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Wnt Signaling Regulates B Lymphocyte Proliferation through a LEF-1 Dependent Mechanism

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Summary

Lymphocyte enhancer factor-1 (LEF-1) is a member of the LEF-1/TCF family of transcription factors, which have been implicated in Wnt signaling and tumorigenesis. LEF-1 was originally identified in pre-B and T cells, but its function in B lymphocyte development remains unknown. Here we report that LEF-1-deficient mice exhibit defects in pro-B cell proliferation and survival in vitro and in vivo. We further show that *Lef1*$^{-/-}$ pro-B cells display elevated levels of *fas* and c-*myc* transcription, providing a potential mechanism for their increased sensitivity to apoptosis. Finally, we establish a link between Wnt signaling and normal B cell development by demonstrating that Wnt proteins are mitogenic for pro-B cells and that this effect is mediated by LEF-1.

Introduction

Lymphocyte differentiation is a complex process that allows stem cells to develop into highly specialized cells. The external signals that regulate cell lineage and stage-specific expression of genes include extracellular growth factors and cell–cell contact. Within cells, these cues are integrated by transcription factors that play a particularly important role in executing a program of differentiation by regulating the expression of genes (reviewed in Reya and Grosschedl, 1998). LEF-1 is a member of the LEF-1/TCF family of HMG transcription factors, which also includes three other members, TCF-1, TCF-3, and TCF-4 (van de Wetering et al., 1991; Korinek et al., 1998). LEF-1 is expressed in developing B and T cells and at multiple sites of organogenesis during embryonic development (Oosterwegel et al., 1991; Travis et al., 1991; van Genderen et al., 1994). LEF-1 has no transcriptional activation potential by itself, but it can act as an architectural protein in the assembly of multiprotein enhancer complexes (Giese et al., 1995). In the T cell receptor α enhancer, for example, LEF-1 regulates transcription in association with ALY and in collaboration with other enhancer binding proteins (Bruhn et al., 1997). In addition, LEF-1/TCF proteins have been shown to interact with β-catenin, an important effector in the Wnt signaling pathway (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996; Brunner et al., 1997; Riese et al., 1997). LEF-1/TCF proteins associate with β-catenin through amino-terminal sequences, and together these proteins mediate a transcriptional response to Wnt signaling.

Wnt proteins represent a growing family of secreted signaling molecules that are expressed in diverse tissues and have been shown to influence multiple processes in vertebrate and invertebrate development (reviewed in Cadigan and Nusse, 1997). Wnt proteins have been shown to regulate pattern formation in *Drosophila* (Siegfried and Perrimon, 1994), axis specification in *Xenopus* (Moon et al., 1997), and organogenesis in the mouse (Monkley et al., 1996; Yoshikawa et al., 1997). In addition, dysregulation of components of the Wnt signaling pathway can have potent oncogenic effects in tissues such as colon and breast (Nusse and Varmus, 1982; Tsukamoto et al., 1988; Korinek et al., 1997; Morin et al., 1997). Wnt signaling involves binding of a Wnt protein to a receptor of the *frizzled* family, inactivation of GSK-3β kinase activity, and stabilization of β-catenin in the cytosol (Willert et al., 1997). Accumulation and nuclear translocation of β-catenin allows for association with LEF-1/TCF proteins and activation of downstream target genes (reviewed in Cadigan and Nusse, 1997; Eastman and Grosschedl, 1999).

Although Wnt functions have been studied in many different tissues, little is known about Wnt signaling in the immune system. The fact that LEF-1 is expressed in developing B and in T cells raises the possibility that Wnt signaling might regulate proliferation and/or differentiation of lymphoid cells. In support of a role of LEF-1/TCF proteins in T cell differentiation and function, targeted inactivation of both *Lef1* and *Tcf1* genes results in an early block in T cell differentiation (Okamura et al., 1998). In addition, *Tcf1*$^{-/-}$ mice show a defect in the function of dendritic cells (Verbeek et al., 1995; Schilham et al., 1998). To define the role of Wnt signaling in the B cell lineage, we adopted two approaches: first, we examined differentiation, proliferation, and survival of B cells in LEF-1-deficient mice, and second, we analyzed the effects of soluble Wnts on pro-B cells in vitro. We find that LEF-1 is expressed in developing pro-B cells in a temporally regulated manner. Furthermore, we show that the absence of LEF-1 leads to reduced proliferation and increased apoptosis of pro-B cells. Finally, we demonstrate that soluble Wnt proteins act directly on pro-B cells to induce entry into the cell cycle and cell proliferation and that the absence of LEF-1 impairs the ability of pro-B cells to respond to Wnt signaling.

Results

To examine the role of Wnt signaling in B cell differentiation, we first analyzed the expression pattern of LEF-1,

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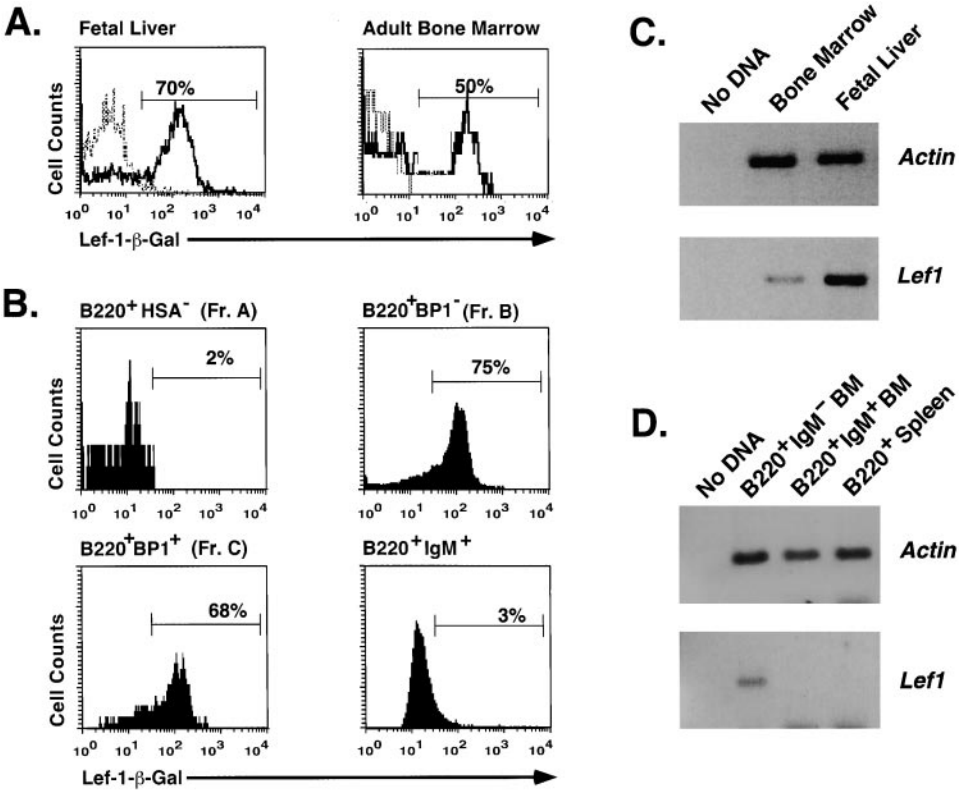

