How Notch and Wnt make T-cells tick
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Chapter 7

Wnt Signaling is not Required for Physiological Th2 Cell Responses but Controls Th2 Effector Cell Viability

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Abstract

Different subsets of T helper (Th) effector cells orchestrate the immune response to different classes of microbes and are also responsible for distinct immune mediated pathologies. Th2 cells direct immune responses against helminth parasites and are involved in asthma. The Wnt signaling pathway has been implicated as a key inducer of Th2 cell responses. Here, we re-examined this issue using both gain and loss of function approaches. We found that deliberate activation of naïve CD4+ T-cells with recombinant Wnt proteins is not sufficient to elicit Th2 cell differentiation. Furthermore, Th2 cell responses to parasite antigens developed normally in mice lacking expression of β-catenin, the major effector of the Wnt-Frizzled pathway. Thus, our data do not support a major role for the Wnt pathway in differentiation of Th2 cells. Instead, we provide evidence that this pathway operates as a selective regulator of already established Th2 effector cells. By systematically analyzing the expression of receptors for Wnts in different CD4+ T-cell subsets, we show that Th2 but not Th1 effector cells express Frizzled receptors (Fzds). Accordingly, only Th2 cells can signal through the pathway. Finally, we show that this signaling serves to control survival of Th2 cells by inducing surface expression of Fas.

Introduction

Naïve CD4+ T-cells can differentiate into several major effector cell types, including those called T helper (Th)1 and Th2. Th1 cells regulate immune defense against intracellular infectious agents, while Th2 lymphocytes direct responses to extracellular parasites1,2. Th1 cells typically produce interferon-γ (IFNγ), while Th2 effector cells secrete interleukin-4 (IL-4), IL-5 and IL-13. Production of these cytokines is central to the function of these subsets of CD4+ T-cells1. Differentiation of these different lineages of Th-cells is governed by dedicated transcription factors. For instance, differentiation of Th2 cells depends on Gata3, which regulates transcription of critical Th2 effector genes both by direct transactivation as well as by epigenetic modification3.

Various immune mediated pathologies are dependent on responses of specific Th-cell subsets. For instance, allergies and asthma are predominantly caused by Th2 cell responses. Such selectivity provides a therapeutic opportunity: if individual Th-cell subsets can be targeted specifically, pathology may be curbed without undesired global suppression of the immune system.

Wnts are locally secreted lipid-modified glycoproteins, present in all metazoan animals. They play roles in cell fate determination, polarity, adhesion, migration, proliferation and cell death4. Wnts are highly conserved between species and share a minimal identity of 18% between each other5. The most characteristic part in their structures is a conserved 22-23-cysteine residue pattern, called Cysteine-Rich Domain (CRD) (Nusse, The Wnt homepage). There are 19 Wnt genes in mice and humans (Nusse, The Wnt homepage). The receptors for Wnts fall into two categories - the Frizzled (Fzd) and the LDL receptor related protein (LRP) families5. It is thought that two of the LRPs (LRP5 and 6) and all of the Fzds (Fzd1-10) can be engaged in a tripartite Wnt binding complex6.

The major effector of the Wnt pathway is β-catenin. In the absence of Wnt signaling, this protein is constitutively phosphorylated by casein kinase I (CKI) or by glycogen synthase kinase 3β (GSK-3β) in a complex with Axin and adenomatous polyposis coli (APC).
As a consequence, β-catenin is polyubiquitinated and targeted for degradation by the proteasome. Upon engagement by Wnts, Fzds activate a protein called dishevelled (Dsh), which prevents this constitutive degradation of the β-catenin protein. When stabilized, β-catenin translocates to the nucleus, where it activates TCF/LEF transcription factor complexes and initiates gene expression. These DNA binding factors act as transcriptional repressors through interaction with Groucho family proteins, but are converted into transactivators upon recruitment of β-catenin. In addition to cytoplasmic β-catenin, there is a pool of β-catenin proteins, which bind E-cadherins, and through α-catenin help organizing the cytoskeleton, thereby affecting cell adhesion and migration.

Wnts have been implicated in the self-renewal and maintenance of hematopoietic stem cells (HSCs), as well as in the maturation of B- and T-cells. There is significant evidence supporting the role of the Wnt pathway in thymocyte development. Thymocytes not only express some of the Fzd receptors, but also secrete various Wnts. Specific deletion of β-catenin in thymocytes leads to a blockade at the CD4+CD8+ double negative 3 (DN3)-to-DN4 transition, whereas β-catenin over-expression allows bypassing of β-selection and therefore causes accumulation of CD4+CD8+ double positive and single positive (DP and SP) thymocytes. On the other hand, artificial increase of the level of the Wnt inhibitor Axin produces reduced thymus size and massive apoptosis of cortical thymocytes.

