Pim-1 Levels Determine the Size of Early B Lymphoid Compartments in Bone Marrow

By Jos Domen, Nathalie M. T. van der Lugt, Dennis Acton, Peter W. Laird, Koert Linders, and Anton Berns

From the Division of Molecular Genetics, The Netherlands Cancer Institute and Department of Biochemistry, University of Amsterdam, 1066 CX Amsterdam, The Netherlands

Summary

The mouse proto-oncogene *Pim*-1, which encodes two cytoplasmic serine-threonine–specific protein kinases, is frequently activated by proviral insertion in murine leukemia virus–induced hematopoietic tumors. Transgenic mice overexpressing *Pim*-1 show a low incidence of spontaneous T cell lymphomas, whereas null mutant mice lack an obvious phenotype. We have analyzed the early B lymphoid compartment from both null mutant and Eμ-*Pim*-1 transgenic mice. The level of *Pim*-1 expression appears to be a determining factor in the ability of these cells to respond to the growth factors interleukin 7 (IL-7) and SF (steel factor). The impaired response in null mutant mice could be rescued by introduction of a functional *Pim*-1 transgene. Moreover, overexpression of *Pim*-1 facilitates the derivation of primitive lymphoid cell lines that are dependent on combined stimulation with IL-7 and SF or insulin-like growth factor 1. These results for the first time identify the involvement of *Pim*-1 in a normal cellular function, as an important regulator of early B lymphopoiesis in mice.

The *Pim*-1 gene was originally identified as a common insertion site in Moloney MuLV (MoMuLV)[1]–induced T cell lymphomas in mice (1, 2). Subsequently, it has also been found activated in MoMuLV-induced B cell lymphomas (3) and Friend virus–induced erythroleukemias (4).

The mouse *Pim*-1 gene encodes two proteins by alternative initiation at AUG (34 kD) or CUG (44 kD). Usage of the latter is not apparent in humans (5). Although the human protein has been reported to phosphorylate tyrosine residues (6), more recent data conclusively show that both the mouse (5) and human (5, 7, 8) Pim-1 proteins exclusively exhibit serine/threonine–dependent protein kinase activity. Both proteins are localized in the cytoplasm and are short-lived, with half-life values of 5–10 min for the 34 kD and ~45 min for the 44-kD protein (5). Both murine and human *Pim*-1 are predominantly expressed in hematopoietic tissues (9, 10) where mRNA and protein are mitogen inducible (11–13).

The gene's oncogenic potential was proven in Eμ-*Pim*-1 transgenic mice, which showed a low incidence of T cell lymphomas (9). The low incidence and variable latency period indicates that overexpression by itself is insufficient for transformation. This was confirmed by exposing mice to viral (MoMuLV) or chemical (*N*-ethyl-*N*-nitrosourea) carcinogenic agents. In both cases, lymphomas developed much faster in transgenic animals (9, 14). In nearly all Eμ-*Pim*-1 tumors, activation of c-*myc* or n-*myc* was observed. The capacity of *Pim*-1 and c-*myc* to synergize in lymphomagenesis was most convincingly shown in crosses of Eμ-myc and Eμ-*Pim*-1 transgenic mice. Coexpression of both transgenes caused the development of pre-B cell leukemias in utero (15), making this the strongest cooperation seen between two oncogenes in vivo to date.

Pim-1 is highly conserved between mammals (2, 10, 16–18). Null mutants, however, display a surprisingly limited phenotype (19, 20) apart from an impaired IL-3 response (21). As our earlier studies showed that *Pim*-1 can predispose to pre-B cell neoplasias, we have analyzed the early B lymphoid compartment from bone marrow of mice expressing different levels of *Pim*-1. Expression level–dependent differences were indeed detected.

Materials and Methods

Mutant Pim-1 Mice. The *Pim*-1 null mutants, lacking part of the coding region, were as described before (21). The null alleles were maintained on a 129/Ola (inbred) or a 129/Ola × BALB/c (outbred) background. The Eμ-*Pim*-1 transgenic, founderline 64 mice have also been described before (10). The transgene consists of a genomic *Pim*-1 clone with two copies of the Ig Eμ enhancer located upstream of the start site and a MoMuLV LTR in the 3'

[1] *Abbreviations used in this paper:* CFC, colony-forming cell; CM, conditioned medium; IGF-1, insulin-like growth factor; LTC, long-term culture; MoMuLV, Moloney murine leukemia virus; SF, steel factor.

1665 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/11/1665/09 $2.00
Volume 178 November 1993 1665–1673

untranslated region and is highly expressed in lymphoid and myeloid cells. The transgene was introduced into (CBA/BrA × C57BL/LiA)F_1 zygotes and the resulting transgenics were backcrossed with (CBA/BrA × C57BL/LiA)F_1, C57BL/LiA, or C57BL/6 mice. The WT-*Pim*-1 transgenic mice, founderline 4550, were made by introduction of an ~11-kb genomic EcoRI fragment of *Pim*-1 into FVB zygotes. The genotype of all the mutant mice was monitored by Southern analysis of tail-tip DNA, according to Laird et al. (22).

Protein Analysis. Analysis of the Pim-1 proteins was essentially as described (5) with some modifications. Briefly, cells were suspended in lysis buffer (20 mM Pipes, pH 7.0, 30 mM NaCl, 5 mM $MgCl_2$, 14 mM β-ME, 1% aprotinin, 1 mM PMSF, 1 mM leupeptin, and 1 μg/μl soybean trypsin inhibitor) and frozen on dry ice. After thawing, the cleared supernatant (10 min microfuge) was used for immunoprecipitation with the anti-*Pim*-1 COOH-terminal peptide serum, bound to protein A–Sepharose beads (Pharmacia Biotech Europe, Brussels, Belgium). Immunoprecipitation buffer was 10 mM sodium phosphate, pH 8.0, 150 mM NaCl, 5 mM CHAPS, and 14 mM β-ME. Kinase assays with antibody-immobilized *Pim*-1 proteins were performed in 20 mM Pipes, pH 7.0, 15 mM $MnCl_2$, 7 mM β-ME, 0.25 mM β-glycerophosphate 0.4 mM spermine, and 10 μCi γ-[^{32}P]ATP (3,000 Ci/mmol) for 30 min at 30°C before being analyzed by 15% SDS-PAGE.

Flow Cytometry. This was done essentially as described (9, 23). Most antibodies used were directly conjugated to fluorochromes or biotinylated and some were used as culture supernatants (CD11b, JORO, ICAM2). Biotinylated monoclonals were revealed by a second incubation with streptavidin-fluorochrome, unconjugated monoclonals by second incubation with mouse anti–rat-κ-FITC. Fluorochromes used are FITC or PE. The cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). Antibodies used are CD45R/B220 (6B2), BP1 (6C3), CD43 (S7), HSA (M1/69), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), TCR-α/β (H57-597), CD44/Pgp-1 (IM7), and Gr-1 (RB6-8C5), all from Pharmingen (San Diego, CA). Sca-1 and PB76 (G-5-2) (24) were a gift from A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia); JORO30-8 and JORO75 (25) were gifts of R. Palacios (Basel Institute of Immunology, Basel, Switzerland); and ICAM-2/Lgp55 (PA3-795.4.16) (26) was a gift from W. Golde (University of Colorado, Denver, CO). Other antibodies used were as described before (9, 23).