Figure 1. Expression of LEF-1 in Early Stages of B Cell Differentiation

The expression of LEF-1 in B cells was determined in heterozygous *LEF-1-lacZ* knockin mice using fluorescein di-β-D-galactopyranoside (FDG), a fluorogenic substrate for β-galactosidase (β-gal).

(A) Expression of LEF-1 in fetal liver and bone marrow. Cells were loaded with FDG, stained for the pan-B cell marker B220, and analyzed by two-color flow cytometry. Histograms are of β-gal expression on $B220^+$ gated cells.

(B) Analysis of LEF-1 expression at distinct stages of B cell differentiation. Cells from E18 fetal liver were loaded with FDG and stained for B220, HSA, and BP-1 (upper and lower left panel), surface molecules that distinguish different stages of early B cell development (Hardy et al., 1991). Splenocytes and bone marrow cells were loaded with FDG and stained for B220 and IgM to determine expression on immature and mature B cells (lower right panel).

(C and D) Analysis of *Lef1* expression by RT–PCR analysis of sorted $B220^+$ cells from E18 fetal liver and adult bone marrow (C) and on sorted $B220^+IgM^-$ and $B220^+IgM^+$ from the bone marrow and spleen (D). Reactions shown are the result of 30 cycles of amplification.

the transcriptional mediator of this signaling pathway. Previously, *Lef1* transcripts have been identified in transformed pre-B cell lines but not in primary cells of the B lineage (Travis et al., 1991). To study the pattern of expression of the *Lef1* gene in vivo, we analyzed fetal and adult B cell compartments in mice in which the bacterial *lacZ* gene had been inserted into one allele of the *Lef1* locus by homologous recombination (Galceran et al., 2000). In these mice, the spatial and temporal expression pattern of LEF-1 protein is mirrored by the expression of *lacZ*, which can be visualized using fluorescein-di-β-D-galactopyranoside (FDG), a fluorogenic substrate for β-galactosidase. Using this approach, we found that LEF-1 is expressed during early B cell development in the fetal liver and adult bone marrow (Figure 1A). While LEF-1 is expressed in the majority of fetal liver B cells, it is expressed in fewer bone marrow B cells (Figures 1A and 1C). Early (pro) B cell differentiation has been previously subdivided into three stages, termed A, B and C, which can be distinguished by expression of the surface markers B220, HSA, and BP-1 (Hardy et al., 1991). Analysis of LEF-1 expression at specific stages of early B cell development shows that LEF-1 is markedly upregulated at the fraction B stage and is also detected in fraction C cells (Figure 1B). In contrast, IgM-positive B cells from the adult spleen and the adult bone marrow do not express significant levels of LEF-1 (Figure 1B, lower panel; data not shown). This expression pattern of the *Lef1* gene was confirmed by RT–PCR analysis of RNA from FACS-sorted B cells (Figures 1C and 1D).

To address the possibility that the stage-specific expression pattern of LEF-1 reflects a role of LEF-1 and Wnt signaling in primary B cells, we examined the presence of various B cell populations in $Lef1^{-/-}$ mice. These mice have been previously shown to display multiple nonimmunological defects, including a complete block in hair, tooth, and mammary gland development and an absence of specific neurons in the brain (van Genderen et al., 1994; Galceran et al., 2000). However, the immune system had not been analyzed in these mice. Since $Lef1^{-/-}$ mice die by 2 weeks after birth, most of our analysis was restricted to the fetal liver, which supports B cell development from embryonic days 13 (E13) to 18.

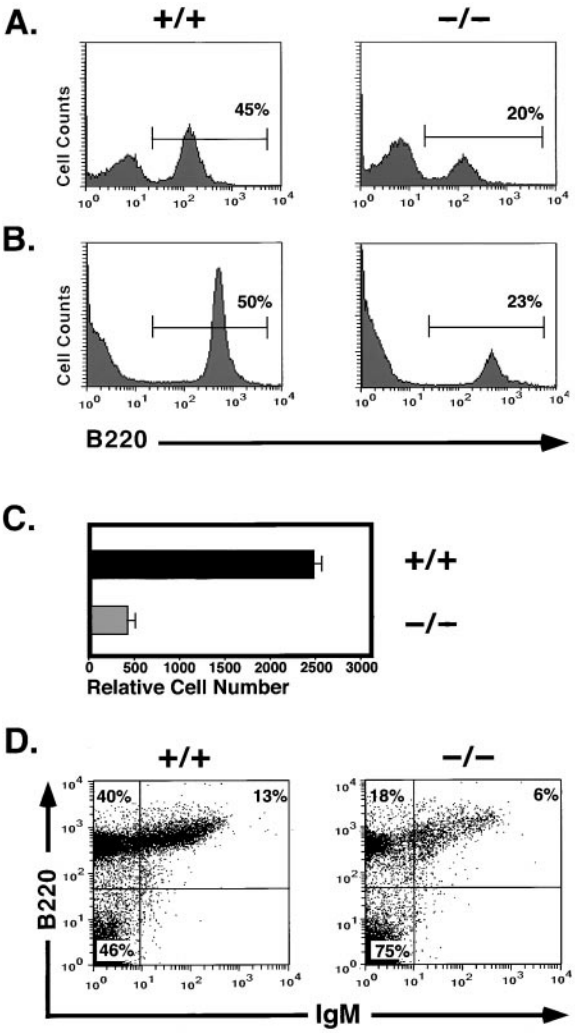

Figure 2. Loss of LEF-1 Leads to Reduced Numbers of Developing B Cells but Allows Normal Differentiation of IgM-Positive Cells

(A and B) Cells from the fetal liver (A) and perinatal bone marrow (B) of wild-type (+/+) and LEF-1-deficient (−/−) mice were isolated and stained with anti-B220 and analyzed by flow cytometry.

