normal B-cell physiology, suggests that in CLL, NOTCH1 activation is the consequence of a dysregulated physiologic signal (13). The genes upregulated by NOTCH1 transcriptional activity were mainly involved in the survival and proliferation of mature B cells by supporting BCR and cytokine signaling and their downstream effectors (13). Consistent with this notion, NOTCH1 activity has been previously shown to synergize with BCR signaling to enhance B-cell activation, suggesting that NOTCH1 signaling might amplify BCR-mediated positive selection events (108).

All these abnormal proliferative signals along with epigenetic changes such as aberrant DNA methylation (109, 110) might cause genomic instability in CLL cells and render DNA more prone to genetic lesions, which represent other important leukemogenic events (25, 111). Genetic lesions include both chromosomal abnormalities such as deletions of chromosomes 13q, 11q, and 17p, trisomy 12, and gene mutations. The most frequent mutations in CLL were found within *TP53*, *NOTCH1, SF3B1*, *MYD88*, *ATM*, and *BIRC3* genes involved in the regulation of key biologically relevant pathways such as DNA repair and cell-cycle control, NOTCH1 signaling, inflammatory pathways, Wnt signaling, and RNA splicing and processing (112). As outlined in this review, *NOTCH1* was the most commonly mutated among these genes, with a high frequency in aggressive subsets of *IGHV*-unmutated CLL expressing particular stereotyped

BCRs (113–115). These findings suggest the potential role of specific stereotyped BCR patterns in promoting the occurrence or selection of *NOTCH1* mutations influencing the outcome of CLL. Interestingly, whereas *TP53* mutations were found mainly as subclonal events that expanded over time favoring CLL progression and therapy resistance (33, 92, 116), *NOTCH1* mutations were either clonal, representing early events in CLL development, or sublconal, indicative of an occurrence at late steps in CLL development and of a selection during disease progression (33, 112). In a temporal study investigating the clonal architecture in CLL, the acquisition of *NOTCH1* mutations was classified as late event being preceded by trisomy 12, del(17p), and del(11q) initial hits (117). Our data indicated that subclonal *NOTCH1* mutations did not appear as temporary events as they identified high-risk patients (66). Rasi et al. reported that subclones harboring *NOTCH1* mutations displayed sensitivity to chemotherapy and did not gain a competitive advantage over the wild-type clones (93). Consistent with these studies, Ouillette et al. presented data supporting that *NOTCH1* mutations did not confer strong selective growth advantages on CLL cells and were not preferentially associated with CLL relapse or CLL clonal dominance (118). Altogether, these findings indicate the need for further investigations to support the role of NOTCH1 alterations in the transformation process and clonal evolution of CLL deriving from a mature B cell.

NOTCH1 Alterations in CLL Hematopoietic Stem/Progenitor Cells: A New Theory on CLL Cellular Origin

Recent findings revolutionized the concept that CLL was a disease arising from a mature B cell, indicating the involvement of a hematopoietic stem cell (HSC) in the transformation process. The first report which challenged the theories on the origin and pathogenesis of CLL demonstrated that HSCs purified from the bone marrow of CLL patients and transplanted into xenograft models had a propensity to generate a CLL-like MBL (119), which is considered the pre-leukemic state of CLL (120, 121). In transplanted mice, CLL-HSCs gave rise to higher number of pro-B cells compared with healthy HSCs, suggesting that the CLL-HSCs were skewed toward a B-cell lineage commitment. In addition, the lymphoid expansions occurring in mice were clonally unrelated to the original CLL patient indicating a *de novo* generation that was probably initiated by genetic abnormalities already acquired at the long-term self-renewing CLL-HSC level (119).

The involvement of NOTCH1 alterations in CLL initiation was shown for the first time by a genomic analysis that revealed the presence of *NOTCH1* mutations in early hematopoietic progenitor cells of CLL patients with *NOTCH1* mutation (122). HSCs and the early hematopoietic progenitors of CLL patients also carry mutations in *SF3B1*, *BRAF*, *EGR2*, *MYD88*, and *NFKBIE* genes that are known to be mutated not only in CLL but also in other hematological malignancies (123–125). The evidence that *NOTCH1* and the above indicated mutations occurred in a progenitor able to undergo both lymphoid and myeloid differentiation also suggested that they might contribute to a pre-leukemic HSC stage, similar to genetic alterations observed in myeloid neoplasms (126, 127). The mechanism through which pre-leukemic HSCs contribute to CLL development is still unknown. We speculate that *NOTCH1* mutations might lead to the development of pre-leukemic mature B cells and increase the possibilities of acquiring further oncogenic events within a specific microenvironmental context or a transformation-permissive cellular background. Recent NGS studies demonstrated that *NOTCH1* mutations appeared at the CD34+/CD19− progenitor and CD34+/CD19+ pro-B cell level (128). This observation suggests that *NOTCH1* mutations may contribute to the expansion of early CLL hematopoietic progenitors representing one of the factors associated with the larger number of pro-B cells detected in the bone marrow of CLL patients compared with healthy donors (119).

Interestingly, we recently showed that CD34+/CD38− cells from CLL patients expressed NOTCH1 receptor and displayed higher levels of the active NOTCH1-ICD than healthy donors, independent of the *NOTCH1* mutational status (129). These data suggested that NOTCH1 activation started in the CLL stem cell compartment anticipating the occurrence of the mutation. Further studies are needed to understand the mechanisms underlying NOTCH1 activation in CLL-HSCs. A role may be played by various NOTCH1 ligands expressed in bone marrow mesenchymal stromal cells of CLL patients (130). However, whether their expression levels in these cells are higher than those in the normal bone marrow cells, and the specific NOTCH1 ligand that is involved in activating NOTCH1 signaling in CLL-HSCs, remain to be defined.

The discovery of NOTCH1 activation in CLL-HSCs and the presence of deregulated pre-BCR signaling driven by *BRAF* and *EGR2* mutations in early CLL hematopoietic progenitors (122) led us to hypothesize that NOTCH1 alterations might cooperate with aberrant pre-BCR signals to favor the occurrence of the premalignant mature B clones. Therefore, the cooperation between NOTCH1 and BCR might be important even for early CLL leukemogenesis.

Although the sequence of the events underlying CLL development is far from being clear, all the findings above suggest a model for CLL development in which NOTCH1 alterations are involved in crucial steps of both HSCs differentiation and mature B-cells activation (**Figure 6**).

All these evidences may have therapeutic implications, suggesting that an anti-NOTCH1 treatment might be able to kill not only mature CLL cells but also their corresponding leukemia stem cells, favoring disease eradication for a definitive cure.

Upstream Signaling Pathways Promoting NOTCH1 Activation in CLL

Little is known regarding the upstream pathways responsible for the deregulated NOTCH1 activation in CLL. Several lines of evidence suggest that NOTCH1 activation in CLL cells is under the control of microenvironmental conditions through ligand-dependent mechanisms. Indeed, the NOTCH1 ligands expressed on stromal cells in the bone marrow (130) or in lymph nodes increased NOTCH1 activity and mediated CLL survival

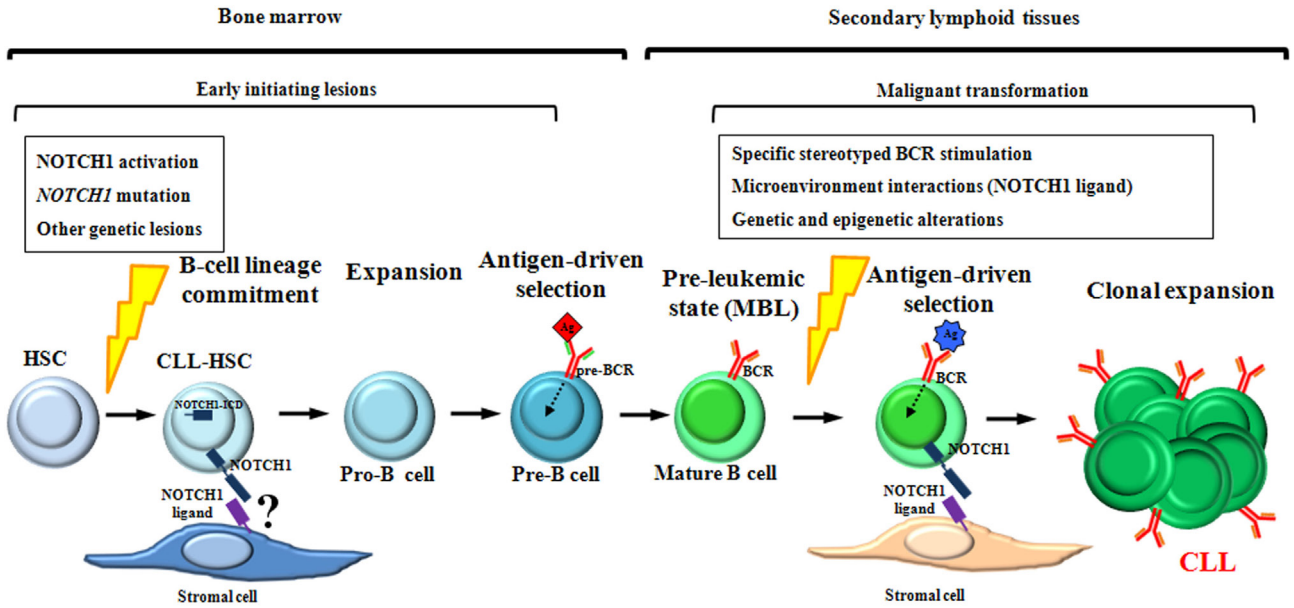

FIGURE 6 | Schematic representation of NOTCH1 involvement during chronic lymphocytic leukemia (CLL) development. NOTCH1 activation and mutation are early oncogenic events acquired within hematopoietic stem cells (HSCs) and maintained in their hematopoietic progeny. As suggested by xenograft models, CLL-HSCs are skewed toward B-cell differentiation giving rise to premalignant B cells that resemble human MBL, the pre-leukemic state of CLL. We hypothesize that NOTCH1 alterations, together with other oncogenic events, contribute to the generation of pre-leukemic mature B cells by favoring the expansion of pro-B cells and by cooperating with pre-BCR signaling during the antigen-driven selection of pre-B cells. In lymphoid tissues, antigenic stimulation of specific stereotyped BCRs, in combination with NOTCH1-related microenvironment interactions and additional genetic lesions, might have a role in the transformation process of pre-leukemic mature B cells into CLL.

regardless of *NOTCH1* mutational status (107). The importance of microenvironmental signals in inducing NOTCH1 cascade is further documented by the evidence that CLL cells in the lymph nodes frequently express NOTCH1-ICD independent of *NOTCH1* mutation (69, 131), especially within the proliferation centers, which represent the key microanatomical sites where CLL cells interact with accessory cells and acquire chemoresistance. Microenvironmental interactions are critical for NOTCH1 activation not only in *NOTCH1* wild-type but also in *NOTCH1*-mutated patients (107).

It has been shown in several malignancies that there is a complex crosstalk between NOTCH1 and the transcription factor nuclear factor-kappa B (NF-κB) (132), another key pathway involved in cancer (133) and in CLL pathogenesis (134). Furthermore, NF-κB can indirectly trigger the NOTCH1 signaling pathway by inducing the expression of the NOTCH1 ligand JAGGED1 during B cell activation (135). In a previous study aimed to explore the mechanisms underlying NOTCH1 activation in CLL cells devoid of *NOTCH1* mutations, we excluded the possibility that genetic lesions of NF-κB regulators or *JAGGED1* and *JAGGED2* genes were involved in NOTCH1 activation in these leukemic cells (136).

Secchiero et al. suggested a potential role of the tumor suppressor protein p53 in the induction of NOTCH1 pathway in CLL (137). They showed that the anti-proliferative and pro-apoptotic agent nutlin-3 resulted in a p53-dependent increase of NOTCH1 mRNA and protein levels in CLL cells. These results, along with the findings that γ-secretase inhibitors (GSIs) increased the cytotoxicity of nutlin-3, suggested that the p53-mediated expression of NOTCH1 initiated an antiapoptotic feedback mechanism limiting the cytotoxic actions of nutlin-3. The p53 dependence of nutlin-induced NOTCH1 expression was confirmed by the observation that the increase in NOTCH1 occurred in *TP53* wild-type myeloid and lymphoid leukemic cell lines but not in cell lines lacking a functional *TP53* gene. Furthermore, whereas the silencing of p53 expression abrogated the induction of NOTCH1 by nutlin-3, the silencing of NOTCH1 enhanced the cytotoxic effect of nutlin-3, highlighting that NOTCH1 was an antiapoptotic target of p53 in both lymphoid and myeloid leukemia cells (137).

There is evidence that various recurrent gene mutations lead to a dysregulated NOTCH1 activation in CLL. In a transcriptomic analysis aimed to characterize the functional impact of *SF3B1* mutations on CLL, Wang et al. identified NOTCH1 signaling as one of the pathways affected by this mutation (138). Specifically, they demonstrated that *SF3B1* mutations increased NOTCH1 signaling through altered splicing of *DVL2*, a core canonical Wnt pathway member and negative regulator of NOTCH1 activation. Mutations in the *FBXW7* gene can also deregulate the NOTCH1 signaling pathway in CLL. *FBXW7* encodes an E3 ubiquitin ligase that regulates the stability of NOTCH1-ICD by targeting it for ubiquitination and degradation (139). Inactivating mutations of *FBXW7* are observed in 2.5% of CLL cases (36), suggesting a role of genetic aberrations

in the NOTCH1-ICD degradation machinery in CLL pathogenesis (32, 140). It has been recently reported that even *MED12* mutations contributed to activate NOTCH1 signaling in CLL (38). MED12, MED13, CDK8, and cyclin C form a four-subunit kinase module that is associated with a 26-subunit mediator core complex, which regulates many transcriptional programs important for development and/or tumorigenesis (141). CDK8 represses NOTCH1 signaling-driven transcription by phosphorylating the PEST domain of NOTCH1-ICD, an event required for its ubiquitination by the E3 ligase FBXW7 and subsequent degradation (142). It has been proposed that the increased levels of NOTCH1-ICD detected in CLL cells in the context of *MED12* mutations are mediated by an aberrant CDK8 kinase activity (38). Inactivating mutations of the *SPEN* gene, which were detected in approximately 1% of CLL cases, also contributed to increase NOTCH1 activation (20, 32). SPEN is a co-repressor of RBPJ, the nuclear effector of the NOTCH1 pathway, and a putative negative regulator of NOTCH1 signaling (143).

These latter studies provided important information about the genetic background in which NOTCH1 activation occurred in CLL, but the current knowledge about the regulation of NOTCH1 signaling in CLL does not explain the mechanisms underlying the constitutive expression and activation of NOTCH1 in peripheral blood CLL cells lacking *NOTCH1* mutation. We hypothesize that one of these mechanisms may involve NOTCH1-ligand interactions between CLL cells themselves, given that they also constitutively express the ligands JAGGED1 and JAGGED2 (19, 144). However, these interactions may also not contribute to NOTCH1 activation, because, as reported in other cell types, when NOTCH1-ligand interactions occur within the same cell (*cis*-interactions), they may lead to suppression rather than activation of NOTCH1 signaling (145, 146). Another possible mechanism underlying the constitutive NOTCH1 activation in circulating CLL cells may be ligand independent through a disrupted endosomal trafficking and an aberrant regulation of NOTCH1 receptor during its recycling, ubiquitination, and degradation (147–149). Finally, we cannot exclude the possibility that other pathways relevant for CLL pathogenesis, including the NF-κB signaling (150) and those triggered by stimulation of BCR, cytokine/chemokine receptors, or CD40 molecule, are involved in activating NOTCH1 in CLL. In this context, we demonstrated that interleukin-4, a T cell-derived cytokine involved in CLL pathogenesis and known inducer of CLL cell survival (151), potentiated NOTCH1 expression and activation in promoting its prosurvival effect (152).

Pathogenic Role of *NOTCH1* Stabilizing Mutations in CLL

The loss of the PEST domain by *NOTCH1* mutations has been predicted to result in NOTCH1-ICD impaired degradation with its consequent stabilization and increased NOTCH1 signaling (29). One of the first demonstrations of the functional impact of *NOTCH1* mutations in CLL was provided by Arruga et al., who revealed the presence of the truncated NOTCH1-ICD protein encoded by the mutant *NOTCH1* allele in CLL cells. Compared with *NOTCH1*-wild-type cases, *NOTCH1*-mutated CLL cells displayed a more intense activation of the NOTCH1 pathway that conferred a marked resistance to drug-induced apoptosis (107). However, although it is well documented that *NOTCH1* mutation stabilizes NOTCH1 signaling in CLL by increasing the stability of the truncated NOTCH1-ICD (107), the molecular mechanisms underlying this effect are poorly defined. In this context, we demonstrated that, in *NOTCH1*-mutated CLL, the loss of the PEST domain altered the phosphorylation status of mutated NOTCH1 protein and generated a phosphorylated NOTCH1-ICD form that accumulated in the nucleus, leading to increased NOTCH1 signaling and prolonged CLL cell survival (153). It has been previously shown in other cell types that, whereas phosphorylation of PEST domain targeted NOTCH1-ICD for proteasomal degradation, attenuating its signaling (142), phosphorylation in regions upstream of the PEST domain could increase NOTCH1 signaling (154) and even mediate NOTCH1-dependent oncogenesis (155). We also demonstrated that NOTCH1-ICD phosphorylation is reversed by Idelalisib (153), a selective inhibitor of PI3Kδ, currently used for CLL therapy (83, 87, 156). These data suggest a possible involvement of the PI3Kδ oncogenic pathway in the phosphorylation of mutated NOTCH1-ICD, and, more important, that this event may represent a new potential therapeutic target in *NOTCH1*-mutated CLL.

Downstream Effects of NOTCH1 Alterations in CLL

NOTCH1-Dependent Transcriptional Program

To shed light on the NOTCH1-controlled biological functions in CLL, a recent study investigated the NOTCH1-dependent CLL transcriptional signature. This comprehensive analysis revealed that NOTCH1 activation, independent of mutational status, leads to upregulation of a high number of transcripts, including known NOTCH1 targets, such as *HES/HEY* family members, *NRARP* and *DTX1*, antiapoptotic and cytokine-chemokine genes, as well as genes involved in immune and signaling pathways relevant for the development and activation of B cells (13) (**Figure 7**). Among these latter, there are BCR-associated pathway genes, including upstream pathway members (e.g., LYN, SYK, BLK, BLNK, and CR2), and downstream effectors, such as MAPK and NF-κB cascade members. Notably, NOTCH1 signaling also induces its own transcript and that of its ligand JAGGED1, that is known to be expressed in CLL cells (19), suggesting a positive feed-forward loop in NOTCH1 activation.

Effects on Antiapoptotic Pathways

Recent advances demonstrated a close relationship between oncogenic NOTCH1 signaling and the control of cell survival, proliferation, homing, and chemoresistance (**Figure 7**). Among antiapoptotic effectors downstream of NOTCH1 signaling, there is the transcription factor NF-κB whose overexpression and increased activity play a pivotal role in CLL pathogenesis by promoting tumor cell proliferation and survival (157–159). We provided the first evidence that in CLL cells, NOTCH1 signaling increased the activity of NF-κB and the expression of cellular inhibitor of apoptosis protein 2 (c-IAP2) and X-linked inhibitor of apoptosis protein(XIAP) (19). A role of NOTCH1 in activating NF-κB has been also demonstrated in recent studies performed in CLL cells with *NOTCH1* mutation (160). In addition, a

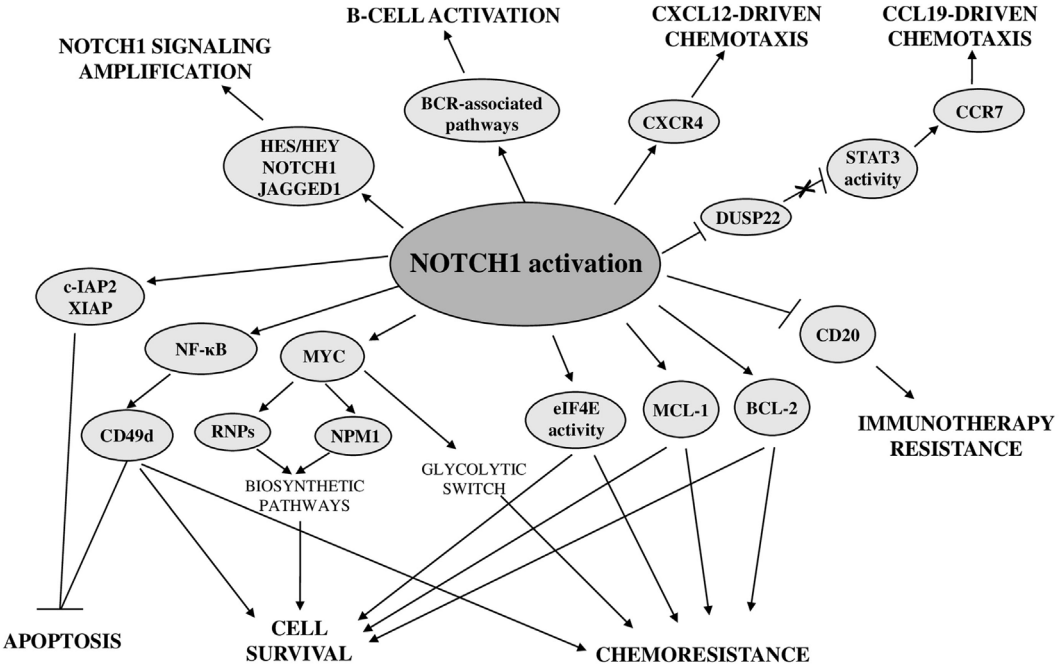

FIGURE 7 | Biological consequences of deregulated NOTCH1 in chronic lymphocytic leukemia.

NOTCH1-dependent activation of NF-κB has been shown to drive the expression of CD49d, a well-known key regulator of microenvironmental interactions and negative prognosticator in CLL (59). The effect of NOTCH1/NF-κB axis on CD49d expression is stronger in *NOTCH1*-mutated CLL cells, but it is not restricted to this CLL subset. The higher levels of CD49d expression and NF-κB activation in *NOTCH1*-mutated compared with *NOTCH1*-wild-type CLL are likely due to the higher NOTCH1 activation induced by mutations (7, 29, 107). Another important mechanism by which constitutive NOTCH1 signaling promotes CLL cell survival is by sustaining the expression of the antiapoptotic myeloid cell leukemia 1 (MCL-1) protein and the activity of the translational regulator eukaryotic translation initiation factor 4E (eIF4E) (152), which are both implicated in CLL pathogenesis (161, 162). NOTCH1 signaling does not regulate MCL-1 expression at the transcriptional level but by preventing its proteasomal degradation, indicating that this effect may be mediated by a non-canonical NOTCH1-ICD-activated signaling rather than by NOTCH1-ICD transcriptional activity (152).

Another antiapoptotic target of NOTCH1 signaling in CLL is BCL-2 (13), a factor with a well-established role in CLL pathogenesis (85). The increased NOTCH1 activation, induced in CLL cells cultured with bone marrow mesenchymal cells, confers chemoresistance by upregulating BCL-2 expression (130).

Effects on CLL Therapy Resistance

NOTCH1 mutations are enriched among chemorefractory CLL patients indicating a potential relationship between deregulated *NOTCH1* and response to treatment. *In vitro*, NOTCH1 activation promotes CLL cell survival by sustaining the expression of MCL-1 and the activity of eIF4E, proteins that contribute to chemotherapy resistance (152). In addition, CLL cells from patients with mutated *NOTCH1* show a marked resistance to *in vitro* fludarabine-induced apoptosis, which is abrogated in the presence of NOTCH1 inhibitors (107, 163). All these findings highlight the importance of targeting NOTCH1 signaling for CLL treatment, especially in combination with agents, such as fludarabine, whose poor efficacy is mainly due to the elevated MCL-1 expression and eIF4E activity detected in these leukemic cells (162, 164).

The presence of *NOTCH1* mutations has been also associated with a relative resistance to anti-CD20 immunotherapy in a prospective clinical study comparing the effectiveness of the FC regimen versus the FC plus rituximab regimen (12). These clinical data are sustained by biological evidences that *NOTCH1*-mutated CLL cells are characterized by lower CD20 expression and lower lysis induced by anti-CD20 exposure *in vitro* (65). These studies showed that accumulation of mutated NOTCH1-ICD in the nucleus was responsible for a dysregulation of histone deacetylase (HDAC)-mediated epigenetic repression of CD20 expression, by altering the balance of the two functions of RBP-Jk as transcriptional activator when complexed with NOTCH1-ICD, or transcriptional repressor when complexed with HDACs. Specifically, in *NOTCH1*-mutated CLL cells, RBP-Jk was less complexed with HDACs which so were more bound to the CD20 promoter, resulting in epigenetic silencing of gene expression.

Effects on Pathways Regulating CLL Homing and Cell Growth

A NOTCH1-ICD-dependent epigenetic modulation of gene expression also affects other targets by influencing signaling pathways regulating growth and homing of CLL cells. It has been

demonstrated that NOTCH1 signaling reduced the expression of the tumor suppressor gene *DUSP22* through a methylation-dependent mechanism. *DUSP22* downregulation led to constitutive activation of signal transducer and activator of transcription 3 (STAT3) signaling which increased the expression of CCR7 promoting CCL19-driven chemotaxis (165).

Analysis of NOTCH1-dependent transcriptional signature showed that even *CXCR4* is a direct target of NOTCH signaling in CLL (13). This gene encodes a chemokine receptor, highly expressed in CLL cells, relevant for their chemotaxis toward microenvironmental cells producing the CXCL12 ligand (166). The CXCL12/CXCR4 axis is crucial for the dissemination of CLL cells to lymphoid organs and has been shown to be associated with poor prognosis (167).

Another target gene of NOTCH1 transcriptional activity is *CCND3* (13) that encodes a cyclin involved in G1/G2 transition, suggesting a role of NOTCH1 in CLL cell proliferation. Gene expression profiling studies demonstrated that NOTCH1 activation might confer cell growth and/or proliferation advantages to CLL cells even by upregulating genes related to ribosome biogenesis such as nucleophosmin 1 (*NPM1*) and ribosomal proteins (*RNPs*) (70). These effects were mainly associated with *NOTCH1* mutation but were also observed in *NOTCH1*-wild-type CLL cells cocultured with JAGGED1-expressing stromal cells. Bioinformatics analyses and *in vitro* activation/inhibition of NOTCH1 signaling suggested a role of MYC as a mediator of NOTCH1 effects on NPM1 and RNP expression in CLL cells. Chromatin immunoprecipitation experiments performed on NOTCH1-ICD transfected CLL-like cells showed the direct binding of NOTCH1 to the *MYC* promoter and transfection with MYC-specific small interfering RNA reduced NPM1 expression, confirming that MYC was a transcriptional target of the NOTCH1 activation complex in CLL. Furthermore, the evidence that modulation of NOTCH1 signaling directly influences MYC transcript levels corroborates the hypothesis that a mutation-dependent increase of NOTCH1 activation may be responsible for a higher MYC-dependent transcription of *NPM1* and *RNPs* (168). Activation of a NOTCH1–c-Myc axis is also involved in a glycolytic switch induced in CLL cells by stromal cells, contributing to stroma-mediated chemoresistance (169). Targeting glucose metabolism may represent a new therapeutic approach for CLL with deregulated NOTCH1 to overcome stromal cell-mediated drug resistance in this disease.

FUTURE PERSPECTIVE: THERAPEUTIC TARGETING OF NOTCH1 IN CLL

Despite increasing insight into its tumor biology, CLL remains an incurable disease. Currently, immunochemotherapy is the standard of care for treatment-naïve patients (170), as it significantly improves clinical outcome. However, this approach is associated with adverse events and yields poor results in those patients with high-risk features. BCR and BCL-2 inhibitors are revolutionizing the treatment landscape of this disease (79, 83, 85), indicating that a molecularly targeted therapy can lead to high-rate improvement of outcome in CLL. However, several issues limit the advances of new CLL inhibitors, including the inability to eradicate the tumor and resistance/progression, suggesting the need for alternative treatment approaches.

The growing evidence for a critical role of the NOTCH1 pathway in CLL makes this cancer gene a target to design a custom-made treatment for this blood disease. The most promising opportunity derived from the discovery of NOTCH1-deregulated signaling and mutations in CLL is the development of anti-NOTCH1-targeted therapies. Notably, a broad number of patients may benefit of an anti-NOTCH1 therapy, given the importance of enhanced NOTCH1 signaling in CLL, even without carrying a *NOTCH1* mutation (13).

The NOTCH1 pathway is highly regulated at multiple steps; thus, a number of genetic and pharmacological strategies are available to block or silence this signaling network. Currently, GSIs are the most extensively explored anti-NOTCH1 molecules in different cancers. Treatment with GSIs induces apoptosis by inhibiting the proteolytic system responsible for the activation of NOTCH1 receptors. A considerable number of early phase trials demonstrated the anti-cancer efficacy of GSIs in solid tumors. In CLL, the use of GSI-I exhibited cytotoxicity on leukemic cells coupled with downregulation of NOTCH1 activity *in vitro* (171). Similarly, the combination of the clinically relevant GSI PF-03084014 and fludarabine demonstrated antitumor effects in primary *NOTCH1*-mutated CLL cells (163).

Concerns about off-target toxicity (172, 173) delayed the clinical translation of GSI-based therapy, including CLL treatment, and suggested the need of more selective antagonists. Several antibodies blocking the activity of individual NOTCH1 receptors have been developed for the treatment of solid tumors, demonstrating limited toxicity compared with GSIs. A humanized antibody targeting NOTCH1 (OMP-52M51) did not affect intestinal goblet cells' differentiation in pre-clinical studies (174, 175) and entered phase I trials in solid tumors and relapsed/refractory lymphoid malignancies (NCT01778439, NCT01703572). Although promising, translation of these results into novel therapeutic approaches for CLL with aberrant NOTCH1 activation will require further investigation, given a limited value shown in the contest of T-ALL (175).

Recently, the search for alternative approaches to a GSI-based NOTCH1 inhibition led to the identification of new NOTCH1 modulators in T-ALL (176). In CLL, we use a similar approach to demonstrate the capacity of the small molecule bepridil to preferentially target NOTCH1 over NOTCH2 and induce apoptosis *in vitro* and *in vivo* (177). However, bepridil is a known calcium channel blocker with potential toxic effects in non-diseased cells that may limit its repositioning in CLL targeted therapy.

On-target delivery remains the principal obstacle in developing anti-NOTCH1 drug approaches. As for most cancer mutations and deregulated pathways, NOTCH1 is not an obvious drug target, given its role in different biological processes and cell types (15, 178). This represents a potential limit for an anti-NOTCH1 therapy, as ubiquitous targeting of NOTCH1 in non-leukemic cells may trigger toxic effects. In CLL, nanoparticle-based drug delivery platforms have emerged as suitable vehicles for specific targeting cytotoxic drugs against the CD20 expressed on neoplastic

B cells (179). The use of nanotechnology represents a potential approach (180) to help improving the selectivity, effectiveness, and safety of molecules that inhibit NOTCH1.

Finally, the efficacy of NOTCH1 targeting may be also reduced by intraclonal heterogeneity of the CLL clones. Indeed, subclones with genetic lesions other than *NOTCH1* mutations frequently coexist in CLL (117). This limit implies the need to test combinations of anti-NOTCH1 molecules with drugs targeting different components of the molecular network in CLL cells. Preliminary evidence shows that GSIs enhance the antileukemic activity of the BTK inhibitor Ibrutinib in CLL cells (181). We believe that the future of CLL treatment lies in the association of small molecule inhibitors targeted at the BCR pathway and the antiapoptotic BCL-2 protein. Upcoming research efforts will need to investigate the value and potential integration of NOTCH1 targeted agents into this CLL treatment algorithm.

AUTHOR CONTRIBUTIONS

ER and PS organized the plan and structure of the manuscript, and all the authors contributed to the redaction.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00229/full#supplementary-material.

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REVIEW
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Notch Signaling as a Regulator of the Tumor Immune Response: To Target or Not To Target?

*Mahnaz Janghorban[1,2], Li Xin[1,3], Jeffrey M. Rosen[1,3] and Xiang H.-F. Zhang[1,2,3,4]**

[1] *Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, United States,* [2] *Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, TX, United States,* [3] *Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX, United States,* [4] *McNair Medical Institute, Baylor College of Medicine, Houston, TX, United States*

The Notch signaling pathway regulates important cellular processes involved in stem cell maintenance, proliferation, development, survival, and inflammation. These responses to Notch signaling involving both canonical and non-canonical pathways can be spatially and temporally variable and are highly cell-type dependent. Notch signaling can elicit opposite effects in regulating tumorigenicity (tumor-promoting versus tumor-suppressing function) as well as controlling immune cell responses. In various cancer types, Notch signaling elicits a "cancer stem cell (CSC)" phenotype that results in decreased proliferation, but resistance to various therapies, hence potentially contributing to cell dormancy and relapse. CSCs can reshape their niche by releasing paracrine factors and inflammatory cytokines, and the niche in return can support their quiescence and resistance to therapies as well as the immune response. Moreover, Notch signaling is one of the key regulators of hematopoiesis, immune cell differentiation, and inflammation and is implicated in various autoimmune diseases, carcinogenesis (leukemia), and tumor-induced immunosuppression. Notch can control the fate of various T cell types, including Th1, Th2, and the regulatory T cells (Tregs), and myeloid cells including macrophages, dendritic cells, and myeloid-derived suppressor cells (MDSCs). Both MDSCs and Tregs play an important role in supporting tumor cells (and CSCs) and in evading the immune response. In this review, we will discuss how Notch signaling regulates multiple aspects of the tumor-promoting environment by elucidating its role in CSCs, hematopoiesis, normal immune cell differentiation, and subsequently in tumor-supporting immunogenicity.

Keywords: Notch, cancer stem cell, immune response, immune-suppressive microenvironment, Notch therapy

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****Correspondence:***
Xiang H.-F. Zhang
xiangz@bcm.edu

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INTRODUCTION

The Notch pathway is regulated by short-range cell–cell signaling activated by interaction of one of the Notch receptors (Notch1–4) with different types of "canonical" ligands (Jagged1, Jagged2, DLL1, DLL3, or DLL4) [reviewed in Ref. (1)] or non-canonically through activation of other pathways such as NFκB, Wnt, TGF-β, and STAT3 [reviewed in detail elsewhere (2–4)]. The canonical Notch pathway is activated by a sequence of proteolytic events following binding of the ligand to the Notch receptor. First, the Notch receptor is cleaved by ADAM metalloproteases at the S2 site, generating a membrane-anchored Notch extracellular truncation fragment, which is further cleaved by the γ-secretase complex at S3 and S4 sites (1). Following γ-secretase cleavage, the Notch intracellular domain (NICD) releases and translocates to the nucleus where it associates with

CSL—the transcriptional repressor CBF1/suppressor of hairless/Lag-1—(or the human homolog RBPJ—recombining binding protein suppressor of hairless). This is accompanied by recruitment of many transcriptional co-activators such as mastermind like (MAML1–3) to initiate the transcription of target genes (1). Because of lack of a DNA-binding motif, Notch binds to its canonical CSL (RBPJ) complex, or other pathway co-activators/repressors. Thus, Notch can regulate other target genes controlled by the TGF-β, NFκB, mTORC2, PI3K, and HIF1α pathways in the cytoplasm and/or nucleus. Although target gene expression is cell-type and context dependent, Hes and Hey families are the most characterized target genes of Notch signaling pathway (**Figure 1**) (5, 6).

This review is focused on the role of Notch signaling as a regulator of the tumor immune response. We will first describe the role of Notch during normal immune cell homeostasis and activation of effector cells, and then discuss the interplay between tumor cells [cancer stem cells (CSCs)] and immune cells in the tumor microenvironment. This information will need to be taken into consideration when designing new therapeutic strategies for Notch inhibition.

NOTCH IN NORMAL IMMUNE CELL HOMEOSTASIS

Notch signaling is one of the key regulators of hematopoiesis, immune cell differentiation, and inflammation and is implicated in various autoimmune diseases and tumor-induced immunosuppression. Notch can control the differentiation and function of both innate and adaptive immunity including dendritic cells (DCs), natural killer (NK) cells, and various T cell types [Th1, Th2, and the regulatory T cells (Tregs)].

Normal Immune Cell Differentiation

Numerous studies have investigated the role of Notch during embryonic and adult hematopoiesis. Various Notch ligands promote self-renewal of hematopoietic stem cells (HSCs) and suppress differentiation. Notch1 expression has been identified in bone marrow progenitor cells. In addition, while Jagged-1 expression in osteoblasts correlates with increased numbers of HSCs, canonical Notch signaling seems to be dispensable for adult hematopoiesis in bone marrow (7–9). More importantly, Notch signaling plays an essential role during T cell lineage commitment. Notch acts as a checkpoint to ensure T cell lineage differentiation by opposing the commitment to other cell lineages, such as B cells, myeloid cells, and DCs. The role of Notch signaling during each stage of immune cell development has been reviewed in detail elsewhere (10).

Notch1 regulates T cell lineage commitment from the common lymphoid progenitor cells and suppresses B cell development in the bone marrow (11, 12). Notch1 knockdown completely blocks T cell development and increases the accumulation of ectopic B cells in the thymus (12, 13). Moreover, Notch1 [and maybe also Notch 2 (14)] regulates the early phases of T cell differentiation in the thymus (through DLL4), but its expression needs to be decreased before T cells can fully differentiate (15). Upon migration of immature B cells from bone marrow to spleen, an increased level of Notch2 expression regulates the maturation of a subset of B cells that reside in the marginal zone, MZB cells. However, Notch2 does not control other mature B cells including follicular B cells and plasma cells (16). Moreover, *in vitro* studies

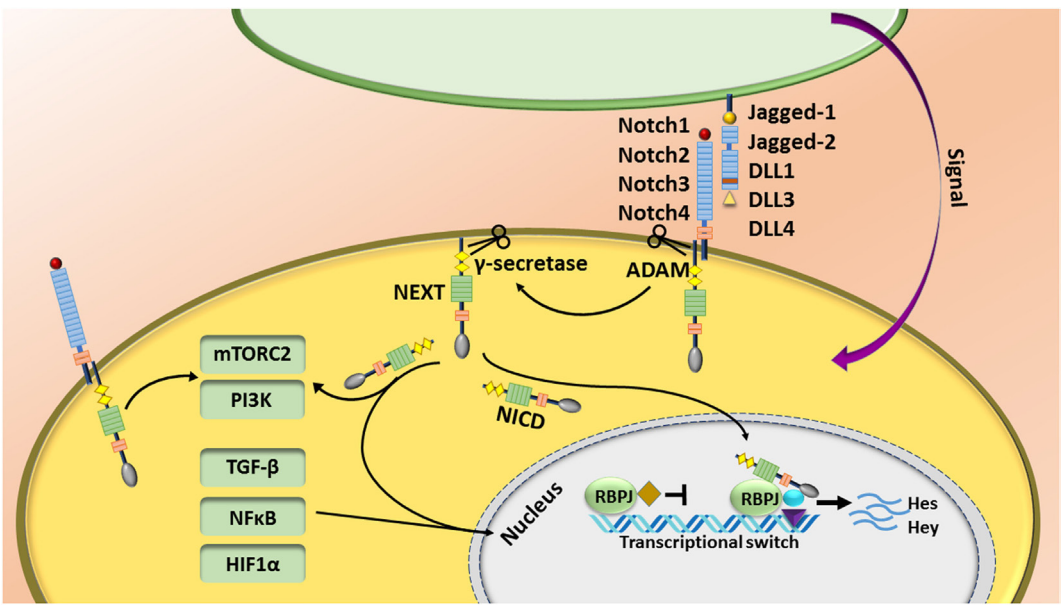

FIGURE 1 | The Notch pathway is regulated by short-range cell–cell signaling. Notch is activated by interaction of one of the Notch receptors with its ligands and induces a sequence of proteolytic event leading to production of the Notch intracellular domain (NICD). NICD translocates to the nucleus, binds to the RBPJ complex, and recruits other transcriptional co-activators to initiate the transcription of target genes. Notch also regulates other target genes controlled by the TGF-β, NFκB, mTORC2, and HIF1α pathways.

have shown that Notch signaling enhances T- and NK cell differentiation from human hematopoietic progenitor cells ($CD34^+$), while inhibiting B cell differentiation (14, 17). Notch also has opposing roles in controlling cell fate decisions between two different types of NK cells, i.e., conventional NK cells versus innate lymphoid cell (ILC)-derived natural cytotoxicity receptor (NCR) $NKp44^+$ group (NCR^+ILC3)—at different maturational stages of progenitor cells. This is dependent on the type of the progenitor cells. Notch can augment the differentiation of one type of these NK cells while suppressing the other types (14).

Notch also regulates the differentiation of myeloid cells. Notch signaling (transient activity) has been shown to mediate myeloid differentiation by increasing mRNA levels of the myeloid-specific transcription factor PU.1 (18). Notch1 and Notch2 are highly expressed in monocytes and in combination with GM-CSF and TNFα skew cell fate decision of DCs over macrophages (19). DLL and Jagged ligands appear to elicit opposite effects in myeloid cells, where fibroblasts expressing DLL1 promote differentiation of DCs and activation of Notch, although Jagged-1 promotes immature myeloid cells (20). In the spleen, Notch2 (probably through DLL1, as expressed in the marginal zone) controls the survival of DCs (also identified as $Cx3cr1^{low}$ $Esam^{high}$ DC subset), which is required for efficient T cell priming (21). Altogether, these studies have demonstrated spatiotemporally regulated roles of Notch in immune cell differentiation.

Effector T Cell Differentiation

During the immune response, antigen-presenting cells (APCs) activate naïve T cells and trigger their clonal cell expansion into various T helper cells dictated by different sets of signaling pathways and cytokines. Notch signaling controls many aspects of effector T cell differentiation including $CD4^+$ T helper cells—Th1, Th2, Th9, and Th17—Tregs, and $CD8^+$ T cells [reviewed in Ref. (22)]. Functionally, Th1 cells are required for clearance of intracellular pathogens and viruses and mediating autoimmune diseases. Th2 cells mediate immunity against helminth parasites and allergic reactions. Th17 cells are critical for controlling extracellular bacterial and fungal infections and mediating autoimmunity (22, 23). Tregs are involved in the regulation of peripheral self-tolerance and tumor immunosuppression (24).

A low level of expression of Notch1 and Notch2 has been detected in naïve $CD4^+$ and $CD8^+$ T cells and their expression is activated through many canonical and non-canonical mechanisms such as T cell receptor (TCR) signaling and different cytokines (22, 25). The role of Notch in regulating Th1 and Th2 differentiation versus function is somewhat controversial. Notch appears to act as an unbiased amplifier of these Th programs by sensitizing cells to their microenvironmental cues, but lacks the direct capacity of instructing specific Th differentiation (23). Notch directly regulates gene expression of master regulators of Th1: T-bet and interferon-γ (IFNγ) (23), Th2: IL4 (also in NKT cells) and GATA3 (26–29), and Th17: IL17 and Rorγt (23, 30). Therefore, depending on the strength of the upstream inflammatory signaling, Notch may serve as a hub to regulate and also synergize with key signaling pathways important for Th commitment such as mTOR–AKT and NFκB to regulate Th differentiation (22). However, alternatively, there are other studies that have shown a more direct role of Notch in the control of the types immune cell responses, e.g., both *in vitro* and *in vivo* studies have shown a greater association of DLL family ligands with the development of IFNγ-secreting Th1 cells and Th17, while Jagged family ligands elicit Th2, Th9, and Treg responses (10, 22, 27). Notch also controls the survival and maintenance of memory $CD4^+$ T cells which are essential for preventing recurrent infection (31). The studies highlight the complexity of the Notch signaling pathway during immune cell response.

Regulatory T cells are an immunosuppressive subpopulation of $CD4^+$ cells that express Forkhead box P3 (FoxP3) and are generated from naïve $CD4^+$ T cells following stimulation with TGF-β1 (32). Tregs are involved in the regulation of peripheral self-tolerance, tissue repair, and the control of pro-inflammatory immune responses, as well as the prevention of the immune response to tumors (24). Both Jagged-1 and Jagged-2 increase the generation of Tregs, e.g., Jagged-2 expression on hematopoietic progenitor cells increases the expansion of Tregs (33). Bone marrow mesenchymal stem cells educate DCs to promote a Treg expansion *via* Jagged-1 (34). Interestingly, upon Th2 stimulation, bone marrow-derived DCs express Jagged-2 (33), which can potentially regulate Treg function. Notch-1 and TGF-β cooperatively regulate the master regulator of Tregs, Foxp3 gene expression and hence directly induce peripheral Tregs (32). Altogether, Notch signaling is important in the regulation of Tregs, which can contribute to tumor-induced immunosuppression as discussed later in this review.

$CD8^+$ T cell differentiation is also regulated by Notch signaling. Naïve $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs) upon recognition of antigens presented by MHC class 1 APCs. CTLs exert their functions by secreting IFNγ, transporting perforins and granzymes to lyse target cells, and inducing apoptosis through FAS-FAS ligand (FASL) (10). Notch1 directly binds to the promoter of EMOES—one of the master regulators of CTL differentiation, perforin, and granzyme B—and therefore enhances CTL differentiation (35). DLL1 expressing DCs activate Notch2 in $CD8^+$ T cells and promote T cell cytotoxicity by increasing the expression of granzyme B (36). Moreover, activated $CD8^+$ T cells choose between short-lived terminal effector cells (TECs) or memory precursor cells (MPCs). Notch signaling controls the fate decision of TECs over MPCs (37, 38), providing more evidence illustrating the complexity of Notch regulation of different cell fate decisions and functions.

Interestingly, recent studies have discovered a new subset of DCs that express high levels of DLL4 under inflammatory conditions (39, 40) [reviewed in Ref. (41)]. Immature DCs are fully differentiated through activation of pattern-recognition receptors including toll-like receptors (TLRs). Immature DCs express low levels of DLL4 and upon activation by TLR7/8, express high levels of DLL4 (41). It seems that at some point during DC differentiation, DLL4 expression is elevated and that $DLL4^+$ DCs have a greater ability than $DLL4^-$ DCs to promote the generation of Th1 and Th17 T cells producing IFNγ and IL-17, respectively (39, 40). Interestingly, inhibiting DLL4 abrogates efficient effector T cell function (42, 43). $DLL4^+$ DCs are also important for promoting the differentiation and expansion of $CD8^+$ T cells (41). Altogether, these results show that Notch plays an important role in regulating normal immune cell differentiation and

the regulation of immune cell function. The role of Notch in the tumor immune response will be discussed in more detail below.

NOTCH IN THE CANCER IMMUNE RESPONSE

It is now well appreciated that inflammatory responses play key roles at different stages of tumor development, from initiation to malignant conversion, invasion, and metastasis, as well as therapy resistance and recurrence (44). Depending on its type, tumor-induced inflammation consists of innate immune cells including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells (MDSCs), DCs, and NK cells and adaptive immune cells such T cells (effector cells—Th cells and Tregs—and NKT cells) and B cells (44). The interplay between tumor cells and immune cells in the tumor microenvironment dictates the overall immune surveillance and responses to therapies, and subsequently clinical outcome and patient survival. Notch regulates many components of the tumor microenvironment including immune cells as well as fibroblasts, endothelial, and mesenchymal cells (**Figure 2**) (42, 43, 45, 46).

