Mechanisms of Notch signaling specificity in lymphocytes and their leukemic counterparts
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Chapter 7

General Discussion
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Signaling pathways have evolved to tightly control cellular development and function in response to external stimuli. When such pathways are aberrantly (in)activated in immune cells, protection from infections cannot be facilitated, and harmful, undesired immune responses or leukemia can be the consequence.

A signaling cascade that plays a central role in the hematopoietic system is Notch. This pathway appears to be strikingly simple, yet, paradoxically, exerts a plethora of functions and can have diverse or even opposite cellular effects. Considering that transcriptional changes in the signal receiving cell are the main output of the pathway, a major research question is how cellular context, timing and signal strength are integrated in Notch target gene regulation. Different mechanisms can be envisioned that govern Notch target gene expression and different cellular outcomes. Throughout this thesis, we studied such mechanisms in the context of (normal) lymphocyte development and function and their leukemic counterparts.

In the following, we will discuss our findings about different mechanisms of Notch signaling specificity from a genetic, developmental and biomedical perspective.

Notch induces multiple T helper cell differentiation programs

The fundamental paradox of the pathway is impressively illustrated by its role in the polarization of Th cell fates: Despite being seemingly simple, Notch signaling has been implicated in the differentiation of virtually all known Th cell lineages\(^1,2\). Until now, the mechanisms by which Notch can induce these distinct lineages are largely unknown.

Since the only redundancy of Notch pathway components lies in the receptor and ligands, one could envision that different receptor/ligand interactions might result in distinct Th cell lineages. Indeed, it initially appeared that the different families of ligands might generate some lineage specificity, with Dll inducing Th1 cells and Jag mediating Th2 cell differentiation\(^3\). However, we now showed that this distinction is not exclusive, since one ligand could induce multiple different Th cell fates (chapter 6). Also, we did not find evidence supporting the notion that different receptors were involved, as all lineages depend on the same receptors, Notch1 and Notch2\(^4,5\) (chapter 6).

How then does Notch regulate adoption of different Th cell fates? As the mechanisms driving Th2 cell differentiation by Notch are reasonably well understood, we here initially focused especially on the mechanisms driving Th1 cell differentiation. It was proposed that Notch might use non-canonical signaling to mediate Th1 cell induction\(^6,7\). However, our data demonstrate that this is not the case, as Notch mediated Th1 differentiation depended on
RBPJ, as does Th2 cell differentiation (chapter 6). Canonical, RBPJ dependent signaling also underlies differentiation of Th9, Th22, Treg and Tfh cells by Notch (Helbig & Amsen, unpublished data), indicating that Notch regulates all these Th cell fates by direct transcriptional mechanisms. Indeed, Notch directly activates the lineage specific effector genes Il-4 and Gata3 in Th2 and Il-17 and Rorc, the gene encoding RORγt, in Th17 cells. Therefore, it is likely that Th1 effector genes are also directly regulated by Notch. We found that the Ifng gene contains evolutionarily conserved RBPJ binding sites in known enhancer regions and is rapidly upregulated upon stimulation with Notch ligands (chapter 6). While preparing this manuscript, Ifng and Tbx21 were confirmed as direct targets of Notch in Th1 cells in a Chromatin Immunoprecipitation study. Thus, Notch activation induces different Th cell fates using the same canonical signaling events, which directly activate lineage specific effector genes. Therefore, the outcome of Notch activation in naïve CD4+ T cells must be determined by other means. Crosstalk with other pathways may to some degree be involved, as has been demonstrated for Smad3 signaling in Th9 differentiation.

Interestingly, a recent report found that Notch regulated expression of the key transcription factor genes in the Th1, Th2 and Th17 programs regardless of the cytokines present. These findings suggest that Notch may not instruct one Th cell fate over alternate ones, as was proposed previously (discussed in). Earlier studies have demonstrated that addition of exogenous cytokines can overcome cytokine production defects in Notch loss of function models. Importantly, Bailis and colleagues found that Notch inactivation by GSI affected Th cell differentiation when polarizing cytokines were diluted, but not in the presence of highly concentrated cytokines. This was true for Th1, Th2 and Th17 cells. They therefore conclude that Notch mediated differentiation occurs predominantly when cytokine levels are low.

Our data also show that Notch activates critical T helper cell differentiation genes. Like Bailis et al., we find evidence that Notch directly controls expression of the signature Th1 gene Ifng. Furthermore, we show that Notch induces expression of a number of cytokine receptor genes. Correspondingly, activation of Notch sensitizes CD4+ T cells to various cytokines. Thus, activation of Notch made these cells much more responsive to IL-4 and allowed induction of Th17 cells by IL-6 even in the absence of (deliberately added) TGFβ. Responsiveness to IL-12 was not enhanced, on the other hand. Together with the study by Bailis, our results suggest that Notch acts as a general enabler of CD4+ effector T cell differentiation. Furthermore, our results show that the precise T helper lineage differentiation program chosen depends to a large degree on the cytokines present. However, Notch activation may also intrinsically promote differentiation of Th1 cells, as this was not affected by the presence of IL-12. This mechanism provides at least a partial explanation for the different reported outcomes of Notch signaling. Pathogen specific T cells which have received a Notch stimulus use these cytokines more effectively than bystander T cells. Expression of ligands
for Notch on APC responds to microbial triggers. This role of Notch may provide the molecular underpinning for the earlier reported requirement for cis delivery of antigen receptor ligand and instructive signals by one and the same APC (Sporri et al): only T cells directly interacting with APC that have phagocytosed microbial cargo present microbial peptides and express the Notch ligands required to jump start T helper cell differentiation.

In a more general sense, these results illustrated how the single Notch pathway can control very distinct types of differentiation, by enabling broad differentiation capacity and allowing additional signals to impart direction to the response.

