Notch1 and IL-7 Receptor Signalling in Early T-cell Development and Leukaemia

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Abstract

Notch receptors are master regulators of many aspects of development and tissue renewal in metazoans. Notch1 activation is essential for T-cell specification of bone marrow-derived multipotent progenitors that seed the thymus, and for proliferation and further progression of early thymocytes along the T-cell lineage. Deregulated activation of Notch1 significantly contributes to the generation of T-cell acute lymphoblastic leukaemia (T-ALL). In addition to Notch1 signals, survival and proliferation signals provided by the IL-7 receptor (IL-7R) are also required during thymopoiesis. Our understanding of the molecular mechanisms controlling stage- specific survival and proliferation signals provided by Notch1 and IL-7R has recently been improved by the discovery that the IL-7R is a transcriptional target of Notch1. Thus, Notch1 controls T-cell development, in part by regulating the stage- and lineage- specific expression of IL-7R. The finding that induction of IL-7R expression down- stream of Notch1 also occurs in T-ALL highlights the important contribution that deregulated IL-7R expression and function may have in this pathology. Confirming this notion, oncogenic IL7R gain-of-function mutations have recently been identified in childhood T-ALL. Here we discuss the fundamental role of Notch1 and IL-7R signalling pathways in physiological and

pathological T-cell development in mice and men, highlighting their close molecular underpinnings.

Keywords: Thymus, T-cell lineage, Notch, IL-7R, lympho-myeloid progenitor, leukaemia

1. From Early Thymic Progenitors to Mature T Cells

All blood lineages but T cells derive in situ within the bone marrow (BM) from resident haematopoietic stem cells (HSCs) that undergo a maturation process characterised by the progressive loss of developmental potentials and the activation of lineage-specific transcriptional programs. T lymphocyte development is an exception, as it occurs within a dedicated lymphoid organ, the thymus. T cells develop from BM-derived progenitors that have lost self-renewing capacity and are thus distinct from HSCs, but still display multi-lineage potential (Fowlkes 1985; Shortman and Wu, 1996; Weerkamp et al. 2006; Blom and Spits 2006). Within the thymus, these haematopoietic progenitors undergo tightly regulated proliferation, maturation and selection processes that require their dynamic relo- cation within specific niches of the thymic microenvironment (Takahama 2006; Ciofani and Zúñiga-Pflücker 2007; Bhandoola et al. 2007; Rothenberg 2007; Anderson et al. 2007; Petrie and Zúñiga-Pflücker 2007). Both surface molecules and soluble factors from thymic stromal cells, mainly thymic epithelial cells (TECs), provide inductive signals that are essential for directing thymic immigrants along the T-cell linage. Specifically, interactions of Notch receptors on thymic precursors with their ligands expressed on TECs, and signalling mediated by the interleukin-7 receptor (IL-7R) in response to TEC-derived IL-7 (Fig. 1), are crucial events that regulate thymopoiesis in both mouse and man (Shortman et al. 1990; Blom and Spits 2006; Maillard et al. 2005). Deregulation of any of these pathways is, thus, linked to the emergence of T-cell pathologies, largely T-cell immunodeficiencies and leukaemias. The details of how Notch and IL-7R signalling control physiological and leukaemic T-cell development in humans rep- resent the scope of this review.

1.1 Two Intrathymic Checkpoints for Cellular Expansion of Developing T Cells

The particular identity of the thymic immigrants has been elusive owing to the rarity of these cells. Extensive studies lend support to the current notion that in both mouse and man the thymus is seeded by haematopoietic progenitors with a distinctive lineage marker-negative (Lin-) ckit+ Flt3+ IL-7R-/lo phenotype (Allman et al. 2003; Porritt et al. 2004; Sambandam et al. 2005; Tan et al. 2005; García-Peydró et al. 2006; Blom and Spits 2006), equivalent to that of lymphoid- primed multipotent progenitors (LMPPs) in the BM (Adolfsson et al. 2005). After thymus arrival, these cells, commonly referred to as the earliest thymic progenitors (ETPs) (0.01–0.03 % of neonatal thymocytes) (Allman et al. 2003; Benz et al. 2008), can be identified within the most immature CD4- CD8- double negative (DN) thymocytes (termed DN1), characterised as CD44+ CD34+ in humans (Márquez et al. 1995; Carrasco et al. 2002; Spits 2002) and CD44+ CD25- in mice (Shortman and Wu 1996). Human ETPs express low levels of the myeloid marker CD33 (Márquez et al. 1998; García-Peydró et al. 2006), while mouse ETPs express PSGL1 and the chemokine receptor CCR9, both involved in thymus seeding (Benz and Bleul 2005; Gossens et al. 2009; Love and Bhandoola 2011). ETPs have been referred to as ‘‘canonical’’ T-cell progenitors because they robustly generate DN2 downstream progeny (Allman et al. 2003; Porritt et al. 2004; Benz et al. 2008) (Fig. 2), which is characterised in humans by up-regulation of CD1a (CD44+ CD25+ in mice) and commitment to the T-cell lineage (Galy et al. 1993). The ETP to DN2 transition is marked by a massive cellular expansion, which depends on IL-7R-mediated signalling (Fig. 1) and represents a fundamental checkpoint in T-cell development (Shortman et al. 1990; Plum et al. 1996). Thereafter, DN2 thymocytes progress to the DN3 stage, composed of CD4+ immature single positive (ISP) thymocytes in humans (Kraft et al. 1993) (CD44- CD25+ in mice). At this stage, proliferation stops and gene rearrangement at the TCRd, c and b loci is completed (Ramiro et al. 1996; Blom et al. 1999). DN thymocytes that succeed in TCR and TCR rearrangements will give rise to TCRγδ T cells, while those expressing a functional TCRβ chain will accomplish progression beyond the DN3 stage by signalling through a highly conserved TCRβ–pTα (pre-TCR) complex (von Boehmer and Fehling 1997; Ramiro et al. 1996; Carrasco et al. 1999, 2001). Cell surface pre-TCR expression promotes cell survival and proliferation and induces progression to the CD4+ CD8+ double positive (DP) stage by a process known as β-selection (von Boehmer et al. 1998), which represents the second checkpoint of intrathymic expansion in T-cell development (Fig. 1). Thereafter, the pre-TCR is down-regulated and DP thymocytes stop

cell division, undergo rearrangements at the TCRα locus (von Boehmer and Fehling 1997; Trigueros et al. 1998; Carrasco et al. 2001) and finally express a mature TCRαβ that will allow their positive selection and differentiation into mature CD4+ or CD8+ single positive (SP) thymocytes. SP thymocytes that survive negative selection migrate to the periphery as MHC-restricted self-tolerant T-cells (Takahama 2006; Anderson et al. 2007). Therefore, thymopoiesis encompasses two checkpoints of cellular expansion sequentially controlled by the IL-7R and the pre-TCR, whose expression has to be tightly regulated to ensure physiological proliferation of developing thymocytes.

1.2 IL-7R Expression Marks a Lymphoid/Myeloid Developmental Split in Human Thymopoiesis

Understanding how ETPs differentiate along the T-cell lineage has been challenging owing to discrepancies about their level of commitment and lineage potential. The so-called classical model proposed that the thymus is colonised exclusively by common lymphoid progenitors (CLP) harbouring T, NK and B lymphocyte potential (Kondo et al. 1997). However, different studies challenged this view (Allman et al. 2003; Adolfsson et al. 2005; Kawamoto and Katsura 2009; Doulatov et al. 2010). Early work showed that, in addition to lymphoid potential, ETPs have the potential to generate dendritic cells (DCs) (Ardavin et al. 1993; Márquez et al. 1995), which is associated in humans to significant macrophage, some granulocyte and little, if any, erythroid potential (Kurtzberg et al. 1989; Márquez et al. 1995; Res et al. 1996; García-Peydró et al. 2006; Weerkamp et al. 2006). The multiple lineage potential of human ETPs has been revealed also in clonal assays (Márquez et al. 1998; Hao et al. 2008), indicating that at least some human thymus settling progenitors have combined lympho-myeloid lineage potential.