Tumor Initiation and Cancer Stem Cells (CSCs)

Immune cells contribute to and enhance tumor initiation and progression through various mechanisms including activating chronic inflammation and tissue repair, angiogenesis, and the induction of pre-malignant cell proliferation, and/or CSCs. CSCs or tumor-initiating cells are a subpopulation of cancer cells that mediate primary tumor formation and metastasis, as well as resistance to therapies through self-renewal activities and immune evasion. Moreover, they are associated with cancer progression, resistance and recurrence, and clinical outcome in cancer patients (47). Elevated Notch pathway activity has been detected in the CSC subpopulation of many cancer types including medulloblastoma, breast, intestine, prostate, and colon cancer, pancreatic ductal adenocarcinoma (PDAC), and squamous cell carcinoma (47–52). The role of Notch in breast cancer and breast CSCs is

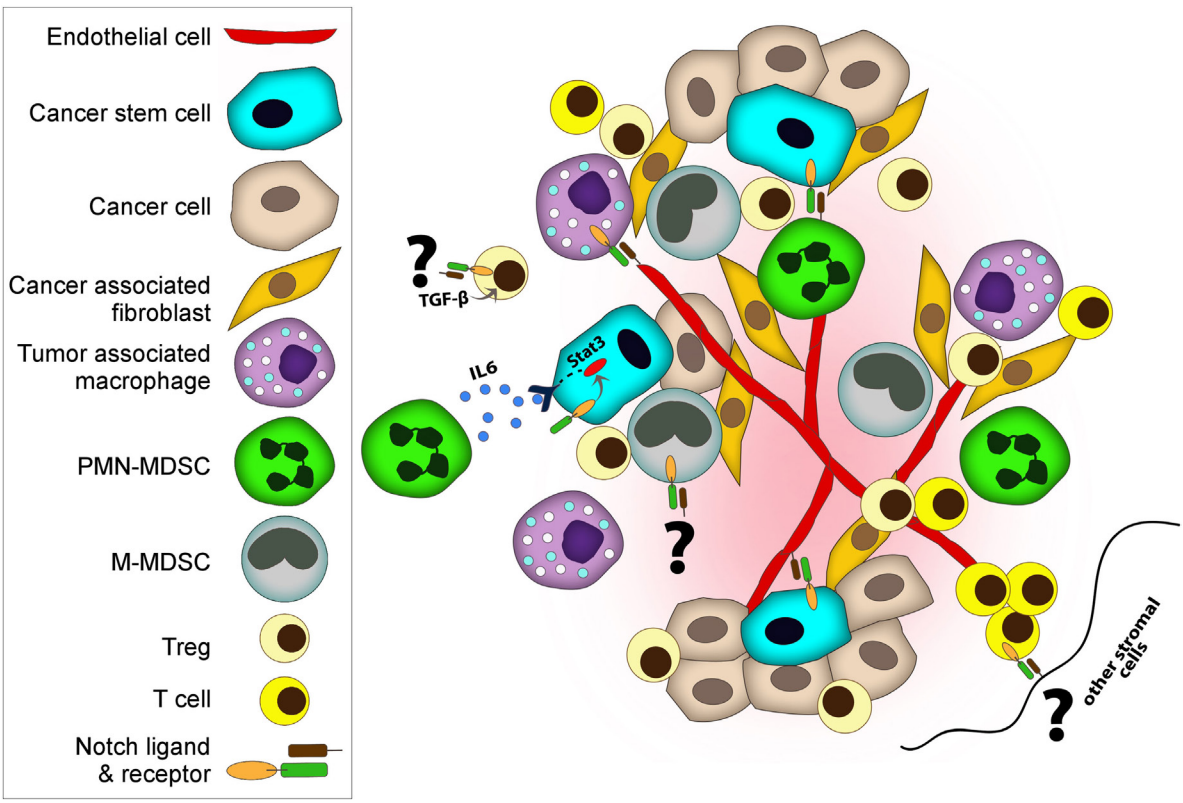

FIGURE 2 | Notch regulates both cancer cells and cancer stem cells (CSCs) and many components of tumor microenvironment including immune cells, fibroblasts, and endothelial. Notch co-operates with various key signaling pathways to exert its functions. Tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs) promote tumor progression by various mechanisms including suppressing immune cells, supporting CSC, and enhancing angiogenesis and metastasis. (i) Deregulated Notch activity in MDSC results in expansion of M-MDSC. (i and ii) MDSCs induce Notch signaling in cancer cells and promote CSC capacity. (iii) MDSCs can also promote CSCs through IL-6/STAT3 activation and nitric oxide/Notch cross-talk signaling. Notch helps sustain STAT3 signaling which is important for CSC maintenance. (iv) Similar to MDSCs, regulatory T cells (Tregs) also promote evasion of immune surveillance. Notch-1–TGF-β signaling cooperatively regulates Foxp3 gene expression, and hence directly induces peripheral Tregs. (v) On the other hand, DLL-1 expressing dendritic cells or stromal cells can activate Notch in cytolytic T cells and enhance antitumor activities. (vi) Moreover, endothelial cells contribute to tumor progression and metastasis. Notch1 controls macrophage recruitment to endothelial cells and facilitate vessel branching, which can increase metastasis. (vii) In addition to facilitating the invasion of cancer cells, endothelial cells play a role as CSC niches by releasing supportive factors or by direct cellular contact.

very well studied. Notch plays a crucial role both in initiation and progression of breast cancer (53). Both Notch1 and Notch4 are found to have differential activities in breast cancer cell lines and patient samples, with Notch4 being the major receptor in the CSC populations of luminal and basal breast cancer cell lines (54, 55). Notch4 and Notch3 are expressed at higher levels in poorly differentiated basal breast cancers and are associated with poor overall survival (54–56). By using a Notch antagonist-γ-secretase (GSI), CSC populations were decreased *in vivo*. An additive effect was detected with GSI and Docetaxel, suggesting that combination therapies with Notch targeted therapies might be beneficial in treating heterogeneous cancer cell populations (55). Moreover, early-phase clinical trials of GSI in breast cancer have provided a limited clinical benefit which can be explained by its activity against CSCs (57). Both Jagged-1 and Jagged-2 have been shown to regulate Notch signaling in breast cancer (56, 58). High expression of Jagged-1 has been detected in aggressive tumors especially triple-negative breast cancer (TNBC) and associated with increased tumor relapse, drug resistance, and metastasis (53). Several studies have shown that Jagged-1 is elevated in endocrine-resistant luminal breast cancers leading to an increase in CSC activity (59). Jagged-2 is also upregulated by hypoxia and results in increased CSCs (60).

Besides the cell intrinsic effects, Jagged-1 expression induced by endocrine therapy resistance affects the tumor microenvironment by induction of macrophage differentiation toward tumor-associated macrophages (TAMs) (61). TAMs are the most frequently found immune cells within the tumor microenvironment that play an important role in suppressing immune surveillance (44). TAMs acquire an anti-inflammatory phenotype, which express immunosuppressive cytokines including IL-10 and TGF-β as well as high expression of arginase-1, which promote cell proliferation, tissue remodeling, and angiogenesis (44). By contrast, macrophages, activated by IFNγ and microbial products, secrete pro-inflammatory cytokines including IL-1b, IL-12, IL-6, TNF-α, and inducible nitric oxide synthase, which are capable of killing pathogens and inducing antitumor immune responses (44). In contrast to these results, forced expression of Notch in macrophages can repress TAM activity by upregulating miRNAs including miR-125 and miR148a-3p, and therefore enhance antitumor capacity (62–64). In addition to TAMs, MDSCs promote tumor progression by various mechanisms including suppressing immune cells and enhancing angiogenesis and metastasis (65). MDSCs are immature myeloid cells that in mice are characterized by either having monocytic characteristics M-MDSCs or neutrophilic characteristics polymorphonuclear (PMN)-MDSCs (65). Notch activity (Hes1 expression) is lower in MDSCs, especially in PMN-MDSCs of patients with renal cell carcinoma and in conditioned media from cultures of breast and lung cancer cell lines. This is caused by an inhibitory phosphorylation of the NICD by casein kinase 2, which disrupts the Notch transcriptional complex (66). In addition, inhibition of Notch promotes PMN-MDSCs over M-MDSCs and these cells had less immunosuppressive capacity when compared with the M-MDSCs when using a lower ratio of MDSCs to cancer cells (67). However, another study showed that deregulated Notch activity can cause myelopoiesis and expansion of MDSCs; this was caused by accumulation of a S2-cleaved Notch receptor, without S3 cleavage, through increased function of ADAM metalloproteases at the S2 site, or inhibition of γ-secretase (**Figure 2**i) (68). It is not clear if differentiation of M-MDSCs was preferred over PMN-MDSCs in this study and whether the immunosuppressive capacity of MDSCs was affected. These apparently conflicting data suggest that the temporal and special regulation of Notch signaling as well as presence of specific cytokines can impact myeloid differentiation and macrophage polarization during tumor initiation.

We previously have shown that MDSCs are recruited to the tumor microenvironment through activation of the mTOR pathway and production of G-CSF. Furthermore, MDSCs induced Notch signaling in cancer cells and promoted CSC capacity (**Figure 2**ii) (69). This type of positive feedback loop between cancer cells, immune cells, and CSCs has been observed previously. MDSCs can also promote CSCs through IL-6/STAT3 activation and nitric oxide/Notch cross-talk signaling. Notch helps sustain STAT3 signaling (**Figure 2**iii) (70). IL-6-STAT3 activation also results in both the expansion of MDSCs and their circulation in various cancer types (71). Moreover, cancer cells increase Jagged-1 and Jagged-2 expression in MDSCs through NFκB-P65 signaling which results in tumor-induced T cell tolerance (72). The presence of MDSCs (CD33 staining and a G-CSF gene signature) correlates with CSC properties in clinical specimens and predicts poor survival outcome (69, 70). Therefore, targeting MDSCs can be beneficial both by decreasing immunosuppression and inhibiting CSCs.

Similar to MDSCs, Tregs also promote evasion of immune surveillance and are associated with tumor invasiveness and poor clinical outcome. Notch1, which is a key regulator of luminal estrogen receptor (ER^+) breast cancers is inversely correlated with the aggressive TNBC/basal-like breast carcinomas and infiltrating $Foxp3^+$ Tregs (73). However, Notch-1–TGF-β signaling cooperatively regulates Foxp3 gene expression, and hence directly induces peripheral Tregs (**Figure 2**iv) (32). Given that Notch4 and Notch3 are expressed at higher levels in poorly differentiated basal breast cancers (54–56) it will be important to elucidate the association of different Notch pathways with Tregs during cancer formation. Moreover, it will be important to understand the regulation of different Notch ligands in different cancer types and the recruitment of Tregs. Both Jagged-1 and Jagged-2 increase the generation of Tregs (33) and both of these ligands are highly expressed in TNBC, CSCs, and the treatment-resistant populations (53, 59, 60). Interestingly, in an experimental model of autoimmune diabetes, it was demonstrated that Notch3 expression in the lymphoid organs results in generation of Tregs. These Tregs secrete suppressive cytokines such as IL-4 and IL-10 and express cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (74), a receptor shown to block T cell co-stimulation, by competing with CD28 for B7 ligand, and therefore abrogate an activated T cell response (75).

Notch also regulates the antitumor immune response. Increased T cell numbers, specifically activated $CD8^+$ CTLs and Th1 cells, correlate with better survival in many cancers (44). Both Notch1 and Notch2 were shown to directly regulate CTL-specific gene expression including granzyme B (35, 36). Notch2, but not Notch1-deficient $CD8^+$ T cells were unable to

expand and suppress tumors in mice (76). In addition, Notch2 agonists or DLL-1 expressing DCs or stromal cells enhanced CTL activity and eradicated tumors (76, 77). Using a mouse model of lung cancer, systemic administration of multivalent forms of DLL-1 enhanced the Th1 response through STAT1/STAT2/T-bet resulting in an increase in T cell infiltration into tumors and $CD8^+$ memory cells, as well as a decrease in Tregs, and tumor vascularization (78). In addition, progression-free survival was increased when the multivalent DLL-1 was combined with EGFR-targeted therapy, Erlotinib, as a result of augmented tumor-induced T cell immunity (78). The soluble clustered DLL1 acts as an activator of Notch receptors, whereas soluble forms of DLL1 (or other Notch ligands) act as inhibitors of Notch signaling (77). In a recent study, a role for Notch in generating antigen-specific stem cell memory T (T_{SCM}) for adoptive immunotherapy cells has been described. T_{SCM} cells were generated from activated $CD4^+$ and $CD8^+$ T cells by co-culturing with stromal cells that expressed DLL1 (79). These long-lived and highly proliferative memory T cells were shown to lose the markers for exhausted T cells, programmed cell death protein 1 (PD-1), and CTLA-4 and to elicit antitumor activities (**Figure 2**v) (79).

A subset of DCs with high DLL4 expression has been described recently. $DLL4^+$ DCs were essential for an effective antitumor response. Under low doses of antigen, DLL4-Notch signaling acts as a co-stimulator to potentiate phosphatidylinositol 3-OH kinase (PI3K)-dependent signaling downstream of the TCR-CD28, and therefore enhances $CD4^+$ T cell to elicit an effective antitumor response (80). This subset of $DLL4^+$ DCs has also been found in human peripheral blood under inflammatory conditions and was shown to be more efficient in promoting Th1 and Th17 differentiation (40). However, its role in cancer patients has not yet been studied. This is very important because there are now several blocking antibodies against DLL4 being tested in clinical trials. Additional evidence for the essential role of Notch in regulating DC-dependent antitumor immune response comes from a study where RBP-J-deficient DCs were shown to be incapable of inhibiting tumor growth due to their decreased capacity to activate and/or recruit T, B, and NK cells (81). Therefore, it is important to understand which specific combination of Notch ligands and receptors contribute to the heterogeneous population of tumor and tumor microenvironment.

Angiogenesis and Metastasis

Tumor progression and the initiation of invasion and metastasis are supported by angiogenesis. DLL4–Notch1 signaling was shown to coordinate the formation of the endothelial "tip cells" in relation to the "stalk cells" required for the correct sprouting and branching patterns during angiogenesis (82). Notch1 controls macrophage recruitment during retinal angiogenesis in mice and these macrophages interact with the DLL4-positive tip cells to facilitate the bridging between sprouts or vessel anastomosis (**Figure 2**vi) (42). Endothelial cells are suggested to play a role as CSC niches by releasing supportive factors or by direct cellular contact (**Figure 2**vii) (83). Endothelial cells were shown to support glioblastoma multiforme (GBM) CSCs by providing Notch ligands. Furthermore, Notch inhibition in endothelial cells blocked self-renewal of the CSCs and GBM tumor growth (84). In ovarian cancer, Notch (Jagged-1 expression) enhances tumor progression by supporting both cancer cell proliferation, chemoresistance, and endothelial cell regulating angiogenesis (45, 46).

Besides supporting CSCs, endothelial cells regulate the passage of cancer cells and immune cells across the endothelium lumen. Notch signaling is implicated in promoting inflamed endothelium which results in opening of gap junctions and promoting the adhesion of tumor cells (85, 86). This enhances migration of leukocytes, and potentially cancer cells, across endothelium. A recent study has shown that endothelial Notch1 can be activated by tumor cells and myeloid cells at a distant metastatic site (lung) (87). Sustained Notch activation induced inflamed endothelium which expressed the adhesion molecule VCAM1; this further promoted neutrophil infiltration, tumor cell adhesion to the endothelium, and intravasation at the primary site, as well as extravasation to the pre-metastatic niche (87).

Notch is also implicated in regulating the epithelial–mesenchymal transition (EMT) in various cancers including breast, prostate, pancreatic, and squamous cell carcinoma (49, 88–90). In both breast and pancreatic cancers, Jagged-1 expression is associated with EMT including increased Slug gene expression, and inhibiting Notch decreased metastasis (88, 89). In particular, gemcitabine-resistant pancreatic cancer acquired EMT properties and a CSC phenotype through Jagged1–Notch2 (89), suggesting that inactivation of Notch may be a potential therapeutic approach to overcome chemoresistance in invasive and metastatic pancreatic cancer. In another study, Jagged-1 expression was correlated with high tumor grade and vascular invasion, and shorter disease-free survival in breast cancer (91). Elevated Jagged-1 expression also correlates with positive lymph node, metastatic relapse, and a higher number of disseminated tumor cells in bone marrow aspirates (91). Interestingly, in patients with detectable circulating tumor cells (CTCs), more than 85% of CTCs express Jagged-1 (91), suggesting that Notch may be implicated in the survival of disseminated tumor cells and metastasis.

At the metastatic site, tumor-derived Jagged-1 promotes osteolytic bone metastasis in breast cancer (92). Notch activation in osteoblasts induces the expression and secretion of IL-6, which in return supports the growth of tumor cells (92). Meanwhile, in osteoclasts (bone-resorbing cells differentiated from monocyte/macrophage precursors), Notch directly controls the maturation of these cells, and therefore enhances osteolytic function (92). Release of TGF-β as a result of bone destruction triggers a positive feedback loop to sustain Jagged-1 expression in tumor cells and therefore maintains the osteolytic environment (92).

Drug Resistance, Dormancy, and Recurrence

Notch is implicated in drug resistance and survival of dormant cells. Elevated Notch signaling is associated with therapeutic resistance and increased risk for tumor recurrence in breast cancer patients (93). Using a Her2/neu mouse model of mammary gland tumors, Notch signaling was shown to be activated in a subset of dormant residual cells following anti-Her2 therapies. Furthermore, Notch accelerated tumor

recurrence (93). Another study showed that ErbB-2 inhibition by a monoclonal antibody Trastuzumab activated Notch1 in breast cancer cell lines and Trastuzumab-resistant cells showed higher Notch activity (94). In both studies inhibiting Notch impaired tumor recurrence (93, 94). Moreover, Notch signaling has been implicated in endocrine-resistant breast cancer (59, 95). Jagged-1–Notch4 is highly expressed in resistant CSCs resulted from anti-estrogen therapy and combining endocrine therapy with Notch inhibition overcame this resistance (59). The presence of TAMs in the microenvironment correlates with tamoxifen resistance and decreased survival of post-menopausal breast cancer patients (96). Jagged-1 upregulation in endocrine-resistant breast cancer modulates the differentiation and polarization of macrophages to TAMs to promote the metastatic potential of cancer cells (61). Notch activation has also been implicated in resistance against chemotherapy by either inducing a CSC phenotype or promoting intratumoral heterogeneity (90, 97, 98), thus suggesting that combination therapies may be more efficacious.

THERAPEUTIC TARGETING OF NOTCH

The extensive study of Notch pathway regulation has provided us with many potential avenues for Notch modulation including inhibiting ligand–receptor interactions or proteolytic activation of the receptor. GSIs are the best studied small molecules targeting the Notch pathway. GSIs prevent Notch from being cleaved and reduce the levels of intracellular activated Notch (53). There are several GSIs at different stages of clinical trials, including MK-0752 and RO4929097. In preclinical studies, the MK-0752 inhibitor—MRK-003—decreased CSCs in breast cancer and PDAC models (99, 100). Using various other GSIs, including RO4929097, GBM CSCs also were significantly decreased (101). Moreover, about 45% of GBM patients had high Notch pathway activity and were predicted to respond to GSIs (101). GSI monotherapies are associated with gastrointestinal toxicities, but in combination with chemotherapy and glucocorticoids, they can be both more efficacious and less toxic (102). In fact, MK-0752 treatment improved the activity of docetaxel and reduced breast CSCs (57). Moreover, a clinical trial of RO4929097 with chemotherapy (paclitaxel and carboplatin) showed complete pathologic response in 50% of TNBC patients (53). Additional details about different GSI clinical trials have been reviewed elsewhere (103).

Therapeutic antibodies may demonstrate better efficacy and specificity than small molecule inhibitors in cancer therapy. Several blocking antibodies against DLL4 are being tested in phase I clinical trials. Demcizumab (OMP-21M18) may inhibit CSCs and angiogenesis and is being tested in various cancer types including non-small-cell lung cancer, ovarian, and pancreatic cancer (103). Results from phase Ib trial of Demcizumab in combination with chemotherapy showed some clinical benefit, however, it did not meet the expected endpoints (47, 53). Rovalpituzumab tesirine (SC16LD6.5) is an antibody–drug conjugate consisting of DLL3-specific IgG1 monoclonal antibody SC16 and the DNA cross-linking agent SC-DR002 (D6.5) (104). Rovalpituzumab tesirine exhibited encouraging single-agent antitumor activity in small-cell lung cancer patients who express high levels of DLL3 (104).

Antibodies have been developed to target Notch1, Notch2, or Notch3. Tarexumab (OMP-59R5) is an antibody against Notch 2 and Notch3 that can inhibit CSCs and tumor growth (47, 105). Tarexumab is being tested in phase II trials for the treatment of pancreatic cancer and small-cell lung cancer (103). Brontictuzumab (OMP-52M51), an anti-Notch1 antibody, has shown to reduce CTCs and provided some efficacy in patients with metastatic colorectal cancer (103).

CONCLUSION AND FUTURE DIRECTION

Most of the Notch therapeutics have been tolerated by patients. Although they are associated with various adverse effects, these effects are usually manageable. However, the effects of these therapies on tumor immunology are not well studied. Immunotherapies are revolutionizing the treatment of many cancers. Inhibiting negative regulators of immune activation (immune checkpoint) through immune checkpoint blockade therapies (ICBT) has been remarkably effective in several cancer types including metastatic melanoma and non-small cell lung cancer (106, 107). These treatments target negative regulators of T cell activity, thereby unleashing antitumor immunity. Two very successful strategies of ICBT have been achieved by antibodies blocking the CTLA-4 or the PD-1 pathways, either alone or in combination (108). Therefore, it is necessary to understand the effect of Notch therapeutics on tumor immunology. As discussed earlier, both *in vitro* and *in vivo* studies have associated DLL family ligands with the development of IFNγ-secreting Th1 cells and Th17, while Jagged family ligands elicit Th2, Th9, and Treg responses (10, 22, 27). Moreover, DLL and Jagged ligands appear to elicit opposite effects in myeloid cells: DLL1 and DLL4 promote differentiation of DCs while activation of Notch through Jagged-1 promotes immature myeloid cells (20). On the other hand, Notch2 controls the survival of DCs (also identified as $Cx3cr1^{low}$ $Esam^{high}$ DC subset), which is required for efficient T cell priming. Therefore, these results suggest that combining anti-Notch2 and DLL therapies with ICBT might not be beneficial because of reduced T cell priming and activation of Th1, Th17, and $CD8^+$ T cells that happens through DCs. Moreover, Notch1 and Notch2 have been shown to directly regulate CTL-specific gene expression including granzyme B, therefore prolonging administration of these drugs might suppress CTL activity and again dampen the efficacy of ICBT. On the other hand, anti-Jagged therapies look more promising if combined with ICBT, e.g., in a mouse model, anti-Jegged-1/2 both inhibited MDSCs and induced Notch1 in $CD8^+$ T cells, which promoted antitumor T-cell immunity and protective immune memory response (72). The majority of recent studies suggest that because of the broad functions of Notch signaling, we must design better strategies utilizing anti-Notch therapies both by dosage deescalation and by combinations with different therapies as well as designing specific treatment schedules. For example, it may be preferable to use anti-Jagged therapies before ICBT, as the host immune system might be primed to respond better to ICBT or to use even a lower dosage of ICBT and therefore decrease toxicity. Moving forward, there needs to be more

research to investigate the effect of Notch therapies on different immune cell compartments and functions to enable the design of combinatorial treatments.

AUTHOR CONTRIBUTIONS

MJ and XZ designed the outline of the review and MJ composed the manuscript. LX and JR contributed to the content of and edited the manuscript. XZ edited the manuscript, and supervised the writing process.

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Notch Signaling Modulates Macrophage Polarization and Phagocytosis Through Direct Suppression of Signal Regulatory Protein α Expression

Yan Lin[1,2,3†], Jun-Long Zhao[3,4†], Qi-Jun Zheng[5], Xun Jiang[1], Jiao Tian[1], Shi-Qian Liang[3], Hong-Wei Guo[1], Hong-Yan Qin[3], Ying-Min Liang[2*] and Hua Han[3,4*]*

[1] Department of Pediatrics, Tangdu Hospital, Fourth Military Medical University, Xi'an, China, [2] Department of Hematology, Tangdu Hospital, Fourth Military Medical University, Xi'an, China, [3] Department of Genetics and Developmental Biology, Fourth Military Medical University, Xi'an, China, [4] Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, China, [5] Department of Cardiac Surgery, Xijng Hospital, Fourth Military Medical University, Xi'an, China

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***Correspondence:**
Jun-Long Zhao
bio_junlongzhao@163.com;
Ying-Min Liang
liangym@fmmu.edu.cn;
Hua Han
huahan@fmmu.edu.cn

[†]These authors have contributed equally to this work.

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The Notch pathway plays critical roles in the development and functional modulation of myeloid cells. Previous studies have demonstrated that Notch activation promotes M1 polarization and phagocytosis of macrophages; however, the downstream molecular mechanisms mediating Notch signal remain elusive. In an attempt to identify Notch downstream targets in bone marrow-derived macrophages (BMDMs) using mass spectrometry, the signal regulatory protein α (SIRPα) appeared to respond to knockout of recombination signal-binding protein Jk (RBP-J), the critical transcription factor of Notch pathway, in macrophages. In this study, we validated that Notch activation could repress SIRPα expression likely *via* the Hes family co-repressors. SIRPα promoted macrophage M2 polarization, which was dependent on the interaction with CD47 and mediated by intracellular signaling through SHP-1. We provided evidence that Notch signal regulated macrophage polarization at least partially through SIRPα. Interestingly, Notch signal regulated macrophage phagocytosis of tumor cells through SIRPα but in a SHP-1-independent way. To access the translational value of our findings, we expressed the extracellular domains of the mouse SIRPα (mSIRPα^{ext}) to block the interaction between CD47 and SIRPα. We demonstrated that the soluble mSIRPα^{ext} polypeptides could promote M1 polarization and increase phagocytosis of tumor cells by macrophages. Taken together, our results provided new insights into the molecular mechanisms of notch-mediated macrophage polarization and further validated SIRPα as a target for tumor therapy through modulating macrophage polarization and phagocytosis.

Keywords: macrophages, notch, signal regulatory protein α, SHP-1, polarization, phagocytosis

INTRODUCTION

The important roles of microenvironmental elements in tumor development and progression have attracted intensive research attentions recently (1). Macrophages are the predominant population of tumor-infiltrating immune cells and participate in immune regulation in a polarized manner in response to microenvironmental stimuli (2). Classically activated macrophages or M1-polarized macrophages are typically induced by bacterial lipopolysaccharide (LPS) and interferon (IFN)-γ and invoke a type 1 response through secreting cytokines such as interleukin (IL)-12 and increasing

antigen presentation capacity (3–5). Moreover, M1-macrophages display a stronger phagocytic activity to "eat" abnormal cells, including dying erythrocytes and mutant cells. On the other hand, M2-polarized macrophages are prototypically activated by IL-4/IL-13 and exhibit phenotypes roughly opposite to M1 macrophages (6). Tumor-associated macrophages (TAMs) share a part of M2 macrophage properties and promote tumor angiogenesis and immune editing through multiple mechanisms (7, 8). Therefore, reprogramming TAMs from M2 into the M1 activation pattern might be a potential therapy for cancers, given that the critical molecular mechanisms controlling M1/M2 polarization are unveiled.

The Notch-RBP-J (recombination signal-binding protein Jk) signaling pathway plays significant roles in macrophage development and activation (9–19). We have recently demonstrated that Notch blockade by RBP-J disruption skews TAMs toward M2 polarization to facilitate tumor progression (10). On the other hand, forced Notch activation by conditional overexpression of Notch intracellular domain (NIC) in macrophages directs TAMs toward an antitumor phenotype by upregulating a few miRNAs (13, 14). Besides, Notch-mediated macrophage polarization also participates in the regulation of phagocytosis and tissue fibrosis (12). However, the downstream molecular mechanisms of Notch signal remain to be elucidated.

Signal regulatory protein (SIRP) α, also known as SIRPA, SHPS-1, p84, and BIT, belongs to the immunoglobulin (Ig) superfamily. SIRPα is abundantly expressed in macrophages, dendritic cells, neutrophils, and neurons (20). Its best characterized ligand is the ubiquitously expressed "don't-eat-me" signal molecule CD47. When SIRPα binds with CD47 through the Ig-like domains in its N-terminal region, signaling through SHP-1 or SHP-2 is activated in a phosphorylation-dependent manner, followed by a panel of different downstream signaling events (21–23). Kong et al. have demonstrated that SIRPα plays a critical role in regulating innate immune activation (24). Recently, they have further found that SIRPα functions as a significant regulator of TAMs in hepatoma (25).

In this study, we explored the regulatory mechanisms of Notch signal in macrophage polarization. Our results show that Notch signal regulates macrophage polarization at least partly by inhibiting SIRPα. We found that soluble mSIRPα^{ext} polypeptide, a recombinant extracellular fragment of the mouse SIRPα (mSIRPα^{ext}), could promote M1 polarization of macrophages and increase their phagocytic activity to L1210 tumor cells in a CD47-dependent way. Our findings extend the molecular signaling mechanisms downstream to Notch in macrophage polarization and highlight SIRPα as a novel target of Notch-mediated macrophage polarization for tumor therapy.

MATERIALS AND METHODS

Animals

Mice were maintained on the C57BL/6 background in a specific pathogen-free facility. RBP-J-floxed (RBP-J^{f}) mice (12) or ROSA-Stop^{f}-NIC transgenic mice (14) were mated with Lyz2-Cre (#019096, Jackson Laboratory) mice to obtain mice with myeloid-specific Notch blockade (Lyz2-Cre-RBP-J$^{f/f}$, with Lyz2-Cre-RBP-J$^{+/f}$ as a control) or activation (Lyz2-Cre-ROSA-Stop^{f}-NIC, with ROSA-Stop^{f}-NIC as a control) mice, respectively. Mice were genotyped with tail DNA using polymerase chain reaction (PCR) primers listed in Table S1 in Supplementary Material. All animal experiments were approved by the Animal Experiment Administration Committee of Fourth Military Medical University.

Cell Culture and Transfection

L1210 murine leukemia cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were maintained in 5% CO_2 to 95% air. To culture bone marrow-derived macrophages (BMDMs), mononuclear cells were isolated from tibias and femurs of C57BL/6 mice. Cells were cultured at a density of 2×10^6/mL in DMEM containing 10% FBS and 25 ng/mL murine macrophage-colony stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ, USA) for 7 days. In some experiments, IFNγ (20 ng/mL, PeproTech), LPS (50 ng/mL, Sigma, St. Louis, MO, USA), or IL4 (20 ng/mL, PeproTech) was added and cultured for 24 h before further analyses. Macrophages treated with PBS, LPS + IFNγ, or IL4 were named as M^{PBS}, M^{LPS}, or M^{IL4}, according to Epelmann et al. (5). Cells were transfected siRNA with Lipofectamine LTX (Invitrogen) according to the recommended protocol. Three pairs of siRNA for each target were designed and their knockdown efficiency was determined by qRT-PCR and Western blotting. The siRNA with highest efficiency was chosen for further experiments. Lentivirus was packaged by Cyagen Biosciences with commercial service (Guangzhou, China).

Plasmid Construction and Recombinant Protein Purification

Genes encoding the extracellular domain of mouse SIRPα (mSIRPα^{ext}) or CD47 (mCD47^{ext}) was amplified with PCR using a mouse embryo cDNA library as a template. The primers used were listed in Table S1 in Supplementary Material. The amplified fragments were inserted into pET32a(+) plasmid (Novagen, Darmstadt, Germany), and the expressed fusion protein Trx-mSIRPα^{ext} (55.2 kDa) or Trx-mCD47^{ext} (32.5 kDa) was purified as described previously in Ref. (26). pEFBOS-NIC was as described in Ref. (14).

Reverse Transcription (RT)-PCR

Total RNA was extracted from cell samples with the Trizol reagent (Invitrogen). cDNA was prepared with a reverse transcription kit (Takara, Dalian, China) following the supplier's instruction. Quantitative real-time PCR was performed using a kit (SYBR Premix EX Taq, Takara) and the ABI Prism 7500 Real-Time PCR System in triplicates, with β-actin as an internal control. Primers are listed in Table S1 in Supplementary Material.

Western Blot

Cells were harvested and the whole cell lysates were extracted on ice with the RIPA buffer containing a protease inhibitor cocktail (Beyotime, Haimen, China). Lysates were centrifuged and the supernatants were collected. Protein concentration was determined using a BCA protein assay kit (Pierce). Aliquots of protein lysates were separated by SDS-PAGE and blotted onto

polyvinylidene fluoride membrane. Membranes were blocked with bovine serum albumin solution and probed with different primary antibodies and washed, followed by HRP-conjugated secondary antibodies (Table S2 in Supplementary Material). Protein bands were visualized with chemoluminescent reagents (Pierce).

In Vitro Pull-Down Assay

The recombinant Trx-mSIRPα^{ext} protein was cleaved using thrombin (Novagen) in a buffer containing 20 mM Tris–HCl, 150 mM NaCl, and 2.5 mM CaCl2 (pH8.4) at room temperature away from light for 20 h. The ProBond™ purification system (Invitrogen) was used to purify the recombinant mSIRPα^{ext} proteins according to manufacturer's protocols. The S-tagged mSIRPα^{ext} was mixed with purified Trx-mCD47^{ext} protein (ratio 1:1), and the mixture was incubated at 4°C for 2 h. Then anti-His antibodies (Sigma) pre-coupled to the Dynabeads-protein G (Invitrogen) were added and incubated at room temperature for 30 min with rotation. After washing with PBS-0.02% Tween 20, 50 mM glycine eluent was added and incubated at room temperature for 5 min with rotation. Proteins were collected and analyzed using SDS-PAGE with 15% acrylmide, followed by Western blotting with the anti-S tag and anti-His antibodies.

Proteomic Analysis

Four-plex iTRAQ-based quantitative proteomics analysis was carried out using proteins isolated from BMDMs of Lyz2-Cre-RBP-J$^{f/f}$ or control mice. Each sample was labeled using iTRAQ 4-plex kits (AB Sciex Inc., Foster City, CA, USA) according to the manufacturer's instructions. Samples from the control BMDMs were labeled with 114, 115 tags, and samples from the RBP-J knockout BMDMs were labeled with 116, 117 tags, respectively. After labeling, the peptide samples were mixed for further LC-MS/MS analysis. The protein expression level in each sample was quantified and the fold change between control and the RBP-J knockout BMDMs was determined.

Luciferase Assay

The 5′ flanking sequence (−2,615 to +123) of the murine SIRPα gene was amplified by PCR with mouse genomic DNA as a template. The fragment was inserted into pGL3-basic to generate pGL3-mSIRPα-promoter. Different truncated fragments of the 5′ flanking region, as depicted in **Figure 1F**, were also generated by PCR and inserted into pGL3-basic (pGL3-mS-T1, 2,3, or 4). HeLa cells (2×10^4) were transfected with different reporters, NIC overexpression plasmid, and phRL-TK using Lipofectamine 2000™ (Invitrogen). The luciferase activity was assessed 24 h later using Luminoskan Ascent (Labsystems, Helsinki, Finland) and a Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol. All luciferase activity was normalized to the Renilla luciferase activity.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was performed using a kit (Merck Millipore) according to the manufacturer's instructions. BMDMs were polarized with LPS + IFNγ for 24 h and fixed with formaldehyde. Cross-linked immune complexes were sonicated and precipitated with anti-Hes1 antibody. DNA was extracted from the collected samples and analyzed by PCR with the primers listed in Table S1 in Supplementary Material.

Flow Cytometry

L1210 cells (3×10^5) were incubated with mSIRPα^{ext} (10 μg/mL) for 30 min on ice in dark. After washing with PBS, cells were stained with FITC-conjugated anti-S tag antibody (Sigma), followed by FACS analysis using a Calibur™ (BD Immunocytometry Systems, San Jose, CA, USA). BMDMs (3×10^5) were cultured in the presence of PBS (M^{PBS}) or LPS + IFNγ (M^{LPS}) for 24 h, and then incubated with mSIRPα^{ext} (10 μg/mL) at 37°C for 2 h. After washing, cells were stained with anti-Ki67 (Sigma) or anti-Annexin V (BD), followed by FACS analysis. Data were analyzed using the Flowjo™ software.

In Vitro Phagocytosis Assay

L1210 cells were labeled with carboxyfluorescein succinimidyl amino ester (CFSE, Dojindo Molecular Technologies, Inc.) according to the recommended protocol, and loaded onto macrophages. In some cases, L1210 cells were pre-incubated with purified recombinant proteins at the concentration of 10 μg/mL at 37°C for 2 h before coculturing with macrophages. Cells were stained with anti-F4/80, rinsed with PBS, and visualized under a fluorescence microscope (BX51, Olympus). Phagocytosis was quantified by calculating the average number of ingested L1210 cells in macrophages.

Statistics

Statistical analysis was performed with the Graph Pad Prism 5 software. Student's *t*-test or one-way ANOVA test was used for statistical analyses. Data were expressed as means ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

SIRPα Was Involved in Notch Signal-Mediated Macrophage Polarization

In an attempt to identify Notch downstream molecules involved in macrophage activation, we employed mice with myeloid-specific Notch blockade (Lyz2-Cre-RBP-J$^{f/f}$ or RBP-J mcKO, with Lyz2-Cre-RBP-J$^{+/f}$ as a control) or activation (Lyz2-Cre-ROSA-Stop^{f}-NIC or NIC^{mCA}, with ROSA-Stop^{f}-NIC as a control). In an initial proteomic analysis of RBP-J deficient and control BMDMs using mass spectrometry, we found that the protein level of SIRPα was upregulated in RBP-J-deficient BMDMs (Figure S1A in Supplementary Material). To validate this finding, RBP-J mcKO, NIC^{mCA}, and control BMDMs were stimulated with PBS (M^{PBS}), LPS + IFNγ (M^{LPS}), or IL4 (M^{IL4}) (5), and the expression of polarization markers, Hes1 and SIRPα was examined by qRT-PCR or Western blotting. These treatments induced the expression of various macrophage polarization markers (Figure S1B in Supplementary Material). Meanwhile, Hes1 and Hey1 were upregulated in M^{LPS} and downregulated in M^{IL4} macrophages (Figure S1C in Supplementary Material). SIRPα expression was downregulated in M^{LPS} and upregulated in M^{IL4}

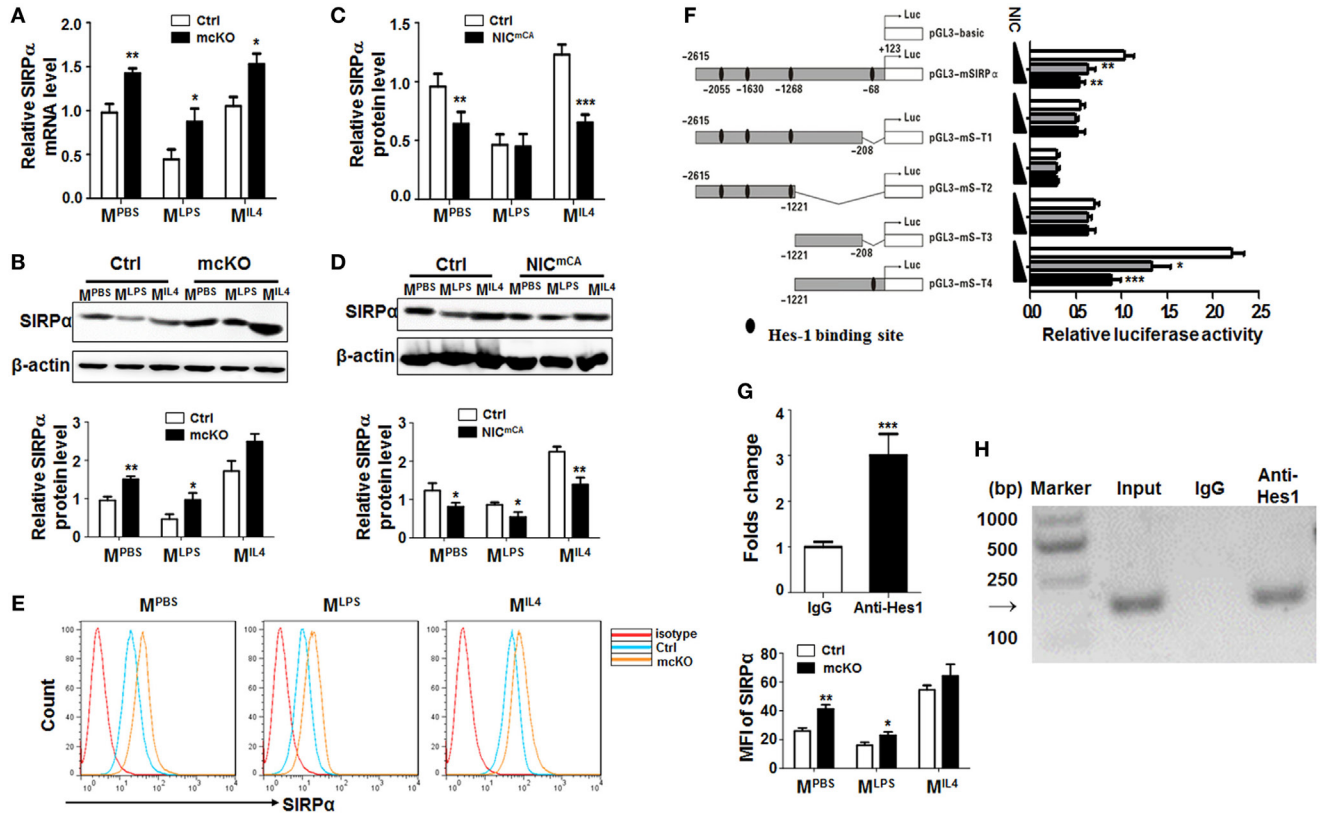

FIGURE 1 | Notch signal regulated Signal regulatory protein α (SIRPα) expression in macrophage polarization. **(A,B)** Bone marrow-derived macrophages (BMDMs) from RBP-J knock out (mcKO) and control (Ctrl) mice were stimulated with PBS, LPS + IFNγ, or IL4 for 24 h. The expression of SIRPα was determined by qRT-polymerase chain reaction (PCR) **(A)** and Western blotting **(B)** ($n = 4$). **(C,D)** BMDMs from Notch signal activation (NIC^{mCA}) and control (Ctrl) mice were treated as in **(A)**. The expression of SIRPα was determined by qRT-PCR **(C)** and Western blotting **(D)** ($n = 4$). **(E)** The surface protein level of SIRPα on BMDMs from RBP-J knock out (mcKO) and control (Ctrl) was detected using FACS ($n = 4$). **(F)** Full length and truncated fragments of SIRPα promoter were inserted into pGL3-basic to generate different reporters as depicted. Filled ellipses represent Hes-binding sites. HeLa cells were transiently transfected with different reporters and NIC-overexpressing vector ($n = 5$). **(G,H)** BMDMs were stimulated and subjected to chromatin immunoprecipitation analysis with IgG or anti-Hes1 antibody. Precipitated chromatin DNA was analyzed with qPCR **(G)** or PCR followed by electrophoresis **(H)** ($n = 4$). Student's *t* test or one-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

BMDMs, as well as in RAW264.7 cells treated in the same way (Figure S1D in Supplementary Material). Moreover, we found that RBP-J deficiency led to upregulated SIRPα expression under different stimuli (**Figures 1A,B**). On contrary, constitutive Notch activation resulted in a tendency of SIRPα downregulation in macrophages (**Figures 1C,D**). Flow cytometry confirmed that the expression of SIRPα was upregulated in RBP-J deficiency BMDMs (**Figure 1E**). These data suggested that Notch signal repressed SIRPα expression in macrophages.

There are four Hes recognition sites (−2,615, −1,630, −1,268, and −68 bp) in the murine SIRPα promoter, suggesting that Notch signal might regulate SIRPα expression through Hes family co-repressors that are downstream to Notch receptors. We, therefore, constructed reporter genes with different truncated fragments of the SIRPα promoter, which were inserted into pGL3-basic (**Figure 1F**). Reporter assay in the presence of NIC overexpression showed that Notch activation could significantly repress luciferase expression driven by SIRPα promoter fragments, and the Hes-binding site at −68 bp was required for Notch activation-mediated repression of the SIRPα promoter (**Figure 1F**). Indeed, a ChIP assay indicated that anti-Hes1 antibody could significantly pull-down the SIRPα promoter fragment surrounding the Hes1 binding site at −68 bp in M^{LPS} (**Figures 1G,H**). These data indicated that Notch signal directly inhibited expression of SIRPα through Hes proteins.

Notch Signal Negatively Regulated SHP-1 Phosphorylation Triggered by SIRPα–CD47 Interaction

Signal regulatory protein α is enriched in myeloid cells, while its ligand CD47 is universally expressed. Interaction between CD47 and SIRPα induces SHP1/SHP2 activation through tyrosine phosphorylation in the SH2 domains (27, 28), leading to inhibited macrophage phagocytosis and pro-inflammatory response. BMDMs from normal mice were transfected with SIRPα siRNA (si-SIRPα) or control oligonucleotide (NC), which were proven to inhibit SIRPα expression efficiently (Figures

S2A,B in Supplementary Material), and stimulated with PBS or LPS + IFNγ for 24 h. Western blotting showed that knock-down of SIRPα by siRNA (si-SIRPα) significantly reduced SHP1 phosphorylation in M^{PBS} and M^{LPS} macrophages (**Figures 2A,B**). On the other hand, SIRPα overexpression obviously increased SHP1 phosphorylation in macrophages (Figure S2C in Supplementary Material; **Figures 2C,D**). We then examined the effect of Notch signal on SIRPα signaling using BMDMs isolated from RBP-J mcKO and control mice and stimulated with PBS, LPS + IFNγ, or IL4. The result showed that Notch signal deficiency significantly increased SHP1 phosphorylation both in M^{PBS} and M^{LPS} macrophages (**Figures 2E,F**). When the expression of SIRPα was knocked down with siRNA, the RBP-J deficiency-induced increase of SHP1 phosphorylation was canceled (**Figures 2E,F**). On the other hand, forced Notch activation by NIC overexpression reduced SHP-1 phosphorylation, which was reversed by SIRPα overexpression mediated by a lentivirus (**Figures 2G,H**). These results indicated that Notch signal could repress SHP-1 activation in macrophages, likely through blocking SIRPα expression.

SIRPα Signaling Modulated Macrophage Polarization

Signal regulatory protein α was differentially expressed in macrophages under different polarization stimuli (Figure S1D in Supplementary Material), suggesting that SIRPα-mediated signaling might be involved in macrophage polarization. To validate this, BMDMs from normal mice were transduced with SIRPα-overexpressing lentivirus and simulated with PBS, LPS + IFNγ, or IL4. qRT-PCR was carried out to access the expression of polarization markers in BMDMs. The result showed that SIRPα overexpression downregulated the expression of M^{LPS} markers TNF-α and IL12 and upregulated M2 markers IL10 and MR under LPS + IFNγ stimulation (**Figure 3A**). Moreover, BMDMs from normal mice were transfected with SIRPα siRNA or NC oligonucleotides and simulated as above. The result showed that the M^{LPS} markers were upregulated while the M^{IL4} markers were downregulated upon SIRPα knockdown (**Figure 3B**). Knockdown of SHP-1 with siRNA exhibited similar effects on macrophage polarization under various stimuli (Figures S2D,E in Supplementary Material; **Figure 3C**). Furthermore, SIRPα

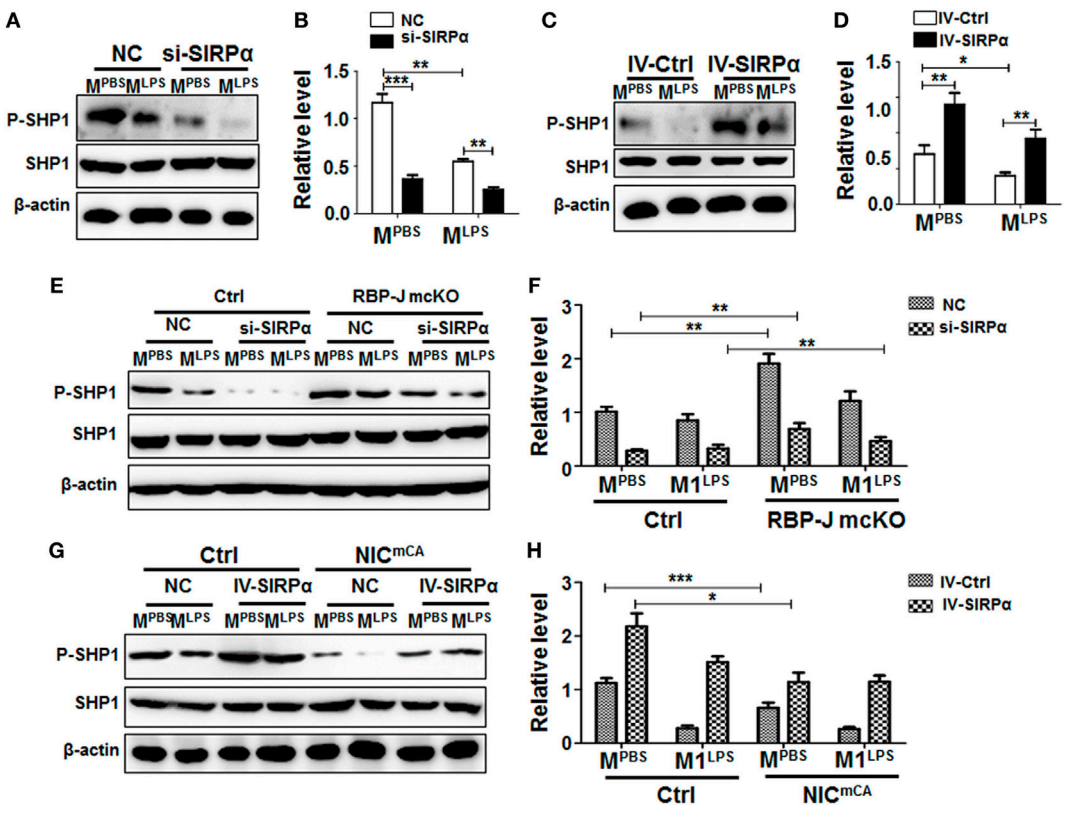

FIGURE 2 | Notch signal regulated signal regulatory protein α (SIRPα) expression and SHP-1 activation. **(A–D)** Bone marrow-derived macrophages (BMDMs) with SIRPα knockdown **(A,B)** or overexpression **(C,D)** were stimulated with PBS or lipopolysaccharide (LPS) + IFNγ for 24 h. Western blotting was carried out to analyze SHP-1 phosphorylation ($n = 4$). **(E,F)** BMDMs from RBP-J deficient (mcKO) and control (Ctrl) mice were transfected with SIRPα siRNA or NC and stimulated with PBS or LPS + IFNγ for 24 h. The phosphorylation of SHP-1 was determined by Western blotting ($n = 4$). **(G,H)** BMDMs from Notch signal activation (NIC^{mCA}) and control (Ctrl) mice were infected with SIRPα-overexpressing lentivirus and stimulated with PBS or LPS + IFNγ for 24 h. The phosphorylation of SHP-1 was determined by Western blotting ($n = 4$). One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; * or #, $P < 0.05$; ** or ##, $P < 0.01$; ***$P < 0.001$.

