[Frontiers in Bioscience 9, 1000-1010, January 1, 2004]

Wnt SIGNALING IN B AND T LYMPHOCYTES

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1. ABSTRACT

Wnt signaling has been shown to be critical for proper embryonic development as well as growth regulation of certain adult tissues. Defects in Wnt pathways have additionally been associated with a number of human cancers. However, it is only recently that a role for Wnts in the immune system has come to be appreciated. Wnts have now been shown to play significant roles in early stage development of both B and T lineage cells. Current studies suggest that proliferation and/or survival of these cells is associated with activation of the 'canonical' Wnt/beta-catenin pathway. Functional Wnt signaling appears to also occur in end stage B (plasma) cells where both the 'canonical' and the Wnt/RhoA pathways are activated. Herein, we review the current understanding of Wnt signaling in B and T cell development and the potential involvement of Wnt cascades in lymphoid neoplasia.

2. INTRODUCTION

Wnts comprise a family of secreted proteins that have been shown to be critical elements in both normal development and a variety of disease conditions. The Wnt family consists of at least 19 members whose individual functions have been difficult to discern in mammalian cells due to general insolubility encountered during attempts at purification. However, genetic approaches in Drosophila have revealed that Wnts are essential for normal embryonic development wherein they determine cell polarity and fate (1, 2). Wnt function is clearly not limited to embryonic development as Wnts have been implicated in growth regulation of a number of adult tissues including, for example, epithelial cell stasis (3) and bone morphogenesis (4, 5). While Wnt signaling has received considerable attention in a number of areas of mammalian cell growth and regulation over the past several years, it is only recently that Wnts have been appreciated as playing a role in the immune system. Herein, we will review evidence for a role of Wnt signaling in B and T lymphocyte development, function and neoplasia. Lymphocytes respond to a vast array of growth factors and cytokines that play critical roles in regulating both development and function(6, 7). Current evidence suggests that in addition to cytokines and growth factors, lymphocytes respond to Wnt signaling at restricted stages of development. Two Wnt signaling pathways have been identified as functional in early stage and terminally differentiated lymphocytes and are referred to as the 'canonical' Wnt/beta-catenin and Wnt/RhoA pathways.

The 'canonical' Wnt/beta-catenin pathway is the most well studied of the Wnt cascades (8). In the absence of Wnt ligand, beta-catenin is phosphorylated by GSK3beta (Figure 1) in a complex including axin and the adenomatous polyposis coli (APC) protein. Phosphorylation targets beta-catenin for subsequent ubiquitination, transportation to the proteosome and degradation. This process prevents the cytosolic accumulation of beta-catenin and maintains beta-catenin at levels that restrict its function as a potential transcription factor. The presence of Wnt ligand leads to binding to a receptor complex containing a seven transmembrane protein, Frizzled (Fz), associated with the low density lipoprotein (LDL) receptor related protein 5 or 6 (LRP5/6) (9-12). Both of these receptor components are required for activation of the 'canonical' pathway as masking of LRP by its biological antagonists, Dickkopfs (Dkk) 1 or 2, prevents signaling through this cascade (13, 14). Signaling can also be blocked by Wnt antagonists termed secreted Fz-related proteins (sFRPs) that have homology to the extracellular domains of Fzs and directly bind to Wnts inhibiting their interaction with receptor (15-20). In the absence of these inhibitors, Wnt binding to Fz/LRP leads to activation of downstream elements termed Dishevelleds (Dvl) which, in combination with other cellular proteins (FRAT), disrupt the GSK3beta/APC/Axin complex. As a result, beta-catenin is not phosphorylated and degraded, but accumulates in the cytoplasm and subsequently translocates to the nucleus where it is found in association with members of the TCF/Lef-1 transcription family. TCF-1 and Lef-1 were originally cloned as T cell specific genes (21-23) and later shown to function as transcriptional activators in association with beta-catenin (24). Lef-1 was also demonstrated to be expressed in certain B lineage cells (23), but TCF-1 appears to be restricted to T cells in adult animals. Two other members of this family have been identified in the mouse, TCF-3 and -4, but expression of both is largely restricted to embryonic development and neither is found in lymphoid tissue or cell lines (25, 26).

