Contrasting Responses of Lymphoid Progenitors to Canonical and Noncanonical Wnt Signals

Sachin Malhotra, Yoshihiro Baba, Karla P. Garrett, Frank J. T. Staal, Rachel Gerstein and Paul W. Kincade

J Immunol 2008; 181:3955-3964; doi: 10.4049/jimmunol.181.6.3955
http://www.jimmunol.org/content/181/6/3955

References
This article cites 60 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/181/6/3955.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Wnt family of secreted glycoproteins has been implicated in many aspects of development, but its contribution to blood cell formation is controversial. We overexpressed Wnt3a, Wnt5a, and Dickkopf 1 in stromal cells from osteopetrotic mice and used them in coculture experiments with highly enriched stem and progenitor cells. The objective was to learn whether and how particular stages of B lymphopoiesis are responsive to these Wnt family ligands. We found that canonical Wnt signaling, through Wnt3a, inhibited B and plasmacytoid dendritic cell, but not conventional dendritic cell development. Wnt5a, which can oppose canonical signaling or act through a different pathway, increased B lymphopoiesis. Responsiveness to both Wnt ligands diminished with time in culture and stage of development. That is, only hematopoietic stem cells and very primitive progenitors were affected. Although Wnt3a promoted retention of hematopoietic stem cell markers, cell yields and dye dilution experiments indicated it was not a growth stimulus. Other results suggest that lineage instability results from canonical Wnt signaling. Lymphoid progenitors rapidly down-regulated RAG-1, and some acquired stem cell-staining characteristics as well as myeloid and erythroid potential when exposed to Wnt3a-producing stromal cells. We conclude that at least two Wnt ligands can differentially regulate early events in B lymphopoiesis, affecting entry and progression in distinct differentiation lineages. The Journal of Immunology, 2008, 181:3955–3964.

More than a decade ago, it was shown that Wnt ligands and their Frizzled (Fzd) receptors are expressed in hematopoietic tissues, where they appear to function as growth factors (1, 2). Subsequent studies implicated them in many other aspects of blood cell formation, and particularly exciting were reports that they can be exploited to propagate stem cells in culture (3–5). However, many questions remain about the importance of particular ones to immune system development in normal adults.

Hematopoietic stem cells (HSC) are normally very rare and are thought to spend most of their time in a quiescent state while residing in specialized stromal cell-containing niches (6). Through mechanisms that are only partially understood, the integrity of stem cells is retained throughout life. That is, they maintain competence to self renew and to generate progenitors capable of making billions of blood cells each day. Stem cells are heterogeneous, and our study focused on Thy1.1 low, RAG-1/GFP-negative HSC enriched among the small lineage marker-negative, Sca-1-positive, c-Kit<sup>high</sup> (LSK) fraction of bone marrow. HSC give rise to multipotent progenitors and several types of lineage-specific cells. For example, early lymphoid progenitors (ELP) can be identified in RAG-1/GFP knock-in reporter mice and represent the most primitive cells with high potency to produce lymphocytes (7, 8). ELP retain some potential for generating non-lymphoid cells, but this is reduced still further in the common lymphoid progenitors (CLP) to which they give rise. CLP are enriched in the Lin– RAG-1/GFP<sup>+</sup> Sca-1<sup>+</sup> c-Kit<sup>low</sup> prolymphocyte (ProL) fraction of bone marrow (7). We have now studied these and other well-characterized hematopoietic cells in relation to Wnt signaling.

The 19 Wnt ligands are 350–450 aa in length and express conserved cysteines as well as sites for N-glycosylation or palmitoylation (9). These modifications guide the shape and hydrophobicity as well as extracellular stability, distribution, and activity of Wnts. Extracellular matrix interactions help to create Wnt activity gradients corresponding to expression levels of Wnt target genes in the responding cells that establish and modulate developmental patterns (10). Wnt signal transduction commences after ligand interaction with membrane-associated Wnt receptors. There are at least 10 seven-pass transmembrane Frizzled (Fzd) receptors, 2 low-density lipoprotein receptor-related proteins (LRP), and a number of extracellular Wnt-modulating proteins such as Kremen, Dickkopf (Dkk), Wnt-inhibitory factor, secreted Fzds, and Norrin (10–12). Depending on the type of ligand-receptor interaction, the presence of intracellular signaling components, and the target cell, three Wnt signaling pathways have been identified. The canonical pathway that has been most studied results in stabilization and nuclear translocation of β-catenin. The Wnt-Fzd-LRP5/6 receptor
complex activates intracellular Dishevelled that inhibits a complex of proteins, including Axin, glycogen synthase kinase 3-β, adenomatous polyposis coli, and casein kinase. This complex normally binds cytosolic β-catenin and targets it for destruction. Stabilized β-catenin translocates to the nucleus, where it interacts with transcription factors such as T cell-specific factor and lymphoid enhancer factor. The two noncanonical pathways, Wnt-Ca²⁺ and Wnt-JNK, do not stabilize β-catenin pools. In these cases, Wnt-Fzd interactions activate membrane-associated G protein complexes and Dishevelled to either increase intracellular Ca²⁺ levels through inositol-3-phosphate or induce the JNK pathway through Rho/Rac GTPases. As a result of those events, noncanonical signals can influence actin-dependent cytoskeletal reorganization (13).