**Notch induces survival and metabolism programs to promote cellular longevity of CD4+ T cells**

Control of viability is another avenue, which allows a pathway to regulate the fates of very different types of cells. In this thesis, we studied the involvement of Notch in the regulation of survival of CD4+ T cells. Effective T cell responses not only require differentiation of naïve CD4+ T helper cells into the Th cell lineage best suited to fight the encountered pathogen, but these cells also need to persist long enough to allow for sterile clearance of the infection, making cellular longevity an important checkpoint. We demonstrate here that Notch functions as a critical regulator of this checkpoint by inducing anti-apoptotic programs that prolong the lifespan of CD4+ T cells (chapter 5).

We found that stimulation of naïve CD4+ T cells with the Notch ligand Dll4 evokes a broad survival program which protects these cells from both extrinsic and intrinsic apoptosis pathways. The induction of this program depends on RBPJ, strongly suggesting that direct transcriptional mechanisms are involved, although the relevant direct targets downstream of Notch remain to be identified. In addition, Notch activation mobilizes metabolic pathways in CD4+ T cells, which allow them to endure the demands associated with extensive proliferation. This way, we have expanded our knowledge of the functions of Notch signaling in the communication between APC and T cells beyond differentiation.

These findings have important biomedical implications: In order to prevent harmful immune responses to innocuous antigens originating from commensal bacteria, food and self antigens expansion of antigen specific T cells needs to be tightly controlled. Owing to this tight regulation, it is difficult to elicit strong T cell responses to protein antigens, a hurdle for vaccination strategies. To overcome this limitation, vaccines rely on the use of adjuvants such as aluminium hydroxide. Importantly, recent reports have shown that the efficacy of T cell responses to recombinant protein antigens used in vaccines does not critically depend on the initial expansion of antigen specific
T cells, but rather hinges on their capacity to survive\textsuperscript{15-17}, a process which we reveal to be regulated by Notch signaling. Moreover, many of the anti-apoptotic genes induced by Dll4 in CD4\textsuperscript{+} T cells are frequently deregulated in different types of cancers, including Notch dependent T-ALL, where these events often correlate with chemotherapy response or resistance\textsuperscript{18-22}. Furthermore, aberrant Notch signaling has been associated with metabolic changes in malignant T cells\textsuperscript{23} (see also chapter 4). Thus, prolonged cellular longevity might also be underlying some of the oncogenic functions of the pathway.

Taken together, our studies in Th cells (chapters 5 and 6) demonstrate that Notch directly induces cellular survival and differentiation programs required for productive immune responses against microbial pathogens. That way, the immune system economically uses the same ancient pathway to mount different immune responses tailored for the encountered infection while limiting harmful cellular responses.

At the crossroads – Notch signaling regulates T cell versus ILC2 development in a signal strength dependent manner

Another important mechanism allowing for different outcomes of Notch signaling is the variation of signal strength. Here, we demonstrate that Notch signaling can induce differentiation of T cells and ILC2 from the same thymic progenitors, and identify Notch dosage as the determinant of this fate decision (chapter 2), with lower doses inducing T cells and stronger signals favoring ILC2 (chapter 2). Thereby, we establish that ILC2 development can be induced by Notch also in humans, and provide another example where Notch signaling specificity can be achieved by modulation of the signal strength.

Exactly how different levels of Notch signaling are achieved in the context of T cell and ILC2 differentiation has to be studied in more detail. It is conceivable that the population of progenitors is heterogeneous with regard to their sensitivity towards Notch activation. Indeed, we did find that the efficacy of ILC2 induction \textit{in vitro} varied between precursors from different donors, suggesting that precursor cell intrinsic differences exist in the ability to differentiate into ILC2 in response to Dll1. This could be a consequence of different upstream responsiveness of Notch to Dll1, due to posttranslational modifications of the receptors or different expression of factors in the pathway. Fringe glycosylases represent one major class of Notch modifiers, which strongly affect Notch signal strength in response to different ligands\textsuperscript{24,25}. These enzymes are expressed in CD34\textsuperscript{+}CD38\textsuperscript{-} progenitors from human cord blood\textsuperscript{26} and might also be present in thymic progenitors. Current techniques have only allowed examination of the expression of these enzymes at the population level. It is therefore conceivable that a hitherto unrecognized heterogeneity in their expression exists, which would evoke
differential sensitivity to Notch ligands among individual cells in a seemingly homogeneous population.

Variation inDll1 induced ILC2 development was greatest among CD34⁺CD1a⁻ progenitors from human fetal thymus (R. Gentek, unpublished results). Preliminary data (R. Gentek) indicate that the capacity to develop into ILC2 upon Notch triggering might correlate with the levels of CD34 expression. Further separation of (fetal) thymic progenitors might provide better insights in these questions. Of course, differential ability to activate Notch is only one of the possible explanations for this variation, as for instance also commitment to other lineages may differ between precursors obtained from different donors. Nonetheless, this variation is at least consistent with the possibility that precursor cell intrinsic differences in Notch responsiveness exist. It would be useful to directly measure activated Notch levels on an individual cell basis to compare cells from different donors and correlate this with the efficacy of ILC2 development.

Apart from cell intrinsic mechanisms, Notch sensitivity could be influenced by factors in specialized niches, as they might be provided for instance by the thymic microenvironment. Interestingly, we found that co-culture with OP9 cells expressing Dll1 was much less efficient at eliciting ILC2 development in human thymocytes than in mice. Moreover, we did not consistently observe ILC2 differentiation upon co-culture with OP9 cells expressing Dll4, which has also been shown to induce ILC2 in mice, or Jag1 and Jag2. Furthermore, the efficiency of ILC2 differentiation was not influenced by the addition of IL-33 (R. Gentek, unpublished results), which drives in vitro ILC2 development in conjunction with Notch in mice. It is possible that the signals provided by these ligands in the OP9 culture system are not strong enough to induce efficient ILC2 differentiation from the progenitors used here. An important consideration in this regard may be that OP9 is a murine cell line and that some of the (co-)signals for ILC2 development delivered by these cells are not (fully) crossreactive between mouse and human.