Colony Assays. Agar cultures of mouse bone marrow cells (27, 28) were performed by plating 5 × 10^4 bone marrow cells from 2–4-mo-old mice in 1 ml medium containing 0.25% agar in 3.5-cm dishes. Cells were plated in Fischer's medium with 20% horse serum (GIBCO BRL, Gaithersburg, MD) in the presence of 20% L cell conditioned medium (M-CSF) or the cells were plated in RPMI 1640 (Flow Laboratories, Irvine, CA) with 20% FCS (GIBCO BRL), 5 × 10^{-5} M β-ME and growth factors (B cells). Growth factors were rIL-7, 10 ng/ml, *Escherichia coli*–produced rrSCF^{164}, 35 ng/ml, gifts of L. M. Souza, and K. Zsebo, Amgen Biologicals (Thousand Oaks, CA), or yeast-derived rMGF, 35 ng/ml, gift of B. Overell, Immunex (Seattle, WA) and LPS (Difco, Detroit, MI), 30 μg/ml. Plates were incubated for 8 d in a fully humidified incubator with a 5% CO_2 atmosphere after which colonies of more than 50 cells were counted. Outbred null mutants and their controls were assayed as pools of two or three bone marrows; all other mice were assayed individually.

Tissue Culture. Whitlock-Witte type long term bone marrow cultures were established and maintained essentially as described (29). For short-term primary B cell cultures 10^6 bone marrow cells, flushed from femurs and tibiae, were incubated in 5 ml long-term culture (LTC) medium plus growth factors in a T25 flask (Costar Corp., Cambridge, MA). LTC medium is RPMI 1640 supplemented with 5% FCS, 5 × 10^{-5} M β-ME, and penicillin and streptomycin. Growth factors used were 10% 3T3-IL7 conditioned medium (CM) and 60 ng/ml steel factor (SF[1]) (rrSCF^{164}). Factor-dependent subcultures and cell lines derived from LTC were grown in LTC medium supplemented with 10% 3T3-IL7 CM, SF (rMGF or rrSCF^{164}, 22 ng/ml), 1% X63-IL3 CM and 1% X63-IL5 CM (30); for short-term stimulations purified rIL-7 was used at 10 ng/ml (Amgen Biologicals). For short-term stimulation of cell lines, cells were spun out of their culture medium, washed in LTC medium without added factors, and resuspended in LTC medium with appropriate factors. Cells were incubated at the indicated densities, usually 10^5 cells per ml in flat-bottomed 96-well plates (Falcon, Lincoln Park, NJ), 200 μl per well. After stimulation, living cells were quantitated using an improved Neubauer hemocytometer (W. Schreck, Hofheim, Germany) in the presence of trypan blue or a Sysmex Toa F800 microcellcounter (Kobe, Japan). In vitro differentiation attempts of LTC-derived cell lines were performed by coculturing these cells with stromal cell lines in the presence or absence of added growth factors. The latter included 10% 3T3-IL7 CM, 10 ng/ml SF, and 30 μg/ml LPS. Stromal cell lines used, which were grown in LTC medium, were PA6 and ST-2 (31), gift of A. Rolink (Basel Institute of Immunology) and RP.0.10 (32), gift of R. Palacios. In vivo differentiation experiments were performed by injecting 5 × 10^6 cells into nonirradiated SCID mice, purchased from Bomholtgard Ltd. (Ry, Denmark). The mice were killed 4–8 wk later, and the hematopoietic organs were analyzed by flow cytometry.

Recombinant Growth Factors. Growth factors were used at the concentrations given, unless indicated otherwise. Recombinant human IL-6 and IL-7, 10 ng/ml, recombinant human IL-8, 50 ng/ml, and rat SCF^{164}, 10 ng/ml, were gifts of Amgen Biologicals. Recombinant mouse MGF, 22 ng/ml, was a gift of Immunex. Recombinant human IL-1α (33), 100 U/ml, was donated by A. Stern (Hoffmann-La Roche, Nutley, NJ). Recombinant mouse IL-9 (34), 5 ng/ml, was a gift of Jacques van Snick (Ludwig Institute, Brussels, Belgium). Media from L929b cells, gift of T. M. Dexter (Paterson Institute, Manchester, UK), were used at 20% as a source of murine M-CSF, CM from X63 cell lines producing IL-3 and IL-5 (30), used at 1%, were donated by A. Strasser, whereas mouse IL-7–producing 3T3 cells, whose CM was used at 10%, were donated by A. Rolink (35). Recombinant mouse leukemia inhibitory factor (ESGRO) was purchased from Amrad (Kew, Australia) and used at 1,000 U/ml, recombinant human insulin-like growth factor 1 (IGF-1) used at 10 ng/ml, was purchased from Boehringer Mannheim (Mannheim, Germany).

Results

B Cell Populations in Bone Marrow. Comparison of the total number of bone marrow cells recovered from *Pim*-1 mutants and their wild-type controls revealed no significant differences. Flow cytometric analysis of the B lymphoid populations present in the bone marrow of the various *Pim*-1 mutant mice showed no clear differences in pre-B cells (B220^{dull} sIg$^-$) (Table 1). Also, a more detailed analysis (36) did not reveal differences (20). Differences were noted in the size of the mature B cell compartment (B220^{bright} sIg$^+$) present in Eμ-*Pim*-1 transgenic mice, which was reduced to half of that seen in wild-type mice (Table 1). *Pim*-1–deficient mice did not differ from their wild-type littermates in this respect.

Table 1. *B Cell Populations in Bone Marrow*

	Total bone marrow	Pre-B cells $B220^{+}sIg^{-}$	B cells‡ $B220^{+}sIg^{+}$
Eμ-*Pim*-1 (n = 8)	9.6 ± 1.4*	15.8 ± 4.9‡	4.6 ± 0.8
Wild type (n = 6)	9.8 ± 1.9	18.8 ± 5.1	8.0 ± 0.9
Pim-1 deficient (n = 8)	6.3 ± 0.6	19.7 ± 4.7	8.2 ± 1.6
Wild type (n = 8)	6.4 ± 1.4	26.1 ± 4.4	6.9 ± 1.2

Distribution of B-lymphoid cells as determined by flow cytometry on bone marrow of mature (1–3-mo-old) mice. Results are given as means ± SD. The data on Eμ-*Pim*-1 transgenic mice were obtained from heterozygotes, homozygous Pim-1-null mutant mice were both outbred (n = 6) and inbred (n = 2).

* Cells ($\times 10^{-7}$) isolated from both femurs and tibiae.

‡ Percent total bone marrow.