Early reports revealed the ability of ETPs to generate multiple lymphoid (T, B and NK) lineages together with DCs in the mouse (Wu et al. 1991; Shortman and Wu 1996; Michie et al. 2000; Akashi et al. 2000; Benz and Bleul 2005). Subsequent studies confirmed the myeloid potential of murine ETPs in both bulk and clonal assays in vitro (Balciunaite et al. 2005; Benz and Bleul 2005; Bell and Bhandoola 2008; Moore et al. 2012) and also in vivo (Wada et al. 2008). However, more recent fate tracing data by Rodewald and colleagues using IL-7Rα-Cre mice showed that the lymphoid-restricted progenitors are

the major route to murine T cells in vivo (Schlenner et al. 2010), suggesting a separate origin of T cells and myeloid cells in the murine thymus. Although the field remains controversial, functional and molecular evidence has lately been provided at the single cell level that the earliest progenitors in the neonatal murine thymus, as well as LMPPs in the BM, have combined granulocyte–macrophage, T lymphocyte and B-cell lymphopoietic potentials, but not megakaryocyte–erythroid lineage potential (Luc et al. 2012; Ceredig 2012), as reported for human ETPs (Weerkamp et al. 2006; García-Peydró et al. 2006).

Realization of the lympho-myeloid potential of ETPs is of crucial relevance for understanding how either T-cell or alternative cell fates are imposed within the thymus. Several studies support that T-cell commitment in mice is a sequential process, involving progressively increasing limitations on the non-T cell potential of ETPs. Loss of B-cell potential may happen immediately after thymus entry or even prior to thymus seeding (Benz and Bleul 2005; Harman et al. 2005; Heinzel et al. 2007), and is followed by loss of myeloid, DC and NK cell potentials (Ciofani and Zúñiga-Pflücker 2007; Bhandoola et al. 2007; Rothenberg et al. 2008; Ceredig 2012). In humans, however, our studies suggest that after loss of B-cell potential progression towards the T-lineage fate involves an early, split of ETPs into two, alternative developmental pathways that proceed through independent myeloid- or lymphoid-primed intermediate progenitors (Fig. 2). This branching point is marked both in vitro and in vivo by down-regulation of CD34 and either the up- or down-regulation of CD44 (Márquez et al. 1995). CD44 up-regulation results, in turn, in up-regulated expression of the myeloid marker CD33 and loss of T-cell potential. These non-T cell progenitors are, however, enriched in myeloid- DC potential and retain some NK cell potential. Conversely, CD44 down-regulation parallels an increased expression of the T-cell marker CD5, loss of myeloid-DC capability and progression towards the T/NK bi-potential lymphoid stage (Márquez et al. 1998; de Yébenes et al. 2002). Expression of CD1a in lymphoid-restricted thymocytes finally marks loss of NK lymphopoietic ability and T-cell commitment (Galy et al. 1993; Sánchez et al. 1994; de Yébenes et al. 2002) (Fig. 2). According to their restricted lineage potentials, intermediate thymic progenitors display exclusive myeloid or lymphoid-associated gene transcription patterns (Martín-Gayo et al. unpublished results). Likewise, myeloid-specific transcriptional priming has recently been reported for myeloid precursors of DCs recently identified in the mouse thymus (Moore et al. 2012).

The myeloid versus lymphoid cell fate decision in human thymus robustly associates with a selective expression of receptors for either granulocyte–macrophage colony-stimulating factor (GM-CSF) or IL-7, respectively, reflecting the cytokine-specific dependence of each developmental pathway (Márquez et al. 1998; de Yébenes et al. 2002, García-Peydró et al. 2006). Therefore, up-regulation of IL-7R marks a key lymphoid-myeloid branching point in human thymopoiesis, which associates with loss of myeloid (macrophage and DC) potentials and with T-lineage specification (Fig. 2).

2. IL-7R Signalling in Early T-cell Development

Different studies have highlighted the conserved non-redundant role played by IL-7 in T-cell development. IL-7 is a cytokine produced by thymic epithelium and BM stromal cells. Binding of IL-7 to the IL-7R, which is composed of the specific α-chain (IL-7Rα) and the common cytokine receptor γc (γc) chain (Fig. 3), results in α and γ chain dimerisation. The, thereby, activated IL-7Rα-γc heterodimer transmits signals for survival and proliferation to T- and also B-lineage cells (Shortman et al. 1990; Akashi et al. 1998; Leonard 2001). Pathways activated downstream of IL-7R include JAK/STAT, PI3K, MAPK and Src kinases, ultimately leading to the expression of target genes such Bcl-2 family members, cyclin D1, SOCS-1 and c-myc (Leonard 2001). Mice deficient in IL-7 or IL-7R have an early block in T-cell development associated with reduced numbers of non-functional T cells and impaired B lymphopoiesis (Sudo et al. 1993; Peschon et al. 1994; von Freeden-Jeffry et al. 1995). Bcl-2 is sufficient to rescue T-cell but not B-cell development in those mice (Maraskovsky et al. 1997, 1998; Akashi et al. 1997), indicating that IL-7 has a pro-survival effect in T-lineage cells. In humans, IL-7R paucity does not affect B lymphopoiesis, but results in severe combined immunodeficiency (SCID) characterised by a complete lack of T cells (Puel et al. 1998; Leonard 2001). Therefore, species-specific lineage-associated IL-7/IL-7R requirements and/or signalling pathways downstream of IL-7R are likely to be responsible for this difference (Pallard et al. 1999).

These results point toward an essential function of IL-7/IL-7R during early T-cell development that supports the survival and the enormous expansion that experiences the intrathymic pool of T-cell-specified DN2 progenitors (Shortman et al. 1990; Peschon et

al. 1994; Plum et al. 1996; Akashi et al. 1998). In addition, signalling through IL-7R at the DN2 stage controls TCRγ locus accessibility and gene rearrangement (Durum et al. 1998). Accordingly, DN2 cells with higher IL- 7R expression levels are biased to the γδ T-cell lineage, at least in mice (Kang et al. 2001). IL-7 is, however, dispensable at or beyond the DN3 stage, although it may be required later on during positive selection of CD8+ thymocytes (Munitic et al. 2004; Yu et al. 2004; Van De Wiele et al. 2004). The stage-specific effect of IL-7 is accomplished by a dynamic regulation of IL-7R expression and function (Munitic et al. 2004). Of the two IL-7R components, the γc is constitutively expressed in thymus-seeding precursors, which have negative or low levels of IL- 7Rα expression, indicating that IL-7Rα is up-regulated at the ETP stage (García- Peydró et al. 2006; Porritt et al. 2004; Allman et al. 2003). Subsequently, IL-7Rα expression increases progressively until the DN2 stage, declines steadily thereafter, and must be terminated between the β-selection and positive selection checkpoints (Munitic et al. 2004; Yu et al. 2004, 2006; Van De Wiele et al. 2004). Besides transcriptional regulation, active suppression of IL-7R signalling induced by suppressor of cytokine signalling (SOCS)-1 guarantees IL-7R-unresponsiveness in pre-selected DP thymocytes (Yu et al. 2006), which is obligatory to allow negative selection and death by neglect to occur at the DP stage. In fact, sustained IL-7R expression in *Il7ra* transgenic mice results in a direct competition between DN progenitors and DP thymocytes for the limiting amounts of endogenous IL-7 within the thymus (Munitic et al. 2004). Finally, restoration of IL-7R surface levels by positive selection will ensure homeostatic proliferation of peripheral mature T cells derived from CD4 and CD8 SP thymocytes (Schluns et al. 2000). Tight regulation of IL-7Ra expression during thymopoiesis, thus, guarantees specific survival and expansion of those intrathymic progenitors that have undergone T-cell specification.

3 Notch Regulation of Early Thymocyte Development

T-cell specification signals uniquely provided by the thymus microenvironment largely rely on the Notch signalling pathway (Takahama 2006; Petrie and Zúñiga- Pflücker 2007; Koch and Radtke 2011). Notch activation is required at sequential intrathymic stages to provide survival, proliferation or metabolic cues as well as differentiation signals, all necessary for efficient T-cell development (Schmitt et al. 2004; Ciofani and Zúñiga-

Pflücker 2007). The Notch pathway is an evolutionary conserved cell signalling system of transmembrane receptors that includes four members in mammals (Notch1–4). Notch1 is the receptor preferentially expressed in developing thymocytes, although all members are expressed in the thymus (Fiorini et al. 2009; Van de Walle et al. 2011; Martín-Gayo et al. unpublished results). Notch receptors engage five mammalian surface ligands of the Delta-like (Dll-1, -3 and -4) and Jagged (Jag1 and 2) families expressed on neighbouring cells (Artavanis- Tsakonas et al. 1999; Bray 2006). In the thymus, all Notch ligands except Dll3 are expressed at different thymic niches. Amongst these ligands, Dll4 is specifically expressed on TECs located in the subcapsular and outer cortical regions, while Dll1 expression seems to be restricted to thymic blood vessels (Mohtashami et al. 2010; Hozumi et al. 2008; Koch et al. 2008 and García-León et al. unpublished results). Both Jag1 and Jag2 are expressed in the thymic medulla, and Jag2 is also found in the inner cortex (Heinzel et al. 2007; Mohtashami et al. 2010; Van de Walle et al. 2011; García-León et al. unpublished results).