Recombinant Wnt proteins and manipulation of Wnt pathway intermediates have been used to artificially expand HSC or progenitors (1, 4, 5, 14, 15). For example, HSC of BCL-2 transgenic mice increased more than 100-fold and retained primitive characteristics when transduced with stable β-catenin (5). Furthermore, HSC were able to reconstitute all hematopoietic cells when exposed to rWnt3a for extended periods in culture and then transplanted (4). Reciprocally, retrovirally introduced Axin inhibited HSC expansion in culture. Glycogen synthase kinase 3-specific inhibitors that stabilize cellular β-catenin were also used to conclude that canonical signaling can enhance engraftment and repopulation ability of stem cells (14). The resulting stabilization of β-catenin resulted in cross-talk between Wnt, Sonic Hedgehog, and Notch pathways. Other studies suggest Wnt contributes to the earliest hematopoiesis in embryos (16), and Vav-directed deletion of β-catenin compromised stem cell competence in adults (17). In additional studies, primitive properties were retained or reacquired by hematopoietic cells receiving Wnt signals (4, 15, 18). Transgenic mice with strong constitutive β-catenin cassette experienced marrow failure, possibly because stem cell differentiation was inhibited (19, 20). Inhibition of human B lymphopoiesis in culture by overstimulation with Wnt3a might be interpreted the same way (21). In contrast to these findings, adult loss-of-function experiments have not demonstrated a clear requirement for Wnt signals in maintaining stem cell integrity in normal mice (22–24). As a possible explanation, one report concluded that simultaneous deletion of β- and γ-catenin genes was not sufficient to ablate all Wnt signaling (24).

Most of the studies noted above pertained to the canonical Wnt pathway. B lymphopoiesis was abnormal in mice whose Wnt5a gene was targeted, and Wnt5a appeared to signal via the noncanonical Wnt/Ca²⁺ pathway to suppress cyclin D1 and interfere with pro-B responses to IL-7 (25). Lymphopoietic abnormalities were intrinsic to hematopoietic cells in Wnt5a−/− mice, even though bone marrow stroma of recipient mice expressed this factor. Furthermore, thymocytes and distal limb buds from Wnt5a−/− mice show increased canonical signaling, suggesting that Wnt5a opposes this pathway in normal situations (26, 27). Another recent study concluded that Wnt5a can antagonize Wnt3a responses and maintain stem cells in a quiescent G₀ state (28). B cell defects and other abnormalities were reported in Fzd9-deficient mice, but no particular signaling pathway was implicated (29).

A case has also been made for Wnt contributions to T lymphopoiesis (26, 30–37). As just one example, overexpression of the Wnt antagonist Dkk1, which prevents ligand-Fzd interaction, blocked T cell formation in fetal thymic organ cultures and blocked development at the double-negative-1 stage (33).

Thus, it is widely believed that Wnts help to maintain “stemness” of HSC, restricting and perhaps even reversing cellular differentiation while allowing a degree of replication. In contrast, some Wnts may induce differentiation of B and T lymphoid cells. Given the complexity of Wnt-related proteins and signaling pathways, much more information is needed about processes that use them. We have now exploited culture models to probe additional aspects of Wnt and focused on discrete early events in B lymphopoiesis. Wnt3a and Wnt5a were selected as prototypical agonists for canonical and noncanonical signaling, respectively. Our experiments suggest that such Wnts can deliver opposing cues to primitive cells, restricting or promoting lineage progression. As with other developmental systems, Wnt can participate in cell fate decisions, a capacity that might be harnessed for regenerative medicine.

### Materials and Methods

#### Mice

All mice were bred and maintained in the Oklahoma Medical Research Foundation Laboratory Animal Resource Center. RAG-1/GFP reporter knock-in mice have been described before (38, 39). Heterozygous F₁ RAG-1/GFP mice, C57BL/6 (B6; CD45.2 alloantigen), B6-Thy1.1 (PL), and B6-BCL-2 mice were bred and maintained in the Laboratory Animal Resource Center. B6-RAG-1/GFP mice were crossed with B6-Thy1.1 knock-in mice to produce animals expressing Thy1.1, RAG-1/GFP, and the CD45.2 alloantigen (RAG-1/PL). BCL-2 transgenic mice were previously described (40).