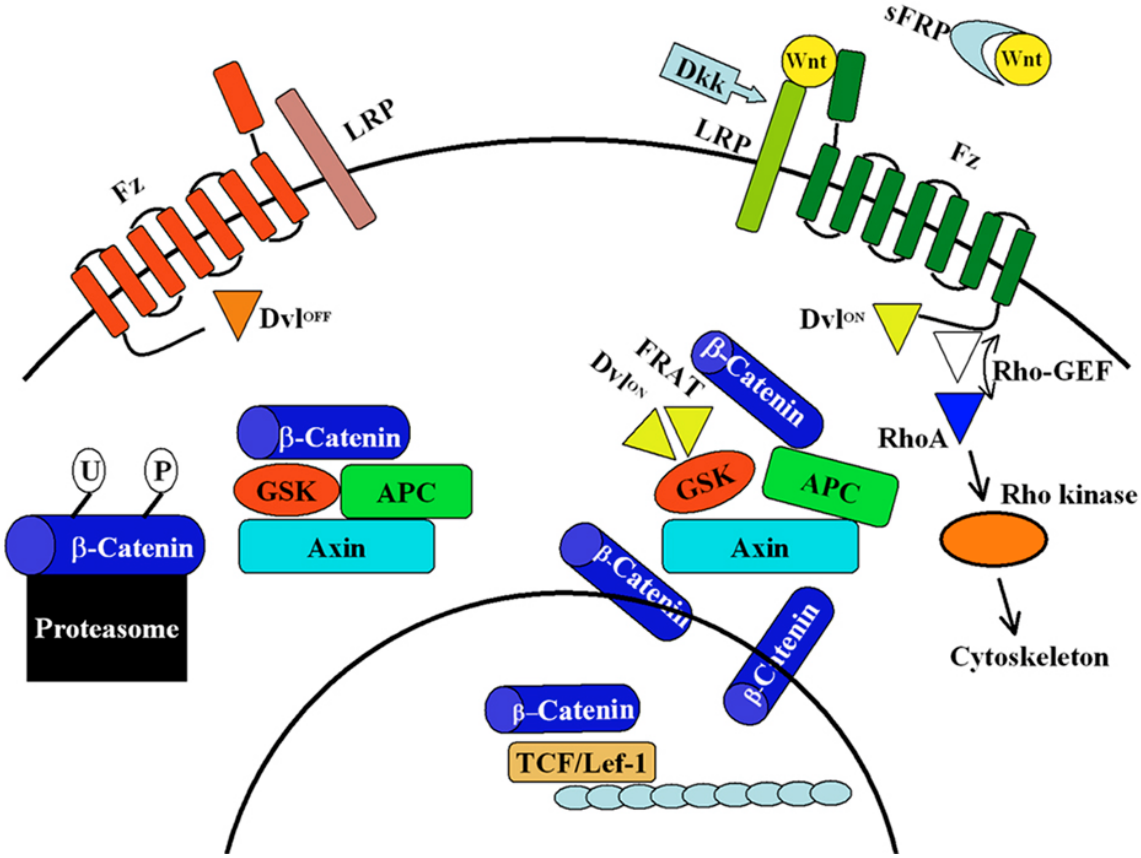

Figure 1. Schematic representation of Wnt signaling pathways characterized in B and T lymphocytes. Left side depicts phosphorylation (P), ubiquitination (U) and proteosome degradation of beta-catenin in the absence of Wnt signaling. Right side describes the downstream events in the two Wnt pathways known to be activated in lymphocytes. In the 'canonical' pathway the GSK3beta/APC/Axin complex is disrupted leading to beta-catenin accumulation and translocation to the nucleus. Activation of the RhoA pathway results in cytoskeletal changes.

TCF/Lef-1 proteins normally serve as transcription repressors (27-29), but when associated with beta-catenin form a transcription activating complex (30). Specific targets of this transcription complex are currently under investigation and likely include genes affecting multiple cellular processes including cell cycle progression and differentiation (see below).

A second Wnt signaling pathway (Wnt/RhoA) has, to date in lymphoid lineages, only been shown to be functional in end stage B (plasma) cells (31). Activation of this pathway does not require the LRP co-receptor as treatment with Dkk antagonists fails to inhibit RhoA activation. Wnt binding to Fz leads to Dvl activation and assembly of a complex including Dvl, Daam1, and Rho-GEF (32, 33). Rho-GEF catalyzes the transition of RhoA-GDP to RhoA-GTP (active form) leading to activation of Rho-associated kinases which play important roles in cytoskeletal organization (34-36). Activation of this latter pathway is related to properties such as cell polarity, motility and tissue invasion. An additional Wnt pathway associated with trimeric G proteins and cellular calcium flux has also been described (37-39), yet currently there are no reports of this pathway being activated in lymphocytes.

3. Wnt SIGNALING IN B CELLS

B lineage cells develop in a multi-step process that proceeds through a relatively well described sequence in which various stages are characterized by expression of specific cell surface markers and characteristic points in immunoglobulin (Ig) gene rearrangement and Ig protein expression. A detailed analysis of B cell development is beyond the scope of the present review and the reader is referred to the following for a more complete discussion (40-42). Stem cells that give rise to the B lineage are initially found in fetal liver and spleen but following birth become restricted to the bone marrow. It is in these organs that the early stages of B cell development occur after which surface Ig positive 'naïve' B cells generally leave the bone marrow and migrate to peripheral lymphoid organs (spleen, lymph nodes) where they may undergo further differentiation. A simplified schematic for B cell development is presented in Figure 2, however it should be remembered that there are likely to be multiple additional transitional stages that exist between those indicated. One of the earliest recognizable phenotypes in B cell development is that of the Pro-B cell which, in the mouse, expresses the B220 surface marker, but no functional