Notch-ligand binding induces sequential conformational changes in the Notch receptor that expose specific sites for two consecutive cleavages mediated first by ADAM (a disintegrin and metalloprotease) members, ADAM10 (Kuzbanian) and ADAM17 (TNF-alpha converting enzyme or TACE), and then by a γ-secretase protein complex (presenilin, APH-1, nicastrin and PEN-2). As a consequence, the intracellular Notch domain (ICN or NICD) migrates to the nucleus where it associates with the transcription factor CSL (CBF1/RBPJk, Su(H) and LAG-1), displaces co-repressors and recruits co-activators including p300/CBP as well as MAML-1–3, thereafter initiating the transcription of target genes (Kopan and Ilagan 2009). Canonical Notch targets are basic-helix-loop-helix proteins (bHLH) such as Hey and Hes (Jarriault et al. 1995). Other Notch targets include CD25 (Adler et al. 2003), pTα (Reizis and Leder 2002) and molecules involved in cell proliferation and survival such as c-myc (Palomero et al. 2006; Weng et al. 2006), cyclin D1 (Ronchini and Capobianco 2001) and p21/Waf (Rangarajan et al. 2001). More recently, IL-7Rα has been identified as a specific Notch1 target in T-lineage cells (González-García et al. 2009).

The capacity of different Notch ligands and receptors to induce T-cell development has been best illustrated by establishment of a co-culture system that uses a stromal cell line (usually OP9 or S17) made to express individual Notch ligands (Jaleco et al. 2001;

Schmitt and Zúñiga-Pflücker 2002), which has been particularly useful for human studies. In this assay, Dll4 appears to be the more effective inducer of T-cell differentiation (Mohtashami et al. 2010) and Dll1 but not Jagged1 can also induce T-cell development from haematopoietic progenitors (Jaleco et al. 2001; Lehar et al. 2005). In contrast, Jag2 appears to have functional similarities to Dll ligands, at least in humans (Van de Walle et al. 2011). These data indicate that different Notch ligands transmit distinct activation signals to T-cell precursors that differentially affect their proliferation and/or differentiation potential, with Jagged ligands (mainly Jagged1) inducing lower Notch activation than Dll ligands (reviewed in Thompson and Zúñiga-Pflücker 2011). Supporting this idea, quantitative Notch signals have been shown to influence the TCRαβ versus TCRγδ decision, likely in combination with TCR signals. TCRγδ development in the mouse seems to be less Notch dependent than TCRαβ differentiation (Washburn et al. 1997; Ciofani et al. 2006; Garbe et al. 2006), a finding that concurs with a higher dependency of the former on Jagged ligands (Jiang et al. 1998). However, sustained Notch1 signalling in humans has been shown to favour TCRγδ development at the expense of TCRαβ generation in vitro (García-Peydró et al. 2003; Van de Walle et al. 2009), although not in vivo in a xenotransplantion assay (García-Peydró et al. unpublished results). These contradictory results underscore the fact that reduction of Notch activation at specific checkpoints is necessary to induce αβ T-cell differentiation at the expense of γδ T cells (Van de Walle et al. 2009). Likewise, they emphasise the potential significance of differentially expressed ligands in microenvironmental niches in thymopoiesis. However, only Dll4 has been shown to provide a relevant T-lineage-inducing Notch signal in vivo (Hozumi et al. 2004, 2008; Koch et al. 2008), while the physiological role played by Dll1 and Jagged ligands in the thymus is still unclear. The possibility that important determinants of cellular outcome, such as Notch ligand density and binding affinity could determine an unknown role for these ligands at specific intrathymic locations is particularly appealing (D'Souza et al. 2008), especially because Dll binding affinity can be modulated by glycosylation mediated by the Fringe family of glycosyltransferases expressed in the thymus (Visan et al. 2006; Stanley and Guidos 2009).

3.1. Notch1 Activation Diverts Early Thymic Progenitors from Alternative Cell Fates and Promotes Cell Growth

The critical role of Notch1 in T cell development was first described by Radtke and co-workers, by generating inducible Notch1 loss-of-function mice (Radtke et al. 1999). Total thymocyte numbers were reduced in those mice and T-cell development was blocked at the most immature T-cell stage, while B cells accumulated in the thymus. Complementary to these findings, constitutive expression of active Notch1 (ICN1) in a transplantation setting impaired B-cell differentiation, induced ectopic development of DP thymocytes and subsequently leukaemia (T-ALL) (Pui et al. 1999; Pear et al. 1996). The obvious conclusion of these studies was that Notch1 signalling critically influences the B versus T lineage choice of CLPs. However, as discussed above, LMPP progenitors rather than CLPs include the canonical T-cell precursors in the postnatal thymus, and thus the role that Notch1 signalling plays in early thymopoiesis has been re-examined.

Earlier gain-of-function approaches in humans assessed the impact on ETP multi-lineage potential of either ligand-independent Notch1 signalling, induced by ectopic expression of active ICN1, or ligand-dependent Notch1 activation, sup- ported by OP9-Dll1 stromal cells (Fig. 4). Both strategies provided evidence that the most prominent function of Notch1 signalling is to inhibit non-T cell fates while supporting the expansion of T-lineage progenitors (De Smedt et al. 2002, 2005; García-Peydró et al. 2003, 2006). In the human thymus, these events critically happen at the lymphoid-myeloid branching point and result in impaired generation of myeloid-primed intermediate progenitors (Fig. 2). Consequently, reduced numbers of macrophages and conventional as well as plasmacytoid DCs are generated from ETPs (De Smedt et al. 2005; García-Peydró et al. 2006; Dontje et al. 2006; Martín Gayo et al. unpublished results). In addition, Notch1 signalling blocks the development of NK cells from T/NK lymphoid progenitors, further enforcing T-cell specification (De Smedt et al. 2005; García-Peydró et al. 2006). Complementary loss-of-function approaches using c-secretase inhibitors (GSI) confirmed these results and showed that increasing thresholds of Notch signalling sequentially suppress B, myeloid/DC and NK cell lineage fates in human thymopoiesis (De Smedt et al. 2005).

Notch-mediated suppression of myeloid cell fate in human lympho-myeloid progenitors is actively induced by repression of myeloid gene transcription that, importantly, seems

to be ligand specific (Martín-Gayo et al. unpublished results). In addition, Notch1 signalling triggers a T-cell lineage gene program in humans (García-Peydró et al. 2006; Van de Walle et al. 2011), coincident with the profile reported in mice (reviewed in Rothenberg et al. 2008; Thompson and Zúñiga- Pflücker 2011), a finding that supports an instructive role of Notch1 activation in T-cell specification. Notably, very recent results have shown that such an instructive role crucially relies on a transcription factor, TCF1, induced by Notch signals. TCF1 imposes the T-cell fate by up-regulating expression of other transcription factors essential for T-cell differentiation such as Gata3 and Bcl11b, and TCR components (Weber et al. 2011). However, ICN1 gene targets that control T-cell proliferation are not similarly triggered by TCF1, and Notch1-induced proliferation may, thus, be TCF-1-independent. Indeed, proliferation induced by Notch1 was shown to rely on unique signals provided by cytokines (Varnum- Finney et al. 2003), specifically by IL-7 (García-Peydró et al. 2006; González-García et al. 2009). Accordingly, Notch1-induced proliferation correlates with up- regulation of IL-7R expression (García-Peydró et al. 2006; González-García et al. 2009). As a whole, these data indicate that acquisition of a functional IL-7R marks the critical checkpoint of inhibition of non-T cell potentials and T-lineage specification of lympho-myeloid progenitors induced by Notch, and also controls the pivotal stage of cellular expansion of T-cell committed progenitors (de Yébenes et al. 2002; García-Peydró et al. 2006).