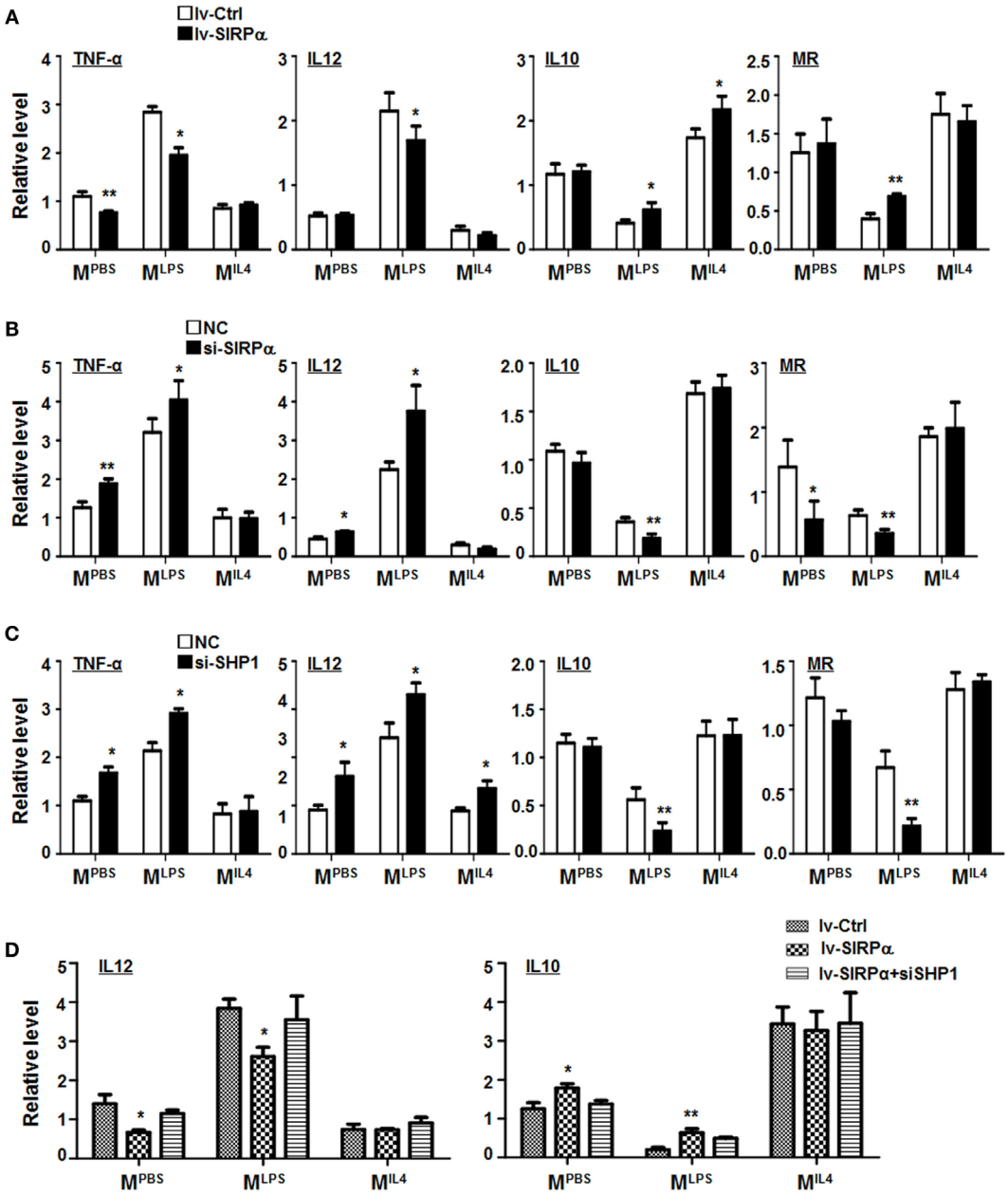

FIGURE 3 | CD47-SIRPα-SHP-1 signal suppressed M1 and promoted M2 polarization. **(A)** Bone marrow-derived macrophages (BMDMs) were transfected with SIRPα or control lentivirus and then cultured with PBS, lipopolysaccharide (LPS) + interferon (IFN)γ, or IL4 for 24 h. The expressions of TNF-α, IL12, IL10, and MR were determined by reverse transcription-polymerase chain reaction (PCR) ($n = 3$). **(B,C)** BMDMs were transfected with siRNA for SIRPα **(B)** or SHP-1 **(C)** followed by stimulation with PBS, LPS + IFNγ, or IL4. The expression of polarization makers was determined by qRT-PCR ($n = 3$). **(D)** BMDMs were transfected with SIRPα or control lentivirus and transfected with SHP-1 siRNA, followed by stimulation with PBS, LPS + IFNγ, or IL4. The expression of IL12 and IL10 was detected by qRT-PCR ($n = 3$). One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$.

was overexpressed in normal BMDMs using lentivirus and transfected with SHP1 siRNA simultaneously, followed by polarization stimulation as above. qRT-PCR showed that knockdown of SHP1 abrogated the effect of SIRPα overexpression on macrophage polarization (**Figure 3D**). These data suggested that the SIRPα-SHP1 signaling repressed M^{LPS} polarization upon LPS + IFNγ stimulation.

SIRPα Partially Mediated the Effect of Notch Signal on Macrophage Polarization

To access the functional significance of SIRPα downstream to Notch signal in regulating macrophage polarization, we cultured BMDMs from RBP-J mcKO mice and treated cells with PBS, LPS + IFNγ, or IL4 in the presence or absence of SIRPα siRNA. qRT-PCR and ELISA were performed to determine the expression

of polarization markers and the production of cytokines. The result showed that RBP-J knockout reduced M^{LPS} and increased M^{IL4} marker expression obviously, while knockdown of SIRPα could partially cancel the effects of Notch signal deficiency (**Figure 4A**). ELISA also showed that blockade of Notch signal suppressed secretion of inflammatory cytokines, such as IL12 and TNF-α, and promoted the production of anti-inflammatory cytokine IL10 (**Figure 4B**). Similarly, inhibiting SIRPα recovered the cytokine production modulated by RBP-J mcKO (**Figure 4B**). These data suggested that SIRPα as a downstream molecule of Notch signal could at least partially mediate the effect of Notch signal on macrophage polarization.

Interaction Between Notch Signal and SIRPα in Regulating Phagocytosis of Macrophages

To look at the role of SIRPα in macrophage phagocytosis, the L1210 murine leukemia cells were labeled with CFSE, and incubated with PBS-, LPS + IFNγ-, or IL4-stimulated BMDMs for 2 h. The result showed that M^{LPS} BMDMs exhibited increased ability to ingest tumor cells as compared with M^{PBS} or M^{IL4} BMDMs (Figures S3A,B in Supplementary Material). Consistent with previous reports, overexpression of SIRPα in BMDMs using lentivirus attenuated tumor cells engulfment (**Figure 5A**), and knockdown of SIRPα with siRNA enhanced BMDM phagocytosis (**Figure 5B**). Consistently, CD47 expressed on L1210 was inhibitory to SIRPα-mediated phagocytosis, because repression of CD47 in L1210 cells significantly increased phagocytosis (Figures S3C,D in Supplementary Material; **Figure 5C**). Compared with untransfected L1210 cells, incubation with CD47-compromised L1210 cells promoted M^{LPS}-like phenotype of BMDMs (**Figure 5D**), which was dependent on SIRPα or SHP1 expression (**Figure 5E**). These results suggested that interaction of CD47 on tumor cells with SIRPα on BMDMs regulated both phagocytosis and polarized activation of macrophages.

RBP-J deficiency or treatment with GSI, an inhibitor of Notch signaling, reduced phagocytosis by BMDMs significantly (**Figures 5F,G**), and this effect of Notch signal blockade was reversed by SIRPα siRNA (**Figure 5G**). These data suggested that Notch signal increased phagocytosis by macrophages likely through repressing SIRPα.

Soluble Extracellular Domain of Mouse SIRPα Could Interrupt the CD47–SIRPα Interaction

It has been shown that blocking CD47–SIRPα interaction could facilitate phagocytosis of tumor cells and promote antitumor immune response. We have also shown that soluble human CD47 could serve as an antagonist to block CD47–SIRPα interaction (26). Expression vector of the extracellular domain of mSIRPα ($mSIRP\alpha^{ext}$) was constructed and expressed in *E. coli* together with the $mCD47^{ext}$ (**Figure 6A**). SDS-PAGE analysis of cell lysates revealed that the $mSIRP\alpha^{ext}$ and the Trx-$mCD47^{ext}$ proteins were successfully expressed with predicted molecular weights of 36 and 32.5 kDa, respectively (**Figure 6B**). Pull-down assay followed by Western blotting showed that the $mSIRP\alpha^{ext}$ protein could be pulled down by the Trx-$mCD47^{ext}$ protein (**Figure 6C**) but not by Trx (not shown), indicating that $mSIRP\alpha^{ext}$ could bind to $mCD47^{ext}$. To examine whether the $mSIRP\alpha^{ext}$ protein could bind to L1210 cells, we incubated the $mSIRP\alpha^{ext}$ fusion protein with L1210 cells, and analyzed the cells using flow cytometry after staining with a FITC-conjugated anti-S tag antibody. The

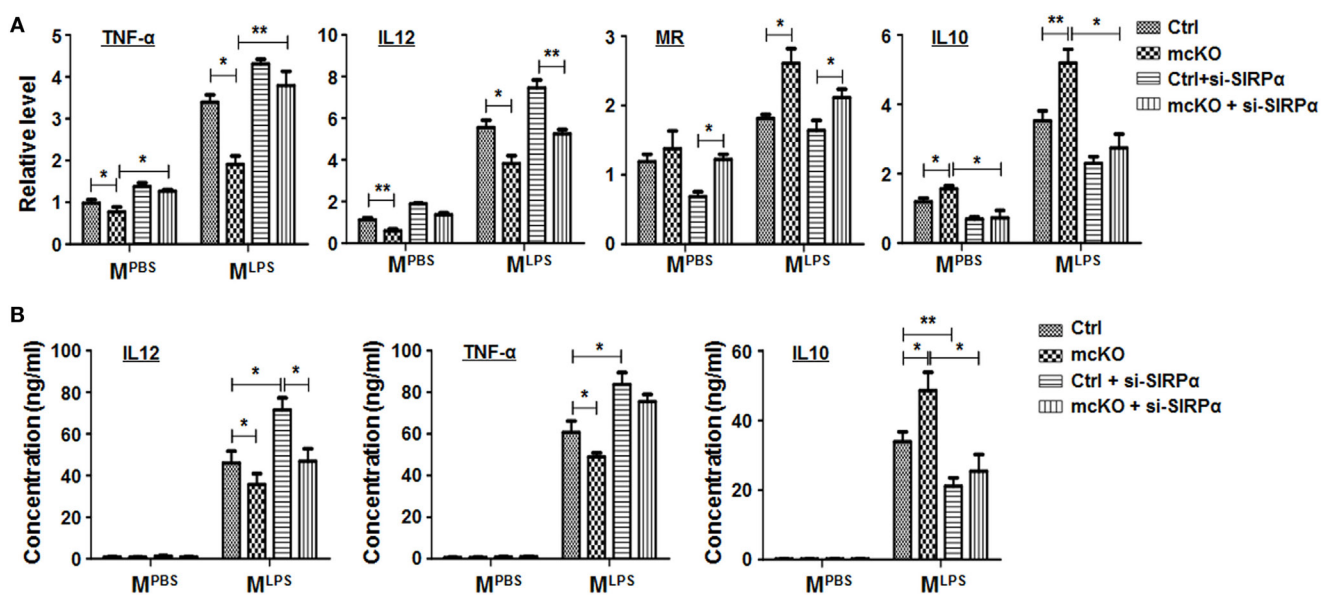

FIGURE 4 | Notch signal regulated macrophage polarization partially through signal regulatory protein α (SIRPα). **(A,B)** Bone marrow-derived macrophagess from RBP-J mcKO and control mice were transfected with siRNA for SIRPα and then cultured in the presence of PBS or lipopolysaccharide + interferon γ for 24 h. The expression of polarization markers **(A)** and the production of cytokines **(B)** were detected ($n = 4$). One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^*P < 0.05$; $^{**}P < 0.01$.

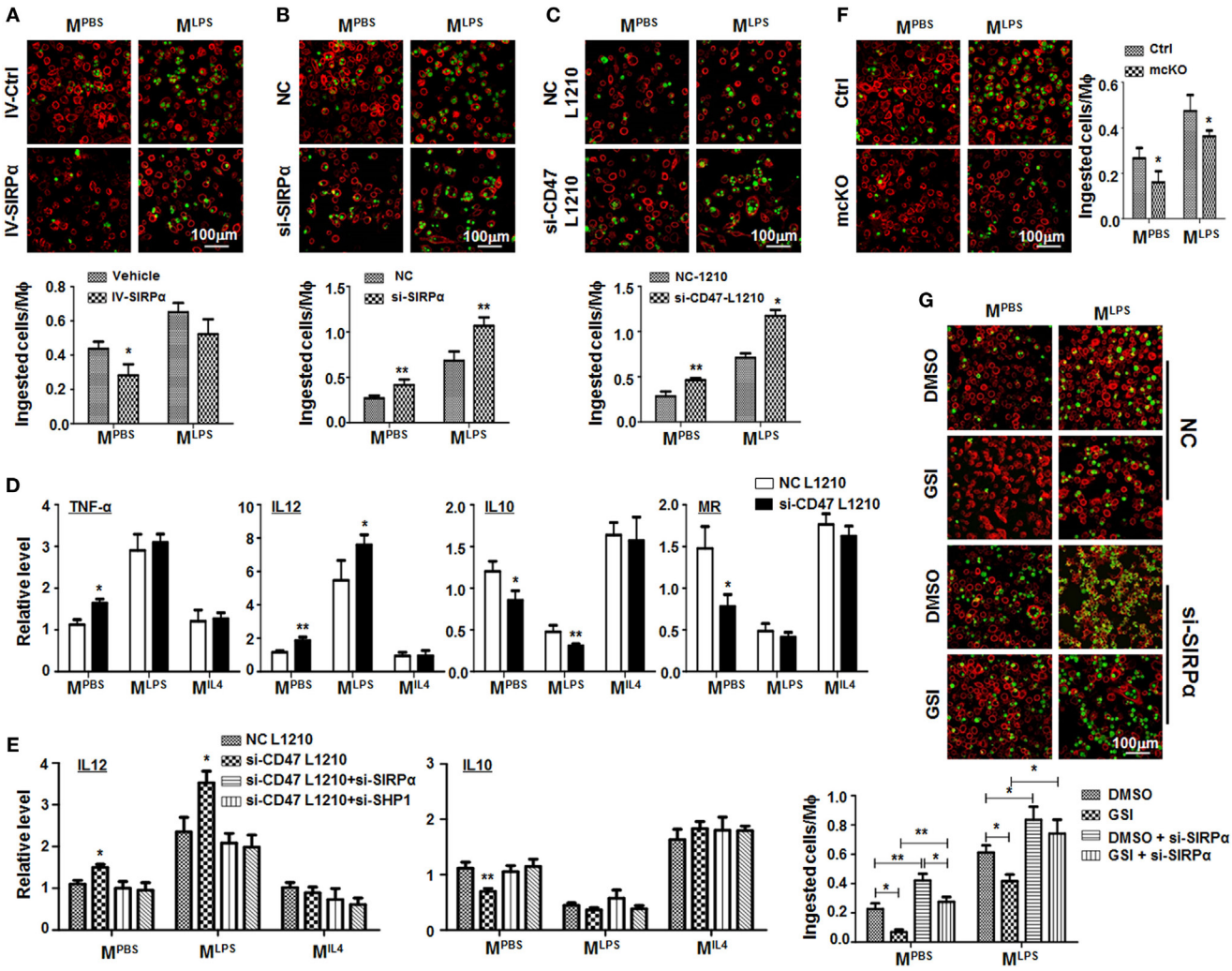

FIGURE 5 | Notch signal increased macrophage phagocytosis through repressing signal regulatory protein α (SIRPα). **(A)** Bone marrow-derived macrophages (BMDMs) were infected with SIRPα overexpression or control lentivirus and stimulated with PBS or lipopolysaccharide (LPS) + interferon (IFN)γ for 24 h. CFSE-labeled L1210 cells were then added and incubated for 2 h. After washing, samples were stained with anti-F4/80 and examined under an immunofluorescence microscope ($n = 5$). **(B)** BMDMs were transfected with si-SIRPα and treated as in **(A)**. The phagocytosis was examined under an immunofluorescence microscope ($n = 5$). **(C,D)** L1210 cells transfected with CD47 siRNA or NC were incubated with PBS- or LPS + IFNγ-stimulated BMDMs. Phagocytosis **(C)** ($n = 5$) and expression of polarization markers **(D)** ($n = 3$) were determined. **(E)** BMDMs were transfected with SIRPα siRNA or NC and stimulated with PBS, LPS + IFNγ, or IL4 and incubated with L1210 cells transfected with CD47 siRNA or NC. The expression of IL12 and IL10 was determined by qRT-polymerase chain reaction ($n = 3$). **(F)** BMDMs from RBP-J mcKO and control mice were treated as in (A). Phagocytosis was examined under an immunofluorescence microscope ($n = 5$). **(G)** BMDMs were treated with DMSO or GSI, and PBS or LPS + IFNγ. Phagocytosis was determined as in **(A)** ($n = 5$). One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^*P < 0.05$; $^{**}P < 0.01$.

result showed that cells incubated with mSIRPα^{ext} exhibited significantly higher fluorescence intensity as compared with the control cells (**Figure 6D**), suggesting that the mSIRPα^{ext} fusion protein could bind to L1210 tumor cells most likely through the interaction with CD47.

mSIRPα^{ext} Polypeptide Could Promote M1 Polarization and Enhance the Phagocytosis of Macrophages

To verify whether mSIRPα^{ext} could block the interaction between CD47 and SIRPα and attenuate SIRPα signaling in macrophages, we incubated BMDMs with mSIRPα^{ext} *in vitro*. This treatment did not affect the proliferation or apoptosis of macrophages (Figures S4A,B in Supplementary Material). However, qRT-PCR showed that mSIRPα^{ext} could upregulate the expression of M^{LPS} markers and downregulate that of M^{IL4} marker IL10 (**Figure 7A**). Moreover, the upregulation of M^{LPS} and downregulation of M^{IL4} markers by mSIRPα^{ext} was canceled by forced overexpression of SIRPα through lentivirus-mediated transfection (**Figure 7B**). We also examined the phagocytosis of L1210 cells by macrophages in the presence of mSIRPα^{ext}. CFSE-labeled L1210 cells were incubated with mSIRPα^{ext} in advanced, and then cocultured with M^{PBS}- or M^{LPS}-stimulated BMDMs in the presence of mSIRPα^{ext}.

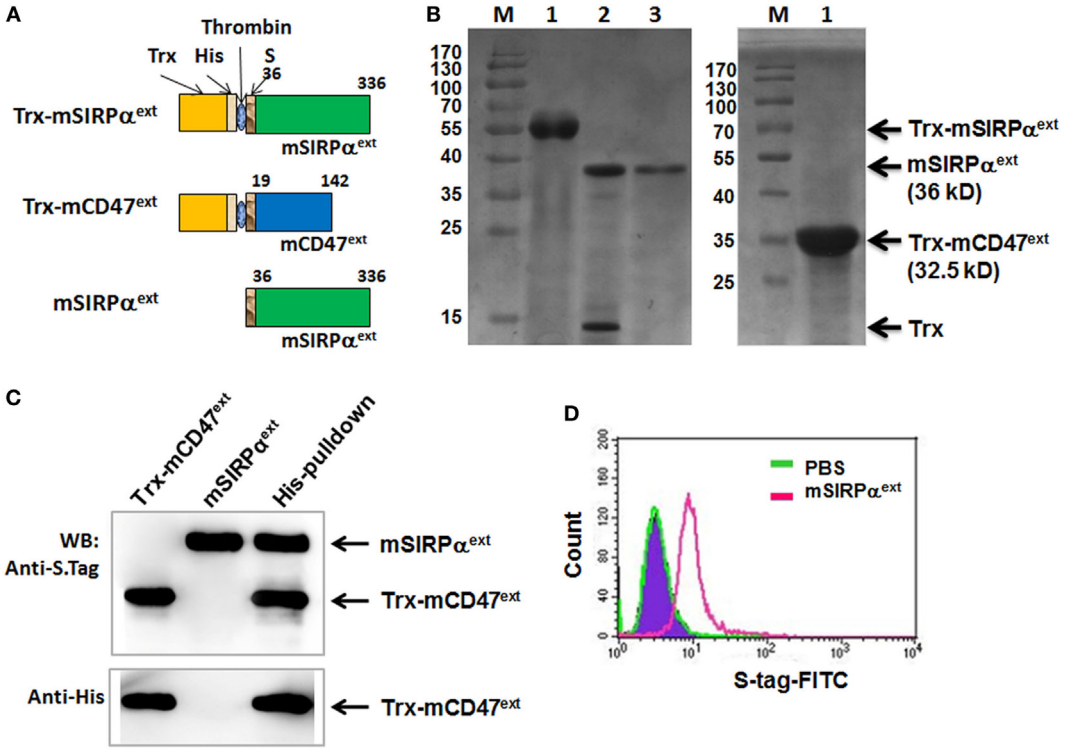

FIGURE 6 | Expression and purification of mSIRPα^{ext} fusion proteins. **(A)** Representative illustrations of the recombinant Trx-mSIRPα^{ext}, Trx-mCD47^{ext}, and mSIRPα^{ext} proteins. The thioredoxin peptide (Trx), His tag (His), S tag, and the site for thrombin-mediated cleavage are indicated. **(B)** SDS-PAGE of the purified Trx-mSIRPα^{ext} (left, lane 1), cleaved Trx-mSIRPα^{ext} (left, lane 2), purified mSIRPα^{ext} (left, lane 3), and Trx-mCD47^{ext} (right, lane 1). The Trx-mSIRPα^{ext}, mSIRPα^{ext}, Trx and Trx-mCD47^{ext} bands are indicated with arrows, with MW shown in parentheses. M, molecular weight marker. **(C)** mSIRPα^{ext} interacted with Trx-mCD47^{ext} in a pull-down assay. The purified mSIRPα^{ext} and Trx-mCD47^{ext} proteins were incubated in PBS at 4°C for 2 h and were precipitated with anti-His antibody pre-coupled with Dynabeads-protein G. Co-precipitated proteins were analyzed by Western blotting with anti-His or anti-S Tag antibody. Data represent three independent experiments. **(D)** L1210 cells were incubated with PBS or mSIRPα^{ext}. After washing, cells were stained with FITC-conjugated anti-S tag antibody, followed by FACS analysis.

The result showed that mSIRPα^{ext} could enhance the phagocytosis of macrophages, and overexpression of SIRPα abrogated this effect (**Figure 7C**). These results suggested that mSIRPα^{ext} fusion protein could block the SIRPα signaling to modulate macrophage activation and phagocytosis, likely *via* competitive interaction with CD47.

DISCUSSION

The Notch signaling pathway modulates various cell fate determination events during development. Moreover, Notch signal is involved in differentiation and plasticity of hematopoietic cells under both physiological and pathological conditions (29, 30). Indeed, there are four types of Notch receptors in mammals, all of which could induce RBP-J-mediated Notch signal activation. We have shown that Notch1 is the most abundantly expressed Notch receptor in BMDMs (14). Several groups have reported that Notch signal regulates myeloid development and macrophage polarization through multiple downstream molecules, such as SOCS3, cylindromatosis, interferon regulatory factor 8, miR-125a, and miR-148a (9–19). Although these studies have pointed to that Notch signal was required for macrophages M1 polarization, Foldi et al. have demonstrated that Notch signal promotes M2 activation of peritoneal macrophages in an *in vivo* model of chitin-induced M2 polarization (31). This probably suggested that the activation modes of macrophages modulated by Notch signal are context-dependent and influenced by specific pathological processes. Therefore, it should be of significance to further identify other potential downstream molecules of Notch signal. To understand the molecular network modulating macrophage activation downstream to Notch signal, we compared protein expression profiles between wild type and RBP-J deficient BMDMs using iTRAQ and found that SIRPα expression increased in the absence of Notch signaling. qRT-PCR and Western blotting validated the conclusion that SIRPα was downregulated by Notch signal activation or M^{LPS} stimulation in macrophages, while M^{IL4} stimulation or Notch blockade upregulated SIRPα. Further experiments showed that Notch activation repressed SIRPα transcription directly through Hes1-binding sites in its promoter region. Functionally, our data suggested that Notch signal modulated macrophage polarization at least partially through regulating the expression of SIRPα.

Signal regulatory protein α is a myeloid-specific receptor of CD47, which is broadly expressed on many types of somatic cells including tumor cells. SIRPα binds with CD47 and delivers a "don't eat me" signal for phagocytic cells to help tumor cells escape

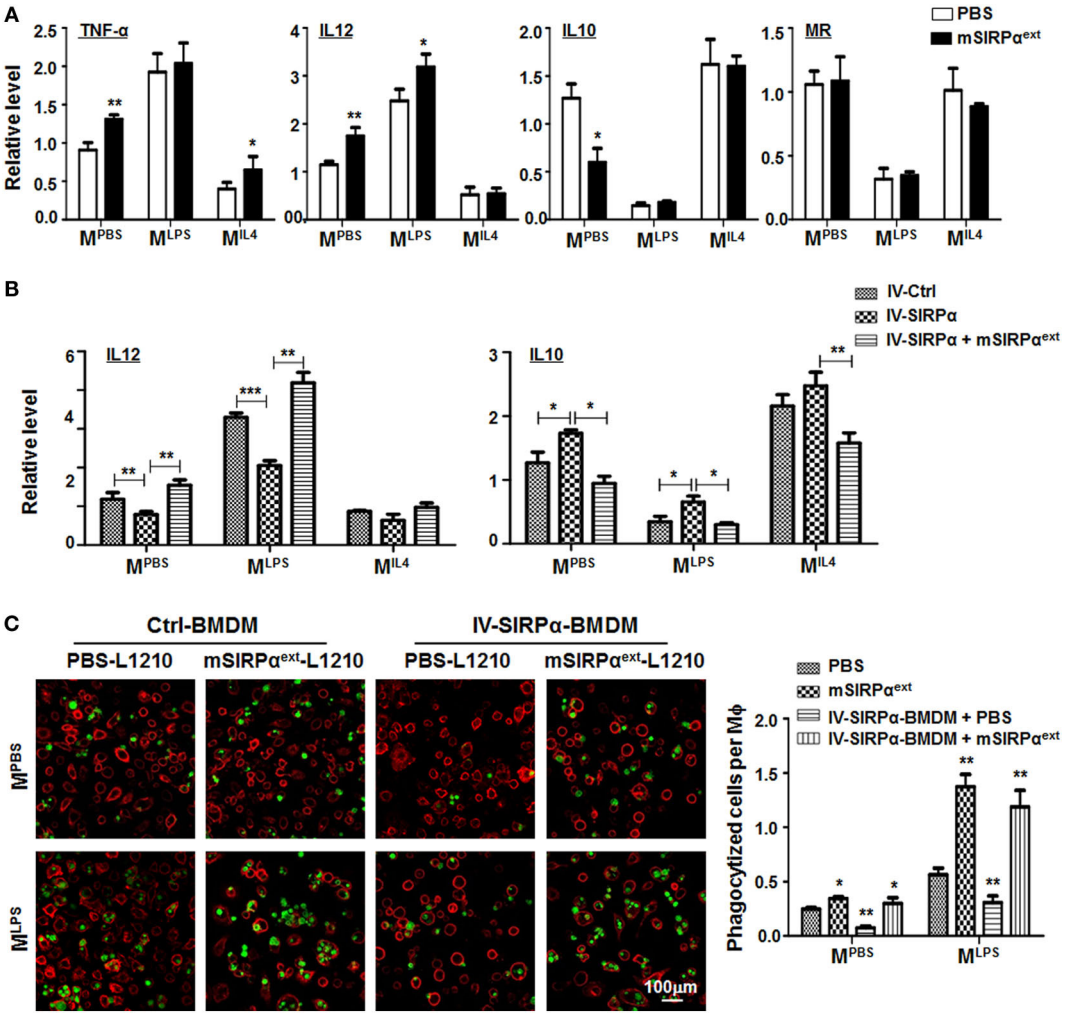

FIGURE 7 | Recombinant mSIRPα^{ext} promoted M1 polarization and enhanced phagocytosis of M1 macrophages *in vitro*. **(A)** Differentially polarized bone marrow-derived macrophages (BMDMs) were incubated with PBS or mSIRPα^{ext} for 6 h. The expression of TNF-α, IL12, IL10, and MR was determined by qRT-polymerase chain reaction (PCR) ($n = 3$). **(B)** BMDMs overexpressing SIRPα were incubated with mSIRPα^{ext} for competitive interaction and stimulated with PBS or lipopolysaccharide (LPS) + IFNγ. The expression of IL12 and IL10 was determined by qRT-PCR ($n = 3$). **(C)** BMDMs were stimulated with PBS or LPS + IFNγ in the presence of mSIRPα^{ext}. CFSE-labeled L1210 cells were then loaded and incubated for 2 h, and examined under an immunofluorescence microscope ($n = 5$). One-way ANOVA test was used for statistical analyses. Bars represent means ± SD, $n = 3$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

from immune clearance (32–36). Being activated by signal from adjacent cells *via* cell contact, SIRPα could induce intracellular downstream signaling by a cascade of phosphorylation modifications. The predominant pathway of SIRPα is mediated by SHP-1 activation, which could further influence NF-κB and Akt signaling and then regulate macrophage immune suppression (37, 38). Recent studies have mainly focused on CD47 expression on tumor cells and have developed cancer immunotherapy targeting CD47 (39–41), but the regulation of SIRPα expression and its role in macrophages in tumor microenvironment have been elusive. The present study suggests that SIRPα functions as an important modulator of macrophage polarization. The expression of SIRPα is significantly different in differentially polarized macrophages. Overexpression of SIRPα in BMDMs promoted M2 polarization, while SIRPα knockdown promoted M1 polarization. Interference of CD47 or SHP-1 of SIRPα signaling in BMDMs also promoted M1 polarization, suggesting that SIRPα regulates macrophage polarization dependent on interaction with CD47 and intracellular SHP-1 signaling.

Macrophages have remarkable potential as mediators of anti-cancer therapies based on their robust ability to ingest tumor cells and modulate tumor microenvironment. The CD47–SIRPα interaction between tumor cells and immune cells represents a critical intercellular communication that inhibits the activation of macrophage-mediated phagocytosis of tumors and thereby acts as a myeloid-specific immune checkpoint (20–23). In this study, we observed that SIRPα overexpression in BMDMs decreased phagocytosis of L1210 leukemia cells, while SIRPα knockdown in BMDMs and CD47 knockdown in L1210 cells both increased phagocytosis by macrophages. Meanwhile,

knock down of SHP-1, which modulates M^{LPS} polarization in BMDMs, did not show significant effect on macrophage phagocytosis (data not shown). These findings might suggest that SIRPα-mediated polarization and phagocytosis could be mediated by different mechanisms. Molecular events downstream to these important processes need further studies. In our study, activation of Notch signaling could suppress SIRPα expression and inhibit M^{IL4} macrophage polarization as well as phagocytosis, which might have important implications in tumor microenvironment.

Blocking CD47 triggers the elimination of cancer cells. Nevertheless, the ubiquitous expression of CD47 on normal cells, such as red blood cells, might create a large antigen sink and unintended binding. The unwanted interaction of CD47 on normal cells could be minimized by reducing the binding strength for CD47 (42). On the other hand, high-affinity SIRPα monomers (~14 kDa) are not sufficient to induce macrophage phagocytosis, instead, act as an adjuvant to lower the threshold for phagocytosis in the presence of a separate, tumor-binding antibody. Agents that block SIRPα directly, such as anti-SIRPα antibodies, also act as adjuvants for tumor-binding antibodies (43, 44). To overcome these limitations, we here validated a soluble extracellular domain of mouse SIRPα (36 KD) and confirmed that $mSIRP\alpha^{ext}$ could not only promote M1 polarization but also increase phagocytosis of L1210 leukemia cells by macrophages. Therefore, $mSIRP\alpha^{ext}$ might have a promising potential for the clinical application as a new therapeutic reagent. More studies are needed to assess its future pharmacological and clinical values.

ETHICS STATEMENT

All animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University and in accordance with the recommendations of Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

AUTHOR CONTRIBUTIONS

HH, Y-ML, and J-LZ designed the research and wrote the manuscript; YL, J-LZ, Q-JZ, XJ, and JT performed experiments; YL, J-LZ, S-QL, H-WG, and H-YQ analyzed data. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01744/full#supplementary-material.

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NOTCH and Graft-Versus-Host Disease

Mauro Di Ianni[1,2†], Beatrice Del Papa[3†], Stefano Baldoni[4], Ambra Di Tommaso[4], Bianca Fabi[4], Emanuela Rosati[5], Annalisa Natale[2], Stella Santarone[2], Paola Olioso[2], Gabriele Papalinetti[2], Raffaella Giancola[2], Patrizia Accorsi[2], Paolo Di Bartolomeo[2], Paolo Sportoletti[3] and Franca Falzetti[3]*

[1] Department of Medicine and Aging Sciences, University of Chieti-Pescara, Chieti, Italy, [2] Department of Hematology, Transfusion Medicine and Biotechnologies, Ospedale Civile, Pescara, Italy, [3] Institute of Hematology-Centro di Ricerche Emato-Oncologiche (CREO), University of Perugia, Perugia, Italy, [4] Department of Life, Health and Environmental Sciences, Hematology Section, University of L'Aquila, L'Aquila, Italy, [5] Department of Experimental Medicine, Biosciences and Medical Embriology Section, University of Perugia, Perugia, Italy

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***Correspondence:**
Mauro Di Ianni
mauro.diianni@unich.it

†These authors have contributed equally to this work.

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In allogeneic hematopoietic stem cell transplantation, which is the major curative therapy for hematological malignancies, T cells play a key role in the development of graft-versus-host disease (GvHD). NOTCH pathway is a conserved signal transduction system that regulates T cell development and differentiation. The present review analyses the role of the NOTCH signaling as a new regulator of acute GvHD. NOTCH signaling could also represent a new therapeutic target for GvHD.

Keywords: NOTCH, graft-versus-host disease, tolerance, graft-versus-leukemia, HSCT

INTRODUCTION

Hematopoietic stem cell transplantation from allogeneic donors is the major curative therapy for hematological malignancies such as acute leukemias (ALs). The development of graft-versus-host disease (GvHD) is the most common complication which dramatically increases post-transplant morbidity and mortality (1). The clinical presentations of GvHD include acute GvHD (aGvHD) which regards 30–50% of transplanted patients and chronic GvHD (cGvHD) which includes 30–70% of patients who underwent allogeneic hematopoietic stem cell transplantation (2, 3). GvHD is triggered by the donor T cells that can cause an inflammatory disease ultimately leading to severe multiorgan damage (liver, gut, and skin) (4–8).

Donor T cells play a crucial role not only in mediating the onset of GvHD but also in eradicating malignancy, the graft-versus-leukemia (GvL) effect as showed by clinical (9, 10) and experimental studies (11–13). Allogeneic T cells recognize host antigens on leukemic cells and leukemia-specific responses may also occur (14). Despite this strong GvL effect exerted by donor T cells, relapse is still the major cause of treatment failure in high-risk AL patients who underwent allogeneic HSCT (15–18). Strategies to separate GvHD and GvL are then under investigation.

The NOTCH signaling pathway relies on the interactions between receptors (NOTCH1–4) and ligands (Jagged1 and -2 or Dll1, -3, and -4) that are expressed on neighboring cells (19). The interactions NOTCH/NOTCH ligand induce proteolytic activation of the receptor by an ADAM family metalloprotease and then by the γ-secretase complex. The sequential cuts lead to the release of the active intracellular NOTCH (ICN) that enters the nucleus and interacts with the DNA binding CSL/RBP-Jk factor, constituting a transcriptional activation complex with a mastermind-like (MAML) family coactivator. This ultimately promotes the transcription of target genes, controlling crucial biologic processes, such as survival, proliferation, and differentiation (20). Besides the canonical ICN/CSL/MAML-dependent transcriptional activation, RBP-Jk-independent non-canonical NOTCH signaling have also described (21, 22).

NOTCH signaling was first studied for its fundamental role in the early step of lymphopoiesis (23) and it has been implicated also in mature T cell function (24–26). More recently, NOTCH signaling has emerged as a new regulator of acute (27–32) and cGvHD (33). In this review, we will focus on NOTCH signaling and aGvHD.

NOTCH SIGNALING IS ACTIVATED DURING GvHD IN DONOR T CELLS

NOTCH and alloimmune responses have been extensively studied in GvHD and in non-GvHd models. In Severe Aplastic Anemia (SAA) mouse model, Roderick et al. (34) showed NOTCH signaling mediate Th1 cell differentiation and T-BET expression. Treatment with γ-secretase inhibitors (GSIs) reduced NOTCH and T-BET expression and rescued mice from SAA.

In the setting of GvHD, the Kean group (35) demonstrated the existence of NOTCH-related signature in alloreactive T cells harvested from a non-human primate model.

The Maillard group reported that NOTCH signaling is a strong regulator of T-cell activation, differentiation, and function during GvHD (28, 36). Murine models of allo-HCT showed that inhibition of canonical NOTCH signaling markedly decreased GVHD severity and mortality (28–30). NOTCH inhibition dramatically reduced the accumulation of alloreactive T cells in the gut. Interestingly, NOTCH-inhibited T cells significantly retained their antileukemic activity. By using humanized antibodies and conditional genetic models, Tran et al. (29) demonstrated that all the effects of NOTCH signaling during GvHD were dependent on NOTCH1/2 receptors in T cells and Dll1/4 ligands in the recipient, with dominant roles for NOTCH1 and Dll4 (29). NOTCH-inhibited T cells acquire a hyporesponsive phenotype in both CD4 and CD8 populations. NOTCH deprived T cells markedly reduced cytokine production but maintain their expansion capacity and their *in vitro* cytotoxic ability (30).

The exact mechanisms of NOTCH modulation in T cells remain to be elucidated. Mochizuki et al. (37) in murine model showed that during GvHD, inflammatory DCs Dll4 ligand positive produce significantly high level of IFN-γ and IL-17. More recently, Chung et al. (27) showed that NOTCH signal are delivered to donor T cells shortly after transplantation and that host stromal cells are the source for NOTCH ligands during *in vivo* priming of alloreactive T cells. Interestingly, Luo et al. (38) have shown in an MHC-mismatched murine all-BMT model, inhibition of NOTCH signaling reduce the incidence of GvHD by reducing DCs and CD8 T cell proliferation and activation.

NOTCH pathway inhibition could be therapeutically targeted by: (1) GSIs that block the proteolytic activation after the NOTCH/NOTCH ligand interaction (39). However, the use of GSIs in murine model of GvHD is associated with a severe toxicity in the gut epithelium (29); (2) monoclonal antibodies directed against NOTCH ligands such as Dll1–4 (29); (3) we recently identified the calcium channel modulator bepridil as a new NOTCH1 pathway inhibitor in Chronic Lymphocytic Leukemia (40). It represents an attractive therapeutic strategy to prevent also GvHD (**Figure 1**).

REGULATORY T CELLS (Tregs) DOWNREGULATE NOTCH SIGNALING IN DONOR T CELLS

Regulatory T cells suppressed alloimmune reactions like, for example, GvHD (41). They also promoted tolerance to allogeneic organ transplants (42). Adoptive Treg/conventional T cell (Tcons)-based immunotherapy in full-haplotype mismatched transplantation practically eliminated acute and cGvHD, supported post-transplant immunological reconstitution and exerted a strong GvL effect (43–48) in high-risk AL patients.

Although the mechanisms underlying Treg suppression of GvHD with no loss of GvL activity remain to be unraveled, the principal hypotheses are based on (a) the Treg/Tcon homing and distribution patterns and (b) different molecular pathways in Tcon activation and proliferation and, consequently, GvL and GvHD. Interestingly, using humanized antibodies and conditional genetic mouse models to inactivate NOTCH signaling in donor T cells markedly reduced GvHD severity and mortality (28–30). NOTCH signaling other than a cell autonomous mechanism can be modulated with an extrinsic signal from an adjacent interacting cell. Current evidence suggests that Tregs and anti-NOTCH1 compounds inhibit the same NOTCH ligands and receptors on Tcons (29, 49). Mimicking the drug-mediated NOTCH1 inhibition (30), Tregs directly inhibited NOTCH1 signaling on Tcons *in vitro* and *in vivo*, with the blockade being observed on CD4 and CD8 cells from mouse lymph nodes (49). Jagged1 and Dll4 NOTCH1 ligands, played major roles (49) with Dll4 being reported to mediate all NOTCH signaling effects in Tcons during GvHD (29). As a GvHD prevention strategy, using alloantigen-specific Tregs which preferentially inhibit alloreactive Tcons to downregulate NOTCH1 clearly offers advantages over administering pharmaceutical agents which exert a total blockade on NOTCH1 signaling on all Tcons.

CD39–NOTCH1 pathway crosstalk was also demonstrated (49). In fact, NOTCH1 expression and signaling on Tcons were restored when CD39 was blocked by the anti-CD39 monoclonal antibody or polyoxometalate-1 (POM-1), the selective CD39 inhibitor (49). Increased cAMP levels were associated with NOTCH1 reduction in Tcons; adding anti-CD39 reduced cAMP levels and reversed the Treg-mediated NOTCH1 reduction. GvHD reappeared in mice after POM-1 administration (49). *In vitro* studies (50–52) showed that blocking Abs or chemical products downmodulated the CD39/adenosine axis and reversed Treg suppression of T cons. Although the Treg mechanisms of action are multiple and partially unclear (53), these data showed that Tregs triggered NOTCH1 downregulation directly in Tcons and acted through the CD39/adenosine axis to inhibit the NOTCH pathway which, in turn, regulates Tcon proliferation (**Figure 1**). This mechanism of action could account for Treg-induced inhibition of Tcon proliferation which was observed by others (30).

Interestingly, in CD4 and CD8 cell populations, NOTCH1 downregulation was more marked in peripheral blood than in bone marrow (54). Tregs were demonstrated to block Tcons in the periphery but not in bone marrow (55). We could speculate that Treg homing patterns play a major role in these results. Tregs could have downregulated NOTCH1 expression in peripheral

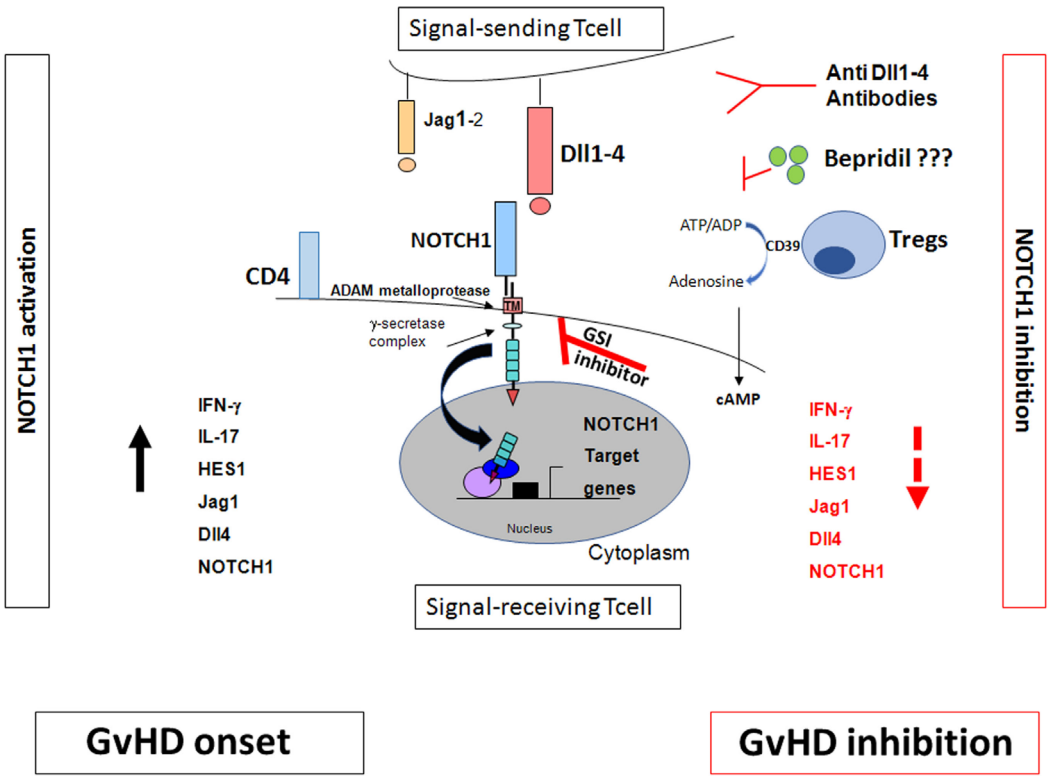

FIGURE 1 | NOTCH signaling and graft-versus-host disease (GvHD). The effects of NOTCH signaling during GvHD are dependent on NOTCH1/2 receptors in T cells and Dll1/4 ligands in the recipient, with dominant roles for NOTCH1 and Dll4. Inhibition of canonical NOTCH signaling by γ-secretase inhibitor (GSI)-inhibitor, anti Dll1–4 antibodies, bepridil, and regulatory T cells (Tregs) markedly decreased GVHD (28–30). Tregs inhibit the NOTCH/NOTCH ligand interactions. They act through the CD39/adenosine axis to inhibit the NOTCH pathway which, in turn, regulates T cell proliferation and consequently inhibits GvHD. Blocking CD39/adenosine axis reverts NOTCH inhibition and favors GvHD onset (49).

tissue because they homed there while, because of different migratory properties, they homed less efficiently, or not at all, to bone marrow. Translation of tissue-specific NOTCH1 expression into a strong GvL effect without GvHD, needs, however, to be elucidated in depth. A Treg-related NOTCH1 blockade could account for clinical and experimental evidence that Tregs prevented GvHD and facilitated a powerful Tcon-dependent GvL effect (44, 45). Consequently Treg-mediated NOTCH inhibition, like drug-induced NOTCH downregulation (28–30) may separate GvHD from GvL. This finding has major implications for adoptive immunotherapy strategies in the field of transplantation for leukemia.

MESENCHYMAL STEM CELLS (MSCs) RECRUIT INDUCED Tregs (iTregs) BY ACTIVATING NOTCH SIGNALING

NOTCH1 signaling is also involved in Treg cell differentiation. Liotta et al. had described Jagged1 involvement in MSC suppression of T-cell proliferation (56). Our group showed when cocultured with CD3+ cells, MSCs induced a T-cell population with a regulatory phenotype (57). When CD4+ T cells were cocultured with MSCs, the NOTCH1 pathway was found to be activated (58). Using GSI-I or the NOTCH1 neutralizing antibody to inhibit NOTCH1 signaling reduced HES1 expression (the NOTCH1 downstream target) and the percentage of MSC-induced CD4+CD25highFOXP3+ cells *in vitro* (58) (**Figure 2**). In human cells FOXP3 is another NOTCH signaling downstream target (58), thus data from murine models were extended (59). NOTCH signaling activation reversed the unstable regulatory/suppressive properties of iTreg cells, ensuring sustained FOXP3 expression and stable Treg-cell phenotypes (58). No crosstalk between NOTCH1 and TGF-β signaling pathways was observed (58). Previous studies had demonstrated TGF-β production was involved in MSCs-mediated Treg cell induction (60, 61) and reported TGF-β/NOTCH1 crosstalk (58) in peripheral Treg cell maintenance. Lack of T-cell receptor stimulation in the work by Del Papa et al. may account for the discrepancy with other reports (58, 62–64). Together, these findings indicated that NOTCH1 pathway activation played a role in MSC-mediated human Treg-cell induction. In conclusion while on one side our observation on MSC-T cell coculture suggest a positive role of NOTCH in the generation of iTregs, on the other side NOTCH inhibition (drug or Treg mediated) in mature donor T cells is associated with reduction in GvHD severity and mortality.

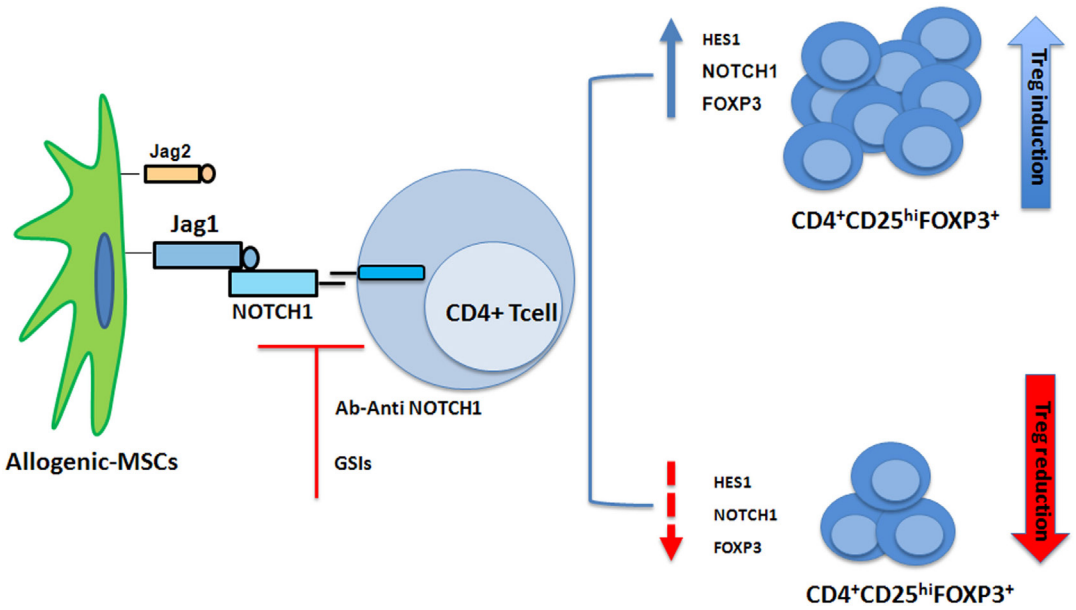

FIGURE 2 | Mesenchymal stem cells (MSCs) recruit induced Tregs (iTregs) by activating Notch signaling. When cocultured with CD4+ cells, MSCs induced a T-cell population with a regulatory phenotype (iTregs) (57). NOTCH1 pathway is activated in CD4+ T cells cocultured with MSCs. Inhibition of NOTCH1 signaling through γ-secretase inhibitor (GSI)-I or the NOTCH1 neutralizing antibody reduced expression of HES1 and the percentage of MSC-induced CD4+CD25highFOXP3+ cells *in vitro* (58).

CONCLUSION AND PERSPECTIVES

Allogeneic immune system played a crucial role not only in mediating the onset of GvHD but also in eradicating malignancy, i.e., the GvL effect. Separating GvHD from GvL represent a major challenge. GvHD prophylaxis and treatment is mainly based on immunosuppressive treatment with drugs such as cyclosporine, tacrolimus, methotrexate, antithymocyte globulin, and glucocorticoids (4). Data reviewed here showed NOTCH1 as a new major regulator of alloreactivity. Triggering NOTCH pathway with pharmacological (GSIs, Ab anti-Notch) or cellular (Tregs) ways might represent a new strategy to separate GvHD from GvL.

AUTHOR CONTRIBUTIONS

MDI organized the plan and structure of the manuscript, and all the authors contributed to the redaction.

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REVIEW
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Cancer Cells Exploit Notch Signaling to Redefine a Supportive Cytokine Milieu

Michela Colombo[1], Leonardo Mirandola[2], Maurizio Chiriva-Internati[2,3,4], Andrea Basile[5], Massimo Locati[6,7], Elena Lesma[1], Raffaella Chiaramonte[1†] and Natalia Platonova[1†]*

[1] Department of Health Sciences, Università degli Studi di Milano, Milano, Italy, [2] Kiromic Biopharma Inc., Houston, TX, United States, [3] Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [4] Department of Gastroenterology, Hepatology and Nutrition, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [5] Department of Oncology and Hemato-Oncology, Università degli Studi di Milano, Milano, Italy, [6] Department of Medical Biotechnologies and Translational Medicine, Università degli Studi di Milano, Milano, Italy, [7] Humanitas Clinical and Research Center, Rozzano, Italy

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Morehouse School of Medicine, United States

***Correspondence:**
Raffaella Chiaramonte
raffaella.chiaramonte@unimi.it

[†]These authors have contributed equally to this work.