#### Isolation of cell populations

Bone marrows from adult, 3- to 5-mo-old, RAG-1/PL, C57BL/6, or B6-BCL-2 mice were flushed from femurs, tibias, and humeri in PBS containing 3% FCS. Stem and progenitor cells were isolated as follows, after ACK lysis and treatment with FcR blocking Ab (2.4G2). These cells were enriched from the bone marrow by incubating with Abs to lineage markers A, B, and C.
CD19 (1D3) and CD45R/B220 (RA3/6B) for B lineage cells, CD11b/ Mac-1 (M1/70), and Gr1 (RB6-8C5) for myeloid lineage cells, Ter119 (Ly-76) for erythroid cells, and CD3 (CD3-/H9255 chain) for T-lineage cells, followed by the magnetic separation of the positively stained cells by BioMag goat anti-rat IgG system (Qiagen). These partially lineage-depleted cells were then washed and stained with biotin antilineage markers (CD19, CD45R/B220, Mac-1, Gr-1, Ter119, CD3, CD8 (Ly-2), and NK1.1 (NKR-P1B and NKR-P1C)), allophycocyanin anti-c-Kit (2B8), PE-Cy5 anti-Sca-1 (D7; eBioscience), and PE-Cy7 anti-Thy1.1 (HIS51; eBioscience). The HSC fraction was obtained from Lin-/H11002 RAG-1/GFP c-Kit/high Sca-1 high Thy1.1 low, ELP were sorted as Lin-/H11002 RAG-1/GFP c-Kit/low Sca-1 low Thy1.1 high, and ProL were sorted as Lin-/H11002 RAG-1/GFP c-Kit/low Sca-1 low Thy1.1 high. The CMP population was defined as Lin-/H11002 RAG-1/GFP c-Kit/low Sca-1/CD45R/B220 high. For Pre-pro-B isolation, cells were stained and sorted as biotin antilineage (Mac-1, Gr-1, Ter119, CD3, CD8, and NK1.1), PE anti-CD19−, and allophycocyanin anti-CD45R/B220−. LSK from C57BL/6 or BCL-2 mice were isolated from Lin-/H11002 c-Kit/high Sca-1/CD45R/B220− gated population. Biotin-conjugated Abs were then stained with fluorochrome-streptavidin (Caltag Laboratories). Unless otherwise mentioned, all Abs were purchased from BD Biosciences. Propidium iodide was used in all isolations to stain for dead cells. Cells were sorted on either FACS Aria (BD Biosciences) or MoFlo (DakoCytomation) flow cytometers. Postsort analyses confirmed isolation purities, and typically >93% purity was obtained after double sorting.

**Retrovirus production and transduction**

Wnt3a, Wnt5a (25) (gift from S. Jones, University of Massachusetts, Worcester, MA), or Dkk1 plasmids were individually cloned into the multiple cloning site of LZRS-IREGFP retroviral vector by restriction digestion and ligation reactions. These inserted and empty (control) vectors (see Fig. 2 A) were transduced into Plat-E (41) virus-packaging cell line (gift from M. Coggeshall’s laboratory, Oklahoma Medical Research Foundation, Oklahoma City, OK) by FuGENE 6 (Roche) method, and transduced cells were selected by 1 g/ml puromycin and 10 g/ml blasticidin, and by sorting for GFP-transduced cells. Virus-containing supernatants were harvested 20 h after changing to fresh medium (X-VIVO 15 medium (Lonza) containing 1% detoxified BSA (StemCell Technologies), 2 mM L-glutamine, 5 × 10−5 M 2-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin), and used immediately for transduction, or stored at −70°C for later use.

For transduction of stem cells (isolated from B6 mice), 100,000 sorted LSK (HSC-enriched cells) were deposited into a well of a 48-well dish.
Cells were immediately transduced with the virus medium in the presence of 20 ng/ml recombinant mouse stem cell factor (SCF), 100 ng/ml Flk2/Flt3 ligand (FL), 20 ng/ml thrombopoietin (TPO), and 16 µg/ml polybrene (Chemicon International), followed by incubation at 37°C for 14–16 h. Subsequently, spin transduction was conducted in a centrifuge at 32°C (1500 rpm) for 1.5 h, and cells were additionally incubated at 37°C for 5 h. Culture medium was then replaced with fresh medium containing virus supernatant, growth factors, and polybrene, and reincubated. A second spin transduction was performed 8 h later, followed by supplementing with fresh culture medium containing the cytokines defined above. After an additional 20 h, GFP+ cells were purified by sorting on MoFlo cell sorter while gating out dead cells. All cytokines were purchased from R&D Systems.

For transduction of OP9 stromal cells, Plat-E-virus-producing cells were grown on OP9 cellular medium for 20 h. Supernatant was then harvested, supplemented with 20 µg/ml polybrene, and used to infect OP9 cells. After 18-h incubation, virus medium was changed with fresh medium, supplemented with polybrene, and reincubated for additional 18 h. Cells were then sorted for GFP+ transduced for four generations on MoFlo cell sorter, and stable GFP+ OP9-LZR (empty vector-transduced), OP9-W3A (Wnt3a-transduced), OP9-W5A (Wnt5a-transduced), and OP9-Dkk1 (Dkk1-transduced) cell lines were generated. Transduced stromal cells secreted the expected factor, and this was confirmed by Western blotting (data not shown). We also determined that the Dkk1-secreted from OP9-Dkk1 was biologically active. That is, it counteracted the influence of Wnt3a on stromal cell expression of adhesion molecules (S. Malhotra and P. W. Kincade, manuscript in preparation).

