STEM CELLS AND DEVELOPMENT
Volume 18, Number 7, 2009
© Mary Ann Liebert, Inc.
DOI: 10.1089/scd.2008.0323

ORIGINAL RESEARCH REPORT

Increased Differentiation of Dermal Mast Cells in Mice Lacking the Mpl Gene

Barbara Ghinassi,[1,2] Maria Zingariello,[1,2] Fabrizio Martelli,[1] Rodolfo Lorenzini,[3] Alessandro M. Vannucchi,[4] Rosa Alba Rana,[2] Mitsuo Nishikawa,[5,*] Giovanni Migliaccio,[6] John Mascarenhas,[7] and Anna Rita Migliaccio[1,7,8]

Thrombopoietin interactions with its receptor, Mpl, play an important role in the regulation of hematopoietic stem/progenitor cell proliferation and differentiation. In this study, we report that the mast cell restricted progenitor cells (MCP) and the mast cell precursors in the bone marrow of wild-type mice express Mpl on their surface. Furthermore, targeted deletion of the Mpl gene in mice decreases the number of MCP while increasing the number of mast cell precursors present in the marrow and spleen. It also increases the number of mast cells present in the dermis, in the peritoneal cavity, and in the gut of the mice. In addition, serosal mast cells from Mpl^{null} mice have a distinctive differentiation profile similar to that expressed by wild-type dermal mast cells. These results suggest that not only does ligation of thrombopoietin with the Mpl receptor exert an effect at the mast cell restricted progenitor cell level, but also plays an unexpected yet important role in mast cell maturation.

Introduction

Mast cells are stem cell–derived hematopoietic cells that have a dual role of immune effector against parasitic invasion as well as mediator of the allergic response [1–2]. These cells arise in the bone marrow and to a lesser extent in the spleen and reside in highly vascularized extramedullary sites, such as the dermis of the skin and the mucosa of the gut [3–5]. Hematopoietic progenitor cells committed to mast cell lineage were first identified in the fetal blood of a mouse [6]. More recent data have identified a progenitor cell population restricted to the "mast cell" lineage, the MCP, in the marrow of adult mice with the phenotype Lin^{neg}c-Kit^{pos}Sca-1^{neg}Ly6c^{neg}FcεRIα^{neg}CD27$^{neg}\beta$7integrin^{pos}T1/ST2^{pos} [7]. The precise relationship of the MCP in the hematopoietic progenitor cell hierarchy is debated [8]. Some investigators have proposed that the MCP is derived from the common myeloid progenitor cell, the CMP [7], while others argue that in addition these cells may derive from the granulocyte-monocyte restricted progenitor cell, the GMP [9]. Under normal circumstances, MCP do not differentiate in the marrow, as indicated by the fact that the frequency of mast cell precursors (c-Kit^{high}CD34^{pos}) in this tissue remains limited (0.02%) throughout adult life [10]. Instead, MCP circulate in the blood to colonize extramedullary sites where they differentiate into tissue-restricted mast cells, each with a specific mast cell protease (MMCP) (for a review on mast cell–specific MMCP, see [11,12]) expression profile [13,14], giving rise to dermal, mucosal, and serosal mast cell populations. The molecular mechanism in normal mice that restricts the mastocytopoietic potential of stem/progenitor cells to extramedullary sites, as well as the factors that guide their differentiation along different lineages is unknown.

There are several similarities between the pathways that control megakaryocytic and mast cell differentiation: Differentiation of both lineages is dependent on the growth factor stem cell factor (SCF) (in synergy with thrombopoietin, TPO [15], and IL-3 [3–5], respectively), and the transcription factors Gata2 and Gata1 [16–18]. In both lineages, the expression of Gata1 is regulated by the first enhancer, the DNase hypersensitive site I, of the gene [16,17], a proposed target for TPO signaling [19,20]. Furthermore, three independent studies have reported that TPO increases the cellular output in

[1]Department of Hematology, Oncology, and Molecular Medicine, [3]Department of Quality and Safety of Animal Experimentation, and [6]Department of Cell Biology and Neurosciences, Istituto Superiore Sanità, Rome, Italy.
[2]Department of Biomorphology, University of Chieti, Chieti, Italy.
[4]Department of Hematology, University of Florence, Florence, Italy.
[5]Department of Kirin Brewery Pharmaceutical Research Laboratory, Gunma, Japan.
[7]Department of Medicine, Mount Sinai School of Medicine, New York, New York.
[8]Myeloproliferative Disorders Research Consortium (MPD-RC).
*Current affiliation: Nishihashimoto Sagamihara-shi, Kanagawa, Japan.

