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Thrombopoietin inhibits murine mast cell differentiation

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Abstract

We have recently shown that Mpl, the thrombopoietin receptor, is expressed on murine mast cells and on their precursors and that targeted deletion of the Mpl gene increases mast cell differentiation in mice. Here we report that treatment of mice with thrombopoietin, or addition of this growth factor to bone marrow-derived mast cell cultures, severely hampers the generation of mature cells from their precursors by inducing apoptosis. Analysis of the expression profiling of mast cells obtained in the presence of thrombopoietin suggests that thrombopoietin induces apoptosis of mast cells by reducing expression of the transcription factor Mitf and its target anti-apoptotic gene Bcl2.

Keywords

Thrombopoietin; Mpl; mast cells; apoptosis; Bcl2; Mitf

INTRODUCTION

Mast cells [1–3] derive from c-Kit^{low}CD34^{low}Sca-1^{pos} stem/progenitor cells present in the marrow that give rise to c-Kit^{high}/T1/ST2^{pos} lineage-restricted progenitor cells (MCP) [4] and then to c-Kit^{high}/CD34^{high} mast cell precursors that circulate in the blood to colonize

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extramedullary sites [5]. Mast cell precursors are characterized by the ability to proliferate extensively, by small cytoplasmic granules and by little or no surface expression of the receptor which attaches to the Fc portion of IgE (FcεRI^{neg}) with high affinity. These cells differentiate, in turn, into tissue-restricted mast cells, dermal, mucosal and serosal, respectively in skin, gut and peritoneal cavity [1–3].

Extrinsic and intrinsic control pathways regulate mast cell differentiation. The extrinsic pathway includes the growth factors SCF and IL-3 [6–8]. SCF is produced by marrow and skin fibroblasts alike [9] and mice carrying alterations in the gene encoding SCF [10], or its receptor c-Kit [11] are mast cell deficient. On the other hand, IL-3 is not produced in normal mice. Mast cells, however, are induced to produce IL-3 in an autocrine/paracrine fashion by IgE/FcεRI interactions [12]. The observation that IL-3-deficient mice have normal numbers of mast cells but produce an inadequate response when challenged with parasites [13] has suggested that IL-3 may be employed to regulate mast cell numbers topically, in response to B cell recruitment to the site of infection [14]. Both SCF and IL-3 must be present in order for mast cell differentiation to occur in bone marrow derived mast cell (BMMC) cultures [6,8]. In 14 days, mast cell precursors with a particular phenotype (c-Kit^{high}CD34^{low}FcεRI^{neg} with small Alcian Blue^{neg} granules) [15], and function (reconstitute dermal and mucosal mast cells when transplanted into mast cell-deficient animals) develop within the cultures [16]. By day 21, they mature into berberine sulphate^{pos}FcεRI^{pos} mast cells, which are biochemically similar to dermal mast cells [6,7,17]. In BMMC cultures, SCF permits proliferation, [18,19] while either SCF [20] or IL-3 [21] contributes to preventing apoptosis. At the intrinsic level, the transcription factors Mitf [22], Gata2 [23,24] and Gata1 regulate mast cell differentiation [25]. Mitf encodes a helix-loop-helix protein that has either positive (MMCP-6) [26] or negative (MMCP-7) [27] effects on the expression of MMCPs. Mitf also controls the expression of the anti-apoptotic Bcl2 gene in these cells [28]. Gata2 and Gata1 are members of the GATA transcription factor family [29]. In mast cell differentiation, as in other hemopoietic lineages, Gata2 expression precedes that of Gata1 [24] and increases cell proliferation rates [23,30]. On the other hand, given the presence of functional Gata1 binding sites in the regulatory region of Carboxypeptidase A (MC-CPA) [31] and that of the α [32] and β [33] chain of FcεRI, Gata1 may play a role in cell maturation.