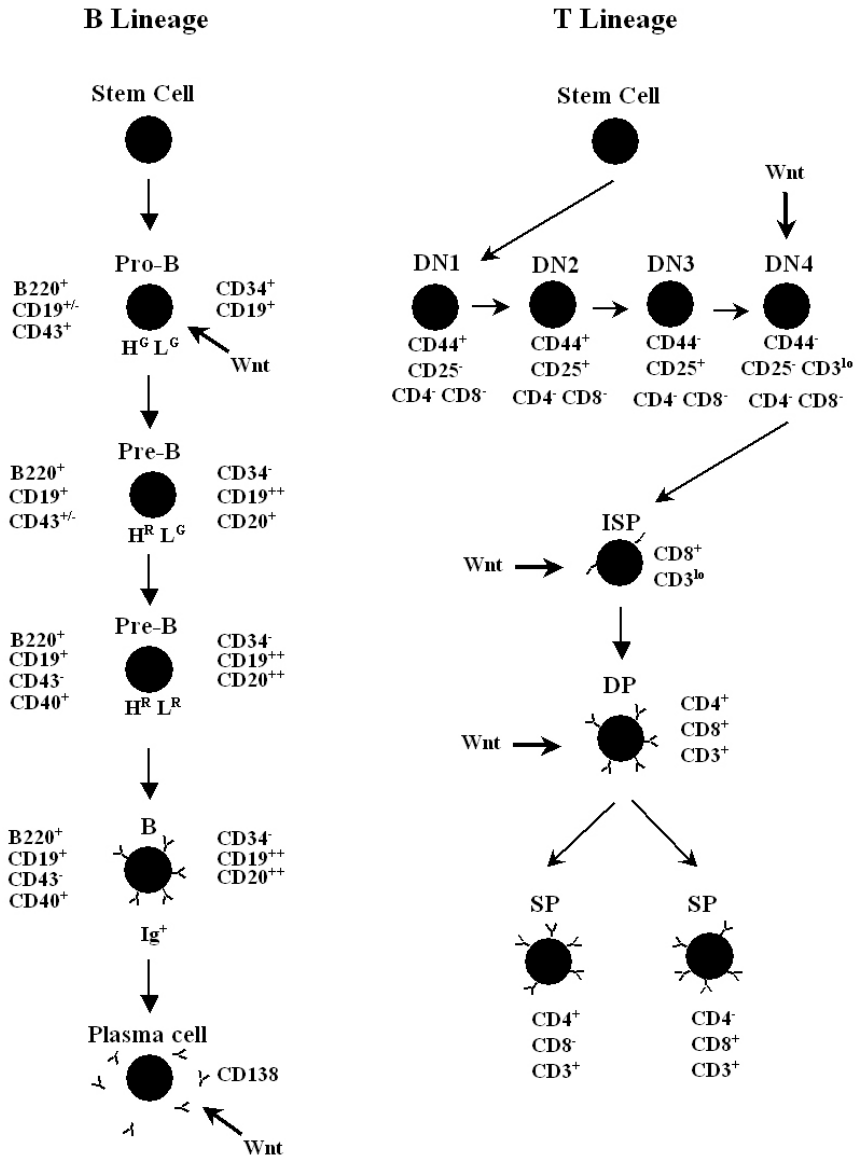

Figure 2. Schematic representation of B and T cell development. For B lineage development murine cell surface markers (B220, CD19, CD43, CD40) are indicated on the left and human (CD34, CD19, CD20) on the right. H^G and L^G indicate that the Ig heavy and light chain genes are in the germline configuration and H^R and L^R that these genes have undergone rearrangement required for production of functional Ig. Abbreviations for T lineage development are as follows: DN, double negative; ISP, immature single positive; DP, double positive; SP mature single positive. Arrows indicate stages at which Wnt signaling has been suggested.

surface Ig. Pro-B cells then proceed through the Pre-B stage wherein heavy and light chain Ig gene segments undergo patterned rearrangements to produce complete transcripts encoding the respective Ig heavy and light polypeptide chains. This process culminates in the B cell stage where Ig molecules consisting of paired heavy and light protein chains are expressed on the cell surface and serve as functional receptors. Upon encountering antigen complementary to the receptor binding site, B cells may undergo further differentiation to end stage plasma cells which lose surface Ig, but now secrete Ig into the surrounding fluids and finally undergo apoptosis.

Currently, there exist a very limited number of studies describing actual or potential Wnt signaling in B lineage cells. Wnt expression in such cells was first described in acute lymphoblastoid leukemia (ALL) characterized as pre-B cell in phenotype and containing a t (1:19) translocation (43). This translocation leads to production of a fusion protein of the E2A transcription factor and Pbx-1, a homeodomain protein that, in complexes, serves to increase DNA binding affinity and enhance transcription. E2A-Pbx-1 was shown to upregulate expression of Wnt-16 which occurred in all patients and cell lines carrying the translocation. It was additionally

demonstrated that 4 members of the Fz family (Fzs 2, 3, 5 and 6) were expressed in ALL and Fzs 2, 3 and 5 in most B lineage cells leading to the suggestion that Wnt-16 might be acting in an autocrine fashion to promote ALL cell growth. It should be noted that a direct proliferative effect of Wnts on these cells has not been demonstrated nor on corresponding normal Pre-B cells.

