Impaired Interleukin-3 Response in Pim-1–Deficient Bone Marrow–Derived Mast Cells

By Jos Domen, Nathalie M.T. van der Lugt, Peter W. Laird, Chris J.M. Saris, Alan R. Clarke, Martin L. Hooper, and Anton Berns

The mouse Pim-1 gene encodes two cytoplasmic serine-threonine–specific protein kinases. The gene has been found to be activated (overexpressed) by retroviral insertion in hematopoietic tumors in mice. Transgenic mice that overexpress Pim-1 (Eu-Pim-1) have a low incidence of spontaneous T-cell lymphomas and an increased susceptibility to Moloney murine leukemia virus and N-ethyl-N-nitrosourea–induced lymphomas. Apart from a slight enlargement of the spleen, no abnormalities were found in prelymphomatous transgenic mice. Inactivation of the Pim-1 gene in the germline of mice resulted in a surprisingly subtle phenotype. Therefore, we investigated whether subtle effects of the absence of Pim-1 could be made visible during in vitro culturing of hematopoietic cells. We found that bone marrow–derived mast cells (BMMC) lacking Pim-1 had a distinct growth disadvantage when grown on interleukin (IL)-3, but not when stimulated by the factors IL-4, IL-9, or Steel factor (SF). This indicates a role for Pim-1 as a modulator of the IL-3 signal transduction pathway.

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PIM-1 is a highly conserved gene1–3 that was originally discovered as a common proviral insertion site in T-cell lymphomas in mice.4 Pim-1 was subsequently shown to be involved in the generation of B-cell lymphomas,5,6 and erythroleukemias7 as well. Characterization of the mouse gene showed an open-reading frame encoding a protein with all of the hallmarks of a protein kinase.1 The gene gives rise to two primary translation products by alternative initiation at CUG and AUG, yielding proteins of 44 and 34 Kd. Both were shown to exhibit protein-serine/threonine kinase activity.8 Relatively high Pim-1 expression is found in hematopoietic tumor cell lines such as thymus, spleen, and fetal liver, but also embryonic stem (ES) cells and testes. Expression is highly inducible by growth factors such as interleukin (IL)-2, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF).11–15 Functionally, this is in line with the very short half-lives of both mRNA15 and protein.10 These observations, in combination with the fact that overexpression in mice contributes to leukemogenesis,6,7,9,16,17 indicate that Pim-1 fulfills a regulatory role in the hematopoietic compartment. However, myeloid and lymphoid cells that overexpress Pim-1 from the Eu-Pim-1 transgene did not show any marked alterations in growth behavior in vitro, such as factor independence, even if combined with an activated c-myc gene (J. Domen, E. Spooncer, and T.M. Duxter, unpublished results), a gene combination that shows dramatic synergistic effects in vivo.8 However, this does not exclude the involvement of Pim-1 in growth factor signaling, since effects may only become apparent under specific conditions. The availability of Pim-1 null mutant mice obtained via homologous recombination in ES cells16 has allowed us to investigate further the involvement of Pim-1 in growth factor responses of factor-dependent hematopoietic cells. We concentrated on bone marrow–derived mast cells (BMMC) because they depend on IL-3,IL9, a factor known to induce Pim-1 mRNA. They also respond to other growth factors, which allows investigation of the involvement of Pim-1 in a series of responses to growth factors, whose activities are not limited to mast cells, but which also play a role in lymphoid proliferation and differentiation.

MATERIALS AND METHODS

Pim-1 mutant mice. Generation of the null mutant mice by homologous recombination in ES cells will be described elsewhere.18 These null mutants, in which the first three exons of Pim-1 have been replaced by a 3-phosphoglycerate kinase (PGK)-neo cassette, have no Pim-1 protein expression, as shown below. The mice used were outbred mice, obtained by crossing 129/Ola and BALB/c mice, followed by back-crossing with 129/Ola. We also used mice derived from an independent targeting event, described previously.20 This null mutation was present in inbred 129/Ola mice. Eu-Pim-1 transgenic mice have been described previously.16 The transgene consists of a genomic Pim-1 clone with two copies of the Ig Eu enhancer upstream and a Moloney long terminal repeat (LTR) in the 3′ untranslated region. The transgene was introduced into (CBA/Bra × C57BL/LJIA)F1 zygotes and the resulting transgenic mice were back-crossed with (CBA/Bra × C57BL/LJIA)F1, C57BL/LJIA, or C57BL/6 mice.