We have recently described that treatment with thrombopoietin (TPO) affects the expression of Gata1 in mice carrying the hypomorphic Gata1^{low} mutation and in their wild-type littermates [34,35]. The effects exerted by TPO in the two animals are paradoxical (it increases and decreases the level of Gata1 expression in wild-type and mutant littermates, respectively) and involve cells of the erythroid, megakaryocytic and mast cell lineage [34,35]. The similarity of the effects of TPO on the expression of Gata1 in the cells of these three lineages, and the notion that Mpl, the TPO receptor, is expressed not only by erythroid cells and megakaryocytes [36] but by mast cells as well [34], suggests to us that TPO/Mpl interactions might regulate mastocytopoiesis in mice. To confirm this hypothesis, we analyze here the effects of in vivo and in vitro TPO-treatments on murine mastocytopoiesis. TPO-treatment of wild-type mice, or addition of TPO to BMMC cultures, favors proliferation of mast cell precursors but reduces the formation of mature cells by inducing their apoptosis. Molecular profiling analysis of cells exposed to TPO, suggests that TPO affects mast cell maturation through a molecular mechanism that involves induction of apoptosis by down modulation of cKit expression on the cell surface and suppression of Mitf and Bcl2 expression, possibly through the SCF/Mitf/PIAS3/STAT3 axis [37].

MATERIALS AND METHODS

Mice

Mpl^{null} mice [38] were provided by Dr. A. Warren (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia) and bred with CD1 females (Charles River, Calco, Italy) at the animal facilities of Istituto Superiore Sanità. Littermates were genotyped by PCR and those found not to carry the mutation used as wild-type controls. All the experiments were performed with 6–10-month old male littermates, according to protocols approved by the institutional animal care committee.

Thrombopoietin treatment

Recombinant murine TPO was injected i.p. into wild-type mice for 5 consecutive days (100 µg/Kg of body weight/daily), as described [35]. Mice were sacrificed either before the first injection or at 7 and 14 days after for further analysis (see below).

Hematological parameters

Blood was collected from the retro-orbital plexus into ethylen-diamino-tetracetic acid-coated microcapillary tubes (20–40 µL/sampling) and hematocrit (Hct) and platelet (plt) counts determined manually [35].

Histological analysis

The ear, spleen and femur were fixed in 10% (v/v) phosphate-buffered formalin (Sigma, St. Louis, MO, USA), paraffin embedded and cut into 2.5–3 µm sections according to standard procedures. Slides of consecutive sections were dewaxed, rehydrated and stained with regular and acidified Toluidine Blue (Multilab, Surrey, England), as described [25]. Cell metachromasia is defined by the color acquired by the cytoplasmic granules after acidified toluidine blue-staining. The granuli of immature and mature mast cells are, respectively, blue (metachromatic^{neg}) and red (metachromatic^{pos}) [25]. TUNEL staining was performed with the “In Situ Cell Death Detection Kit” (Boehringer Mannheim, Mannheim, Germany) using, as negative controls, sections treated with a fluorescent deoxyuridine triphosphate-free TUNEL reaction mixture. Cells obtained by peritoneal lavage were cytopspun onto glass slides (Cytospin 3, Shandon, Pittsburgh, PA) and stained with either May-Grunwald/Giemsa (Sigma) or Alcian Blue. For electron microscopy, samples were fixed in glutaraldehyde (2.5%), post-fixed in OsO₄ and embedded in SPURR resin (Polyscience, Warrington, PA) [25]. Light microscopy was analyzed with a Leica Light Microscope (Leica LTD, Heidelberg, Germany) equipped with a Coolsnap videocamera for computerized images (RS Photometrics, Tucson, AZ, USA), while transmission electron microscopy was performed with the EM 109 Zeiss (Oberkochen, Germany).

Flow cytometry analysis

Cells were labeled with PE-CD117 (that recognizes c-Kit) coupled with either FITC-CD34, -Annexin V or with -CD45R/B220, as a control. Further antibodies used in the study were FITC-Mac3, -CD61, and -CD71 and PE-Ly-6G (Gr1), -CD41, -TER119. The expression of FcεRI was revealed by sequential incubations with the monoclonal mouse anti-DNP-IgE (clone SPE-7, Sigma) and FITC-conjugated rat anti-mouse IgE [25]. Unless otherwise stated, all the antibodies were from PharMingen (San Diego, CA) and were incubated at a concentration of 1 µg/10⁶ cells for 30 min on ice. Cell fluorescence was analyzed either with a Coulter Epix Elite ESP (Beckman Coulter, Miami, FL) or a FACS ARIA (Becton Dickinson, Franklin Lakes, NJ). Non-specific fluorescent signals were gated out with appropriate fluorochrome-conjugated isotype controls, and dead cells were excluded by propidium iodide staining.