The sensitivity of hematopoietic progenitors towards Notch might be modulated upon entry of the thymus. Thus, while strong signals are required for ILC2 in the thymic environment to allow distinction with the T cell lineage, lower signals might be sufficient to induce ILC2 development in the bone marrow. Further studies will have to investigate these possibilities and determine the exact requirements for Notch in ILC2 development and function, especially in vivo.

A role for thymic ILC2 differentiation?
We identified an ILC2 population in the human thymus, and demonstrated that human thymic progenitors have the potential to develop into ILC2 upon Notch activation in vitro (chapter 2). These findings are of particular interest considering the ongoing debate about the exact site(s) of ILC2
development.

ILC2 populations have been described in the bone marrow\textsuperscript{28,29} and recent reports claim to have identified a bone marrow ILC2 precursor in mice\textsuperscript{30,31}. However, this LSIG (LinSca1\textsuperscript{hi}Id2\textsuperscript{hi}GATA3\textsuperscript{hi}) population appears to have already committed to the ILC2 fate\textsuperscript{31}, and therefore might represent an immature ILC2 stage rather than a \textit{bona fide} precursor. ILC2 are genetically close to early T cell progenitors\textsuperscript{32}, suggesting that common precursors might exist which adopt the T cell or ILC2 fate depending on microenvironmental signals, including Notch. As T cells develop in the thymus, it seems possible that ILC2 can also develop intrathymically. At apparent odds with this model, mice carrying \textit{FoxN1} mutations (nude mice), which cause defects in thymic development, possess normal numbers of ILC2\textsuperscript{27,33}. While this demonstrates that ILC2 development can occur at extrathymic sites in mice, whether ILC2 differentiation normally also takes place in the thymus was not investigated.

Migration to the thymus is a possible explanation for the thymic population we observed, as ILC2 have also been found in the blood\textsuperscript{34}, facilitating homing to different sites. However, the thymus is a sterile internal organ, and migration to this site seems unlikely for cells that are known to function in border protection. Instead, the presence of ILC2 suggests that ILC2 might indeed develop locally in the thymus (as well). This notion is further strengthened by the finding that ILC2 are also present in human fetal thymus, and at higher frequencies than after birth (M. Munneke, unpublished observations). It is possible that primitive ILC2 differentiation occurs in the (fetal) thymus and is gradually taken over by the bone marrow, whose development succeeds the thymus\textsuperscript{35}. However, our data indicate that thymic ILC2 differentiation persists long after the onset of marrow hematopoiesis, which takes place around the 11\textsuperscript{th} week of fetal development\textsuperscript{35}.

Considering the dependence on strong Notch signals, the thymus might offer optimal conditions for ILC2 development: Thymic epithelial cells as well as other components of the stroma, such as thymic DCs, express Notch ligands\textsuperscript{26,36}. Furthermore, soluble factors which modify the amplitude of Notch signaling\textsuperscript{37} are secreted by epithelial and non-epithelial cells\textsuperscript{38}. In this regard, it is noteworthy that induction and outgrowth of the thymic epithelial anlage do not depend on \textit{FoxN1}\textsuperscript{39,40}. Mutations in \textit{FoxN1}, which cause the ‘nude’ mice phenotype and DiGeorge syndrome, instead affect epithelial cells. It is tempting to speculate that the thymic rudiment in nude mice allows for (some) ILC2 development, and thus contributes to the normal numbers of ILC2 found in these mice.

Thus, although it is now widely believed that ILC2 development does occur in the bone marrow in mice, whether ILC2 can also develop at distinct sites is not clear. Thymic progenitors retain the capacity to develop into ILC2 in both mice and humans, and the identification of a thymic ILC2 population
indicates that ILC2 can indeed also develop intrathymically in humans. Nonetheless, our data do not rule out that the thymic ILC2 population is a result of migration to this organ. In future studies, the possibility of thymic ILC2 development could be addressed more directly using labeled human hematopoietic progenitors and human thymic grafts in immunodeficient (Rag2^{-/-}IL-2Rγ^{-/-}) mice, similar to what has been reported for the development of plasmacytoid dendritic cells (pDCs).

**Old and new paths to lymphocyte diversity – on the developmental and evolutionary origin of ILC(2) and T cells and the role of Notch**

The different ILC lineages share a number of features, on the basis of which it has been postulated that they have a common developmental origin: ILC derive from the CLP and, with the exception of IL-7R group 1 ILC such as NK cells, require IL-7 for homeostatic survival. Importantly, all ILC depend on the transcriptional repressor Id2.

Id proteins are helix-loop-helix proteins that can be considered the natural antagonists of the class of E (E box binding) protein transcription factors. In contrast to E proteins, Id proteins lack a basic DNA binding domain, characterizing them as transcriptional repressors. Id proteins heterodimerize with E proteins through their helix-loop-helix domains, preventing E proteins from binding to DNA and thus, essentially exerting a dominant-negative effect on E protein function. E proteins are known to regulate cell fate decisions in the hematopoietic system. The accurate initiation of lineage specific programs therefore critically depends on the ratio of E proteins and the counteracting Id proteins. Of the four mammalian E proteins, the E2A splice forms E12 and E47 are of particular importance for commitment to and differentiation of B and T cells, as they regulate the gene networks of transcription factors governing these processes.