Once T-cell specification is induced, definitive T-cell differentiation and growth functions triggered downstream of Notch1 become uncoupled events. It has been shown that enforced expression of active Notch1 in human ETPs is itself sufficient to block non-T cell development and to trigger an almost unlimited cellular expansion in vitro in response to IL-7; however, it is insufficient to induce TCR rearrangements and to complete T-cell maturation of DN2 thymocytes, unless co cultured with stromal cells (García-Peydró et al. 2006). Likewise, a DN2 developmental arrest has recently been observed when murine fetal liver progenitors are activated with immobilised Dll4 plus cytokines under stroma-free conditions (Ikawa et al. 2010). These results suggest a requirement of additional inductive signals provided by stromal cells supporting a complete differentiation program along the T-cell lineage. Alternatively, Notch1 signalling could provide self- renewal capacity to DN2 cells, and simultaneously arrest development beyond the DN2 stage, thus allowing for segregation of proliferation and differentiation processes during thymopoiesis. Supporting the latter possibility, an early

decrease in Notch activation is required for human thymic precursors to complete ab T-lineage differentiation in vitro (Van de Walle et al. 2009). Moreover, in vitro production of mature ab-lineage cells from murine thymic precursors is contingent to decreased IL-7 responsiveness and self-renewal arrest beyond the DN2 stage (Balciunaite et al. 2005). Diminished IL-7R signalling was recently shown to be necessary for DN2 mouse thymocytes to up-regulate Bcl11b, a transcription factor that is essential to drive full development along the T-cell lineage (Ikawa et al. 2010; Li et al. 2010). Therefore, it is likely that physiological progression through the DN2- determination step is instructed by environmental signals in the thymus, such as limited IL-7 availability and/or reduced IL-7R expression. According to this view, Bcl11b deficiency selectively impairs development of ab but not cd T cells (Ikawa et al. 2010), which develop from DN2 thymocytes expressing either low or high IL-7R levels, respectively (Kang et al. 2001). Collectively, these data suggest that regulation of Bcl11b expression is an early T-cell developmental checkpoint controlled by IL-7R-mediated signalling.

3.2. Notch1 Signalling Controls T-Lineage-Specific IL-7R Expression in Early Thymopoiesis

The stage- and lineage-specific role of IL-7 during thymopoiesis indicates that strict mechanisms control the dynamic intrathymic regulation of IL-7R expression. Likewise, regulatory mechanisms may control the differential expression of IL-7R in T- and B-lineage cells during lymphopoiesis. In mouse early lymphoid/B-cell progenitors, IL-7Rα gene (*Il7ra*) transcription is specifically regulated by the Ets transcription factor PU.1 (DeKoter et al. 2002). PU.1 is expressed very early in thymopoiesis as well, but PU.1 down-regulation is obligatory for T-cell fate specification and progression along the T-cell lineage (Anderson et al. 2002). PU.1 function in mature T-cells seems to be replaced by the Ets transcription factor GA binding protein (GABP), but its role during early thymopoiesis is less clear (Xue et al. 2004). In B lymphopoiesis, however, GABP cooperates with PU.1 and regulates IL-7Ra expression in pre-B and committed B-cells (DeKoter et al. 2007). While these data support the existence of specific regulators of IL-7Rα expression in B-cell development, the nature of equivalent regulators in the T-cell lineage has been an open question for years. Recently, molecular studies from our group have shown that Notch1 accomplishes this function at least in humans (González-García

et al. 2009). Both gain- and loss-of-function approaches have shown that expression of IL-7Rα in developing T-lineage cells critically depends on Notch1 activation. Notch1 regulates IL-7Rα expression at the transcriptional level and, notably, in a T-lineage-specific manner, since IL-7Rα gene (I*L7R*) transcription can be inhibited by ectopic expression of a dominant negative form of the MAML-1 co-activator (dnMAML-1) in T- but not B-lineage cells (González-García et al. 2009). Chromatin immunoprecipitation and luciferase reporter assays have further established that *IL7R* gene expression is directly induced by active Notch1 in T-cell lines and DN2 thymocytes, indicating that IL7R is a transcriptional target of Notch1. Supporting the participation of CSL in Notch1-induced IL7R transcription, we have identified a conserved CSL-binding site in the IL7R promoter (Fig. 5) and show that either site-directed mutagenesis or CSL- deficiency impairs IL7R promoter activity induced by active Notch1. Therefore, T- lineage-specific IL7R transcription induced by Notch1 is CSL/MAML-1-depen- dent (González-García et al. 2009). More recently, ICN1 has been shown to interact with an IL7R gene enhancer in a human T-cell line, suggesting that additional mechanisms of regulation of IL-7Rα expression mediated by Notch1 could exist (Wang et al. 2011).

The physiological role of Notch1 in the regulation of IL-7Rα expression in human thymopoiesis is supported by studies showing that, which encodes the IL-7R effector bcl-2, Notch1 activity parallels expression of IL7R as well as BCL2 from the ETP to DN3 stages. Conversely, transcription of NOTCH1 and IL7R decreases concordantly beyond the DN3 stage and remains low throughout the rest of thymocyte development. Accordingly, defective Notch1 activation selectively results in a compromised expansion of the DN1-3 compartments, which can be rescued by ectopic IL-7Rα expression, suggesting that Notch1 signals are no longer required once T-cell specification and IL-7R expression have been induced. However, IL-7R is unable to replace Notch1 signals at the β-selection checkpoint (González-García et al. 2009), a finding that concurs with the reported requirement of Notch1 and pre-TCR signalling during β-selection (Wolfer et al. 2002; Ciofani et al. 2004; Ciofani and Zúñiga-Pflücker 2005; Maillard et al. 2006; Taghon et al. 2009). Therefore, Notch1 signals control thymocyte proliferation at two sequential checkpoints. First, a functional IL-7R is up-regulated on T-cell specified pro-genitors and second, expression of a pre-TCR complex is induced that supports metabolism, survival and proliferation of committed T-cell progenitors as well as

progression to the DP stage independently of IL-7R. Between both proliferation phases, decreased Notch1 signalling seems to be required to induce down-regulation of IL-7R expression and Bcl11b up-regulation, which will finally allow T-cell-specified progenitors to complete differentiation along the ab lineage, as discussed above.

4. Notch1 Signalling in T-ALL

T-cell acute lymphoblastic leukaemia (T-ALL) is a lymphoproliferative disorder accounting for 10–15 % of pediatric and 25 % of adult ALL cases, which results from the malignant transformation of normal developing T cells in the thymus (Pui et al. 2004). Aberrant Notch1 signalling was initially described in human T-ALLs (<1 %) with rare chromosomal translocations that generate a truncated Notch1 isoform lacking the extracellular domain (TAN1) under the transcriptional control of the TCRβ enhancer (Ellisen et al. 1991). Subsequently, the group of Aster provided evidence that Notch1 signals play a more prominent role in leukaemogenesis than initially suspected, as activating NOTCH1 mutations were found in more than 50 % of human T-ALLs (Weng et al. 2004). These mutations involve the extracellular heterodimerisation (HD) domain and, less frequently, the C-terminal PEST domain of Notch1. HD mutations increase ADAM cleavage and subsequent ligand-independent receptor activation, while PEST mutations increase the stability and half-life of ICN1. Other Notch1 activating mutations as well as mutants of genes that regulate turnover of ICN1 such as Fbw7, which encodes a ubiquitin ligase involved in ICN1 degradation, have been subsequently identified in mouse models of T-ALL and T-ALL patients (reviewed in Aifantis et al. 2008; Li and von Boehmer 2011; Aster et al. 2011; Koch and Radtke 2011). Thus, there is an increasing interest in under- standing the role of Notch1 in the pathogenesis of T-ALL, with the final aim of identifying novel therapies which target Notch1 signalling.