BMMC cultures. BMMC cultures were initiated by flushing bone marrow cells from femurs and sometimes tibiae into RPMI1640 plus 15% fetal calf serum (FCS) plus 5 × 10−3 mol/L β-mercaptoethanol plus 20% WEHI-3b–conditioned medium (CM) as a source of IL-3. Cultures were initiated at 1 × 106 cells/mL. Nonadherent cells were subcultured weekly at 2 × 105 cells/mL. After 4 weeks, the cultures contained an almost homogeneous population of BMMC, as judged from May–Grunwald/Giemsa– or Alcian blue/safranin-0–stained (BDH, Poole, UK) cytospins.

Short-term growth factor stimulations. BMMC from established cultures (>4 weeks in culture) were collected by centrifugation, washed three times in medium without IL-3, and resuspended (at twice the final cell concentration) in RPMI1640 plus 5% or 15% FCS plus 5 × 10−3 mol/L β-mercaptoethanol. One hundred micro-

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liters per well of growth factor in the same medium was distributed on flat-bottom 96-well plates. A 100-μL cell suspension was added to each well and the plates were incubated for the indicated amount of time at 37°C in a fully humidified incubator with a 5% CO₂ atmosphere. Final cell densities were determined with an improved Neubauer hemocytometer in the presence of trypan blue or a hybridized essentially as described.16 A mouse Pim-1 cDNA (283 liters per well of growth factor in the same medium was distributed to each well and the plates were incubated for the indicated amount of time at 37°C in a fully humidified incubator with a 5% CO₂ atmosphere. Final cell densities were determined with an improved Neubauer hemocytometer in the presence of trypan blue or a hybridized essentially as described.16 A mouse Pim-1 cDNA (283)

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Fig 2. Dose-response of Pim-1-deficient BMMC to IL-3 and SF. (A) IL-3 dose-response of BMMC derived from three sibling mice, homozygous (422T, -/+), heterozygous (424H, -/-), or wild-type (423W, +/+), with respect to the targeted Pim-1 allele. Cultures were initiated at a density of 3 × 10⁶ cells/mL and allowed to grow for 7 days with the amount of WEHI-3b-CM as indicated. (B) Dose-response of purified recombinant murine IL-3 of a Pim-1-deficient (6Ti) and a wild-type (AWi) BMMC culture. Initial density was 2 × 10⁶ cells/mL and stimulation was for 7 days. (C) IL-3 dose-response of two cultures, homozygous-targeted (92Tt, +/+), or wild-type (92Wi, -/-). The cultures were incubated with the indicated amounts of X63-IL-3-CM as a source of IL-3. Initial density was 2 × 10⁶ cells/mL and the cells were allowed to grow for 3 days. The experiment was performed in duplicate, SDs are shown when larger than the marker symbols. (D) As (C), but the cells were stimulated with soluble recombinant rat stem cell factor-164 (rsSCF-164).

or SF; withdrawal of both growth factors leads to cell death (Fig 3). Cell death induced in hematopoietic cells by lack of growth factor stimulation is usually through apoptosis. This is also the case for these cultures, as judged from the appearance of oligonucleosome-length DNA fragments and the delay of cell death in the presence of cycloheximide (data not shown), both of which are hallmarks for apoptosis. Interestingly, cells derived from Pim-1-deficient BMMC cultures die more slowly upon removal of IL-3 than wild-type BMMC (Fig 3A and B). This difference is less clear when cells are deprived of SF (Fig 3B). In the latter case, cell death also occurs more rapidly.