Considering that ILC depend on Id2, whereas E protein function is essential to B and T cell development, it has been suggested that the expression of Id2 might be a distinguishing criterion between a common ILC precursor and progenitors with B and T cell potential. However, the exact onset and regulation of Id2 expression during lymphocyte development is not known, and our knowledge of the further differentiation of ILC downstream of the CLP or a common ILC precursor is still limited. Nonetheless, recent studies challenge the model that B/T cell precursors are separated from a common ILC precursor by the expression of Id2, a notion which is supported by our finding that thymic progenitors can develop into both T cells and ILC2 (chapter 2).

Thus, an updated model of lymphocyte development is emerging: Induction and concerted action of E2A and the lineage defining transcription factor Pax5 induces B cell commitment and differentiation of the CLP. Upon (early) encounter of Notch signals, the developmental potential of the CLP derived population is restricted to the T and ILC lineages (including NK cells,
ILC2 and LTi cells), whereas CLP that have not received Notch stimulation at an early stage give rise to ILC3. According to recent studies in mice, the progenitors downstream of the CLP can be distinguished on the basis of \(\alpha_4\beta_7\) integrin expression: The \(\alpha_4\beta_7^+\) population bears developmental potential for T cells, ILC2, NK and LTi cells, whereas \(\alpha_4\beta_7^-\) cells are precursors to ILC3\(^\text{45}\). However, the human progenitor(s) resembling the murine \(\alpha_4\beta_7^+\) and \(\alpha_4\beta_7^-\) population have yet to be determined.

Further development of both T cells and the different ILC lineages depends on lineage defining transcription factors and, importantly, the availability of Notch signals. Intriguingly, ILC3 are reported to directly derive from the progenitor initially not primed by Notch (the \(\alpha_4\beta_7^-\) cells in mice), but do require Notch and Id2 expression at a later stage for full maturation\(^\text{51}\). On the other hand, these cells can also develop from LTi cells, and ILC3 can in turn give rise to IFN\(\gamma\) producing ILC1 under certain conditions, for instance upon retrieval of IL-7\(^\text{52}\), indicating that there is substantial plasticity within group 3 ILC as well as between ILC3 and ILC1. Exactly what determines terminal differentiation and/or plasticity of ILC, and whether Notch signaling plays a role in these processes, remains to be determined.

Interestingly, it has been reported that restriction to the T/NK/ILC2/LTi (ILC3) lineages following early Notch activation (marked by murine \(\alpha_4\beta_7\) expression in mice) coincides with the upregulation of Id2\(^\text{53}\). This is in line with the notion that B cells diverge from the CLP prior to this stage, and thus prior to Id2 expression, which is detrimental to B cell development\(^\text{54}\). However, this model implies that Id2 is expressed in progenitors bearing T cell potential. In this regard, it is important to note that early stages of T cell development appear to not be influenced by Id2 overexpression\(^\text{55}\). Our data on human ILC2 differentiation support a model in which T cell and ILC2 progenitors separate late during development. A precursor with potential for both lineages is likely contained within the cells seeding the thymus, as the earliest thymic progenitors differentiate into ILC2 in response to Dll1 induced Notch signaling, while they gradually lose this capacity upon commitment to the T cell lineage in both mice\(^\text{27}\) and humans (chapter 2).

The factors governing the decision between the T cell and ILC2 fates remain to be identified. However, our data indicate that the dosage of Notch signaling is a critical determinant of cell fate at the (hypothetical) T/ILC2 branch point. Importantly, (mature) ILC2 and (early) T cell progenitors share the expression of a gene set classically attributed to T cell progenitors\(^\text{32}\). Thus, while this genetic program remains largely active in mature ILC2, further transcriptional changes occur during later steps of T cell maturation. In humans, Notch signals are high at early stages of T cell development, but have to decrease subsequently to allow for the generation of \(\alpha\beta\) T cells\(^\text{56}\). Indeed, we demonstrate here that sustained high levels of Notch favor ILC2 over T cell development (chapter 2). As discussed above, the exact factors determining differential signal strength remain to be identified. In the light
of the developmental model discussed above, we speculate that differences in Id2 expression may account for differential action of E proteins between (later) T cell progenitors and ILC2.

A candidate target of Notch which is essential to both (early) T cell stages as well as ILC2, is Tcf1. Notch directly activates Tcf7, the gene encoding Tcf1, through a distant acting enhancer and forced expression of Tcf1 induces T cell development even in the absence of Notch. Tcf1 expression is downregulated after the DP stage of T cell development, but is required for (final) maturation and function of ILC2. Intriguingly, a recent report showed that also Notch dependent development of NCR+ ILC3 requires Tcf1, indicating that this signaling axis might be of general importance for Notch mediated ILC differentiation. Taken together, although much remains to be learned about the exact factors driving ILC development and the developmental divergence of T cells and ILC(2), Notch signaling is emerging as a factor commonly involved in ILC. Our data indicate that one mechanism by which Notch can induce multiple different cells fates is differential signal strength. Considering the analogy between ILC and Th cells and our findings that Notch can induce genetic programs mediating the differentiation of various Th cells (chapter 6), mechanisms other than signal strength, for instance external cytokine stimuli, are likely to also play a role.