Cell Purification

Mast cells were purified by sorting, as described [25]. Briefly, peritoneal cells were first incubated with FITC-conjugated CD45R and immunodepleted with a monoclonal mouse anti-fluorescein antibody-coated MACS microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), as described by the manufacturers. The B220-negative cell fraction was then incubated with PE-CD117, and CD117^{high} cells, ($\approx 50\%$ of B220^{neg} cells) isolated with the FACS ARIA ($>95\%$ CD117^{high} after reanalysis). Cells in the prospective stem/progenitor cell gate [39] were sorted with the FACS ARIA from the marrow of wild-type mice incubated with PE-CD117 and FITC-CD34. The sorted CD117^{pos}/CD34^{pos} cells were $>90\%$ pure by FACS reanalysis and gave rise to >90 CFU-GM-derived colonies/100 plated cells in clonogenic assay [40].

Culture of bone marrow derived mast cells (BMMC)

BMMC were established from marrow (and spleen) light density cells ($1-2 \times 10^5$ cells/mL) or sorted CD117^{pos}/CD34^{low} cells ($0.2-2 \times 10^4$ cells/mL), as described [11,25,40]. The cultures were stimulated with SCF (100 ng/mL) and IL-3 (10 ng/mL) with or without the presence of TPO (50 ng/mL), and replenished with fresh growth-factors and demipopulated every 3–4 days, as necessary, to keep the cell concentration $<10^6$ cells/mL.

Serotonin-release assay

Peritoneal B220^{neg} cells or day 21 BMMC-derived cells (1×10^6 /mL) were incubated for 6 hours at 37 °C with ³H-serotonin [5-hydroxy(³H)tryptamine trifluoroacetate, 2 μ Ci/mL] (84.0 Ci/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK), washed twice and either lysed with Triton X-100 (1% v/v), as a measure of the total amount of ³H-serotonin incorporated, or incubated again (20×10^6 cells/mL) for 1 hour on ice with the monoclonal mouse anti-DNP-IgE (10 μ g/mL). After incubation, aliquots of 0.4×10^6 cells were resuspended in 50 μ L and stimulated for 15 min at 37 °C with the rat monoclonal α IgE (2 μ g/mL, R35–72, PharMingen). Reactions were terminated with 50 μ L of cold Hank's balanced salt solution, cells removed by centrifugation and the level of ³H-serotonin in the supernatants measured with the Packard 1600TR liquid scintillator counter (PerkinElmer Life Science Inc, Boston, MA) and expressed as a percent of the total amount of ³H-serotonin incorporated (according to the algorithm: cpm in supernatants $\div$ cpm in Triton lysates $\times$ 100) [5,25].

RNA isolation and quantitative RT-PCR

Total RNA was prepared with Trizol (Gibco BRL) and retrotranscribed with random primers using the SuperScript III kit (Invitrogen Life Technologies, Bethesda, MD). Gene expression was quantified with the Taqman PCR kit (PE Applied Biosystems, Foster City, CA) and pre-developed custom-made oligos, whose sequence is available upon request, using either the ABI PRISM 7700 or the 7300 Sequence Detection System (Applied Biosystem). GAPDH was also quantified in each reaction. Results were analyzed with the SDS program (v1.9, Applied Biosystem) and expressed in arbitrary units, using the amplification of GPDH as calibrator, according to the following algorithm: $\Delta Ct = [CtX - CtGPDH]$, where Ct is the threshold cycle of the gene analyzed, and were presented as $2^{-\Delta Ct}$.

Statistical analysis

Statistical analysis was performed by analysis of variance (Anova test) using Origin 3.5 software for Windows (Microcal Software Inc., Northampton, MA).