Wnt signaling in normal B lineage cells has, in fact, only been demonstrated at the Pro-B cell stage. Reya *et al.* (44) in a study of B lineage cells from normal and Lef-1$^{-/-}$ mice have shown that Lef-1 was expressed in the majority of normal murine fetal liver Pro-B cells, but in far fewer bone marrow Ig^{+} B cells. Highest expression was observed in the latter stages of Pro-B cell differentiation and very little expression was noted in IgM^{+} B cells from either spleen or bone marrow. In comparison, Lef-1$^{-/-}$ mice show a decrease in B lineage cells in both fetal liver and perinatal bone marrow with the majority of B lineage cells present classified as Pro-B in phenotype. The decrease in B lineage cells correlated with a marked increase in Pro-B cell apoptosis and decrease in the number of cells entering cell cycle indicating a role for Lef-1 in Pro-B cell proliferation. The decreased number of Lef-1$^{-/-}$ B lineage cells were, however, capable of normal differentiation suggesting that Lef-1 function was limited to proliferation raising the possibility that Wnt signaling through Lef-1 was an important aspect of Pro-B cell growth and survival. This suggestion was confirmed by the demonstration that Wnt-3a treatment led to a 4-fold increase in normal Pro-B cell proliferation, but had little effect on Lef-1$^{-/-}$ Pro-B cells. Furthermore, Wnts 2b, 5a and 10b mRNA were found to be expressed in bone marrow indicating the likely exposure of Pro-B cells to Wnts in the normal physiological setting (45). Lef-1 mRNA and protein expression have also been demonstrated in Pre-B cell lines (23, 46), but functional Wnt signaling has not been described in these lines or corresponding normal populations. It is possible that Lef-1 expression in Pre-B lines may be related to transformation or these lines, as well as normal Pre-B cells, may lack appropriate receptors to activate the 'canonical' signaling pathway. Interestingly, upon transfection of reporter constructs, the Lef-1 promoter appears to be highly active in B and T cell lines, but Lef-1 is not expressed in corresponding normal mature B and T cells suggesting that active repression regulates expression in these cells (47).

While Wnt signaling has been clearly demonstrated in Pro-B cells similar studies have not been reported for Pre-B cells or Ig expressing mature B cells. In fact it is unlikely that Ig^{+} B cells respond to Wnts through the 'canonical' pathway as neoplastic counterparts of these cells (B cell lymphomas) do not proliferate upon treatment with Wnt-3a conditioned medium and do not express LRP co-receptors (31). Additionally, populations of normal spleen and lymph node cells that consist almost entirely of mature B and T cells similarly do not proliferate in response to Wnt-3a (Qiang and Erman, unpublished observations) and Lef-1 is not expressed in normal Ig^{+} lymphocytes (44). However, it appears as though access to the 'canonical' pathway may be accomplished in Ig^{+} B cells by other mechanisms. Crosslinking of the Ig receptor has been shown to result in beta-catenin stabilization and transcriptional activation through TCF/Lef-1 binding sites (48). In this pathway, stabilization of beta-catenin is mediated by PI-3K activation of PKC which, in turn, inhibits GSK3beta in a manner analogous to Dvl following activation by Wnts. It should be cautioned that the above observations do not rule out the possibility of Wnt signaling in Ig+ B cells occurring through non-'canonical' pathways.

Given the absence of Wnt signaling in Ig^{+} B cells, it is somewhat surprising that end stage B (plasma) cells (Figure 2) as represented by corresponding tumors present in the human disease, multiple myeloma, do respond to Wnt-3a (31). Plasma cell lines express multiple Fz receptors (as many as 9 in a single line) and all express either the LRP 5 or 6, or both, co-receptors. In contrast, only Fz-3 is expressed to a significant extent in Ig^{+} B cell lymphomas. Treatment of plasma cell lines with Wnt-3a leads to increases in Dvl-2, phosphorylation of Dvl-3, stabilization of beta-catenin and transcriptional activation presumably through an Lef-1 mediated process as Lef-1 is the only member of the TCF/Lef-1 family expressed in these cells. Earlier studies had suggested that Lef-1 was not expressed in plasma cell tumors (23) and it is possible that the greater sensitivity of RT-PCR as opposed to Northern analysis provides an explanation for the discrepancy between these two reports. An alternative explanation is that there exists a species specific difference in that Lef-1 expression in the earlier studies assessed murine plasma cell tumors whereas the later analysis employed human plasma cell lines. Thus, in human plasma cells, activation of the 'canonical' Wnt/beta-catenin pathway follows an identical pattern to that established in other systems. It is noteworthy that no increase in proliferative capacity is associated with activation of this pathway. It is possible that any increase is masked by other factors present in the conditioned media or plasma cells may already be proliferating in response to endogenously expressed Wnts. The latter interpretation is consistent with the observation of several different Wnt messages being expressed by these cells (Qiang and Rudikoff, unpublished observations) and the high basal level of beta-catenin when compared to B cell lymphomas.