The main targets of aberrant Notch1 activation leading to leukaemia are Notch-associated signalling pathways that control survival and proliferation in normal T-cell development. As highlighted above, the IL-7R and pre-TCR are the major Notch-dependent pathways that accomplish this function in thymopoiesis. Historically, the pre-TCR pathway was suggested first to interact with Notch1 signals in T-cell oncogenesis, as mice transplanted with BM progenitors expressing active NOTCH1 alleles rapidly developed an aggressive

T-cell leukaemia (Pear et al. 1996; Pui et al. 1999), but only when pre-TCR signalling was intact (Allman et al. 2001). Likewise, T-ALL development in Notch3 transgenic mice was shown to be dependent on pre-TCR expression (Bellavia et al. 2002). However, a cooperative rather than absolute requirement of pre-TCR in Notch-induced leukaemogenesis has been proposed later (Campese et al. 2006). Regarding possible molecular mechanisms underlying this cooperation, it was found that the PTCRA gene encoding pTα is a transcriptional target of Notch (Reizis and Leder 2002). In addition, other pre-TCR components including CD3ε and TCRβ seem to be regulated by Notch signals as well, further suggesting that the Notch pathway could be upstream of pre-TCR assembly and expression (Aifantis et al. 2008). Nonetheless, both pathways activate common transcription factors and kinases, and share BCL-2A1 and cyclin D3 as common targets involved in G1/S cell cycle progression. This would suggest that they can act in parallel but converge at signalling intermediates in T-ALLs (Aifantis et al. 2008). Supporting this possibility, c-myc, a crucial regulator of cellular metabolism and cell cycle progression, whose expression picks around the b-selection checkpoint, has been identified as a key Notch target that cooperates with ICN1 in Notch1-dependent leukaemogenesis (Weng et al. 2006; Palomero et al. 2006). Importantly, Notch1 and c-myc activate common targets required for growth of leukaemic cells, suggesting a feed-forward loop in leukaemogenesis (Palomero et al. 2006). Notch activation positively regulates activity of the mTOR pathway in a c-myc-dependent manner (Chan et al. 2007). The PTEN/PI3K/Akt/mTOR pathway is a major pre-TCR-associated pro-oncogenic pathway regulated by Notch1 in T-ALL. Seminal work by the group of Ferrando showed that Notch1 is a negative regulator of PTEN, and identified recurrent PTEN inactivating mutations in T-ALLs that conferred GSI resistance (Palomero et al. 2007). The consequence of PTEN loss is a deregulated balance among activation/inhibition of PI3K and aberrant activation of Akt, a major inducer of proliferation and survival in T cells. In addition, Pi3K or Akt mutations have been identified in a high proportion of T-ALLs. Notch1 can also induce PI3K/Akt-dependent proliferation by inhibition of p53, another tumor suppressor downstream of pre-TCR signaling (Mungamuri et al. 2006). Other key downstream effectors of pre-TCR signalling activated by Notch1 in T-ALL include the NF-κB and NFAT pathways (Vilimas et al. 2007; Ciofani and Zúñiga- Pflücker 2005; Aifantis et al. 2001). Finally, it has been suggested that Notch promotes inhibition of the transcriptional activity of the E2A proteins in T-ALL, through a mechanism that involves

pre-TCR-mediated ERK-dependent up-regulation of the E2A inhibitors Id1 or Id3, while pre-TCR-mediated induction of Id3 represses E2A-dependent transcription of Notch1 under physiological conditions (reviewed in Li and von Boehmer 2011). Therefore, multiple signalling pathways driven by Notch1 and pre-TCR interact synergistically to promote transformation to T-ALL (reviewed in Koch and Radtke 2011; Li and von Boehmer 2011). These data highlight the requirement of Notch1 down-regulation after b-selection to avoid the oncogenic properties of Notch signalling.

4.1. Notch1 and IL-7R: Independent or Complementary Pathways in T-ALL?

As discussed above, another pathway critically involved in physiological growth of developing thymocytes is the IL-7R signalling pathway. Several results have suggested that IL-7 and IL-7R may contribute to T-cell leukaemia progression. In mice, expression of an IL-7 transgene results in lymphoma development (Rich et al. 1993), and AKR/J mice that show an up-regulated expression of IL-7Ra in the thymus develop spontaneous thymomas (Laouar et al. 2004). More importantly, human T-ALLs commonly express functional IL-7R that significantly contributes to T-ALL proliferation in response to exogenous IL-7 (Dibirdik et al. 1991; Barata et al. 2005). Notably, PI3K is a major effector of IL-7-induced viability and proliferation of T-ALLs (Barata et al. 2004), a finding that places the PI3K/Akt pathway at the crossroads of Notch1 and IL-7R signalling in T-ALL. Other effectors of IL-7R signalling including Bcl2 and cyclin D1 are also over- expressed in T-ALL (Barata et al. 2005). The recent identification of IL7R as a downstream target of Notch1 activity in normal human T-cell development might suggest a functional link between the Notch1 and IL-7R pathways in T-cell leu-kaemogenesis. Confirming this possibility, we found that IL7R is transcriptionally regulated by Notch1 activity also in T-ALL. IL-7Rα expression is, specifically, down-regulated in T-ALL but not B-ALL cell lines when Notch1 signalling is inhibited by GSI treatment or ectopic expression of dnMAML-1 (González-García et al. 2009). Notch inhibition results in decreased proliferation and cell cycle arrest, likely involving PTEN up-regulation (Palomero et al. 2007). Significantly, impaired proliferation of these T-ALL cell lines can be rescued by ectopic expression of IL-7Rα, which results in a selective growth advantage of IL-7Rα-expressing T-ALL cells in response to IL-7

(González-García et al. 2009). Therefore, IL-7/IL-7R signalling is able to support the survival and expansion of leukaemic cells with impaired Notch1 signalling. More importantly, our recent studies indicate that IL-7R is an important mediator of cell growth in primary T- ALLs as well (González-García et al. unpublished results).

Overall, these results support a cooperative role of Notch1 and IL-7R pathways in supporting leukaemogenesis. Likewise, they point to the IL-7/IL-7R pathway as a potential candidate to induce and/or maintain T-cell leukaemogenesis independently of Notch signals. The latter possibility is further supported by the observation that 18 % of adult and 2 % of pediatric T-ALL cases have activating mutations in JAK1 (Flex et al. 2008), which encodes a tyrosine kinase that directly binds IL-7R and promotes signalling (Fig. 3). Therefore, it can be hypothesised that IL7R itself might be a target of activating mutations in T-ALLs. Based on analyses of the complete coding sequence of IL7R in pediatric T-ALL samples, others and we have recently provided evidence that heterozygous oncogenic gain- of-function mutations do, in fact, occur in around 10 % of T-ALLs (Zenatti et al. 2011; Shochat et al. 2011). Notably, IL7R mutations do not occur in the cytoplasmic tail that recruits signalling effectors, but consist of in-frame insertions or deletions insertions in the juxtamembrane-transmembrane domain at the interface with the extracellular region. The vast majority of IL7R mutations create an unpaired cysteine residue that results in disulfide-bond-mediated homo-dimerisation of IL-7Rα chains able to signal in the absence of cc and IL-7 ligand binding (Fig. 6). Interestingly, IL7R mutations were also found by Shochat and coworkers in B-ALL samples, suggesting a general strategy for mutational activation of type I cytokine receptors in leukaemia. Therefore, although therapeutic strategies directed to Notch1 inhibition, particularly treatment with GSI, initially emerged as a promising therapy (Weng et al. 2004), recent knowledge of the molecular pathology of T-ALL open new avenues for the design of specific targeted therapies.

5. Conclusions

The study of the molecular mechanisms underlying T-cell development and transformation highlights the close relationship between Notch signalling pathways involved in T-cell physiology and pathology. The fundamental function of Notch1 in

thymopoiesis is to drive T-cell specification from multipotent precursors seeding the thymus and to support further progression along the T-cell lineage. These processes are associated with a unique role of Notch1 as a crucial regulator of cellular expansion at two critical checkpoints. Firstly, Notch1 controls IL-7R-dependent expansion of the T-cell-specified progenitor pool, and thereafter Notch1 cooperates with the pre-TCR to trigger expansion of progenitors that successfully progress along the T-cell maturation pathway. However, deregulated Notch1 activation at these stages results in T-cell transformation and leukaemia. Therefore, the identification of Notch1 signalling effectors involved in physiological proliferation of developing T-cells is crucial for designing new therapeutic strategies that target relevant oncogenic pathways in T-ALL. The identification of the IL-7R as a downstream transcriptional target of Notch1 both in physiology and pathology, together with the finding of oncogenic gain-of-function IL-7R mutations in T-ALL, open new possibilities for the development of specific targeted therapies. Ongoing preclinical studies exploring the efficacy of new therapies targeting the IL-7R in vivo in xenograft models of human T-ALL offer promise for the development of more effective T-ALL treatments in the near future.