RESULTS

TPO-treatment decreases mast cell differentiation in mice

Figure 1 and Table 1 present the number and differentiation profile of dermal mast cells observed in mice treated with TPO. The platelet number in blood represents control for the efficacy of the TPO-treatment.

As expected [35], TPO-treatment significantly increases the number of platelets present in the blood at day 7 (1.8×10^6 vs 0.9×10^6 platelets/ μ L, respectively, $p < .01$). The number of circulating platelets remains higher, although no longer statistically different, than normal up to 14 days but returns to pre-treatment levels by day 21 (Table 1 and data not shown).

TPO-treatment increases the number of mast cell precursors (metachromatic^{neg} after Toluidine Blue) by 3-fold at day 7–14 and that of mature mast cells (Alcian Blue/Safranin^{pos}) by 2-fold at day 14 in the dermis of the ear (Figure 1, Table 1). Many of the cells, however, undergo apoptosis. In fact, while TUNEL staining labels the nuclei of a limited number of cells (< 20 cells/ mm^2) in the dermis of the ear from untreated mice, numerous cells in the dermis just below the epithelium of the ear are TUNEL^{pos} 14 days after TPO-treatment (600 ± 43 cells/ mm^2 , Figure 1 and Table 1).

TPO-treatment also increases the total cell number (by 2–4-fold) and the frequency of CD117^{high}Fc ϵ R1^{pos} cells (by 3–40-fold) present in the peritoneal cavity at day 7–14 (Figure 2A, Table 1). All these values return to pre-treatment levels by day 21 (data not shown). Similar to what had been observed in the dermis, numerous serosal mast cells from TPO-treated mice are apoptotic. In fact, Annexin V^{pos} cells are rare ($\approx 1\%$) among CD117^{high} cells in the peritoneal cavity of untreated mice, but represent $\approx 60\%$ of those from TPO-treated animals (Figure 2A and Table 1). The pattern of TUNEL and Annexin V staining of dermal and serosal mast cells is consistent with the hypothesis that TPO triggers apoptosis of mast cells in vivo between day 7 and 14 of treatment.

TPO-treatment greatly affects the morphology of serosal mast cells that become smaller and contain fewer granules than the corresponding cells from untreated controls (Figure 2A). These morphological changes are associated with numerous alterations in differentiation profile. By flow cytometry, serosal mast cells from mice after 7 days of TPO-treatment express 10-fold less CD117 (800 vs 8,700 AFU/cell, respectively) and 1-log more Fc ϵ RI (1.1×10^3 vs 0.1×10^3 AFU/cell, respectively) than normal (Figure 2A). By real time RT-PCR, serosal mast cells from TPO-treated mice express normal levels of Mpl, c-Kit and Gata2, but significantly lower levels of Bcl2 and Mitf (both by 30–50%, $p < .05$ –.01), MMCP-6 and MMCP-7 (both by ~ 1 -log) and significantly higher levels of Gata1 (by 50% at day 7) (Figure 2B).

Finally, after IgE/ α IgE stimulation, serosal mast cells from TPO-treated mice release significantly less (by 50%) serotonin than normal cells (Figure 2C).

TPO inhibits mast cell differentiation in BMMC cultures

The effects of TPO on mast cell differentiation are further analyzed in BMMC seeded with marrow cells from wild-type (and Mpl^{null}, as negative control) mice.

TPO has no effect on the number ($6.2 \pm 0.7 \times 10^7$ vs $7.2 \pm 0.5 \times 10^7$ cells/flask by day 21, $p > .05$) and morphology (Figure 3A) of cells obtained in BMMC cultures from Mpl^{null} mice. In contrast, in BMMC cultures from wild-type mice, TPO increases the number of mast cell precursors obtained at day 14 ($4.1 \pm 0.2 \times 10^7$ vs $2.3 \pm 0.2 \times 10^7$ cells in flasks containing or not TPO, respectively, $p < .05$) by ~ 2 fold but reduces that generated at day 21 ($14.2 \pm 3.2 \times 10^7$ vs $22.5 \pm 5.5 \times 10^7$ cells/flask, respectively, $p < .05$) by ~ 2 fold. Furthermore, while more than 90%