Several studies have addressed the role of Wnt signaling in mature CD4+ T-cells. Wnt was reported to control migration by upregulating the expression of the two metalloproteinases – Mmp2 and Mmp9. Furthermore, both positive and negative effects of the Wnt pathway on CD4+ T-cell survival have been reported. On the one hand, expression of a stabilized, and therefore constitutively active form of β-catenin induced Bcl-XL mRNA upregulation and promoted Treg cell survival. On the other hand, stabilized β-catenin caused death in Staphylococcal enterotoxin B (SEB) challenged and TCR restimulated CD4+Vβ8+ lymphocytes. Finally, the Wnt pathway has been implicated in the generation or cytokine producing effector Th-cells: On the one hand, it was reported that TCF1 and β-catenin are critical for initiation of Th2 cell responses. Thus, TCF1 was found to bind one of the promoters of the Gata3 gene and deficiency for TCF1 or genetic inhibition of the interaction between β-catenin and TCF1 resulted in greatly reduced Th2 cell responses. Another study showed β-catenin can interact with the chromatin organizer SATB1 and may regulate expression of the Gata3 gene and hence Th2 cell responses. In contrast Lef1, which also interacts with β-catenin to activate transcription, inhibited production of IL-4, IL-5 and IL-13. Thus, although all these studies concluded that components of the Wnt pathway affect Th2 cell responses, there is no consensus whether this constitutes a positive or negative role. To some degree, these apparent discrepancies might be explained by the fact that TCF1 and LEF1 can act both as repressors and as activators depending on the presence or absence of β-catenin. Definitive proof for these functions would require the use of genetic loss of function experiments. Although such experiments have been performed, these have not been definitive. Thus, the models used have either relied on siRNA mediated knock down, which frequently have off target effects, or on genetic modifications which severely perturbed development of thymocytes and therefore may have yielded indirect phenotypes on Th-cell responses.

We here revisit the function of the Wnt pathway in CD4+ T-cells. By using β-catenin conditional knockout animals, we show that this pathway is not required for Th2 cell...
differentiation and cytokine production both in vitro as well as in vivo. However, we do find show that responsiveness to Wnt is a specific property of Th2 but not Th1 effector cells, as only the former express Frizzled receptors (Fzds). Finally, we show that this Wnt responsiveness regulates survival of Th2 cells by inducing expression of Fas.

Results and Discussion

**β-catenin is not required for thymic T-cell differentiation after CD4 is expressed.** The Wnt pathway has been implicated as an important regulator of Th2 cell responses. Critical evidence for this point consists of the reduction in such responses in mice carrying genetic deficiencies in the Wnt signaling pathway. Although consistent with a role for the Wnt pathway in Th2 cell responses, thymocyte development is already severely perturbed in these mice. It is therefore not currently possible to distinguish between a direct effect from Wnt signaling on Th2 cell responses and an indirect effect caused by atypical differentiation of CD4+ T-cells before induction of the Th2 cell response. To re-examine this issue, we generated mice in which the Wnt pathway was disabled in T-cells after the Wnt/TCF-1 dependent stages. As the canonical Wnt signaling pathway converges on β-catenin, we crossed mice with floxed β-catenin alleles to animals expressing the Cre enzyme under the control of the CD4 promoter (hereafter referred to as β-catenin knockout mice, for simplicity). Expression of this Cre transgene occurs after β-selection during thymocyte development and full deletion of floxed alleles does not occur until the CD4+ CD8+ stage of development. Analysis of cell lysates showed that both mature CD4+ and CD8+ T-cells in these mice lacked expression of β-catenin. However, overt defects in thymocyte development were not obtained by this strategy, as evidenced by the normal proportions of CD4+ and CD8+ T-cells in both Thymus and Spleen.