These notions further underline that T cells and ILC are closely related, in terms of function, developmental requirements and the expression of genes classically attributed to early T cell progenitors. In fact, ILC in many ways look like T cells without antigen receptors. It is, therefore, attractive to hypothesize that ILC are evolutionary precursors of T cells. Adaptive immune cells with Immunoglobulin-like antigen receptors emerged with the diversion of jawed (Gnathostomes) from jawless vertebrates (Agnathans), and indeed, homologues of recombination activating genes first appear in cartilaginous fish (Chondrichthyes), the first jawed vertebrates. The latter, which are represented by current day hagfish and lampreys, possess cells which resemble B and T lymphocytes, but use a mechanism different from B and T cell receptor rearrangements to generate antigen receptors. It has been speculated that IL-17 producing ILC appeared first during evolution with the appearance of the lymphotoxin-β receptor in mammals. However, considering that even the earliest jawed vertebrates, Chondrichthyes, are already fully equipped for an adaptive immune response, it is possible that (primitive) ILC existed long before. Notably, FoxN1 expressing thymus like structures were recently identified in the gills of lampreys. Considering the data discussed here, these structures could allow for the development of ILC(2) like cells in specified niches.
Identification of ILC2 like leukemia and a possible oncogenic role for Notch

Our finding that ILC2 development is favored by strong Notch activation (chapter 2) led us to hypothesize that oncogenic activation of Notch, by its very nature an exaggerated signal, might elicit ILC2 like leukemia (ILC2-LL) from hematopoietic progenitors. Since ILC2 were only recently described, their malignant counterparts have likely not been identified as such and are currently misannotated. In chapter 3, we addressed the possibility that oncogenic Notch induces ILC2-LL, and identified candidates for this malignancy by gene expression analyses.

We found that T-ALL derived, constitutively active mutant NOTCH1 alleles give rise to ILC2 development in human thymocytes. Furthermore, using gene expression profiling guided by the current knowledge about the ILC2 lineage, we were able to identify ILC2-LL in different cohorts of T-ALL and, strikingly, AML patients. It will remain to be established to what degree these cells constitute the true malignant counter part of ILC2 or are of mixed lineage instead. More confidence on this would require a more profound definition of normal ILC2, for instance provided by genome wide expression signatures from these cells. Furthermore, it would be important to conduct a immunophenotypic analysis as well as a functional studies of cytokine production (IL-5, IL-13) and responsiveness to relevant cytokines (IL-25, IL-33, TSLP). Yet, the data presented here underline the usefulness of gene expression studies for the identification of (new) leukemic entities, especially in the light of our currently limited knowledge about ILC. Gene expression profiling has been valuable for detailed stratification of both solid tumors and hematological malignancies in the past decade. Ever since an early seminal study demonstrating that ALL and AML specimens could be distinguished based on their genetic signatures, such approaches have been successfully used for the identification of previously unrecognized subgroups. Additionally, expression signatures of already defined subsets can be exploited for predictive purposes, which has immediate clinical implications. With the increased application of next generation sequencing, the importance of gene expression profiling for leukemia classification will likely increase even further.

Accurate classification of hematopoietic malignancies according to the normal lineage they most closely resemble is of clinically important, as different subtypes display distinct clinical behavior, and the treatment choice often differs largely between these groups. Defining new leukemic entities therefore has immediate clinical implications, especially for heterogeneous diseases like T-ALL and AML, and within subgroups of otherwise weakly defined, atypical malignancies.

Our analyses imply Notch signaling in candidate ILC2 malignancies, supporting the hypothesis that Notch has an oncogenic function in these
cells. Notch is generally believed to have a tumor suppressor function in myeloid leukemia\(^{67,68}\) and mutations activating the pathway are very rare. Interestingly, the rare mutations that have been described are associated with poor prognosis and appear to be limited to atypical cases of AML\(^{69,70}\). We reveal that a previously described atypical group of AML, characterized by activating mutations in \(NOTCH1\) that cause epigenetic silencing of \(CEBPA\)^{70}, might represent ILC2 like rather than myeloid leukemia. This offers the exciting possibility that Notch signaling could potentially be targeted therapeutically in these leukemias. Although current strategies to target Notch are inefficient or suffer from adverse, dose limiting side effects, alternative approaches are heavily investigated\(^{71}\).

Our data specifically identify a subset of \(CEBPA\) silenced leukemias that were originally annotated as atypical AML, yet bear activating mutations in \(NOTCH1\), as ILC2-LL. Considering that these malignancies also express the myeloid antigens CD13 and CD33, it is possible that they represent truly bilineal leukemias. However, whether normal ILC2 express CD13 and CD33 is currently unknown. A bilineal nature of these candidate ILC2-LL would be consistent with the hypothesis that these malignancies result from the transformation of early progenitors, as both CLP and early thymic progenitors are known to retain myeloid potential\(^{72,73}\). Lineage infidelity might be the consequence of aberrantly activated signaling pathways which partially ‘override’ differentiation programs. Indeed, we found that strong, sustained Notch activation gives rise to ILC2 even from thymic progenitors that have already committed to the T cell lineage (chapter 2). It is becoming increasingly recognized that hematopoietic lineages are rather plastic, as is the case for instance for T helper cell subsets\(^{74,75}\). Similarly, lineage plasticity is heavily debated for ILC lineages, in particular within the ILC3 subset and between those and ILC1\(^{76}\). Such lineage plasticity might also be underlying cases of bilineal/biphenotypic leukemias, or even rare cases of ‘lineage switch’ leukemias, and might also be reflected in the ‘aberrant’ expression of CD13 and CD33 by \(CEBPA\) silenced candidate ILC2-LL. Importantly, however, this leukemic entity was significantly different from both typical AML and T-ALL\(^{70,77}\), supporting the notion that these indeed are malignancies of ILC rather than myeloid cells or T cells. Such lineage plasticity considerations further underline the need for accurate leukemia classification according to the normal hematopoietic cell type those malignancies resemble most closely, for which gene expression profiling is a valuable tool.

Taken together, we present evidence for the existence of ILC2-LL, and demonstrate that these are likely characterized by oncogenic activation of Notch signaling. These findings have potential implications for patient stratification, treatment choice and possibilities of therapeutic targeting, and therefore warrant further research into malignancies of ILC(2).
Dimeric Notch signaling – A dual track to lymphocyte development and malignancies?