Plasma cell lines additionally represent the only B or T lineage cells in which activation of non 'canonical' Wnt signaling pathways has been observed (31). Herein, Wnt-3a treatment leads also to formation of a Dvl/Daam-1/ Rho-GEF complex which activates RhoA and downstream Rho-associated kinases (32). Activation of the Wnt/RhoA pathway in myeloma cells leads to striking morphological changes resulting in rearrangement of the actin cytoskeleton and adhesion of these normally suspension growing lymphocytes to culture plates. The Wnt/RhoA pathway does not require the LRP co-receptor as masking LRP with Dkk antagonists inhibits beta-catenin stabilization, but not RhoA activation. However, the morphological changes can be completely blocked by inhibitors of Rho-associated kinases. This pathway and its associated morphological effects appear to be important in promoting both motility and tissue invasive properties (Qiang et al., in preparation). Again it should be noted that Wnt signaling in plasma cells has only been demonstrated

in tumor lines and it remains to be determined whether similar signaling occurs in normal plasma cells. If the data derived from tumor lines is reflected in normal lymphoid cell counterparts, it would appear that functional Wnt signaling occurs early (Pro-B stage) in B cell development, but that receptors are likely down regulated and signaling lost at the Ig^+ B cell stage. Functional signaling then re-occurs in end stage plasma cells concomitant with re-expression of appropriate receptors.

Regardless of whether Wnt signaling is limited to plasma cell tumors or reflects normal plasma cell signaling, it is still intriguing to consider the possible role of Wnts in the corresponding disease (multiple myeloma). The morphological changes and increased adhesion associated with Wnt responsiveness strongly suggest enhanced interaction with neighboring stromal cells which is critical in disease progression (49, 50). Thus, Wnt signaling through myeloma plasma cells may either directly or indirectly (as a result of interactions with bone marrow stroma) affect disease manifestation. In this context, it is noteworthy that bone lesions are the most common pathological feature of multiple myeloma resulting from an imbalance in the normal levels of bone forming osteoblasts and bone degrading osteoclasts. Wnts have been shown to play a major role in bone matrix deposition associated with osteoblast generation (5, 51-54) and it will be important to determine whether Wnt signaling through myeloma plasma cells affects bone metabolism. While exploration of the role of Wnt signaling in myeloma is at a very early stage, there are a number of observations that suggest potentially important and complex roles in such diseases. Array analysis of malignant plasma cells from myeloma patients and IL-6 dependent myeloma cell lines (55, 56) has revealed that among the most upregulated genes in this disease are Dkk-1 and FRZB/sFRP-3. Dkk-1 as noted above binds to, and masks, the LRP co-receptor inhibiting signaling through the 'canonical' pathway. sFRPs constitute a family of 5 secreted proteins that have cysteine rich domains homologous to the extra cellular domains of Fzs (16-20). sFRPs thus bind to Wnts (Figure 1) and prevent interaction with cellular Wnt receptors. It is thus possible that, if the mRNA levels in these patients correspond to functional secreted protein, myeloma cells are producing inhibitors that would interfere with Wnt signaling in an autocrine manner as well as in other neighboring cells. Such down regulation may affect the homeostasis of bone metabolism leading to increased osteoclast formation and bone destruction (57) as well as other processes important in tumor cell growth and disease progression. Potential Wnt signaling playing a role in lymphoid neoplasia may not be limited to multiple myeloma as Wnt-3 (58) and Lef-1 (59) have been shown to be highly upregulated in B cell chronic lymphocytic leukemia. It will thus be important in the future to fully assess the potential role of Wnt signaling in a variety of B lineage neoplasias as a possible contributor to both neoplastic cell growth and alteration of the tumor micro-environment.

4. Wnt SIGNALING IN T CELLS

In a manner analogous to B cell development, T cells also pass through a complex series of differentiation steps leading to the formation of mature effectors found in peripheral lymphoid organs. A detailed analysis of T cell development is beyond the scope of this presentation and numerous reviews (60, 61) have dealt with this subject. T lineage stem cells are produced in the bone marrow and migrate to the thymus where differentiation and proliferation of thymocytes occur. Various stages in this process (Figure 2) can be identified based on expression of cell surface markers and the T cell antigen receptor (TCR). Among such surface proteins used to define developmental stages are the CD4 and CD8 co-receptors that associate with the TCR (CD3) in mature T cells. The first stages of thymocyte development are characterized by a lack of expression of either CD4 or CD8 and, as such, cells are designated in immunological jargon as 'double negative' (DN). DN cells pass through four distinct stages (DN1-4) that can be differentiated by expression of two additional markers CD25 and CD44. DN4 cells transition to a stage in which low levels of CD8 are expressed in conjunction with an incomplete TCR leading to the designation immature single positive (ISP). ISP cells proliferate rapidly and generate the major thymocyte population (greater than 85% of cells) expressing both CD4 and CD8 and referred to as double positive (DP). DP cells complete rearrangement of TCR genes and express mature TCR on the surface leading to a multifaceted selection process during which cells with TCRs that do not recognize 'self' are essentially left out of the process and die by neglect from lack of a positive signal. Cells expressing TCRs that react strongly with 'self' and are potentially autoreactive undergo apoptosis (negative selection). Cells with receptors that recognize 'self' at some lower, but as yet undefined level, permitting recognition but not stimulating autoreactivity, are positively selected (61). Such cells will subsequently be capable of recognizing foreign antigen presented by 'self' (major histocompatability complex) and be appropriately stimulated to induce effector functions. Cells surviving this dual selection lose expression of either CD4 or CD8 generating populations that are referred to as single positive (SP) and also express high levels of the TCR ($CD4^+$ $CD3^+$ or $CD8^+$ $CD3^+$). SP cells leave the thymus and seed peripheral lymphoid organs such as spleen and lymph nodes where they function as effectors of T cell immunity.