(C) Comparison of live B cells in fetal liver of wild-type and LEF-1-deficient mice. Fetal liver cells were isolated and stained for B220 and for fragmented DNA using the TUNEL assay, and the relative numbers of $B220^+$ $TUNEL^-$ cells from wild-type (+/+) and LEF-1-deficient (−/−) mice are shown.

(D) Cells from perinatal bone marrow were stained with antibodies specific for surface antigens B220 and IgM and analyzed by flow cytometry.

In addition, we analyzed cells from the perinatal bone marrow to determine the status of IgM-positive cells, which cannot be detected in significant numbers in the fetal liver. Flow cytometric analysis indicated that the total number of $B220^+$ cells was consistently reduced by at least a factor of two in both the fetal liver (Figure 2A) and the perinatal bone marrow (Figure 2B). Significantly, the difference in the numbers of $B220^+$ cells in wild-type and LEF-1-deficient mice was 6- to 8-fold if dead and dying cells were excluded from the analysis (Figure 2B). The majority of the $B220^+$ cells we detected in the E18 fetal liver were $CD43^+$, placing them in the pro-B cell compartment. Although in certain cases, E18 fetal liver has been found to contain as much as 10% IgM^+ cells (Ceredig et al., 1998), our mice consistently display less than 5% IgM^+ cells.

To determine whether this reduction in the size of the B cell compartment was due to a defect in differentiation, we analyzed bone marrow from mice at postnatal day 13 (P13). While the absolute numbers of IgM^+B220^+ cells were found to be reduced, the ratio of IgM^- to IgM^+ B lymphocytes remained unchanged in the Lef-1-deficient marrow (Figure 2D). In addition, PCR analysis of the rearrangement status of the immunoglobulin heavy and light chain genes in the fetal liver of E18 wild-type and *Lef1*$^{-/-}$ mice revealed normal rearrangement in the mutant B cells (Reya et al., 1999; data not shown). The normal differentiation of B cells was also confirmed by adoptive transfer of fetal liver cells from wild-type and mutant mice. In such transfer experiments, fetal liver B cell precursors developed into IgM^+ cells and responded normally to mitogens (Reya et al., 1999; data not shown). Thus, B cell differentiation is not obviously affected by the absence of LEF-1.

The reduced numbers of pro-B cells in the fetal liver of *Lef1*$^{-/-}$ mice could be due to a decrease in cell proliferation and/or a decrease in cell survival. To examine cell survival, we analyzed fetal liver sections from wild-type and *Lef1*$^{-/-}$ mice for the presence of cells undergoing apoptosis. Using the TUNEL assay, which detects cells containing fragmented DNA, we found a marked (up to 20-fold) increase in the number of apoptotic cells in the *Lef1*$^{-/-}$ fetal liver relative to that in the wild-type tissue (Figure 3A). The discrete appearance of apoptotic cells in the tissue sections, as well as the size of the dying cells, suggested that the dying cells were of hematopoietic origin. To determine whether the apoptotic cells were B cells, we carried out the TUNEL assay by flow cytometry and specifically analyzed the number of B220-positive cells that contained fragmented DNA (Figure 3B). This analysis revealed a 3-fold increase in the number of apoptotic cells in the $B220^+$ compartment of *Lef1*$^{-/-}$ mice but no increase in the $B220^-$ compartment of these mice, consistent with the lineage-specific expression pattern of LEF-1 (Figures 3B and 3C). This increase in the number of apoptotic cells, observed by flow cytometry, was less pronounced than that detected by the TUNEL assay of fixed tissue. The difference may be explained, in part, by a loss (up to 4-fold) of fragile apoptotic cells during the processing of cells for flow cytometry, which does not occur in the analysis of fixed tissue samples. To confirm that TUNEL-positive cells actually reflect ongoing apoptosis, we utilized Annexin V, which binds to the exposed phosphotidyl serine in the membranes of apoptotic cells. Using this assay, we detected a 3-fold increase in the number of Annexin V^+ and Propidium Iodide $(PI)^-$ cells in the $B220^+$ cell population of LEF-1-deficient mice (Figure 3D). Annexin V staining is detectable at early stages of apoptosis when cells are PI negative, whereas late apoptotic cells, which are PI positive, are not included in this analysis to avoid contamination with necrotic cells.

To determine the molecular basis for the reduced survival of developing B cells, we examined wild type and *Lef1*$^{-/-}$ mice for the expression of genes involved in the regulation of apoptosis. Semiquantitative RT–PCR