When BMMC cultures were established from mice overexpressing Pim-1 from the Eμ-Pim-1 transgene, a clear difference in the growth was noted between cultures derived from B.CBA mice (transgensics and sibling wild types) and cultures derived from 129/OLA and 129/OLA × BALB/c mice (null mutants and sibling wild types). The B.CBA cultures grew relatively poorly and contained more dead cells. However, when BMMC carrying the Eμ-Pim-1 transgene were compared with their wild-type littermates, no clear differences were apparent. Despite the overexpression, the cells remained dependent on IL-3 and did not grow faster than cells derived from wild-type littermates. Both sets of cultures contained similar amounts of dead cells when growing on IL-3, with 87% ± 9% of the cells alive in transgenic cultures, and 84% ± 10% alive in wild-type cultures (means ± SD; 15 cell counts on cultures from three different mice per group). Transgenic and wild-type cultures did not clearly differ in their response to SF (data not shown).
Responses of BMMC to growth factor combinations. Subsequently, we determined the effects of stimulation with more than one growth factor. Several growth factors can enhance the IL-3-mediated proliferative response. BMMC, wild-type or homozygous with respect to the Pim-1 null allele, were stimulated with IL-2, IL-3, IL-4, IL-5 (X63-CM), or IL-9. Without added growth factors the cells die, albeit less rapidly in cultures lacking Pim-1 than in wild-type cultures (Fig 4). IL-2 (and IL-5, data not shown), fail to induce growth, showing that stimulation by X63-CM is specific for the interleukin construct with which they were transfected. IL-4 and IL-9 alone also do not induce growth, although they do retard cell death. This is most apparent in the wild-type culture, where cell death occurs faster. In the presence of IL-3, both factors reproducibly induce a growth response, which appears not to be influenced by the presence or absence of Pim-1. IL-3 alone induces growth as before, i.e., stronger in the wild-type culture. This response cannot be increased in either culture by doubling the amount of IL-3 used. The responses of cultures overexpressing Pim-1 did not differ significantly from that of wild-type cultures (data not shown).

Furthermore, when BMMC were treated simultaneously with IL-3 and SF (Fig 5), they exhibited the same growth rate as with SF alone, but Pim-1-deficient cells grew to higher densities, something which has not been observed in single-factor stimulations. Cells from cultures overexpressing Pim-1 grew less well than wild-type cells under these conditions. This again confirms that Pim-1 specifically influences certain aspects of the IL-3 response.

Pim-1 expression levels in mutant mast cells. To ascertain that the differences observed in growth induced by IL-3 correlated with the presence of Pim-1, we quantitated the Pim-1 mRNA and protein levels (Fig 6). We found high Pim-1 mRNA expression in wild-type BMMC, comparable to the levels seen in lymphocytes. Even higher levels are seen in BMMC carrying the Ep-Pim-1 transgene (transgenic mRNA is characterized by a slightly lower mobility). No mRNA was found in BMMC from homozygous mutant mice, while intermediate levels were present in heterozygous BMMC (Fig 6A). To verify that the protein levels correlate with the mRNA levels, we determined Pim-1-specific kinase activity. We failed to detect any autophosphorylation of Pim-1 in lysates from BMMC homozygous for the
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**Fig 5.** Combined stimulation of BMMC by IL-3 and SF. Means ± SD are shown. (A) Time course of growth of three Pim-1-deficient cultures (---) and three wild-type cultures (-- -- --), which are stimulated by 25% WEHI-3b-CM (IL-3) and 74 ng/mL SF (MGF). (B) Time course of growth of two B.CBA wild-type (---) and two Ep-Pim-1 cultures (---)

**Pim-1 null allele** (Fig 6B). The 34-, 35-, and 44-Kd Pim-1 proteins could readily be detected in lysates from wild-type and heterozygous cultures, with the latter again showing intermediate levels. Transgenic cultures clearly overexpressed the Pim-1 proteins.

**Pim-1 expression in response to different growth factors.** Pim-1 expression is highly inducible by a variety of growth factors and mitogens, including IL-3,13,14 We investigated whether growth of wild-type cultures on the two growth factors that can sustain BMMC proliferation, IL-3 and SF, differed in the Pim-1 expression levels they induced. Figure 7 shows that they indeed do. While cells grown on IL-3 contain high Pim-1 protein levels, as shown in a kinase assay, cells grown on SF alone contain only low levels of the Pim-1 proteins. When both growth factors are combined, the levels are similar to those seen in cells grown on IL-3 alone, indicating that SF is not able to downmodulate Pim-1 induction by IL-3. Neither did combined stimulations with IL-3 and IL-4 or IL-3 and IL-9 lead to differences in Pim-1 protein level.