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Notch signaling is a well-known key player in the communication between adjacent cells during organ development, when it controls several processes involved in cell differentiation. Notch-mediated communication may occur through the interaction of Notch receptors with ligands on adjacent cells or by a paracrine/endocrine fashion, through soluble molecules that can mediate the communication between cells at distant sites. Dysregulation of Notch pathway causes a number of disorders, including cancer. Notch hyperactivation may be caused by mutations of Notch-related genes, dysregulated upstream pathways, or microenvironment signals. Cancer cells may exploit this aberrant signaling to "educate" the surrounding microenvironment cells toward a pro-tumoral behavior. This may occur because of key cytokines secreted by tumor cells or it may involve the microenvironment through the activation of Notch signaling in stromal cells, an event mediated by a direct cell-to-cell contact and resulting in the increased secretion of several pro-tumorigenic cytokines. Up to now, review articles were mainly focused on Notch contribution in a specific tumor context or immune cell populations. Here, we provide a comprehensive overview on the outcomes of Notch-mediated pathological interactions in different tumor settings and on the molecular and cellular mediators involved in this process. We describe how Notch dysregulation in cancer may alter the cytokine network and its outcomes on tumor progression and antitumor immune response.

Keywords: Notch, cytokine, chemokine, cancer, immune response, VEGF, inflammation, senescence

INTRODUCTION

The critical events in tumor development and progression include heterotypic interactions between neoplastic cells and normal components of the tumor niche. This crosstalk causes the activation of several signaling pathways that, in turn, promote tumor growth, survival, drug resistance, bone resorption, and metastases.

The interplay between tumor cells and immune system has a crucial role in this process. Indeed, tumor development causes a dysregulation of the physiological cytokine milieu, affecting the effectors of cellular and innate immunity, ultimately tipping the balance between immunosuppression and immune stimulation that sustains the disease progression (1).

Recently, Notch signaling has emerged as a key regulator of the cellular relationships within the tumor microenvironment (TME). The Notch system comprises a family of transmembrane receptors

(Notch1–4), activated by the interaction with five membrane-bound ligands (Jagged1–2 and Dll1–3–4) present on adjacent cells. Ligand binding results in Notch cleavage by two proteases, ADAM and γ-secretase. These cleavages release Notch intracellular domain (ICN) from the plasma membrane, allowing it to translocate into the nucleus, where it regulates the transcription of a plethora of target genes in a transcriptional complex with the CSL (CBF-1/suppressor of hairless/LAG-1, also known as RBP-Jk), mastermind-like (MAML1–3) coactivator, and other proteins (2). Besides this canonical Notch pathway, in oncogenesis and inflammation, it has been described a non-canonical Notch signaling which is γ-secretase independent (3). Notch signaling is tightly controlled by several mechanisms, including degradation mediated by the proteasome and lysosome machineries (4, 5). The cancer-related aberrant activation of Notch pathway affects the biology of the single tumor cell and its interaction with the surrounding microenvironment (6).

In this review, we analyze how the dysregulation of the Notch pathway in the tumor niche skews the local cytokine milieu (**Table 1**), shaping the immunological landscape, and we describe the outcomes of this process on tumor growth, progression, senescence, and metastases illustrating the different molecular mechanisms and mediators operating in the distinct cellular contexts.

NOTCH SIGNALING PROMOTES AN IMMUNOSUPPRESSIVE TME

The TME is characterized by the prevalence of anti-inflammatory, immunosuppressive cytokine milieu. The production of an immunosuppressive secretome often requires Notch signaling activation. In this chapter, we explore the role of Notch as a positive regulator of the most important anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β), interleukin 10 (IL-10), interleukin 4 (IL-4), and IL-6. The role of CXCL12 and of receptor activator of nuclear factor kappa-B ligand (RANKL) will be discussed as well.

TRANSFORMING GROWTH FACTOR-β

Transforming growth factor-β is expressed at high levels in several malignancies, where it correlates with poor prognosis (40). The main source of TGF-β in cancer is tumor and stromal cells, but it may also be released following bone extracellular matrix remodeling mediated by bone-associated tumors (41).

Transforming growth factor-β supports tumor progression through several mechanisms. The activation of TGF-β receptor promotes chemoresistance and angiogenesis in breast, prostate, gastric, and colon cancer. In addition, TGF-β is also a key player in epithelial-to-mesenchymal transition (EMT) (42).

Transforming growth factor-β is best known for its potent immunosuppressive activity that affects both cells of the innate and adaptive immunity (43–46).

The crosstalk between TGF-β and Notch triggers the TGF-β immunosuppressive activity in several contexts. TGF-β is a well-known inhibitor of DC maturation and, upon stimulation of TGF-βRI receptor, the active form of Notch1 can boost TGF-βRI signaling in DCs by binding Smad3. The interaction of Notch1 with Smad3 promotes the translocation of the latter into the nucleus and induces the transactivation of Smad target genes (7, 42). Moreover, Ostroukhova et al. demonstrated that T-reg cell-derived TGF-β inhibited the activation of effector T cells through the Notch target, HES1. *In vivo* experiments confirmed that this inhibitory effect of Tregs on the activation of effector T cells may be reverted by the treatment with anti-Notch1 antibodies (8).

In lung carcinoma, Notch mediates the pro-tumoral effect of TGF-β secreted by CD11b+ Ly6C+ Ly6G− myeloid-derived

TABLE 1 | Effects of Notch signaling on the cytokine milieu and the immune system.

Notch pathway member	Cytokine	Main functions	Cancer type	Immune mediators	Reference
Notch1	TGFβ	Immunosuppression, anti-inflammatory, epithelial-to-mesenchymal transition, angiogenesis	–	DC, Treg	(7, 8)
Dll4	TGFβ	as above	Lung carcinoma	MDSC	(9)
Notch3, Jagged1	IL-6	as above	Breast cancer	MDSC	(10–12)
Unknown	CXCL12	Migration, proliferation, angiogenesis	Multiple myeloma	M2	(13, 14)
Unknown	CXCL12	as above	Ovarian cancer	T lymphocyte	(15, 16)
Unknown	CXCL12	as above	Hepatocellular carcinoma	Treg, M2	(6, 17)
Dll family, Jagged1/2	IL-10	as above	–	Th1	(18)
Unknown	IL-10	as above	Melanoma, lung carcinoma	TAM	(19–21)
Dll family	IL-10	Immunosuppression, anti-inflammatory	–	DC, Th1	(22, 23)
Jagged1/2, Notch1	IL-4	as above	–	Th2, DC	(24–26)
Dll4	IL-4	Immunosuppression	–	TAM	(27)
Dll4	IL-17	as above	–	γδT cell	(28)
Unknown	IL-17	as above	Oral cancer	CD4+ T, Th17	(29)
Notch1, Jagged2	CCL5	Proliferation, invasion, metastasis	Breast cancer	TAM M2	(30)
Jagged1	IL-1β, CCL2	Pro-inflammatory, proliferation	Breast cancer	TAM	(31, 32)
Notch1	CCL2	Proliferation	Lung carcinoma	Mo-MDSC macrophage	(33)
Jagged1	IFN-γ	Killing immunological functions	–	DC, T cell	(34)
Jagged2	IFN-γ	as above	Lymphoma	NK	(35)
Notch1, Notch2	IFN-γ	as above	–	CD4+ T, CD4+ Th1, CD8+ T	(36–38)
Dll1	VEGF	Angiogenesis, immunosuppression	Lung carcinoma	T cell	(39)

suppressor cells (MDSCs). MDSCs are a heterogeneous population of immature myeloid cells that can inhibit T cell responses. In lung carcinoma, MDSCs suppress CD4+ and CD8+ T cell activity (47), secrete TGF-β, which promotes neoplastic cells proliferation and the expression of Dll4. MDSC-derived Dll4 activates Notch in lung carcinoma cells, boosting TGF-β signaling by binding and activating Smad proteins. Consistently, lung cancer cells treated with the Notch inhibitors, DBZ and DAPT, showed a reduced response to TGF-β and a decreased cell growth, indicating that at least in part TGF-β pro-tumorigenic functions are Notch dependent, and suggesting that targeting Notch may represent a promising therapeutic strategy to antagonize TGF-β (9).

Finally, it is worth mentioning that the cooperation between TGF-β and Notch pathway, on top of altering the immune surveillance, promotes EMT (6, 42) in different malignancies, such as ovarian cancer (48) and squamous cell carcinoma (49). Here, high levels of ICN1 seem to cooperate with the TGF-β pathway in the tumor milieu, favoring Smad2/3 phosphorylation, and finally promoting EMT and the survival of tumor-initiating cells (49). The molecular basis of this process is not fully understood, but its implications in cancer progression are clear. EMT process modifies tumor cell behavior, reducing the adhesion to neighboring cells, promoting the invasion through the basement membrane, and finally allowing metastatic dissemination (50).

Finally, TGF-β may also positively regulate the Notch pathway through different mechanisms (**Figure 1**). In breast cancer bone metastasis, Jagged1 acts as a downstream mediator of TGF-β oncogenic signal, contributing to a positive feedback

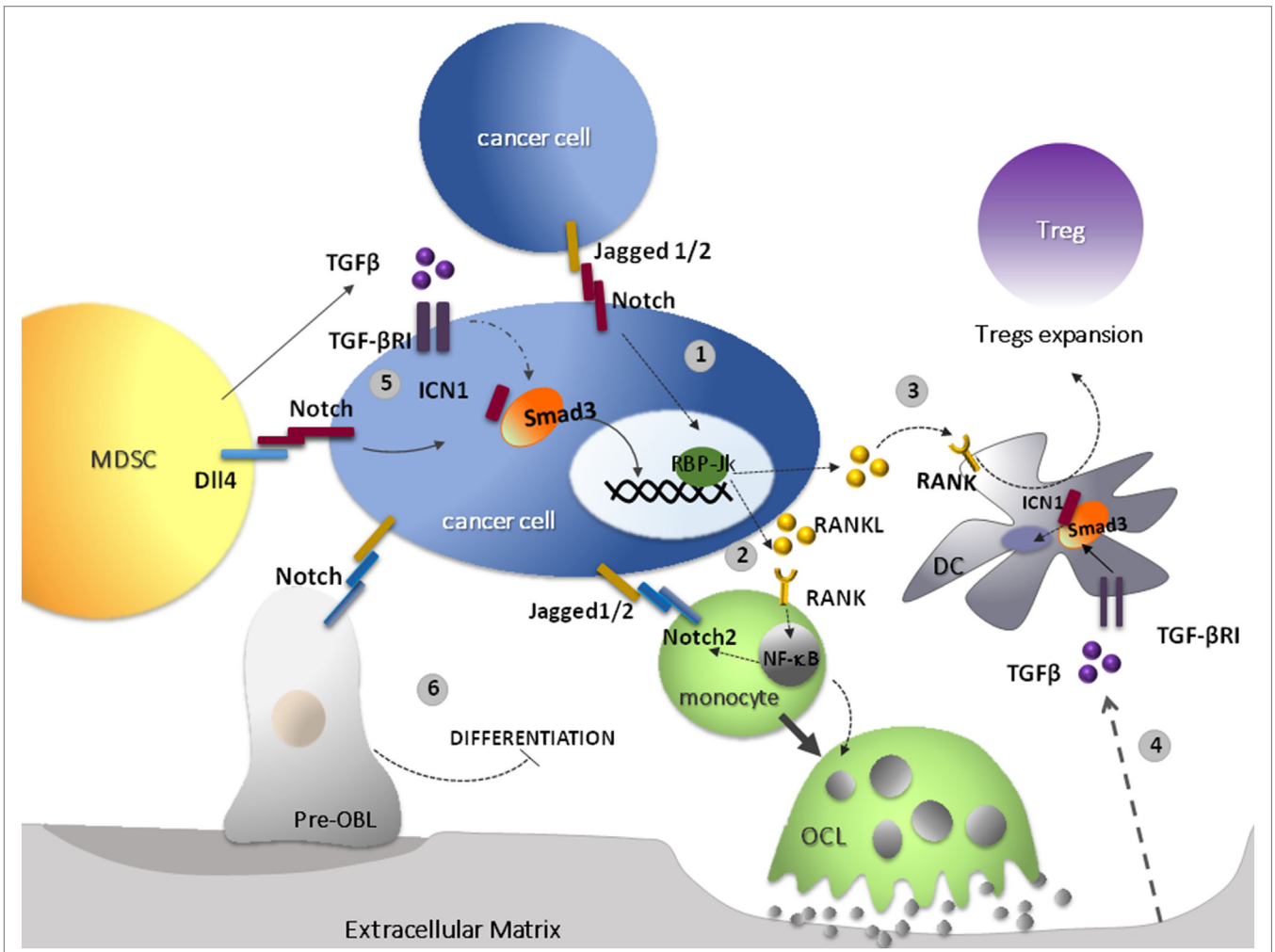

FIGURE 1 | Transforming growth factor-β (TGF-β) and receptor activator of nuclear factor kappa-B ligand (RANKL) cooperate to suppress the immune response in the bone marrow. 1. In bone-associated cancers, the activation of Notch may be promoted by Jagged1/2 ligands overexpressed by cancer cells; one of the outcomes of Notch overactivation is to increase RANKL expression (52). 2. RANKL represents the main osteoclastogenic factor and promotes osteoclasts (OCLs) differentiation and bone resorption (53). 3. In addition, RANKL plays immunoregulatory functions. RANKL may activate its receptor RANK, which is overexpressed by DCs and, in turn, boosts DCs ability to induce the expansion of the local Treg population promoting tolerance to tumor antigens (54). 4. One of the outcomes of the increased bone resorption is the release of TGF-β from the extracellular matrix (55). 5. TGF-β can be also secreted by tumor and stromal cells and by myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment (TME). Its immunosuppressive effects may be promoted by Notch signaling (see text for details) (41, 51). 6. In specific contexts, such as breast cancer-derived bone metastasis, TGF-β released by cancer cells mediates bone remodeling and stimulates the overexpression of Jagged1 in tumor cells. Jagged1 present on cancer cell surface, in turn, triggers Notch activation in OCLs and osteoblasts (OBLs), promoting the development of tumor-associated bone disease (56).

in cancer-mediated bone destruction. Cancer-derived TGF-β mediates bone remodeling and stimulates the overexpression of Jagged1 in tumor cells. In turn, Jagged1, located on the cancer cell surface, triggers Notch activation in osteoclasts (OCLs) and osteoblasts (OBLs). The net effect of this process is OCLs differentiation and activation, and OBLs inhibition (51). This is in agreement with the evidence that Jagged1 forced expression can restore the ability of xenografted breast cancer cells to form bone lesions in Smad knock-out mice (10).

INTERLEUKIN 6

IL-6 has been proposed as a therapeutic target in several tumors, since it represents one of the most abundant soluble factors in the TME (57). It is associated with poor prognosis and is present at high concentrations in the serum of patients with different malignancies, including multiple myeloma, breast, colon, gastric, pancreatic, esophageal, hepatic, cervical, and renal cancer (55, 58).

IL-6 signaling has been shown to promote tumorigenesis by regulating cancer metabolism, increasing cancer cell growth and self-renewal, as well as resistance to apoptosis, boosting invasiveness and metastasis, regulating angiogenesis (57), and sustaining RANKL expression and bone resorption (59). IL-6 may also regulate the immune system by playing a role as pro-inflammatory and anti-inflammatory cytokine (59). Here, we will focus on the immunosuppressive effect, more frequently described in cancer, while we will refer to the pro-inflammatory, immune-activating effect of IL-6 in the chapter on cancer cell senescence.

The activation of Notch pathway induces the expression of IL-6 in malignant cells of different tumors, i.e., in colon cancer, stimulates tumor cell proliferation (60), and in luminal breast cancer, it promotes self-renewal and drug resistance (61). In other malignancies, such as multiple myeloma (62) and gastric cancer (63), Notch ability to drive IL-6 secretion has been observed also in the surrounding stromal cells of the TME. The increase of IL-6 in the TME promotes tumor cell growth and disease progression (**Figure 2**).

The interplay between IL-6 and Notch has been studied in depth in multiple myeloma and breast cancer. Myeloma cells colonize the bone marrow (BM), which represents a safe harbor, where tumor cells find an ideal environment for their proliferation and survival (66). In the BM of multiple myeloma patients, IL-6 is produced by tumor cells, BMSCs, and cells of the myeloid lineage, such as eosinophils, macrophages, DCs, and mast cells (67). Recently, we demonstrated that in multiple myeloma the overexpression of Notch ligands, Jagged1 and Jagged2, combined with the expression of Notch receptors, activates the endogenous Notch signaling, which drives IL-6

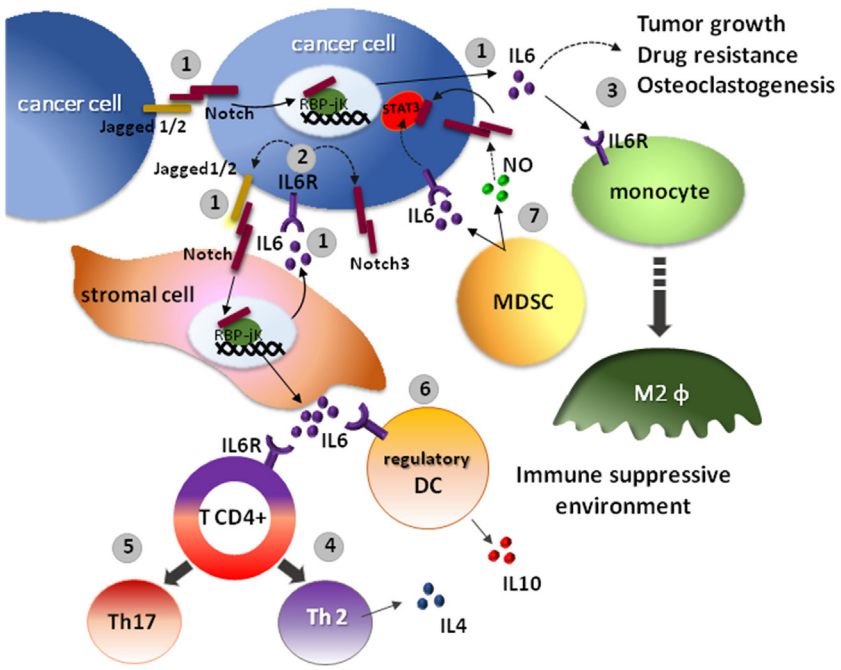

FIGURE 2 | The interplay between IL-6 and Notch affects the anticancer immune response. 1. The overexpression of Notch ligands, Jagged1 and Jagged2 by tumor cells promotes the activation of the Notch signaling in the tumor microenvironment (TME), boosting IL-6 secretion by the same cancer cells and by the neighboring stromal cells (62). 2. On the other hand, IL-6 increases the expression of Jagged1 and Notch3 in tumor cells (64). 3. High IL-6 levels in the TME promote tumor cells growth, resistance to therapy, osteoclastogenesis, and contributes to the development of an immunosuppressive niche. Indeed, the activation of IL-6R on immune cells causes the polarization of M2 macrophages. 4. The differentiation of CD4+ T cells into interleukin 4 (IL-4)-producing Th2 cells. 5. The increased differentiation of pro-inflammatory Th17 cells. 6. The development of interleukin 10 (IL-10)-producing regulatory DCs (65). 7. An alternative source of IL-6 is represented by myeloid-derived suppressor cells (MDSCs). MDSCs support tumor stem cell maintenance by a combined action of IL-6 and nitric oxide (NO). Indeed, MDSCs, through the release of IL-6, promotes the phosphorylation of signal transducer and activator of transcription 3 (STAT3) essential for maintenance of cancer cell stemness, and by producing NO indirectly activates the Notch pathway. Notch activation, in turn, causes prolonged STAT3 activation (12).

secretion. Moreover, myeloma cell-derived Jagged may activate Notch receptors in BMSCs *via* heterotypic interaction and promote IL-6 secretion, ultimately causing IL-6 levels to increase in the BM microenvironment (62). In myeloma progression, IL-6 promotes tumor cells growth, osteoclastogenesis, resistance to therapy (62, 67, 68), and, importantly, contributes to the development of an immunosuppressive milieu in the BM niche (67). The mechanism underling the immunosuppressive activity of IL-6 in multiple myeloma is complex and still poorly understood. The outcomes of IL-6 activity on immune cells include favoring the polarization of M2 macrophages, inhibiting Th1 differentiation, and redirecting CD4+ T cells differentiation into IL-4-producing Th2 cells, promoting the differentiation of immature DCs in IL-10-producing regulatory DCs (65). Moreover, IL-6, together with TGF-β, affects the balance between Tregs and Th17 cells, reducing the tumor-suppressive Tregs and promoting the differentiation of pro-inflammatory, Th17 cells (67). Nonetheless, the final outcome of Th17 cells in multiple myeloma is not clear. In different tumor settings, Th17 cells may either positively regulate immune surveillance or promote tumor cells survival (67, 69). Moreover, IL-6 favors the polarization of M2 macrophages. These cells play a crucial role in connecting cancer with inflammation and support tumor cells proliferation, invasion, and metastasis development, promote angiogenesis, and hamper T-cell-mediated antitumor immune response, thus sustaining tumor progression (70).

In breast cancer, high IL-6 is associated with poor prognosis (56). Several biological effects triggered by IL-6 are mediated by Notch signaling activation. Indeed, IL-6 requires Notch3 activity to promote cancer cell invasion and self-renewal (11, 71). This is not the only way by which IL-6 and Notch cooperate in this malignancy. Interestingly, MDSCs are another source of IL-6 in the tumor niche. These cells contribute to tumorigenesis by suppressing T cell activation and promoting stem-like properties of breast cancer cells. These effects are mediated by MDSCs ability to promote the interplay between the Notch signaling and IL-6-dependent signal transducer and activator of transcription 3 (STAT3) activation in cancer cells. MDSCs produce IL-6, which promotes the phosphorylation of STAT3, and the production of nitric oxide, in turn activating Notch signal, which causes prolonged STAT3 activation and supports cancer cell stemness (12).

The crosstalk between Notch pathway and IL-6 in breast cancer cells seems to be mediated also by NF-κB. Indeed, the activation of the non-canonical Notch signaling mediated by two components of the NF-κB cascade, IKKα and IKKβ, has been reported to upregulate IL-6 expression (72). The relevance of the non-canonical-Notch/NF-κB/IL-6 axis stems from the evidence that, while canonical Notch4 is necessary for the development of mammary glands, non-canonical Notch4 signaling is related to breast cancer tumorigenesis (73).

The interplay between Notch and IL-6 is even more complicated in breast cancer-associated bone metastasis. Here, the overexpression of Jagged1 activates Notch signaling in BMSCs, promoting the secretion of IL-6. In turn, IL-6 increases the expression of Jagged1 and Notch3 in tumor cells (64) and stimulates tumor growth and drug resistance (10).

Although the above reported findings do not provide a direct evidence, they allow us to hypothesize that the remodeling of the immune system may represent one of the mechanisms through which IL-6 and the Notch pathway cooperate to promote multiple myeloma and breast cancer progression.

CXCL12

CXCL12, known also as stromal-derived factor 1 (SDF1), binds two chemokine receptors: CXCR4 and CXCR7. We will focus on CXCR4 since it represents the most widely expressed chemokine receptor in human malignancies and it is a crucial player in the plasticity and alteration of the TME both in hematologic tumors, such as multiple myeloma, acute myeloid leukemia, T cell acute lymphoblastic leukemia (T-ALL), and in solid tumors such as ovarian, prostate, colon, brain, breast, and bladder cancer (74–76).

CXCR4 signaling is upregulated by hypoxia or in response to steroid hormones and it is associated with an invasive and metastatic phenotype (77) due to its involvement in several aspects of tumor development and progression such as cell migration, proliferation, resistance to apoptosis, angiogenesis, and development of metastasis (77–80).

In addition, the CXCR4/CXCL12 axis plays also a key role in inducing TME tolerogenic polarization in different types of cancers, although the exact mechanism has not been elucidated. Feig et al. demonstrated that in pancreatic cancer the blockade of CXCL12 produced by tumor-associated fibroblasts promotes CD3+ T-cells recruitment and restores the sensitivity to the antagonists of the checkpoint inhibitors programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (81). Accordingly, Chen et al. showed that CXCR4/CXCL12 blockade synergized with anti-PD-L1 immunotherapy in advanced hepatocellular carcinoma (82), while a similar mechanism was recently reported in an *in vivo* model of colorectal cancer (83). This synergy is relevant since, although checkpoint inhibitors have emerged as effective new therapeutic approaches in cancer, the response rate in patients is still variable and could benefit from a combinatory therapy (84).

The cooperation between Notch and CXCR4/CXCL12 has been reported in hematologic and solid malignancies. We recently showed that the expression of CXCR4 and CXCL12 in multiple myeloma cells is positively regulated by Notch signaling and may be impaired by γ-secretase inhibitors (13). The activation of Notch signaling in multiple myeloma is due to the contemporary expression of Notch ligands and receptors (13). Distinct reports indicate that activated Notch promotes CXCR4 gene expression by binding to CXCR4 regulative regions and transactivating its transcription (13, 85). CXCR4/CXCL12 blockade results in a decreased tumor cell proliferation and survival and, importantly, in the loss of myeloma cells ability to colonize the BM *in vivo* (13).

The interaction between Notch, CXCL12, and CXCR4 might also have a further outcome since high CXCL12 levels in the multiple myeloma niche increase the M2 macrophage population in the immune cell infiltrate. Indeed, CXCR4 directs the recruitment of monocyte precursors at the tumor site, and M2 macrophages from the BM of myeloma patients express higher

levels of CXCR4 compared with patients with the benign form of monoclonal gammopathy of uncertain significance and healthy individuals (14). Recently, Fabbri et al. demonstrated that also in B-cell chronic lymphocytic leukemia Notch1 is able to directly regulate CXCR4 expression (86), while in other hematological malignancies characterized by Notch1 hyperactivation, such as T-ALL, no Notch1-dependent regulation has been observed, but a cooperation the two pathways. Indeed, CXCR4 genetic deletion in murine hematopoietic progenitors abrogated ICN1 ability to induce leukemogenesis (87), but DAPT treatment failed to inhibit CXCR4 expression either in cell lines or primary cells (88), suggesting that an indirect and more complex mechanism of cooperation between these two pathways may be crucial in promoting tumor progression.

Among solid tumors, ovarian cancer shows a cooperation between Notch and CXCR4 signaling. Indeed, DAPT-mediated Notch inhibition causes a decrease in tumor cells growth and migration through the downregulation of CXCR4 and CXCL12 expression (15). By regulating this chemokine system, Notch might influence also the immunosuppressive function exerted by CXCR4 signaling in ovarian cancer. Indeed, CXCL12/CXCR4 blockade reduces infiltrated Tregs, increases the presence of IFN-γ+/IL-10+ T CD4+ and CD8+ lymphocytes, and supports spontaneous humoral and cellular antitumor responses (16). Similarly, in hepatocellular carcinoma, characterized by persistent Notch activation (6), hypoxia may induce CXCL12 upregulation, that in turn promotes the recruitment of Tregs and M2-type macrophages (17). This suggests that the collaboration between CXCR4/CXCL12 and Notch might induce an immunosuppressive TME involving various types of immune cells among which Tregs and M2 macrophages.

RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND

RANKL is a member of the tumor necrosis factor (TNF) family of cytokines. Its deregulation is particularly relevant in bone-associated cancers (primary or secondary) due to its involvement in the maturation of monocyte in OCLs (53) and the resulting associated osteolysis. The increase in RANKL levels characterizes almost all bone-associated cancers such as multiple myeloma and metastases derived from primary tumors which spread to the skeleton, i.e., carcinomas of the prostate, breast, lungs, thyroid, bladder, and kidneys as well as melanoma (89).

Indeed, one of the outcomes of cancer cells localization in the BM is the unbalance between bone destruction and formation due to altered differentiation and activity of OBLs and OCLs (90). This dysregulation is caused by an increased secretion of RANKL by neighboring stromal cells and infiltrating Th17, Tregs and DCs (91), and leads to the development of osteolytic lesions that, not only affect patient's quality of life, but also promote tumor growth, survival, metastasis formation, and the development of pharmacologic resistance (89, 90, 92, 93) (**Figure 1**).

Notch pathway dysregulation is involved in several bone-associated tumors. Results from our group and the group of Kang (10, 52) showed a similar situation in multiple myeloma and breast cancer. In both cases, tumor cells overexpress the Jagged ligands and are able to activate the Notch2 receptor in OCL precursors promoting their differentiation, that finally results in increased bone resorption and in the development of bone disease (10, 52). We also showed that in myeloma cells Notch activity positively influences the release of *RANKL*, while Notch inhibition, mediated by gamma secretase inhibitor (GSI) or Jagged ligands knockdown, downregulates RANKL secretion with consequently decreased OCL differentiation and activity (52).

Myeloma-derived RANKL promotes osteoclastogenesis by activating in OCL precursors two major pathways essential for their differentiation. NF-κB is triggered by RANK and Notch signaling is promoted as a consequence of an increase in the expression of Notch2, that in turn is activated by Jagged ligands expressed on myeloma cells (52, 94).

Another Notch-dependent source of RANKL in the BM niche is represented by osteocytes. In these cells, Notch signaling can be activated by the interaction with myeloma cells. As a consequence, Notch activity hampers osteocytes viability and promotes RANKL and sclerostin secretion that, in turn, supports the recruitment of OCL precursors (95).

Although Notch may act as a regulator of the balance between OCL and OBL/osteocyte activity, up to now it has not been investigated if Notch controls also other RANKL activities. Indeed, RANKL is involved in the shaping of the immune system operated by cancer cells in different tumors. RANKL favors the expansion of the local Treg population in bone metastatic prostate cancer (96), promotes M2–macrophages polarization in breast cancer models of lung metastasis (97, 98), interferes with NK anticancer activity in acute myeloid leukemia (51), is necessary for T cell tolerance in a melanoma model (99), where successful results were obtained by a combinatory treatment of RANK/RANKL blockade with anti-CTLA-4 (100). The positive results of preclinical studies induced to design clinical trials to evaluate the potential combinatorial effect of anti-RANKL monoclonal antibodies and immune checkpoint inhibitors (101). Interestingly, all tumors in which RANKL exerts an immunosuppressive activity share a recognized oncogenic activity of Notch, suggesting a collaboration of Notch and RANK also in hampering the antitumor-immune response.

INTERLEUKIN 10

Interleukin 10 is an immunosuppressive anti-inflammatory cytokine produced by various types of cells during the immune response. IL-10 signaling requires the assembly of a two-receptor complex consisting of two copies each of IL-10R1 and IL-10R2 chains; IL-10 receptor binding activates the JAK signal transducer and STAT pathway (102).

Interleukin 10 is produced by Th2 cells and monocytes, as well as by subsets of T cells, namely CD4+ CD25+ Foxp3+ (Tregs) and CD4+ CD25− Foxp3− type 1 regulatory (Tr1), Th1 and CD8+ T cells, B cells, macrophages, DCs, eosinophils, and mast cells (103). Tumor cells produce large amounts of IL-10 that contributes to tumor progression; in most types of cancers, serum IL-10 levels correlate with disease severity (104, 105). In TME, IL-10 secreted by immune and malignant cells activates an autocrine

loop that relies on IL-10 receptor and induces the upregulation of oncogenes, including cancerous inhibitor of protein phosphatase 2A and MYC (106).

Interleukin 10 released in TME exerts its immunosuppressive function in different ways: (1) unbalancing Th1 vs Th2 tumor-specific immune responses; (2) mediating the differentiation and activation of Tr1 cells involved in immunosuppression (107, 108); (3) inhibiting the production of pro-inflammatory cytokines and mediators such as IL-1, IL-6, IL-12, and tumor necrosis factor α (TNFα) by macrophages and DCs; (4) preventing the differentiation of DCs from monocytes and their maturation (109). In particular, IL-10 downregulates MHC-II on DCs and the co-stimulatory molecules CD80 and CD86 on macrophages (110); therefore, DCs display a defective antigen presentation and fail to activate cytotoxic T cells (111). Collectively, these effects promote the progression in different tumor such as ovarian carcinoma, lymphoma, and melanoma (102, 112).

Recently, an antithetic immunostimulatory function of IL-10 has been reported, too. Indeed, IL-10 may also promote the proliferation of CD8+ T cells (113), the differentiation of plasma cells along with the prolongation of their survival, the proliferation of NK cells, and their production of IFN-γ upon stimulation with IL-18 (114, 115). It is possible that the overall effect of IL-10 depends on the specific tumor type and TME, therefore a targeted therapy directed to IL-10 should carefully consider the possible dual immunosuppressive and immunostimulatory role of this cytokine.

The Notch–IL-10 axis is generally involved in self-limitation of immune response. Rutz et al. showed that Notch signaling, in synergy with IL-12 or IL-27, stimulates Th1 cells to release large amounts of IL-10, that contribute to self-limitation of Th1 immunity by hampering the inflammatory potential of Th1 cells (22). Interestingly, only Dll, but not Jagged ligands expressed by DCs are able to trigger Notch receptors located on T cells surface and activate IL-10 production *in vitro* and *in vivo* (22, 23).

Recently, a negative feedback regulation of hepatic inflammation mediated by the Notch–IL-10 axis was also reported. Hepatic inflammation is associated with the expression of Dll and Jagged ligands in liver sinusoidal endothelial cells that, in turn, activates Notch signaling in Th1 cells, with a consequent increase in HES1 and Deltex-1 expression (18). Notch activation triggers the production of IL-10 in Th1 cells causing their switch from an inflammatory to an immunosuppressive function. Consistently, Notch-deficient CD4+ T cells express lower IL-10 levels in the presence of liver sinusoidal endothelial cells, leaving the expression of Th1 cytokines, such as IFN-γ and TNFα, unaltered (18). We speculate that this mechanism of self-limitation of T-cell response in inflamed liver might also occur in hepatocellular carcinoma since it arises in more than 90% of cases as consequence of hepatic injury and inflammation (116).

So far, we have reported an inhibitory role of the Notch–IL-10 axis on the immune system mediated by Th1 cells and T cells activation, but unexpectedly, this axis also acts to switch tumor-associated macrophages (TAMs) to the inflammatory, antitumor phenotype M1, thereby increasing the antitumor immune response. Indeed, the conditional expression of ICN in macrophages of a transgenic murine model induces the conversion of TAM from M2 to M1 phenotype by inducing the expression of miR-125a, resulting in TNFα and IL-12 secretion and reduced release of IL-10 and TGF-β. These macrophages exhibited strong antitumor activities in transplanted tumors (19). Conversely, Notch blockade by GSI, small interfering RNA, or RBP-Jk deletion switches macrophages to M2 phenotype, characterized by the ability to produce IL-10 and an attenuated capacity of activating Th1 cells (20, 21). Consistently, T cells activated by RBP-Jk−/− macrophage showed a reduced cytotoxic activity against melanoma cells when compared with wild-type macrophages (20).

INTERLEUKIN 4

In the TME, IL-4 is produced by tumor cells, mast cells, activated Th2 cells, eosinophils, basophils, and MDSCs (117, 118). A close relationship between tumor progression and IL-4 produced by tumor-infiltrating Th2 lymphocytes has been found in several malignancies such as non-small cell lung carcinoma, breast cancer, renal cell carcinoma, prostate cancer, and others (117). Moreover, enhanced expression of the IL-4 receptor (IL-4R) has been reported in various neoplastic tissues, i.e., glioblastoma, malignant melanoma, head and neck cancer, renal cell carcinoma, breast, prostate, ovarian cancer, and bladder cancer (117, 119).

Interleukin 4 signaling supports cancer cell proliferation and survival (120). Moreover, IL-4 contributes to suppress the anti-tumor immune response by acting at different levels on adaptive and acquired immune system (121).

Although no evidence has been reported of an interaction between Notch and IL-4 in cancer cells, Notch signaling plays a key role in activating IL-4 expression in different cellular components of the TME. A Notch/RBP-Jk binding site has been identified in the 3′ end of the IL-4 gene, suggesting that the Notch pathway has the ability to directly regulate IL-4 expression (122); moreover, Notch has been shown to control IL-4 secretion in myeloid progenitors and NK cells probably due to the presence of two RBP-Jk binding sites in the conserved non-coding sequence-2, located downstream the IL-4 gene (123). The cooperation between Notch and IL-4/IL-4R pathway also contributes to the differentiation and activation of immunosuppressive Th2 cells. The activation of Notch signaling by DC-expressed Jagged2 induces Th2 cells differentiation by boosting the expression of GATA3, IL-2/IL-2Rα, and IL-4 (24, 124). A previous work from Fang et al. suggest that Notch1 is the receptor involved in this process, since ICN1 directly regulates GATA3, which is a master regulator of Th2 differentiation and promotes IL-4 transcription by coordinating chromatin remodeling (25, 125). Sauma et al. demonstrated that IL-4 produced by Th2 cells may also promote a positive feedback loop on T cell polarization sustaining Jagged2 expression in DCs (24).

The existence of a crosstalk between Notch and IL-4 signaling during DCs differentiation is supported by the findings of Cheng et al., who showed that Jagged1-induced Notch activation causes the accumulation of immature DCs, because of the lack of IL-4, which is required for their differentiation (26). Conversely, Dll4-mediated Notch activation in macrophages may have an antitumor effect as shown by the evidence that Dll4-mediated Notch1 activation hampers IL-4-induced M2 polarization and

promotes M1 macrophage apoptosis; the authors suggest that the interaction between Notch and IL-4 pathway may involve HES1 ability to bind STAT3, finally inhibiting IL-4R signaling (27).

NOTCH SIGNALING STIMULATES CANCER-ASSOCIATED PRO-INFLAMMATORY CYTOKINES

Cancer is tightly associated with chronic inflammation. The ability of infiltrated immune cells to promote tumor growth, progression, and immune surveillance may be mediated by several pro-inflammatory cytokines and chemokines, including TNFα, interleukin 17 (IL-17), IL-1, and CCL2 and CCL5. In the following sections, we will describe the interaction of Notch signaling with such cytokines and the outcome of their interplay on the immune response.

TUMOR NECROSIS FACTOR α

Tumor necrosis factor α is a pro-inflammatory and immunomodulatory cytokine, member of the TNF/TNF receptor superfamily (126). TNFα is one of the most strong activators of NF-kB pathway (127). TNFα may be produced in response to inflammation and infection by macrophages, lymphocytes, fibroblasts, and keratinocytes, but also tumor cells may be a relevant source (128).

The pro-inflammatory and immunomodulatory effect of TNFα is at the basis of its pro-tumor activity, observed in different malignancies including cutaneous, ovarian, pancreatic cancer, and tumors of the pleural cavity and the bowel (128). High levels of TNFα in the serum have a poor prognosis in ovarian, renal, pancreatic, prostate, breast cancer, and chronic lymphocytic leukemia (126). The mechanism underlying TNFα pro-tumor activity was nicely described in ovarian cancer. TNFα released by tumor cells and cells of TME acts through its receptor TNFR1 and further reinforces TNFα expression and the inflammatory and immune-modulatory network including CXCL12, CCL2, IL-6, VEGF, and macrophage inhibitory factor (129). This induces the differentiation of myeloid progenitors to endothelial cells, extracellular matrix remodeling, and recruiting leukocytes at tumor site for local immunosuppression (128). TNFα immunosuppressive function includes downregulation of TCR signaling and DC function, promotion of T cell apoptosis, activation of Tregs, induction of tumor cell dedifferentiation with a consequent reduced expression of immunogenic antigens and impaired recognition by cytotoxic T cells, impaired differentiation of immature MDSCs with increased suppressive activity resulting in T and NK cell dysfunction and finally inducing other cytokines that can inhibit cell-mediated immunity (130–134).

An interplay of Notch pathway and TNFα has been described in several studies (**Figure 3**). TNFα stimulation may result in the transcription of important Notch target genes mediated by the activity of NF-kB. In a mouse model of pancreatic cancer, TNFα induces the activation of Ikkβ, a component of the NF-κB signaling, that promotes the expression of Notch target genes HES1 by inducing histone H3 phosphorylation at the HES1 promoter

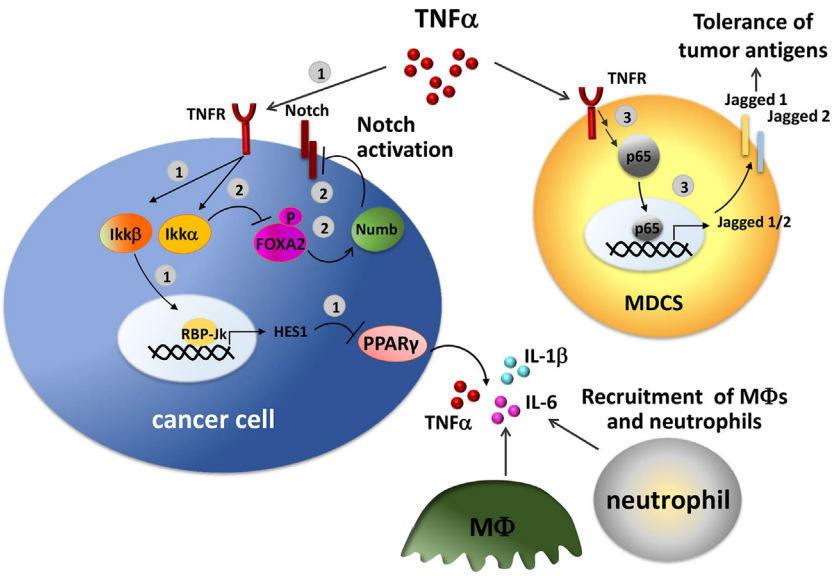

FIGURE 3 | Crosstalk between Notch and tumor necrosis factor α (TNF-α) in the tumor environment. TNF-α induces Notch activation through NF-κB pathway in tumor cells. 1. TNF-α activates Ikkβ inducing the expression of the Notch target gene HES1. HES1, in turn, inhibits the expression of the anti-inflammatory receptor peroxisome proliferator-activated receptor γ (PPARγ) with a consequent increased production of TNF-α, IL-6, and IL-1β, that recruit macrophages and neutrophils in the tumor site (135). 2. TNF-α promotes Notch signaling by activating Ikkα, that in turn phosphorylates and inhibits FOXA2, thereby reducing the transcription of the target gene Numb, a Notch repressor (136). 3. In tumor-infiltrating myeloid-derived suppressor cells (MDSCs), NF-κB-p65, a key mediator of TNF-α/TNFR signaling, transactivates Jagged1 and 2 promoters and induces their expression stimulating the tolerogenic activity of tumor-associated MDSCs (137).

resulting in transcriptional activation. This, in turn, inhibits the expression of the anti-inflammatory nuclear peroxisome proliferator-activated receptor γ (PPARγ), reinforcing the inflammatory loop (135). PPARγ repression in pancreatic cancer cells results in the constitutive production of pro-inflammatory cytokines, including TNFα, IL-6, and IL-1β, relevant in the recruitment of macrophages and neutrophils into the tumor site. Consistently, *in vivo* treatment with a PPARγ agonist, rosiglitazone reduces macrophage infiltration (135).

Evidence obtained in liver cancer clearly shows a cooperation between TNFα and Notch in inflammation-mediated cancer pathogenesis. TNFα regulation of Notch1 signaling is mediated by Ikkα-induced phosphorylation of FOXA2. This causes the inhibition of FOXA2 activity as a transcription factor and consequently decreases the expression of its target genes including Numb. Numb is a well-known Notch repressor, thereby the consequence of its inhibition is the increased activation of Notch1, that is associated with tumorigenesis (136). Indeed, by *in vivo* studies on mice that received transplanted tumors harboring Numb knockdown Hep3B cells infected with retrovirus expressing FOXA2 continued to show tumor growth even in the presence of FOXA2. Moreover, a link of IKKα-mediated FOXA2 phosphorylation to hepatocellular carcinoma tumorigenesis was supported by higher levels of IKKα, phosphorylated FOXA2, and activated Notch1 in hepatocellular carcinoma specimens respect to normal liver tissues (136).

Further studies performed on murine models of Lewis lung carcinoma, colon carcinoma, thymoma, and melanoma, showed that the expression of NF-kB-p65, a key mediator of TNFα, is associated to increased levels of Jagged1 and 2 in tumor-infiltrating MDSCs (137), suggesting that TNFα may positively regulate the expression of Jagged ligands. Jagged1 and Jagged2 widely affect immune system regulation as shown *in vivo* with humanized anti-Jagged1/2-blocking antibody CTX014. This treatment affected the accumulation and tolerogenic activity of MDSCs in tumors and inhibited the expression of immunosuppressive factors arginase I and inducible nitric oxide synthase (137). As a consequence, tumor-induced T-cell tolerance was reduced and the infiltration of reactive CD8+ T cells was increased thus enhancing the *in vivo* efficacy of T-cell-based treatment (137).

Finally, one elegant study showed a complementary effect of Notch and TNFα in multiple myeloma-induced bone disease (95). The authors showed that co-cultured multiple myeloma cells and osteocytes reciprocally activated Notch signaling. Notch activation in osteocytes induced apoptosis that in a second phase was amplified by high levels of TNFα secreted by MM cells. The cooperation of the two pathways is further confirmed by the evidence that single treatment with GSI-XX or anti-TNFα only partially inhibited cell death, whereas the combined treatment completely prevented osteocyte apoptosis. The increased apoptosis levels in osteocytes not only reduce their bone deposition activity but also increase active bone matrix degradation since it is also associated with the enhanced expression of the key osteoclastogenic factor RANKL (95). This evidence suggests that multiple myeloma cells exploit the collaboration of Notch and TNFα signaling pathways to induce bone resorption in multiple myeloma and possibly RANKL-mediated immunosuppression.

INTERLEUKIN 17

Interleukin 17 is a family of pro-inflammatory cytokine (including IL-17A to F) mainly produced by Th17 cells, a lineage of T helper cells defined by their ability to produce IL-17, IL-21, and IL-22 (138, 139). Other immune cells may contribute to IL-17 levels, including CD8+ T cells, NK cells, γδ T cells, and neutrophils (140). IL-17 engages one of its five cell surface receptors (IL-17 receptor A to E) and triggers the production of various pro-inflammatory cytokines and chemokines that recruit monocytes and neutrophils to the site of inflammation (141).

The most important role of IL-17 is attributed to its ability to stimulate various cell types to produce pro-inflammatory cytokines and chemokines by activating the NF-κB pathway (142–144).

The presence of Th17 cells and the expression of IL-17 have been found in almost all tumors (145, 146). Nonetheless, the role of IL-17 in cancer is controversial, since both pro-tumoral and antitumoral effects have been reported, possibly due to its pleiotropic activity. IL-17 may promote tumorigenesis in several types of cancer and in different ways: (1) by inhibiting tumor apoptosis and promoting tumor proliferation (147–149); (2) by inducing tumor–associated stroma to release of the pro-tumoral cytokine IL-6 (150); (3) by recruiting macrophages and MDSCs to the tumor site (151, 152); (4) by promoting Tregs infiltration into tumor tissue through upregulation of CCL17 and CCL22 (153); (5) by promoting angiogenesis through the increase of VEGF production (154); and (6) by stimulating tumor cells to express matrix metalloproteinase-2 (MMP-2) and MMP-9 involved in cancer cell invasion (155).

Despite these pieces of evidence, recent studies on cancer patients highlight an antitumoral role of IL-17. Indeed, the 5-year survival rate in patients with gastric adenocarcinoma (156), esophageal squamous cell carcinoma (157), chronic lymphocytic leukemia (158), ovarian cancer (159), and cervical adenocarcinoma (160) displaying increased IL-17 levels was reported to be significantly higher than survival in patients with lower IL-17 expression. The possible mechanisms underlying this effect could rely on the positive regulation of the adaptive immune response *via* stimulation of the production of cytokines and chemokines such as IFN-γ, CXCL9, and CXCL, recruitment of CD4+, CD8+ T cells (159), DCs (157), and neutrophils (161) to tumor sites, stimulation of NK cell activity (157), generation and activation of CTLs (162).

The complex array of IL-17 effects on the immune system response might explain its dual behavior and, probably, its overall outcome may depend on tumor cell type and the surrounding TME, including the pattern of immune cells and cytokines. On the other side, it cannot be ruled out that opposite effects of IL-17 reported for the same type of tumor can be due to differences in the animal models used or the number of cases, or to possible different targets of the performed investigation, including IL-17 produced only by Th17 cells or by the whole the set of IL-17-producing subsets of cells.