Cell lines, cultures, and flow cytometry

To evaluate B and myeloid lineage development, 1,000 double-sorted stem or progenitor cells were seeded into triplicates, on a monolayer of OP9-control/OP9-W3A/OP9-W5A or OP9-Dkk1 in well of a 24-well plate. This coculture was maintained in OP9 medium (α-MEM medium (Invitrogen), 10% FCS, 2 mM L-glutamine, 5 × 10^{-5} M β-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin) in the presence of SCF (20 ng/ml), FL (5 ng/ml), and IL-7 (1 ng/ml) for the indicated time. At the end of culture, cells were counted, excluding single cells, and subjected to flow cytometry procedures for identification and analysis. For longer term cultures (21 days), 5,000–10,000 hematopoietic cells were harvested from each coculture well and seeded on a fresh monolayer of stroma. Fluorescent labeled anti-CD45.2 (clone 104; eBioscience) Ab was used to positively distinguish hematopoietic cells from stromal cells.

For stroma-free cultures to evaluate B and myeloid lineage development, 1,000 double-sorted stem or progenitor cells were seeded into wells of a 96-well dish. Cells were cultured in X-VIVO 15 medium containing 1% detoxified BSA, 20 ng/ml SCF, 100 ng/ml FL, 1 ng/ml IL-7, 5 × 10^{-5} M β-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, for indicated periods of time.

For the two-step cultures outlined below, stem or progenitor cells were initially cocultured on appropriate OP9 stroma for the indicated time, isolated by cell sorting, and then cultured in the second medium.

To examine myeloid/erythroid potential, cells of each sorted fraction were mixed with IMDM-based methylcellulose medium (100 cells/ml) supplemented with 50 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, and 3 U/ml recombinant human erythropoietin (MethoCult GF 3434; StemCell Technologies). After 11 days, colonies were enumerated and classified according to shape and size under an inverted microscope.

For stroma-free erythroid cultures, 2000 cells were seeded into wells of a 96-well dish. Cells were cultured in IMDM medium containing 0.5% detoxified BSA, 18% FCS, 20 ng/ml SCF, 50 ng/ml FL, 10 ng/ml IL-3, 50 ng/ml TPO, and 5 U/ml erythropoietin for the indicated time.

In all cases in which flow cytometry was used, propidium iodide was used to exclude dead cells. Flow cytometry was performed on a BD FACS LSR-II (BD Biosciences), and data analysis was done with either BD FACS Diva (BD Biosciences) or FlowJo software (Tree Star).

Analysis of gene expression

Semi-quantitative RT-PCR was used to assess mRNA expression of early hematopoietic genes. mRNAs were isolated from sorted cells using MicroPoly (A) Purist (Ambion) and converted to cDNA with murine Moloney leukemia virus reverse transcriptase (Invitrogen). RT-PCR was performed in buffer containing 200 µM dATP, dGTP, dTTP, 100 µM dCTP, and 0.5 µCi [32P]dCTP. Aliquots were removed at cycles 25, 28, and 31 for β-actin and cycles 32, 35, and 38 for all others to ensure that PCR remained within the exponential range of amplifications. Aliquots (5 µl) were denatured in a formamide-loading buffer and applied to a 6% polyacrylamide gel containing 7 M urea. Incorporation of [α-32P]dCTP into PCR product bands was quantified by PhosphorImager (Molecular Dynamics). Primer sequences and amplification conditions are available from the authors on request.

Real-time PCR to assess expression of Wnt family genes on stem and progenitor populations was done using preoptimized SYBR Green 96-well plate pathway-specific reverse-transcriptase Profiler primer array (Catalog APM-043C; Superarray). mRNAs were isolated from highly purified stem cells, ELP, ProL, or CMP and converted to CDNA, as described above. RT-PCR was performed using reagents supplied with the primer array and as described in the array user manual using ABI PRISM 7500 (Applied Biosystems). A melting curve program was run immediately after the PCR for the entire 96-well plate, and only wells that showed a single peak (indicates specific amplification) following melting were included in analysis. All wells were also visually inspected for signs of evaporation. Data analysis for gene expression was calculated as 2^{-ΔΔCT} (threshold cycles) values, with β-actin as endogenous housekeeping control. Results from this experiment are available from the authors on request.

Cytoplasm tracer dye (DDAO-SE) labeling

CellTrace Far Red DDAO-SE dye (Catalog C34553; Invitrogen Life Technologies) was used as a cytoplasm-labeling dye to analyze cell divisions by FACs. HSC were incubated with 10 µM DDAO-SE (DMSO stock) in PBS (containing 0.1% detoxified BSA) for 10 min at 37°C in a water bath. Stain was then quenched by adding 5 vol of ice-cold culture medium to the cells and incubated for 5 min on ice. Cell pellets were washed three times before seeding on appropriate matrices, as indicated. Intensity of the DDAO-SE signal that corresponded to the proliferation status of cultured cells was monitored at the times indicated by flow cytometry.