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Figure legends

Fig. 1 Overview of thymic T-cell development. Bone marrow-derived lympho-myeloid progenitors enter the thymus through blood vessels located at the cortico–medullary

junction (CMJ) and give rise to the pool of (ETPs). Subsequently, ETPs initiate migration towards the external thymic cortex and interact with Notch ligands expressed on cTECs by means of Notch1 expression. Notch1 signalling induces T-cell commitment, IL-7R expression and progression through the CD4- CD8- DN2 and DN3 stages. Interaction of IL-7R on developing thymocytes with locally produced levels of IL-7 triggers a first wave of expansion of the T-cell progenitor pool. DN2 or DN3 thymocytes that succeed in TCRγ and TCRδ rearrangements give rise to TCRγδ T cells, while successful rearrangement at the TCRβ locus enables the expression of a TCRβ-pTα pre- TCR complex at the DN3 stage. PreTCR signalling promotes cell survival and extensive proliferation as well as progression to the CD4+ CD8+ DP stage. This process, known as β- selection, represents the second checkpoint of intrathymic expansion in T-cell development. At this stage, proliferation stops and DP thymocytes undergo rearrangement at the TCRα locus and express the TCRαβ. TCRαβ-expressing DP thymocytes then undergo positive and negative selection processes by means of interactions with cTECs or medullary epithelial cells (mTECs) and DCs, respectively. Selected cells exit the thymus through blood vessels at the CMJ as CD4+ or CD8+ SP thymocytes to establish the peripheral T-cell pool.

Fig. 2 Proposed model of lymphoid and myeloid development in human thymus. Human ETPs are lympho-myeloid precursors that can generate separate lymphoid- or myeloid-primed intermediate progenitors, characterised by the selective expression of either the IL-7R or the GM–CSFR, respectively. Lymphoid progenitors display T- and NK-cell potentials, while myeloid progenitors have lost T-cell potential, retain some NK-cell potential, and are enriched in myeloid (macrophage and DCs) potentials. Notch1 signalling diverts ETPs away from the myeloid pathway by blocking the generation of myeloid-primed progenitors, thus enforcing the lymphoid cell fate. Simultaneously, Notch1 induces the expression of IL-7R, which marks the lymphoid–myeloid branching point and triggers proliferation of lymphoid-primed progenitors. Thereafter, sustained Notch1 signalling blocks NK development from bipotent T/NK lymphoid progenitors and favours development along the T-cell lineage. This process is characterised by the progressive loss of CD34, CD44 and CD33 and the sequential acquisition of CD1a by CD4- CD8- DN thymocytes (DN2 stage) and CD4 molecules (DN3 stage). Successful TCRβ gene rearrangement at the DN3 stage results in TCRβ association with the

invariant pTα chain and formation of a pre-TCR complex at the DN4 stage. The pre-TCR cooperates with Notch1 signals at the β-selection checkpoint to induce a second wave of intrathymic proliferation and progression to the DP stage. Further rearrangement at the TCRα locus in DP thymocytes enables the expression of a mature TCRαβ. DP cells that survive TCRαβ-mediated positive and negative selection give rise to CD4+ or CD8+ SP thymocytes that migrate to the periphery

Fig. 3 IL-7 receptor signalling pathway. IL-7 receptor α chain (IL-7Rα) and γ-common (γc) chains components of the IL-7R undergo dimerisation upon IL-7 binding. Thereby, the IL-7R heterodimer triggers activation of JAK3 and JAK1 kinases bound to the intracellular domain of cc or IL-7Rα, respectively. JAK1 induces phosphorylation of the IL-7Rα intracellular domain and promotes recruitment of PI3K and STAT proteins. Phosphorylation of STAT proteins by JAK results in their dimerisation and translocation to the nucleus and finally in the transcription of target genes including Bcl-2, SOCS-1, CyclinD1 and c-myc. In addition, PI3K recruited to the intracellular domain of IL- 7Rα becomes activated and phosphorylates Akt, which thereafter promotes cell survival through degradation of pro-apoptotic proteins such as Bad and Bax and glucose uptake through the expression of GLUT1. IL-7R signalling also involves activation of the ERK pathway

Fig. 4 Experimental strategies used to analyse the impact of Notch1 signalling in human T-cell development. Ligand-independent constitutive Notch signalling is induced in human ETPs by retroviral transduction using a bicistronic vector encoding active Notch1 (ICN1) and green-fluorescent protein (GFP) as a cell tracer. For ligand-dependent Notch activation, ETPs are co-cultured onto OP9 stromal cells ectopically expressing the Dll1 Notch ligand (OP9–DL1). In both systems, cells are cultured in the presence of a multilineage-cytokine cocktail that supports the simultaneous differentiation of lymphoid (T and NK cells) and myeloid (macrophages and DCs) lineages from ETPs.

Fig. 5 Schematic representation of human IL7R and mouse *Il7ra* gene promoters. Human *IL7R* and mouse *Il7ra* gene promoters expand about 2,000 base pairs (bp) upstream of the transcription initiation site. Both promoters share two TATA-like boxes and an ETS transcription factor- binding site. The human *IL7R* promoter also contains a CSL-binding site that is conserved in the mouse *Il7ra* promoter. The physiological also relevance of the latter has yet not been confirmed.

Fig. 6 Mechanisms of IL-7R activation under physiological and pathological conditions. a. Under physiological conditions, double negative thymocytes (DN Thy) express both IL-7Rα and γc chains in monomeric conformation. Binding of IL-7 produced by TECs to the IL-7R components promotes their heterodimerisation, and thereby triggers IL-7R signalling leading to survival and proliferation. **b.** In pathological conditions, as in T-ALL, IL7R mutations at the juxtamembrane domain result in the formation of stable IL-7Rα-IL-7Rα homodimers, which promote signalling in the absence of IL-7, thus inducing uncontrolled survival and proliferation.

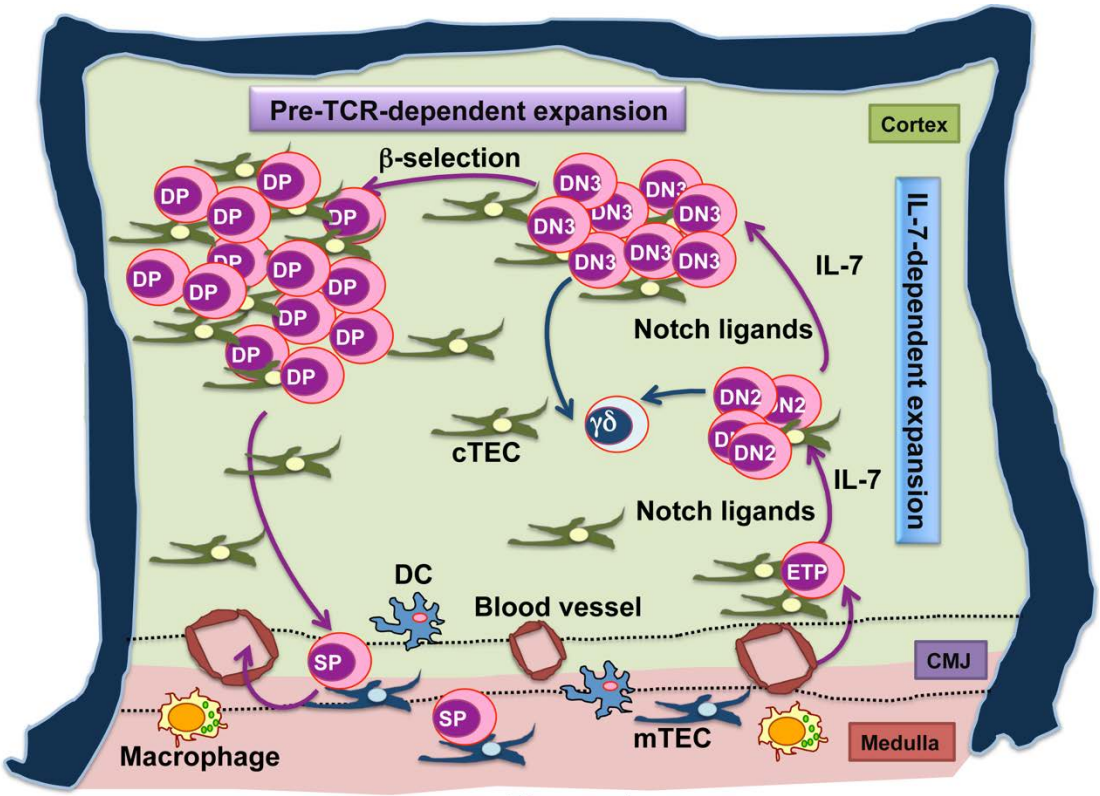

Pre-TCR-dependent expansion
Cortex
β-selection
DP
DN3
IL-7
Notch ligands
IL-7-dependent expansion
γδ
cTEC
DN2
IL-7
Notch ligands
DC
ETP
Blood vessel
CMJ
SP
Macrophage
mTEC
Medulla