Notch regulates IL-17 expression affecting the immune cell response (**Figure 4A**). Here, we will report a picture of the mechanisms and the outcomes in different immune cell types involved in the antitumor response. Dll4-triggered Notch activation of CD4+ T cells under Th17 skewing conditions (stimulation

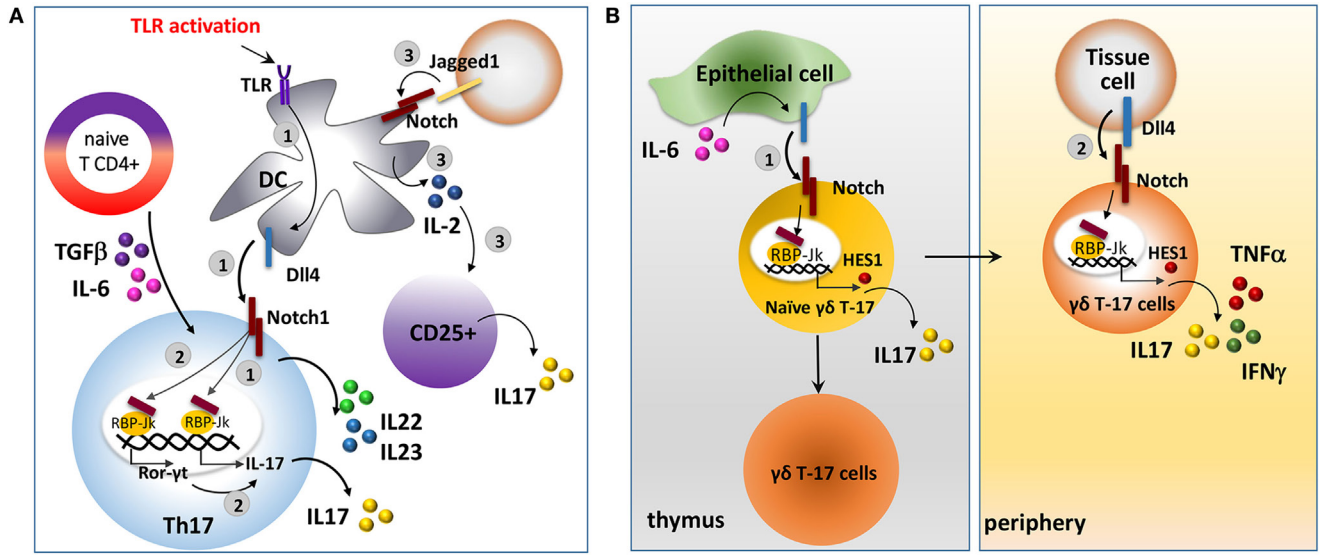

FIGURE 4 | The interaction between Notch and interleukin 17 (IL-17) in different immune cellular settings. **(A)** Notch signaling stimulates Th17 cell differentiation. 1. Dll4, upregulated on DC cells upon TLR activation, in cooperation with skewing cytokines, IL-6 and transforming growth factor-β (TGF-β), activates Notch signaling in naive CD4+ T cells inducing the production of IL-17 and Th17-related cytokines, IL-22 and IL-23, and the consequent differentiation in Th17 cells. Dll4-mediated Notch activation in naive CD4+ T cells results in the direct transactivation of IL-17 promoter by activating the RBP-Jk/CSL transcription factor (163–165). 2. In addition, ICN may also stimulate Th17 cell differentiation by transactivating the promoter of Ror-γt, a transcription factor required for Th17 differentiation and a main regulator of IL-17. 3. Jagged1-conditioned DCs produce IL-2 that is required for the production of IL-17 by CD25+ cells (166). **(B)** Notch signaling in γδ T-17 cells. 1. Dll4, upregulated on thymic epithelial cells following IL-6 induction, activates Notch signaling in γδ T-17 cells resulting in expression of HES1, that in turn induces the production of IL-17 and the development of γδ T-17 cells (28, 167). 2. High level of Dll4 in peripheral tissues, such as the intestine and lung, is enable to activate Notch in γδ T-17 cells (28). This results in the production of IL-17, IFN-γ, and tumor necrosis factor α (TNFα) along with the proliferation and activation of peripheral γδ T-17 cells and enhancement of their antitumor cytolytic effect (29).

with IL-6 and TGF-β) is associated with the production of IL-17 and Th17-related cytokines, IL22 and IL23. Mechanistically, Dll4-stimulation mediates the direct transactivation of IL-17 promoter *via* RBP-Jk activation. In addition, ICN may also bind and transactivate the promoter of retinoic acid-related orphan receptor (Ror-γt), a transcription factor required for Th17 differentiation and the main regulator of IL-17 (163, 164). Overall, this leads to increased IL-17 production and enhanced differentiation of Th17 cell population. Accordingly, Notch signaling blockade significantly reduced IL-17 production, even under Th17 skewing conditions (163). Osborne's group confirmed that IL-17 is a direct transcriptional target of Notch in Th17 cells and that the differentiation of these cells requires Notch activation (165).

Notch signaling is also involved in promoting an alternative DC phenotype able to stimulate T cells to release IL-17. Indeed, DCs are known to express both Notch receptors and ligands (122). The group of Dallman showed that Jagged1 may trigger DC maturation in an alternative way from the LPS-toll-like receptor signaling (166). Jagged1-conditioned DCs promotes survival, proliferation and increases the suppressive ability of Tregs. Moreover, Jagged1-conditioned DCs produce IL-2 that stimulates CD25+ T cells to produce IL-17 (166).

The activity of Notch signaling in promoting IL-17 expression links Notch to the differentiation of another population of T lymphocytes, γδ T-17 cells, a subset of γδ T cells that shares many of the Th17 phenotypic markers and effector cytokines (IL-17 and IL-22) and has important functions in inflammation and antitumor immunity (168–170).

Notch activation is known to be involved in thymic determination and regulation of the innate function of γδ T-17 cells in thymus and periphery (28) (**Figure 4B**). Specifically, HES1 induces γδ T-17 cell development and IL-17 production (28). In the thymus, IL-6 stimulates thymic epithelial cells to express Dll4 resulting in Notch activation in γδ T-17 cells (167). In peripheral tissues, such as the intestine and lungs, the high levels of Dll4 expression (171), stimulate a Notch-dependent increase of IL-17 γδ T cells (28).

The only evidence that links Notch activity to the antitumor functions of γδ T-17 in cancer has been reported by Gogoi et al. These authors showed that Notch stimulates the activation and proliferation of peripheral γδ T-17 cells. Notch inhibition, mediated by GSI, reversed this effect and blocked γδ T-17 cell production of cytokines including IFN-γ, TNFα, and IL-17, resulting in the decrease of γδ T-17 cell antitumor cytolytic effect on oral cancer cell lines (29).

CCL5

The inflammatory chemokine CCL5 and/or its receptor, CCR5, are expressed in various human cancers, including breast cancer, prostate cancer, ovarian and cervical cancer, gastric and colon cancer, melanoma, multiple myeloma, Hodgkin's lymphoma, and

T-acute lymphoblastic leukemia (88, 172–174). Further source of CCL5 in TME may be infiltrating leukocytes, BMSCs, mesenchymal stem cells, or tumor-associated fibroblasts (174).

CCL5 may enhance tumor development in multiple ways: acting on tumor cells by inducing proliferation, invasion, and metastasis, shaping the TME by stimulating the activation of carcinoma-associated fibroblasts (CAFs) and OCLs (in bone-associated cancer or bone metastasis), and shaping the immune infiltrate toward immunosuppression by promoting the apoptosis of cytotoxic CD8+ T cells, the recruitment of TAMs, MDSCs, eosinophils, mastocytes, CD4+ T cells, and T regulatory cells (174).

Notch has been reported to positively regulate CCL5 expression in multiple myeloma-associated BMSCs (173) and in breast cancer (30). As reported above, BMSC-derived CCL5 together with other chemokines results in enhanced myeloma cell viability and migration (173). A recent report demonstrates that the axis CCL5/CCR5 play a key role in a metabolic feedback loop between breast cancer cells and macrophages with important outcomes on immune system infiltrate (30). Cancer cells may have a high rate of glycolysis even in the presence of oxygen; this effect is known as aerobic glycolysis or Warburg effect (175) and results in the production of high levels of lactic acid, which in turn decreases pH in TME that may confer a proliferative advantage to cancer cells. Lactic acid produced by breast cancer cells supports TAM M2 polarization and their production of CCL5 by increasing Notch1 and Jagged2 mRNA and protein expression (30). In return, TAM-derived CCL5 induces breast cancer cell migration, EMT, and promotes aerobic glycolysis *via* AMPK signaling activation, resulting in increased metastatic ability (30).

In conclusion, the crosstalk between lactate, Notch signaling, and CCL5 has several deleterious outcomes: increased metastatic ability and TAM recruitment. In addition, although no direct evidence has been reported, it is conceivable that the increased acidification and decreased glucose availability greatly influence T cell metabolic fitness (176), switching the infiltrated T cell populations from cytotoxic to regulatory. Indeed, lactate, directly and indirectly, affects T cell proliferation and activation; while low glucose levels hamper the activation of effector T cells and induce their apoptosis, thereby favoring the increase of Tregs, that is not reliant on high rates of glucose metabolism (176).

NOTCH MAY SHAPE THE COMPOSITION OF THE IMMUNE CELL INFILTRATE BY REGULATING IL-1β AND CCL2

IL-1α and IL-1β are pro-inflammatory cytokines, members of the interleukin 1 family. IL-1α is mainly secreted by macrophages, neutrophils, and endothelial cells in the acute inflammatory response where it collaborates with TNFα to promote systemic inflammation and fever. Notably, both IL-1α and IL-1β are crucial components of the pro-inflammatory secretory profile of senescent cancer cells as detailed below.

IL-1β is predominantly produced by activated macrophages and adipocytes in the TME, although cancer cells may contribute to increasing its levels (31, 177). IL-1β production is a two-step process involving the production of an inactive IL-1β proprotein (pro-IL-1β), followed by its activation induced by caspase-1, a component of the activated multiprotein complex called inflammasome together with the Nod-like (NALP) and apoptosis-associated speck-like (ASC) (178).

IL-1β has a pleiotropic and controversial role in cancer. It is a crucial mediator of the innate immune response, promotes tumor growth, angiogenesis, and metastasis in several tumor types including breast cancer, melanoma, non-small-cell lung carcinoma, and colorectal adenocarcinoma (177).

Breast cancer represents one of the better-studied models and expresses all the members of the IL-1 system, including IL-1α and β, antagonist IL-1Ra, and receptor IL-1R (177, 179, 180).

IL-1β transcription is regulated by Notch in cancer cells, differently from its regulation in the cells of myeloid lineage, where its expression is stimulated by the engagement of toll-like receptors or endogenous danger signals. Studies in breast cancer cells clearly demonstrate that Jagged1, ICN1, and ICN3 are required for IL-1β transcriptional activation occurring at the RBP-Jk DNA binding site at −2,085 from the translation start site (31). Despite this direct transcriptional regulation, Zheng et al. suggest that IL-1β transcription may be triggered by a more complicated mechanism relying on the activation, mediated by phosphorylation, of STAT3 and requires an active Notch1 signaling (181).

Through the regulation of IL-1β, Notch may shape the immune infiltrate at the tumor site, affecting both the innate and the adaptive antitumor immune response. IL-1β is a pleiotropic cytokine and its role in cancer might be context dependent even if this statement is still under discussion (182) since opposite outcomes have been reported ranging from cancer protection to cancer progression.

A protective role for IL-1β is reported in models of colon cancer associated to colitis, possibly due to the concomitant production of IL-18, relevant for intestine healing (183), or in a model of epithelial skin carcinogenesis, where the inflammasome adapter ASC may play a protective role in keratinocytes. By contrast, in the same model of skin carcinogenesis, ASC plays as a tumor promoter in myeloid cells (184), and IL-1β and inflammasome are crucial for mesothelioma development (185) and murine mammary carcinoma progression mediated by myeloid recruitment (186).

The pivotal role or Notch in the modulation of immune cells infiltrating the TME has been mainly studied in breast cancer. Notch regulates the recruitment of TAMs in two different ways. It promotes the expression of IL-1β and CCL2 and supports monocyte adhesion to blood vessel endothelium in synergy with CCL2 that promotes chemotaxis and extravasation (31).

Tumor-associated macrophage infiltration is associated with poor prognosis (187) and sustains a cytokine milieu abounding of TGF-β, IL-1β, and CCL2 that collaborate to promote monocytes recruitment and promotes an immunosuppressive TME. The underlying mechanism is complex and involves a synergy of tumor cells and TAMs. These cells secrete TGF-β that promotes Jagged1 expression in tumor cells. Tumor-derived Jagged, in turn, boosts the expression of IL-1β and CCL2 in the same tumor cells and in TAMs (32). In addition, Notch activation in tumor cells potentiates TGF-β signaling by promoting the secretion of the urokinase-type plasminogen activator, that allows the maturation

of the immature form of TGF-β released by TAMs and further sensitize tumor cells to TGF-β through the upregulation of TGFβR1 (31).

A further reinforcement of an immunosuppressive TME in breast cancer may be induced by high levels of IL-1β. Indeed, high IL-1β levels have been reported to be associated with impaired activation of CD8+ T cells and systemic expansion and polarization of immunosuppressive neutrophils (32, 188). The expansion of this population seems to be owed to IL-1β ability to activate IL-17-producing γδ T cells responsible for increased systemic levels of G-CSF, a cytokine known for its role in granulopoiesis (188).

The relevance of the interplay between Notch and IL-1β is strengthened by the evidence that effective IL-1β and CCL2 antagonists are currently in clinical review to treat benign inflammatory disease, and their transition to the cancer clinic has been proposed (31).

The nasty outcomes of the cooperation between Notch and IL-1β in cancer may be potentiated by body metabolism, specifically by leptin, a hormone whose levels are significantly increased with obesity. Also in this case, most studies focus on breast cancer, where leptin acts as a positive regulator of Notch expression and activation in estrogen responsive and triple-negative breast cancer (TNBC) cells through canonic JAK2/STAT, MAPK1/2K 1/2, and PI3K/AKT, and non-canonic signaling pathways JNK and p38 MAP kinases (189, 190).

Here, besides the reported Notch-mediated increases of the expression of IL-1β, VEGF, and VEGFR2, the authors demonstrate that IL-1β signaling is required for the positive regulation of Notch receptors induced by leptin (190); moreover, beside the immunosuppressive effect of Notch signaling reported above, Notch, IL-1β and leptin crosstalk outcome mediates other key features including cell proliferation, survival, migration, and angiogenesis in breast cancer (190) and likely in other tumors including pancreatic and endometrial cancer (189, 191).

Inflammatory TME plays a key role in the self-renewal of cancer stem cells (CSCs). In particular, the interplay between Notch and IL-1β in TME is reported also to affect CSCs in TNBC resulting in increasing their self-renewal. Indeed, metastatic TNBC cells in the brain express high levels of IL-1β that stimulates the neighboring astrocytes to express Jagged1. This in turn triggers Notch signaling upregulation in CSCs enhancing their self-renewal (192).

Concerning CCL2, its production from different cell types, such as fibroblasts, OBLs, endothelial cells, and smooth muscle cells, is thought to promote cancer growth and metastasis (193). Notch pathway has been frequently reported to positively regulate CCL2 expression (31), although this effect seems to be cell type specific, indeed in experimental liver fibrosis and patients with acute-on-chronic liver failure Dll4 is inversely associated to CCL2 (194) and in the melanoma cell line M624 silencing of the Notch coactivator MAML1 results in CCL2 mRNA and protein upregulation (195). The outcome of Notch-mediated positive regulation of CCL2 in breast cancer cells in synergy with IL-1β has already been described (31). Further noteworthy outcomes of the Notch/CCL2 axis are important in the nasty communication between tumor cells and BMSCs in the primary tumor site and in the metastatic one. Multiple myeloma cells primarily reside at the BM, where they get an advantage and induce BMSCs to a pro-tumor behavior in different ways, including conveying extracellular vesicles containing different stimuli among which miRNAs. Tumor-derived miR-146a may induce the activation of Notch1 in BMSCs stimulating them to secrete CCL2 and several cytokines including IL-8, IL-6, CXCL1, IP-10, and CCL5 that enhance myeloma cell viability and migration (173). An elegant study by Yumimoto et al. explored the mechanisms of cancer metastasis, showing that lung metastases are promoted by BMSCs migrated to the lungs, and identified a trigger of the metastatic process in the low expression of the tumor suppressor FBXW7 in TME, a condition associated with poor prognosis in breast cancer patients (33). The FBXW7 role is based on its ability to downregulate Notch signaling since it mediates a key step in the degradation of ICN (and other oncogenes), acting as substrate recognition component in the SCF-type ubiquitin ligase complex. *In vivo* experiments showed that loss of FBXW7 in BMSCs results in the accumulation of ICN1, that in turn promotes the secretion of CCL2. CCL2 is a chemotactic stimulus for the recruitment of Mo-MDSCs and macrophages that, in turn, induce the metastatic site to sustain the growth of tumor cells that have already colonized the lungs (33).

NOTCH AND IFNγ COOPERATE TO SHAPE THE IMMUNE CELL LANDSCAPE

IFN-γ is a key promoter of macrophage activation and induction of MHC-II expression. The most important sources of IFN-γ are cells of T lineage including CD8+ T cells and Th1 cells, NK and NK T cells belonging to the adaptive or the innate immune system (196).

The antitumor activity of IFN-γ stems from several distinct mechanisms:

(1) tumor-directed anti-proliferative and pro-apoptotic actions, based on STAT1 activation and the expression of, respectively, cell cycle inhibitors such as p21 and p27, or apoptotic mediators including caspase-1 or Fas and Fas ligand (197);
(2) inhibition of angiogenesis, indirectly induced by a family of interferon-induced chemokines with potent angiostatic actions, i.e., IP-10, Mig, and I-TAC (197);
(3) potentiation of the killing immunological functions, including (a) development of antitumor adaptive immune response mediated by (i) IFN-γ ability to direct the appropriate Th1/Th2 balance by stimulating Th0 cell polarization toward Th1 and inhibiting Th2 cell differentiation; (ii) IFN-γ-mediated stimulation of MHC-I expression by tumor cells with the consequent increase of tumor-antigen presentation; (iii) activation of the tumor cell killing mediated by T CD8+ cells; (b) the activation of the host antitumor innate immune response, mediated by macrophages and NK cells (197).

IFN-γ has been reported to be a direct transcriptional target of Notch; a study performed on Th1 cells demonstrated that Notch is recruited to the RBP-Jk-binding elements at an enhancer site

of the IFN-γ gene (198). We will briefly describe the role played by Notch in regulating the important activity of IFN-γ in the regulation of antitumor innate and adaptive immune response by examining the outcome on the key cell types involved: DCs, NKs, CD8+, and CD4+ T cells.

Concerning DCs, Notch and IFN-γ collaborate to promote their maturation and the ability to activate the different T cell subsets. Notch positively regulates DC maturation; indeed, Notch ligand Jagged1 induces the upregulation of maturation markers, IL-12 production, and DC ability to promote T cell proliferation and maturation in effector T cell as demonstrated by IFN-γ production (34). Moreover, the CD80/CD86 triggered upregulation of IL-6 secretion, necessary for full T cell activation, occurs through the collaboration of Notch and PI3K signaling (199). Finally, Notch signaling increases the expression of MHC complexes in DCs, necessary for T cell activation.

A great part of the role of Notch signaling in antitumor response is due to DC ability to activate Notch signaling in interacting lymphocytes through the expression of Notch ligands. Among the physiological stimuli promoting the expression of Notch ligands in DCs, GM-CSF and CpG DNA have been reported to increase Jagged2 expression (35), while LPS induced Jagged1 and Dll4 production (200). DC-derived Notch ligands participate in the instruction of T helper cells to commit to the Th1 (201), Th2 (122), Th17 (163), or Treg (202) lineage.

Recent studies showed that NK cells can be activated by DCs and macrophages (35, 203, 204) and that Jagged and Dll ligands can promote the development or activation of NK cells *in vitro* (205, 206). Kijima and colleagues (35) confirmed *in vivo* that DCs can increase NK cell cytotoxicity by stimulating the activation of Notch2 on NK cells through the ligand Jagged2, whose expression may be stimulated by GM-CSF and CpG DNA. Importantly, Jagged2 stimulated NK cells to increase IFN-γ secretion and cytolytic activity resulting in decreased tumor burden in a murine lymphoma model (35).

The generation of cytotoxic CD8+ T cells is essential for tumor control. DCs provide key signals to induce the priming and activation of CD8+ T cell. Notch pathway has an important role in both these processes in human CD8+ T cells. Indeed, the activation of Notch2 signaling on CD8+ T cells, mediated by Dll4 or Jagged1 expressed by DCs, is required for the activation and proliferation of human CD8+ T cells and for the release of effector cytokines including IFN-γ, along with TNFα, perforin, and granzyme B (36, 37, 200, 207).

Auderset et al. reported that Notch pathway is involved also in the differentiation and activity of the Th1 subset of CD4+ T cells (38). Specifically, Notch signaling mediated by Notch1 and/or Notch2 induces IFN-γ secretion by CD4+ Th1 cells. Interestingly, the involvement of Notch signaling seems to provide a possible alternative stimulus for Th1 cell differentiation in the absence of the skewing cytokine IL-12 as demonstrated by RBP-Jk ablation or in mice expressing a dominant negative MAML transgene (208). Indeed, upon LPS stimulation CD8− DC subtype induces MyD88-dependent expression of Dll4, which in turn may engage Notch on Th1 cells inducing IFN-γ expression, differently from LPS effect on CD8+ DCs that results in increased IL-12 expression (209).

In contrast to the collaboration of IFN-γ and Notch in promoting Th1 cell differentiation, IFN-γ and Notch play antithetic roles in Th2 cell polarization. IFN-γ may potentiate the Th1 shift by an inhibitory effect on Th2 cell response, while essentially Notch antagonizes IFN-γ-induced inhibition of Th2 cell differentiation. A confirmation of this role comes from the evidence that upon IFN-γ neutralization Notch activation becomes dispensable (198).

VEGF AND NOTCH COOPERATE TO INDUCE TUMOR ANGIOGENESIS AND EVASION FROM THE IMMUNE SYSTEM

VEGF regulates different aspects of tumor progression including angiogenesis, but although its role in cancer progression is much wider. Recently, an immunosuppressive function of VEGF has emerged that protects cancer cells from the increased recruitment of immune cells at the tumor site promoted by neoangiogenesis. Indeed, if tumor vasculature is key in providing tumor cells with oxygen, nutrients, and glucose, along with an escape to enter blood circulation allowing tumor metastasis, it also maximizes the exposure of tumor cells to the antitumor activity of immune cell populations. Thereby, we will detail the interaction of VEGF with Notch signaling in regulating these important aspects of tumor progression.

VEGF is known as a major pro-angiogenic signaling pathway involved in developmental, physiological, and tumor-associated angiogenesis. VEGF family consists of six ligands (VEGF-A, -B, -C, -D, -E, and placental growth factor) with different affinity to the four VEGF receptors: VEGFR1–2–3 and neuropilin 1 (210). This family regulates almost all steps of new vessels formation including sprouting and intussusceptive angiogenesis, vessel maturation, and differentiation into arterioles, venules, and capillaries (211).

Aberrant sprouting angiogenesis is characteristic of tumor vasculature. VEGF and Notch cooperate to control the sprouting of new vessels by tightly regulating the balance between tip and stalk cells (212) as detailed in **Figure 5**. Thereby, it is evident that increased levels of Notch signaling or VEGF levels in cancer may locally alter the vasculature.

Almost all tumors express VEGF and high intratumor and serum levels of this cytokine are associated with poor prognosis in cancer patients (218, 219). The release of VEGF by tumor cells is activated by distinct microenvironmental cues, including hypoxia and inflammatory cytokines, or by deregulated oncogenes and pathways (220). The outcome is an "angiogenic switch" with the formation of new vasculature around the tumor, that promotes its growth, invasion, and metastasis (221). Moreover, new blood vessels have an immunosuppressive effect by expressing inhibitory molecules such as programmed cell death ligand 1 (PDL1) and PDL2, indoleamine 2,3-dioxygenase, the adhesion molecule CD31 that may inhibit T cell activation and immunosuppressive cytokines such as IL–10, IL-6, and TGF-β (222). Also, tumor vasculature and high VEGF levels may shape the TME by controlling immune cells extravasation, i.e., promoting the migration of Treg cells while hampering the infiltration of

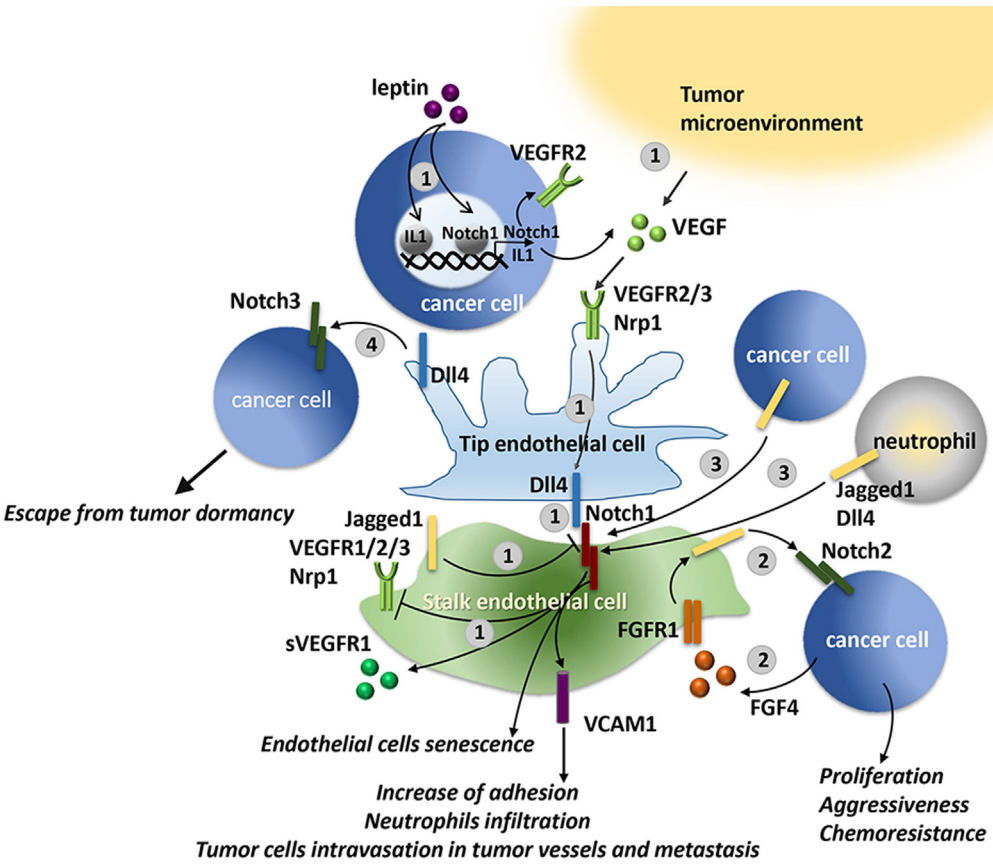

FIGURE 5 | VEGF and Notch interaction in physiological and tumor angiogenesis. Notch controls tip/stalk endothelial cells balance in tight cooperation with VEGF. 1. VEGF may be produced by cancer cells, for example, in breast cancer cells, leptin through IL-1 and Notch1, induces VEGF/VEGFR-2 upregulation (190), or by the neighboring cells of the tumor microenvironment (TME). VEGF engages VEGFR2/3 and neuropilin 1 (Nrp1) receptors on endothelial cells, activating a signaling that promotes Dll4 expression and determines the differentiation toward a tip cell type. Dll4 expressed on tip cells triggers Notch signaling in the neighboring cells suppressing the tip cell phenotype and inducing the differentiation in stalk cell. Notch activation in stalk cells either decreases VEGFR1/2/3 expression or stimulates antiangiogenic soluble splice variant of VEGFR1 (sVEGFR1) leading to a reduced sensitivity to VEGF (213). Oppositely to Dll4, Jagged1 expressed by stalk cells antagonizes Dll4-mediated Notch signaling in stalk cells, thereby increasing tip cell number and sprouting (214). 2. B-cell lymphoma cells release FGF4 that engages FGFR1 on the endothelial cells. FGFR1 activation induces Jagged1 upregulation that, in turn, triggers Notch2 signaling in lymphoma cells promoting their proliferation, tumor aggressiveness, and chemoresistance (215). 3. Notch1 signaling in endothelial cells may be activated by tumor-derived ligands, i.e., Jagged1 and Dll4 expressed by lung carcinoma and melanoma cells, or by Jagged1 expressed by neutrophils. Notch1 activation promotes endothelial cell senescence and expression of the adhesion molecule, VCAM1, that promotes neutrophil infiltration and tumor cell metastasis (216). 4. In models of tumor dormancy of colorectal carcinoma and T cell acute lymphoblastic leukemia (T-ALL) it was shown that Dll4 expressed on tumor endothelial cells can trigger Notch3 pathway activation in tumor cells conferring tumorigenic properties to the dormant cells (217).

effector T cells and thereby favoring an immunosuppressive cytokine milieu (222).

Tumor vasculature is triggered by the crosstalk between VEGF and Notch signaling within the tumor-associated endothelial cell (213). High levels of VEGF in TME, derived from the tumor, endothelial, stromal cells, and immune cells such as macrophages and Tregs, induce an aberrant activation of Notch signaling in tumor-associated endothelial cells that promotes the formation of new altered vessels by replicating a dysregulated version of physiologic angiogenesis (213, 223, 224).

Studies on mouse tumor models confirmed the involvement of VEGF/Notch axis in the formation of tumor endothelium that supports tumor growth. As reported in **Figure 5**, tumor-derived VEGF induces Dll4 expression in tumor vessels resulting in increased number of stalk cells, characterized by high Notch activity, at the expense of the tip cells. As a consequence, the formed vasculature displays a reduced density, but enhanced vessel diameter and perfusion, and thereby support tumor growth (225, 226). Conversely, as expected, the inhibition of Notch in mouse models of glioma, lymphoma, fibrosarcoma, colorectal, lung, and mammary gland tumors, by systematic retroviral delivery of soluble blocking version of Dll4 or anti-Dll4 antibodies increased VEGFR expression in endothelial cells, vessel branching and density of the tumor vasculature that led to reduced tumor growth due to poor perfusion of tumor vessels and increased hypoxia (226, 227). Consistently, a Notch1 decoy that inhibits the interaction Notch–Dll caused a hypersprouting phenotype, stimulated dysfunctional tumor angiogenesis, and hampered tumor growth

in xenograft mouse models of mammary, pancreatic, lung tumors, and melanoma (228). The relevance of the cooperation between VEGF and Dll4-mediated Notch signaling is highlighted by the fact that blocking of Notch signaling through Dll4 neutralizing antibody increases sensitivity to anti-VEGF therapy and reduces tumor growth (225, 227).

High expression of Jagged1 in tumor endothelium destabilizes the tip/stalk balance resulting in a hybrid tip/stalk phenotype leading to enhanced sprouting angiogenesis that promotes tumor growth (214). In accordance, it was reported that in ovarian cancer murine model targeting Jagged1 in tumor-associated stroma mainly composed of endothelial cells and fibroblasts, led to reduced tumor microvessel density and tumor growth (229). Consistently, Cao et al. demonstrated that B-cell lymphoma cells through FGF4/FGFR1 signaling upregulated Jagged1 on adjacent endothelial cells; in turn, Jagged1 activated Notch2 signaling in the lymphoma cells promoting tumor aggressiveness and chemoresistance (215). In line with this evidence, in models of tumor dormancy of colorectal carcinoma and T-ALL, Indraccolo et al. demonstrated that endothelial Dll4 regulated Notch 3 signaling in tumor cells allowing the escape from tumor dormancy (217).

Besides the aberrant Jagged1 expression in tumor-associated endothelial cells, also tumor cells overexpress Jagged1 that plays role in tumor sprouting angiogenesis and in the release of pro-inflammatory chemokines by endothelial cells. Zeng et al. showed that in head and neck squamous cell carcinoma tumor-derived Jagged1 triggered the activation of Notch in neighboring endothelial cells, stimulated the sprouting of capillary-like formations and significantly increased neovascularization and tumor growth *in vivo* (230). Consistently, it was demonstrated that Jagged1 and Dll4 expressed by lung carcinoma and melanoma cells and Jagged1 expressed by neutrophils triggers Notch1 activation in endothelial cells inducing their senescence along with the expression of chemokines and the adhesion molecule VCAM1, that favor neutrophil infiltration, tumor cell intravasation in tumor vessels and metastasis (216).

Kitajewski's group showed that in mammary gland tumor murine model, in which Jagged1 tumor expression was upregulated by ectopic expression of FGF4, Notch inhibition through Notch1 decoy disrupted tumor angiogenesis and delayed the growth of murine Mm5MT xenografts (231). The same group later showed that the interplay between Jagged1 and VEGF promotes tumor endothelial branching along with vascular mural maturation that requires the involvement of Jagged1 (228). Using Notch1 decoy which specifically inhibits Jagged-class mediated Notch activation, the authors showed that Jagged ligands positively regulate angiogenesis by suppressing sVEGFR1 and promoting the interaction between mural cells and endothelial cells (228). Thereby, selective Jagged blockade using a Notch decoy increases sVEGFR1 levels, suppressing sprouting and perfusion, and disrupts pericyte and vascular smooth muscle cell coverage in tumor endothelium of mouse models of mammary, pancreatic, lung tumors, and melanoma, resulting in inhibited tumor growth (228).

A recent study on invasive mammary micropapillary carcinomas hypothesized also a cooperation of VEGF and Notch in tumor lymphangiogenesis. Here, the active form of Notch1 is expressed in extra-tumoral lymphatic endothelial cells together with a receptor of VEGF-C, VEGFR3, involved in lymphatic endothelial cell proliferation, tumor lymphatic invasion, and tumor metastasis (232).

So far, we have described a cooperative activity where VEGF released in TME positively stimulates tumor angiogenesis by regulating Notch signaling in endothelial cells. But, Notch activation in tumor cells and neighboring cells may positively regulate the levels of VEGF released in the TME, with a consequent stimulation of angiogenesis, tumor, and stromal cells.

In breast cancer cells, Notch signaling is necessary for leptin-induced expression of VEGF and VEGFR2 (as detailed above) suggesting that Notch is a downstream mediator of leptin-mediated regulation of breast cancer cell growth and tumor angiogenesis (190). Consistently, Notch1 blockade results in downregulated secretion of VEGF associated with a reduction of tumor angiogenesis and tumor cell invasive abilities (233). In pancreatic tumor cells, exogenous Jagged-1 expression induced VEGF secretion and increased the invasive phenotype of pancreatic cancer cells (234). The Notch–VEGF axis is also exploited by tumor cells to shape the surrounding BMSCs and activate their angiogenic effect as demonstrated by two studies on multiple myeloma. These groups demonstrate that Jagged2, overexpressed by myeloma cells, induces Notch activation in BMSCs, which in turn activates VEGF secretion. Secreted VEGF promotes angiogenesis and acts as a growth factor for myeloma cell stimulating its proliferation (235, 236).

Finally, we must mention that Notch and VEGF signaling cooperated in promoting the "vascular niche" formation necessary for CSC expansion. This aspect has been mainly explored in CNS tumors. Studies on glioblastoma multiforme (GBM) confirmed that Notch plays a role in endothelial control of CSCs. Indeed, the inhibition of Notch signaling blocks GBM CSC self-renewal by decreasing the number of endothelial cells. In turn, the CSC niche promotes angiogenesis, by CSC-mediated release of VEGF or through pro-angiogenic cytokines produced by tumor-infiltrating lymphocytes, such as Th17 cells, and macrophages (237).

As anticipated VEGF functions in cancer are not restricted to tumor angiogenesis. Indeed, VEGF produced in the TME sustains tumor progression also playing an immunosuppressive role by regulating various types of immune cells, such as DCs, T cells, macrophages, and MDSCs (238). Since many kinds of immune cells express VEGF receptors, functions of these cells can be regulated by tumor-derived VEGF. For instance, through the activation of VEGFR1, VEGF inhibits the maturation and activation of DCs with a consequent reduced CD8+ T cell response against tumors such as colorectal, gastric, lung, and breast cancer (239–243).

VEGF contributes to suppress antitumor immune response interfering with CD4+/CD8+ T cell development and differentiation (244). Tumor-derived VEGF facilitates the infiltration and proliferation of Tregs by engaging VEGF receptors, localized on the surface of Tregs (245, 246). It should be noted that infiltrated Tregs recruited to hypoxic areas may also contribute

to increasing VEGF levels in the TME sustaining also tumor angiogenesis (223).

VEGF may affect the myeloid immune cell landscape. Indeed, VEGF may attract immature myeloid cells from the BM into the tumor where, in cooperation with other factors, such as IL-10 and TGF-β, induces these precursors to differentiate into M2 macrophages (247) or may also directly recruit macrophages to the tumor site (248). Tumor-derived VEGF stimulates MDSCs expansion through binding to VEGRF1; consistently, bevacizumab treatment of patients with renal cell cancer showed decreased the number of MDSCs in the peripheral blood (249).

Although the respective functions of Notch and VEGF as immunomodulators are quite well studied along with their interplay in directing tumor angiogenesis, little is known about their interaction and collaboration in tumor immune escape. A study performed by Huang et al. confirmed the hypothesis that their cooperation may contribute to the tumor evasion of immune cell surveillance by demonstrating that Notch-VEGF crosstalk affects the immunosuppressive function of T-cells (39). In a mouse model, the chronic infusion of VEGF, that mimicked the pathophysiologic VEGF concentrations observed in patients with advanced-stage cancer, reduced the levels of Dll1 and Dll4 observed in BM cells (39). Low levels of Dll ligands resulted in the suppression of T cell function, observed as a decrease of the T/B cells ratio in the spleen. Conversely, the selective activation of Dll1-mediated Notch signaling in BM precursors in tumor-bearing mice resulted in the increase of tumor-infiltrating T cells and enhanced activation of Th1-type IFNγ-producing T cells, resulting in tumor growth inhibition.

Finally, VEGF may promote immunosuppression also by contributing to activate the immune checkpoints effector, PD-1. PD-1 is a T cell surface receptor that limits T cell response intensity to avoid autoimmunity. PD-1 regulates two different steps in T cell activation. During DC-mediated activation of T cells, PD-1 engaged by its ligand, PDL1, expressed on DCs may promote apoptosis in effector T-cells and survival in regulatory T cells, resulting in self-tolerance and suppression of T cell cytotoxic activity. Thereby, high levels of PD-1 are involved in immune evasion *via* induction of T cell exhaustion and tolerance for tumor antigens (250). Tumor-derived VEGF has been shown to enhance expression of PD-1 on activated CD8+ cells of colon carcinoma murine models through VEGFR2 signaling, which could be reverted by antiangiogenic agents targeting VEGF-A–VEGFR (251). In addition, VEGF may also induce the expression of PDL1 on tumor-associated myeloid DCs, thus impairing DC-mediated T-cell stimulation (252).

Although a direct collaboration of VEGF with Notch has not been reported yet in the regulation of immune checkpoint, the two pathways clearly act synergistically, since also Notch signaling may play a role in T cell exhaustion. As a matter of fact, Notch binds to PD-1 promoter stimulating its transcription in activated CD8+ T cells. Thereby, Notch signaling inhibition, induced by DAPT or SAHM1, affects PD-1 expression, and restores the function of effector T cell (253).

Overall, shreds of evidence presented here indicate that the interaction between Notch and VEGF influences various cell type in TME promoting tumor cell growth, angiogenesis, metastasis, and escape from the antitumor immune response. Future works are needed to further elucidate a role of the crosstalk between Notch and VEGF with particular attention to tumor-associated immune suppression since uncoupling this interaction may increase the potential of immunotherapy to circumvent the evasion of antitumor immune response.

NOTCH REGULATES THE CYTOKINE PROFILE ASSOCIATED TO CANCER CELLULAR SENESCENCE AND ITS OUTCOME ON INNATE AND ADAPTIVE IMMUNE SYSTEM

The evidence reported in the previous paragraphs indicates that a complex connection exists between Notch signaling levels in tumor and healthy cells populating the TME, the cytokine milieu and different outcomes on tumor progression. Recently, it was reported that Notch signaling ability to control the profile of cytokines secreted by tumor cells may be involved also in cancer cellular senescence.

Cancer-associated cellular stress events, such as DNA damage or activation of oncogenes, may result in cellular senescence. Cellular senescence is a process that leads cells to a permanent proliferative arrest during aging, embryogenesis, and cancer development.

Senescent cancer cells do not proliferate, and as such they do not contribute to the increase in tumor burden, but they may remain metabolically active and secrete proteins with either tumor-suppressing or tumor-promoting activities (254, 255), collectively reported as senescence-associated secretory profile (SASP). SASP is composed of inflammatory cytokines such as TGF-β, IL-1, IL-6, IL-8, growth factors, including VEGF and IGF1, and MMPs similar to those involved in early stage inflammation (255).

On the whole, the phenomenon of senescence may have beneficial effects for the host, such as terminal arrest of cancer cell proliferation and/or its clearance by immune cells recruited through the released cytokines, but often detrimental outcomes may prevail since the secreted senescence-associated cytokines feed non-senescent neighbors inducing their growth or creating an immunosuppressive environment that hampers the antitumor activity of the immune system, resulting in unchecked tumor progression.

Notch activity plays a leading role in cancer cell senescence, primarily through the isoforms, Notch1, Notch3, and the ligand Jagged1 (256, 257). Notch1 involvement has been studied extensively and therefore its role is better defined. Several reports associate Notch1 activity with the onset of senescence in cancer, but the temporal regulation is complicated by the sequence of two distinct phases, necessary for the full acquisition of senescence, and differently regulated by Notch activity. *In vitro* and *in vivo* experiments from Hoare et al. (256) show that Notch1 activity is dynamically upregulated during the first phase of induction in both oncogene-induced senescence (OIS) and DNA damage-induced senescence (DDIS) and returns to basal levels when senescence is completely established (**Figure 6**). These two

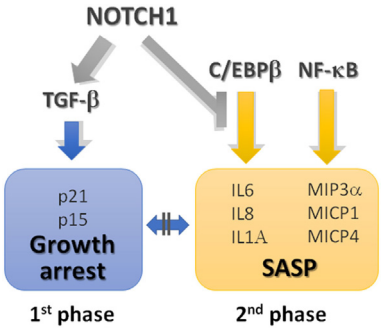

FIGURE 6 | Notch signaling regulates cancer cell senescence and the associated secretome profile. Notch activity is dynamically regulated during the acquisition of the full cell senescence. Through an elegant work on cancer models of oncogene-induced senescence (OIS), Notch-induced senescence (NIS), and DNA damage senescence induced in human diploid fibroblasts, and confirmed in *in vitro* models of pancreatic and liver cancer, Hoare et al. demonstrated that the first phase of senescence is characterized by the activation of Notch with the consequent promotion of transforming growth factor-β (TGF-β) expression and the inhibition of transcriptional activity of C/EBPβ transcriptional activity. This is unleashed with Notch downregulation during full senescence acquisition resulting in the expression of pro-inflammatory senescence-associated secretory profile (SASP) (256). See text for details.

phases, characterized, respectively, by high and low levels of Notch activity, also display two distinct secretomes: the first characterized by anti-inflammatory cytokines and the second by pro-inflammatory cytokines and matrix-modifying enzymes (i.e., MMP1, 3, 10). The mechanism underlying the switch between the two phases involves the interplay between Notch1 and CCAAT/enhancer-binding protein β (C/EBPβ) (256), as depicted in **Figure 6**. In this first phase, high Notch signaling inhibits C/EBPβ transcriptional activity and promotes the secretion of TGF-β. This cytokine mediates immunosuppression along with the growth arrest observed in senescence, in part through the induction of p15 and p21. Upon full senescence achievement, the decrease of Notch activity releases the transcriptional activity of C/EBPβ that, together with NF-κB, coordinates the secretion of pro-inflammatory SASP including IL-6, IL-8, and the master senescence regulator IL-1A (255).

While OIS and DDIS require a biphasic modulation of Notch signaling, tumor cells showing steady high Notch signaling levels are characterized by a different form of senescence, known as Notch-induced senescence (NIS). NIS produces an unusual immunosuppressive cytokine profile characterized by increased TGF-β, growth arrest, and inhibition of pro-inflammatory cytokines (69). Accordingly, Kagawa et al. (258) reported evidence that the ectopic expression of ICN1 induced cellular senescence by inhibiting cell proliferation, finally resulting in irreversible cell-cycle arrest in G0/G1 phase, through the increased expression of p16INK4A and p21 and Rb dephosphorylation. Similarly, Notch3 increased expression in replicative senescence and in DDIS was associated to increased p21 (257).

In addition, the lack of inflammatory secretome associated to NIS was shown to affect *in vivo* lymphocyte recruitment at the tumor site, at least in part by hampering lymphocyte adhesion to endothelial cells and preventing extravasation (256). This is important, since the local infiltration of innate and adaptive immune system is key to the clearance of senescent cancer cells. Several evidences confirm that Notch signaling antagonizes the formation of an immune-stimulating microenvironment associated to senescence. Indeed, Kang et al. (259) recently reported that inhibition of Notch activity, obtained through the use of DN-MAML, results in: (1) senescent phenotype with the release of immune-stimulating cytokines and chemokines that favor the formation of an immunostimulatory microenvironment, including IL-8, IL-1α, IL-1β (260), IFN-γ, IFN-β, CCL2 (195), CCL5, CXCL9, CXCL10, and CXCL11, CCL18; (2) the tumor infiltration by immune cells, such as DCs, NKs, and CD8+ T cells, in *in vivo* syngeneic animal models.

Another important key feature regulated by Notch signaling is the transmission of senescence from the senescent cancer cells to the neighboring non-senescent ones, caused by two distinct mechanisms: secreted cytokines or cell–cell contact.

The first mechanism involves Notch1-driven TGF-β and induces senescence in a paracrine manner, at least in part by promoting of the expression of the cyclin-dependent-kinase inhibitors p15 and p21 in neighboring cells. The second mechanism involves a cell–cell communication mediated by Notch signaling. Notch activation occurring during OIS or NIS results in increased Jagged1 expression. Jagged1 expressed by senescent cancer cells acts as a master mediator of senescence-lateral induction by activating Notch receptors expressed by neighboring cells (256).

The evidence that many of the Notch target genes encoding for cytokines, i.e., IL-1β, IL-6, IL-8, CCL2, and CCL5 (31, 33, 173, 195), above reported in different tumor settings are negatively regulated by Notch during cancer cell senescence underlines that Notch signaling outcome is highly context dependent.

Senescence may be transmitted to nearby cancer cells, but also to the healthy cells of the surrounding TME. Indeed, Procopio et al. (261) recently uncovered the diffusion of senescence from cancer cells to CAFs through a different mechanism associated with reduced levels of the RBP-Jk transcription factor. RBP-Jk down-modulation is exhibited by CAFs derived from different tumors, including skin squamous cell carcinoma (262), head/neck (263), breast (264), and lung (265) cancers, in comparison to normal fibroblasts. RBP-Jk knockdown in primary fibroblasts from dermis, oral mucosa, breast, and lung, induces cellular senescence, and increases the pro-inflammatory cytokines, such as IL-6. In this scenario, Notch role appears to be further complicated by p53. RBP-Jk acts as a repressor for p53, with which shares DNA binding sites on regulative regions of senescence-associated target genes, including p21, and possibly inflammatory cytokines, such as IL-6. Low levels of RBP-Jk allow p53-mediated transcription and, additionally, the repressor-like activity of RBP-Jk may be unleashed by Notch1 activation, also induced by nearby Jagged-bearing cells (261).

Although several facets remain to be elucidated, including the interplay of Notch activity with other signaling pathways involved in the regulation of senescence, Notch activity in cancer cell senescence is crucial in diffusing senescence from cancer cells to other malignant cells and to non-cancerous cells, and in

TABLE 2 | Overview of the last clinical trials that targets cancer-altered cytokines.

Cytokine	Cancer type	Clinical trial phase	Drug	ClinicalTrials.gov Identifier or reference[a]
TGFβ	Prostate cancer	Phase 2	Galunisertib	NCT02452008
TGFβ	Advanced metastatic carcinoma	Phase 1	Galunisertib	NCT02423343
TGFβ	Hepatocellular carcinoma	Phase 2	Galunisertib	NCT01246986 (266)
TGFβ	Metastatic cancer, pancreatic cancer	Phase 1/2	Galunisertib	NCT01373164 (267)
IL-6	Patients with hormone refractory prostate cancer	Phase 2	CNTO 328 (anti IL-6 monoclonal antibody) alone or in combination with n combination with mitoxantrone	NCT00433446, NCT00385827 (268)
IL-6	Patients with unresectable or metastatic kidney cancer	Phase 2	Siltuximab (CNTO 328, anti IL-6 monoclonal antibody)	NCT00311545
IL-6R	Subjects with metastatic HER2 positive breast cancer	Phase 1	Anti-IL-6R monoclonal antibody tocilizumab in combination with trastuzumab and pertuzumab in	NCT03135171 (269)
IL-6	Subjects with relapsed or refractory multiple myeloma	Phase 2	CNTO 328 (anti-IL-6 monoclonal antibody) in combination with bortezomib	NCT00401843 (270)
IL-6	Subjects with newly diagnosed, previously untreated multiple myeloma requiring systemic chemotherapy	Phase 1b/2	Siltuximab (CNTO 328) with lenalidomide, bortezomib, dexamethasone	NCT01531998 (271)
CXCR4	Acute myeloid leukemia	Phase 1/2	Plerixafor	NCT01435343 (272)
CXCR4	Refractory acute lymphoblastic leukemia	Phase 1	Plerixaflor	NCT01319864 (273)
RANKL	Breast cancer	Early phase 1	Denosumab	NCT02900469
RANKL	Bone metastases	Phase 1	JMT103	NCT03550508
TNF1α	Advanced cancer		Infliximab	(274)[a]
IL-17	Patients with relapsed and/or refractory multiple myeloma	Phase 1/1b	CJM112/anti-IL-17A antibody alone or in combination with drug: PDR001, anti-PD1 antibody	NCT03111992 (recruiting)
CCL2	Solid tumors	Phase 1	Carlumab	NCT01204996 (275)
CCL2	Prostate cancer	Phase 2	Carlumab	NCT00992186 (276)
VEGF	Many cancer types	Phase 1/2/3	Bevacizumab and others anti-VEGF drugs	(277, 278)

[a]*Due to the very high number of clinical trials targeting these cytokines, here we referred to review papers that provide an overview of the different experimentations.*

providing a senescence-associated anti-inflammatory secretome that contributes to hamper the antitumor immune response. Overall, the evidence indicates that a Notch-directed therapeutic approach is a unique opportunity to re-establish the local antitumor response of the immune system.

CONCLUSION

Tumor cells are characterized by their ability to shape the surrounding microenvironment, altering the behavior of neighboring normal cells to sustain tumor growth, drug resistance, neoangiogenesis, and bone destruction. In addition, malignant cells cause an immune imbalance in the TME, impairing the functions of cells involved in innate and adaptive immune response, leading to immunesuppression, and promoting inflammation. Overall, these processes contribute to determine the fatal outcome of several malignancies.

In this context, increasing attention has recently been paid to the cytokine network in the TME and to its ability to redefine immune cells differentiation, as testified by increasing number of clinical trials involving drugs or monoclonal antibodies targeting cytokines or their receptors (see **Table 2**).

Here, we have provided a full overview about the pleiotropic role of Notch signaling dysregulation in tuning the expression and activity of a plethora of cytokines involved in the pathological interaction between tumor and TME.

This evidence together with the key role played by Notch dysregulation in several malignancies, suggests that a Notch-targeted approach may be sufficient to restore the physiological cytokine milieu, overcoming tumor-driven immune reprogramming, and improving patient's overall survival.