The Wnt/β-catenin pathway is neither required nor sufficient for Th2 cell differentiation in vitro. To examine whether the Wnt pathway is necessary for Th2 cell differentiation, we first resorted to widely used in vitro differentiation culture systems, in which Th2 cell differentiation is induced by the combination of TCR stimulation and exogenous IL-4. siRNA mediated knock down of β-catenin was previously found to reduce production of Th2 effector cytokines under these conditions. These culture conditions indeed induced high percentages of wild type CD4+ T-cells to differentiate into cells, which secreted high amounts of IL-4, but not IFNγ. Deficiency for β-catenin did not reduce either the proportion or the amount of IL-4 produced by Th2 effector cells generated this way. Likewise, no effect was found from β-catenin deficiency on IL-12 mediated differentiation of Th1 cells. Although IL-4 is a potent stimulus for Th2 cell differentiation, it is not required for many Th2 responses in vivo. Furthermore, addition of IL-4 may override the requirement for physiological differentiation signals. An example of this was found when the contribution of the Notch pathway in Th2 cell differentiation was examined. Therefore, we examined whether deliberate activation of the Wnt pathway might be sufficient to elicit Th2 cell differentiation by adding recombinant Wnt3a to CD4+ T-cell differentiation cultures. To determine whether this recombinant Wnt3a was capable of activating the Wnt signaling pathway, we examined whether addition of this factor resulted in nuclear accumulation of β-catenin protein. As positive controls, we activated mouse L-cells, known to signal through the Wnt pathway, under these conditions (Nusse, The Wnt homepage). In addition, we cultured cells in the presence of the non-specific activator of the Wnt cascade – LiCl. LiCl indirectly stabilizes β-catenin by inhibiting GSK-3β, a critical factor for its
Fig. 1. β-catenin is neither required nor sufficient for Th2 cell induction in vitro. (A and B) Naive CD4+ T-cells from C57BL/6 wild type - β-cateninfl/fl CD4-CRE (WT) (black bars) and their conditional knockout littermates - β-cateninfl/fl CD4-CRE (KO) (white bars) animals were polarized (Th1/Th2) in vitro in the presence of APCs. After five days, viable cells were purified by fycoll. (A) Subsequently, cells were re-stimulated for 48 hours with plate-bound anti-CD3. Cytokine production was measured by ELISA (representative of n=3 experiments). (B) Alternatively cytokine production was determined by intracellular flow cytometry. (C) Naive CD62L+CD44-CD25-CD49b+CD4+ T-cells were activated under Th0 conditions (no cytokines added) with plate bound antibodies to CD3 and CD28 alone or in the presence of the Wnt synthesis inhibitor IWP2 with or without recombinant Wnt3a. Differentiation was measured after 4 days by intracellular flow cytometry.

degradation. As a negative control, we used KC. Addition of recombinant Wnt3a clearly resulted in nuclear accumulation of β-catenin in CD4+ T-cells stimulated with antibodies to CD3 and CD28 (Fig. S2). Therefore, this set up can be used to test the consequences of deliberate activation of the Wnt pathway on Th-cell differentiation. To exclude possible indirect effects from Wnt pathway activation in APC we used a reductionist in vitro approach, in which rigorously purified CD62L+CD44-CD25-CD49b+naive CD4+ T-cells were activated with antibodies to CD3 and CD28 in the presence of absence of recombinant Wnt3a. Furthermore, T-cells could potentially produce Wnt proteins themselves, in which case addition of extra Wnt might not have much of an effect. For that reasons, we performed our differentiation cultures in the presence of IWP2, an inhibitor of Wnt synthesis. Using this approach, we did not find any effect from addition of Wnt3a on the proportion of CD4+ T-cells differentiating into either IL-4 producing cells or IFNγ producing cells (Fig. 1C). We conclude that activation of the Wnt pathway in CD4+ T-cells is not sufficient to induce Th2 cell differentiation in this reductionist setting, therefore.

The Wnt/β-catenin pathway is not required for Th2 cell responses in vivo. Although our in vitro culture experiments failed to reveal a major role for the Wnt pathway in differentiation of Th2 cells, it is possible that the conditions used did not fully mimic physiological Th2 cell differentiation. We therefore tested the requirement of the pathway using a powerful in vivo model for physiological Th2 cell responses. The major role for Th2 cells is to direct immune responses against parasitic infections. The eggs from the helminth parasite Schistosoma mansoni elicit strong Th2 cell responses in vivo and Th2 cell inducing activity is found in soluble extracts from such eggs (soluble egg antigen-SEA). Indeed, immunization of wild type mice with SEA resulted in the development of CD4+ T-cells, which transcribed the Th2 effector genes IL-4, IL-5 and IL-13 specifically upon restimulation with SEA in vitro (Fig. 2). Immunization of β-catenin knockout mice with SEA resulted in generation of effector cells, which produced similar amounts of mRNA transcripts for these Th2 effector cytokines. Thus, β-catenin is not required for the differentiation of Th2 effector cells in response to parasite antigen in vivo.
Th2 but not Th1 cells express RNA from Wnt receptors Fzd4, 6 and 7. Although the experiments described above did not support a major role for Wnt signaling in differentiation of Th2 cells, we considered the possibility that this pathway might serve a role in already established effector cells. To explore this possibility, we made a systematic analysis of the presence of the Wnt receptors in various CD4+ T-cell subsets. Since no good antibodies are available against mouse Fzds (we have found that commercial antibodies did not label well on western blots, AA, RAF and DA, unpublished results), we performed real time PCR reactions for all mouse Fzd receptors on cDNA prepared from \textit{in vitro} polarized Th1 and Th2 cells. Of the ten existing Fzd proteins, only Fzd4, 6 and 7 could