of the cells obtained after 21 days in BMMC cultures (both wild-type and Mpl^{null}) are mature cells ($c\text{-Kit}^{high}CD34^{high}Fc\epsilon RI^{high}$; Figure 3A see also [11, 13, 25]), only a minority ($\approx 25\%$) of those developed at day 21 in the presence of TPO express CD34, and none are $Fc\epsilon RI^{pos}$ (Figure 3A). In addition, wild-type BMMC-derived cells obtained in the presence of TPO have few cytoplasmic granules (Figure 3A).

Table 2 presents the expression profile of wild-type mast cells obtained under BMMC conditions. All the genes analyzed are expressed by wild-type BMMC-derived cells at levels either slightly (*Mitf*, *Bcl2* and *MMCP-6*, by 2-fold, $p < .05$) or greatly (*c-Kit*, *Gata1*, *Gata2*, *MC-CPA* and *MMCP-7* by 2-log, $p < .01$) higher than those expressed by the corresponding serosal mast cells (Figure 2B and Table 2). The expression profile of BMMC-derived cells from cultures seeded with Mpl^{null} mice is similar to that of the corresponding wild-type cells, with the exception of a lower expression (by ~ 1.5 -log, $p < .01$) of *MMCP-7* (Table 2). TPO does not affect the expression profiling of Mpl^{null} BMMC-derived cells but significantly reduces the levels of *c-Kit*, *Mitf*, *Gata2*, *Bcl2*, *MMCP-6* and *MMCP-7* expressed by wild-type BMMC-derived cells (Table 2).

Finally, following $IgE/\alpha IgE$ stimulation, BMMC-derived wild-type mast cells obtained in the presence of TPO incorporate and release 30% less serotonin than cells obtained in control cultures without TPO (Figure 3B).

Effect of TPO on mast cell differentiation from purified murine stem/progenitor cells

To further assess whether TPO directly affects mast cell differentiation and to define the differentiation stage targeted by this growth factor, the effect of TPO on the number and phenotype of cells obtained in BMMC cultures seeded with wild-type $CD117^{pos}/CD34^{pos}$ progenitor cells was analyzed under conditions of limiting dilution ($200\text{-}2 \times 10^4$ cells/mL) (Figure 4). By day 14, purified $CD117^{pos}/CD34^{pos}$ cells generate cells with the $CD117^{high}CD34^{neg/low}Fc\epsilon RI^{neg}$ (Figure 4C) $CD41^{neg}/CD61^{low}$, $Ter119^{low}/CD71^{neg}$, $Gr1^{neg}$ and $Mac3^{neg}$ (not shown) phenotype. As such, these cells represent a homogeneous population of mast cell precursors completely devoid of megakaryocytic, erythroid, granulocytic or monocytic cells. By day 21, the number of cells present in the cultures increases in proportion to the number of cells originally seeded (Figure 4B). These day 21 BMMC-derived cells have the morphology of large granulated Alcian Blue^{pos} cells with the antigenic profile of mature mast cells $CD117^{high}CD34^{neg/high}Fc\epsilon RI^{high}$ (Figure 4B, C). The presence of TPO exerts no effect on the number and phenotype of mast cell precursors generated in these cultures at day 14 (Figure 4B and C), but dramatically decreases the number of cells obtained at day 21 (Figure 4B). This result confirms that TPO hampers the transition from precursor cells to mature mast cells occurring between day 14 and 21 of BMMC culture.