Unfortunately, the majority of Notch-directed approaches are based on GSIs, whose "pan Notch"-blocking activity results in patient's gastrointestinal toxicity due to intestine metaplasia (279, 280). Thus, a better dissection of the specific contribution of the different Notch ligands and receptors to the dysregulation of the cytokine milieu would help to better refine therapeutic strategies directed to restore the antitumor immune response, avoiding GSI-related side effects. At this purpose, recently a new generation of drugs has been developed such as monoclonal antibodies targeting Notch1 (281), Notch2/3 (282), Dll4 (283), or small molecules targeting Jagged1/2 (284, 285). These novel therapeutic strategies promise to specifically inhibit the dysregulated members of the Notch pathway, contributing to restore the normal activity of the immune system, and finally hampering tumor progression.

AUTHOR CONTRIBUTIONS

All the listed authors have provided a substantial, direct, and intellectual contribution to the work and approved it for the publication.

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NOTCH Signaling in T-Cell-Mediated Anti-Tumor Immunity and T-Cell-Based Immunotherapies

*Michelle A. Kelliher and Justine E. Roderick**

Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA, United States

The NOTCH (1–4) family of receptors are highly conserved and are critical in regulating many developmental processes and in the maintenance of tissue homeostasis. Our laboratory and numerous others have demonstrated that aberrant NOTCH signaling is oncogenic in several different cancer types. Conversely, there is also evidence that NOTCH can also function as a tumor suppressor. In addition to playing an essential role in tumor development, NOTCH receptors regulate T-cell development, maintenance, and activation. Recent studies have determined that NOTCH signaling is required for optimal T-cell-mediated anti-tumor immunity. Consequently, tumor cells and the tumor microenvironment have acquired mechanisms to suppress NOTCH signaling to evade T-cell-mediated killing. Tumor-mediated suppression of NOTCH signaling in T-cells can be overcome by systemic administration of NOTCH agonistic antibodies and ligands or proteasome inhibitors, resulting in sustained NOTCH signaling and T-cell activation. In addition, NOTCH receptors and ligands are being utilized to improve the generation and specificity of T-cells for adoptive transplant immunotherapies. In this review, we will summarize the role(s) of NOTCH signaling in T-cell anti-tumor immunity as well as TCR- and chimeric antigen receptor-based immunotherapies.

Keywords: NOTCH, T lymphocytes, cancer, immunotherapy, anti-tumor response

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***Correspondence:**
Justine E. Roderick
justine.roderick@umassmed.edu

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INTRODUCTION

There are four NOTCH receptors (NOTCH1–4) in mammals, which are ubiquitously expressed. Activation of the NOTCH signaling occurs after engagement of a NOTCH receptor with one of its membrane bound Delta-like ligands 1,3,4 (DLL1, DLL3, DLL4) or Jagged ligands 1,2. In some contexts, NOTCH can become activated through ligand-independent mechanism(s) leading to a variety of human diseases (1). After ligand engagement NOTCH undergoes a series of proteolytic cleavages, resulting in an activated NOTCH intracellular domain (NICD), which translocates into the nucleus to activate gene transcription. Given that NOTCH signaling is critical in regulating cell fate decisions in many tissue types, it is not surprising that NOTCH activity is deregulated in several malignancies (2–4). The first evidence for the involvement of NOTCH signaling in cancer was discovered in T-cell acute lymphoblastic leukemia (T-ALL), where activating mutations were identified in NOTCH1 (5). Our laboratory showed that oncogenic NOTCH1 regulates MYC expression and leukemia-initiating cell activity and demonstrated the efficacy of NOTCH1 inhibitors in pre-clinical T-ALL models (6–9). Activating mutations in *NOTCH1* have also been identified in chronic lymphocytic leukemia, non-small cell lung carcinoma, and translocations involving NOTCH1/2 in patients with triple negative breast cancer (10–13). While mutations in NOTCH receptors are rare in other tumor types, NOTCH is aberrantly activated in several malignancies, including colorectal and pancreatic

cancer, melanoma, adenocystic carcinoma, and medulloblastoma through a variety of mechanisms (2, 4). Conversely, loss of function mutations in *NOTCH1/2/3* have also been identified suggesting NOTCH can also function as a tumor suppressor (2, 3).

While progress has been made in how NOTCH signaling contributes to malignant transformation, the role of NOTCH activity in anti-tumor immune responses is less clear. While several cell types contribute to anti-tumor responses, CD4 T-helper 1 (TH1) cells and CD8 cytotoxic T-lymphocytes (CTL), are critical in mediating anti-tumor immunity due to their ability to recognize tumor antigens and mediate tumor killing. Several studies have shown that NOTCH is required for activation and effector function of CD4 and CD8 T-cells (14). Tumor cells can dampen T-cell responses by producing immunosuppressive cytokines, expressing inhibitory ligands, and recruiting immunosuppressive myeloid and lymphoid cells into the tumor microenvironment (15). Given that NOTCH is required for T-cell activation and effector function it is reasonable to hypothesize that NOTCH contributes to T-cell anti-tumor responses and that tumor cells may evade T-cell mediated killing by suppressing NOTCH activation. Consistent with this hypothesis, new data suggest that NOTCH activation is suppressed in tumor-infiltrating T-cells and that NOTCH re-activation induces potent anti-tumor T-cell responses in mouse cancer models (16–20).

Adoptive transplants of tumor antigen-specific T-cells is one immunotherapy used to overcome the limitations of endogenous T-cells and enhance anti-tumor responses. Tumor antigen-specific T-cells are either isolated from the tumor site or engineered with synthetic T-cell receptors (sTCRs) or chimeric antigen receptors (CARs) specific for tumor antigens (21, 22). Recently, NOTCH signaling has been utilized to improve the generation and efficacy of adoptive T-cell therapies (ACT) (23, 24). Furthermore, newly developed synthetic NOTCH receptors (synNOTCH) have been engineered to enhance the specificity of CAR T-cells (25–27). These studies highlight the importance of studying NOTCH responses in T-cell-mediated anti-tumor immunity in order to design more effective T-cell-based immunotherapies.

NOTCH SIGNALING IS REQUIRED FOR T-CELL ACTIVATION AND EFFECTOR FUNCTION

NOTCH signaling has been extensively studied in T-cell development, activation, and effector function. Upon TCR-stimulation naïve CD4 T-cells differentiate into multiple subsets of T-helper (TH) cells (14, 28). TH subsets are designed to recognize and fight distinct types of infection and are characterized by their specific cytokine profile. NOTCH activation has been shown to play a role in the differentiation of TH1, TH2, TH9, TH17, T-regulatory cells, and follicular TH cells (14, 28). TH1 cells mediate anti-tumor responses in conjunction with CTLs. Genetic deletion or pharmacologic inhibition of NOTCH1 signaling with gamma-secretase inhibitors (GSIs) decreases the numbers of activated TH1 cells *in vitro* and in mouse models of TH1-driven autoimmune disease (29, 30). NOTCH directly stimulates the transcription of the TH1 master transcriptional regulator T-BET (*TBX21*) as well as the TH1 signature cytokine interferon-gamma (*IFNγ*) (29–31).

CD8 naïve T-cells differentiate into CTLs upon early TCR stimulation, and then terminal effector cells or memory precursor cells (14). Recent evidence shows that conditional deletion of *Notch1* or inhibition of NOTCH signaling with GSIs diminishes the production of CTL effector molecules, including IFNγ, tumor necrosis factor alpha, granzyme B, and perforin, as well as a reduction in the CD8 transcription factors T-BET and eomesodermin (EOMES) (32–36). In addition to playing a role in activating effector T-cells NOTCH is also important in the maintenance and generation of memory T-cells (35, 37). While these studies provide compelling evidence that NOTCH signaling regulates T-cell effector activation, it remains unclear how NOTCH dictates such a multitude of responses in T-cells. Data from several studies suggest that NOTCH ligands may dictate T-cell effector responses.

NOTCH LIGANDS DICTATE T-CELL FATE

NOTCH ligands have been shown to have diverse effects on T-cell effector function. In CD4 T-cells, activation of the TCR in the presence of DLL1/4 skews toward a TH1 fate and inhibits TH2 differentiation (38, 39). Conversely, Jagged1/2 ligands may be important for TH2 differentiation, but appear to have no role in TH1 differentiation (38, 39). The role of DLL1 in CD8 T-cell activation and differentiation is unclear (38, 39). One study found that DLL1 overexpression in dendritic cells results in increased levels of granzyme-B expression in alloantigen stimulated CD8 T-cells (32). However, a prior study reported that CD8 T-cells stimulated with DLL1 and alloantigens resulted in decreased IFN-γ production and increased IL-10 production, suggesting a suppressive role for DLL1 in CD8 activation (40). Additional studies are needed to clarify the effects of DLL1 and other NOTCH ligands on the activation and effector function of CTLs.

These studies suggest that T-cell effector function mediated by NOTCH is determined by the stimulating ligand, this is further supported by data demonstrating that ligand expression on antigen-presenting cells (APC) is dictated by the engaging stimulus. For example, APC exposed to allergens upregulate Jagged1/2 expression inducing a TH2 response whereas viral infection stimulates DLL1/4 expression on APC and a TH1 response (41, 42). However, some studies demonstrate normal T-cell polarization and effector function in the absence of NOTCH ligands, favoring a model in which NOTCH enhances T-cell activation and proliferation, however, cytokines instruct T-cell fate (39). Understanding how NOTCH ligands dictate effector function will be critical to maximize the therapeutic potential of NOTCH-based immunotherapies.

TUMOR CELLS AND THEIR MICROENVIRONMENT SUPPRESS THE EXPRESSION OF NOTCH RECEPTORS AND LIGANDS

Full-length NOTCH receptors are normally expressed on naïve mouse T-cells and activated in response to antigen; however,

T-cells isolated from tumor bearing mice have decreased expression of NOTCH (1–4), (18, 19). Consistent with this reduction in NOTCH levels, significant decreases in NOTCH target genes (*Deltex1*, *Hey1*, and *Hes1*) are also observed in tumor-associated T-cells (19), suggesting that tumor-associated T-cells have repressed NOTCH signaling and potentially decreased effector function.

Reduction in NOTCH1/2 levels was found to be mediated in part by tumor-infiltrating myeloid-derived suppressor cells (MDSCs) (18). MDSCs are a heterogeneous population of immature myeloid cells that are recruited to sites of inflammation and the tumor microenvironment to prevent immune-mediated damage (43). MDSCs are recruited by multiple factors including vascular endothelial growth factor (VEGF), IL-1β, and IL-6 (44). Coculturing of MDSC with activated T-cells reduced the expression of full length and intracellular NOTCH1/2 (18). MDSC isolated from cancer patients have been shown to suppress T-cell activation (45, 46), however, whether MDSC suppress *via* effects on NOTCH signaling is not known.

In addition to reducing NOTCH1/2 levels, reductions in NOTCH ligand expression on T-cells and other immune cells has also been observed in murine tumor models (16, 19). Reduced expression of DLL1/4 in the bone marrow of tumor bearing mice inversely correlated with increased VEGF levels in one study (16). VEGF has been shown to potentiate T-cell anti-tumor responses, suggesting that expression of this growth factor by cancer cells may inhibit T-cell responses by downregulating DLL1/4 (47). MDSC isolated from the tumor site have decreased DLL1/4 and increased Jagged1/2 expression (18). Given that DLL1/4 induce TH1 and CTL effector function, this could be an additional mechanism, whereby the tumor microenvironment impairs/disables NOTCH signaling. While these studies demonstrate that NOTCH activity is impaired in tumor-infiltrating T-cells in mouse cancer models, precisely how NOTCH receptor/ligands are downregulated is unclear. Furthermore, there is as yet, no direct evidence that NOTCH signaling is impaired in T-cells from cancer patients.

ACTIVATION OF NOTCH RECEPTORS AND THEIR LIGANDS INCREASES T-CELL-MEDIATED ANTI-TUMOR RESPONSE

Conditional activation of NOTCH1/2 in CD8 T-cells induces a robust and sustained anti-tumor response, resulting in increased IFNγ production and reduced tumor burden (18, 20). Similarly, treatment of tumor bearing mice with an agonistic NOTCH2 antibody enhanced CD8 T-cell cytotoxicity and reduced tumor size (20). Consistent with this finding, conditional deletion of *Notch2* in CD8 T-cells potentiated tumor growth in mice and reduced overall survival (20).

Constitutive expression of DLL1 on bone marrow and dendritic cells was also reported to enhance T-cell infiltration into tumors, suppress tumor growth and increase the survival of mice transplanted with murine tumor cell lines [Lewis Lung Carcinoma (LLC), D459 Fibrosarcoma, and EL4 T cell Lymphoma] (16, 20). Increased DLL1 but not Jagged2 expression on dendritic cells stimulated T-cell cytotoxicity and increased IFN-γ levels (20). Moreover, therapeutic administration of a multivalent, clustered form of DLL1 (c-DLL1) arrested tumor growth and prolonged survival of mice transplanted with LLCs or D459 tumor cells (16, 17). The c-DLL1 was shown to bind and activate NOTCH (1–4), resulting in increased NOTCH target gene expression (16, 17). Administration of c-DLL1 stimulated IFN-γ production and increased tumor-infiltrating antigen-specific T-cells (16, 17). Tumor regression in c-DLL1 treated mice appears to be T-cell mediated, since c-DLL1 treatment had no effect on tumor growth in *Rag1*$^{-/-}$ recipients or in mice treated with anti-CD8 antibody (16). Furthermore, adoptive transfer of tumor antigen-specific T-cells from c-DLL1-treated mice were sufficient to attenuate tumor growth in immunocompromised NOD-SCID mice (17).

The proteasome inhibitor bortezomib was shown to enhance T-cell-mediated anti-tumor responses in part by restoration of NOTCH receptors and ligand mRNA expression (19). Bortezomib treatment led to increased expression of CD25, CD44, IFNγ, and granzyme B in CD8$^+$ T-cells isolated from mice engrafted with cancer cell lines (19, 48). Combination treatments consisting of bortezomib and adoptive T-cell transfer reduced tumor burden and prolonged survival in human renal carcinoma xenografts (48). Whether bortezomib treatment regulates NOTCH activity directly or if these effects are secondary is unknown. Together these studies support the concept that activating NOTCH enhances T-cell anti-tumor immunity and prolongs tumor-free survival. While the development of NOTCH agonist antibodies and c-DLL1 therapies appear to be a promising approach to enhance T-cell anti-tumor immunity, the potential effects on NOTCH driven malignancies needs to be considered.

CURRENT CHALLENGES IN ACT

Adoptive T-cell therapies involves the generation of tumor antigen-specific CTLs *in vitro*, which are then infused back into the patient where they kill tumor cells. Tumor-specific T-cells are generated by selection and expansion of tumor-infiltrating lymphocytes (TIL), or by transduction of sTCR or CAR (21, 22). ACT using TILs has been a successful treatment option for melanoma, however, this approach could only be used on patients whose T-cells could be isolated and cultured (49, 50). CAR T-cell therapies have yielded exceptional clinical results in B-ALL (51–53), but identification of tumor-specific antigens is needed in order to expand CAR T-cell therapies to additional malignancies. Both approaches need improvement because the generation of TIL and CAR T-cells is time consuming and T-cell numbers are limiting. Furthermore, while the T-cells used for ACT have enhanced tumor antigen recognition, they are still susceptible to immunosuppressive factors in the tumor microenvironment.

NOTCH LIGANDS IN T-CELL-BASED IMMUNOTHERAPIES

Generation of CAR-specific T-cells from induced pluripotent stem cells (iPSC) from cancer patients is one approach currently being utilized to overcome limited numbers of patient T-cells (24). Using this approach iPSC are differentiated into T-cells by culturing on stroma expressing the NOTCH ligand DLL1 (24). Similar approaches have been used to generate CAR T-cells from hematopoietic stem cells (54). Researchers have also used pluripotency and reprogramming factors to expand human tumor-specific T-cells (55, 56). While this strategy produces unlimited tumor-specific CTLs, the TCR repertoires are often limited. To overcome this obstacle, investigators have begun to test the efficacy of T-stem cell memory (T_{SCM}) cells in adoptive T-cell transplants. T_{SCM} cells have the ability to function as memory T-cells by responding rapidly to antigen, however, they are not terminally differentiated and therefore possess an enhanced capacity for self-renewal and proliferation (57). T_{SCM} cells have been characterized in mice and humans and found to persist years after primary infection or vaccination. The current model to generate T_{SCM} cells is by stimulating naïve T-cells in the presence of Wnt3A or inhibitors of glycogen synthase kinase-3b (57). Adoptive T-cell transplants with CAR T-cells generated from T_{SCM} cells results in more potent anti-tumor responses than CAR T-cells generated from other T-cell types (57). Recent work by Kondo et al. exploit NOTCH pathway activation to generate T_{SCM} cells from activated mouse and human T-cells referred to as iT_{SCM} cells (23). iT_{SCM} cells re-capitulate the features of T_{SCM} cells including rapid response to antigen re-stimulation and increased self-renewal capacity. iT_{SCM} cells also exhibit decreased expression of the T-cell inhibitory receptors programmed cell death-1 (PD-1) and cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4), allowing for enhanced survival and activation in the tumor microenvironment (23). Unlike traditional T_{SCM} cells generated from naïve T-cells, iT_{SCM} cells are derived from activated T-cells and therefore could be generated from TILs, eliminating the need for transduction with sTCRs or CARs. These alternative methods to generate T-cells for ACT may provide greater anti-tumor immunity by increasing T-cell longevity and yield.

SYNTHETIC NOTCH RECEPTORS GENERATE POTENT CAR SPECIFIC T-CELLS

While current methods have markedly enhanced anti-tumor reactivity, CAR T-cells are still restricted to endogenous T-cell responses have limited capabilities to overcome the immunosuppressive microenvironment. To overcome this, researchers generated CAR T-cells with synthetic NOTCH receptors (synNOTCH), which allow for specific cytotoxic responses (26, 27). NOTCH receptors are single pass transmembrane proteins composed of an extracellular ligand-binding domain, a transmembrane region, and an intracellular signaling domain. synNOTCH receptors contain the transmembrane domain, however, they have synthetic extracellular ligand domains and intracellular transcriptional domains (26, 27). In recent work by Roybal et al., human T-cells were engineered to express synNOTCH receptors, where the extracellular ligand domain of NOTCH was replaced with CARs targeting tumor antigens, CD19 or HER2 (27). Following CAR engagement, the synNOTCH receptor undergoes transmembrane cleavage, releasing the synthetic NICD. NICD then translocates to the nucleus to activate gene transcription. Unlike normal NICD which recognizes and binds CBF1/RBP-Jkappa sites, synthetic NICD is replaced with an intracellular transcription activation domain (Gal4-VP64 or tTA) that in turn drives a distinct reporter expressed in the synNOTCH expressing cell (26, 27). synNOTCH receptors have been engineered to drive the expression of several cytotoxic factors that enhance T-cell anti-tumor responses, including expression of the death ligand TRAIL, the cytokine IL-12, and the transcription factor T-BET. In addition, synNOTCH receptors can drive the production of antibodies to PD-1 and CTLA-4 to overcome inhibitory ligand expression by cancer cells or express IL-10 and PD-L1 to reduce inflammation generated by enhanced T-cell cytotoxicity. synNOTCH-engineered T-cells have shown efficacy in conventional humanized xenograft models (27). Using these synNOTCH receptors to customize CAR T-cell responses will enhance anti-tumor activity, and armor the T-cells against the immune suppression mediated by the tumor microenvironment.

CONCLUSION/FUTURE PERSPECTIVES

Activation of T-cell effector function in an immunosuppressive microenvironment is a critical component of effective T-cell-mediated anti-tumor immunity. Tumor cells and their microenvironment suppress T-cell responses in part by repressing NOTCH receptors and ligands and consequently T-cell effector function. While in-depth characterization of tumor cells has led to the development of targeted therapies, characterization of tumor-infiltrating T-cells from patients is still lacking. Several studies have begun to establish gene signatures that represent a variety of immune populations and demonstrated that these signatures can be predictive of clinical outcome and response to immune therapy (58, 59). A similar approach could be taken to determine if NOTCH receptors and ligands are suppressed in T-cells isolated from cancer patients. Therapies that activate/maintain NOTCH signaling were shown to improve T-cell-mediated tumor clearance, prolonging the survival of tumor bearing mice. However, the efficacy and safety of this approach in patients remains unclear.

NOTCH ligands may also serve as tools to improve the generation and efficacy of T-cells used for ACT. T_{SCM} cells may overcome the obstacles currently facing ACT, including increasing anti-tumor responses and decreasing immunosuppression. The use of synNOTCH CAR T-cells is particularly intriguing as the cytotoxic response of these cells can be tailored to provide an enhanced and specific anti-tumor response. Future studies examining combinations of synNOTCH T-cells on the anti-tumor immune responses and their effects on endogenous tumor-infiltrating T-cells should provide insight.

While these findings highlight the exciting potential to improve T-cell-based immunotherapies, there are still many

questions regarding the clinical relevance and application of these approaches. In addition, the safety and efficacy of these NOTCH strategies need to be evaluated to ensure that sustained NOTCH activation does not result in leukemic transformation or potentiate tumor growth. One major limitation in accomplishing these goals is the lack of a primary derived xenograft mouse model with a humanized immune system. Continued research will provide a better understanding as to how NOTCH signaling contributes to T-cell anti-tumor responses and uncover new approaches to improve T-cell-based immunotherapies.

AUTHOR CONTRIBUTIONS

JR wrote the manuscript with help from MK.

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Notch and NF-κB: Coach and Players of Regulatory T-Cell Response in Cancer

Francesca Ferrandino[1], Paola Grazioli[2], Diana Bellavia[1], Antonio Francesco Campese[1], Isabella Screpanti[1] and Maria Pia Felli[2]**

[1] Department of Molecular Medicine, La Sapienza University, Rome, Italy, [2] Department of Experimental Medicine, La Sapienza University, Rome, Italy

The Notch signaling pathway plays multiple roles in driving T-cell fate decisions, proliferation, and aberrant growth. NF-κB is a cell-context key player interconnected with Notch signaling either in physiological or in pathological conditions. This review focuses on how the multilayered crosstalk between different Notches and NF-κB subunits may converge on Foxp3 gene regulation and orchestrate $CD4^+$ regulatory T (Treg) cell function, particularly in a tumor microenvironment. Notably, Treg cells may play a pivotal role in the inhibition of antitumor immune responses, possibly promoting tumor growth. A future challenge is represented by further dissection of both Notch and NF-κB pathways and consequences of their intersection in tumor-associated Treg biology. This may shed light on the molecular mechanisms regulating Treg cell expansion and migration to peripheral lymphoid organs thought to facilitate tumor development and still to be explored. In so doing, new opportunities for combined and/or more selective therapeutic approaches to improve anticancer immunity may be found.

Keywords: Notch, NF-κB, regulatory T cells, Foxp3, cancer

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***Correspondence:**
Isabella Screpanti
isabella.screpanti@uniroma1.it
Maria Pia Felli
mariapia.felli@uniroma1.it

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INTRODUCTION

Regulatory T (Treg) cells are a heterogeneous population of T lymphocytes. Human and mouse Tregs act as gate-keepers of multiple immune reactions, suppressing unwanted immune responses such as autoimmunity, allergy, or transplant rejection (1–3). Treg cells (Tregs) are a first line of host-defense against infection, and prevent activation and expansion of autoreactive T cells. Infiltration of Tregs is associated with a decreased ratio of cytotoxic $CD8^+$ T cells to Tregs (4), tumor progression (5), and poor prognosis in a number of cancers (6–8).

NF-κB transcription factors critically integrate the etiological mechanisms establishing inflammation as underlying malignancy (9), mainly orchestrating immune responses (10). NF-κB, triggered by multiple signaling pathways, in turn serves as a cell-intrinsic player in Treg development and function (**Table 1**). It also contributes as a multifaceted regulator being triggered and targeting gene expression regulation (17).

Natural Treg (nTreg) arising in the thymus very early after birth and induced Treg (iTreg) in the periphery are both influenced by Notch signaling, notably in a cell context-dependent pathway (23). Notch signaling promotes the generation and function of nTreg, but its inhibition enhances Treg functions and protects mice from graft-vs.-host disease (24, 25). In intact thymic medulla, Tregs during their development require RelB-dependent functions of medullary thymic epithelial cells, which also provide co-stimulatory molecules and MHC class I/II (18).

TABLE 1 | Function of distinct NF-κB subunits in physiological T-reg activity and in cancer.

NFκB Subunits	Physiological functions in Tregs	Pathological functions in Tregs	References
RelA/p65	• Development (nTregs). • Acquisition/maintenance of mature Tregs identity and function.	• Ablation results in autoimmune syndrome. • Cooperatively with CSL up-regulates Foxp3 expression (nTregs).	(11–16)
RelB	• Not-intrinsically required for development or suppressive function. • Development in intact thymic medulla (nTregs) by a Treg-extrinsic mechanism. • Peripheral Treg homeostasis under p100 control.	• Loss induces systemic autoimmunity and expansion of Foxp3$^+$ Tregs (Treg-extrinsic mechanism). • Mediates SDF1/CXCR4 axis at the tumor site (Treg-extrinsic mechanism).	(11, 17–19)
c-Rel	• Development (nTregs). • Maintenance of numbers and identity (nTregs). • Homeostatic expansion (iTregs).	• Inhibition of antitumor responses. • Migration to inflamed tissues and tumors (aTreg). • Maintenance of numbers and identity at the tumor site (aTreg). • Loss induces mild autoimmunity.	(11, 13, 20–22)

Treg identity is (Gitr$^+$CD25$^+$Foxp3$^+$); nTregs, natural Tregs; iTregs, induced Tregs; aTregs, activated Tregs; CSL-the transcriptional repressor CBF1/suppressor of hairless/Lag-1 (or the human homolog RBPJk-recombining binding protein suppressor of hairless); Stromal cell-derived growth factor 1 (SDF1)/CXCR4; p100 subunit encoded by the NF-kB2 gene.

Many authors have contributed to unveiling the key features of both Notch and NF-κB pathways in Treg biology, also in the context of a tumor. Here we will focus on some important clues related to the functional plasticity of the two signaling pathways, and to their interplay still unexplored in the regulation of Treg expansion and function in cancer.

THE NF-κB TEAM IN Treg BIOLOGY

The mammalian NF-κB family is composed of five members, p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52, which originate a collection of homodimers and heterodimers (26), that are tightly controlled and sequestered into the cytoplasm by IκB, NF-κB inhibitory proteins.

NF-κB activation occurs through two pathways depending on the components of the IκB kinase (IKK) complex: the canonical heterotrimer IKKα/IKKβ/IKKγ and the alternative IKKα/IKKα homodimer (17), which is required for the homeostasis of Tregs and for the expansion of both regulatory and effector CD4$^+$ T cells (27). Next, IKKβ-dependent serine-phosphorylation and ubiquitin-dependent degradation of IκBα initiate canonical NF-κB dimer (p50/p65) activation and nuclear entry (17). Notably, p65 and c-Rel (encoded by *Rela* and *Rel*, respectively) drive the acquisition/maintenance of Treg identity (Gitr$^+$CD25$^+$Foxp3$^+$) and function (11). In contrast, the conditional deletion of RelB in Foxp3$^+$ Tregs does not alter the number and function of this subset, even though the germline deletion of RelB induces autoimmunity and an expansion of Foxp3$^+$ Tregs (**Table 1**), mainly due to T cell-extrinsic mechanisms (19).

In the context of T cells, multiple extracellular signaling cascades including Notch (28, 29) can converge on the canonical NF-κB pathway. This may also be triggered by the pre-T-cell receptor (pre-TCR) (30) whose functional cooperation with constitutive Notch3 expression is involved in the pathogenesis of a Notch3-induced T-cell acute lymphoblastic leukemia (T-ALL) (31) characterized by a wide CD4$^+$CD25$^+$Treg expansion (32, 33).

Regarding the alternative signaling pathway, NF-κB-induced kinase (NIK) phosphorylates to activate IKKα, which promotes p100 (encoded by *NF-κB2*) precursor protein processing. This then generates the main "alternative" complex p52/RelB that crucially controls lymphoid organogenesis and cell migration (34).

Interestingly, Murray et al. genetically manipulated the NIK expression in mice and demonstrated that the NIK deletion in T cells specifically impairs the maintenance of peripheral Foxp3$^+$ Tregs, thus suggesting a Tregs intrinsic function for the noncanonical pathway (35). Alternatively, the lineage-specific constitutive activation of NIK in Treg cells induces an alteration of their functions and gene signature (Gitr$^+$CD25$^+$Foxp3$^+$), leading to the development of an autoimmune syndrome (36).

In mature T cells, upon the engagement of the TCR/CD28 complex, PKCθ and the CARMA1/BCL10/MALT1 (CBM) protein complex are recruited to finally induce NF-κB activation (37) (**Figure 1**). Mutations of TCR signalosome (CBM-PKCθ-IKKβ) components selectively impact nTreg biology, whereas conventional T-cell development seems to be less affected (38–41). Notably, Notch1 can also initiate NF-κB activation via cytosolic interactions with T-cell signalosome components (42). PKCθ-selective transport to lipid rafts within the immunological synapse (43) will recruit IKK to the CBM and trigger IKK activation; this pathway is negatively regulated by the deubiquitinase CYLD. *CYLD*-deficient mice display constitutive NF-κB activation in thymocytes and peripheral T cells. The Treg frequency is enhanced although Tregs are less functional than the *wild-type* counterparts (44). Recently, it was demonstrated that another negative regulator of NF-κB—ubiquitin-editing enzyme A20—restricts nTreg development; however, A20-/- Tregs are completely functional *in vivo* (45). Interestingly, while A20 terminates NF-κB signaling, CYLD prevents spontaneous NF-κB activation. Notch3 overexpression in combination with the pTα/preTCR function increases Lck-dependent PKCθ translocation to the cell membrane, triggering PKCθ/IKKβ-axis hyperactivation (46). Intriguingly, PKCθ and CYLD are antagonistic partners in the NF-κB activation in T cells (47). However, PKCθ is involved in Treg cell differentiation *in vivo*, but it is dispensable for Treg-mediated suppression (48); therefore, the balance between the positive (PKCθ) and/or negative (CYLD,

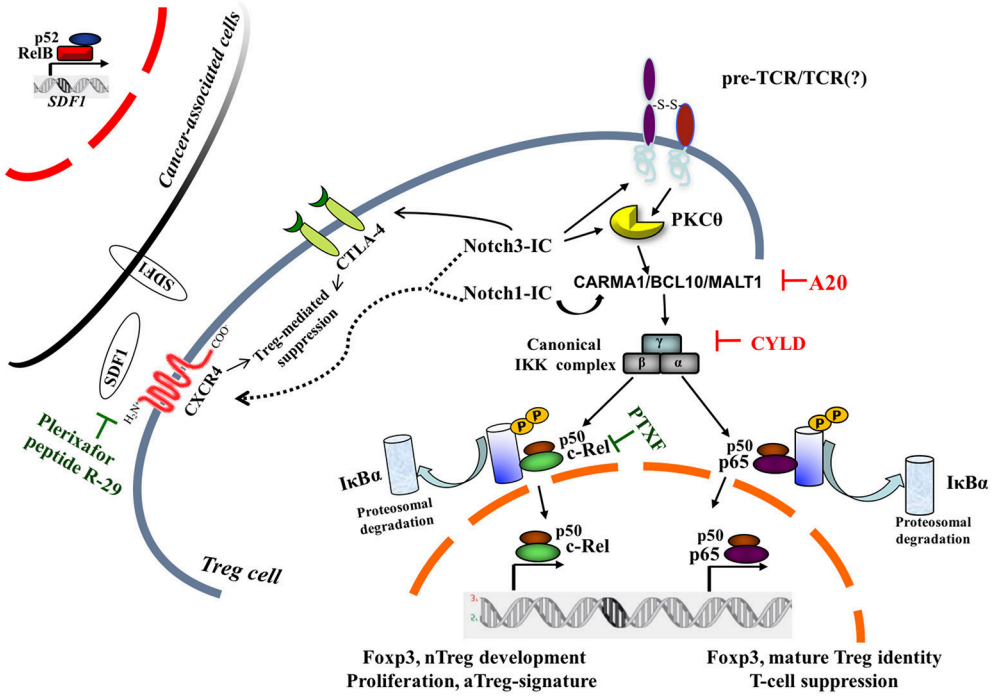

FIGURE 1 | Canonical NF-κB pathway is central to intrinsic Notch1- and Notch3-modulated Treg cell function within tumor microenvironment. Two NF-κB negative regulators, A20 and CYLD, on removal of nonproteolytic K63-linked polyubiquitin chains from signaling molecules, interfere with the preTCR/TCR pathway, leading to NF-κB activation. For a pharmacological approach, pentoxifylline (PTXF) that selectively degrades c-Rel is indicated, as well as inhibitors of Treg-mediated suppression activity by CXCR4 antagonists, such as plerixafor (AMD3100) or peptide R-29. The dotted line refers to hypothetical Notch1- and/or Notch3-induced CXCR4 modulation in Treg cells, whereas the black curved-line indicates the Notch3-enhanced CTLA4 expression in N3-ICtg Tregs (36). Cancer-associated cells once activated in a tumor microenvironment can express many proinflammatory genes, including stromal cell-derived factor 1 (SDF1), the cognate ligand of CXCR4, partly in an NF-κB-dependent manner (23). pTα-chain (preTCR) and T-cell receptor (TCR); IκBα, inhibitor of NF-κBα.

A20) regulators of NF-κB may govern the generation and function of Tregs (**Figure 1**).

Post-translational modifications can also fine-tune the transcriptional activity of nuclear NF-κB to modulate its interaction with coactivators, corepressor, IκB proteins, and the binding to heterologous transcription factors (enhanceosomes), thus shaping NF-κB-dependent gene programs (10).

In particular, the phosphorylation of serine 276 and additional residues are critical for CBM recruitment and the transcriptional activity of p65 (10). Notably, IKKβ-mediated phosphorylation of p65/serine 536 has been shown to require PI(3)K-Akt activity, an emerging node for crosstalk between NF-κB and PI(3)K-Akt pathways, whose balance is important in promoting selection into the Treg-cell lineage (49) (and references therein). Interestingly, the phosphorylation of the p65/serine 536 residue is strongly promoted in Notch3-induced T-ALL (50).

NF-κB: A FORWARD PLAYER OF Tregs ACTIVITY IN CANCER

The infiltration of Tregs into various tumor tissues promotes tumor progression by limiting the antitumor immune response and the supporting tumor immune evasion (4, 6, 51). Tregs exert these functions, as a combined result of efficient migration into the tumor site, local expansion of specialized subsets, and *de novo* generation within the tumor, all of which are still poorly unveiled. A highly immunosuppressive Treg subtype, expressing tumor-necrosis-factor-receptor 2 with activated NF-κB/p65 has been abundantly recognized in human ovarian cancers (52). In human hepatocellular carcinoma, the decreased survival rate was associated to a higher level of peripheral blood Tregs; similar observations have been reported in chronic lymphocytic leukemia (CLL) patients (53). However, controversial is the role of high Treg infiltration as a prognostic parameter in colorectal cancer (54, 55).

In mice, the resting Tregs (rTregs) resident in lymphoid tissues prevent lymphoproliferative disease and autoimmunity, and are maintained by the Foxo1-activated transcription function (5, 56). On the contrary, the "effector-memory like" activated Treg subset (aTreg) migrates to the inflamed tissues and tumors and potently inhibits antitumor responses (20–22), essentially associated to the c-Rel function (**Table 1**) (57).

Tregs typically suppress T-cell proliferation and cytokine production in target $CD4^+$ T cells. This inhibition is achieved by reducing nuclear NF-κB/p65 accumulation (58).

Reversibly, in mice, the inhibition of the canonical NF-κB pathway by the "super repressor" IkBSR-enforced expression

or the IKKβ loss impairs Tregs development (39), whereas the genetic ablation of canonical NF-κB proteins (c-Rel) profoundly reduces the numbers of $CD4^+Foxp3^+$ Tregs in the neonatal and adult thymus and in peripheral lymphoid organs (59, 60).

During the development of nTregs inside the thymus, both the nuclear localization and activity of c-Rel and RelA have been described in the transition from $CD4^+CD8^+$ (DP) to Treg precursors generation ($CD25^{hi}Gitr^{hi}Foxp3^-CD4^+$) (12). Elegant studies by Gosh et al. demonstrated that canonical NF-κB members have unique but partially redundant roles in Treg biology, with c-Rel being critical for thymic Treg development and p65 essential for mature Treg identity and maintenance of immune tolerance (11, 13). Indeed, c-Rel loss decreases the number of nTregs and the expression of Treg signature genes (Gitr, CD25, Foxp3) involved in the maintenance of Treg identity (11), whereas mice harboring the p65 ablation in Tregs develop a lethal autoimmune syndrome. However, in the tumor context, the same group demonstrated that melanoma growth is drastically reduced in mice lacking c-Rel, but not p65, in Tregs. Strikingly, the selective degradation of c-Rel, by pentoxifylline, delays tumor growth by altering Treg function and identity (**Figure 1**) and potentiates anti-PD-1/PD-L1 therapy (57). Therefore, c-Rel modulates activated Treg functions.

As for the alternative pathway of NF-κB activation, conditional NIK overexpression in T cells expands both the Treg and the activated conventional T-cell subsets; however, Tregs are largely nonfunctional allowing conventional T cells (Tconvs) to escape suppression, thus inducing a lethal inflammation in mice (61). Recently, it was demonstrated that the conditional deletion of the p100 gene in Tregs causes a massive inflammation due to the impaired suppressive function of *NF-κB2/p100*-deficient Tregs, revealing an increased nuclear translocation of RelB responsible for the accumulation of Tregs *in vivo* (**Table 1**) (62). To date, it remains to be elucidated if the modulation of the alternative pathway of NF-κB leads to similar effects in cancer.

Notch AND Notch/NF-κB SIGNALING CROSSTALK AS A PLAYMAKER OF Tregs IN CANCER

The Notch signaling pathway has been repeatedly associated with different aspects of Treg biology (63), but the potential effect of Notch and its privileged crosstalk with the canonical NF-κB pathway on Treg behavior in cancer is still poorly understood.

Recent evidence has demonstrated that elevated Notch signaling positively modulates peripheral Treg numbers and function in different tumor microenvironments, as demonstrated in the head and neck squamous-cell carcinoma (HNSCC) (64) and even associated to the pathological aggressiveness in human pancreatic (intraductal papillary mucinous) tumors (65).

The study reported in (65) demonstrated that the enhancement of Tregs in the peripheral blood samples of patients affected by a pancreatic tumor fairly correlated to the higher expression of Notch1 and Notch2, while the elevated expression of the Notch/ligand, Jagged1, was related to recurrence (65). Accordingly, in HNSCC, Notch inhibition reduced Tregs, myeloid-derived suppressor cells, tumor-associated macrophages, and the expression of immune checkpoint molecules in the circulation and in the tumor (64). More selectively, Notch1 has been associated to Tregs infiltration in a subset of human breast luminal tumors (66).

Life-and-death decisions in Tregs are influenced by Notch subcellular localization. In fact, when in cytosol, Notch1 protects Tregs from apoptosis induced by cytokine withdrawal (67). The microenvironment can even modulate Notch localization in Treg. In a nutrient-limiting condition, sirtuin 1 stabilizes the Notch intracellular domain (N-ICD) proximal to the plasma membrane and promotes the survival and function of Tregs (68). Therefore, tumor microenvironmental changes may tune noncanonical Notch1 signaling in Treg activities.

Canonical and noncanonical Notch signaling play key roles, often in conjunction with NF-κB, in the Treg-dependent immunological response to the cancer (69, 70). Upon ligand binding, the Notch extracellular subunit is released and trans-endocytosed by the ligand-expressing cell, and this probably activates the genetic programs in stromal cells apt to modulate either thymocyte development (i.e., $oxp3^+$ nTregs) or the tumor microenvironment. In the receptor-bearing cell, three subsequent proteolytic cuts release N-ICD. Subsequently, N-ICD translocates to the nucleus and interacts with the DNA-binding CSL/RBP-Jk factor (71). This drives N-ICD to the target gene promoter, where it recruits mastermind-like (MAML) and additional coactivators, finally driving target gene expression in a wide spectrum of tissues or in a tissue-restricted way. In fact, Notch1-IC can directly bind on RelB and p52 promoters potentially recruiting the MAML1/CSL complex (72).

The crosstalk of Notch with NF-κB in T-cell development (73) as well as in Notch-induced T-cell leukemogenesis has been extensively reported by our group that generated a Notch3 transgenic mice (N3-ICtg) (28, 31, 46, 50). Intriguingly, this murine model is also characterized by enhanced $CD4^+CD25^+CTLA4^+$ Tregs generation (32), suggesting that Notch/NF-κB crosstalk may modulate Treg behavior in cancer.

Notch and NF-κB, both activated in several cancer scenarios, display a multilayered crosstalk. Directly, Notch1 modulates the expression of NF-κB subunits in T-cell leukemia (74) or, indirectly it binds to NF-κB subunits to modulate the transcriptional outcomes in a specific context and cell type (75). Upstream, Notch1 may associate with IKKα, activating NF-κB in cervical cancer cells (76). Unlike Notch1, neither the upregulation of NF-κB subunit expression by Notch3 hyperactivation nor a direct binding between these two partners has been reported so far.

In a different context, the noncanonical Notch1 signaling, independently from RBP-jk, but likely through NF-κB, regulates the activation and proliferation of $CD4^+$ T cells and the differentiation of iTreg lineage (77).

Conversely, NF-κB can trigger Notch ligands, Jagged1 (78) and Jagged2 (79), both increasing Tregs generation (80) and recently found upregulated in hair-follicle-resident Tregs that form an immune-privileged niche for stem

cell biology. Few papers correlated the two ligands to $CD4^+CD25^+Foxp3^+$ expansion in inflammation (81) and in pancreatic tumors (65), thus suggesting Jagged as an important area of investigation in cancer-associated Tregs. Already in clinical trials, therapeutic antibodies inhibiting ligand/receptor interactions would be informative and a valuable drug in cross-signaling between Tregs, stroma, and Notch-expressing cancer cells.

To exploit their effects on tumor progression, Tregs need to migrate into tumor sites. In this context, it has been recently demonstrated that Tregs homing to the bone marrow is CXCR4-mediated (29, 30, 82) (and references therein). In fact, CXCR4 is critical for Notch3-enhanced T-cell leukemia propagation (83) and in the maintenance in the bone marrow of Notch1-induced T-ALL cells (84) that are characterized by the constitutive activation of NF-κB (28, 50, 85). In the neoplastic context, CXCR4 expression has been linked to NF-κB signaling activation (86). Additionally, CXCR4 antagonism (AMD3100) (**Figure 1**) reverts the suppressive activity of activated Tregs ($CTLA4^+/CXCR4^+/PD\text{-}1^+/ICOS^+$) in renal cancer (87) or reprograms Tregs in human mesothelioma (88). Therefore, we can suggest a Notch/CXCR4 connection in potentiating Treg activities, resulting in a protective immunosuppressive environment for T-ALL cells.

FACTORS PLAYING ON Foxp3 PROMOTER

In the primary $CD4^+$ environment, Foxp3 expression marks the commitment to $CD4^+CD25^+Foxp3^+$ Tregs (89) and is required for suppressive activity and transcriptional repression (90). Foxp3 regulates gene expression either by associating with other nuclear factors (91, 92) or antagonizing the NF-AT function by directly competing for DNA binding to consensus forkhead binding sites adjacent to NF-AT (93). Furthermore, Foxp3 over-expression may indirectly impair the translocation of NF-κB into the nucleus by increasing IκB-α stability, thus preventing p65 nuclear entry (94).

On the other side, multiple signaling pathways converge on Foxp3 modulation (93, 95, 96). Three different groups highlighted the central role of the canonical c-Rel transcription factor in Foxp3 gene expression (59, 97, 98). Indeed, c-Rel cooperatively with NF-AT binds to the Foxp3 promoter to form a Foxp3-specific enhanceosome (c-Rel/p65/Smad3/NFATc2/CREB) and recruits chromatin-modifying complexes to the regulatory sequences shortly before the appearance of $Foxp3^+$ thymocytes in the $CD4^+$ T-cell compartment (98).

Dispensable for nTregs development, TGFβ signaling critically regulates peripheral Treg (iTreg) number and functionality and induces Foxp3 expression (99, 100), whereas c-Rel is required only for the optimal homeostatic expansion of iTregs. Indeed, CD28 co-stimulus preferentially triggers RelA to activate Foxp3, at least in human iTregs (101).

Finally, the Foxp3 promoter behaves as an integration site between canonical NF-κB and different signaling pathways (102) that could cooperatively or antagonistically influence Tregs behavior in tumor microenvironments.

Notch3 AND NF-κB KICK-STARTERS IN Foxp3 PROMOTER ACTIVATION

Several papers have highlighted the multiple roles served by Notch and/or NF-κB pathways in regulating Foxp3 gene expression (63, 102).

Our group revealed the importance of Notch signaling activation in driving Tregs generation and functions by demonstrating the higher levels of Notch3 in $CD4^+CD25^+$ with respect to $CD4^+CD25^-$ T cells (32). Moreover, we also showed that Notch3/preTCR cooperation increases both Foxp3-expressing Treg population numbers and Foxp3 expression, as well as enhances *in vivo* activity of nTregs (33).

Other groups demonstrated that Notch1, together with TGFβ, regulates Foxp3 expression and the maintenance of peripheral iTregs (103).

Notch and NF-κB can regulate multiple steps in different T-cell subsets, but neither the mere absence of NF-κB (104) nor the Notch deregulation alone (14) impair numbers and frequencies of the total $CD4^+$ T-cell compartment.

However, we demonstrated that the Notch3 hyperactivation in the N3-ICtg murine model of T-ALL requires PKCθ signals to upregulate Foxp3 core-promoter and to regulate $Foxp3^+$ T-cell generation and suppressive function (14). Therefore, Notch3 and PKCθ converge on the hyperactivation of the canonical NF-κB pathway that rules over the developmental aspects and the activity of Tregs in the tumor microenvironment (11). Interestingly, constitutive NF-κB activation in two different *CYLD*-deficient murine models enhances Foxp3 expression and increases the total amount of $Foxp3^+$ Tregs in the thymus and lymph nodes (44, 105).

Standing the PKCθ/CYLD antagonism, we can hypothesize that the PKCθ hyperactivation observed in N3-ICtg thymocytes may suppress the CYLD function, thus further sustaining NF-κB activation, in agreement with Notch/Hes1-induced CYLD repression and reduced expression of this IKK negative regulator in primary T-cell leukemia (85).

The enhanced generation of Tregs in the thymus is strictly linked to Foxp3 induced by NF-κB family partners sequentially activated. This picture can be further complicated by the arrival of Notch3 signals that can recruit on the Foxp3 promoter a new complex binding the p65/CSL-nested site close to the transcription start site of the Foxp3 promoter (**Table 1**) (14). More importantly, we can suggest that this Notch/p65 cooperation can be active also in the regulation of Foxp3 signaling in cancer cells (106), as recently described in thyroid cancer and T-ALL (15, 16).

Compendiously, the crosstalk between hyperactive Notch3 and canonical NF-κB pathways upregulates Foxp3 expression, thus enhancing the suppressive function of Tregs against protective antitumor immune responses in tumor microenvironments.

CONCLUSION AND PERSPECTIVES

The increased number of Tregs within peripheral blood, lymphoid tissue, and the tumor microenvironment is frequently associated with poor prognosis in several cancers (i.e., ovarian, gastric, breast, and renal cancer). Specifically targeting the Treg compartment while sparing other T-cell populations, which may be useful in tumor immune response, is difficult. Many chemotherapeutic agents (cytostatic drugs) impinge on the increased proliferative rate of Tregs in cancer patients but still with a limited selectivity (107). Further research is required to develop Treg-specific depletion strategies to favor immune response against malignant cells.

In this mini-review, we discussed the intricate network that governs Foxp3 transcription and Treg generation and function, particularly emphasizing the role played either by Notch or by NF-κB signaling, or newly, by their convergence in T-cell leukemia. The multilayered Notch/NF-κB interplay may suggest new issues to be targeted in "cell-intrinsic" mechanisms driving Foxp3-mediated activities of Tregs. In the future, we need to explore the relative role of crosstalk between specific Notch receptors and NF-κB subunits within the subsets of tumor-associated Tregs and importantly their interplay with cancer and microenviromental cells. Therefore, selective γ-secretase-inhibitors or therapeutic antibodies with Notch-specific affinity may suppress the selected Tregs, thus contributing to combined chemotherapy. Innovative cancer immunotherapies target Treg surface receptor and effector T cells, possibly impinging on the abnormal NF-κB-mediated Tregs activity (52). Therapeutic Notch modulation could enhance the efficacy of immunotherapy firstly acting as the immune modulator by reinforcing the T cells' antitumor effector function and secondly behaving as NF-κB partner by impinging on the intrinsic mechanisms of Tregs and cancer-associated cells. Therefore, elucidating the role of both pathways could be a valuable tool to design specific treatment plans aimed to decrease drug dosage and toxicity. Notch and NF-κB profiles may contribute to identify patients and tumors likely to respond to immunotherapy and to provide a new alternative approach to nonresponders. Promising therapies implied that Notch modulation (anti-Jagged1/2) combined with novel immune checkpoint blockade therapies (108).

Still unresolved is the wide partnership of ubiquitous Notch and NF-κB subunits in regulating Foxp3 and Tregs transcriptional programs, and even more the reason why the hyperactivation of either Notch or NF-κB signaling pathway is insufficient to generate fully mature Tregs. The knowledge of specific NF-κB subunits that are upregulated in cancer-associated Tregs will have a clear impact in the development of selective immunomodulatory therapeutics that target NF-κB, by performing a subunit-specific inhibition in Tregs, as suggested by Pentoxyphylline, an FDA-approved drug.

Treg targeting approaches may also include a strategy to interfere with microenvironmental signals, mostly represented by the chemokine receptor/ligand system, as CXCR4-mediated Treg homes to the tumor. It will be insightful also to decipher the cross-signaling in regulating the Foxp3 expression in different Notch-governed cell contexts such as in T-ALL cells (16, 109). The final aim of all these studies would be to define innovative anticancer therapeutic approaches with genetically modified Tregs (110) to treat cancer.