Finally, a mechanism underlying potentially separable functions of Notch is provided by its dimerization on suitable regulatory elements found in some targets\(^78,79\) NOTCH1 homodimerization involves three residues in the ANK domains of neighboring NICD molecules\(^78\). Studies in mice showed that T cell development and induction of T-ALL critically depend on this signaling event\(^80\). Inhibition of dimerization failed to interfere with most functions of Notch, on the other hand. Therefore, targeting the dimeric interface was suggested as a promising new therapeutic approach, predicted to reduce adverse effects by selectively affecting oncogenic, but not the many other functions of Notch. Targeting of the protein-protein interactions constituting the dimer interface is now technically feasible, as demonstrated in seminal studies using either small molecules\(^81\) or peptides\(^82\). However, it had not been shown whether dimeric Notch signaling also operates in humans. Given the important biomedical implications, we studied the role of this very specific signaling mode in human lymphocyte development and T-ALL (chapter 4).

Requirements for Notch dimerization in human lymphocyte development

Our studies revealed that, in addition to signal strength (chapter 2), NOTCH1 homodimerization is an important mechanism underlying Notch mediated ILC2 differentiation (chapter 4). Using a mutant affecting one of the critical NOTCH1 homodimer interface residues, R1985, we consistently obtained much fewer ILC2 when compared to wild-type NICD1. The responsible targets in Notch mediated ILC2 differentiation remain to be identified. Considering that we also showed that ILC2 development is favored by strong Notch activation, it is important to note that the dimerization mutant does not affect general Notch signaling and overall signal strength (chapter 4). Therefore, Notch mediates human ILC2 development through at least two independent molecular mechanisms, high dosage and dimerization. Notch may induce a program encompassing both dimerization dependent and independent genes to direct the ILC2 fate. Alternatively, the two may be linked: the chance to simultaneously bind two Notch molecules in one region is lower than the chance to bind a single molecule. It is therefore conceivable that strong signal strength is required for ILC2 development to reach a sufficiently high concentration of NICD molecules to allow effective simultaneous binding to SPS elements.

These data demonstrate that, as a mechanism, dimeric Notch signaling is conserved between mice and humans, a finding which is of biomedical relevance by itself. To our surprise, however, our study revealed that human T cell development does not require dimeric Notch signaling, in sharp contrast to murine T cell differentiation. We found that dimerization mutant (R1985A) NICD1 induced T cell differentiation with similar efficiency as wild-
type NICD1, likely because this mutant was capable of inducing expression of PTCRA, the gene encoding the pre-TCR alpha chain. Expression of this chain is an important prerequisite to formation of a functional pre-TCR complex and thus, successful completion of β-selection. In striking contrast, murine thymocytes expressing dimerization mutant NICD1 fail to induce ptcra, and are consequently blocked at the β-selection checkpoint. We demonstrate that differences in the composition of the human and murine PTCRA enhancer likely account for this discrepancy.

A (critical) role for NOTCH1 homodimerization in human T-ALL?

In mice, T cell leukemogenesis critically depends on dimeric Notch signaling: mice reconstituted with wild-type NICD1 expressing cells rapidly developed aggressive T-ALL with complete penetrance, and this was completely abrogated when mice received cells expressing dimerization mutant NICD1. The same report showed that continued growth of two murine T-ALL lines requires dimeric Notch signaling.

Since directly investigating the onset of T-ALL is not possible in humans, we studied whether NOTCH1 homodimerization is equally essential for maintaining growth of a panel of human T-ALL lines (chapter 4). Given that therapies per definition do not target the induction phase of leukemia, we contend that studying the effects on growth of established T-ALL is clinically relevant. Unexpectedly, we found that all tested lines grew independently of dimeric Notch signaling. Importantly, this was also the case for a number of murine T-ALL lines different from the ones reported by Liu and colleagues. We therefore firmly establish that dimerization independent growth is very common amongst T-ALL lines of murine and human origin.

The Notch target responsible for dimerization dependent growth of the mouse T-ALL lines T6E and G4A2 is c-myc, a potent oncogene which is also essential for human T-ALL. A possible explanation for dimerization independence might therefore be that human T-ALL lines can induce this gene via different pathways. Translocations are probably not an alternative mechanism for c-myc activation, as dimerization-independence was also observed in T-ALL lines established from Rag2 mice, in which rearrangements are less likely. However, c-myc is downstream of a multitude of cellular pathways other than Notch, such as canonical Wnt signaling. Intriguingly, activation of Wnt through stabilization of β-Catenin is one of the mechanisms involved in T-ALL induced by loss of PTEN, which the murine lines we studied here were deficient for. Furthermore, c-myc is regulated post-transcriptionally, for instance by microRNAs. Finally, c-myc protein levels are controlled post-translationally, for example through the Ubiquitin ligase Fbxw7, which is frequently inactivated in murine and human T-ALL as a consequence of mutations in the fbxw7 gene or via repression by the oncogenic transcription factor Tal1.
Of note, although several possible CSL binding sites have been identified near the proximal c-myc promoter and in a putative enhancer, the exact mechanisms driving transcription of c-myc by dimeric complexes have not been identified so far. It is possible that expression of c-MYC can also be induced by NOTCH1 monomers in humans, similar to what we describe here for PCTRA. This notion is supported by the finding that R1985A fully rescued growth of human T-ALL lines, although GSI treatment strongly reduces c-MYC levels in these cells. Direct measurements of the c-MYC levels will be important to determine whether expression of this factor is indeed independent of NOTCH1 dimerization in humans. Another Notch target regulated by a combination of monomeric and dimeric complexes is HES1, which is essential for maintaining growth of TALL1, one of the T-ALL lines studied here. Hence, the residual sensitivity of HES1 for dimeric Notch could also support dimerization independent growth of these cells.