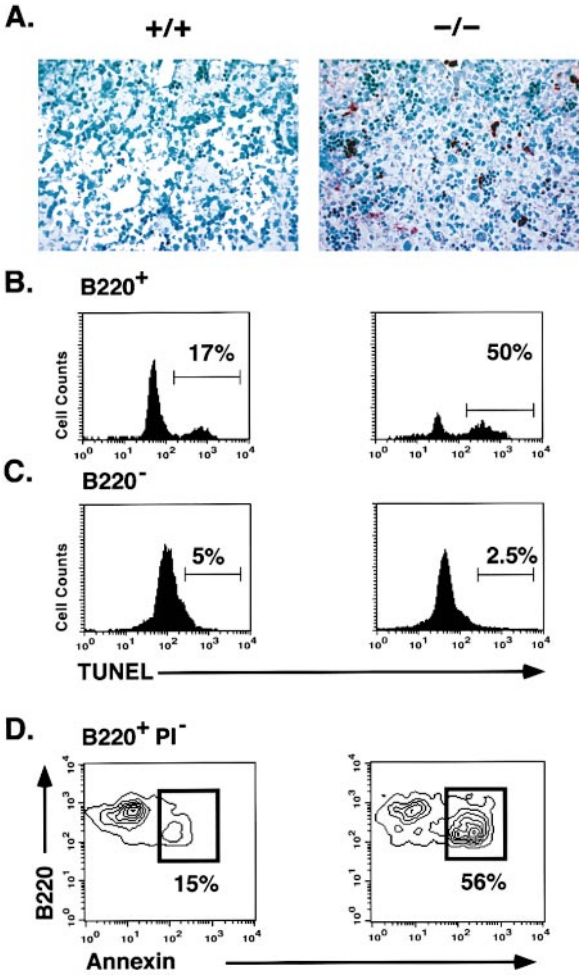

Figure 3. Loss of LEF-1 Results in Increased Apoptosis of Developing B Cells

(A) TUNEL assay of apoptosis in fetal liver tissue. Fetal livers from wild-type (+/+) and LEF-1-deficient (−/−) mice were isolated, fixed in formalin, paraffin embedded, and sectioned at 3–5 μm/section. Sections were stained using the TUNEL assay, which allows detection of cells containing fragmented DNA. Sections were counterstained with 0.5% methyl green. TUNEL-positive cells appear reddish brown.

(B and C) Flowcytometric analysis of apoptotic B and non-B cells. Fetal liver cells from E18 wild-type and *Lef1*$^{-/-}$ mice were stained with antibodies directed against the B cell–specific surface antigen B220, fixed in paraformaldehyde and ethanol, and then stained for fragmented DNA using the TUNEL assay.

(D) Analysis of apoptosis by Annexin V staining. Fetal liver cells from wild-type and *Lef1*$^{-/-}$ mice were stained with antibodies to B220, followed by Annexin V and propidium iodide. Cells in the B220^{+}PI^{-} gate (thereby excluding necrotic and late apoptotic cells) were analyzed for Annexin V staining, and the number of positive cells (early apoptotic) is represented.

analysis of sorted pro-B cells (fraction B) from *Lef1*$^{-/-}$ and wild-type fetal liver revealed a marked increase in the expression of *fas* and c-*myc* in the mutant cells (Figure 4). The *fas* gene is generally not expressed in early stage B cells, but it is expressed in activated mature B cells and can act there as a potent mediator of cell death. Similarly, overexpression of c-*myc* has been implicated in cell death, particularly in cell death that is

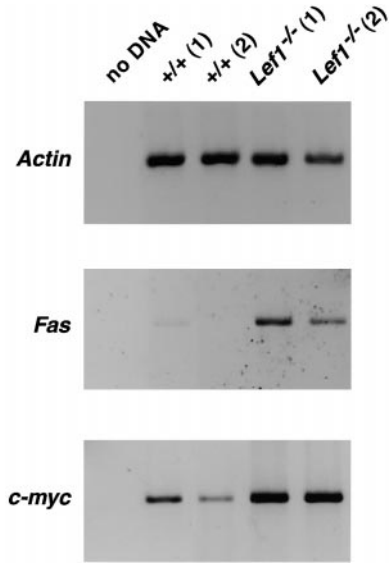

Figure 4. Increased Apoptosis in *Lef1*$^{-/-}$ Developing B Cells Is Accompanied by Upregulation of *fas* and *c-myc*

Equivalent pro-B cell fractions were sorted from E18 fetal liver of wild-type and *Lef1*$^{-/-}$ mice and RNA was prepared and used for RT–PCR analysis. Reactions shown are the result of 30 cycles of amplification. In parallel experiments, the amount of input cDNA was varied to ensure linearity of amplification.

mediated by fas upon growth factor withdrawal (Evan et al., 1992; Shi et al., 1992). In contrast, the levels of expression of other genes implicated in apoptosis, such as *Bcl-2*, *Bcl-x*, and *p53*, were not altered in the mutant mice (data not shown). Taken together, these data indicate that two genes, implicated in apoptosis, are dysregulated in the absence of LEF-1.

We also examined whether the reduction in the size of the B cell compartment may involve a decrease in the proliferation of developing B cells. In a thymidine incorporation assay of *Lef1*$^{-/-}$ fetal liver pro-B cells that had been purified by flow cytometry, we observed a 50% decrease in DNA synthesis relative to pro-B cells from wild-type mice (Figure 5A). In addition, we measured the DNA content of the cells using propidium iodide staining and found that 50% fewer LEF-1-deficient B cells were in cycle (S/G2/M) as compared to wild-type cells (Figure 5B). These data suggest that B cells depend on LEF-1 for proliferation during early stages of development.

The pro-B cell stage of differentiation is critically dependent upon the cytokine interleukin-7 (IL-7) for proliferation and cell survival (Grabstein et al., 1993; Peschon et al., 1994; Fisher et al., 1995; von Freeden-Jeffry et al., 1995). Thus, one mechanism by which LEF-1 deficiency may result in reduced proliferation and survival is a reduced responsiveness of pro-B cells to IL-7. To test this possibility, we cultured fetal liver cells from wild-type and mutant mice in the presence of IL-7 and monitored the proportion of cells responding to IL-7 by determining incorporation of bromodeoxyuridine (Figure 6). Cells from wild-type and *Lef1*$^{-/-}$ mice responded similarly, suggesting that LEF-1 is not required for IL-7-mediated cell proliferation.