AUTHOR CONTRIBUTIONS

FF researched the literature and wrote the initial draft of the manuscript. PG performed the literature review and helped in editing the table. AFC and IS critically revised the manuscript. DB performed the literature review and helped in editing the figure. MPF wrote the manuscript and edited the figure and table.

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NF-κB1 Regulates Immune Environment and Outcome of Notch-Dependent T-Cell Acute Lymphoblastic Leukemia

Paola Grazioli[1†], *Andrea Orlando*[2,3†], *Nike Giordano*[2], *Claudia Noce*[2], *Giovanna Peruzzi*[3], *Gaia Scafetta*[4], *Isabella Screpanti*[2*] *and Antonio Francesco Campese*[2*]

[1] *Department of Experimental Medicine, Sapienza University, Rome, Italy,* [2] *Department of Molecular Medicine, Sapienza University, Rome, Italy,* [3] *Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy,* [4] *Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University, Rome, Italy*

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***Correspondence:**
Isabella Screpanti
isabella.screpanti@uniroma1.it
Antonio Francesco Campese
antonello.campese@uniroma1.it

†These authors have contributed equally to this work

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive pediatric malignancy that arises from the transformation of immature T-cell progenitors and has no definitive cure. Notch signaling governs many steps of T cell development and its dysregulation represents the most common causative event in the pathogenesis of T-ALL. The activation of canonical NF-κB pathway has been described as a critical downstream mediator of Notch oncogenic functions, through the sustaining of tumor cell survival and growth. The potential role of Notch/NF-κB partnership is also emerging in the generation and function of regulatory T cells (Tregs) in the context of cancer. However, little is known about the effects of combined mutations of Notch and NF-κB in regulating immune-environment and progression of T-ALL. To shed light on the topics above we generated double-mutant mice, harboring conventional *knock-out* mutation of NF-κB1/p50 on the genetic background of a transgenic model of Notch-dependent T-ALL. The immunophenotyping of double-mutant mice demonstrates that NF-κB1 deletion inhibits the progression of T-ALL and strongly modifies immune-environment of the disease. Double-mutant mice display indeed a dramatic reduction of pre-leukemic $CD4^+CD8^+$ (DP) T cells and regulatory T cells (Tregs) and, concurrently, the rising of an aggressive myeloproliferative trait with a massive expansion of $CD11b^+Gr\text{-}1^+$ cells in the periphery, and an accumulation of the granulocyte/monocyte progenitors in the bone-marrow. Interestingly, double-mutant T cells are able to improve the growth of $CD11b^+Gr\text{-}1^+$ cells *in vitro*, and, more importantly, the *in vivo* depletion of T cells in double-mutant mice significantly reduces the expansion of myeloid compartment. Our results strongly suggest that the myeloproliferative trait observed in double-mutant mice may depend on non-cell-autonomous mechanism/s driven by T cells. Moreover, we demonstrate that the reduction of $CD4^+CD8^+$ (DP) T cells and Tregs in double-mutant mice relies on a significant enhancement of their apoptotic rate. In conclusion, double-mutant mice may represent a useful model to deepen the knowledge of the consequences on T-ALL immune-environment of modulating Notch/NF-κB relationships in tumor cells. More importantly, information derived from these studies may help in the refinement of multitarget therapies for the disease.

Keywords: Notch, NF-κB1, T-ALL, Tregs, tumor environment, myeloproliferation

INTRODUCTION

The canonical Notch signaling represents a highly conserved pathway starting with interaction between transmembrane receptors (Notch1–4, in mammals) and ligands (Jagged−1 and −2 and Dll−1,−3, and −4, in mammals), that are expressed on neighboring cells. This event leads to the release of a functional intracellular domain (ICN), that translocates into the nucleus, where it combines with the DNA-binding factor CSL/RBP-Jk and other co-factors and regulates transcription of numerous genes (1). Notch impinges on a plethora of transduction pathways, the combination of which produces pleiotropic effects and context dependent modulations of the signal (2). This variability enables Notch to regulate disparate processes in health and disease, including cancer, where it may act as an oncogene or, conversely, as a tumor suppressor (3). In the hematopoietic system, Notch receptors exert fundamental roles in multiple steps of T cell development and in differentiation of T cell subsets (4, 5), including immunosuppressive $Foxp3^+$regulatory T cells (Tregs) [as reviewed in (6)]. Not surprisingly, deregulated activation of Notch1 or Notch3 causes the development of "T-cell acute lymphoblastic leukemia (T-ALL)", in both mice and humans (7–11). T-ALL represents an aggressive pediatric malignancy that derives from the transformation of hematopoietic progenitors and has no definitive cure (3).

NF-κB is a large family of inducible transcription factors that controls important cellular processes, such as differentiation, survival, and proliferation. NF-κB canonical pathway starts with the activation of the Iκ*B* kinase (IKK) complex by different stimuli that leads to the degradation of Iκ*B*, the NF-κB inhibitor. Then, the NF-κB dimers, mainly represented by the association of RelA/p65 with NF-κB1/p50, are able to translocate into the nucleus and to activate many targets (12). NF-κB controls T cell development and differentiation at multiple stages (13) and represents a crucial downstream effector of Notch signaling in both physiological and pathological conditions. The functional relationship of Notch with NF-κB has been well established in T-ALL onset and progression (8, 14–18), in addition to the interaction of Notch with other important oncogenic mediators, such as pre-TCR/pTα and Ikaros (19–23). Recently, literature is pointing out the function of Notch in shaping tumor microenvironment (24). In particular, Notch is involved in the regulation of tumor immune response (25–28). However, details about the influence of Notch and/or NF-κB on the immune-environment of T-ALL are very limited (29–34). A possible role for Notch/NF-κB partnership has been also suggested in Tregs in the context of cancer (35). Notably, increased percentages of Tregs has been associated with poor prognosis in T-ALL patients (36). However, no studies as yet addressed the effects of Notch/NF-κB interaction on Tregs in T-ALL.

Previously, we demonstrated that *lck*-driven deregulation of Notch3-ICN inside T cell compartment of transgenic (*N3tg*) mice induces an aggressive disease with the features of juvenile T-ALL and characterized by the accumulation of pre-leukemic $CD4^+CD8^+$ (DP) T cells in the periphery (8). The expansion of tumor cells is sustained by the constitutive activation of the p65/p50 NF-κB complex (8). Moreover, T-ALL development in *N3tg* mice is associated to enhanced generation of "natural" Tregs (37). Importantly, deletion of the PKCθ kinase, which mediates activation of canonical NF-κB, reduces incidence of leukemia in *N3tg* mice (14). Finally, we also reported that Notch3, PKCθ, and p65/NF-κB co-operate in modulating Foxp3 transcription in Tregs (38).

However, how the deletion of NF-κB components may affect disease progression and Treg behavior in Notch-dependent T-ALL has not yet been investigated. To this end, we generated double-mutant mice, harboring NF-κB1/p50 deletion on a T-cell targeted Notch3-transgenic background. The characterization of this model suggests that inhibition of NF-κB1 delays the progression of T-ALL and modifies immune-environment of the disease, by inducing a dramatic reduction of DP T cells and Tregs and concurrently the rising of an aggressive T-cell dependent myeloproliferative trait.

MATERIALS AND METHODS

Mice

We intercrossed *N3tg* (8) and $p50^{-/-}$ (39) mice, both on C57BL/6 background, to generate $N3tg/p50^{-/-}$ double-mutant mice, that were bred and housed under specific pathogen-free conditions. The $p50^{-/-}$ mice were obtained from Jackson Laboratories, Bar Harbor, ME, USA. All mice were monitored daily and euthanized upon disease detection (8), as evidenced by enlarged spleen, hunched posture, ruffled fur, reduced mobility, and/or labored breathing. Experimental groups were based on age and genotype of mice. The Foxp3EGFP reporter mice (38), are "knock-in" mice on C57BL/6 background, overexpressing an IRES-EGFP cassette in the 3′ untranslated region of Foxp3 gene. The number of mice used in each experiment was reported in figure legends. Animal studies were approved by the local Animal welfare committee and were carried out in accordance with the recommendations of the Italian national guidelines for experimental animal care and use and of the European Directive 2010/63/EU.

Cell Preparation and Flow Cytometry

Single-cell suspensions were prepared from thymus, spleen, bone-marrow, or peripheral blood in 1x PBS supplemented with 2% FBS and erythrocytes were lysed with ammonium-chloride-potassium lysing buffer, as previously reported (40). Freshly isolated cells were stained with surface markers for 30 min on ice using the following antibodies: CD4 (RM4-5), CD8a (53–6.7), CD11b (M1/70), Gr-1(RB6-8C5), all from BD Biosciences. For Treg detection cells were stained with the following surface antibodies: CD4 (RM4-5), CD8a (53-6.7), CD25 (PC61) (all from BD Biosciences) and then with the intracellular Foxp3 antibody (FJK-16s) (eBioscience), by using Foxp3/Transcription Factor Staining Buffer Set (eBioscience), following manufacturer's instructions. Samples above were run on a FacsCalibur (BD Biosciences) and analyzed with the CellQuest Pro software (BD Biosciences).

To evaluate early myeloid progenitors distribution, bone-marrow cells were stained with the APC mouse lineage Ab cocktail (BD Biosciences), and with the APC-CD4, APC-CD8a,

and APC-IL7Ra antibodies (BD Biosciences), to determine the Lineage negative (Lin^-) subset, and then with the APCH7-cKit (2B8), PerCPCy5.5-Sca-1(D7), FITC-CD34 (RAM34), and PE-FcgRIII/II (2.4G2) antibodies (BD Biosciences). Analysis of apoptosis was performed in gated $CD4^+CD8^+$ DP T subset by staining cells with surface markers V450-CD4 (RM4-5) and PE-CD8a (53–6.7), and then by labeling cells with BUV395-Annexin V and 7-AAD (BD Biosciences), as previously described (41). In order to evaluate apoptosis in Tregs, cells were stained with surface markers PerCPCy5.5-CD4 (RM4-5) and FITC-CD8a (53–6.7), then labeled with Fixable Viability Stain 780 and APC-Annexin V (both from BD Biosciences), just prior to fixation/permeabilization and staining with PE-Foxp3 (FJK-16s), performed as above. Proliferation of $CD4^+CD8^+$ DP T cells was assessed by intracellular staining with the BV510-Ki-67 (B56) antibody (BD Biosciences). For cell cycle analysis 7AAD (Sigma) was used at 25 mg/ml with RNase (Sigma) 40 mg/ml. All intracellular stainings were performed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience). For the intracellular staining with anti-pSTAT5/pY694 (47/STAT5; BD Bioscience) T splenocytes were isolated by using Pan T cell isolation kit (Miltenyi), following manufacturer's instructions. Before the staining, the cells were either left unstimulated or stimulated with increasing doses of rhIL2 (Peprotech) for 15 min at 37°C and then, were fixed and permeabilized with Transcription Factor Phospho Buffer Set (BD Bioscience) according to the manufacturer's instructions. Samples were run on BD LSRFortessa equipped with DIVA software (BD Biosciences) and data were elaborated using FlowJo software (TreeStar).

In vivo T-Cell Depletion

N3tg/p50$^{-/-}$ double-mutant mice at 3 weeks of age were injected intraperitoneally with 250 μg of InVivoPlus anti-mouse CD8α (2.43 clone), plus 250 μg of InVivoPlus anti-mouse CD4 (GK1.5 clone) or with 500 μg of InvivoPlus RatIgG2b (LTF-2 clone) isotype control (all from BioXCell), resuspended in 200 μl/mouse of PBS 1x, twice a week. After 3 weeks of treatment mice were sacrificed and characterized by FACS analysis, as described above. In particular, to evaluate the distribution of T cell subsets we used the following surface antibodies: CD4 (RM4-4, BD Bioscience) and CD8β (H35-17.2, eBioscience).

Cell Sorting

For sorting experiments, bone-marrow samples were obtained from *wt*, *N3tg,* and *N3tg/p50*$^{-/-}$ mice, as above. Mononuclear cells were isolated by ficoll gradient and stained with a "lineage cocktail" containing PE-conjugated monoclonal antibodies against CD11b, Gr-1, Ter119, CD45R, CD3ϵ, CD4, and CD8a. "Lineage negative" (Lin^-) cells were purified with the FACSAria cell sorter (BD Biosciences; purity ≥ 98%).

In other sets of experiments, thymus or spleen cell suspensions were obtained and stained with anti-CD4 and anti-CD8 surface markers, as above. Then $CD4^+CD8^+$ (DP), $CD4^+CD8^-$, or $CD4^-CD8^+$ T cells from *wt*, *N3tg,* and *N3tg/p50*$^{-/-}$ mice, or $CD4^+CD8^-EGFP^+$ Tregs from spleen of Foxp3EGFP reporter mice (see above), were isolated (purity ≥ 98%) using a BD FACSAria III (BD Biosciences), equipped with FACSDiva software (BD Biosciences), as previously described (42).

Colony Forming Unit Assay

For myeloid colony forming unit assay, Lin^- cells (5×10^3 cells/well), purified as above, were plated in triplicate in methylcellulose-based semisolid medium (Methocult M3236, Stem Cell Technology) with FCS and the following cytokines: IL-3 (2 ng/ml), IL-6 (2 ng/ml), SCF (50 ng/ml), G-CSF (50 ng/ml), and GM-CSF (10 ng/ml). After 7–10 days colonies were analyzed and counted and cells were harvested, controlled for the expression of myeloid markers by FACS analysis and then replated (10×10^3/well) to assess survival.

Cell Culture

All the cell culture samples were cultured at 37°C and 5% CO_2 in complete medium, that is RPMI-1640 medium (GIBCO), supplemented with 10% FBS, 10 U/ml penicillin and streptomycin, 2 mM glutamine. In particular, total cells obtained from bone-marrow of *wt* mice (0.25×10^6/well) were co-cultured 1:1 in 96 well plates with total T splenocytes from *wt*, *N3tg,* or *N3tg/p50*$^{-/-}$ mice. T splenocytes were isolated by using Pan T cell isolation kit (Miltenyi), following manufacturer's instructions. After 24 and 48 h of co-culture, cells were counted and stained with CD11b and Gr-1 antibodies for FACS analysis, as above. In some experiments, the co-culture assay was conducted by using $CD4^+CD8^+$ (DP), $CD4^+CD8^-$, or $CD4^-CD8^+$ T splenocytes sorted from double-mutant mice at purity ≥ 98%, as described previously. To check for the role of cell-to-cell contact mechanisms, we performed the co-culture experiments above in 96 well plates in the presence of transwell inserts (pore diameter, 0.4 μm, Corning). Finally, for testing the possible role of Tregs, total cells obtained from bone-marrow of *wt* mice (0.25×10^6/well) were co-cultured 1:1 in 96 well plates with total T splenocytes from *N3tg/p50*$^{-/-}$ mice, without or with different numbers of $CD4^+CD8^-EGFP^+$ Tregs ($1 \times 10^4/3 \times 10^4/6 \times 10^4$), purified from spleen of Foxp3EGFP reporter mice (see above).

In another set of experiments, freshly isolated thymocytes (0.5×10^6/well) were cultured in 96 well plates in the following conditions: untreated, upon TCR activation with coated anti-CD3 antibodies (145-2C11) 3 μg/ml, or in the presence of rhIL2 (Peprotech) 50 U/ml or murine IL-15 (Peprotech) 50 ng/ml. Then, apoptosis was assessed at 48 h by FACS analysis, as described above.

Finally, T splenocytes from *wt, N3tg,* or *N3tg/p50*$^{-/-}$ mice (0.5×10^6/well), purified as above, were cultured in 96 well plates and stimulated or not with coated anti-CD3 antibodies (145-2C11) 3 μg/ml plus rhIL2 (Peprotech) 50 U/ml, in triplicates. Then, at 24 and 48 h, cells were counted and stained to assess Treg numbers by flow cytometry, as described above.

mRNA Analysis

$CD4^+CD8^+$ thymocytes purified as above were processed to extract total RNA with TRIzol reagent (Invitrogen), and reverse transcription was performed with High-Capacity cDNA Reverse Transcription Kit (ThermoFisher), according to the

manufacturer's protocol. TaqMan quantitative real-time PCR (qPCR) was performed on cDNA using the StepOnePlus™ Real-Time PCRSystem (ThermoFisher), following instructions of manufacturer. Taqman Gene Expression Master Mix and Taqman Gene Expression Assays on demand for Bcl2 (Mm00477631_m1), A1 (Mm03646861_mH), and Hprt (Mm01545399_m1) were purchased from ThermoFisher. Relative quantification was carried out using the comparative ΔΔCt method. Hprt expression was used to normalize the expression levels of mRNAs.

Western Blotting

Total protein extracts were prepared from sorted DP T thymocyte samples and Western blotting analysis was conducted, as previously described (43), using anti-p21 antibody (C-19, sc-397, Santa Cruz), and anti-Caspase-3 or anti-cleaved Caspase-3 antibody (#9665 and #9661, respectively, both from Cell signaling). Anti-β-actin antibody (Sigma-Aldrich), was used to normalize protein expression levels. Densitometric analysis was performed using ImageStudio software (LI-COR Biosciences).

Statistics

Results were expressed as means ± SD. We performed unpaired two-tailed Student's test. Differences were considered significant when $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$. Kaplan-Meier survival analysis was performed comparing kinetics of disease development in *N3tg/p50*$^{-/-}$, *N3tg*, *wt*, and *p50*$^{-/-}$ mice. *P*-value was calculated by Log rank (Mantel-Cox) test. Statistical analysis was performed using Prism, GraphPad software.

RESULTS

Deletion of NF-κB1/p50 Delays Progression of Notch3-Dependent T-ALL and Induces Myeloproliferation

In order to analyze the effects of canonical NF-κB pathway modulation on Notch3-dependent T-ALL we generated *N3tg/p50*$^{-/-}$ double-mutant mice, by intercrossing NF-κB1/p50$^{-/-}$ mice (39), with *N3tg* animals (8). Surprisingly, the follow-up of *N3tg/p50*$^{-/-}$, *N3tg*, and relative control mice (*wt* and *p50*$^{-/-}$), revealed that *N3tg/p50*$^{-/-}$ double-mutant mice had a median survival of 65.5 days; in contrast, *N3tg* mice showed a median life span of 109.5 days (**Figure 1A**). Notably, *N3tg/p50*$^{-/-}$ mice presented clinical signs that were different from typical features of T-ALL, routinely observed in *N3tg* animals (8). At the end point, *N3tg/p50*$^{-/-}$ mice appeared indeed smaller in size with respect to *wt* or single mutant controls (not shown). Moreover, disease of *N3tg/p50*$^{-/-}$ mice at 8–9 weeks of age was accompanied by splenomegaly, though less pronounced than that observed in *N3tg* mice (**Figure 1B** and not shown). Finally, the thymus of double-mutant mice was dramatically reduced in size (**Figure 1C** and not shown), starting at 4–5 weeks of age.

To clarify the nature of double-mutant mice pathology we performed immunophenotypic analysis of hematopoietic cell subsets in different organs from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice, by FACS analysis. Regarding the T cell compartment, we focused on immature CD4^{+}CD8^{+} (DP) T-cell population. These cells are normally confined to the thymus, while their presence in the periphery represents a reliable marker to follow T-ALL progression (44–46). CD4^{+}CD8^{+} (DP) T cells were highly decreased in percentages and numbers in both spleen (SPL; **Figures 2A,B**) and bone-marrow (BM; **Figures 2C,D**) of

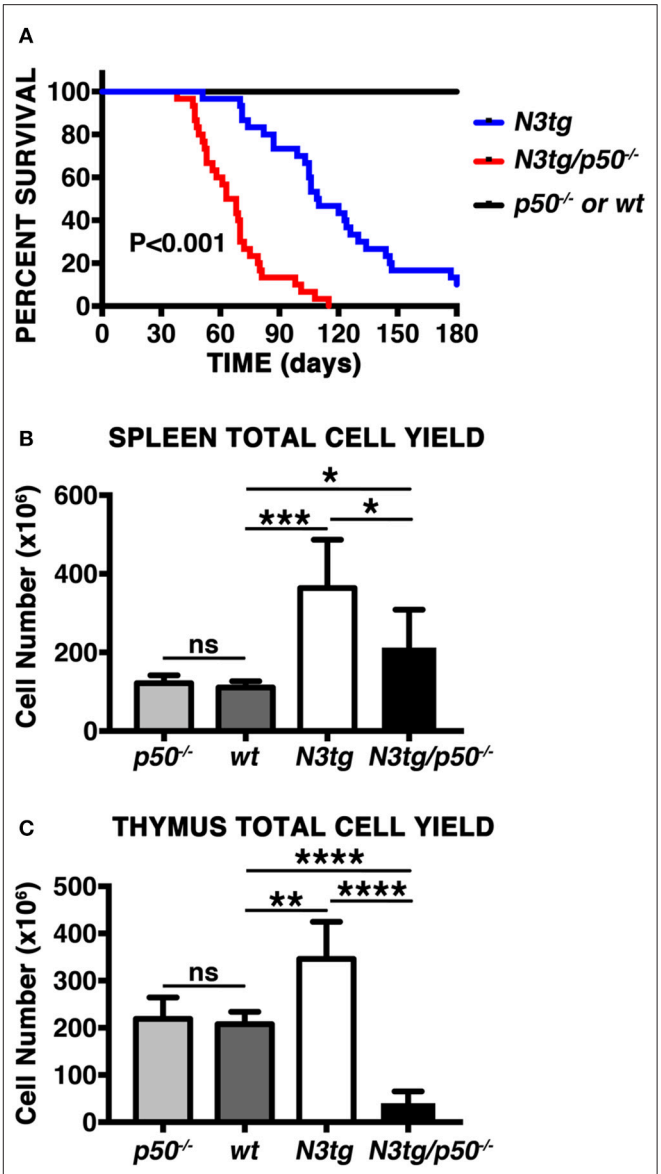

FIGURE 1 | NF-κB1 deletion modifies T-ALL features in *N3tg/p50*$^{-/-}$ vs. *N3tg* mice. **(A)** Kaplan-Meier survival plot showing disease latency in *N3tg/p50*$^{-/-}$ ($n = 30$), *N3tg* ($n = 30$), *p50*$^{-/-}$ ($n = 15$), and *wt* ($n = 15$) mice. *P*-value was calculated by Log rank (Mantel-Cox) test. **(B)** Total cell counts of the spleen from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice at 8–9 weeks of age. **(C)** Total cell counts of the thymus from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice at 4–5 weeks of age. In **(B,C)** the values are presented as mean ± SD of at least five independent experiments ($n \geq 5$ mice per group). ns, not significant; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$ represent significant differences between the indicated groups.

N3tg/p50$^{-/-}$ vs. *N3tg* mice at 8–9 weeks of age, whereas they were virtually absent in organs from *p50*$^{-/-}$ and *wt* controls (not shown). Conversely, the analysis of myeloid cell distributions revealed marked expansion of CD11b^{+}Gr-1^{+}cells in the spleen (**Figures 3A,B**), as well as in the BM (**Figure 3C**, *upper panel*) and peripheral blood (**Figure 3C**, *lower panel*) of *N3tg/p50*$^{-/-}$ mice at 8–9 weeks of age, when compared with *N3tg*, *p50*$^{-/-}$, and *wt* counterparts. Collectively, our results indicate that the deletion of NF-κB1 in *N3tg* mice induces a delay of T-ALL progression on one hand, and promotes myeloproliferation on the other hand, thus affecting the composition of T-ALL immune-environment.

Expansion of "Granulocyte/Monocyte Progenitor"(GMP) Subset in the Bone-Marrow of *N3tg/p50*$^{-/-}$ Mice

The accumulation of CD11b^{+}Gr-1^{+} cells suggested the presence of alterations in the myeloid cell development of

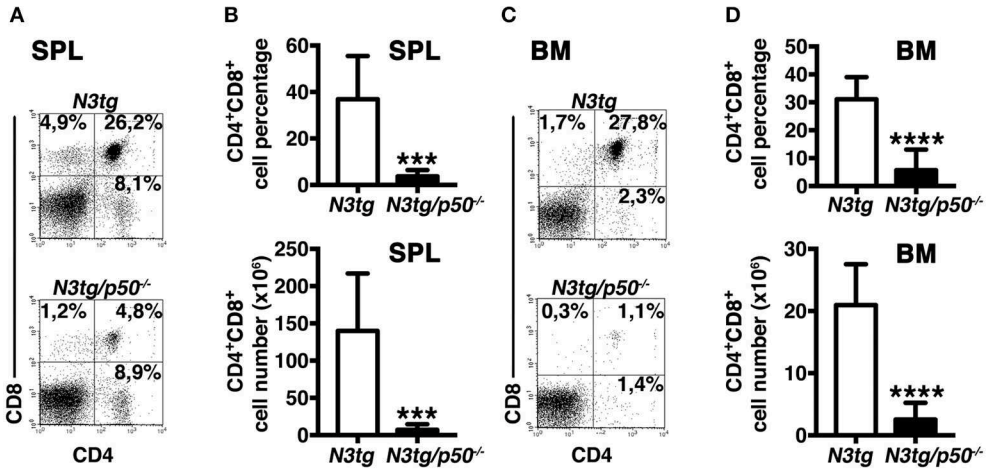

FIGURE 2 | Reduced expansion of CD4^{+}CD8^{+} (DP) T cells in *N3tg/p50*$^{-/-}$ mice. Representative dot plots showing CD4 vs. CD8 distributions in the spleen **(A)** and BM **(C)** of *N3tg/p50*$^{-/-}$ vs. *N3tg* mice at 8–9 weeks of age, as assessed by flow cytometry. Numbers inside each cytogram represent the percentages of different cell subsets. **(B)** Percentages and absolute numbers of DP T cells in the spleen (SPL) of *N3tg/p50*$^{-/-}$ vs. *N3tg* mice, measured as in **(A)**. **(D)** Percentages and absolute numbers of DP T cells in the bone marrow (BM) of *N3tg/p50*$^{-/-}$ vs. *N3tg mice*, measured as in **(C)**. In **(B,D)** the values are presented as mean ± SD of five independent experiments ($n \geq 5$ mice per group). ***$P \leq 0.001$ and ****$P \leq 0.0001$ represent significant differences between the indicated groups.

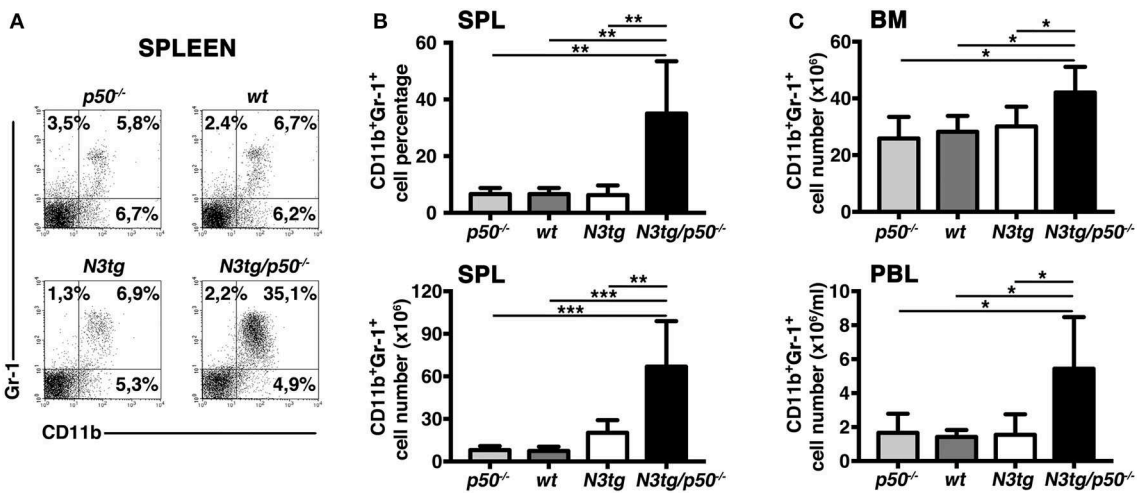

FIGURE 3 | Enlargement of CD11b^{+}Gr-1^{+} subset in *N3tg/p50*$^{-/-}$ mice. **(A)** Representative dot plots showing CD11b vs. Gr-1 distributions in the spleen of *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice at 8–9 weeks of age, as measured by FACS analysis. Numbers inside each cytogram represent the percentages of different cell subsets. **(B)** Percentages (*upper panel*) and absolute numbers (*lower panel*) of CD11b^{+}Gr-1^{+} cells in the spleen from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice, measured as in **(A)**. **(C)** absolute numbers of CD11b^{+}Gr-1^{+} cells in the BM (*upper panel*) and peripheral blood lymphocytes (PBL) (*lower panel*) from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice, measured as in **(A)**. In **(B,C)** data represent mean ± SD of five independent experiments ($n \geq 5$ mice per group). *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ represent significant differences between the indicated groups.

N3tg/p50$^{-/-}$ double-mutant mice. Thus, we analyzed the distribution of myeloid progenitor subsets [as defined by the differential expression of CD34 and FcγRII/III markers inside the Lin$^-$ckit$^+$Sca1$^-$ compartment, see (47) and **Figure 4A** legend], in the bone-marrow of mice of different genotypes at 4–5 weeks of age. We revealed that double-mutant mice displayed a significant increase in the percentages (**Figures 4A,B**, *left panel*), and absolute numbers (**Figure 4B**, *right panel*) of "granulocyte/monocyte progenitor" (GMP) subset with respect to both *N3tg* and *wt* littermates, whereas the progenitor distribution in the BM of *p50*$^{-/-}$ mice was similar to *wt* controls [(48) and not shown]. To confirm these data, we performed a myeloid "colony forming unit" assay with Lineage negative (Lin$^-$) cells, purified from the bone-marrow of *N3tg/p50*$^{-/-}$, *N3tg,* and *wt* mice, and plated on semi-solid methylcellulose medium in the presence of factors stimulating the differentiation of progenitors toward a myeloid fate (see the Materials and Methods section). As depicted in **Figure 4C**, the number of colonies/plate (CFUs) obtained at the P2 and P3 re-plating points, was significantly higher in *N3tg/p50*$^{-/-}$ when compared to *N3tg* and *wt* mice.

Interestingly, immature CD4$^+$CD8$^+$ (DP) T cells are still largely represented in the BM of double-mutant mice at 4–5 weeks of age (**Figure 4D**), thus leaving open the possibility

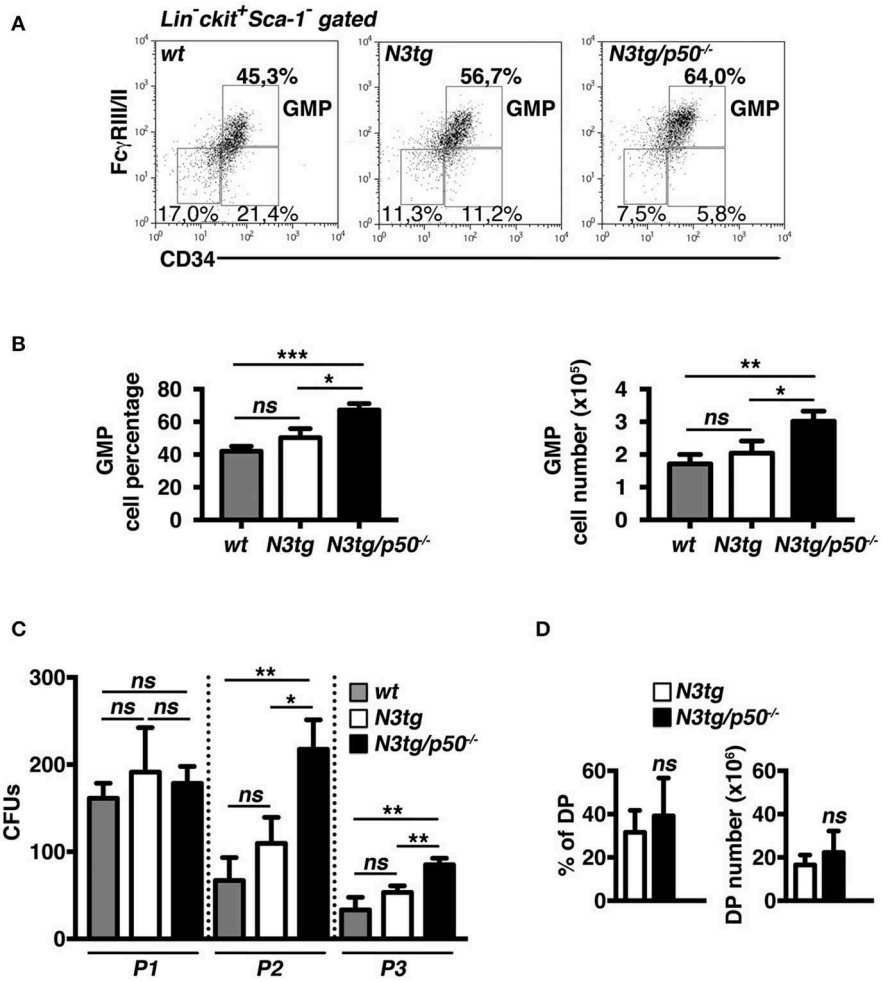

FIGURE 4 | GMP progenitors accumulate in the BM of *N3tg/p50*$^{-/-}$ mice. **(A)** Representative dot plots showing CD34 vs. FcγRIII/II distribution inside Lin$^-$cKit$^+$Sca-1$^-$ gated cells in the BM of *N3tg/p50*$^{-/-}$, *N3tg,* and *wt* mice at 4–5 weeks of age, as assessed by flow cytometry analysis of bone-marrow progenitor subsets (GMP, Granulocyte/Monocyte progenitors: CD34$^+$FcγRII/III$^+$; CMP, Common Myeloid progenitors: CD34$^+$FcγRII/III^{low}; MEP, Megacariocyte/Erithroid progenitors: CD34$^-$FcγRII/III^{low}), Numbers inside each cytogram represent the percentages of different subsets. **(B)** Percentages (*left panel*) and absolute numbers (*right panel*) of GMPs in the BM of *N3tg/p50*$^{-/-}$, *N3tg* and *wt* mice, measured as in **(A)**. **(C)** Colony forming assay of purified BM Lin$^-$ cells plated on semi-solid methylcellulose medium in the presence of factors stimulating the differentiation of progenitors toward a myeloid fate. Graph represents the mean number of colonies per plate (CFUs) obtained at any of the three re-plating points examined (P1, P2, and P3), for *N3tg/p50*$^{-/-}$, *N3tg*, or *wt* cells. In **(B,C)** data represent mean ± SD of three independent experiments ($n = 3$ mice per group). ns, not significant; $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ represent significant differences between the indicated groups. **(D)** Percentages (*left panel*) and absolute numbers (*right panel*) of DP T cells in the BM of *N3tg/p50*$^{-/-}$ vs. *N3tg mice* at 4–5 weeks of age, as measured by flow cytometry analysis of CD4 vs. CD8 distributions. The values are presented as mean ± SD of five independent experiments ($n = 5$ mice per group). ns, not significant differences between the groups.

that these immature *N3tg/p50*$^{-/-}$DP T cells may participate in inducing *in trans* the alteration of myeloid cell development described above.

T Cells Sustain the Expansion of Myeloid Compartment in *N3tg/p50*$^{-/-}$ Mice

To test the hypothesis that T cells may influence the myeloproliferation observed in double-mutant mice, we designed experiments of *in vivo* T-cell depletion, by the combined administration of anti-CD4 and anti-CD8 antibodies to *N3tg/p50*$^{-/-}$ mice, starting at 3 weeks of age. After 3 weeks, *N3tg/p50*$^{-/-}$ treated mice displayed a significant reduction of percentages (**Figures 5A,B**, *upper panels*) and absolute numbers (**Figure 5B**, *lower panels*) of all the examined T cell subsets in the BM and spleen, with respect to the *N3tg/p50*$^{-/-}$ controls, as expected. Notably, T-cell depleted mice were characterized by a remarkable decrease in the proportion (**Figures 5C,D**, *upper panels*) and absolute numbers (**Figure 5D**, *lower panels*) of the CD11b^{+}Gr-1^{+} subset in the same organs as above, when

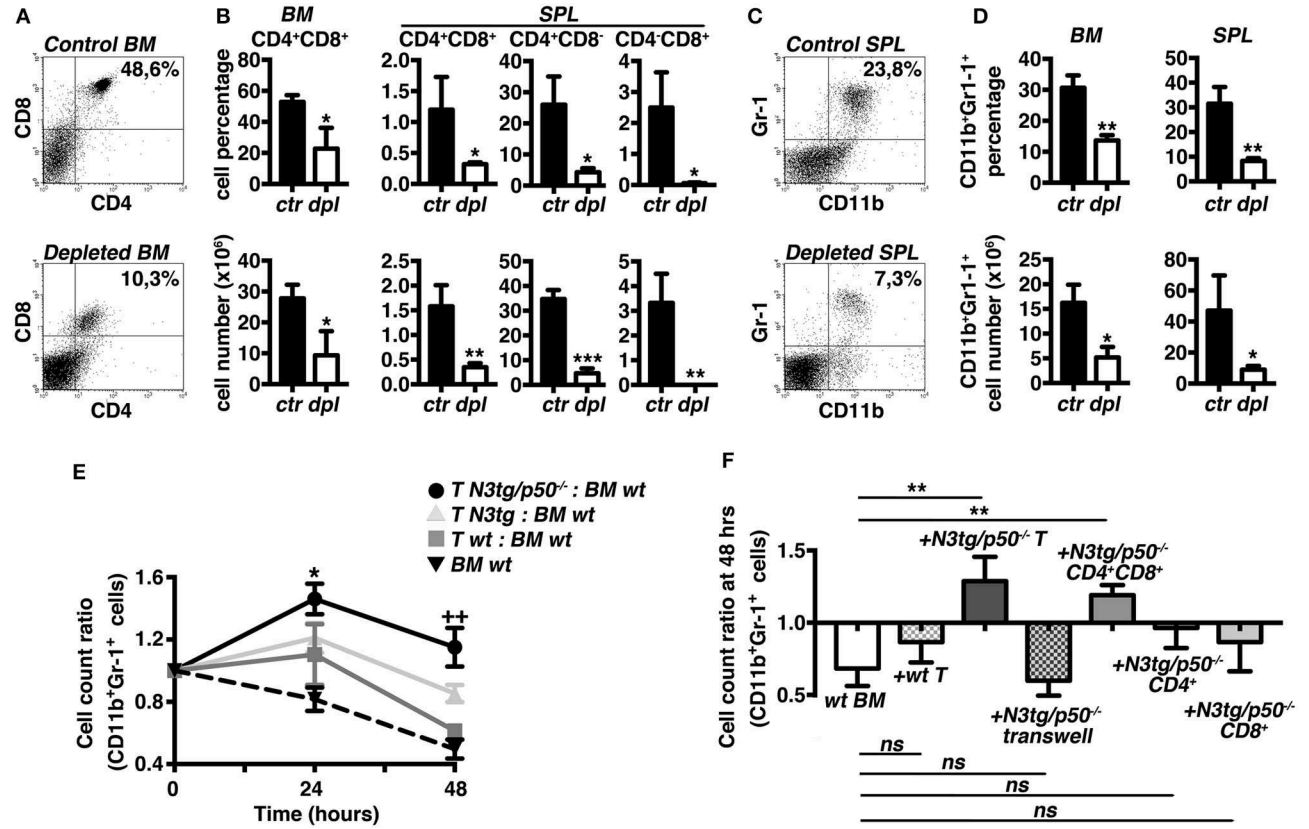

FIGURE 5 | T cell compartment drives the expansion of myeloid cells in *N3tg/p50*$^{-/-}$ mice. Three weeks-old *N3tg/p50*$^{-/-}$ mice were injected i.p. with anti-CD4 plus anti-CD8 depleting antibodies or with relative isotype controls, bi-weekly for 3 weeks. **(A)** Representative dot plots showing CD4 vs. CD8 distributions in the BM of control (*upper panel*) or T-cell depleted (*lower panel*) *N3tg/p50*$^{-/-}$ mice, as measured by FACS analysis. Numbers inside each cytogram represent the percentages of DP T cells. **(B)** Percentages (*upper panels*) and absolute numbers (*lower panels*) of BM CD4^{+}CD8^{+} (DP) T cells and of (DP) T, CD4^{+}CD8,$^{-}$ and CD4^{-}CD8^{+} splenocytes from control vs. T-cell depleted *N3tg/p50*$^{-/-}$ mice (*ctr* and *dpl*, respectively), measured as in **(A)**. **(C)** Representative dot plots showing CD11b vs. Gr-1 distributions in the SPL of control (*upper panel*) or T-cell depleted (*lower panel*) *N3tg/p50*$^{-/-}$ mice, as measured by FACS analysis. Numbers inside each cytogram represent the percentages of CD11b^{+}Gr-1^{+} cells. **(D)** Percentages (*upper panels*) and absolute numbers (*lower panels*) of CD11b^{+}Gr1^{+} cells in the BM and spleen from control vs. T-cell depleted *N3tg/p50*$^{-/-}$ mice (*ctr* and *dpl*, respectively), measured as in **(C)**. In **(B,D)** data represent mean ± SD of three independent experiments ($n = 3$ mice per group). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ represent significant differences between the T-cell depleted samples (*dpl*) and the relative controls (*ctr*) **(E)** The graph represents the ratio of the CD11b^{+}Gr1^{+} cell count at 24 or 48 h to the related CD11b^{+}Gr1^{+} cell count at 0 h in the co-cultures of *wt* BM cells with T splenocytes from *N3tg/p50*$^{-/-}$, *N3tg* or *wt* mice and in the *wt* BM cells cultured alone, as a basal control. Data represent mean ± SD of three independent experiments ($n = 3$ mice per group), each in triplicates. $^{*}P \leq 0.05$ and $^{++}P \leq 0.01$represent significant differences, at 24 and 48 h, respectively, between the values observed in co-cultures of double-mutant T splenocytes with *wt* BM cells (*T N3tg/p50*$^{-/-}$*: BM wt*) and the values observed in the other co-culture samples (i.e., *T N3tg: BM wt*; *T wt: BM wt*; *BM wt* alone, as a basal control) **(F).** The graph represents the ratio of the CD11b^{+}Gr1^{+} cell count at 48 h to the related CD11b^{+}Gr1^{+} cell count at 0 h in *wt* BM cells cultured alone (*wt BM*), in the co-cultures of *wt* BM cells with T splenocytes from *wt* mice (+*wt T*), or with T splenocytes from *N3tg/p50*$^{-/-}$ mice in the absence or presence of transwell inserts (+*N3tg/p50*$^{-/-}$*T* and + *N3tg/p50*$^{-/-}$*T transwell*, respectively), and finally, in the co-cultures of *wt* BM cells with (DP) T, CD4^{+}CD8^{-} or CD4^{-}CD8^{+} T splenocytes purified from double-mutant mice (+*N3tg/p50*$^{-/-}$*CD4*$^{+}$*CD8*$^{+}$, +*N3tg/p50*$^{-/-}$*CD4*$^{+}$, and +*N3tg/p50*$^{-/-}$*CD8*$^{+}$, respectively). Data represent mean ± SD of three independent experiments ($n = 3$ mice per group), each in triplicates. ns, not significant; $^{**}P \leq 0.01$ represents significant differences between the indicated groups. In **(E,F)** all the cells were from mice at 4–5 weeks of age.

compared to the controls. Our results strongly suggest that T cells participate in sustaining the myeloid proliferation of double-mutant mice.

To gain more insight into the T/myeloid cell interaction in *N3tg/p50*$^{-/-}$ mice, we performed *in vitro* co-culture experiments of total *wt* BM cells with T cells purified from the spleen of double-mutant, *N3tg* or *wt* mice at 4–5 weeks of age. Strikingly, we observed a significantly higher CD11b^{+}Gr-1^{+} cell count ratio in the co-cultures with *N3tg/p50*$^{-/-}$ T cells compared to the co-cultures with *N3tg* or *wt* T cells, at any time point considered (**Figure 5E**), indicating that *N3tg/p50*$^{-/-}$ T cells may sustain the growth of CD11b^{+}Gr-1^{+} cells *in vitro*. Then, we repeated the co-culture assay by using transwell inserts or by using different subsets of double-mutant T splenocytes, instead of total T splenocytes (**Figure 5F**). The results indicated that transwell inserts strongly inhibited the effect of *N3tg/p50*$^{-/-}$ T cells on CD11b^{+}Gr-1^{+} cell growth, thus suggesting that it requires cell-to-cell contact. Furthermore, we revealed that only CD4^{+}CD8^{+} (DP) T cells from double-mutant mice are able to drive the expansion of myeloid compartment, whereas CD4^{+}CD8^{-} and CD4^{-}CD8^{+} T subsets do not influence this process, significantly. Overall, our *in vivo* and *in vitro* combined approaches suggest that the myeloproliferative trait of *N3tg/p50*$^{-/-}$ mice may rely on a mechanism exerted *in trans* by *N3tg/p50*$^{-/-}$ DP T cells and that requires cell-to-cell contact.

Profound Alterations of T Cell Compartment in the Thymus and Spleen of *N3tg/p50*$^{-/-}$ Mice

The thymus represents the natural environment in which DP T cell develop under the control of many pathways, including Notch and NF-κB. The reduction of DP T cell subset in the spleen and BM of double-mutant mice, coupled with thymus regression, suggested that such phenomena may rely on alterations of T cell development. This prompted us to analyze T cell compartment in more details. However, to minimize interference by T-ALL or myeloproliferation, we performed our studies in young mice, at 4–5 weeks of age, when *N3tg/p50*$^{-/-}$ mice presented no evident symptoms of illness.

Percentages (**Figures 6A,B**) and absolute numbers (**Figure 6C**) of CD4^{+}CD8^{+} (DP) T cells were remarkably reduced in the thymus of *N3tg/p50*$^{-/-}$ mice, when compared with those of *N3tg*, *p50*$^{-/-}$ and *wt* mice. Concurrently, double-mutant mice presented increased percentages of CD4^{+}CD8^{-}, CD4^{-}CD8^{+}, and CD4^{-}CD8^{-} thymocytes (**Figures 6A,B**). However, in terms of absolute numbers, the reduction observed in *N3tg/p50*$^{-/-}$ vs. *N3tg* mice was not limited to DP T cells, but concerned also other subsets, with particular regard to CD4^{+}CD8^{-} T cells (**Figure 6C**). Interestingly, the expansion of all thymocyte subsets that characterize *N3tg* vs. *wt* mice [(8) and **Figure 6C**], was abrogated in double-mutant mice. Moreover, the effects of NF-κB1 deletion on thymocytes emerged specifically in the Notch3 transgenic background, being absent in *p50*$^{-/-}$ mice, when compared to *wt* controls. In summary, our results suggest that deletion of NF-κB1 may revert the consequences of Notch deregulated activation inside the thymic T-cell compartment.

Similar conclusions derived from analysis of splenic DP T cell compartment, that appeared significantly restricted in *N3tg/p50*$^{-/-}$ vs. *N3tg* mice, in both percentages (**Figures 7A,B**) and absolute numbers (**Figure 7C**). These results suggest that the delay of T-ALL progression observed in *N3tg/p50*$^{-/-}$ vs. *N3tg* mice at 8–9 weeks of age (see **Figure 2**), is already effective at an initial stage of the disease. We noted a slight decrease of CD4^{+}CD8^{-} T cell percentages and absolute numbers (**Figures 7D,E**, respectively, *left panels*), in *N3tg* mice, with respect to those of *p50*$^{-/-}$ and *wt* controls, though these reductions were recovered in double-mutant animals. Furthermore, percentages and absolute numbers of CD4^{-}CD8^{+} T subset (**Figures 7D,E**, respectively, *right panels*), were reduced in *N3tg/p50*$^{-/-}$ and *N3tg* mice in a similar way.

Thymic and Splenic Treg Numbers Are Greatly Reduced in *N3tg/p50*$^{-/-}$ Mice

Notch signaling deregulation in T-cell compartment correlates with the expansion of Tregs during T-ALL development in *N3tg* mice (37), depending in part on the activation of canonical NF-κB pathway (38). Besides, canonical NF-κB subunits, such as c-rel and p65, have been described as crucial in Treg development, function, and homeostasis, including in the context of cancer (49–52). Moreover, c-rel has a crucial role in promoting the formation of an enhanceosome specific for the *FoxP3* promoter and that also includes p65, whereas p50 does not activate FoxP3 promoter (52). Notably, a major role of NF-κB1 in Treg biology has been excluded (49, 52, 53), though p50 modulation seems to exert detrimental effects on Tregs in particular conditions, such as in p50/c-Rel double *knock-out* mice (53, 54) or in mice carrying a mutation of p105 (the p50 precursor) that blocks its degradation (55). In this context, *N3tg/p50*$^{-/-}$ model allowed us to verify if NF-κB1 may cooperate with Notch3 in regulating Treg behavior in T-ALL environment. To this end, we examined CD4^{+}CD8^{-}Foxp3^{+} Treg distributions in the thymus and spleen of *N3tg/p50*$^{-/-}$ mice, by flow cytometry. In the thymus, percentages of Tregs were normal, relatively to those of controls (**Figures 8A,B**), while their absolute numbers declined (**Figure 8C**). This event translated in the spleen of *N3tg/p50*$^{-/-}$ mice, where Tregs diminished significantly in percentages (**Figures 8D, E**), as well as in absolute numbers (**Figure 8F**). Notably, Treg subset was exclusively reduced when NF-κB1 ablation occurred in the *N3tg* background, suggesting that Notch and NF-κB1 may co-operate in regulating Treg numbers specifically in the context of T-ALL.