Figure 1

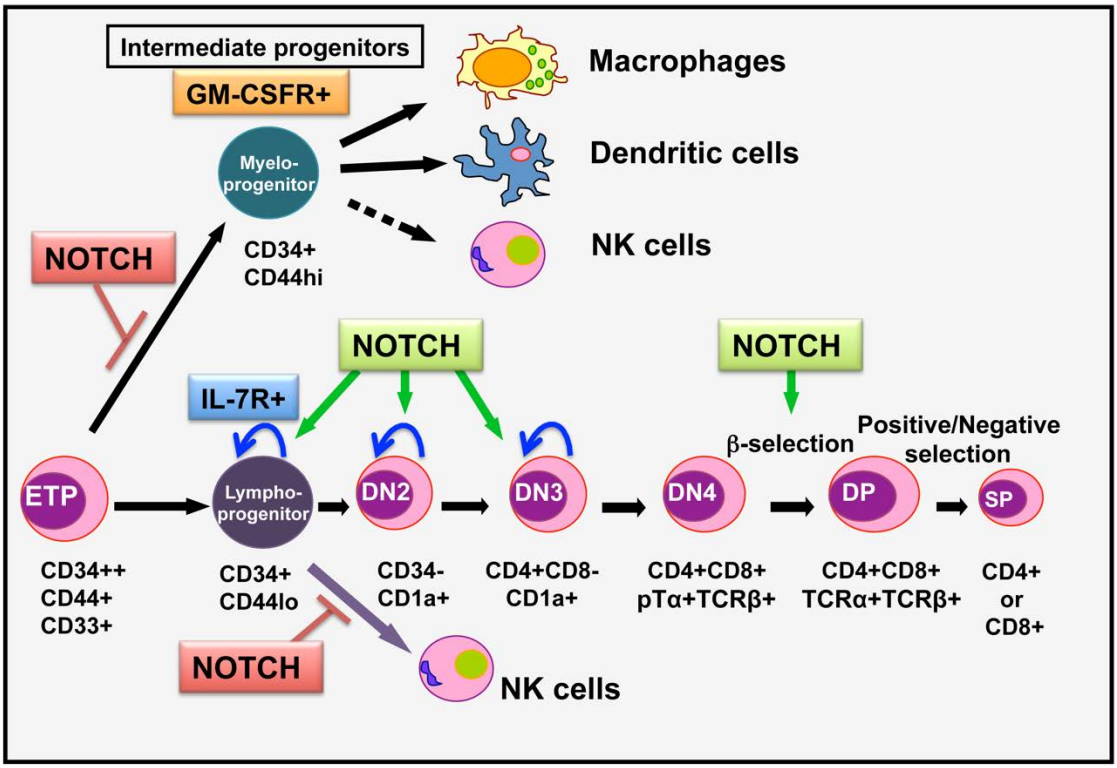
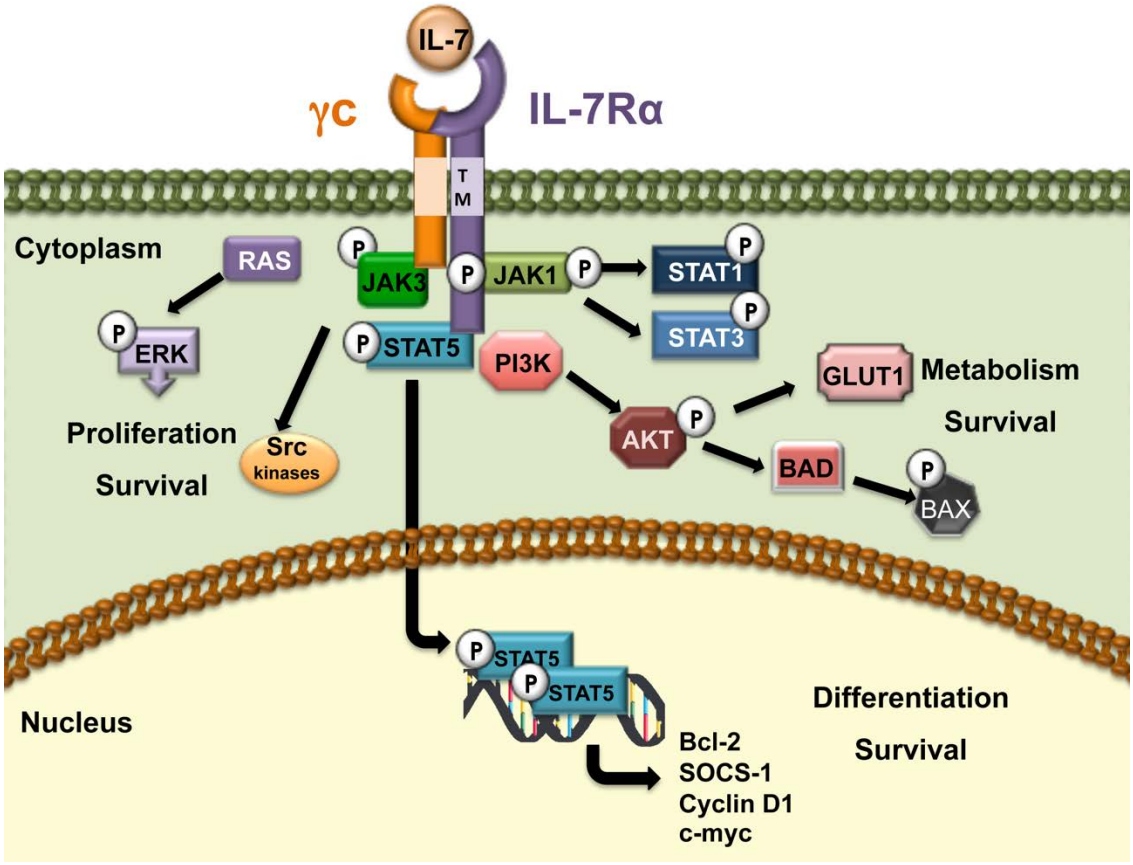

Intermediate progenitors
GM-CSFR+
Myelo-progenitor
CD34+
CD44hi
Macrophages
Dendritic cells
NK cells
NOTCH
NOTCH
NOTCH
IL-7R+
β-selection
Positive/Negative selection
ETP
Lympho-progenitor
DN2
DN3
DN4
DP
SP
CD34++
CD44+
CD33+
CD34+
CD44lo
CD34-
CD1a+
CD4+CD8-
CD1a+
CD4+CD8+
pTα+TCRβ+
CD4+CD8+
TCRα+TCRβ+
CD4+
or
CD8+
NOTCH
NK cells

Figure 2

Figure 3


IL-7
γc
IL-7Rα
T
M
Cytoplasm
RAS
JAK3
JAK1
STAT1
STAT3
ERK
STAT5
PI3K
GLUT1
Metabolism
Survival
AKT
Proliferation
Survival
Src
kinases
BAD
BAX
Nucleus
STAT5
STAT5
Differentiation
Survival
Bcl-2
SOCS-1
Cyclin D1
c-myc