BMMC cultures seeded with human $CD34^{pos}$ cells [21–23] and genetic alterations of TPO signaling are associated with the development of myeloproliferative disorders expressing both megakaryocytic and mast cell abnormalities [24]. This would suggest that TPO, a growth factor produced by the osteoblasts [25] and present in the marrow stem cell niche [26], might be involved in the regulation of mast cell differentiation. Wild-type serosal murine mast cells have been shown in previous work to express the mRNA for Mpl, the receptor for TPO [20], and in vivo mouse treatment with TPO or addition of this growth factor to bone marrow–derived murine mast cell cultures strongly decrease the generation of mature mast cells by inducing apoptosis [27]. To further clarify the effects of TPO on mast cell differentiation, we show that MCP and mastocytic precursors present in the marrow of mice express Mpl on the cell surface. Next, we determined that targeted deletion of the Mpl gene in mice has effects on mast cell differentiation opposite to those described previously with TPO treatment (27): decreasing the number of MCPs present in marrow and spleen, and increasing the number of mast cell precursors in the connective tissue, in the mucosa, and in the peritoneal cavity. In addition, mast cells derived from Mpl^{null} mice have a protease expression profile similar to that of dermal rather than serosal wild-type mast cells. Finally, we show that, in mice, TPO is expressed by cells from multiple organs including, in addition to liver and marrow, spleen, kidney, and gut. Moderate bleeding, or the presence of the Mpl^{null} mutation, decreases the level of TPO protein detected in blood while altering the levels of TPO mRNA expressed in most of the other tissues. Altogether, these results confirm that TPO plays an important role in selectively increasing dermal versus mucosal mast cells.

Materials and Methods

Mice

Mpl^{null} mice [28] were provided by Dr. W. Alexander (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia) and bred with CD1 females (Charles River Laboratories, Inc., 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. In selected experiments, wild-type mice were bled by harvesting 400 µL of blood through the retro orbital plexus with a glass Pasteur pipette. All the experiments were performed with 6- to 10-month-old male littermates, according to protocols approved by the institutional animal care committee.

Histological analysis

The ear, spleen, femur, and stomach were fixed in 10% (v/v) phosphate-buffered formalin (Sigma, St. Louis, MO), 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) and safranin-counterstained Alcian Blue (Bio-Optica, Milano, Italy), as described in [16]. 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 blue ($metachromatic^{neg}$) and red ($metachromatic^{pos}$), respectively [16]. Light microscopy was analyzed using a Leica Light Microscope (Leica Microsystems Ltd., Heidelberg, Germany) equipped with a Coolsnap videocamera for computerized images (RS Photometrics, Tucson, AZ) while transmission electron microscopy was performed using the EM 109 Zeiss (Carl Zeiss Optronics GmbH, Oberkochen, Germany).

Flow cytometry analysis and cell purification

Cells were labeled with PE-CD117 (that recognizes c-Kit) coupled with either fluorescein isothiocyanate (FITC)–CD34, FITC–Annexin V, or FITC–CD45R/B220 as control. Further antibodies used in this study are FITC-Mac3, -CD61, -T1/ST2 (MD Biosciences Inc., St. Paul, MN) 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 antimouse IgE [6]. Expression of Mpl was analyzed by three color flow cytometry using a biotinylated Mpl antibody (AMM2; Kirin Pharmaceuticals, Takasaki, Japan) and APC-CD117, coupled either with FITC-FcεRI (eBiosciences, Inc., San Diego, CA) or FITC-T1/ST2 [29]. In these experiments, aspecific antibody binding was reduced by preincubating the cells with a FcγRII/III blocker (CD16/CD32). Unless otherwise stated, all the antibodies were from PharMingen (PharMingen, San Diego, CA) and were incubated at a concentration of 1 µg/10^6 cells for 30 min on ice. Cell fluorescence was analyzed either with a Coulter Epix Elite ESP (Beckman Coulter, Inc., Miami, FL) or a FACS ARIA (Becton Dickinson, Franklin Lakes, NJ). Nonspecific fluorescent signals were gated out with appropriate fluorochrome-conjugated isotype controls and dead cells were excluded by propidium iodide staining. Serosal mast cells were purified as described in [16]. Briefly, peritoneal cells were first incubated with FITC-conjugated CD45R/B220 and immunodepleted with a monoclonal mouse anti-fluorescein antibody–coated MACS microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The B220-negative cell fraction was then incubated with PE-CD117, and $CD117^{high}$ cells (≈50% of $B220^{neg}$ cells) isolated with the FACS ARIA (>95% $CD117^{high}$ after reanalysis).