The Apoptotic Rate of DP T Cells and Tregs From *N3tg* Mice Is Enhanced in the Absence of NF-κB1

In the attempt to explain the reduction of DP T cells and Tregs in *N3tg/p50*$^{-/-}$ mice, we analyzed apoptosis of these populations, by 7AAD/Annexin V assay on freshly isolated cells. CD4^{+}CD8^{+} (DP) T subset from *N3tg* mice presented an accumulation of Annexin V^{+} cells in both the thymus (**Figures 9A,C**) and

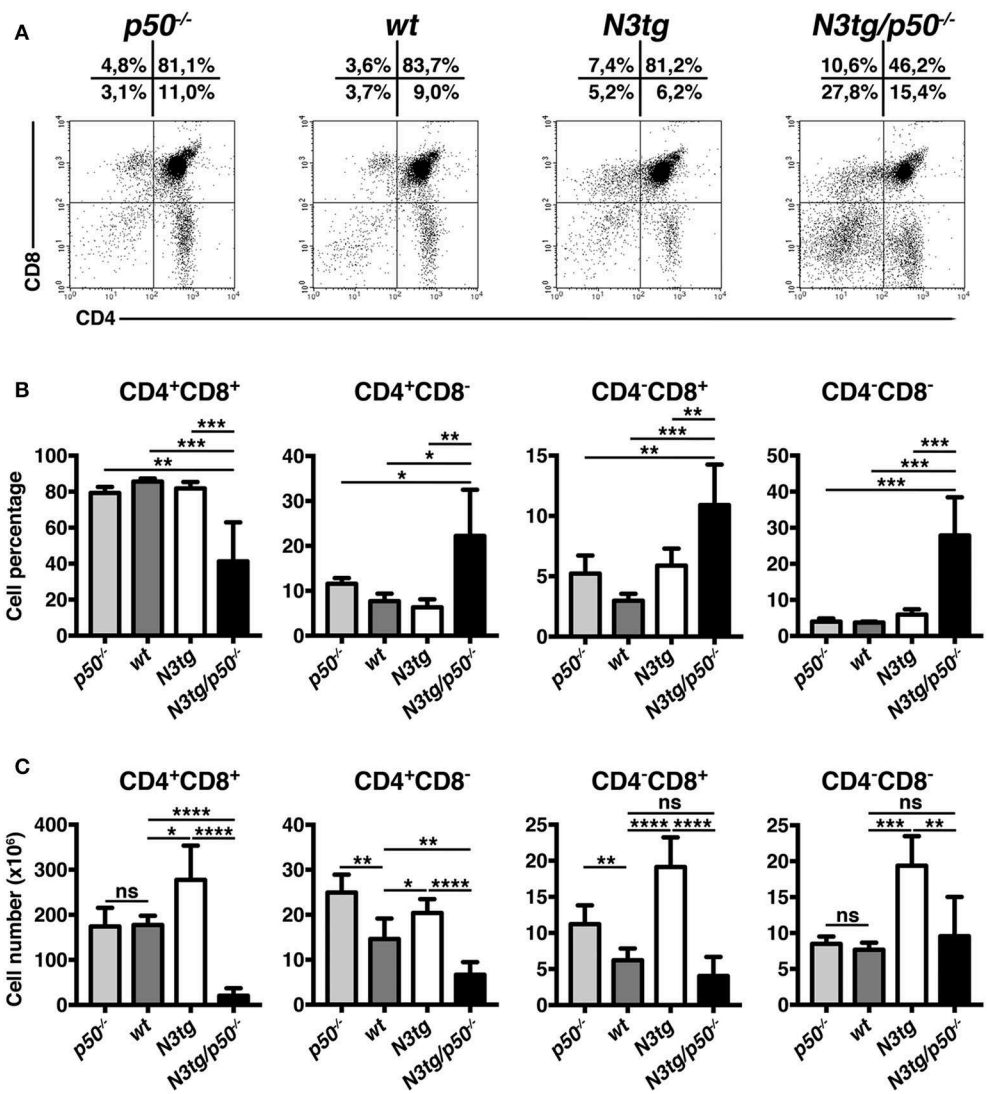

FIGURE 6 | *N3tg/p50*$^{-/-}$ mice display altered distributions of thymocyte subsets. **(A)** Representative dot plots showing CD4 vs. CD8 distributions in the thymus of *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice at 4–5 weeks of age, as measured by FACS analysis of CD4 vs. CD8 distributions. Numbers over each cytogram represent the percentages of different T cell subsets. **(B)** Percentages and **(C)** absolute numbers of thymocyte subsets in *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice, measured as in **(A)**. In **(B,C)** the values are presented as mean ± SD of five independent experiments ($n \geq 5$ mice per group). ns, not significant; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$ represent significant differences between the indicated groups.

spleen (**Figures 9B,C**), in comparison to *wt* DP thymocytes, used as a control. Interestingly, similar results were reported in transgenic mice with an *lck*-driven constitutive activation of Notch1 intracellular domain (56). However, the percentage of apoptotic cells was even more increased in DP T cells from *N3tg/p50*$^{-/-}$ mice (**Figure 9**), suggesting that Notch and NF-κB1 may cooperate in regulating survival of DP T cells. Then, we analyzed if the increase of apoptotic rate described above may depend on a T cell intrinsic mechanism or whether it is a secondary effect of the myeloproliferation on microenvironment. To this end, we performed *in vitro* experiments to measure the percentages of apoptotic cells in DP thymocytes from *N3tg/p50*$^{-/-}$, *N3tg,* and *wt* mice that were cultured in the following conditions: untreated, upon TCR activation via anti-CD3 stimulation or in the presence of survival cytokines such as IL-2 or IL-15. Interestingly, significant differences in the percentages of Annexin V^{+} cells persisted among the three groups of DP thymocytes (*N3tg/p50*$^{-/-}$ > *N3tg* > *wt*) in all the conditions tested (**Figure 9D**), in a similar extent to what observed *in vivo*, thus indicating the cell-autonomous nature of this event.

Inside Treg subset, the proportion of apoptotic cells was increased in CD4^{+}CD8^{-}FoxP3^{+} cells from both the thymus (**Figure 10A**, *left panels* and **Figure 10C**, *upper panel*) and spleen (**Figure 10B**, *left panels* and **Figure 10C**, *lower panel*) of *N3tg/p50*$^{-/-}$ mice. However, percentages of Annexin V^{+} Tregs

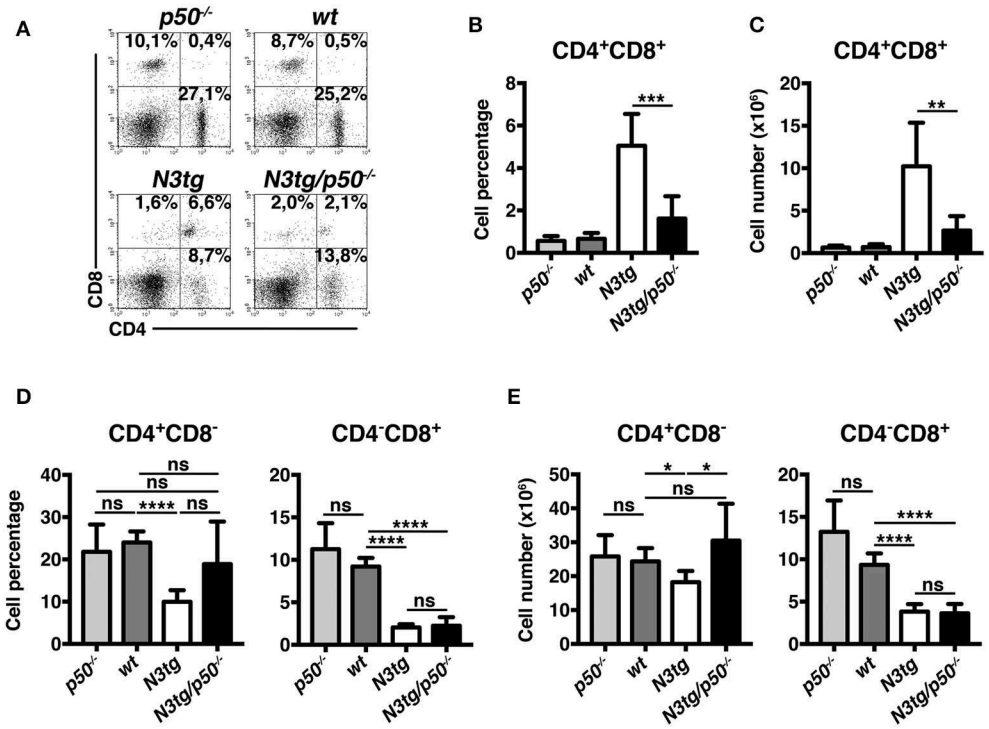

FIGURE 7 | NF-κB1 deletion decreases $CD4^+CD8^+$ (DP) T cell numbers in the spleen of *N3tg/p50*$^{-/-}$ vs. *N3tg mice*. **(A,B)** Percentages and **(C)** absolute numbers of DP T cells in the spleen of *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice at 4–5 weeks of age, as measured by FACS analysis of CD4 vs. CD8 distributions. In **(A)** numbers inside each cytogram represent the percentages of different cell subsets. **(D)** Percentages and **(E)** absolute numbers of $CD4^+CD8^-$ and $CD4^-CD8^+$subsets in the spleen of *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$ and *wt* mice, measured as in **(A)**. In **(B–E)** results are shown as mean ± SD of five independent experiments ($n = 6$ mice per group). ns=not significant; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$ represent significant differences between the indicated groups.

in *N3tg* mice were comparable to those of *wt* controls, and this apparently rules out a pro-apoptotic role of Notch signaling activation in Tregs. Importantly, no differences were noted in the apoptotic rate of $CD4^+CD8^-FoxP3^-$ T cells from mice of different genetic backgrounds (**Figures 10A,B**, *right panels*), used as an internal control. We also excluded defects in the proliferation rates of Tregs that increased in a similar way in both *N3tg* and *N3tg/p50*$^{-/-}$ mice, when compared to those of *wt* counterparts, as measured by Ki67 staining (not shown). In summary, our results suggest that the decrease of DP T and Treg cells in double-mutant mice relies on the enhancement of their apoptotic rate.

Tregs From *N3tg/p50*$^{-/-}$ Mice Present Altered Responsiveness to IL-2

The IL-2/IL2R system is extensively involved in regulating many aspects of Treg biology, including the survival. Interestingly, in Tregs from double-mutant mice we did not observe a defective expression on a per cell basis of the CD25 receptor (**Figure 11A**, *left* panel), or of FoxP3 (as a specific target of the IL-2 signaling in Tregs), (**Figure 11A**, *right* panel), as evaluated by calculating their Mean Fluorescence Intensity (MFI). Nevertheless, we revealed an altered response to IL-2 of *N3tg/p50*$^{-/-}$ Tregs *in vitro*. Indeed, the Treg cell count ratio increased significantly upon IL-2 stimulation in *wt* samples and even more in *N3tg* samples, with respect to the untreated controls, whereas no differences were noted in IL-2-treated with respect to untreated double-mutant Tregs (**Figure 11B**). These data suggest that the reduced number of Tregs in double-mutant mice could be related to the impaired response to IL-2. To corroborate our findings, we examined Tregs for the expression of the activated form of STAT5 (pSTAT5), a critical downstream target of IL-2 signaling in Treg development and function. We stimulated T splenocytes with increasing doses of rhIL-2, and then revealed the expression of pSTAT5, by FACS analysis. Data obtained evidenced that *N3tg/p50*$^{-/-}$ mice present a slight but significant decrease in the proportion of $pSTAT5^+$ cells inside the $CD4^+CD8^-Foxp3^+$ Treg compartment, upon activation with high doses of IL-2 (50 or 200 U/ml; **Figure 11C**), when compared to *wt* controls. These results suggest the presence of an altered IL-2/STAT5 signaling in double-mutant Tregs.

Recently, it was reported that the lack of Tregs in FoxP3-deficient "scurfy" mice induces indirectly a deregulated myelopoiesis, resembling the myeloproliferative trait of our *N3tg/p50*$^{-/-}$ mice (57). Interestingly, *N3tg/p50*$^{-/-}$ young mice (at 4–5 weeks of age), present a dramatic reduction of Treg percentages (**Figures 11D**, **E**, *upper panel*), and absolute numbers (**Figure 11E**, *lower panel*), in the BM, comparable to what

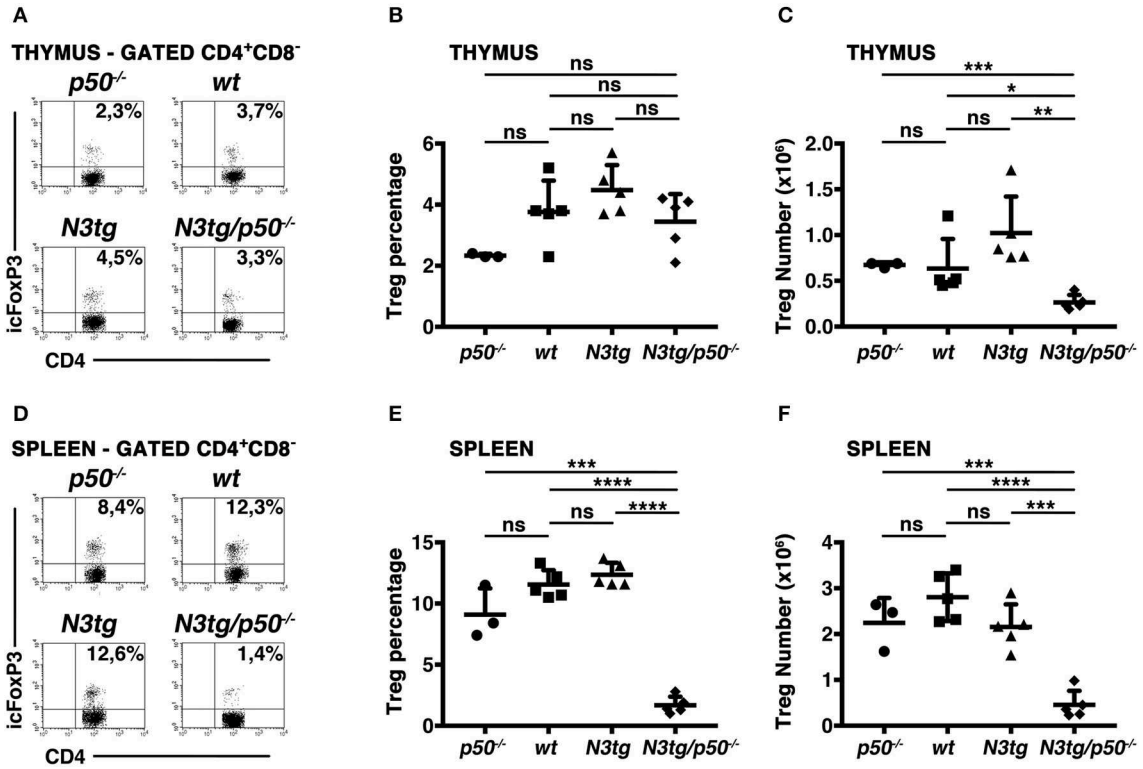

FIGURE 8 | Tregs are reduced in the thymus and spleen of *N3tg/p50*$^{-/-}$ double-mutant mice. **(A)** and **(D)** Representative FACS analysis of CD4^{+}CD8^{-}Foxp3^{+} Tregs in the thymus and spleen, respectively, of *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$ and *wt* mice at 4–5 weeks of age. Numbers inside each cytogram represent the percentages of Foxp3^{+} cells inside gated CD4^{+}CD8^{-} cells. **(B)** and **(C)** Mean percentage values and absolute numbers, respectively, of CD4^{+}CD8^{-}Foxp3^{+} Tregs in the thymus from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice, assessed as in **(A)**. **(E,F)** Percentages and absolute numbers, respectively, of CD4^{+}CD8^{-}Foxp3^{+} Tregs in the spleen from *N3tg/p50*$^{-/-}$, *N3tg*, *p50*$^{-/-}$, and *wt* mice, assessed as in **(D)**. In **(B,C,E,F)** the values are presented as mean ± SD of three independent experiments with *N3tg/p50*$^{-/-}$ ($n = 5$), *N3tg* ($n = 5$), *p50*$^{-/-}$ ($n = 3$), and *wt* ($n = 5$) mice. ns, not significant; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$ represent significant differences between the indicated groups.

observed in their thymus and spleen (see **Figure 8**). Thus, we investigated the possible role of the Treg lack inside T cell compartment of *N3tg/p50*$^{-/-}$ mice in promoting the CD11b^{+}Gr-1^{+} cell growth observed in our co-culture system. To this aim, we performed *in vitro* co-culture experiments of total *wt* BM cells with T cells purified from the spleen of double-mutant mice at 4–5 weeks of age, in combination with different numbers of FoxP3^{+} Tregs, purified from the spleen of the Foxp3EGFP "knock-in" reporter mice (38). Interestingly, the presence of Tregs in the co-culture does not influence significantly the positive effect of *N3tg/p50*$^{-/-}$ T cells on myeloid cell growth (**Figure 11F**). Thus, it seems unlikely that the lack of Tregs could represent a crucial event in promoting myeloproliferation of double-mutant mice.

Apoptosis Induction in DP T Cells From *N3tg/p50*$^{-/-}$ MiceCorrelates With Suppression of the p21$^{Waf1/Cip1}$ Protein Expression

In the attempt to find mechanistic basis for the enhancement of apoptosis in DP T cells from double-mutant mice, we first considered the Bcl-2 protein family, that exerts essential function in cell death regulation. In particular, Bcl-2 and A1 members have antiapoptotic roles and represent important targets of NF-κB in lymphocyte development and hematological malignancies (16, 58). Moreover, the overexpression of Bcl-2 and A1 has been associated to survival of T-lymphoma cells in *N3tg* mice (8). On this premise, we purified DP thymocytes from *N3tg*, *N3tg/p50*$^{-/-}$, and *wt* mice at 4–5 weeks of age and evaluated the expression of Bcl-2 and A1 mRNAs. Surprisingly, the expression of both Bcl-2 and A1 was increased in samples from *N3tg* DP thymocytes, when compared to *wt* DP thymocytes, and the deletion of NF-κB1 magnified this effect in double-mutant counterparts (**Figure 12A**). Then, we explored the possibility that NF-κB1 may affect the expression of "cyclin-dependent kinase inhibitor" p21$^{Waf1/Cip1}$. This protein represents a main factor in promoting cell cycle arrest, upon various stimuli. However, p21$^{Waf1/Cip1}$ also acts as an inhibitor of apoptosis in many cell types and through different mechanisms [as reviewed in (59)]. Intriguingly, we detected a downregulation of p21$^{Waf1/Cip1}$ protein expression levels in samples of DP thymocytes from *N3tg/p50*$^{-/-}$ mice (**Figure 12B**), when compared with both *N3tg* and *wt* samples. We also studied the possible effects of

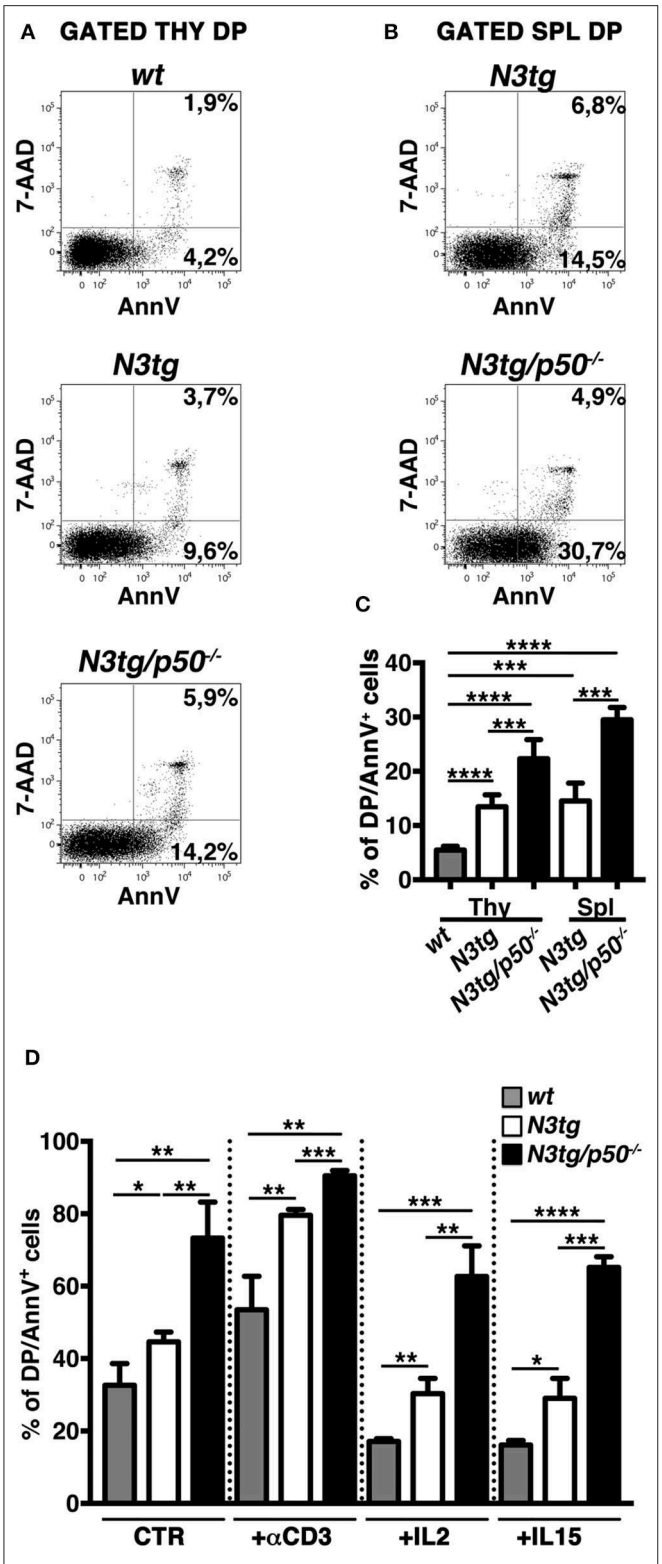

FIGURE 9 | Increased apoptotic rate of $CD4^+CD8^+$ (DP) T cells from *N3tg/p50*$^{-/-}$ mice. Representative dot plots of 7AAD/Annexin V distributions inside gated $CD4^+CD8^+$ (DP) T cells from thymus **(A)** and spleen **(B)** of *N3tg/p50*$^{-/-}$ vs. *N3tg* mice at 4–5 weeks of age. DP thymocytes from *wt* littermates were used as a control. Percentages of both 7 AAD^-Annexin V^+ early apoptotic cells and $7AAD^+$Annexin V^+ late apoptotic cells are indicated inside each cytogram. **(C)** The graph illustrates the percentages of Annexin V^+ cells inside DP T compartment from thymus (Thy) and spleen (Spl) of *N3tg/p50*$^{-/-}$ and *N3tg* mice at 4–5 weeks of age, as well as from thymus of *wt* controls. The values are presented as mean ± SD of four independent experiments ($n \geq 4$ mice per group). $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ represent significant differences between the indicated groups. **(D)** The graph reports the percentages of DP/Annexin V^+ cells, assessed by flow cytometry, in thymocytes from *N3tg/p50*$^{-/-}$, *N3tg*, and *wt* mice cultured for 48 h in the following conditions: untreated (CTR), upon TCR activation with anti-CD3 stimulation (+αCD3), or in the presence of survival cytokines such as IL-2 (+IL2) or IL-15 (+IL15). The values are presented as mean ± SD of three independent experiments ($n = 3$ mice per group), in triplicates. ns, not significant; $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$ represent significant differences between the indicated groups.

(Continued)

$p21^{Waf1/Cip1}$ downregulation on both the cell cycle and the proliferation of double-mutant DP thymocytes with respect to *N3tg* and *wt* mice counterparts. We did not observe any significant alteration in the distribution of DP T thymocytes of different genotypes in the different phases of the cell cycle, as assessed by 7-AAD staining (not shown), as well as in the percentages of proliferating DP thymocytes, as assessed by the staining with the Ki-67 proliferation marker (**Figure 10C**, *upper panel*). However, the expression of Ki-67 on a per cell basis was significantly increased in double-mutant DP thymocytes (**Figure 12C**, *lower panel*). Finally, we revealed that the decrease of the $p21^{Waf1/Cip1}$ protein in double-mutant DP thymocytes does not translate into differences in the protein expression levels of activated cleaved caspase3 or pro-caspase3 (**Figure 12D**). Overall, our results suggest that NF-κB1 deletion may promote apoptosis in *N3tg* DP T cells through mechanism/s that are dependent on $p21^{Waf1/Cip1}$ expression.

DISCUSSION

The central role of Notch and NF-κB in the development and progression of T-cell acute lymphoblastic leukemia is well-established (8, 14–18). Nevertheless, little is currently known about combined effects of deregulating these pathways on T-ALL environment. Here, we report that the deletion of NF-κB1/p50 subunit in a murine model of Notch-dependent T-ALL shapes the immunological environment of the disease and influences its outcome. Double-mutant mice show indeed an inhibition of T-cell leukemia progression, evidenced by a strong reduction of pre-leukemic $CD4^+CD8^+$ (DP) T cells in the periphery. At the same time, they develop a dramatic expansion of immature $CD11b^+Gr\text{-}1^+$ myeloid cells. Surprisingly, the deletion of NF-κB1/p50 induces the overall effect of reducing survival of *N3tg/p50*$^{-/-}$ mice, with respect to that of *N3tg* animals. It is likely that this effect depends on myeloproliferation. Interestingly, myeloproliferation has already been associated with dysregulation of Notch signaling in hematopoietic system of different murine models, including those of Notch-dependent leukemia (29, 32). Though, Notch has

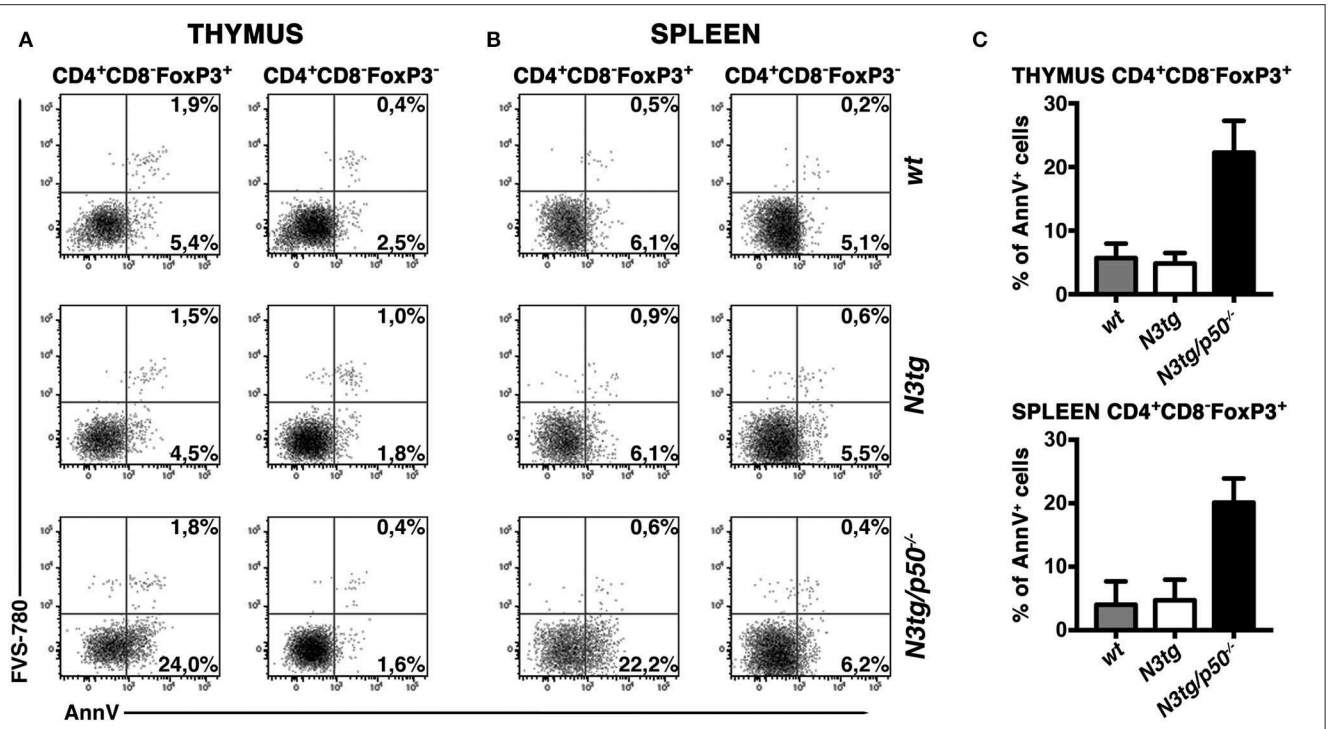

FIGURE 10 | Tregs from *N3tg/p50*$^{-/-}$ double-mutant mice show enhanced apoptosis. Representative dot plots of FVS-780/Annexin V distributions inside gated CD4^{+}CD8^{-}Foxp3^{+} Tregs or gated CD4^{+}CD8^{-}Foxp3^{-} cells, from thymus **(A)** and spleen **(B)** of *N3tg/p50*$^{-/-}$, *N3tg,* and *wt* mice at 4–5 weeks of age. Percentages of both FVS-780^{-}Annexin V^{+} early apoptotic cells and FVS-780^{+}Annexin V^{+} late apoptotic cells are indicated. **(C)** The graph illustrates the percentages of Annexin V^{+} cells inside CD4^{+}CD8^{-}Foxp3^{+} Tregs from thymus (*upper panel*) and spleen (*lower panel*) of *N3tg/p50*$^{-/-}$, *N3tg*, and *wt* mice at 4–5 weeks of age. The values are presented as mean ± SD of two independent experiments ($n = 2$ mice per group).

been described as a tumor suppressor in myeloid malignancies [reviewed in (60)]. Together, these observations would support NF-κB as a downstream mediator of Notch signaling in this context.

In order to better characterize the myeloproliferative trait of double-mutant mice, we demonstrate that these mice display an alteration in the myeloid cell development with a significant expansion of the "granulocyte/monocyte progenitor" (GMP) subset in the bone-marrow. Notably, T-cell depletion *in vivo* significantly reduces myeloproliferation in double-mutant mice. Furthermore, T cells from *N3tg/p50*$^{-/-}$ mice are able to improve the growth of CD11b^{+}Gr-1^{+} myeloid cells *in vitro*, through a mechanism exerted by *N3tg/p50*$^{-/-}$ DP T cells and that requires cell-to-cell contact. Finally, no ectopic expression of the T cell targeted Notch3-ICN transgene, was observed in the myeloid compartment of *N3tg/p50*$^{-/-}$ mice (PG and AC, unpublished results). Altogether, these results suggest that the myeloproliferation observed in our model relies on non-cell-autonomous processes driven by *N3tg/p50*$^{-/-}$ T cells. However, a more precise definition of the mechanisms involved in this effect deserves additional studies in the future.

NF-κB1 deletion seems to affect immune-environment of *N3tg* mice through a second way, namely the impairment of T cell development. *N3tg/p50*$^{-/-}$ mice present a significant reduction in size of the thymus that reflects the marked decrease in numbers of all thymocyte subsets and mainly of DP T cells. Overall, the enforced expansion of thymic populations that characterize *N3tg* mice vs. *wt* controls [(8) and **Figure 6C**] is abrogated in *N3tg/p50*$^{-/-}$ mice, thus suggesting a specific involvement of NF-κB1 in mediating this effect of Notch3 dysregulation. The DP T cell compartment is significantly reduced also in the periphery of double-mutant mice, confirming the role of NF-κB1 in sustaining survival of DP T pre-leukemic cells. Instead, NF-κB1 deletion influences marginally the distribution of CD4^{+}CD8^{-} and CD4^{-}CD8^{+} T cells in the spleen of *N3tg/p50*$^{-/-}$ mice, indicating the presence of compensatory mechanism/s in this process.

There is a third way through which the absence of NF-κB1 may modify T-ALL immune environment, that is represented by the notable reduction of Treg subsets in *N3tg/p50*$^{-/-}$ mice. Importantly, *p50*$^{-/-}$ mice present no major alterations in Treg numbers and function in both the thymus and periphery (49, 52, 53). However, p50 may affect Treg subset under certain conditions (53–55) and its function in Tregs in the context of cancer has not been extensively addressed, so far. To this regard, we revealed the presence of a striking effect of NF-κB1 deletion on Treg survival, that emerges exclusively in the *N3tg* leukemic background. Interestingly, DP thymocytes have been described as the subset in which development of Treg precursors starts (61). Thus, it is possible that the reduction

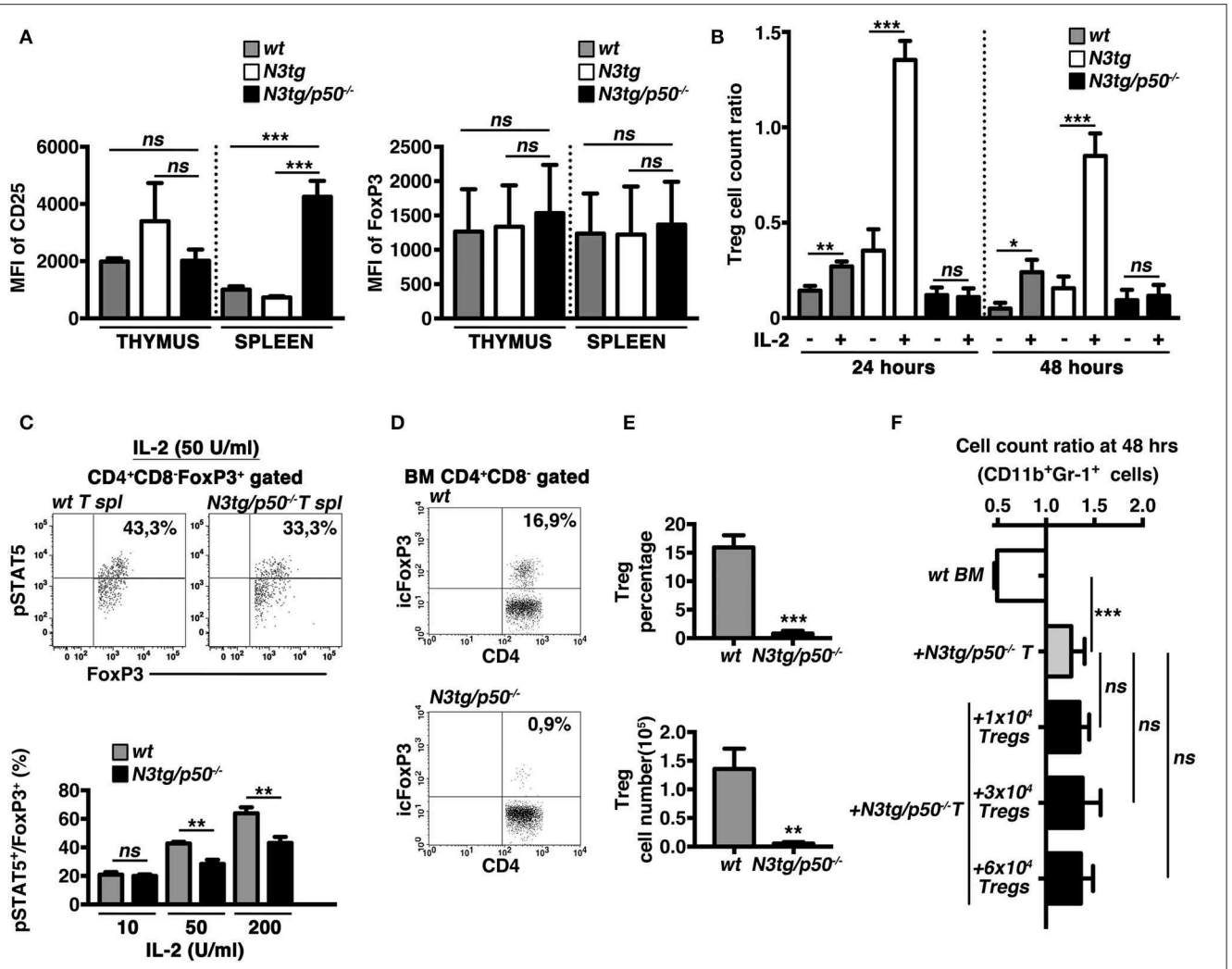

FIGURE 11 | The IL-2/STAT5 signaling is affected in Tregs from *N3tg/p50*$^{-/-}$ mice. **(A)** The histograms represent the MFI of CD25 (*left panel*) and Foxp3 (*right panel*) in gated CD4$^+$CD8$^-$FoxP3$^+$ subsets from thymus and spleen of *wt*, *N3tg*, and *N3tg/p50*$^{-/-}$ mice at 4–5 weeks of age. **(B)** The graph represents the ratio of the Treg cell count at 24 or 48 h and the related Treg cell count at 0 h in culture samples of T splenocytes from *N3tg/p50*$^{-/-}$, *N3tg*, or *wt* mice at 4–5 weeks of age, in the absence (–) or presence (+) of anti-CD3/IL-2 stimulation. In **(A,B)** the values are presented as mean ± SD of three independent experiments ($n = 3$ mice per group). ns, not significant; $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ represent significant differences between the indicated groups. **(C)** In the *upper panels*, representative FACS analysis of pSTAT5 expression inside CD4$^+$CD8$^-$FoxP3$^+$ subset of total T splenocytes (*T spl*) from *N3tg/p50*$^{-/-}$ and *wt* mice at 4–5 weeks of age, upon activation with 50 U/ml of rhIL-2. Numbers inside each cytogram represent the percentages of pSTAT5$^+$FoxP3$^+$cells inside gated CD4$^+$CD8$^-$FoxP3$^+$ subset. In the *lower panel* the graph represents the percentages of pSTAT5$^+$FoxP3$^+$cells inside CD4$^+$CD8$^-$FoxP3$^+$ compartment of total T splenocytes from *wt* and *N3tg/p50*$^{-/-}$ mice at 4–5 weeks of age, following the activation with rhIL-2, at the indicated doses. Samples stained without rhIL-2 stimulation served as negative control (not shown). The values are presented as mean ± SD of three independent experiments ($n = 3$ mice per group), in triplicates. ns, not significant; $^{**}P \leq 0.01$ represents significant differences between the indicated groups. **(D)** Representative dot plots of icFoxP3/CD4 distributions inside gated CD4$^+$CD8$^-$ T cells from BM of *N3tg/p50*$^{-/-}$ vs. *wt* mice at 4–5 weeks of age, as assessed by FACS analysis. Percentages of FoxP3$^+$CD4$^+$ cells are indicated inside each cytogram. **(E)** Mean percentage values (*upper panel*) and absolute numbers (*lower panel*), of Foxp3$^+$ Tregs inside the CD4$^+$CD8$^-$subset in the BM from *N3tg/p50*$^{-/-}$ and *wt* mice, assessed as in **(D)**. The values are presented as mean ± SD of three independent experiments ($n = 3$ mice per group). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ represent significant differences between the *N3tg/p50*$^{-/-}$ samples and the relative *wt* controls. **(F)** The graph represents the ratio of the CD11b$^+$Gr1$^+$ cell count at 48 h to the related CD11b$^+$Gr1$^+$ cell count at 0 h in *wt* BM cells cultured alone (*wt BM*), as a basal control, in the co-cultures of *wt* BM cells with T splenocytes from *N3tg/p50*$^{-/-}$ mice (+*N3tg/p50*$^{-/-}$ *T*), as well as in the co-cultures of *wt* BM cells with T splenocytes from *N3tg/p50*$^{-/-}$ mice in the presence of different numbers of CD4$^+$CD8$^-$EGFP$^+$ Tregs purified from the spleen of the Foxp3EGFP "*knock-in*" reporter mice (+*N3tg/p50*$^{-/-}$*T* + 1 × 10^4/3 × 10^4/6 × 10^4 Tregs). Data represent the mean ± SD of three independent experiments ($n = 3$ mice per group), each in triplicates. ns, not significant; $^{***}P \leq 0.001$ represents significant differences between the indicated groups.

of Tregs that we noted in double-mutant mice depends on the massive reduction of DP thymocytes. The impairment of Treg subsets could also depend on the altered response of *N3tg/p50*$^{-/-}$ Tregs to the IL-2, depending, at least in part, on a reduced activation of STAT5, though the exact mechanisms underlying this effect remain to be elucidated. IL-2 was reported as an essential growth factor for Tregs that is critically required for their homeostasis and metabolic

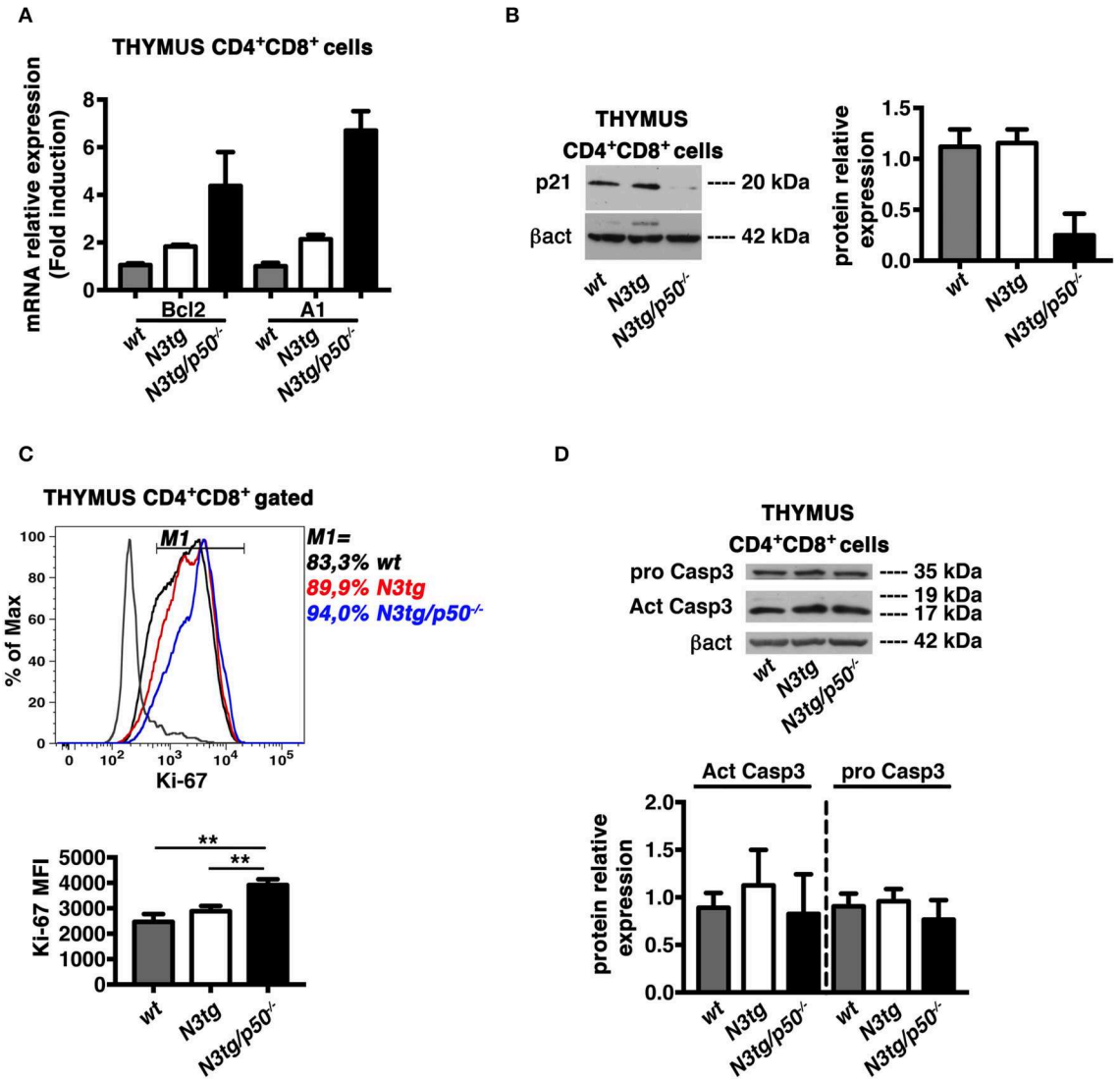

FIGURE 12 | NF-κB1 deletion increases $CD4^+CD8^+$ (DP) T cell apoptosis in *N3tg/p50*$^{-/-}$ vs. *N3tg* mice, through a $p21^{Waf1/Cip1}$-dependent mechanism. **(A)** Relative mRNA expression levels of Bcl-2 and A1 evaluated by real-time RT-PCR in DP T cells isolated from thymus of *N3tg/p50*$^{-/-}$, *N3tg*, or *wt* mice at 4–5 weeks of age. The expression levels of Bcl-2 or A1 in *wt* DP thymocytes were set as 1. Mean values ± SD are shown and they were obtained from two independent experiments ($n = 2$ mice per group), each in triplicate. **(B)** In the *left panel*, representative Western blot analysis of $p21^{Waf1/Cip1}$ protein expression levels in whole-cell extracts of DP T cells isolated from thymus of *N3tg/p50*$^{-/-}$, *N3tg*, or *wt* mice at 4–5 weeks of age. Anti β-actin (β-act) antibody was used as loading control. In the *right panel*, the densitometric analysis of protein expression levels are shown. The values are presented as mean ± SD of two independent experiments ($n = 2$ mice per group). The $p21^{Waf1/Cip1}$ protein expression levels in *wt* DP thymocytes were set as 1. **(C)** In the *upper panel*, histogram of Ki-67 expression inside gated $CD4^+CD8^+$ (DP) T cells from thymus of *N3tg/p50*$^{-/-}$ (blue line), *N3tg* (red line), and *wt* (black line) mice at 4–5 weeks of age, assessed by flow cytometry. The staining negative control is also shown (gray line). M1 gate defines the percentages of Ki-67$^+$ cells. Data are representative of three independent experiments. In the *lower panel*, the graph illustrates the MFI of the Ki-67 marker inside $CD4^+CD8^+$ (DP) thymocytes in the same samples as in the *upper panel*. The values are presented as mean ± SD of three independent experiments ($n = 3$ mice per group). $^{**}P \leq 0.01$ represent significant differences between the indicated groups. **(D)** In the *upper panel*, representative Western blot analysis of pro-caspase-3 and active caspase-3 protein expression levels in whole-cell extracts of DP T cells isolated from thymus of *N3tg/p50*$^{-/-}$, *N3tg*, or *wt* mice at 4–5 weeks of age. Anti β-actin (βact) antibody was used as loading control. In the *lower panel*, the densitometric analysis of protein expression levels of active caspase-3 and pro-caspase-3 protein are shown. The values are presented as mean ± SD of two independent experiments ($n = 2$ mice per group). In the graphs the protein expression levels in *wt* DP thymocytes were set as 1.

fitness (62). Moreover, NF-κB regulates multiple aspects in the biology of Tregs, including their survival and also their function in tumor environment [as reviewed in (35)]. Thus, it is likely that the deletion of NF-κB1 in the *N3tg* background could influence Treg survival, by inducing alteration of the IL-2 response.

From a mechanistic perspective, our results indicate that the decrease of DP T cells and Tregs in *N3tg/p50*$^{-/-}$ mice arises from

an enhancement of apoptotic rate of these subsets. Importantly, the enhancement of apoptosis in T cells from *N3tg/p50*$^{-/-}$ seems to represent an intrinsic event, independent from changes in the microenvironment induced by the expansion of myeloid cells. The apoptosis increase, indeed, persists when double-mutant DP T thymocytes are cultured *in vitro*, also upon TCR activation with anti-CD3 or in the presence of pro-survival cytokines, such as IL-2 or IL-15.

Interestingly, the increase of apoptosis in DP T cells from transgenic mice with dysregulated activation of Notch-ICN was already reported, even if without any definitive explanation (46, 56). Furthermore, it was shown that canonical NF-B pathway activation plays a crucial role in the selection processesof DP thymocytes (13) and promotes their apoptosis (63). Overall, based on our results and literature data, we can speculate that the increased apoptosis of DP T cells from *N3tg* mice, compared to that of *wt* controls, may derive from Notch-dependent constitutive activation of NF-κB. However, Notch activation in thymocytes of *N3tg* mice also promotes other mechanisms (8), that disrupt growth regulation and produce the net result of favoring tumor cell survival. Conversely, the deletion of NF-κB1 in *N3tg/p50*$^{-/-}$ mice can shift the balance toward cell death, possibly through the dramatic decrease of $p21^{Waf1/Cip1}$ protein expression. Many literature data hypothesize that $p21^{Waf1/Cip1}$ protects thymic tumor cells from apoptosis (64) and that NF-κB-dependent induction of $p21^{Waf1/Cip1}$ expression may represent an anti-apoptotic mechanism of resistance in cancer cells, including T-ALL cells (65). Moreover, pharmacological suppression of $p21^{Waf1/Cip1}$ protein by flavopiridol treatment has been experimented on T-ALL Jurkat cells as a successful antileukemic therapy, when combined with HDAC-inhibitors (66). It is noteworthy that the $p21^{Waf1/Cip1}$ protein is involved in the regulation of apoptotic processes that could be dependent or independent from caspase (59, 67). Intriguingly, we reported here that the decrease of $p21^{Waf1/Cip1}$ protein expression in double-mutant DP thymocytes does not impinge on the expression of the active caspase-3 protein. Furthermore, the regulation of apoptosis by $p21^{Waf1/Cip1}$ may occurr independently from its role in the cell cycle (59, 67). To this regard, we observed that the downregulation of $p21^{Waf1/Cip1}$ protein in double-mutant DP thymocytes correlates with an enhanced expression of the Ki-67 proliferation marker on a per cell basis, without any significant alterations of DP thymocyte distribution in the different phases of the cell cycle.

It is important to note that a causative link between Treg reduction and T-ALL progression remains not formally proven in *N3tg/p50*$^{-/-}$ mice. However, Treg accumulation has been highlighted as a negative event in human T-ALL prognosis (36). Hence, it is possible that the inhibition of T-ALL progression observed in *N3tg/p50*$^{-/-}$ mice is not exclusively due to the induction of apoptosis inside pre-leukemic DP T cells, but also depends on the dramatic depletion of Tregs. In this context, we also suggest here that the lack of Tregs does not have a crucial role in driving myeloproliferation of *N3tg/p50*$^{-/-}$ mice, as instead demonstrated in FoxP3-deficient "scurfy" mice (57).

In conclusion, *N3tg/p50*$^{-/-}$ double-mutant mice may represent a novel model to achieve a better understanding of how combined mutations of Notch and NF-κB1 may impact on both the progression of T-ALL and the composition of T-ALL immune-environment in the attempt to identify innovative multiple target therapy for the disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the local Animal welfare committee and was carried out in accordance with the recommendations of the Italian national guidelines for experimental animal care and use and of the European Directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

PG and AO designed and performed the experiments, analyzed the data, and wrote the first draft of the paper. NG, CN, and GS performed the experiments. GP analyzed the data. IS critically revised the manuscript. AC supervised the experiments, analyzed the data, and wrote the manuscript.

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