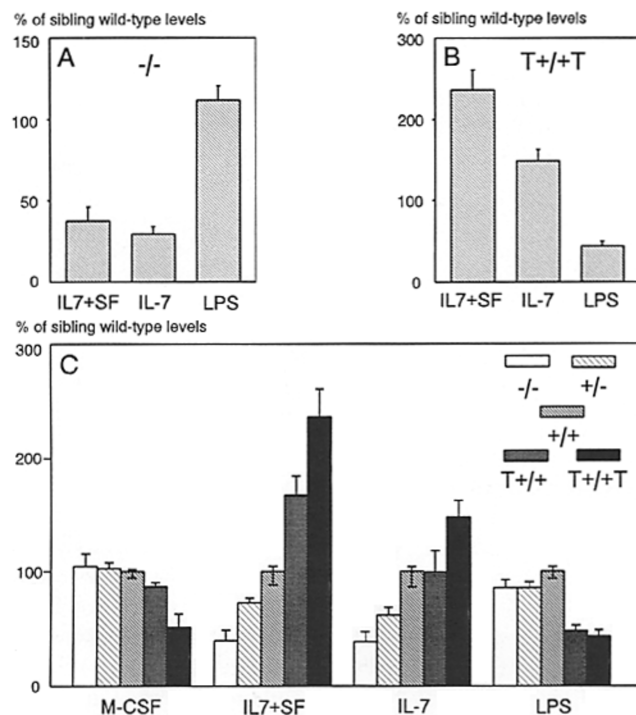

Figure 1. B lymphoid colony assays on bone marrow of mutant *Pim*-1 mice. The results, means ± SEM, are given relative to the number of colonies induced from wild-type bone marrow per experiment. (*A*) Colonies seen in the bone marrow of 19 outbred mice, homozygous for the *Pim*-1 null allele. (*B*) The same stimulations performed on bone marrow from five mice homozygous for the Eμ-*Pim*-1 transgene. (*C*) The number of CFC found in four different mutant *Pim*-1 mice in response to stimulation with lymphoid and a myeloid control (*M-CSF*), growth factors. Assayed were five homozygous targeted (−/−), five heterozygous targeted (−/+), and five wild-type (+/+) mice, all 129/Ola inbred. Variation for the latter group is indicated by the lower error bars. Results also given are for 12 wild-type (+/+, upper error bars), 12 heterozygous transgenic (T+/+), and five homozygous Eμ-*Pim*-1 transgenic mice. The transgene is present on a B.CBA background. Similar numbers of CFC were found in wild-type mice of the various genetic backgrounds.

Bone Marrow Growth Factor Response in Colony Assays. The number of different B lymphoid colony-forming cells (CFC) present in bone marrow from mice lacking (Fig. 1 *A*) or overexpressing *Pim*-1 (Fig. 1 *B*) were determined. In bone marrow of *Pim*-1–deficient mice, the number of colonies obtained with either IL-7, which induces pre-B cell colonies (28), or with IL-7 and SF, which also stimulates more primitive cells to differentiate into pre-B cells (37), is reduced to approximately one third of wild-type levels. A reduction is also seen in colony size (see also below). In contrast, mature B cell colonies formed upon stimulation with LPS (38) are normal both in number and size.

In Eμ-*Pim*-1 transgenic mice, there is an increase in the number of colonies formed in response to the combination of IL-7 and SF. A moderate increase is seen in stimulations with IL-7 alone, whereas there is actually a decrease in the number of LPS-responsive CFC. The latter is corroborated by flow cytometry (see above). No clear effect on colony size was noted. Interestingly, homozygous transgenic mice clearly differed in the severity of their phenotype from the heterozygous mice. A similar effect has been noted for the phenotype described before for these mice. T cell lymphomagenesis, with homozygous mice displaying a much higher incidence (~40% per yr) than heterozygous mice (5–10% per yr).

Whereas a strict correlation is seen between *Pim*-1 expression levels (shown below) and the number of colonies induced by IL-7 and SF (Fig. 1 *C*). This is not seen for the induction of myeloid (macrophage) colonies by M-CSF (39). A possible exception are homozygous transgenic mice, where the numbers are reduced.

The data show that in *Pim*-1–deficient mice, the number of early, IL-7 + SF responsive, B lymphoid cells is reduced whereas the numbers of mature, LPS responsive, B cells in bone marrow are normal. In mice overexpressing *Pim*-1 the opposite is seen, and the numbers of the IL-7 + SF responsive early B cell progenitors are increased, whereas the number of LPS-responsive B cells, is reduced.

Bone Marrow Growth Factor Response in Liquid Cultures. Because pre-B cell colonies showed differences in size, we monitored their growth potential by directly stimulating bone marrow in liquid cultures (37). Clear differences were noted when unfractionated bone marrow from different *Pim*-1 mutants was stimulated with IL-7 + SF or IL-7 alone. The growth of bone marrow cells from mice heterozygous for the *Pim*-1 null allele was significantly reduced when compared with that of wild-type littermates, whereas a more drastic reduction was seen with the bone marrow of homozygous null mutant mice (Fig. 2). Subculturing (for up to several weeks) did not improve growth in the *Pim*-1–deficient cultures, although wild-type cultures continued to grow with similar kinetics (data not shown).

Bone marrow of Eμ-*Pim*-1 transgenic mice showed an increased response with IL-7 + SF (Fig. 2), whereas the effect of IL-7 only did not differ significantly from those of wild-type littermates. Upon subculturing, differences in growth rate disappeared (data not shown). This indicates that the increased production initially seen reflects differences in the