The identification of a role for Wnt signaling in T lineage cells has developed from the characterization of downstream elements in this pathway rather than the study of Wnt ligands and their receptors. Two transcription factors in the 'canonical' Wnt pathway, TCF-1 and Lef-1, were originally cloned based on restricted expression limited to T and Pre-B cells (21-23, 62). Both TCF-1 and Lef-1 were shown to be expressed in thymus and T cell lines by Northern analysis and found to activate the TCR alpha chain enhancer. As described above, Lef-1 has also been detected in several stages of B cell differentiation, but TCF-1 appears restricted, among lymphoid cells, to the T lineage. Using beta-catenin as bait in a two hybrid system, Behrens *et al.* (24) identified Lef-1 as a binding partner. Beta-catenin and Lef-1 could be co-immunoprecipitated and expression of Lef-1 led to translocation of beta-catenin to the nucleus and co-localization of the two proteins. A biological relevance for this interaction was demonstrated by the observation that injection of Lef-1 into Xenopus

Table 1. Wnt expression in T lineage cells

	Wnts							
	1	3/3a	4	5a	5b	6	10a	10b
Staal *et al.* (69)								
Neo-natal thymus	+		+++					
Mulroy *et al.* (70)								
Adult thymus	+++	+++	+++	++		+	++	
DN	+++	+++	+++					
Balciunaite *et al.* (71)								
Adult thymus	+++	++	+++		+++			+++
DN3	-	-	+++		++			+++
DN4	-	-	+++		+++			+
DP	-	-	+++		+++			+/-
SP ($CD4^+$)	-	+	+++		+/-			+/-
SP ($CD8^+$)	-	+/-	+++		+			+
Lymph node T cells	-	+++	+		+			+/-
Thymic epithelium								
Precursor	+/-	+	+++		+/-			+/-
Mature	+++	++	++		+/-			++

embryos led to axis duplication which was dependent on binding of Lef-1 to beta-catenin. These findings provided a link between beta-catenin and the TCF/Lef-1 family of transcription factors (and potentially the Wnt signaling pathway).

Functional roles for TCF-1 and Lef-1 in T cell development have been examined using corresponding knock out mice. Lef-1 appears to be expressed at relatively low levels during thymocyte development and primarily in the late DN and ISP (Figure 2) stages (63). Lef-$1^{-/-}$ mice exhibit normal thymocyte differentiation although a number of developmental defects in other organs are observed (64). In contrast, TCF-1 is expressed at all stages of thymocyte development with highest levels in ISP cells and TCF-$1^{-/-}$ mice have pronounced defects in thymocyte expansion (63). Thymii from these mice are greatly reduced in size with deceases in thymocyte numbers ranging from 10 to 100 fold. The DN population appears to be unaffected in very young mice (< 1 month) however mice greater than 1 month of age lack the DN2 population and almost none of the DN cells are in cycle (65). Furthermore, the ISP population in TCF-$1^{-/-}$ mice is increased by 3-5 fold. However, ISP cells which normally are rapidly dividing and give rise to the predominant DP population, for the most part do not enter cell cycle. As a result, large decreases are seen in the DP and mature SP populations. Those SP cells that do develop and migrate to the periphery appear phenotypically and fuctionally normal indicating that the TCF-1 defect does not prevent normal development, but appears to inhibit ISP cell division and the generation and/or survival of DP thymocytes. Defects in thymocyte development appear to extend to DN cells as animals age. Subsequent studies using transgenic mice in which TCF-1 constructs, either containing or lacking the beta-catenin binding domain, were expressed on the TCF-$1^{-/-}$ background (66) have provided supporting data for the suggested role of TCF-1. Thymocyte populations from transgenic mice expressing TCF-1 lacking the beta-catenin binding domain were phenotypically indistinguishable from $TCF^{-/-}$ animals (low cycling of ISP cells and reduction of DPs) and additionally evidenced a high rate of apoptosis in the DP population. Thymocytes from mice expressing TCF-1 with the beta-catenin binding domain showed partial restoration of ISP cycling and rescue of the DP population from apoptosis.

While the above studies defined a role for beta-catenin and TCF-1 in thymocyte development, a role for Lef-1 was not obvious as Lef-$1^{-/-}$ animals exhibit normal thymocyte development and Lef-1 transcripts are far less abundant. However, as these two factors share a high degree of homology throughout their entire protein sequences, overlapping functions might be expected. This issue was addressed by Okamura *et al.* (67) in an analysis of double (TCF-$1^{-/-}$Lef-$1^{-/-}$) knockout mice. These mice die at birth but thymii can be harvested from embryos and thymocyte development assessed in organ culture (68). The total number of thymocytes in the TCF-$1^{-/-}$Lef-$1^{-/-}$ mice was 14 fold less than in the TCF-$1^{-/-}$ alone. Additionally, virtually no DP ($CD4^+CD8^+$) or mature SP ($CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$) cells were observed and a partial blockage was noted at the DN3 ($CD44^-CD25^+$) stage. It thus appears as though there is partial redundancy in the effects mediated by these two factors. However, it should be cautioned that the double knockout represents a highly abnormal physiological setting and a significant developmental effect for Lef-1 is only seen in the absence of TCF-1. On the other hand, the full normal physiological role of either factor may only be realized in the presence of the other due to required complementation.