![Fig. 2 - β-catenin is not required for Th2 responses to parasite antigen \textit{in vivo}](image)

Littermate control β-catenin\(^{+/+}\) CD4-CRE (black bars-WT) and β-catenin\(^{0/0}\) CD4-CRE (white bars-KO) mice were immunized with SEA. Splenic CD4\(^+\) T-cells were re-stimulated with SEA \textit{in vitro}. Th2-specific cytokine mRNA expression was determined by quantitative RT-PCR (n=3).

![Fig. 3 - Preferential expression of Fzds on Th2 effector cells](image)

(A) Naive CD4 (CD4\(^+\) CD62L\(^{+}\) CD44\(^{low}\)) were purified and polarized \textit{in vitro} into Th1 and Th2 lymphocytes in the presence of APCs for five days, after which viable cells were isolated. Relative levels of Fzd genes in cDNA from naive CD4\(^+\) T-cells (light grey bars), Th1 cells (dark grey bars) and Th2 cells (black bars) were determined by quantitative RT-PCR and normalized against HPRT (representative of n=5 experiments). (B) Total RNA was isolated from Th1 and Th2 cells (generated in the presence of plate-bound anti-CD3 and anti-CD28) at different days (d) during polarization. Quantification for Fzd-4, Fzd-6 and Fzd-7 mRNA abundance was performed as in (A). (C) Th1 and Th2 cells were generated as in (A) from C57BL/6 (black bars), β-catenin\(^{+/+}\) CD4-CRE (grey bars-WT) and their conditional knockout littermates - β-catenin\(^{0/0}\) CD4-CRE (white bars-KO) mice and re-stimulated with or without Wnt3a for three hours. Quantification for Fzd-4, Fzd-6 and Fzd-7 mRNA abundance was performed as in (A). (D) Relative levels of the Wnt1 gene were determined by quantitative RT-PCR and normalized against HPRT. Memory, naive and (CD25\(^+\)) regulatory T-cells were used as comparison controls (n=3 experiments).
be detected. Strikingly, these Wnt receptors were selectively present in Th2 but not in Th1 lymphocytes, indicating that not all CD4+ T-cell subsets may be equally responsive to Wnt (Fig. 3A). Expression of the different Fzds appeared late in Th2 cell differentiation cultures, suggesting that responsiveness to Wnt is a property of fully differentiated Th2 cells (Fig. 3B). Although the Wnt pathway may promote expression of Wnt receptors in a positive feedback mechanism, expression levels of Fzds 4, 6 and 7 were comparable in wild type or β-catenin deficient CD4+ T-cells (Fig. 3C).

What might be the origin of the Wnt proteins to activate the pathway in Th2 cells? There are numerous sources for Wnts in mice. One possibility could be that T-cells themselves produce these proteins. Gene chip experiments in our lab have shown that the only Wnt RNA expressed by Th1/2 cells encodes Wnt1. These results were confirmed through RT-PCR (Fig. 3D). Since Wnt1 is redundant with Wnt3a, these results are consistent with the possibility that T-cells may in an auto- and/or paracrine manner be a source for activation of the pathway in Th2 cells, which have the receptors necessary for signaling.

Wnt activation leads to nuclear β-catenin translocation in Th2 but not in Th1 cells. Since Th2 but not Th1 lymphocytes express the receptors for Wnts, we expected that Th2, but not Th1 cells could signal through the Wnt pathway. Indeed, we found that Wnt3a induced nuclear translocation of β-catenin only in Th2 and not in Th1 cells (Fig. 4A). This translocation was likely caused by stabilization, as (both nuclear and cytoplasmic) protein, but not mRNA levels of β-catenin were changed post stimulation (Fig. 4B and S2). Although Wnt3a and Wnt5a are both capable of activating the pathway in thymocytes, Wnt5a did not induce nuclear translocation of β-catenin in Th1 or Th2 cells, suggesting the existence of a previously unrecognized layer of specificity. Strikingly, even LiCl, which acts downstream of the receptors, induces marked stabilization of β-catenin in Th2, but hardly in Th1 cells. Thus, the selective use of the Wnt pathway by Th2 cells seems to be a property that is regulated at multiple levels.