DISCUSSION

We had previously shown that *Mpl*, both as mRNA and protein, is expressed in murine mast cells [34]. Here we show that forced activation of *Mpl* by TPO administration impairs mast cell differentiation in vivo and in vitro. In vivo, TPO apparently increases the number of dermal (Figure 1) and serosal (Figure 2) mast cells (Table 1). The majority of mast cells observed in TPO-treated mice, however, is either TUNEL^{pos} or Annexin V^{pos} and is therefore in apoptosis (Figure 1 and 2A). In addition, TPO-treated mast cells have impaired morphology (Figure 2A), expression profiling (Figure 2B), and serotonin releasing activity (Figure 2C). These effects are mimicked by TPO in vitro in BMMC cultures. In fact, addition of TPO to these cultures transiently increases, by ≈ 2.5 fold, the number of mast cell precursors generated by day 14, but decreases the overall number (Figure 3) and maturation state [low *Fc\epsilon RI* expression (Figure 3A), poor *MMCPs* profiling (Table 2) and low serotonin releasing activity (Figure 3B)] of cells present after 21 days in BMMC cultures. Although apoptosis is not formally assessed, mast

cells obtained in vitro in the presence of TPO, as those obtained in vivo, express low levels of Bcl2, implying the presence of high death rates in TPO-stimulated cultures.

As already observed for the *Mpl*^{null} mutation, TPO-treatment induces changes in expression profiling consistent with the biological alterations induced in the cells. TPO-treatment modestly (≈ 2 -fold) increases Gata1 and its target Fc ϵ RI [32,33], but strongly reduces the expression of cKit on the cell surface and Mitf and its target genes, Bcl2 [28] and MMCP-6 [26]. This last result indicates the SCF/Mitf/PIAS3/STAT3/Bcl2 network [37] as a possible downstream effector of the TPO induced apoptosis of mast cells.

According to two independent studies, BMMC cultures seeded with human CD34^{pos} cells increase cellular output when treated with TPO [41,42]. The increases in the number of mast cell precursors induced by TPO in human [41,42] and murine (this manuscript) BMMC cultures are similar and probably due to expansion of the stem/progenitor cell compartments. Furthermore, TPO-exposed human mast cells, as the murine cells described here, express lower levels of Fc ϵ RI [42]. Although the expression of *Mpl* in human mast cells is debatable [41, 42], *Mpl* expression has been determined in murine cells [34]. In contrast with the human studies, however, the major effect of TPO observed here is induction of apoptosis. It is possible, though, that the effects of TPO on mast cell maturation are species-specific. Further studies in humans, comparing mast cell differentiation in normal donors and in patients carrying either an inherited [43] or acquired [36,44] mutation of *Mpl*, or of its downstream target Jak2, will clarify the full spectrum of TPO activity in human mastocytogenesis.

TPO is not only an important regulator of megakaryocytopoiesis, but it also serves as an important component of the stem cell niche [45]. As recently shown by Suda et al [46], TPO, produced by the osteoblasts on the marrow endosteum, regulates, together with SCF, the stem cell activity in the niche. The high apoptotic rates displayed by mast cells from TPO-treated mice suggest that TPO might represent the factor preventing stem cell differentiation toward the mast cell lineage in the niche. Therefore, TPO may act as the physiological regulator limiting the proliferation of mast cells to extramedullary sites. On the other hand, the observation that TPO treatment alters Gata1 and Mitf expression, as well as the expression of their target genes MC-CPA and MMCP-6, suggests that TPO might be involved in the regulation of the lineage switch between serosal and dermal mast cell differentiation in response to parasite infection [47]. This interesting possibility will be addressed in dedicated experiments that will couple the use of prospectively isolated progenitor cells with functional studies in the C57BL/6-Kit^{W-sh} transplantation model [4].

In conclusion, our data are consistent with the regulatory model of mast cell differentiation described in Figure 5, according to which TPO boosts the number of mast cell precursors but prevents their final differentiation output. It achieves the former by promoting expansion of the hematopoietic stem/progenitor cell compartments and the latter by prohibiting the transition between precursor and mature cells.

Acknowledgments

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Figure 1. The apparent increased number of immature (metachromatic^{neg} after Toluidine-Blue) and mature (Alcian-Blue-Safranin^{pos}) dermal mast cells observed in mice after TPO-treatment is associated with high levels of apoptosis (TUNEL^{pos})

Toluidine-Blue (A–C), Alcian-Blue/Safranin (D–F) and TUNEL (G–I) staining of ear sections from untreated (A,D,G) and TPO-treated [day 7 (B,E,H) and 14 (C,F,I)] wild-type mice. Magnification 40x (panels) and 100x (inserts).