Statistical analysis

Statistical significance of differences between groups was assessed with Student’s t test, performed using GraphPad Prism software (version 5.01; GraphPad). A result was considered significant if the p value was <0.05.

Results

Autocrine production of Wnt3a can completely block lymphohematopoiesis

Overexpression of Wnt-related molecules such as β-catenin has been used to probe consequences for hematopoiesis (15, 19, 20, 42). However, there is little information about the potential for...
stem and progenitor cells to respond to their own regulators, as suggested by the findings discussed above. We found no evidence that Wnt3a is constitutively made by stem or progenitor cells (data not shown, and see Materials and Methods), but this molecule is made in bone marrow and has been extensively used to elicit canonical Wnt pathway signals (4, 21, 43, 44). Therefore, we retrovirally introduced a Wnt3a expression cassette to the stem cell-enriched LSK fraction of bone marrow before culturing the GFP+/H11001 cells under serum-free, stromal cell-free conditions (Fig. 1). Flow cytometry analyses were conducted 11 days later, and this revealed dramatic suppression of differentiation as a result of Wnt3a. That is, numbers of total nucleated cells were greatly reduced relative to cells transduced with the control vector, and very few myeloid or lymphoid lineage cells were produced. Because stem and progenitors were the only cells present in these defined cultures, we can conclude that strong autocrine Wnt pathway signaling compromises hematopoiesis.

Stromal cell presentation of Wnt family molecules suggests that this family has regulatory potential

Matrix-binding Wnt ligands normally act at short range, and potential target cells in bone marrow reside within a complex multicellular niche (9, 45). Therefore, we stably overexpressed three Wnt family molecules in OP9 stromal cells before using them in coculture experiments (Fig. 2). This stromal cell clone was selected because of its inability to make CSF-1, whereas Wnt3a, Wnt5a, and Dkk1 were used as canonical ligand, noncanonical ligand, and antagonist, respectively. Flow cytometry performed after 11 days revealed that the differentiation of highly purified HSC to CD19+/H11001 B lineage lymphoid cells was completely blocked by exposure to stromal cell-produced Wnt3a. CD45R/B220+

FIGURE 4. Wnt ligands preferentially affect early events. Contour plots (A) show B-lymphoid lineage development after 11 days of coculture on the indicated Wnt family protein-secreting cell line. The same experimental results were calculated and shown as average yields per input progenitor with SE bars (B). Shown is one representative of four experiments. N.D. indicates not done. Asterisks indicate statistical significance as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.0001.
CD19^-CD11c^-CD11b^-NK1.1^- plasmacytoid dendritic cells (pDC) are similar in some ways to B cells, and their formation was also completely inhibited (Fig. 2). Although percentages of B220^-CD19^-CD11b^-CD11c^- conventional dendritic cells (cDC) and B220^-CD19^-CD11b^+CD11c^- myeloid cells were not compromised by Wnt3a, their absolute numbers were significantly reduced in parallel with total nucleated cells (Fig. 2C). Very similar results were obtained using stem cells from BCL-2 transgenic mice (data not shown). Differentiation patterns were quite different in Wnt5a-producing cultures, in which numbers of CD19^+ B lineage cells increased 5-fold. The effect was selective inasmuch as there were no remarkable changes in myeloid or dendritic cells. Control experiments established that DKK1-producing stromal cells were insensitive to canonical Wnt3a signals and could inhibit its effect (see Materials and Methods). Therefore, it was surprising that hematopoiesis was largely unaffected by coculture on Dkk1-transduced stromal cells (Fig. 2). In four independent experiments, lymphocyte yields were slightly higher in OP9-Dkk1 cultures than on control stromal cells, but this trend did not reach statistical significance. We conclude that Wnt3a and Wnt5a have opposing influences on B lymphopoiesis.

Wnt signaling preferentially regulates early events, and in opposing ways

We discovered that whereas B lymphopoiesis was totally blocked in Wnt3a-expressing short-term cultures (Fig. 2), this was not the case at longer culture intervals (Fig. 3). In fact, the most notable influence of Wnt3a at 21 days was an increase in numbers of CD11b^- myeloid cells. It seemed possible that some lymphoid progenitors escaped an early checkpoint and became progressively less Wnt responsive with differentiation. This was investigated by initiating cultures with stem cells and lymphoid progenitors (Fig. 4). All produced at least some B lineage cells within 11 days, and yields were higher, as expected, when cultures were started with pre-pro-B cells. Dramatic results were obtained when the cells were exposed to Wnt3a. That is, ProL, and pre-pro-B cells could still generate CD19^+ B lineage cells even in the presence of Wnt3a, whereas lineage progression from earlier stages was totally blocked (Fig. 4). Many, but not all CD19^- ProL acquired CD19 when exposed to Wnt3a-producing stromal cells, and lymphocyte yields from CD19^- pre-pro-B cells were only slightly affected by Wnt3a. We conclude that only stem cells and early lymphoid progenitors are completely sensitive to canonical Wnt pathway signaling.