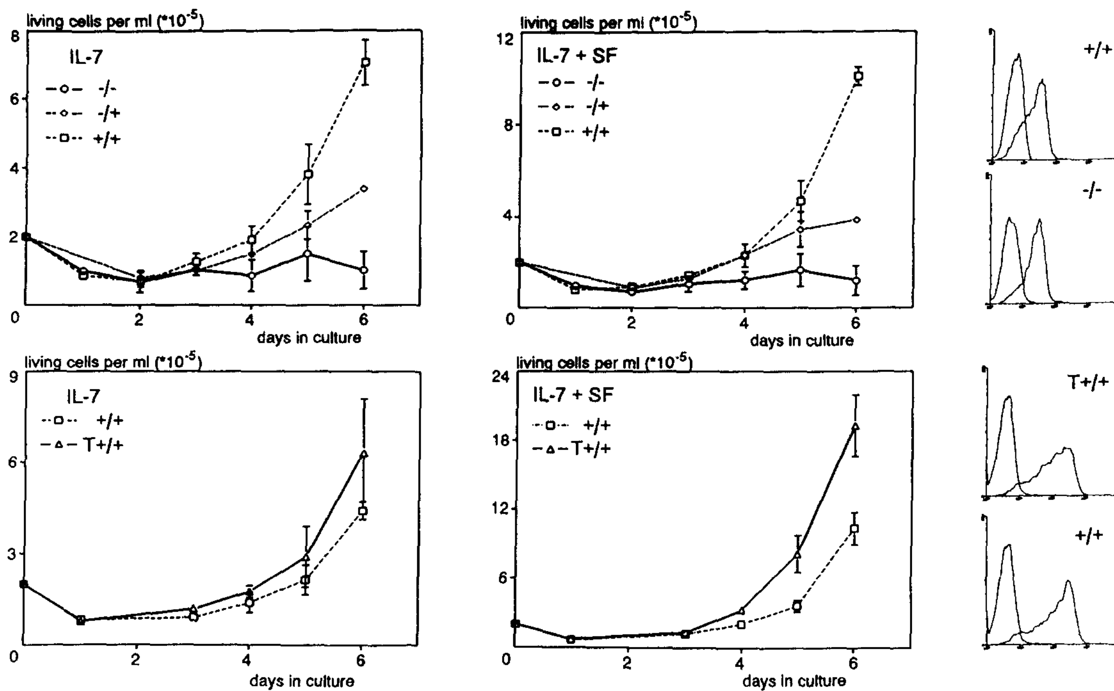

Figure 2. Stimulation of *Pim*-1 mutant bone marrow cells by B lymphoid growth factors in liquid culture. Growth was stimulated with 10% 3T3-IL-7 CM with or without SF (60 ng/ml). Living cells were quantitated daily in the presence of trypan blue. SD are shown when larger than the marker symbol. Top panels show stimulations recorded in cultures from five wild-type, two heterozygous null mutant, and five homozygous null mutant mice. (*Left*) Stimulation with IL-7; (*middle*) with IL-7 + SF; and (*right*) flow cytometric analysis of two cultures stimulated with IL-7 + SF. Left peaks show PBS control whereas the right peaks represent staining with FITC-conjugated B220. Cells were cultured 10 d in the presence of IL-7 and SF. Bottom panels show similar stimulations using the bone marrow from three wild-type and three heterozygous Eμ-*Pim*-1 transgenic mice. Stimulation was with IL-7 (*left*) or IL-7 + SF (*middle*). (*Right*) The B220 surface phenotype of two cultures. Left peaks show PBS control whereas the right peaks show staining with biotinylated B220 revealed by streptavidin-PE. Higher fluorescence intensity than in top panel is caused by biotin-streptavidin amplification. Cultures were stimulated 8 d with IL-7 and SF.

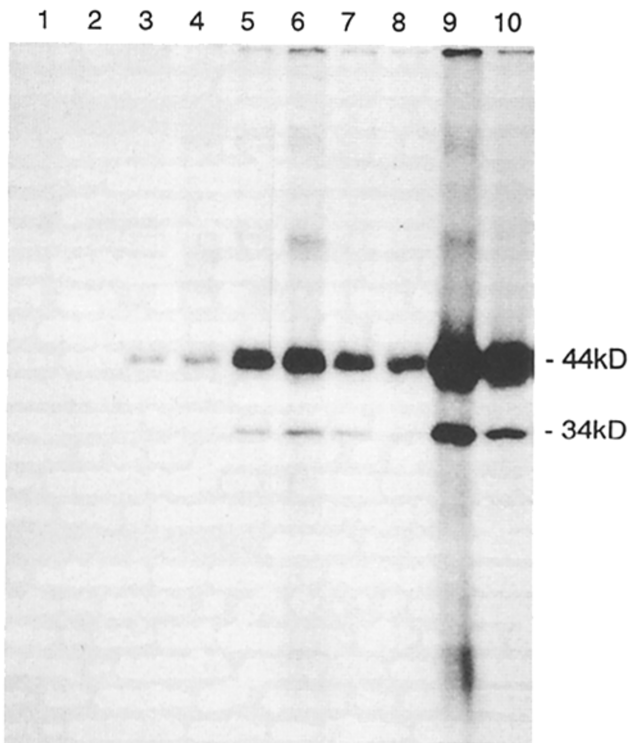

Figure 3. Pim-1 protein expression levels in mutant Pim-1 mice. Pim-1 levels were determined by autophosphorylation with ^{32}P of the antibody-immobilized proteins in a kinase assay. The 44-kD Pim-1 protein is overrepresented since this is a much better substrate for autophosphorylation (5). 2.5 × 10^6 primary pre-B cells, as in Fig. 2, were analyzed. (Lanes *1* and *2*) Homozygous null mutants (lanes *3* and *4*) heterozygous null mutants; (lanes *5–8*) wild-types; and (lanes *9* and *10*) heterozygous Eμ-*Pim*-1 transgenics. The cultures assayed in lanes *1–6* are derived from inbred 129/Ola mice and were stimulated with IL-7, whereas the cultures assayed in lanes *7–10* are derived from B.CBA mice and they were stimulated with IL-7 and SF.

number of responsive cells present in bone marrow rather than differences in growth rate.

Flow cytometric analysis of the cells obtained in these cultures (Fig. 2) shows that virtually all of the cells produced are pre-B cells ($B220^+$, sIg^-). This also holds true for all Pim-1 mutant cultures, including those with a severely impaired response to IL-7, demonstrating that, as in the colony assays, Pim-1 null mutants can still recognize and use IL-7.

Pim-1 Expression in Murine Pre-B Cells. To determine whether these differences correlated with the Pim-1 protein levels, cells were harvested after 7–10 d of stimulation and the Pim-1 proteins were immunoprecipitated and labeled with ^{32}P in an autophosphorylation reaction (5). As shown in Fig. 3, the amount of Pim-1 protein detected in this way correlated with

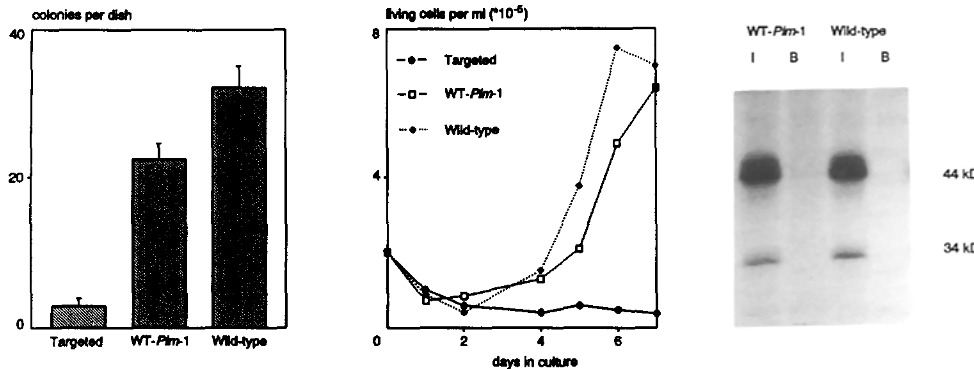

Figure 4. Rescue of the null mutant phenotype by introduction of the WT-*Pim*-1 transgene. Bone marrow cells from mice, two per group, homozygous for the *Pim*-1 null allele, with or without the WT-*Pim*-1 transgene, and wild-type, were tested for the presence of CFC responsive to IL-7 (*left*), the growth response to IL-7 in liquid cultures (*middle*), and the Pim-1 protein levels in 5 × 10^6 pre-B cells from IL-7–stimulated cultures (*right*). (Lanes *I*) Immunoprecipitations; and (lanes *B*) immunoprecipitations in the presence of excess amounts of blocking peptide. The proteins are visualized by autoradiography after autophosphorylation in the presence of γ[^{32}P]dATP.

the *Pim*-1 genotype. No clear differences in *Pim*-1 expression were noted between cells stimulated with IL-7 or IL-7 plus SF.