Because our study was restricted to established T-ALL lines, we cannot exclude that primary T-ALL are dependent on dimeric Notch signaling. Recently improved techniques to culture and genetically modify primary human T-ALL cells might allow addressing this possibility in the future. However, we demonstrate that dimerization independence is a common feature of most T-ALL cell lines, suggesting that mechanisms circumventing the need for dimeric Notch signaling are rather easily activated. We therefore consider it unlikely that targeting the NOTCH1 homodimer will have the desired therapeutic effect in (human) T-ALL.

In contrast to the lack of requirement for dimeric Notch in established T-ALL, we found that expansion of primary thymocytes does partially depend on dimeric Notch. Most notably, dimerization mutant NICD1 inhibited thymocyte expansion in a dominant-negative fashion when endogenous Notch was activated through ligand binding. These findings could be explained by a model in which monomeric and dimeric complexes induce antagonistic pathways, in addition to a role for Notch dosage: Until a certain dosage, monomers activate growth-promoting programs. Monomeric signals that have exceeded the limit tolerated by thymocytes liberate growth-inhibitory mechanisms instead. These are normally counteracted by dimeric Notch signaling, which predominantly induces growth-promoting (or survival) programs. Expression of dimerization mutant NICD1 from the retroviral vector may by itself elicit levels below the threshold for the postulated mobilization of growth inhibitory pathways. When endogenous Notch is activated by Dll1, however, the levels exceed this threshold. Because the counterbalancing activity of the dimers is inhibited by the dimerization mutant, the net result is inhibition of growth.

As it can be argued that exposure to Notch ligands is the physiological context for Notch mediated oncogenic transformation, we do believe our data support a role for NOTCH1 homodimerization in the onset of T-ALL. Similarly, an oncogenic role for dimeric Notch signaling is also conceivable.
for ILC2 like leukemia, considering that Notch signaling appears to be involved in these malignancies (chapter 3), while dimeric Notch signaling is important for ILC2 development (chapter 4).

Future studies could benefit from the use of humanized immune system mice to further elucidate the requirements for and the mechanisms underlying NOTCH1 homodimerization in the context of human T-ALL. This is of great importance in the light of possible therapeutic exploitation, which appears to be generally possible by small molecules or peptides. Furthermore, careful analysis of the gene expression programs mobilized by monomers and dimers will be necessary to determine whether growth inhibitory pathways are indeed specifically induced by dimerization mutant NICD1, as we postulate here, and if so, which ones.

More complicated than it looks - Regulation of Notch target gene expression by (homo)dimerization

Our studies illustrate that the mechanisms underlying dimeric Notch signaling are still poorly understood. In the following, we will discuss some of the questions which have remained unanswered thus far.

One potential caveat studies regarding the role of NOTCH dimerization presented here and done by others is the possibility that the R1985 mutant have residual ability to homodimerize. Although we did not find evidence for this in transient transfection experiments, mechanisms may exist in vivo that enhance dimerization and therefore still allow some dimerization dependent activity to be exhibited by this mutant. Important in this regard may be the contribution of K1946 and E1950 to the dimer interface. Based on the capacity to drive reporter gene expression under the control of the stereotypical SPS motif derived from the HES1 promoter, R1985 appears to be the most important residue. The striking differences found between this mutant and wild-type NICD1 confirm the critical importance of this residue. Yet, K1946 and E1950 contribute to the dimeric interface with the formation of salt bridges, which is of functional importance, as indicated by the partial abrogation of dimerization dependent target gene expression by their mutation (R. Gentek, unpublished data). Furthermore, mutation of the analogue of R1985 diminishes homotypic association of the Drosophila ANK domain in solution, but does not completely abrogate it. Thus, although the Arginine at position 1985 undoubtedly is critical, R1985A might allow for residual dimerization. In this regard, it is important to note that thus far, all studies have relied on mutational analysis of one dimerization residue at a time. In order to gain more insights into the mechanisms underlying Notch dimerization, future studies will have to further mutate the dimer interface, combining mutations of R1985 and the residues forming salt bridges. Nonetheless, the fact that the R1985A mutant clearly was ineffective at promoting differentiation of human ILC2 does show that even if it has residual homodimerization capacity in vivo, this ability must be much
reduced even in that setting.

A major open question concerning NOTCH1 homodimerization is where the assembly of dimeric complexes takes place. Homotypic association of Drosophila ANK domain fragments was detected in a cell free system by analytical ultracentrifugation\textsuperscript{104}. This interaction critically depends on the residue corresponding to mammalian R1985\textsuperscript{104}, indicating that Notch homodimerization can indeed occur in solution, but with very low affinity ($K_d$ in the millimolar range)\textsuperscript{104}. However, the multimeric intermediate claimed to be required for the assembly of functional NTCs is not affected by the R1985A mutation\textsuperscript{105}, suggesting that homodimers are not formed freely in the nucleoplasm. Thus, it appears most likely that dimerization of pre-bound monomeric NTCs occurs on the DNA. This does not necessarily involve classical SPS motifs, as dimerization may occur between CSL binding elements at distant sites due to chromatin looping.