TPO ELISA

Bone marrow washes were prepared by gently flushing the cavity of two femurs with 400 µL of phosphate buffered saline containing 0.1% Tween as described in [30]. The same buffer was used to prepare cell washes from liver and spleen. Blood was collected from the retro orbital plexus of the animals with a heparin-coated Pasteur pipette and platelet-poor plasma prepared by centrifugation at 5000 rpm (platelet contamination of the samples <9%). The levels of TPO present in plasma and cell washes were measured using a commercial ELISA kit (Quantikine Immunoassay mouseTPO; R&D Systems, Inc., Minneapolis, MN).

RNA isolation and quantitative RT-PCR

Total RNA was prepared with Trizol (Gibco-BRL, Grand Island, NY) and reverse transcribed with random primers

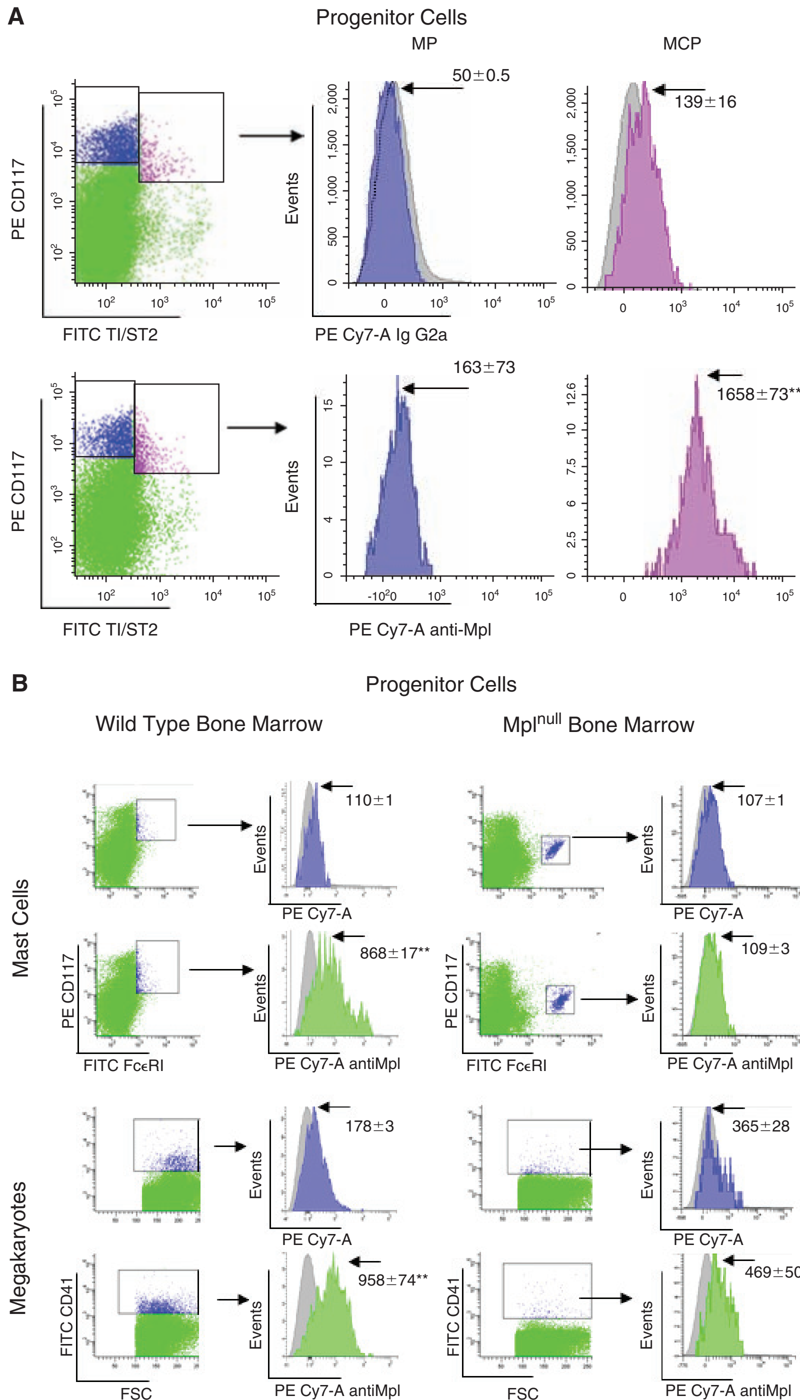

FIG. 1. The Mpl receptor is expressed on mast cells and mast cell–restricted progenitor cells present in the murine marrow. (**A**) Fluorescence-activated cell sorter (FACS) analysis with a biotinylated-irrelevant isotype (PE-Cy7-A) (top panels) or biotinylated-AMM2 (PE-Cy7-A anti-Mpl) antibody (bottom panels) of wild-type progenitor cells (c-Kit^{pos}). Progenitor cells were divided into myeloid restricted (MP, blue) and mast cell–restricted (MCP, red) progenitor cells on the basis of T1/ST2 staining [7]. The gating defining MP and MCP was confirmed by a functional assay based on the ability of prospectively isolated cells to generate myeloid or mast cell precursors, respectively, in culture [29]. The cells were incubated with the antibodies in the presence of a FcγRII/III blocker. The mean (±SD) peak fluorescence intensities (in arbitrary