Rescue of the Impaired Response in Pim-1–deficient Mice by Introduction of a Transgene. To prove that the defects noted in *Pim*-1–deficient mice resulted from the absence of *Pim*-1 and not from another, possibly linked, mutation present in the embryonic stem cells, we crossed the *Pim*-1–deficient mice with mice carrying a wild-type *Pim*-1 transgene, WT-*Pim*-1. In the resulting mice, which were homozygous for the *Pim*-1 null allele and heterozygous for the WT-*Pim*-1 transgene, the IL-7 response had indeed been rescued (Fig. 4). Flow cytometric analysis of the cultures (data not shown) confirmed the presence of the expected population of pre-B cells (B220$^+$, sIg$^-$). When Pim-1 protein levels were analyzed in cells derived from these cultures (Fig. 4) close to normal levels of both *Pim*-1 proteins were seen in cells derived from null mutant mice carrying the WT-*Pim*-1 transgene. This conclusively shows that the impaired IL-7 response is caused by the absence of the Pim-1 proteins.

Whitlock-Witte Type Long-Term B Lymphoid Cultures Can be Established from Mutant Pim-1 Mice. In view of the fact that the drastically reduced growth factor responses of early B cells of *Pim*-1–deficient mice in vitro are not reflected in reduced populations in vivo, we studied more complex culture systems, to see whether compensating mechanisms which normalize B cell levels in vivo could be mimicked in vitro. For this purpose, Whitlock-Witte type long-term B lymphoid cultures (29) were established from bone marrow of the different *Pim*-1 mutants and their wild-type controls. In this system, whole bone marrow is cultured without added growth factors and prolonged B lymphopoiesis takes place, supported by stromal cells. All the *Pim*-1 mutants tested, ranging from homozygous for the null allele to heterozygous for the Eμ-*Pim*-1 transgene, were able to establish such

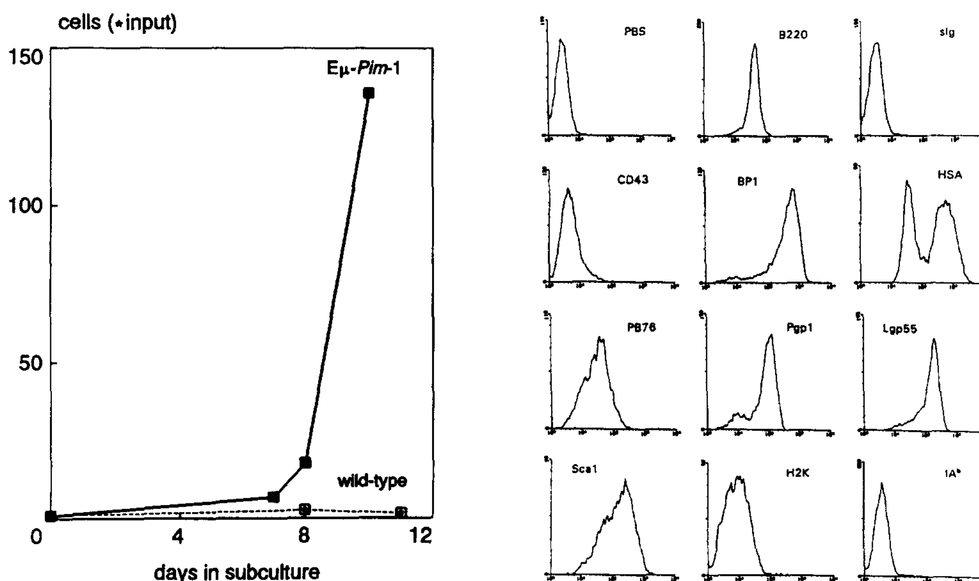

Figure 5. Growth factor response of cells produced by long-term B lymphoid cultures. (*Left*) Nonadherent cells, produced by established Whitlock-Witte cultures derived from wild-type or heterozygous Eμ-*Pim*-1 transgenic mice, cultured in the presence of IL-3, IL-5, IL-7, and SF. Cells were quantitated using a hemocytometer in the presence of trypan blue. Shown is the establishment of WSC905. (*Right*) Expression of surface markers, determined by flow cytometry, on WSC905. Similar expression patterns were seen on agar-derived clones from this cell line.

cultures. There are no obvious differences between the various genotypes, showing that the differences described above cannot be seen when complex cell–cell interactions are allowed between stromal and lymphoid cells.

Characterization of Cell Lines Derived from Whitlock-Witte Cultures. We tested nonadherent cells from such cultures for their response to various growth factors. In the absence of growth factors, and even in the presence of long-term cultures-CM, the cells died rapidly. However, in the presence of certain growth factors, rapid and continuous growth, allowing cloning in soft agar, was induced in cells derived from all four independent Eμ-*Pim*-1 transgenic cultures tested, but not from wild-type cultures (Fig. 5). The surface markers of these cells showed a pre-B cell phenotype (Fig. 5). No staining was observed for CD11b, GR-1, JORO-30-8, JORO 75, CD3, CD4, and CD8. CD43 (S7) expression was either low or absent. Cell identity was confirmed by RNA and DNA analysis, which showed high expression of the transgene, λ5 and *rag*-1, and rearrangements of the Ig H chain, but not L chain or TCR β chain gene (data not shown). Attempts to grow cells with a similar phenotype directly from bone marrow using the same growth factors failed.