Although Wnt5a consistently augmented B lymphopoiesis in cultures initiated with HSC and evaluated after 11 days, lymphocyte numbers were equivalent to controls by 21 days (data not shown). Again, we asked whether this might be ascribed to differential sensitivity of target cells. Enhanced B lineage lymphocyte differentiation was only seen in short-term cultures of HSC, and there was no noticeable influence on two types of lymphoid progenitors (Fig. 4B). These findings indicate that, in this system, the influence of Wnt5a is also confined to the most primitive cells.

Progenitors that are not fully lymphoid committed lose differentiated properties in response to Wnt signaling

Artificial stimulation of the canonical Wnt pathway can restrict differentiation of primitive hematopoietic cells (5, 15, 20). We wondered whether the same might be achieved on exposure to authentic Wnt. Many stem cells retained primitive staining characteristics after 2 days on Wnt3a-producing stromal cells, and acquisition of lineage markers was partially inhibited even at 11 days (Fig. 5, A and B). Consistent with a previous study involving rWnt3a protein (4), we found that numbers of transplantable stem cells were retained on OP9-Wnt3a cells (data not shown). We consistently recovered fewer nucleated cells from such cultures, and DDAO-SE dye dilution analyses suggested that stem cell proliferation was inhibited in Wnt3a-producing stromal cell cocultures (Fig. 5C).

There is also evidence that Wnt signals can destabilize hematopoietic progenitors (18). That is, introduction of constitutively active β-catenin to lymphoid or myeloid progenitors allowed them to each be reprogrammed to new fates. To investigate this phenomenon further, stem cells, ELP, and ProL were sorted from RAG-1/GFP reporter mice and stimulated for 18 h in stromal cell cocultures before RT-PCR analyses (Fig. 6A). Although fresh lymphoid progenitors contained RAG-1 transcripts and continued to contain substantial levels after culture on vector control-transduced stromal cells, this was extinguished when ELP were placed on Wnt3a-producing cells (Fig. 6A). The same was true for progenitors representing the ProL stage. Remarkably, mRNAs corresponding to other transcription factors (Ebf, Pax-5, and Aiolos) were not substantially changed at this early time point (data not shown). RAG-1/GFP levels normally increase when ELP are cultured on Wnt5a-producing cells (Fig. 6B). These findings are one representative of four (A and B) or three (C) experiments.
stromal cell-free progenitor assays (47–49). Numbers of non-lymphoid cells produced from lymphoid progenitors increased on exposure to Wnt3a-producing stromal cells. This was recorded by flow cytometry in which yields of CD19<sup>−</sup> B220<sup>−</sup> CD11c<sup>−</sup> CD11b<sup>+</sup> cells dramatically increased (Fig. 7A). This corresponded to small, but consistent increases in numbers of myeloid colony-forming cells, and the colonies tended to be larger from progenitors that had been exposed to Wnt3a (Fig. 7B, and data not shown).

ELP and even some ProL reacquired the RAG-1/GFP<sup>−</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> c-Ki<sup>h</sup> Thy1.1<sup>low</sup> staining properties of stem cells (Fig. 8A). Even more surprisingly, cells expressing the Ter119 erythroid marker emerged when ELP were exposed to Wnt3a-producing stromal cells for 3 days and then stimulated with erythropoietic factors (IL-3, TPO, SCF, FL, and erythropoietin; Fig. 8B).

These observations suggest that Wnt3a presented by stromal cells in vitro transiently and partially arrests lymphopoiesis. In contrast to results obtained with rWnt3a (4), we found that stem cell proliferation was inhibited. Additionally, lymphoid-specified progenitors undergo a degree of de-differentiation.

**Discussion**

These new findings suggest ways that Wnt family proteins might differentially regulate B lymphopoiesis and provide a basis for reconciling previous reports. For example, the timing of ligand exposure and stage of differentiation were found to be important variables. From a large list of potentially important molecules, two ligands were shown to be capable of opposing actions and merit further study in that context. Although Wnt3a caused preservation and even reacquisition of HSC properties, it did not appear to be a growth factor in short-term cultures.

Hematopoietic cells express a bewildering number of Wnt ligands, receptors, coreceptors, inhibitors, and signaling intermediates. The only differentiation-related trend in our screen for Wnt family gene expression in hematopoietic stem and progenitor cells was a tendency for Fzd receptors to decline (data not shown, and see Materials and Methods). As others have concluded from less comprehensive analyses (21, 33, 50), hematopoiesis could potentially be regulated by autocrine and paracrine Wnt-dependent mechanisms. Wnt3a has been extensively used as a canonical Wnt pathway ligand (4, 21, 43, 44), but it is noteworthy that we could not detect transcripts in highly enriched stem cells. A similar screen of stromal cells indicated that they may also produce and respond to multiple Wnt family molecules (data not shown).