units), observed in three separate experiments, are indicated. **Significantly ($P < 0.01$) higher than isotype controls. (**B**) FACS analysis with a biotinylated-irrelevant isotype (PE-Cy7-A) or biotinylated-AMM2 (PE-Cy7-A antiMpl) antibody of mast cells (CD117^{pos}FcεRI^{pos}) and megakaryocytes (CD41^{pos}) from the marrow of wild-type (left) and Mpl^{null} (right) littermates. The cells from Mpl^{null} mice represent negative controls. The cells were incubated with the antibodies in the presence of a FcγRII/III blocker. The mean (±SD) peak fluorescence intensities (in arbitrary units), observed in three separate experiments, are indicated. **Significantly ($P < 0.01$) higher than isotype controls (gray histograms).

using the SuperScript III kit (Invitrogen Life Technologies, Bethesda, MD). Gene expression was quantified using the Taqman PCR kit (PE Applied Biosystems, Foster City, CA) and predeveloped custom-made oligos, whose sequence is available upon request, using either the ABI PRISM 7700 or the 7300 Sequence Detection System (Applied Biosystems) [27,29]. GAPDH was also quantified in each reaction. Results were analyzed by using the SDS program (v1.9; Applied Biosystems) 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 are presented as $2^{-\Delta Ct}$.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using Origin 6.1 software for Windows (Microcal Software Inc., Northampton, MA).

Results

Mpl is expressed by mast cell restricted progenitor cells (MCP) and precursor cells present in the mouse bone marrow

By using flow cytometry analysis, the anti-Mpl antibody clearly labels above isotype control levels myeloid

