How Notch and Wnt make T-cells tick

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Chapter 8

General Discussion
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The immune system has coevolved with pathogens challenging us every day. In this arms race, where one side constantly tries to outsmart the other, the immune system made use of conserved building blocks to create new means of defense. How the immune system uses two such building blocks, the Wnt and Notch pathway, to generate optimal T-cell responses is the topic of this thesis.

The studies described provide insight into the various functions of Notch signaling in lineage differentiation of CD4+ T-cells as well as in modulating the size of the immune response. A role for Notch in CD8+ T-cell responses with regard to the binary cell fate decision of short-lived effector cell (SLEC) versus memory precursor cell (MPEC) development is described and the role of Wnt signaling in driving Th2 cell differentiation is readdressed.

Notch and the adjuvant effect

CD4+ Th-cells activate and direct immune effector cells in order to combat infections. To this end, CD4+ T-cells must first differentiate from naïve cells into various Th-cell lineages with specific effector functions\(^1\), tailored for optimal defense against the pathogen encountered. Sensing of the pathogen is done by APCs, not T-cells. Thus, it is critical that the information about the type and severity of an infection is passed on to T-cells accurately. A pathway critically involved in mediating this information between APC and T-cell is the Notch signaling pathway. In this thesis, we identified a novel function for Notch in the communication between APC and T-cell, not directly related to differentiation. Instead, we showed that Notch regulates the magnitude of the immune response by promoting cellular longevity. The data presented in chapter 2 demonstrate that stimulation of naïve CD4+ T-cells with the Notch ligand DLL4 induces a broad anti-apoptotic program, which protects these cells against both intrinsic (mitochondrial) and extrinsic (death receptor mediated) apoptosis pathways. Furthermore, cellular metabolism is stimulated allowing the cells to better cope with the metabolic demands associated with rapid proliferation during an immune response. The anti-apoptotic program induced by Notch involves a transcriptional mechanism, as evident by the dependence on RBPJ. Which of the induced factors are direct targets for activation by Notch-RBPJ and whether non-canonical and/or non-transcriptional mechanisms are also involved, remains to be investigated.

Effective T-cell responses require both strong expansion of antigen-specific clones as well as persistence of the expanded repertoire for long enough to achieve sterile cure. Both aspects are tightly controlled to avoid potentially harmful responses to innocuous antigens, such as those associated with food and commensal bacteria. The downside of this tight control is that it has been difficult to elicit strong T-cell responses to recombinant protein antigens for the purpose of vaccination, necessitating the use of adjuvants\(^2\). Much attention has been given to the requirement for classical costimulation, for instance through activation of CD28, to allow expansion of CD4+ T-cells\(^3\). However, it has turned out that initial expansion in response to protein antigens occurs normally \textit{in vivo} even in the absence of adjuvant\(^4–6\). Instead, the major checkpoint in these settings has proven to be the ability of the expanded cells to survive. Our findings have identified Notch as a regulator of this late acting quality control mechanism. Ligands for Notch are expressed on APC upon engagement of pattern recognition receptors by PAMPs\(^7\). Therefore, Notch activation can be used by the immune system to determine if the presence of an antigen forms a threat and deserves the generation of an aggressive immune response. A benefit from using a
membrane-bound stimulus like a Notch ligand (as opposed to cytokines) is, that bystander cells do not profit from this survival signal, meant to expand a pool of T-cells specific for the current threat.

**Notch controls CD4⁺ effector Th-cell differentiation**

Differentiation of naïve CD4⁺ T-cells into the various Th-cell subsets is an important step in directing the immune response to the various types of pathogens. Mounting the wrong type of immune response will fail to protect and might cause damage to the host. Therefore, differentiation of Th-cells is a well-controlled process. The influence of cytokines on the differentiation of CD4⁺ T-cells has been studied intensively. Additionally, it has been appreciated that membrane bound signals serve a function in the process of CD4⁺ Th-cell differentiation. One such signal is provided by Notch ligands, expressed on APCs.