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**Fig. 4. Wnt3a induces β-catenin nuclear translocation in Th2 but not in Th1 cells.** Naïve CD4+ T-cells were polarized into Th1 or Th2 effector cells for 5 days. (A) Nuclear extracts were isolated from cells stimulated with Wnt3a, Wnt5a, LiCl or without stimulation in serum-free media for four hours. Protein levels were determined via western blotting (representative of n=5 experiments). (B) (Top) nuclear and cytoplasmic extracts or (bottom) mRNA from Th1 and Th2 effector cells cultured with or without Wnt3a in serum-free media for four hours. Protein levels were determined via western blotting (top). Relative β-catenin mRNA expression was obtained after reverse transcription, real-time PCRs and normalization against HPRT (bottom) (representative of n=3 experiments).
**Wnt signaling is important for Th2 survival.** The strikingly selective ability of Th2 cells to induce nuclear translocation of β-catenin suggests that Th2 effector cell behavior might be regulated by this transactivator. We examined this possibility using a genetic system to activate β-catenin specifically in Th2 cells. In β-catenin conditional constitutively active (CA) mice, LoxP sites have been placed around the third exon of the β-catenin gene, which encodes the region necessary for destabilization of the protein. Therefore, upon Cre mediated deletion, an artificially stable β-catenin protein is created, resulting in constitutive signaling⁴². Deletion of the floxed third exon in mice by CD4-CRE caused excessive growth of the thymus and resulted in a short life span of the animals and absence of naïve peripheral CD4⁺ T-cells (A.A. and R.A.F., unpublished results, ²⁵). To circumvent this issue, we therefore isolated naïve CD4⁺ T-cells from CA-β-catenin mice and deleted exon 3 of the gene during Th1 and Th2 effector cell cultures in vitro by retroviral transduction with a CRE vector⁴³. This experimental approach results in specific activation of β-catenin directly in the CD4⁺ effector T-cells generated in vitro, without indirect effects from perturbed earlier development. Stabilization of β-catenin resulted in decreased production of IL-4 by Th2 cells (Fig. 5A). This effect was not specific for Th2 cells, as also production of IFNγ in Th1 cells was reduced by expression of stabilized β-catenin. We reasoned that the apparent lack of specificity for IL-4 or IFNγ might be explained by a general negative effect on cellular viability due to activation of β-catenin. Indeed, restimulation of effector cells with antibodies to CD3 resulted in dramatically reduced viability in those cells expressing CA-β-catenin, but not in untransduced control cells or cells expressing control vector. Loss of viability upon restimulation of effector CD4⁺ T-cells is known as activation induced cell death. It is caused by induction of FasL expression on the surface of the effector cells, which then commit “fratricide” by engaging Fas molecules on neighboring cells. This process is generally much

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Fig. 5. Activation of the Wnt pathway leads to Fas-induced cell death in Th2 cells. Naïve CD4⁺ T-cells from mice bearing CA-β-catenin were activated by TCR crosslinking in the presence of APCs. The third exon of the gene was deleted by infection with a Cre-IRES-GFP retroviral vector 24 hours after initial activation. At day five, effector cells were purified by fycoll and FACSsorting of the GFP positive cells. GFP-positive and negative cells were then cultured separately. (A) Cells were re-stimulated with PMA and ionomycin for five hours. Cytokine production was measured by intracellular staining for IFNγ and IL-4. (B) Number of viable GFP positive cells was determined as percentage of all cells (live – 7-AAD-negative and dead – 7-AAD positive) after one (24hrs) or two days (48 hrs) of culture post re-stimulation with plate bound anti-CD3 (representative of n=2). (C) Expression of Fas was measured on day 5 by flow cytometry on GFP⁺ cells from Cre (filled area) or empty vector (grey line) transduced cells. (D) 16 hours post stimulation with anti-Fas antibodies, cell death was measured by staining for AnnexinV. Survival rates of Cre transduced Th2 cells (CRE) and empty vector (EV) bearing Th2 cells were compared.
Wnt Signaling is not Required for Physiological Th2 Cell Responses but Controls Th2 Effector Cell Viability

stronger in Th1 cells than in Th2 cells, presumably due to the lower surface levels of Fas on the latter cells (Fig. 5B and S3A). In line with this Th2 cells are less sensitive to cross-linking of Fas with antibodies to Fas (Fig. S3B). As Fas may be a target of the Wnt pathway, a likely hypothesis was that activation of β-catenin would elevate expression of this death receptor and thus render the cells more susceptible to FasL induced apoptosis. Indeed, we found that Fas was expressed at markedly higher levels on the surface of Th2 cells bearing CA-β-catenin than on control Th2 cells (Fig. 5C). Correspondingly, expression of CA-β-catenin resulted in significantly increased sensitivity to crosslinking of Fas (Fig. 5D). These results show that activation of β-catenin regulates Th2 effector cell viability, presumably at least in part through elevating expression of Fas.