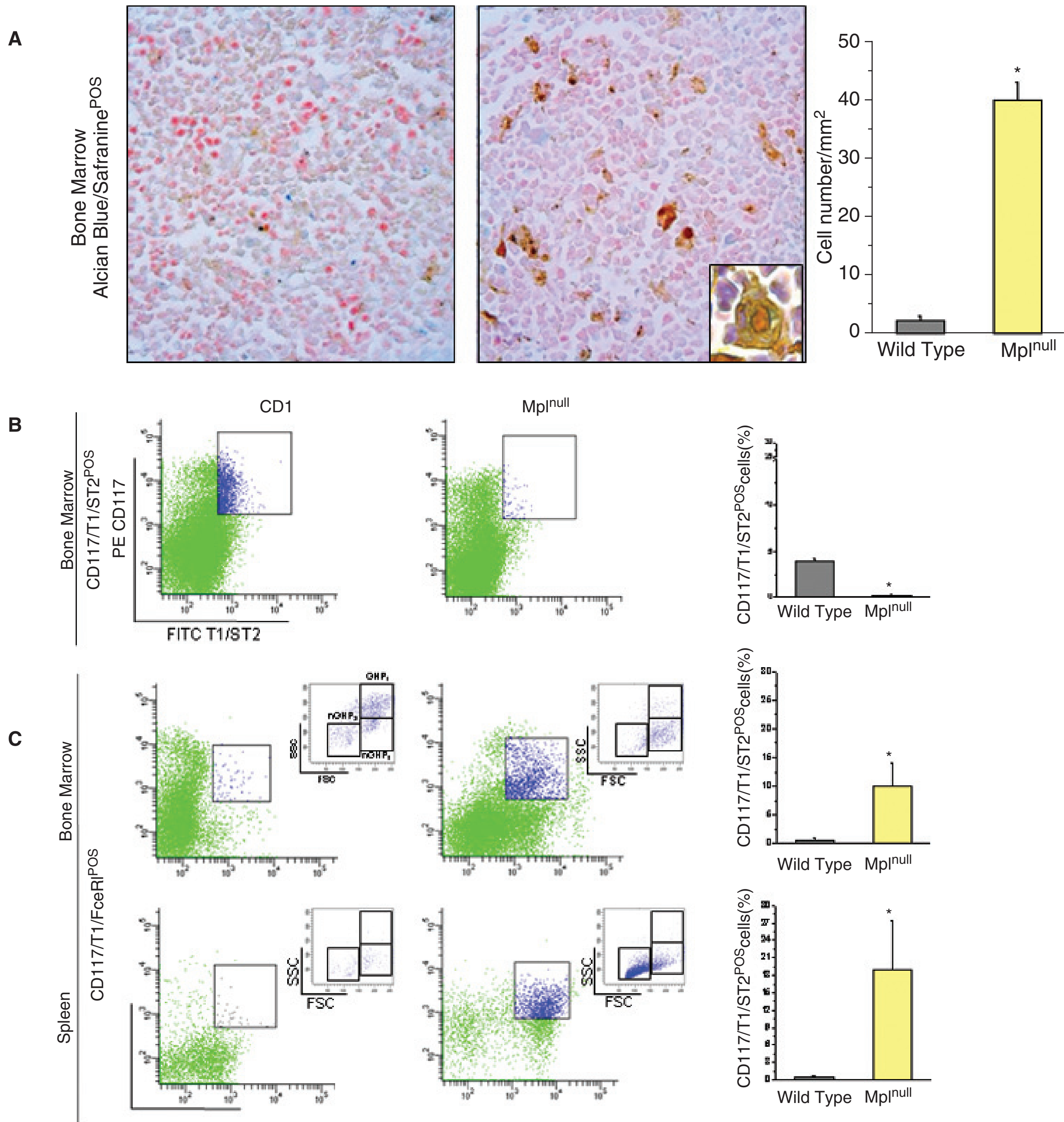

FIG. 2. The marrow from Mpl^{null} mice contains few mast cell restricted progenitor cells (MCP) (CD117^{pos}/T1/ST2^{pos}) but numerous mast cells (CD117^{pos}FcεRI^{pos}). (**A**) Alcian Blue/Safranine staining of marrow sections from wild-type and Mpl^{null} mice. The insert in the panel of the right represents a representative Alcian Blue/Safranine positive cell. Magnification: (**A** and **B**) 10× and (*insert*) 40×. (**B–C**) FACS analysis for CD117/T1/ST2 (**B**) and CD117/FcεRI expression (**C**) of marrow and spleen cells from wild-type and Mpl^{null} littermates, as indicated. CD117^{pos}/T1/ST2^{pos} and CD117^{pos}FcεRI^{pos} profiles are defined according to the gates indicated by rectangles. Side (SSC) and forward (FSC) scattogram analyses divide CD117^{pos}FcεRI^{pos} cells into nGHP$_S$, nGHP$_L$, and GHP$_L$, corresponding, respectively, to nongranular-small, nongranular-large, and granular-large mast cells, as described in [10]. The mean (±SD) frequency of Alcian Blue/Safranine^{pos} (**A**), CD117^{pos}T1/ST2^{pos} (**B**), and CD117^{pos}FcεRI^{pos} cells observed in at least three separate experiments are presented on the right. Values statistically different ($P < 0.01$) from wild-type controls are indicated by asterisks. The values for wild-type mice used in these experiments are the same as those published in [27].

progenitors (MP, CD117^{pos}/T1/ST2^{pos}) and MCP (CD117^{pos}/T1/ST2^{pos}) from wild-type mice. The levels of Mpl expression on the surface of MCP are significantly higher than those expressed by MP (1658 ± 73 vs. 163 ± 73, respectively) (Fig. 1A). The anti-Mpl antibody clearly labels above isotype control also mast cell precursors (CD117^{pos}/FcεRI^{pos}) in the

marrow of wild-type mice (Fig. 1B). The levels of expression on Mpl on the surface of these precursors is lower than those expressed by the MCP (868 ± 71) and comparable to those expressed by wild-type megakaryocytes (CD41^{pos} cells) analyzed as positive controls (958 ± 74) (Fig. 1B). In contrast, Mpl is not detectable on the surface of megakaryocytes and mast cell precursors from the marrow of Mpl^{null} mice used as negative control (Fig. 1B).