The Notch pathway has been implicated previously in the differentiation of a variety of CD4⁺ Th-cell lineages, including Th1, Th2 and Th17. Functional relevance has been demonstrated for some, but not all lineages in vivo. However, so far it is not clear how the direction of the differentiation in response to Notch is determined. The two families of Notch ligands seem to provide some kind of lineage specificity as Jagged ligands were implicated in the differentiation of Th2 cells and DLL ligands in Th1 cell differentiation. Still, these ligands do not provide exclusive lineage determination signals, as we found that one ligand can induce multiple lineages (chapter 3). Furthermore, induction of all lineages seems to depend on the same Notch receptors (Notch1 and Notch2). Thus, how the Notch signaling pathway can induce so many diverse and sometimes even opposing cell fate decisions remains puzzling. It had been speculated that canonical versus non-canonical Notch signaling would provide an explanation, although this idea has not been supported by direct evidence. In particular, such an explanation has been invoked to explain induction of Th1 versus Th2 cell differentiation by Notch. Thus, Th2 cell differentiation would depend on canonical (RBPJ-dependent) signaling, whereas non-canonical pathways would lead to Th1 cell differentiation. However, our experiments in chapter 3 show that RBPJ is required also for Th1 cell induction, disproving this hypothesis. As almost all of the other lineages seem to depend on canonical (RBPJ-dependent) signaling as well, other means must exist to determine the direction of differentiation in response to Notch stimulation. It seems very likely that interaction with other signaling pathways will hold some of the key. Our results support the notion that Notch may act as a general enhancer of effector cell differentiation by directly transactivating expression of effector cell genes. Thus, we show that the *Ifng* gene, arguably the most critical Th1 effector gene, is rapidly transcribed upon stimulation of Notch and that many enhancers of this gene contain conserved RBPJ binding sites, suggesting that this gene is indeed a direct target of Notch. Chromatin immunoprecipitation experiments will now be required to formally document a physical connection between Notch and this gene. Such direct control of Th1 effector function would echo earlier findings in Th2 cells, where Notch directly transactivates the *Gata3* and *Il4* loci and in Th17 cells, where the RORγt gene has been identified as a direct target. Interestingly, the data presented in chapter 3 demonstrate that Notch signaling also upregulates expression of a variety of cytokine and chemokine receptor genes on CD4⁺ T-cells, consequently making these cells more receptive to stimulation by soluble factors present during differentiation. It is tempting to speculate that this finding provides at least a partial explanation for how the direction of the differentiation could be determined. Depending on the factors present...
in the medium in vitro (e.g. different types of serum) or the cytokines produced during an immune response in vivo the T-cells may differentiate in a specific direction. Having received stimulation via Notch would provide them with a benefit compared to bystander T-cells (which have not been in contact with a Notch ligand expressing APC and might not have the proper TCR to detect the present infection), allowing pathogen specific T-cells to more effectively make use of the cytokines present.

Thus, our data demonstrate that the immune system uses the Notch pathway not only to convey quantitative but as well qualitative information about the present infection.

**Tfh cells constitute yet an additional effector cell type controlled by Notch**

The broad inflammatory disease in scurfy mice represents an interesting model to study key components important for cytokine production by CD4+ T-cells. Scuffy mice suffer from severe multi-organ inflammation due to excessive responses of multiple CD4+ Th-cell lineages, which occur due to the absence of functional Treg cells. Since Notch signaling has been implicated in regulating the differentiation of many CD4+ Th-cell subsets, this model was exploited in chapter 4 to address the question of how important RBPJ-dependent Notch signaling is for the development of the different Th-cell subsets in vivo. CD4+ T-cells from scurfy mice with CD4-specific deficiency for RBPJ showed reduced production of multiple cytokines, including the Th2 cytokine IL-4 and the Th1 cytokine IFNγ. Most remarkably, however, chapter 4 provides evidence for a hitherto unknown function of this pathway in Tfh cells. Tfh cells are specialized at providing help to B-cells. Their numbers are increased in scurfy mice and so are antibody titers. In contrast, scurfy mice carrying RBPJ-deficient CD4+ T-cells have strongly reduced isotype class switched serum antibody levels (chapter 4). Strikingly, RBPJ-deficiency did not abrogate the production of all antibody isotypes, but seemed specific for the Th2 dependent isotypes IgG1 and IgE. This is likely partially explained by the strong reduction in IL-4 production by RBPJ-deficient CD4+ T-cells. However, an additional explanation presented itself as RBPJ-deficient scuffy mice contain strongly reduced numbers of fully mature CXCR5+, IL-21 producing Tfh cells. CXCR5 is required for proper localization in germinal centers, where isotype class switching takes place and IL-21 is an important growth factor for B-cells. It is not clear as yet why this seemingly general defect in Tfh cells is not reflected in reduced titers of all antibody isotypes. One explanation for this could be that the unaffected isotypes represent maternal antibodies, given that their levels are not significantly elevated in scurfy mice, in sharp contrast to IgG1 and IgE. However, previous studies on RBPJ-deficiency in T-cells also found selective suppression of IgG1 and IgE but not other isotypes, in systems where such specificity could not be explained by a role for maternal antibodies. An intriguing possibility is that Notch exerts specific control over a sub-lineage of Tfh cells, namely those dedicated to the promotion of Th2 dependent antibodies. Indeed, the 3' enhancer HS5/CNS2, which was previously shown to be Notch responsive, was recently shown to be specifically active in Tfh cells and not in tissue Th2 cells. Furthermore, the presence of multiple potential RBPJ binding sites in genes associated with Tfh cells raises the possibility that Notch directly regulates the expression of these factors. Clearly, additional experiments are required to further characterize this role of Notch in Tfh cells. However, our results in chapter 4 did add yet another set of effector functions to the list of T-cell functions
controlled by Notch.