**Mucosal-type mast cells in vivo.** BMMC resemble the mucosal-type mast cells present in mice. When these were quantitated in Alcian blue-stained midjejunal sections (Table 1), no significant differences were observed between Pim-1 null mutant and wild-type mice. To investigate whether the impaired IL-3-induced proliferation would

**Fig 6.** Pim-1 mRNA and protein levels in wild-type and mutant BMMC. The left panel shows a Northern blot. Control lane 2M3 contains RNA from the highly Pim-1-expressing, v-abl-transformed, pre-B-cell line 2M3, lane --/--- from the homozygous mutant BMMC culture 218T, lane --/+ from the heterozygous mutant BMMC culture 219H, lane +/+ from the wild-type BMMC culture B.CBA, and lane T +/+ from a heterozygous Ep-Pim-1 transgenic culture, 4770. Equal amounts of RNA were loaded as judged from the ethidium bromide-stained ribosomal bands. The right panel shows protein expression, assayed by in vitro autophosphorylation of the Pim-1 proteins. Lanes --/--- show immunoprecipitations of lysates made from the homozygous mutant BMMC culture 477T, lanes --/+ from the heterozygous mutant BMMC culture 425H, and lanes +/+ from the wild-type BMMC culture 100W. The leftmost panel shows immunoprecipitations from a B.CBA wild-type culture, +/+ , and a culture heterozygous for the Ep-Pim-1 transgene, T +/+ . The lanes marked I show normal immunoprecipitations, the ones marked B (block) show immunoprecipitations in the presence of an excess of the peptide against which the serum was raised. Note that the intensity of 44 Kd versus the 34-/35-Kd bands does not reflect their true molar ratio, because the 44-Kd protein is autophosphorylated much more efficiently.10
compromise the mast-cell response in vivo, homozygous targeted and wild-type mice were infected with the nematode parasite *N. brasiliensis*, to which a partially IL-3-dependent mast-cell response is mounted, resulting in a 25- to 40-fold increase in the number of intestinal mast cells in BALB/c mice.\(^{19,23}\) Quantitation of serum IgE levels (not shown) and mast cells in jejunal sections (Table 1) showed a normal response to the infection. No differences were observed between the two groups of mice.

**Discussion**

The data presented here show that BMMC lacking *Pim-1* have an aberrant response to IL-3 in vitro. The response of other factors tested (IL-4, IL-9, and SF) is not detectably altered in the absence of *Pim-1*. The IL-3 response of BMMC overexpressing *Pim-1* does not differ significantly from that of wild-type cultures. Combined stimulation with IL-3 and SF allows null-mutant cultures to reach higher cell densities than wild-type cultures, while transgenic cultures grow to lower densities than wild-type cultures. The difference in cell death observed when the cells are growth factor-deprived is intriguing, but it cannot explain the differences observed in IL-3-induced proliferation, since there are no clear differences in the percentage of dead cells present in IL-3-stimulated growing or resting cultures. The difference in apoptosis may be caused by a difference in cycling speed. It has been noted before that aberrant cell-cycle control, as, for example, caused by constitutive *c-myc* expression, can lead to accelerated apoptosis when dependent cells are deprived of IL-3.\(^{29}\) The virtual absence of *Pim-1* expression in wild-type BMMC maintained on SF is interesting, explaining the lack of difference in this response between null-mutant and wild-type cultures. This implies a role for *Pim-1* as a modulator of the IL-3 response.

IL-3, produced by activated T cells, keratinocytes,\(^{30}\) activated mast cells,\(^{31,32}\) and stroma cells,\(^{33}\) is a potent growth factor, acting on a wide range of myeloid and lymphoid cells. It can stimulate both self-renewal and differentiation of multipotent cells into all myeloid lineages. As a mitogen, it is generally more powerful for early progenitor cells than for more mature cells. However, the factor is also capable of potentiating some mature cell functions.\(^{30}\) Infusion of IL-3 into mice leads to an increase in myeloid progenitors in, and size of, the spleen, while bone marrow cellularity decreases.\(^{34,35}\) Only a modest increase is found in mature peripheral blood cells. Apart from stimulating myeloid cells, IL-3 has also been reported to be able to stimulate lymphoid cells in vitro.\(^{36,37}\) However, it is unclear whether this is important in vivo.\(^{38}\)