**FIGURE 6.** The canonical Wnt3a ligand extinguishes expression of RAG-1 in lymphoid progenitors. Semiquantitative RT-PCR analyses of RAG-1 transcripts were performed on HSC, ELP, or ProL (A). Transcripts represent gene expression of either freshly isolated bone marrow cells (Marrow) or cells that were cocultured overnight on OP9-control (Control) or OP9-Wnt3a (W3A) cells. Overlay histograms (B) show RAG-1/GFP protein levels of freshly isolated GFP<sup>−</sup> cells (blue) or ELP (red) compared with ELP that were cultured for 2 days on OP9-control (black, solid) or OP9-Wnt3a (gray shaded overlay) stromal cells. Shown is one representative of three experiments.

**FIGURE 7.** Wnt3a stimulates myeloid differentiation from lymphoid progenitors. Total myeloid (CD19<sup>−</sup> B220<sup>−</sup> CD11b<sup>−</sup> CD11c<sup>−</sup>) cell yields/input of HSC, ELP, and ProL are shown for 11 days of coculture on OP9-control or OP9-Wnt3a (A). Myeloid colony-forming efficiencies (CFU) are shown for HSC, ELP, or ProL that were either freshly isolated or pre-stimulated on OP9-control or OP9-Wnt3a for 3 days and sorted before reculture in methylcellulose medium (B). Shown is one representative experiment of three. Asterisks indicate statistical significance as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.0001.

**FIGURE 8.** Lymphoid-committed progenitors acquire alternate lineage fates in response to Wnt3a. HSC, ELP, or ProL were placed in 11-day cocultures on OP9-control or OP9-Wnt3a stromal cells (A). The recovered cells were then assessed for a stem cell (Lin<sup>−</sup> c-Ki<sup>h</sup> Sca-1<sup>−</sup> Thy1.1<sup>low</sup>) phenotype. Results are shown as total HSC yields/input. ELP were pre-cultured on OP9-control or OP9-Wnt3a stromal cells for 3 days and sorted before reculture under stromal cell-free erythroid-supportive conditions (B). The contour plots show erythroid (Ter119<sup>+</sup> CD11b<sup>−</sup> Gr-1<sup>−</sup>) differentiation potential in one representative of three experiments. Asterisks indicate statistical significance as follows: **, p < 0.01; ***, p < 0.0001.
Our gain-of-function experiments began by artificially expressing Wnt3a in hematopoietic cells and then culturing them under defined conditions. The aim was to test the possibility that stem and progenitors can be self-regulated by this mechanism. We recorded almost complete inhibition of myeloid and lymphoid differentiation, whereas the residual cells lacked markers associated with these lineages. These results show that autocrine stimulation is possible, and are consistent with many studies in which differentiation was blocked by β-catenin (15, 19, 20). Although some level of Wnt signaling might be beneficial to stem cells, it is clear that excessive stimulation would lead to marrow failure.

We then prepared cultures in which selected Wnt molecules were produced by OP9 stromal cells that were unable to overexpress CSF-1 and cause macrophage overgrowth (44). This system is more likely to reflect physiologic conditions than prior studies with retrovirally introduced or recombinant materials, and there was no concern about Wnt protein degradation. Our unpublished experience suggests Wnt is more effective when presented in this way, and the stromal cells provide differentiation factors needed to support dendritic cell formation. As when the ligand was produced by stem cells, stromal cell Wnt3a completely blocked the generation of B lineage lymphocytes. Consistent with their similarities to B cells (51), pDC also did not emerge. Although CD11b^+CD11c^− myeloid cells decreased, CD11b^+CD11c^+ cDC were produced relatively normally. This differential sensitivity is interesting inasmuch as it suggests Wnt can participate in lineage choice decisions. That would be consistent with the ability of Wnt to direct differentiation options in neuronal (52), bone (53), and cardiac tissues (54). Identical results were obtained with marrow from BCL-2 transgenic rather than normal mice. This was done to approximate conditions used in another Wnt investigation (4) and suggests Wnt3a does not inhibit by inducing apoptosis.

Numbers of pro-B and pre-B cells were expanded in fetal Wnt5a-deficient mice, and it was proposed to function as a B lineage suppressor (25). Therefore, it was surprising that production of B lineage lymphocytes was elevated 5-fold on Wnt5a-producing stromal cells. This effect was only observed when cultures were initiated with HSC; lymphoid progenitors displayed no sensitivity in our model, and there was no influence of Wnt5a on myeloid or dendritic cells. Consistent with our findings, Wnt5a-deficient mice, and it was proposed to function as a B lineage tumor suppressor (25, 56). Furthermore, Wnt5a signaling pathways (25, 56). Numerical recombination projects relating to Wnt signaling in hematopoietic cells (26–28). Particularly interesting in this regard is the ability of Wnt5a to down-regulate β-catenin and thus inhibit T lymphopoiesis (26). Again, this suggests how Wnt family molecules can function as counteracting regulators and participate in lineage fate decisions.