**Notch: the key to effector and memory CD8 T-cell development?**

CD8+ T-cells exhibit direct cytolytic activity and are involved in responses against intracellular pathogens. There is particular interest in strategies to optimize CD8+ T-cell responses against viruses like influenza A, which develop mutants capable of escaping immunoglobulin mediated defense by altering their coat, but have much less variation in their nuclear proteins targeted by CD8+ T-cells. Additionally, CD8+ T-cell responses have the potential to be exploited in the treatment of cancer, making the understanding of the details leading to optimal CD8 responses of particular interest.

In any infection, the adaptive immune system must strike a balance between committing resources towards immediate protection on the one hand, and laying the foundation for long term protection on the other. This issue has been studied extensively in CD8+ T-cell responses. Two types of cells are generated early in a CD8+ T-cell response: terminally differentiated SLECs for immediate protection and MPECs, which give rise to long-lived memory cells. Generating an appropriate number of SLECs to successfully combat an infection requires accurate sensing of the severity of an infection. This task, again, is taken by APCs which have to pass the information on to T-cells in the form of inflammatory signals. Greater inflammation signals greater infectious load and translates into the development of more SLECs. **Chapter 6** shows that Notch signaling is critically involved in this process. Notch receptor levels increase on CD8+ T-cells in response to inflammatory signals. Importantly, no SLECs are generated in the absence of Notch signaling. The precise mechanisms used by Notch for promoting differentiation of terminally differentiated effector cells are not yet clear. However, essential CD8+ effector cell genes, such as the **perforin** and **granzyme b** genes, were shown to be direct targets of Notch signaling previously, suggesting a rather direct mechanism. The most straightforward interpretation of these results is that Notch engagement drives adoption of the SLEC fate and that MPEC development occurs as a default pathway in the absence of Notch activation. However appealing such a model may be, one particular finding reported in **chapter 6** seems inconsistent with such a simple model. Although an elevated proportion of Notch-deficient antigen-specific CD8+ T-cells does develop into MPECs, survival of these cells is compromised at later stages in the response. One possible explanation could be Notch has different functions at different stages of the response. Notch signaling at the beginning of the response might lead to adoption of the SLEC fate, whereas MPECs would have to engage Notch at a later stage to be permitted to survive. Alternatively, Notch may act as a rheostat, such that strong Notch signaling translates into development of SLECs, whereas weaker Notch signals would prime MPECs for survival. Such a quantitative model would fit with published findings that a certain degree of inflammation is required for the generation of MPECs with long-term survival capacity.

**Wnt signaling in Th2 cell differentiation**

Like the Notch pathway, the Wnt signaling pathway is an evolutionary conserved building block used in many differentiation processes. It has been proposed that Wnt signaling would be critically involved in the differentiation of CD4+ T-cells by inhibiting Th1 and inducing Th2 differentiation. Critical support for this notion consisted of experiments...
using mice with genetic deficiencies in the Wnt pathway. As T-cell development in such mice is severely perturbed during thymocyte development, it was possible that the results obtained were caused by indirect effects rather than a direct role of this pathway on Th-cell differentiation. In chapter 7 this issue has been revisited using both gain and loss of function approaches. The results presented argue against a key function of Wnt signaling in Th2 cell differentiation. In vitro, recombinant Wnt did not affect differentiation of either Th1 or Th2 cells. Furthermore, Th2 responses to parasite antigens developed normally in mice lacking β-catenin, the major effector of canonical Wnt signaling. Nonetheless, we found evidence that Wnt signaling does have a role in Th2 cells. Analysis of Th1 and Th2 cells revealed that only the latter express receptors for Wnt (Frizzled). Hence, only these cells can respond to Wnt signals. We found that these signals are used to modulate survival of Th2 effector cells by inducing expression of Fas. Induction of cell death via Fas may prevent excessive T-cell responses. It has long been known that Th1 effector cells express higher surface levels of Fas and are much more susceptible to Fas mediated cell death than Th2 cells. Our data now reveal a mechanism allowing similar control of survival in Th2 cells. Since agonists for both Fas and Wnt are available, it would be attractive to explore the possibility to exploit this function of Wnt for therapeutic approaches in Th2 mediated diseases such as asthma and allergies.

In conclusion, we have shown that the ancient Wnt and Notch signaling modules have been coopted by the immune system to direct T-cell responses. Notch has turned out to be particularly dedicated to induction of effector cell differentiation and function. Lack of Notch activation may predispose cells to less terminally differentiated fates, as most prominently suggested by our results on CD8⁺ T-cells. A more restricted role seems to be played by Wnt, which selectively controls survival of a subset of effector cells. With these studies, we have identified some of the cogwheels and springs that control the inner workings of the immune system.

References

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