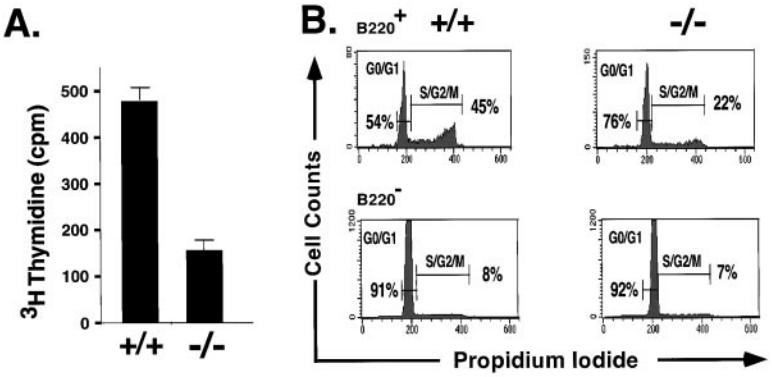

Figure 5. Loss of LEF-1 Leads to Reduced Proliferation of Developing B Cells

(A) To determine the rate of cell proliferation, FACS-sorted fetal liver B cells from wild-type (+/+) and LEF-1 deficient (−/−) mice were aliquoted (triplicate samples of 2×10^5 cells/well) into 96-well plates and cultured for 48 hr. Cells were pulsed with [^{3}H]thymidine for 18 hr, harvested, and assayed for thymidine incorporation by scintillation spectrophotometry. Data shown is representative of three independent experiments.

(B) Cells from the fetal liver of wild-type (+/+) and LEF-1-deficient (−/−) mice were isolated, stained for B220 and intracellularly stained with propidium iodide, and analyzed by flow cytometry to determine the cell cycle status. The average number of B cells in cycle over three independent experiments was 42% ± 3% (+/+) and 20% ± 1.5% (−/−).

Since LEF-1 is a transcriptional mediator of the Wnt signaling pathway (Behrens et al., 1996; Brunner et al., 1997; Riese et al., 1997; Hsu et al., 1998), the B cell defects in LEF-1-deficient mice suggested that Wnt signaling is involved in regulating the proliferation and survival of pro-B cells. To date, however, the only evidence for an influence of Wnt proteins on B cell proliferation is provided by the recent finding that certain leukemic B cell lines overexpress a novel Wnt protein, Wnt 16 (McWhirter et al., 1999). Therefore, we undertook experiments to test directly whether normal developing B cells could respond to Wnt signaling. Initially we tested whether lithium chloride, which inhibits GSK-3β and mimics Wnt signaling by stabilizing free β-catenin (Stambolic et al., 1996), had a functional effect on developing B cells. We found that in the range of 3 mM, LiCl causes a 3-fold increase in proliferation of fetal liver pro-B cells, a response comparable to that induced by IL-7 stimulation (Figure 7A).

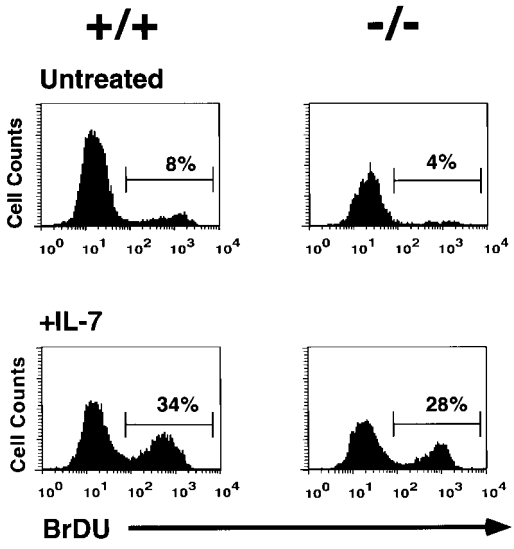

Figure 6. Reduced Cell Proliferation in *Lef-1*$^{-/-}$ Mice Is Not Due to Impaired IL-7 Responsiveness

To determine the response of LEF-1-deficient pro-B cells to IL-7, sorted fetal liver B cells from wild-type (+/+) and *Lef1*$^{-/-}$ mice (triplicate samples of 1×10^6 cells/well) were aliquoted into 48-well plates and stimulated with 30 U/ml IL-7 or cultured unstimulated for 48 hr. Cells were pulsed with BrdU for 18 hr and stained intracellularly with anti-BrDU antibodies and on the surface with anti-B220 antibodies. Stained cells were analyzed by two-color flow cytometry.

Since lithium could augment B cell proliferation, we were interested in determining whether specific Wnt proteins could exert a similar effect. To this end, we first analyzed the expression of *Wnt* genes in the microenvironment of developing B cells. Our analysis revealed that multiple Wnt genes, including *Wnt10B*, *Wnt3A*, and *Wnt5A*, were expressed in bone marrow (Figure 7B). We also determined whether these *Wnt* genes are expressed in stromal cells that support early B cell development. Interestingly, while *Wnt5A* expression could be found in cultured primary bone marrow stromal cells, neither *Wnt10B* nor *Wnt3A* were expressed in those cells (Figure 7B), suggesting that these Wnt proteins are most likely made by hematopoietic cells of the bone marrow.

To perform functional tests with Wnt proteins, we sought a source of soluble Wnt protein. To date, the only successful soluble form of a Wnt protein has been generated by stable transfection of a *Wnt3A* cDNA into L cells (Shibamoto et al., 1998). We were thus able to test the ability of developing B cells to respond to Wnt3A. A simple biochemical read-out of Wnt signaling is the stabilization of β-catenin. We therefore incubated sorted fetal liver pro-B cells in conditioned medium from cells secreting murine Wnt3A (Shibamoto et al., 1998) and assayed pro-B cell lysates for β-catenin stabilization. Free β-catenin could not be detected in cells stimulated with control supernatant but was readily detectable in cells that were exposed to Wnt-containing supernatant (Figure 7C). This result suggested that pro-B cells were able to transduce a Wnt signal at a biochemical level.