While the presence of TCF-1 and Lef-1 and their demonstrated interaction with beta-catenin is suggestive of Wnt signaling, it is possible that this pathway is activated by other ligand/receptors as has been demonstrated in B cells via the B cell antigen receptor (48). It is only in the last two years that functional Wnt signaling has been actually demonstrated in T cells. Staal *et al.* (69) have described the presence of mRNA corresponding to Fzs 5, 7, and 8 in neonatal thymii. As these studies were not performed on sorted populations, it is not clear which thymocyte subsets or thymic epithelial cells actually express these messages. Using transgenic mice carrying a TCF reporter gene linked to beta-galactosidase (LacZ), it was demonstrated that viral transduction of Wnt-1 into thymocytes resulted in increased expression of LacZ in the DN (12%) and ISP (4%) populations. Transduction of either Wnt-1 or Wnt-4 into thymocytes resulted in a recovery of ~ 7-fold more DN cells as compared to controls after 6 days suggesting that Wnts acted as either a growth or survival factor in these cultures. As Fzs 5, 7 and 8 were shown to be expressed in the thymus, additional experiments were performed transducing fetal lymphoid progenitors with constructs encoding the extra-cellular domains of these molecules with the premise that such constructs would be soluble and bind to Wnts in the same manner as the naturally occurring Wnt antagonists, sFRPs (16, 18). Fetal organ culture of transduced thymocytes revealed a block at the DN stage by soluble Fz-7 and at the DN to ISP transition by soluble Fzs-8 and -5. These studies provide a link between Wnt and TCF/beta-catenin indicating that 'canonical' Wnt signaling occurs in thymocyte populations.

The role of Wnt signaling in T lineage cells is likely to be very complex and/or very redundant. Several studies (69-71) have demonstrated that multiple Wnt mRNAs are expressed in both thymocytes, thymic epithelium and peripheral T cells (Table 1). Wnt-4 is the most consistently and highly expressed Wnt in all thymic populations. Wnt-1 expression appears to be high in the total DN population (70), but missing in the DN3 and 4 subsets (71) raising the possibility, if true, of a basis for functional response differences in these subsets. A similar study of Fz expression in these same populations would clearly be of interest as an aid in initial attempts to sort out possible differences in communication between various sub-populations. Although most Wnts appear to be preferentially localized to cell membranes as opposed to being readily secreted, thymocytes are likely to be in contact with an array of these ligands both on neighboring T cells and thymic epithelium. TCF/Lef-1 reporter constructs in thymic epithelium have been shown to be activated by co-culture with thymocytes and this activation can be mimicked by transduction with Wnt-4 (71). Thus, thymocytes can provide Wnt signals to epithelial cells, likely through Wnt-4. The potential importance of highly expressed Wnts-1 and 4 in thymic development is further indicated by the decrease (20-30%) in fetal thymocytes from either Wnt-1 or Wnt-4 knockouts and 50-70% in the double knockout. The presence of all thymic sub-populations in reduced numbers presents a phenotype very similar to that of TCF-1 knockouts (70). Taken together, the above results establish a link between TCF/Lef-1 transcription factors and Wnt/Fz at the cell surface indicating that the 'canonical' Wnt pathway plays an important role in normal thymocyte development. The current studies further suggest that Wnts may act at multiple points in the T lineage including late DN, ISP and possibly DP stages (Figure 2). As yet, Wnt signaling has not been demonstrated in mature T cells. However, it will be interesting to see if the 'canonical' Wnt pathway is accessible through other receptors, such as possibly the TCR, in a manner analogous to the Ig receptor in B cells.

While considerable effort has been directed toward the study of Wnt signaling in normal T cells, almost nothing is known about Wnt pathways in T cell diseases or neoplasias. Chung *et al.* (72) have reported that, in contrast to normal peripheral T cells wherein beta-catenin is not expressed, heterogeneous, but generally high level, expression was observed in T cell leukemia and lymphoma cell lines as well as patients with acute or chronic myelocytic leukemias. Inhibition of beta-catenin function in T cell lines by either dominant negative beta-catenin or dominant negative TCF led to decreases in proliferation and clonogenicity suggesting a role for beta-catenin in the proliferation of these neoplasias. It remains to be determined whether beta-catenin activity is associated with upstream Wnt signaling (presumably through an autocrine mechanism), but the observations are provocative in suggesting a role for beta−catenin/TCF in cancers of the T cell lineage.