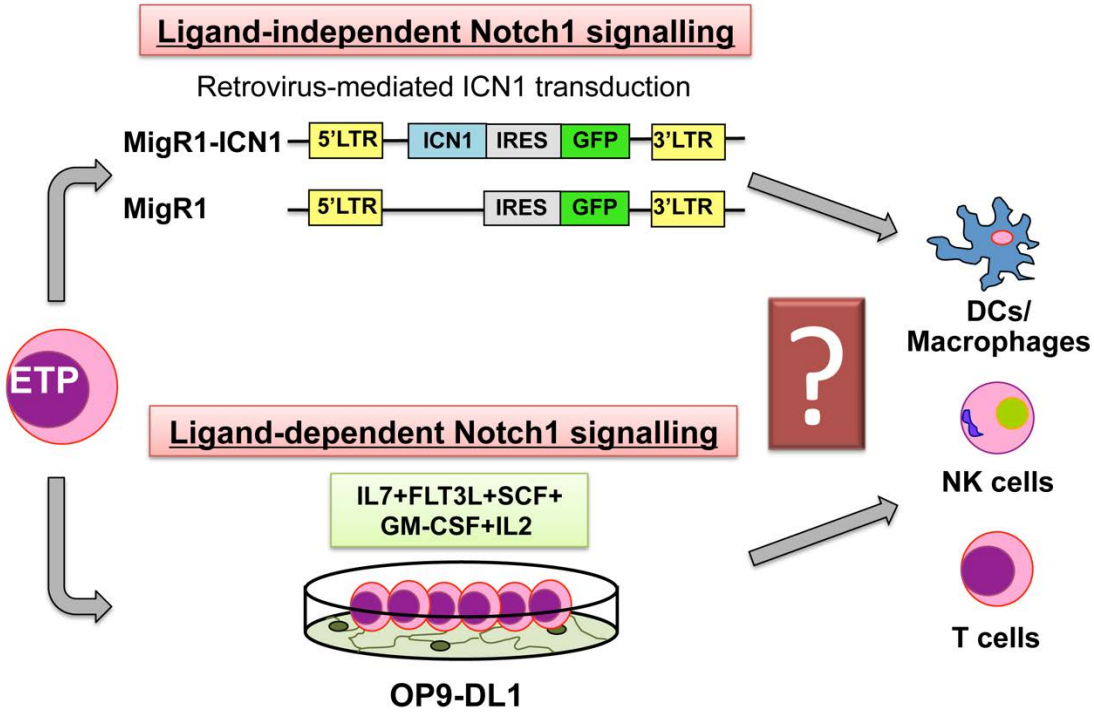

Ligand-independent Notch1 signalling
Retrovirus-mediated ICN1 transduction
MigR1-ICN1
5'LTR
ICN1
IRES
GFP
3'LTR
MigR1
5'LTR
IRES
GFP
3'LTR
DCs/
Macrophages
?
ETP
Ligand-dependent Notch1 signalling
IL7+FLT3L+SCF+
GM-CSF+IL2
NK cells
T cells
OP9-DL1

Figure 4

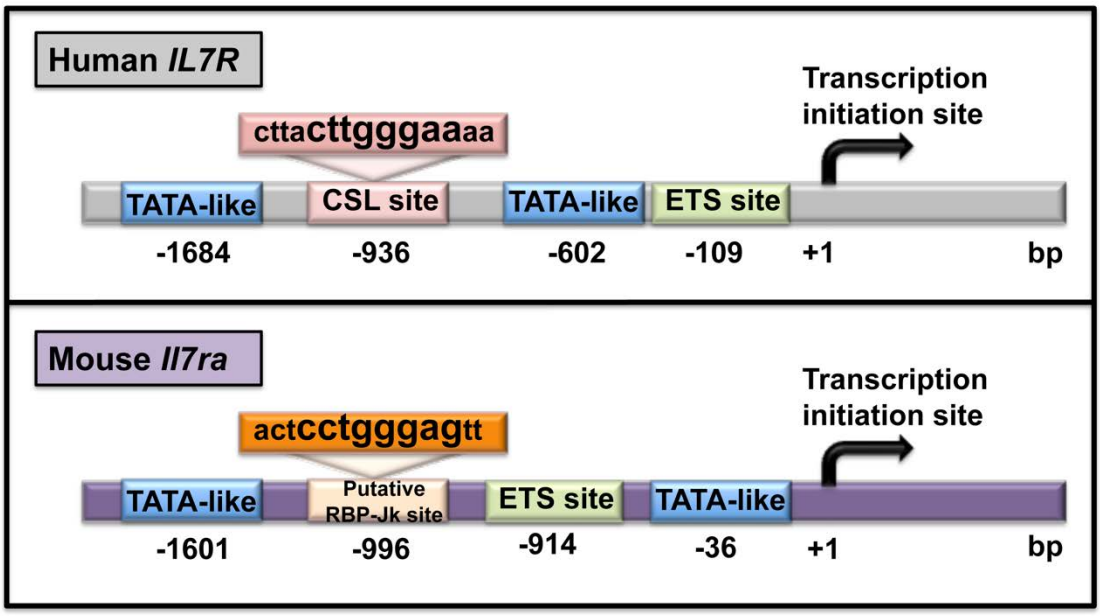

Human IL7R
cttacttgggaaaa
Transcription initiation site
TATA-like
CSL site
TATA-like
ETS site
-1684
-936
-602
-109
+1
bp
Mouse Il7ra
actcctgggagtt
Transcription initiation site
TATA-like
Putative RBP-Jk site
ETS site
TATA-like
-1601
-996
-914
-36
+1
bp

Figure 5

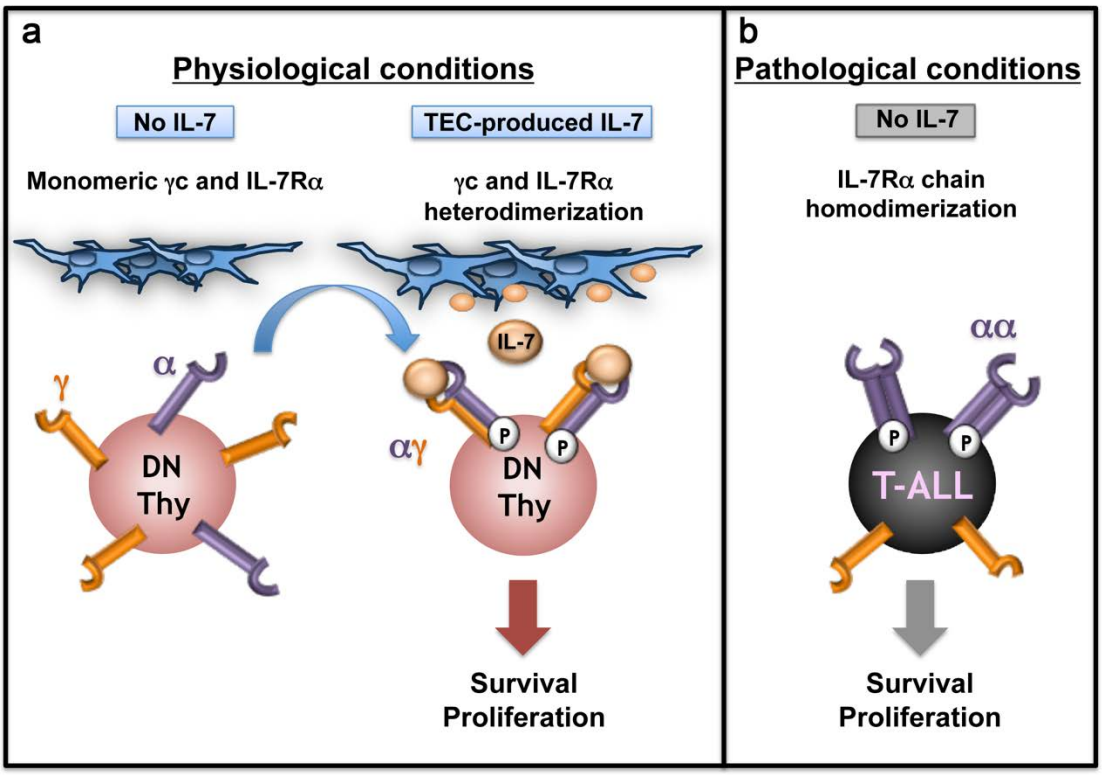

a
Physiological conditions
No IL-7
TEC-produced IL-7
Monomeric γc and IL-7Rα
γc and IL-7Rα
heterodimerization
IL-7
γ
α
αγ
P
P
DN
Thy
DN
Thy
Survival
Proliferation
b
Pathological conditions
No IL-7
IL-7Rα chain
homodimerization
αα
P
P
T-ALL
Survival
Proliferation

Figure 6