Conclusion

Various studies have previously implicated Wnt signaling in control of Th-cell responses, with some studies supporting a suppressive and others supporting a positive function for this pathway in induction of Th-cell differentiation and/or effector cell function. Although providing elegant evidence that this pathway may contribute to regulation of the Gata3 gene, in our view, these studies did not definitively document a requirement for Wnt signaling in Th2 cell responses. Critical experiments to determine whether this requirement exists relied on the use of mice with constitutive deletion of TCF1, mice expressing a β-catenin transgene or mice expressing an ICAT transgene (a genetic inhibitor of the interaction between β-catenin and TCF1). All these mice suffer from severe defects in development of thymocytes. Consequently, it is difficult to determine whether phenotypes obtained with mature CD4+ T-cells from these mice reflect direct functions of TCF1 in Th2 cell differentiation, or indirect effects from perturbed development. Although this caveat does not pertain to the siRNA mediated silencing of β-catenin in mature T-cells used in another study, such knock down can result in off target effects. We therefore examined the requirement for Wnt signaling in Th2 cell responses using mice, in which expression of β-catenin was extinguished at a late stage in thymic development. Indeed, this late deletion allowed thymic development to proceed without overt abnormalities. Using these mice, we did not find that β-catenin is required for either differentiation of Th2 cells or for their effector cell function. We cannot exclude the possibility that molecules, such as γ-catenin/plakoglobin or yet another unknown molecule may substitute for β-catenin, although β-catenin has previously been shown not to be redundant in the T-cell lineage, at least during thymocyte development. However, the fact that addition of recombinant Wnt3a also did not affect Th2 cell induction under otherwise non-skewing conditions seems to argue against a decisive function for this pathway in Th2 cell differentiation.

Although our results did not support a role for the Wnt pathway in differentiation of Th2 cells, we did find that Th2 effector cells are specifically equipped to respond to the Wnt pathway. Expression of several Frzs was high on Th2 effector cells and hardly detectable on naive CD4+ T-cells or on Th1 effector cells. Correspondingly, Th2, but not Th1 effector cells activated β-catenin in response to recombinant Wnt3a. Further support for selective susceptibility of Th2 cells to Wnt signaling was provided by an earlier study, in which it was shown that IL-4 receptor signaling suppresses transcription of short inhibitory isoforms of TCF1. While Wnt signaling may not be required for differentiation of Th2 cells, these findings do suggest that the Wnt pathway may control effector cell function. This function
did not appear to affect production of cytokines. Instead, activation of β-catenin regulates susceptibility of Th2 cells to Fas mediated apoptosis, by inducing expression of this death receptor. In contrast to Th1 cells, Th2 cells have long been thought to be resistant to activation induced cell death, based on results with in vitro generated effector cells. Expression of Fas is high on Th1 cells generated in such cultures and low on Th2 cells. Our results now reveal a regulatory mechanism that allows similar control of viability in Th2 cells. It is intriguing to speculate that this property could be exploited therapeutically to selectively kill Th2 cells in allergic or asthmatic disorders, without eliciting general immune suppression from affecting all other T-cell types.