To determine whether this signal has a functional effect on developing B cells, we examined its effect on cell proliferation. Using BrdU incorporation as an assay, we found that Wnt signaling increased the relative proliferation rate of pro-B cells approximately 4-fold, allowing a majority of developing B cells to enter the cell cycle (Figure 7D). To examine whether this effect of Wnt 3A on pro-B cells is dependent on LEF-1, we compared the ability of wild-type and LEF-1-deficient pro-B cells to

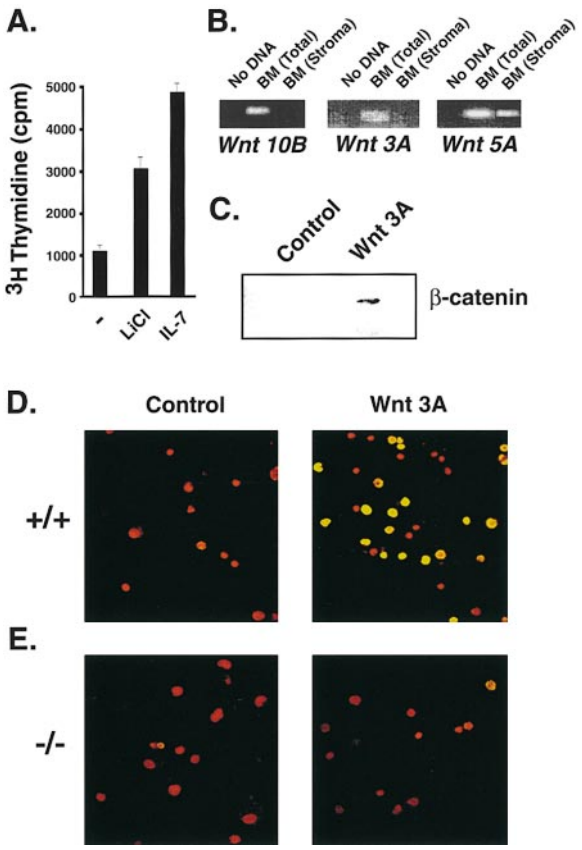

Figure 7. Wnt Signaling Induces B Cell Proliferation in a LEF-1-Dependent Manner

(A) To determine the response of developing B cells to lithium chloride, which mimics a Wnt signal, FACS-sorted fetal liver B cells from wild-type mice (triplicate samples of 2×10^5 cells/well) were aliquotted into 96-well plates and stimulated with 3 mM LiCl for 48 hr. For comparison, cells were cultured without a stimulus or with 30U/ml of IL-7. Cells were pulsed with ^{3}H-thymidine for 18 hr, harvested, and assayed for thymidine incorporation by scintillation spectrophotometry.

(B) Total bone marrow (BM) cells harvested from wild-type mice or cultured bone marrow stroma were used to prepare RNA for RT–PCR analysis to detect *Wnt10B*, *Wnt3A*, and *Wnt5A* transcripts. Reactions shown are the result of 35 cycles of amplification.

(C) To determine the response of developing B cells to Wnt signaling, fetal liver B cells from wild-type mice were stimulated in with Wnt3A-conditioned medium for 2 hr, lysed, and free β-catenin was detected by immunoblot analysis with antibodies against β-catenin (Young et al., 1998).

(D and E) Fetal liver pro-B cells from wild-type (+/+) (D) and LEF-1-deficient (−/−) (E) mice were isolated and cultured for 2 days in Wnt3A-conditioned medium or control medium. Cells were pulsed with BrdU for 18 hr, cytospun, fixed, and intracellularly stained with FITC-conjugated anti-BrdU antibody (green). The cells were counterstained with 7AAD (red) and analyzed by fluorescence microscopy. Cells that incorporated BrdU appear in yellow.

respond to Wnt signaling. The majority of wild-type pro-B cells proliferated in response to Wnt3A, whereas only a small percentage of *Lef1*$^{-/-}$ pro-B cells entered the cell cycle upon addition of Wnt3A-conditioned medium (Figure 7E). Taken together, these data implicate Wnt signaling as an upstream regulator of LEF-1 activity in developing B cells and suggest that a loss of responsiveness to Wnt signaling may account for the defects observed in the LEF-1-deficient mice.

Discussion

In this study, we show that LEF-1 is expressed at specific stages of B cell differentiation and is required for cell proliferation and survival but not for cell differentiation. Furthermore, we find that the loss of LEF-1 leads to upregulation of *fas* and c-*myc* expression, providing a potential explanation for the increased cell death in LEF-1-deficient mice. Finally, we provide evidence that Wnt signaling is mitogenic for early stage B lymphocytes and is dependent on the presence of LEF-1, implicating the loss of normal Wnt signaling in the reduced growth and survival of B cells in LEF-1-deficient mice.

Although LEF-1 is specifically expressed in pro-B and pre-B cells, mice lacking LEF-1 do not show any obvious defect in B cell differentiation. *Lef1*$^{-/-}$ mice show a normal frequency of both D to J and V to DJ recombination and a normal appearance of IgM-positive B cells (Reya et al. 1999). However, we detected a decrease in the expression of *BP-1* (T. R. and R. G., unpublished data) and an increase in the expression of the terminal deoxynucleotidyl transferase (*TdT)* gene (M. O. et al., unpublished data). Although these results suggest that LEF-1 may regulate these genes, neither BP-1 nor TdT proteins are required for the normal maturation of B cells (Komori et al., 1993; Lin et al., 1998). It is also unlikely that changes in the expression of these genes contribute to the cellular defects, such as decreased survival and proliferation, that are observed in the LEF-1-deficient mice. Thus, LEF-1 has either no major role in regulating the differentiation to B220$^+$IgM$^+$ cells or is functionally redundant with other members of this family of transcription factors. Significant levels of *Tcf4* mRNA accumulation can be detected in pro-B cells (M. O. et al., unpublished data). Therefore, TCF-4 may act redundantly with LEF-1 in regulating B cell differentiation, although a deficiency of LEF-1 alone results in functional defects in early B cells. Precedence for redundancy within this family of transcription factors comes from previously observed redundancy between TCF-1 and LEF-1 in the regulation of T cell differentiation (Okamura et al., 1998).

A defect in the survival of lymphocytes in *Lef1*$^{-/-}$ mice was observed in fetal liver pro-B cells. Interestingly, we have also observed increased TUNEL staining of thymocytes in *Lef1*$^{-/-}$ mice (data not shown), suggesting that LEF-1 may play a general role in survival of developing lymphocytes. In pro-B cells, the increased apoptosis may be due to upregulation of *fas* and c-*myc*. Upregulation of *fas* in B cells that normally do not express this gene results in cell death (Garrone et al., 1995; Onel et al., 1995; Wang et al., 1996). Likewise, c-*myc* overexpression has been found to result in cell death upon growth factor withdrawal (Evan et al., 1992). If upregulation of these genes is involved in apoptosis in LEF-deficient B cells, the question arises as to whether these genes are direct targets of LEF-1. Previous reports have suggested that the c-*myc* promoter contains LEF-1 binding sites and that Wnt signaling induces c-*myc* in colorectal cancer derived cell lines (He et al., 1998). In contrast, recent reports indicate that c-*myc* is not regulated by LEF-1/TCF proteins in certain epithelial cell lines (Kolligs et al., 1999), suggesting that it may not be a general target of the Wnt signaling pathway. Our observation that c-*myc* expression is increased in LEF-1-deficient B cells suggests that in this cell type, c-*myc*

may even be negatively regulated by Wnt signaling. Alternatively, the increased expression of c-*myc* in LEF-1-deficient B cells may be an indirect effect of the loss of LEF-1, perhaps related to the reduced proliferation that is observed in these cells.