Figure 2. TPO-treatment reduces the number of serosal mast cells by inducing apoptosis

A) FACS analysis with CD117/Fc ϵ RI, CD117/Annexin-V or CD117/irrelevant antibodies, (negative controls, grey curves and dotted lines) of serosal mast cells from wild-type mice, either untreated or 7 and 14 days after TPO-treatment. Gates defining CD117^{pos} cells are indicated in the first panels. May-Grunwald (A1–A3) staining of representative cells is presented, for comparison. Magnification 40x.

B) Expression of genes encoding growth-factor receptors (Mpl/c-Kit), transcription factors (Mitf/Gata1/Gata2), anti-apoptotic proteins (Bcl2) and MMCPs (MC-CPA/MMCP-6/MMCP-7), in CD117^{high} cells from wild-type mice either untreated or 7 and 14 days after

TPO-treatment. [Mean ($\pm$ SD) of three separate experiments performed in triplicate]. Values statistically different from untreated controls are indicated by * ($p < .05$) and ** ($p < .01$).

C) Serotonin released by serosal mast cells from wild-type mice upon IgE+ α IgE-stimulation, either untreated or 7 or 14 days after TPO-treatment. Results are expressed as percent of total serotonin incorporated (i.e., cells lysed with Triton, 100% $\approx$ 30,000 cpm/ 10^5 cells). [Mean ($\pm$ SD) of three independent experiments performed in duplicate]. * Statistically different ($p < .01$) from controls.

Figure 3. TPO hampers mast cell differentiation in BMMC cultures

A) FACS analysis for CD117/CD34, CD117/FcεRI or CD117/irrelevant antibodies (gray curves), May-Grunwald (A1–A4) and Alcian-Blue (A5–A6) staining of day 21 BMMC-derived wild-type and *Mpl*^{null} cells, stimulated with SCF/IL-3 or SCF/IL-3/TPO, as indicated. Similar results were obtained in 5 separate experiments.

B) Serotonin released upon IgE+αIgE-stimulation by BMMC-derived mast cells obtained either in the absence or presence of TPO, as indicated. Results are expressed as percent of total levels of serotonin incorporated (i.e., cells lysed with Triton). Mean (±SD) of five separate experiments performed in duplicate. * Statistically different ($p < .05$) from cultures without TPO.

Figure 4. TPO hampers mast cell differentiation of purified progenitor cells cultured under conditions of limiting dilution

A) CD117^{low}/CD34^{low} gate used to purify progenitor cells from the bone marrow of wild-type mice and reanalysis of the sorted cells for purity.

B) Total cell number obtained after 14 (left) and 21 (right) days in BMMC cultures seeded with increasing numbers (200-2×10⁴ cells/mL) of CD117^{low}/CD34^{low} cells and stimulated with or without TPO. The morphology of representative cells is presented in the inserts. Similar results were obtained in two additional experiments.

C) FACS analysis for CD117/CD34, FcεRI (grey area) and irrelevant antibody (blue curve) of cells obtained at day 14 and 21 of BMMC culture stimulated with SCF/IL-3. The profile of

the cells obtained in the presence of TPO was similar to that obtained in its absence at day 14 and could not be determined, because of the low cell number, at day 21.

Figure 5. A model for the biological activity of TPO on mast cell differentiation

The model is based on results presented in this paper and in [33] and suggests that, in addition to the proliferative effect exerted at the stem/progenitor cell level, TPO affects mastocytopoiesis by blocking the transition from precursor ($CD117^{pos}CD34^{neg/pos}$) to mature ($CD117^{pos}CD34^{high}Fc\epsilon RI^{high}$) cells. Such inhibition involves repression of *Mitf* expression that alters, in turn, the apoptotic (by reducing *Bcl2* expression) and differentiative (by reducing *MMCP-6* expression) program of the cells [33]. It is suggested that TPO affects *Mitf* expression indirectly by down modulating *cKit* expression on the cell surface and suppressing the *SCF/Mitf/PIAS3/STAT3* network described in mast cell by Levy et al [37].