Materials and Methods

**Reagents and Antibodies.** Anti-CD3 (145-2C11), anti-CD28 (37.1), anti-IL4 (11B11), anti-IFNγ (XMG1.1), NK1.1.(HB101), anti-Th1 (Y19) (all American Type Tissue Culture Collection, Manassas, Virginia), anti-IL4 PE and APC, anti-IFNγ APC and PE, anti-Thy1.1 PE, anti-CD44 Cyochrome, anti-CD62L FITC and APC, anti-CD25 PE, anti-NK1.1 PE, anti-Fas PE (all BD Pharmingen), recombinant mouse IL4, recombinant mouse IL2 (both Pharmingen), recombinant mouse IL12 (a generous gift from Wyeth Research). Biotinylated or non-conjugated anti-IL4, anti-IL5, anti-IL10, anti-IFNγ, anti-igg1 and antibodies for ELISA were purchased from BD Pharmingen. ELISAs were developed with Horseradish Peroxidase Avidin D (Vector Laboratories, Inc., Burlingame, CA) and SureBlue Peroxidase Substrate (KPL, Gaithersburg, MD). Anti-mouse Frizzled-4, -7 and Dkk-3 antibodies were purchased from R&D Systems. Rabbit Anti-Histone Deacetylase 1 (HDAC1) was made by Sigma, Saint Louis, MS, while rabbit polyclonal anti-mouse Tcf7 antibody (N-term) was received from ABGENT, San Diego, CA. Wnt3a proteins were produced in Roel Nusse's lab, Stanford, CA. AIM-V® + AlbuMAX © (BSA) Serum Free Media used during in vitro Wnt3a and Wnt5a stimulation of T-cells was made by Gibco. DNA-free kit was ordered from Ambion. 7% NuPAGE Novex Tris-Acetate Gel was made by Invitrogen, Carlsbad, CA. Qiagen Plasmid Purification Kits and RNase Mini Kits were produced by Qiagen GmbH, Germany. MICROCON YM-30 Centrifugal Filter Devices used in concentrating nuclear and cellular extracts were purchased from Millipore. Poly-carbonate and Collagen coated transwell permeable supports used in our in vitro T-cell migration assays were made by Corning Inc., Lowell, MA.

**Intracellular cytokine staining.** Intracellular cytokine staining was done with BD Cytofix/Cytoperm Plus (with Golgi Stop) (BD Pharmingen) as described in the kit manual.

**Vectors and Constructs.** The hCRE-GFP-RV and GFP-RV vectors were generously provided by W. Paul.

**Mice.** 5- to 8-week-old C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or NCI (Rockville, MD) and maintained in the Yale University Animal Resources Center. Bred in our colony under SPF conditions: CD4-Cre transgenic mice (Taconic). β-catenin floxed mice (B6.129-Catnb1tm2kem/J, stock#004152) were obtained from Jackson Laboratories, Bar Harbor, ME. β-catenin exon3 floxed mice (CA-β-catenin) developed and received from Dr. Makoto Taketo, Sakyo, Kyoto, Japan (Harada et al., 1999).

**Induction of Anti-SEA Responses.** Extracts from Schistosoma mansoni eggs were prepared as described (Boros and Warren, 1970). Water-soluble fraction was injected intraperitoneally (50 µg) twice a week for 3 weeks. After an additional two-week rest period, sera and spleens were collected. To measure T-cell responses, CD4+ T-cells were isolated from spleens and restimulated in vitro with C57Bl/6 splenocytes and 25 µg/ml SEA. Supernatants were collected after 4 days and cytokine concentrations determined by ELISA.

**RT-PCR and Quantitative PCR.** After lysis in Trizol reagent (Invitrogen, Life Technologies) and purification on RneasyTM columns (Qiagen), mRNA was transcribed into cDNA with Oligo(dT)12-18 primers and SuperScript II RNase H- RT Kit as described in the manuals (both from Invitorgen). In some cases, the cDNA was treated with Rnase H (Invitrogen).

Fluorogenic probes were obtained from Applied Biosystems. Quantitative PCR was performed for 40 cycles using 7500 Real Time PCR System (Applied Biosystems). Relative concentrations were
determined on basis of standard curves of pooled cDNA and normalized for HPRT contents using software provided by the manufacturer. HPRT probes and Power SYBR Green PCR Master Mix were purchased from Applied Biosystems.

**Nuclear and Whole Cell Extracts.** Nuclear extracts were made as follows: Cell membranes were lysed in 250μl buffer A (10mM HEPES pH7.9, 3mM MgCl2, 10mM NaCl, 0.1mM EDTA, 300mM sucrose, 0.5mM DTT, supplemented with Complete miniTM protease inhibitor mix (Roche Diagnostics GmbH)) and 0.01% Nonidet P40 (Roche Diagnostics GmbH). The nuclear proteins were extracted with buffer B (20mM HEPES pH7.9, 3mM MgCl2, 420mM NaCl, 0.2mM EDTA, 25% glycerol, 0.5mM DTT, supplemented with Complete miniTM protease inhibitor mix (Roche Diagnostics GmbH)). Whole-cell extracts were made by lysing the cells in 50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% TritonX 100. Protein concentrations were measured with Bio-Rad DC Protein Assay Kit.