Rather than being a direct effect of the loss of LEF-1, the increased cell death observed in LEF-deficient pro-B cells may be a consequence of decreased cell proliferation (Sauter et al., 1999). In support of this possibility, we found that LEF-1-deficient pro-B cells exhibit altered cell cycle progression in vivo and reduced cell proliferation in vitro. Moreover, we observed that Wnt3A induces proliferation of pro-B cells in a LEF-1-dependent manner but has no effect on cell survival. These findings suggest that the primary function of LEF-1 in pro-B cells may be to regulate proliferation. Consistent with this notion, a cell cycle defect has also been observed in developing thymocytes in TCF-1-deficient mice (Verbeek et al., 1995; Schilham et al., 1998). One way in which LEF-1/TCF transcription factors could regulate cell cycle progression is through the induction of *cyclin D1*, which has been recently identified as a direct target of LEF-1/TCF proteins in association with β-catenin (Shtutman et al., 1999; Tetsu and McCormick, 1999). However, examination of sorted pro-B cells from wild-type and LEF-1-deficient mice for the expression of *cyclin D1* by a semiquantitative RT–PCR analysis did not reveal any differences in the expression levels (data not shown). This suggests that the absence of LEF-1 may not be sufficient for downregulation of *cyclin D1* and that other targets may account for the cell cycle defect in *Lef1*$^{-/-}$ mice.

The cell cycle defect in *Lef1*$^{-/-}$ B cells may reflect an inability of these cells to respond to mitogenic Wnt signals in their microenvironment. Wnt proteins have been shown to act as potent growth factors in a variety of cell types and tissues. In particular, Wnt1 can induce proliferation of mammary epithelial cells and fibroblasts (Bradley and Brown, 1995; Bafico et al., 1998; Young et al., 1998), and can synergize with Steel factor to enhance the proliferation of hematopoietic progenitors (Austin et al., 1997). Our observation of pro-B cell proliferation in response to Wnt3A represents the first example of proliferation induced by Wnt proteins in the immune system. Two observations support a role of Wnt signaling in developing B cells. First, incubation of pro-B cells in Wnt3A-conditioned medium results in stabilization of cytosolic β-catenin. Second, Wnt3A-conditioned medium and lithium chloride, which inhibits GSK-3β and mimics Wnt signaling (Stambolic et al., 1996), both augment pro-B cell proliferation. This effect is observed in wild-type but not in LEF-1-deficient pro-B cells, although the mutant cells respond normally to interleukin 7. Thus, the proliferative defect in *Lef1*$^{-/-}$ pro-B cells may reflect a specific defect in Wnt signaling.

We have found that several *Wnt* genes, such as *Wnt10B*, *Wnt3A*, and *Wnt5A*, are expressed in the microenvironment that supports B cell differentiation. Since multiple Wnt proteins act through the same signaling pathway involving β-catenin and the LEF-1/TCF family of transcription factors, it is possible that additional Wnt proteins can influence B cell proliferation during development. Wnt proteins have been shown to act as short-range signaling molecules (Cadigan and Nusse, 1997), and therefore, these proteins may play a role in the dependence of pro-B and pre-B cells on contact with stromal cells (Dorshkind et al., 1992; LeBien, 1998). Indeed *Wnt5A* is expressed in primary stromal cells of the bone marrow, supporting this possibility. Besides establishing a link between Wnt signaling and normal lymphocyte function, our study also has implications for the understanding of Wnt-mediated tumorigenesis. Deregulation of various components of the Wnt signaling pathway have been implicated in tumor formation (Jue et al., 1992; Wong et al., 1994; Lee et al., 1995). In particular, overexpression of *Wnt1* causes mammary epithelial cell tumors, and APC and β-catenin mutations have been identified in many colon carcinomas (Morin et al., 1997). Recently, a novel *Wnt* family member (*Wnt16*) has been found to be overexpressed in leukemic cell lines (McWhirter et al., 1999). This observation, together with our finding that the Wnt pathway regulates the proliferation and survival of normal B lymphocytes, suggests that aberrant Wnt signaling may also contribute to tumorigenesis in the immune system.

Experimental Procedures

Mice

Lef1$^{+/-}$ and LEF-1-β-gal mice were housed and bred in SPF facilities at the animal care facility at the University of California, San Francisco. *Lef1*$^{+/-}$ mice were mated and offspring containing *Lef1*$^{-/-}$ were harvested at E18 or postnatal d1. A PCR-based screening method was used to distinguish wild-type, heterozygous, and homozygous mutant mice. LEF-1-β-gal mice were mated and offspring used at E16–E18. Mice were screened using a 1 mm tail piece stained with X-gal staining solution (100 mM sodium phosphate, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal) for 30 min at 37°C.

Cell Isolation and Tissue Preparation

Fetal liver and fetal thymi were isolated and dispersed in RPMI with 10% FCS. Fetal liver cells were centrifuged over a Lympholyte M gradient (Cedar Lane) at a 1:1 ratio at 1500 rpm for 15 min to remove red blood cells. Cells were subsequently washed and centrifuged at 2000 rpm for 5 min and counted using a hemacytometer before use in experiments. For sectioning, intact fetal liver and thymi were isolated and fixed in formalin solution (Sigma) for 30 min and transferred to fresh formalin for 12 hr. Subsequently, the tissues were embedded in paraffin and sectioned.

