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

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**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)–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'
untranslated region and is highly expressed in lymphoid and myeloid cells. The transgene was introduced into (CBA/B6 x C57BL/129F1)F1, zygotes and the resulting transgenics were backcrossed with (CBA/B6 x C57BL/129F1)F1, C57BL/129F1, 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 MgCl2, 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, 1% 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 MnCl2, 7 mM β-ME, 0.25 mM β-glycerophosphate, 0.4 mM spermine, and 10 μCi γ-[32P]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 a 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). An- cell conditioned medium (LTC) medium plus growth factors in a T25 flask (Costar Corp., Cambridge, MA). LTC medium is RPMI 1640 supplemented with 5% FCS, 5 x 10⁻³ M β-ME, and penicillin and streptomycin. Growth factors used were 10% 3T3-IL7 conditioned medium (CM) and 60 ng/ml steel factor (SF) (rrSCF164). Factor-dependent subcellular and cell lines derived from LTC were grown in LTC medium supplemented with 10% 3T3-IL7 CM, SF (rrMGF or rrSCF164, 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 10³ 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. A. Schreck, Hofheim, Germany) in the presence of trypan blue or a Sysmex Toa P8000 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 x 10³ 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 cytomtry.

**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 SCF164, 10 ng/ml, were gifts of Amgen Biologicals. Recombinant mouse GM-CSF, 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-3 (30), used at 1%, were donated by A. Strasser, whereas mouse IL-3-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⁺ slg⁻) (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⁺ slg⁺) present in B Cell POP-M1 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 | B cells |
|---------------------|-------------------|-------------|---------|
|                     |                   | B220*slg-   | B220*slg* |
| E~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 E~Pim-1 transgenic mice were obtained from heterozygotes, homozygous Pim-1-null mutant mice were both outbred (n = 6) and inbred (n = 2).

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 E~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 (+/+). Bone marrow of Mature (1-3-mo-old) mice. Results are given as means ± SD.

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 over-expressing 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 E~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 unfractonated 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 E~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 E~±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 protein levels were determined by autophosphorylation with 32P of the antibody-immobilized proteins in a kinase assay. The 44-kD Pim-1 protein is over-represented since this is a much better substrate for autophosphorylation (5). 2.5 × 10⁶ 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 E~±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.
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 cultures.

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, A5 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.

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 slg 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 × 10⁶ 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-1 (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⁺, slg⁺, 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,
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), some-
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