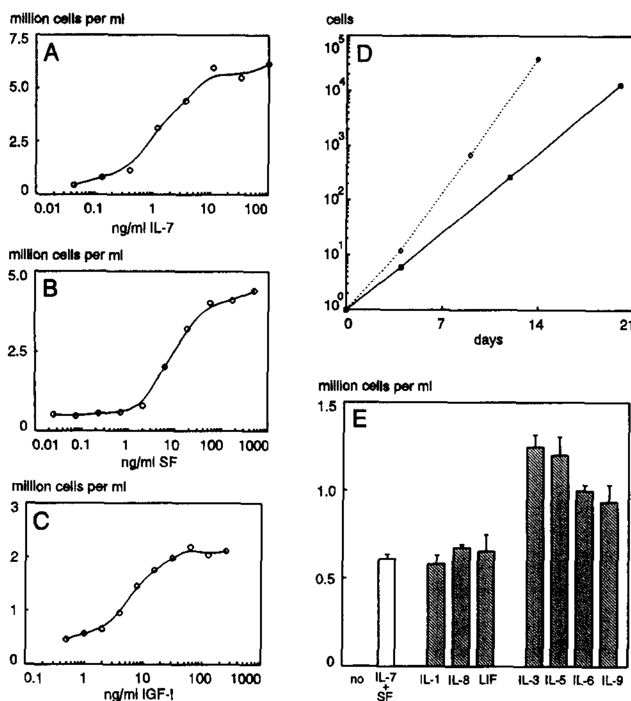

Figure 6. Characterization of growth factor requirements of Eμ-*Pim*-1 transgenic cell lines derived from Whitlock-Witte cultures. Illustrated is cell line WSC704. (*A*) Titration of the proliferative response to IL-7 in the presence of saturating amounts of SF. Stimulation for 3 d, initial density 10^5 cells per ml. (*B*) Titration of the response to SF in the presence of saturating amounts of IL-7, initial density 10^5 cells/ml, stimulation for 3 d. (*C*) Titration of the response to IGF-1 in the presence of saturating amounts of IL-7. Initial density 10^5 cells/ml, stimulation for 4 d. The response of cell line WSC905 is shown. (*D*) Comparison of proliferation in response to IL-7 + SF (*thin dashed line*) or IL-7 + IGF-1 (*thick line*). (*E*) Response to triple growth factor combinations. Stimulation without factors, or saturating amounts of IL-7 and SF, are shown on the left (*white bar*). (*Hatched bars*) Stimulations with IL-7, SF, and the growth factor indicated at the bottom. An increase is seen with some, but not with other growth factors. Means ± SD are shown of a representative assay in duplicate.

The cells are dependent on simultaneous stimulation by IL-7 and SF (Fig. 6 *A* and *B*). IL-7 could not be replaced by any of the other growth factor (combinations) tested. SF, however, could be replaced by IGF-1 (Fig. 6 *C*), although the growth rate was less than in response to IL-7 and SF (Fig. 6 *D*). IGF-1 did not further increase IL-7 + SF induced growth, unlike some other factors (Fig. 6 *E*).

Interestingly, unlike what has been reported for various other progenitor B cell lines (35), these seem to be blocked in differentiation, since attempts to induce sIg expression using cocultivation with the stromal cell lines ST2, PA6, and RPO.10, in the presence or absence of IL-7 and LPS, have failed. A similar lack of differentiation was observed in vivo, when 5 × 10^6 cells were injected into SCID mice.

Discussion

Pim-1 Expression Levels Determine the Size of B Cell Progenitor Compartments in Bone Marrow. Murine *Pim*-1, whose overexpression is associated with a variety of tumors, encodes a serine/threonine–specific protein kinase (5). We have shown previously that aberrant levels of Pim-1 affect IL-3–induced proliferation of bone marrow–derived mast cells in vitro (21). Here we have used mouse mutants to show that it is involved in vivo in the regulation of early steps of B lymphopoiesis. This is illustrated most clearly by the observation that the number of SF and IL-7 responsive early-B lymphoid CFC in bone marrow directly follows the expression levels of *Pim*-1. The growth rate in response to these factors is impaired in the absence of *Pim*-1. We also found that overexpression facilitates the establishment of factor-dependent early lymphoid cell lines that seemed to be blocked in differentiation.

IL-7 is a stromal cell factor with growth-promoting activity for B cell precursors (28–40). More primitive B cells require an additional stimulus, such as SF (41–43) or IGF-I (44). To identify additional important factors, mouse mutants in which the responses to known factors are impaired, can be important tools. *Pim*-1–deficient mice, which show a severely impaired response to IL-7 and SF, but which are capable of normal B lymphopoiesis in vivo and in LTC in vitro, will constitute an excellent model system to search for such factors.

The earliest B cell precursor compartment assayed (IL-7 + SF responsive) was the most profoundly affected. CFU-S assays on Eμ-*Pim*-1 transgenic mice have shown that this very early nonlymphoid committed, precursor population is not increased (Domen, J., E. Spooncer, and T. M. Dexter, unpublished observations), suggesting that these effects of aberrant *Pim*-1 expression do not extend to the most early hematopoietic cell compartment. The number of mature B cells in bone marrow (B220$^+$, sIg$^+$, responsive to LPS), while normal in *Pim*-1–deficient mice, is reduced in Eμ-*Pim*-1 transgenic mice. A decrease in mature B cell numbers has also been observed in Eμ-myc transgenic mice, here concomitant with the increase in pre-B cell numbers (45). The absence of significant changes in the periphery of *Pim*-1 mutants (9,

20) may be explained by an increased life span or proliferation of relatively long-lived mature B cells (46). This is also observed in the other null mutants with impaired B cell production, such as c-*abl* (47) and λ5 mice (48). The lack of correlation between the size of the pre-B cell compartment as defined by flow cytometry and the number of CFC present suggests that only a distinct subset of the pre-B cells are clonogenic precursors. The total number of pre-B cells in the bone marrow may be limited through other mechanisms, like the available bone marrow space.

Intriguingly, in marrows with the highest *Pim*-1 expression, a reduction in M-CSF responsive cells is observed. This reduction may reflect a selective differentiation of common precursors, such as described for fetal liver (49), towards the lymphoid lineages, or a more direct effect on macrophage differentiation, e.g., by affecting common mediators specific for the SF and M-CSF response.

Defects Caused by the Targeted Disruption of Pim-1 Locus Can be Rescued in Trans. The defects seen in the null mutant mice are likely caused by the inactivation of *Pim*-1, as the same phenotype has been observed in mice obtained from different, independently targeted, ES cell clones. However, in order to formally prove that the phenotype observed was the result of *Pim*-1 inactivation, we introduced a wild-type *Pim*-1 transgene. This transgene indeed rescued the effects seen, which can thus be completely ascribed to *Pim*-1.

Pim-1 Functions in a Dose-dependent Fashion. *Pim*-1 exerts its function in a dose-dependent manner, over a large dosage range, both in the assays described and in lymphoma incidence. Dosage-dependent phenotypes are displayed by some protein kinases, like regulators of mitosis in yeast (50), sometimes despite the presence of redundantly acting proteins (51), whereas the activity of other kinases, e.g., most protooncogenes, is strictly regulated (52). The highly inducible and short-lived RNA (11–13) and extremely short protein half-life (5) suggest, in line with the dosage-dependence seen, that Pim-1 might function as a constitutively active enzyme, although it cannot be excluded that activation is required, but not rate limiting.