**In Vitro T-cell Differentiation Experiments.** Naive CD44low CD62Lhigh CD49b− CD25− CD4+ T-cells were purified from spleen and peripheral lymph nodes by positive selection with anti-CD4 beads (Miltenyi, cat♯130-049-201) followed by FACsorting. Cells were cultured in Bruff’s medium (10% FCS, penicillin, streptomycin, and L-glutamine). 10⁵ naive CD4+ T-cells were cultured with 2.5 x 10⁶ irradiated (2000 rad) B10. BR splenocytes obtained by collagenase treatment (Collagenase D, Roche), 10 μg/ml of pigeon cytochrome C (Sigma, cat♯ C-4011) and 20 μg/ml SEA. For Th1 and Th2 cultures, 2 x 10⁵ naive CD4+ cells were activated by 4 x 10⁶ irradiated (2000 rad) T-cell- and NK cell-depleted C57Bl/6 splenocytes with soluble anti-CD3 and anti-CD28 (1 μg/ml each), 10 U/ml IL-2 and 3.5 ng/ml IL-12 and 10 μg/ml anti IL-4 (11B11) (Th1 cultures) or 1000 U/ml IL-4 and 10 μg/ml anti IFNγ (Th2 cultures). After 5 days, viable cells were harvested by ficoll (LSMOL Lymphocyte Separation Medium, Cappel), restimated at 1x 10⁶ cells per well (96-well flat-bottom plate, Falcon) with plate bound anti-CD3 (10 μg/ml). Cytokine concentrations (48 hr supernatants) were determined by ELISA. For intracellular cytokine staining, viable effector cells were isolated by ficoll gradient, stimulated with PMA (50 ng/ml), and ionomycin (0.5 μM) and stained with the BD Cytofix/Cytoperm Plus kit (with Golgi Stop) (BD Pharmingen).

**Retroviral transfections and transductions.** Retrovirus was generated in Phoenix-ECO packaging cell line (a gift of Dr. G. Nolan, Stanford University, Palo Alto, California) after transfecting in the presence of Lipofectamine 2000 (Invitrogen), following the company protocol. CD4+ T-cells were stimulated with irradiated (2000 Gray) T-depleted splenic APCs, 5 μg/ml anti-CD3, 2 μg/ml anti-CD28, and 50 U/ml rIL2 and transduced as described. Viable cells were isolated by ficoll. GFP-positive and/or Thy-1.1-positive (after staining with anti-Thy1.1 PE (Pharmingen)), as well as -negative cells were separated by FACS. The Phoenix-ECO packaging cell line was a gift of Dr. G. Nolan (Stanford University, Palo Alto, California). Virus was made and transductions were performed as described.

**β-catenin stabilization (β-catenin translocation) assay.** Cells were cultured according to cell type/cell line protocols, stimulated with 50ng/ml of Wnt3a, as well as with 20mM LiCl or KCl in serum free media for three to four hours as established by Dr. R.Nusse's lab (Nusse, The Wnt homepage) and nuclear extracts were purified as described above. Western blots on nuclear β-catenin were performed.

**References**


Wnt Signaling is not Required for Physiological Th2 Cell Responses but Controls Th2 Effector Cell Viability


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Fig. S1: Characterization β-catenin knockout mice. (A) Total CD4+ and CD8+ cells were purified from three β-catenin+/+ CD4-CRE (WT) and three β-cateninfl/fl CD4-CRE (KO) mice (separate lanes). Total cytoplasmic extracts were prepared and the levels of β-catenin and β-actin were determined by western blotting. (B) Thymuses and spleens from wild type - β-catenin+/+ CD4-CRE (WT) or conditional knockout littermates - β-cateninfl/fl CD4-CRE (KO) were isolated and analyzed by FACS for the expression of CD4 and CD8.

Fig. S2. Recombinant Wnt3a induces nuclear accumulation of β-catenin. (A) L cells were stimulated with Wnt-3a (50ng/ml), LiCl (20mM, non-specific activator of the Wnt pathway) or KCl (20mM). (B) CD4+ cells were cultured for 3hrs in the presence of Wnt3a (50ng/ml), LiCl (20mM, non-specific activator of the Wnt pathway) or KCl (20mM) in the presence or absence of plate bound anti-CD3 and anti-CD28. Nuclear extracts were made and western blotting was performed for β-catenin, followed by re-probing for histone deacetylase (HDAC).

Fig. S3. Th1 cells express higher surface levels of Fas than Th2 cells. (A) Th1 and Th2 cells were generated and after 5 days surface levels of Fas were determined by flow cytometry. (B) Cells generated as in (A) were treated over night with either isotype control or anti-Fas. Viability of cells was analyzed by flow-cytometry.