While it is unknown what the consequences of absence of IL-3 in vivo are, variations in the response of different mouse strains to IL-3 have been documented. Differences between inbred mouse strains described in the literature include a 10-fold variation in the induction of small mast cells after infusion of IL-3,\(^{39}\) while it has also been reported that some strains of mice are virtually unresponsive to IL-3 in colony assays.\(^{39}\) Such variations between strains and mutants has also been reported for IL-3–induced mast cell growth in vitro.\(^{40}\) We also find differences in the IL-3 response between wild-type mice of the strains used to overexpress *Pim-1*, whose genetic background is mainly C57BL/6-derived, and which performs rather poorly when compared with the strain used to inactivate *Pim-1*, which is present on a 129/OLA background and which grows well. However, the effects attributed to *Pim-1* can be shown in comparisons of littermates that only differ in one, molecularly defined, lesion in the *Pim-1* gene, and consequently the effects described here do not reflect differences in the genetic background, but have to be attributed solely to *Pim-1*. The variations seen between mouse strains with an apparently normal hematopoietic system are in accordance with our finding that, although *Pim-1* appears to be a modulator of the IL-3 response, mice lacking *Pim-1* do not show any obvious hematopoietic abnormalities in vivo and are even able to mount an apparently normal mast-cell response when challenged with nematode parasites. This can be explained by the fact that multiple growth factors act in vivo. For example, for the nematode-induced mast cell response, IL-4 has also been shown to be a major factor.\(^{23}\) In addition,

**Table 1. Mucosal-Type Mast Cells per Villus-Crypt Unit**

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<thead>
<tr>
<th>Condition</th>
<th>Wild-Type</th>
<th><em>Pim-1</em> Null Mutant</th>
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<tr>
<td>Noninfected</td>
<td>0.6 ± 0.5</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td><em>N. brasiliensis</em>–infected</td>
<td>12.3 ± 4.4</td>
<td>12.1 ± 3.9</td>
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Mucosal-type mast cells were counted in 10 villus-crypt units per animal. The total number of animals analyzed per group is listed. Results are means ± SD.
it is important to stress that, as the BMMC cultures show, the capacity to respond to IL-3 in the absence of Pim-1 is only reduced, not absent. The consequences of the absence of Pim-1 for the IL-3 response did not differ between inbred (129/Ola, Fig 2B through D) and outbred (129/Ola × BALB/c, other figures) mice. The latter mice are all derived from the targeting event described by Laird et al., and the inbred 129/Ola null mutant mice were generated from the double-targeted ES cells described previously.

Abrogation of IL-3 dependence of mast cells has been accomplished in a nonautocrine fashion by introducing tyrosine kinase oncogenes such as v- abl, fms, src, and trk into mast cells. Introduction of an activated H-ras gene into mast cells led to an abrogation of IL-3 dependence in an autocrine fashion. Abrogation of IL-3 dependence cannot be accomplished by overexpressing Pim-1 in BMMC.

The reversal of the growth phenotype when cells are stimulated simultaneously with IL-3 and SF is somewhat puzzling, as proliferation in response to SF is normal in Pim-1-deficient BMMC and does not involve induction of Pim-1 expression in wild-type BMMC. Possibly, the mode of action of Pim-1 on IL-3-induced proliferation is dependent on the state of activation of certain other signal transduction pathways, eg, that of SF. In this way, the proteins could contribute to the integration of different growth factor signals. Such a function would be compatible with the subtle effects caused by aberrant Pim-1 expression. Linkage between IL-3 and SF stimulation has been noted before. IL-3 stimulation can lead to downregulation of c-kit [SF-receptor], while incubation of human CD34+ hematopoietic progenitor cells with c-kit anti-sense oligonucleotides specifically inhibits granulocyte-macrophage colony formation induced by IL-3, but not by GM-CSF. The observation that some of the specific tyrosine-phosphorylated target proteins are shared by both pathways is in line with this notion. BMMC stimulated with SF differentiate from mucosal-type mast cells toward connective tissue-type mast cells. When both stimuli are combined, IL-3 appears to be limited to the IL-3 signal transduction pathway. In line with this notion, we have recently shown that aberrant expression of the gene can have similar effects on the responses of B-lymphoid cells.

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