Implications for the Function of Pim-1. The in vitro analysis of B lymphopoiesis in the *Pim*-1 mutant mice presented here has shown for the first time that *Pim*-1 is involved in the responses to different growth factors which act on early B-lymphoid compartments. Absence of *Pim*-1 does not lead to a total lack of responsiveness to the early B-lymphoid growth factors IL-7 and SF, whereas overexpression does not abrogate factor dependency, similar to the effects noted for IL-3 (21). This is unlike what has been reported for protein tyrosine kinases which can abrogate growth factor dependency (53), indicating that *Pim*-1 is a modulator rather than a mediator for the response to these factors. Whereas *Pim*-1–deficient cells show reduced growth rates when propagated in the presence of IL-7 or IL-3, we have not observed increased growth rates in cells overexpressing *Pim*-1. This suggests that *Pim*-1 levels in wild-type cells are already saturating for the proliferation-inducing signals. However, this does not seem to be in agreement with the phenotype of overexpression in vivo, an increasing tendency to develop lymphomas. Therefore, regulation of the size of early compartments by *Pim*-1 probably does not reflect a direct involvement in the proliferative response to growth factors. More likely, *Pim*-1 is involved in transducing signals that can halt or impair differentiation. This would explain the coincidence of *Pim*-1 levels and early B-lymphoid colony forming capacity of bone marrow and the behavior of cell lines obtained from overexpressors. It would also explain how *Pim*-1 functions as an oncogene when overexpressed, as expanded progenitor pools would increase the size of the cell compartment critically important to the accumulation of mutations. Detailed biochemical analysis is needed to fully resolve the mechanism of action of the *Pim*-1 proteins. Furthermore, it remains to be determined if *Pim*-1 fulfills a similar function in the T cell lineage. The involvement of *Pim*-1 in T cell lymphomagenesis indicates that this is probably the case.

J. Domen gratefully acknowledges members of T. M. Dexter's laboratory at the Paterson Institute (Manchester, UK) for the introduction into the various hematopoietic tissue culture techniques. We thank Drs. C. Saris and M. van Lohuizen for critically reading and improving the manuscript; E. Noteboom for help with flow cytometry; A. Beenders, T. Maitment, and N. Bosnie for assistance in animal care; and W. van Aalst and M. Meijer from the Central Laboratory of the Red Cross Blood Transfusion Service for help with the experiments involving SCID mice.

This work was supported by the Netherlands Cancer Society and the Foundation for Medical and Health Research.

Address correspondence to Dr. Jos Domen, Department of Pathology, B-263, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305-5324. Peter W. Laird's current address is Whitehead Institute for Biomedical Research, Rm 455, Nine Cambridge Center, Cambridge, MA 02142.

Received for publication 21 June 1993 and in revised form 13 August 1993.

References

1. Cuypers, H.T., G. Selten, M. Zijlstra, E. Robanus-Maandag, W. Boelens, P. van Wezebeek, C. Melief, and A. Berns. 1984. Murine leukemia virus–induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal regions. *Cell.* 37:141.
2. Selten, G., H.T. Cuypers, W. Boelens, E. Robanus-Maandag, J. Verbeek, J. Domen, C. van Beveren, and A. Berns. 1986. The primary structure of the putative oncogene *pim*-1 shows extensive homology with protein kinases. *Cell.* 46:603.
3. Mucenski, M.L., D.J. Gilbert, B.A. Taylor, N.A. Jenkins, and N. Copeland. 1987. Common sites of viral integration in lymphomas arising in AKXD recombinant inbred mouse strains. *Oncogene Res.* 2:33.
4. Dreyfus, F., B. Sola, S. Fichelson, P. Varlet, M. Charon, P. Tambourin, F. Wendling, and S. Gisselbrecht. 1990. Rearrangements of the *Pim*-1, c-*Myc*, and p53 genes in Friend helper virus–induced mouse erythroleukemias. *Leukemia.* 4:590.
5. Saris, C.J.M., J. Domen, and A. Berns. 1991. The *pim*-1 oncogene encodes two related protein-serine/threonine kinases by alternative initiation at AUG and CUG. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:655.
6. Telerman, A., A. Amson, R. Zakut-Houri, and D. Givol. 1988. Identification of the human *pim*-1 gene product as a 33-kilodalton cytoplasmic protein with tyrosine kinase activity. *Mol. Cell. Biol.* 8:1498.
7. Hoover, D., M. Friedmann, R. Reeves, and N.S. Magnuson. 1991. Recombinant human Pim-1 protein exhibits serine/threonine kinase activity. *J. Biol. Chem.* 266:14018.
8. Padma, R., and L. Nagarajan. 1991. The human *PIM*-1 gene product is a protein serine kinase. *Cancer Res.* 51:2486.
9. van Lohuizen, M., S. Verbeek, P. Krimpenfort, J. Domen, C. Saris, T. Radaszkiewicz, and A. Berns. 1989. Predisposition to lymphomagenesis in *pim*-1 transgenic mice: cooperation with c-*myc* and N-*myc* in murine leukemia virus–induced tumors. *Cell.* 56:673.
10. Meeker, T.C., L. Nagarajan, A. Ar-Rushdi, G. Rovera, K. Huebner, and C. Croce. 1987. Characterization of the human *PIM*-1 gene: a putative proto-oncogene coding for a tissue specific member of the protein kinase family. *Oncogene Res.* 1:87.
11. Dautry, F., D. Weil, J. Yu, and A. Dautry-Varsat. 1988. Regulation of pim and myb mRNA accumulation by interleukin 2 and interleukin 3 in murine hematopoietic cell lines. *J. Biol. Chem.* 263:17615.
12. Wingett, D., R. Reeves, and N.S. Magnuson. 1991. Stability changes in *PIM*-1 proto-oncogene mRNA after mitogen stimulation of normal lymphocytes. *J. Immunol.* 147:3653.
13. Lilly, M., T. Le, P. Holland, and S.L. Hendrickson. 1992. Sustained expression of the *PIM*-1 kinase is specifically induced in myeloid cells by cytokines whose receptors are structurally related. *Oncogene.* 7:727.
14. Breuer, M., R. Siebos, S. Verbeek, M. van Lohuizen, E. Wientjens, and A. Berns. 1989. Very high frequency of lymphoma induction by a chemical carcinogen in *PIM*-1 transgenic mice. *Nature (Lond.).* 340:61.
15. Verbeek, S., M. van Lohuizen, M. van der Valk, J. Domen, G. Kraal, and A. Berns. 1991. Mice bearing the Eμ-*myc* and Eμ-*pim*-1 transgenes develop pre-B-cell leukemia prenatally. *Mol. Cell. Biol.* 11:1176.
16. Domen, J., M. von Lindern, A. Hermans, M. Breuer, G. Grosveld, and A. Berns. 1987. Comparison of the human and mouse *PIM*-1 cDNAs: nucleotide sequence and immunological identification of the in vitro synthesized *PIM*-1 protein. *Oncogene Res.* 1:101.
17. Zakut-Houri, R., S. Hazum, D. Givol, and A. Telerman. 1987. The cDNA sequence and gene analysis of the human pim oncogene. *Gene.* 54:105.
18. Wingett, D., R. Reeves, and N.S. Magnuson. 1992. Characterization of the testes-specific *pim*-1 transcript in rat. *Nucleic Acids Res.* 20:3183.
19. te Riele, H., E. Robanus Maandag, A. Clarke, M. Hooper, and A. Berns. 1990. Consecutive inactivation of both alleles of the *pim*-1 proto-oncogene by homologous recombination in embryonic stem cells. *Nature (Lond.).* 348:649.
20. Laird, P.W., N.M.T. van der Lugt, A. Clarke, J. Domen, J. McWhir, A. Berns, and M. Hooper. 1993. In vivo analysis of *Pim*-1 deficiency. *Nucleic Acids Res.* In press.
21. Domen, J., N.M.T. van der Lugt, P.W. Laird, C.J.M. Saris, A.R. Clarke, M.L. Hooper, and A. Berns. 1993. Impaired IL-3 response in *Pim*-1 deficient bone marrow derived mast cells. *Blood.* 82:1445.
22. Laird, P.W., A. Zijderveld, K. Linders, M.A. Rudnicki, R. Jaenisch, and A. Berns. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* 19:4293.
23. Krimpenfort, P., F. Ossendorp, J. Borst, C. Melief, and A. Berns. 1989. T cell depletion in transgenic mice carrying a mutant gene for TCR-β. *Nature (Lond.).* 341:742.
24. Strasser, A. 1988. PB:76: a novel surface glycoprotein preferentially expressed on mouse pre-B cells and plasma cells detected by the monoclonal antibody G-5-2. *Eur. J. Immunol.* 18:1803.
25. Palacios, R., J. Samarides, D. Thorpe, and T. Leu. 1990. Identification and characterization of pro-T lymphocytes and lineage uncommitted lymphocyte progenitors from mice with three novel surface markers. *J. Exp. Med.* 172:219.
26. Golde, W.T., M. McDuffie, J. Kappler, and P. Marrack. 1990. Identification of a new cell surface glycoprotein with accessory function in murine T cell response. *J. Immunol.* 144:804.
27. Dexter, T.M., J. Garland, D. Scott, E. Scolnick, and D. Metcalf. 1980. Growth of factor-dependent hemopoietic precursor lines. *J. Exp. Med.* 152:1036.
28. Lee, G., A.E. Namen, S. Gillis, L.R. Ellingsworth, and P.W. Kincade. 1989. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β. *J. Immunol.* 142:3875.
29. Whitlock, C.A., and O.N. Witte. 1987. Long-term culture of murine bone marrow precursors of B lymphocytes. *Methods Enzymol.* 150:275.
30. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97.
31. Nishikawa, S.I., M. Ogawa, S. Nishikawa, T. Kunisada, and H. Kodama. 1988. B lymphopoiesis on stromal cell clone: stromal cell clones acting on different stages of B cell differentiation. *Eur. J. Immunol.* 18:1767.
32. Palacios, R., S. Stuber, and A. Rolink. 1989. The epigenetic influences of bone marrow and fetal liver stroma cells on the developmental potential of Ly-1$^+$ pro-B lymphocyte clones. *Eur. J. Immunol.* 19:347.
33. Gubler, U., A.O. Chua, A. Stern, C.P. Hellmann, M.P. Vitek, T.M. Dechiara, W.R. Benjamin, K.J. Collier, M. Dukovich, P.C. Familletti, et al. 1986. Recombinant human interleukin-