Subject to debate is currently also the question whether dimeric Notch signaling is a specific event restricted to a small number of target genes, or might represent a more common signaling mode. This is of immediate biomedical relevance, considering potential approaches to target the dimeric interface. A conclusive answer to this question is unfortunately complicated by our very limited knowledge about dimerization sensitivity on a genome-wide scale. A recent ChIPseq study found that head-to-head orientation and a spacer of 11-21 base pair length, the classical SPS architecture, were the likeliest composition of motifs that contained more than one RBPJ binding site, but the overall frequency of such elements was very low\textsuperscript{106}. However, several studies have experimentally defined dimerization dependent target genes whose CSL binding elements substantially diverge from this classical SPS composition: The sequence of the second site can be non-canonical, comprising a ‘cryptic’ low affinity site for CSL\textsuperscript{79,80}. Moreover, the two sites might be separated by more than a short spacer. In fact, even CSL sites located at very distant sites potentially allow for dimerization, as discussed above. Therefore, restricting genome-wide analyses to classical SPS elements likely underestimates the abundance of dimerization dependent sites. Indeed, using a refined algorithm based on experimental protein binding array data, another group recently found that an overall of 40\% of dynamic sites contain SPS motifs (J. Aster, personal communication/unpublished data). The fact that SPS motifs were selectively enriched at dynamic, but not static sites indicates that these are functionally relevant to target gene expression. Yet, whether these genes indeed strictly depend on Notch dimerization or might be regulated by a combination of dimeric and monomeric complexes as we demonstrate here for PT CRA will need to be determined experimentally. Considering the limited effects dimerization mutant NICD1 has, as also shown in this thesis, it seems unlikely that strict dependence on dimeric Notch signaling is common amongst Notch targets. Yet, it is still possible that dimeric complexes amplify Notch target gene expression more widely. Thus, dimeric Notch signaling might serve a dual function in activating
a specific, small subset of genes that do not get activated by monomeric complexes, and in more broadly fine-tuning expression of Notch targets that do however not critically depend on dimeric complexes. Genome wide transcriptome analysis will yield insights into the general contribution of Notch dimerization to the full array of Notch dependent functions.

Another layer of complexity is added by the possibility that the other Notch isoforms can also homodimerize. This idea is supported by the remarkably high conservation of the ANK domain both between species and isoforms. Indeed, in vitro assays did not detect differences in the capacity of NICD1-4 to form NTCs on DNA containing SPS motifs\textsuperscript{78,79}. However, the dimerization residues are not completely conserved between isoforms, as for instance the analogous residue of NOTCH1 K1946 is an Arginine in NOTCH4. Intriguingly, NICD4 functionally resembles dimerization mutant NICD1 in many ways: Ectopic NICD4 expression does not induce T-ALL in bone marrow transfer experiments in mice and fails to rescue the growth of dimerization dependent murine T-ALL lines from inhibition of γ-secretase or induced withdrawal of c-myc\textsuperscript{107}. Indeed, NICD4 does not induce c-myc expression in mice\textsuperscript{107}. These findings are consistent with the possibility that NICD4 is unable to homodimerize. It is also conceivable that different NICD isoforms can engage in dimerization through heterotypic contacts between their ANK domains. In support of this possibility, NICD2 and NICD3 have been identified as direct binding partners of NICD1 in T-ALL cells\textsuperscript{108}. NICD4, however, did not bind to NICD1 in these cells, further support (albeit circumstantial) for the notion that this isoform might not be capable of ANK domain mediated dimerization, be it of heterotypic or homotypic nature.

A central question is how transcriptional activation by dimeric and monomeric Notch differ molecularly. We think it likely that dimeric and monomeric NTCs provide different surfaces for co-factors. Different co-factor requirements could explain why a monomeric NTC containing dimerization mutant NICD transactivates from single CSL binding sites equally well as wild-type, but is defective on SPS motifs. Strikingly, as demonstrated here (chapter 4), mutation of one of the CSL binding sites in a SPS motif does not render dimerization mutant NICD1 capable of transactivating this motif, although in theory a normal single binding site is created. This demonstrates that the coactivators recruited by monomeric Notch are not capable of transactivating from such elements. It seems likely that this largely depends on the context of the enhancer/promoter involved. Apparently some specific activity is required for its transcriptional activation and this activity is likely recruited only by dimeric Notch. In addition to enabling selective target gene expression, co-factors might also function to stabilize dimeric complexes. Comparing the proteome binding to wild-type NICD and dimerization defective mutants in a similar approach to previously published work\textsuperscript{108} will enhance our understanding of the molecular mechanisms underlying target gene expression by Notch in general, and dimeric complexes in particular.
Concluding remarks

The studies presented in this thesis addressed (molecular) mechanisms underlying the striking ability of the Notch pathway to elicit very different responses. We established a new role for Notch signaling in promoting cellular metabolism and longevity of T cells, which has important implications for both vaccination strategies as well as approaches to target Notch in their leukemic counterparts. Our data shed further light on how Notch performs an ‘old’ function by demonstrating that Notch directly activates multiple different T helper cell programs depending on the cytokines present; a mechanism which economically uses the same pathway to enable immune responses tailored to the threat encountered. We provide another example of different outcomes by Notch signaling in the same cell by showing that Notch induces both T cell and ILC2 development from thymic progenitors depending on the strength of the signal. In line with these findings, our data support an oncogenic role for Notch in ILC2 like leukemia, a previously unrecognized class of malignancies resembling ILC2, which we here identified in cohorts of T-ALL and AML patients using a bioinformatic approach to gene expression analyses. Finally, we demonstrate that NOTCH1 homodimerization is a conserved mechanism of selective target gene expression also in humans. However, unlike in mice, this signaling mode is not critical in mediating human T cell development and sustained T-ALL growth, predicting that targeting of dimeric Notch complexes is unlikely to have desired therapeutic success.

In conclusion, our studies provide further examples for and mechanisms underlying the basic paradox that this seemingly simple pathway has diverse and in some cases opposite effects. In the light of approaches to exploit this pathway therapeutically, it is of critical importance to understand the molecular mechanisms underlying the different functions of Notch, a puzzle to which we add some pieces with this thesis.
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