Table 1

Frequency of mast cell precursors (methacromatic^{neg} after Toluidine-Blue), and of mature (Alcian-Blue/Safranin^{pos} or CD117^{pos}/FcεRI^{pos}) mast cells and of apoptotic (TUNEL^{pos} or Annexin-V^{pos}) cells in dermis of ear and in peritoneal cavity from wild-type mice, either untreated or at day 7 and 14 of TPO-treatment, as indicated. The hematocrit (Hct) and the number of platelets in the blood of the different experimental groups are also reported, as control.

	Blood		Ear (cells/mm ²)		Peritoneal Cavity			
	Hct	Ptl/μL (×10 ⁶)	Toluidine Blue ^{pos}	Alcian-Blue/Safranin ^{pos}	TUNEL ^{pos}	Total Cell No. (×10 ⁶)	CD117 ^{pos} FcεRI ^{pos} (%)	Annexin V ^{pos} (%CD117 ^{pos} cells)
Untreated	47.8±0.8	0.9±0.2	320±28	267±41	15±1	8.1±2.4	1.0±0.1	1.3±0.1
Day 7 of TPO	46.8±3.5	1.8±0.3	904±220[*]	310±25	40±20	18.3±1.1^{**}	46.2±2.6^{**}	61.4±3.5^{**}
Day 14 of TPO	41.3±4.0	1.5±0.8	827±67^{**}	633±72[*]	600±43^{**}	25.3±1.5^{**}	2.9±0.2[*]	63.4±3.6^{**}

Results are represented as mean (±SD) of at least 5 independent animals for experimental group.

The values for untreated controls are the same as in Table 1. Values statistically different

* p<.05 and

** **p<.01) from normal controls are indicated in bold. The majority (98%) of CD117^{pos} cells are also FcεRI^{pos} (see also Figure 1–2).

Table 2
Levels of gene expression in mast cells obtained in BMMC from wild-type and Mpl^{null} mice in the presence or absence of TPO.

	Wild-type Mice		Mpl ^{null} Mice	
	BMMC	BMMC+TPO	BMMC	BMMC+TPO
	2 ^{-ΔCt}	2 ^{-ΔCt}	2 ^{-ΔCt}	2 ^{-ΔCt}
<i>Receptors</i>				
Mpl	3.0±2.0×10 ⁻⁴	2.1±0.8×10 ⁻⁴	b.d.	b.d.
c-Kit	0.8±0.1	4.1±0.3×10⁻¹*	0.3±0.02 **	0.3±0.01
<i>Transcription factors</i>				
Mitf	3.0±1.0×10 ⁻²	2.2±0.1×10⁻²*	3.1±0.02×10 ⁻²	3.5±0.1×10 ⁻²
Gata1	5.0±1.0×10 ⁻²	3.7±0.4×10 ⁻²	2.2±0.4×10⁻²*	2.2±0.6×10 ⁻²
Gata2	1.0±0.1	0.48±0.01 **	2.1±0.2×10⁻²**	n.d.
<i>Anti-apoptotic factors</i>				
Bcl2	1.0±0.1×10 ⁻²	0.7±0.1×10⁻²*	2.1±0.2×10⁻²*	1.6±0.5×10 ⁻²
<i>Proteases</i>				
MC-CPA	15.4±2.7	9.6±0.3 **	10.7±2.2	10.2±1.8
MMCP-6	2.8±0.1	7.3±0.2×10⁻¹**	3.1±0.1 *	3.1±0.1
MMCP-7	5.5±0.3×10 ⁻¹	2.5±0.2×10⁻¹**	1.5±0.1×10⁻¹**	1.4±0.1×10 ⁻¹

Values are presented as the mean (±SD) of three different experiments performed in triplicate. Values observed in standard Mpl^{null} BMMC were statistically compared with those obtained in wild-type cultures. Values observed in BMMC supplemented with TPO were compared with the corresponding cultures that did not contain the growth factors. Statistically significant differences are indicated in bold and with

* (p<05) or

** (p<01), respectively.

b.d. = below detection; n.d. = not done.