5. PERSPECTIVES

Considering the large number of cytokines and growth factors that are known to regulate B and T cell development, it is almost amusing that nature has chosen to also add Wnts to the mix. Not only do Wnts affect both B and T cell development, but are critical for the generation of mature effector populations in both lineages. Interestingly, Wnts affect these lineages at relatively early (Pro-B and DN, ISP T cell) stages of development and appear not to impact mature effector cells. The possible exception is the above noted response of end stage plasma cell tumors (31). It remains to be determined if a similar response occurs in normal plasma cells although this is a difficult question to answer due to the number of plasma cells required to perform such experiments. What do Wnts actually do in lymphocyte development? In the case of Pro-B cells, Wnts clearly have been shown to directly promote proliferation (44). Additionally, while Wnts do not increase proliferation of plasma cell lines, they act as chemotactic factors and promote invasion (Qiang and Rudikoff, in preparation). For T cells, the role of Wnts is less clear. Based on the observations that, although the total number of T cells in TCF-1$^{-/-}$ mice are reduced, mature functional T cells are still produced, it has been suggested that Wnts do not affect differentiation (63, 69). However, the data do not rule out the possibility that a partial block in differentiation occurs and only some cells pass through and develop to maturity. The blockage at the late DN and ISP stages could also be associated with either/or both proliferation and apoptosis. These questions will need to be addressed by

direct treatment of purified populations with soluble Wnts. Such experiments have, to date, not been possible due to the unavailability of requisite reagents, but it is anticipated that these difficulties will be overcome in the near future by use of appropriate expression systems (73).

While the dissection of the role of Wnts in lymphocyte development is in a relatively early stage, a number of obvious questions arise some of which are experimentally approachable and others more problematic. Surprisingly little is known about downstream targets activated by beta-catenin/TCF/Lef-1 complexes. In other systems myc (74) and cyclin D1 (75, 76) have been identified as being Wnt/beta-catenin regulated. These two genes are affected by numerous growth factors and would generally be placed in the category of common 'proliferation associated' factors. Additional Wnt targets include molecules involved in cellular adhesion such as CD44 (77) and fibronectin (78). In the immune system, FoxN1 a member of the Forkhead family of transcription factors, has been identified as a Wnt regulated element (71) . Expression of this gene is required for normal formation of the thymic epithelium and in its absence T cell precursors are not recruited to the primordial thymus leading to athymia and the nude phenotype (79, 80). However, other immunologically important targets remain to be identified and such studies are likely to be under way using array analysis as one currently available approach.

Other important questions, such as specificity and regulation, are common to all biological systems. The lack of soluble Wnts has severely hampered such analyses and the fact that most cells characterized appear to express multiple receptors (Fzs) has complicated attempts at this type of analysis. Why, for example, do Pro-B and plasma cells respond to Wnt-3a yet other B-lineage stages do not? A straightforward explanation would be differential expression of receptors. If plasma cell lines are representative of normal plasma cells and B cell lymphomas of normal B cells than this explanation holds true as B cell lymphomas do not express the LRP co-receptor required for 'canonical' Wnt responses and do not respond to Wnt-3a. However, B cell lymphomas also express far fewer Fzs than plasma cells and thus regulation may be more complex than simply LRP expression (31). These observations would predict significant variation in Wnt receptor expression throughout lineage development and is a testable hypothesis using sorted populations of both B and T cells.

It should be cautioned that variation in receptor expression may only be one means of regulating Wnt responses as further complexity is suggested by responses observed in the thymus. Thymic epithelial cells have been shown to activate the 'canonical' Wnt/beta-catenin pathway in response to Wnts expressed on neighboring thymocytes (71). Transfection of epithelial cells with Wnt-4, which is expressed at high levels in thymocytes, led to similar activation indicating that Wnt-4 was capable of activating this pathway in the epithelial cells. However, thymic epithelial cells also express high levels of Wnt-4 raising the question of why constitutive signaling is not occurring either in an autocrine manner or between neighboring epithelial cells and if Wnt-4 produced by epithelial cells can be used to induce signaling in adjacent thymocytes. Thus, is the context of Wnt presentation also critical in determining response outcomes? It should also be noted that nearly all Wnt studies in lymphocytes have focused on the 'canonical' pathway and, with the exception of the RhoA pathway in plasma cells, little is currently known about activation of other Wnt pathways in B or T lineage cells. Finally, what are the potential roles of Wnt signaling in lymphoid neoplasia? Aberrant expression of Wnts has been suggested to play a role in Pre-B cell ALL (43) and normal Wnt signaling may promote the survival and invasive properties of multiple myeloma cells, yet a function for Wnts in other lymphoid diseases is largely unexplored. The intriguing observation that components of the Wnt signaling cascade are highly upregulated in several lymphoid malignancies (55, 56, 59) further raises the possibility that Wnts may modulate the tumor microenvironment in ways that influence disease progression either directly or indirectly. Thus, Wnt biology has now clearly become an important contributor to normal lymphoid development and, most likely, aspects of lymphoid neoplasia. The scientific community has largely been forced to come to terms with the immunological language (i.e. Pre-B, double negatives, immature single positives, etc.) and now it appears as if the immunological community will have to deal with Wnts, Frizzleds, Dishevelleds, etc.

6. ACKNOWLEDGEMENTS

We thank Jeffrey Rubin for helpful discussions and comments.

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Key Words: Signal Transduction, Wnt, beta catenin, B cell, T cell, Review

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