1α: purification and biological characterization. *J. Immunol.* 136:2492.
34. Renauld, J.-C., A. Goethals, F. Houssiau, H. Merz, E. van Roost, and J. van Snick. 1990. Human P40/IL-9: expression in activated CD4+ T cells, genomic organization and comparison with the mouse gene. *J. Immunol.* 144:4235.
35. Rolink, A., A. Kudo, H. Karasuyama, Y. Kikuchi, and F. Melchers. 1991. Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells *in vitro* and *in vivo*. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:327.
36. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213.
37. McNiece, I.K., K.E. Langley, and K.M. Zsebo. 1991. The role of recombinant stem cell factor in early B cell development. *J. Immunol.* 146:3785.
38. Kincade, P.W., C.J. Paige, R. Michael, E. Parkhouse, G. Lee. 1978. Characterization of murine colony-forming B cells. I. Distribution, resistance to anti-immunoglobulin antibodies and expression of Ia antigens. *J. Immunol.* 120:1289.
39. Stanley, E.R., L.J. Guilbert, R.J. Tushinski, and S.H. Bartelmez. 1983. CSF-1—a mononuclear phagocyte lineage-specific hemopoietic growth factor. *J. Cell. Biochem.* 21:151.
40. Namen, A.E., A.E. Schmierer, C.J. March, R.W. Overell, L.S. Park, D.L. Urdal, and D.Y. Mochizuki. 1988. B cell precursor growth promoting activity. *J. Exp. Med.* 167:988.
41. Martin, F.H., S.V. Suggs, K.E. Langley, H.S. Lu, J. Ting, K.H. Okino, C.F. Morris, I.K. McNiece, F.W. Jacobsen, E.A. Mendiaz et al. 1990. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell.* 63:203.
42. Williams, D.E., J. Eisenman, A. Baird, C. Rauch, K. Van Ness, C.J. March, L.S. Park, U. Martin, D.Y. Mochzuki, H.S. Boswell et al. 1990. Identification of a ligand for the c-*kit* proto-oncogene. *Cell.* 63:167.
43. Huang, E., K. Nocka, D.R. Beier, T.-Y. Chu, J. Buck, H.-W. Lahm, D. Wellner, P. Leder, and P. Besmer. 1990. The hematopoietic growth factor KL is encoded by the SI locus and is the ligand of the c-*kit* receptor, the gene product of the W locus. *Cell.* 63:225.
44. Landreth, K.S., R. Narayanan, and K. Dorshkind. 1992. Insulin-like growth factor-I regulates pro-B cell differentiation. *Blood.* 80:1207.
45. Langdon, W.Y., A.W. Harris, S. Cory, and J.M. Adams. 1986. The c-*myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. *Cell.* 47:11.
46. Förster, I., and K. Rajewsky. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. USA.* 87:4781.
47. Schwartzberg, P.L., A.M. Stall, J.D. Hardin, K.S. Bowdish, T. Humaran, S. Boast, M.L. Harbison, E.J. Robertson, and S.P. Goff. 1991. Mice homozygous for the abl^{m1} mutation show poor viability and depletion of selected B and T cell populations. *Cell.* 65:1165.
48. Kitamura, D., A. Kudo, S. Schaal, W. Müller, F. Melchers, and K. Rajewsky. 1992. A critical role of λ5 protein in B cell development. *Cell.* 69:823.
49. Cumano, A., C.J. Paige, N.N. Iscove, and G. Brady. 1992. Bipotential precursors of B cells and macrophages in murine fetal liver. *Nature (Lond.).* 356:612.
50. Russel, P., and P. Nurse. 1987. Negative regulation of mitosis by $wee1^+$, a gene encoding a protein kinase homolog. *Cell.* 49:559.
51. Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach. 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell.* 64:1111.
52. Cooper, J.A., K.L. Gould, C.A. Cartwright, and T. Hunter. 1986. Tyr^{527} is phosphorylated in $pp60^{c-src}$: implications for regulation. *Science (Wash. DC).* 231:1431.
53. Cleveland, J.L., M. Dean, M. Rosenberg, J.Y.J. Wang, and U.R. Rapp. 1989. Tyrosine kinase oncogenes abrogate interleukin-3 dependence of murine myeloid cells through signaling pathways involving c-*myc*: conditional regulation of c-*myc* transcription by temperature-sensitive v-*abl*. *Mol. Cell. Biol.* 9:5685.