Dkk1 associates with LRP5, LRP6, and Kremen coreceptors, effectively acting as an inhibitor of canonical Wnt stimulation (57). There is additional evidence that it can block noncanonical signaling, and Dkk1 may itself function as a ligand (58, 59). Although we are certain that Dkk1 made by our transduced OP9 stromal cells was biologically active (see Materials and Methods), there were no statistically significant consequences for lymphohematopoiesis in cocultures. We obtained similar results when HSC from fetal liver were cultured on OP9-Dkk1. To the extent that this model reproduces conditions in vivo, the findings do not prove that Wnt is needed for normal, steady-state blood cell formation. However, the observations also do not preclude its possible importance during disease circumstances or when there are unusual demands for blood cells.

Results from Wnt studies may be easier to interpret if considered in terms of kinetics and maturation stage. Although Wnt3a completely arrested B lymphopoiesis in 11-day cultures, similar populations of lymphocytes were present in Wnt3a and control cultures 10 days later. Moreover, total numbers of lymphocytes were suppressed only by ~30% at that time. This result is subject to several possible interpretations. For example, the level of Wnt3a might decay with time or be insufficient to prevent lineage progression. The Wnt3a/GFP expression cassette stably produced GFP over many passages, and transduced stromal cells never lost their ability to suppress B lymphopoiesis in fresh cultures. In fact, our experimental design involved harvest and replating of hematopoietic cells onto new OP9-Wnt3a cells after the first 11 days. Alternatively, progenitors might become tolerant after repeated exposure to Wnt3a. Insufficient numbers of stem cells would be present after short-term culture to directly address this possibility. It also seemed possible that a few escaping progenitors might differentiate, giving rise to cells that are less Wnt3a responsive. This would be consistent with the observation noted above that Fzd receptor levels are lower on progenitors than stem cells. Indeed, we found that progression in the B lymphocyte lineage correlated with decreasing responsiveness to Wnt3a or Wnt5a.

Consistent with this idea, stromal cell-derived Wnt3a caused short-term retention of undifferentiated, stem cell-like populations in our cultures. Cells that had multilineage potential in transplantation assays were recovered after 5 days, whereas no activity was found in vector control-transduced OP9 stromal cell cocultures (data not shown). This is in agreement with reports that rWnt3a protein allowed propagation of stem cells (4). Furthermore, artificial stimulation of the canonical Wnt signaling pathway allowed propagation of multipotential hematopoietic cells for many months (15). However, we believe it unlikely that Wnt is a hematopoietic growth factor. As discussed above, our cultures were almost entirely composed of lymphocytes at longer intervals, and no attempt was made to investigate stem cell potential. We found no evidence that Wnt3a supports stem cell proliferation, and, in fact, there was less than normal expansion in 3-day cultures. The same tendency was reported in previous studies (21, 28, 44). Although not cited as an important variable, two previous studies were done with BCL-2 transgenic rather than normal bone marrow (4, 5) and used different culture conditions. It remains unclear whether and how Wnt signaling can be exploited to expand stem cells therapeutically.

One approach to regenerative medicine involves de-differentiating and reprogramming specialized cell types. In that context, it is interesting that expression of stable β-catenin caused lymphoid and myeloid progenitors to become lineage unstable (18). We now show that the same is true in cocultures with Wnt3a-producing stromal cells. That is, RAG-1-producing lymphoid progenitors became RAG-1− within 18 h under these conditions. It will be important to find rapid changes in transcription factor levels that would account for RAG-1 down-regulation. Common lymphoid progenitors within the ProL fraction of bone marrow have very little myeloid differentiation potential (47–49). Thus, it is significant that increased numbers of myeloid cells were recovered from OP9-Wnt3a stromal cell cocultures. ELP are much more lymphoid biased than stem cells or multipotent progenitors (46), and their myeloid potential also increased. Normally incapable of clonal proliferation in response to recombinant growth factors, some ProL reacquired this potential in response to Wnt3a signaling. Two additional findings suggest that Wnt3a can reverse hematopoietic differentiation. Cells with a Lin− Sca-1^+ c-Kit^high Thy1.1^low
RAG-1- phenotype expanded in OP9-Wnt3a cocultures. This was true even when cultures were initiated with RAG-1 ProL sorted to high purity, diminishing the possibility that a primitive subset of cells preferentially expanded. Even more impressive was acquisition of the potential for erythroid lineage differentiation. This is thought to represent the earliest of lineage choice events in stem cell differentiation (60). In contrast to these results with Wnt3a, Wnt5a-producing cultures always generated pure lymphocytes (Fig. 4, and data not shown).

It remains unclear whether Wnt signaling is essential for blood cell formation in normal bone marrow. That issue is difficult in part because what is observed represents the net influence of multiple Wnt pathways. However, our new findings show how members of this complex family have the potential to enhance or repress progression in the B lymphocyte lineage. Moreover, they might someday be exploited to confer primitive properties on differentiated cells.

Acknowledgments

We acknowledge Dr. Stephen Jones (University of Massachusetts Medical School) for providing the Wnt5a vector, and Dr. Irving Weissman (Stanford University) for BCL-2 transgenic mice.

Disclosures

The authors have no financial conflict of interest.

References