Flow Cytometry

Cells from the fetal liver and thymus were prepared as above, and 10×10^5 cells were resuspended in 50 μl FACS buffer (PBS/5% FCS/0.1% sodium azide) in a 96-well U-bottomed ELIZA plate. Cells were incubated with 1 μg anti-B220 PE and/or anti IgM FITC (PharMingen) for 30 min prior to washing twice with FACS buffer and centrifuged at 2000 rpm for 2 min after each wash. Cells were then resuspended in 200 μl FACS buffer and analyzed on the FACSCalibur (Becton Dickinson). All data was analyzed using CellQuest software (Becton Dickinson).

β-gal Detection of LEF-1-β-gal Fetal Liver Lymphocytes

Fetal liver cells were isolated from LEF-1-β-gal mice and prepared as described above to remove red blood cells. After surface staining for appropriate markers, cells were resuspended in 10×10^6 cells/100 μl in PBS and incubated at 37° for 10 min. Subsequently, these cells were incubated in an equal volume of 2 mM fluorescein di-β-D-galactopyranoside (Molecular Probes) at 37°C for 2 min before addition of PBS incubation on ice for 1 hr. Cells were then centrifuged and resuspended in 200 μl FACS buffer and run on the FACSCalibur (Becton Dickinson).

Measurement of Cell Cycle Progression

Freshly isolated cells were washed and surface stained with anti-B220, washed with 2 ml phosphate-buffered saline (PBS), and resuspended in 500 μl ice-cold PBS containing 0.06% paraformaldehyde. Cells were fixed on ice for 1 hr, centrifuged at 2000 rpm for 7 min, and resuspended in 500 μl PBS containing 0.2% Tween 20. Cells were then incubated in a 37°C water bath for 15 min, washed with PBS containing 5% FCS and 0.1% sodium azide, and resuspended in staining solution (PBS containing 50 μg ribonuclease A, 10 μg/ml propidium iodide, and 0.1% sodium azide). After staining for at least 2 hr on ice, samples were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson), using CellQuest (Becton Dickinson) software.

Proliferation Assays

Assay medium was RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM 2-mercaptoethanol (GIBCO–BRL). Triplicate cultures of 2×10^5 cells were incubated with 200 μl assay medium (in the absence of stimulus or in the presence of IL-7 or LiCl) in 96-well flat-bottomed microtiter plates. After 48 hr of culture in a 37°C/10% CO_2, cells were pulsed with 1 μCi of [^{3}H]thymidine (NEN) and incubated for an additional 16 hr before being harvested. Radioactivity incorporated was measured by liquid scintillation spectrophotometry.

TUNEL Analysis

In situ TUNEL was carried out on 5 μm paraffin sections of fetal liver or thymus. Sections were stained using the Genzyme TACS in situ apoptosis detection kits (Genzyme) using TdT as the enzyme and DAB substrate for detection. Genzyme TUNEL protocol was followed except for the following modifications: quenching was carried out with 3% H_2O_2 in methanol and TdT labeling with 2 μl TdT per sample for 2 hr at 37°. Slides were incubated in streptavidin-HRP solution for 30 min. During development, 350 μl stock DAB solution was used, and slides were incubated for 20 min before washing and counterstaining with 1% methyl green solution for 10 s. TUNEL detection by flow cytometry was carried out using Apo-Direct (PharMingen) with 1×10^6 cells from the fetal thymus or fetal liver after they had been surface stained with antibodies of interest. Annexin V staining was carried out after initial staining with anti-B220 (PharMingen) using AnnexinV staining kit (Genzyme). Cells were subsequently run on FACSCalibur (Becton Dickinson) and analyzed using CellQuest software.

BrdU Analysis

Assay medium was RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM 2-mercaptoethanol (GIBCO–BRL). Duplicate cultures of 1×10^6 cells were incubated with 500 μl assay medium in 48-well flat-bottomed microtiter plates. After 48 hr of culture in a 37°C/10% CO_2, cells were pulsed with BrdU (Sigma) and incubated for an additional 16 hr before being harvested. Cells were then washed and fixed in 1% paraformaldehyde for 20 min on ice, followed by fixation in 100% ice-cold ethanol overnight. Cells were then washed in PBS, stained with anti-BrdU-FITC (PharMingen), and analyzed on the FACSCalibur (Becton Dickinson) or by fluorescence microscopy.

RT–PCR

Fetal liver cells were FACS sorted using the FACStar (Becton Dickinson) into eppendorf tubes containing 10 μg yeast tRNA. Each sample shown represents one fetal liver; no samples were pooled. RNA for RT–PCR was purified from the fetal liver samples with Trizol (GIBCO–BRL), and resuspended in DEPC-treated water. The samples were treated with RQ1 RNase-free DNase (Promega) for 20 min at 37°C before inactivation by phenol/chloroform extraction and ethanol precipitation. The fetal liver RNA was then reverse transcribed with MMLV-RT (GIBCO–BRL) for 1 hr at 37°C and inactivated for 5 min at 95°C. The cDNA reactions were brought up to a final volume of 100 μl and 5 μl used in an RT–PCR reaction. RT–PCR reactions were standardized by levels of β-actin. Standard RT–PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, using 0.1 μl of Taq polymerase (Boehringer Mannheim) per reaction for the number of cycles indicated in the legend to Figure 4. To ensure that amplification occurred in the linear range, aliquots were withdrawn at 27, 30, and 33 cycles for *fas* and c-*myc* and at 20 or 22 cycles for actin. Furthermore, in parallel experiments, the amount of input cDNA was varied to ensure that the increased amounts of input cDNA correlated with increased amounts of PCR product. Primers for RT–PCR reactions were designed using Geneworks software: Actin (X03765) (1) 5′-GACGACATGGAGAAGAT CTGG, (2) 5′-TGTGGTGGTGAAGCTGTAGC; Fas (GenBank accession number M83649) (1) 5′-CACACTCTGCGATGAATAGC, (2) 5′-ATT GGTACCAGCACAGGAGC; c-myc (GenBank accession number X01023) (1) 5′-TCCTGTACCTCGTCCGATTC, (2) 5′-TTCCAAGAC GTTGTGTGTCC. All PCR reactions were run on 2% agarose gels with ethidium bromide and visualized by UV transillumination. Images were captured with a gel documentation system (Alpha Innotech).

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