In conclusion, in contrast with the restriction of Mpl expression that occurs during lymphoid commitment [31], the expression of Mpl increases during the transition from MP to MCP and decreases when MCP mature into mast cell precursors to levels comparable to those expressed by megakaryocytes.

Targeted deletion of the Mpl gene increases the number of mast cells and of MCPs present in the marrow

The observation that mast cells and their progenitors express Mpl prompted us to analyze whether targeted deletion of this gene would alter mastocytopoiesis in mice. The number and morphology of mast cells and of their progenitors present in bone marrow and spleen of wild-type and Mpl^{null} mice is compared in Figure 2.

As expected on the basis of results with other progenitor cell types [28], the marrow from Mpl^{null} mice contains lower numbers of MCP (Fig. 2B). At the precursor level, marrow sections from Mpl^{null} mice do not contain granulated cells detectable by Toluidine Blue staining ([10,32] and data not shown), but contains numerous cells stained golden-brown by Alcian Blue/Safranine, indicating the presence of mast cells with an immature phenotype in these mutants (40 ± 3 vs. 2 ± 1 cells/mm^2 in Mpl^{null} and wild-type marrow, respectively) (Fig. 2A).

The mast cell nature of the Alcian Blue/Safranine^{pos} cells is confirmed by flow cytometry for CD117 and FcεRI coexpression. The CD117^{pos}FcεRI^{pos} cells comprise only 0.2% of the total bone marrow cell population in wild-type mice. As reported previously [10,32], these cells are divided, by side and forward scatter analysis, into nongranular-small (nGHP$_S$) and granular-large (GHP$_L$) cells (Fig. 2C). Also the spleen from normal mice contains few (0.5%) CD117^{pos} FcεRI^{pos} cells (Fig. 2C). In contrast, the bone marrow and spleen from Mpl^{null} mice contain 10 times more (10–19%) CD117^{pos}FcεRI^{pos} cells than the corresponding tissues from wild-type littermates (Fig. 2C). The immature nature of the CD117^{pos}FcεRI^{pos} cells in the marrow is confirmed by the fact that these cells express high levels of mast cell carbonic peptidase A (MC-CPA) ($2^{-\Delta Ct} = 6.4 \pm 0.2 \times 10^{-2}$) but barely detectable levels of MMCP-6 and MMCP-7.

Targeted deletion of the Mpl gene increases the number of dermal, mucosal, and serosal mast cells and alters the differentiation profile of serosal mast cells

Mpl^{null} mice contain 60% more of immature (metachromatic^{neg} after Toluidine Blue) and ≈2.5-fold more of mature (Alcian Blue/Safranine^{pos}) mast cells in the dermis of the ear than wild-type animals (Fig. 3). On the other hand, the frequency of TUNEL^{pos} dermal mast cells in the ear from Mpl^{null} and wild-type littermates is comparable (Fig. 3C). By electron microscopy, dermal Mpl^{null} mast cells contain more highly electron dense granules than the corresponding wild-type cells (Fig. 4). Increases in the frequency of immature and mature mast cells are also observed in the connective region of the stomach of Mpl^{null} mice (Fig. 5). Quantitative measurements of the longer diameter of the mast cells indicated that Mpl^{null} dermal, mucosal, and serosal mast cells are ~2-fold bigger than the corresponding wild-type cells (Fig. 6).

Mpl^{null} mice also contain numbers of CD117^{high}FcεRI^{pos} mast cells higher (by 3- to 6-fold) than normal in the peritoneal cavity (Fig. 7). The increase is due both to higher frequency of CD117^{high}FcεRI^{pos} cells (4.3 ± 2.3% vs. 1.0 ± 0.1%, respectively) and to higher numbers of cells present in the peritoneal cavity (≈15 × 10^6 vs. 8 × 10^6 cells/mouse, respectively). Serosal Mpl^{null} mast cells are bigger (Fig. 6), contain more granules, react more strongly with Alcian Blue (Fig. 8), and express higher level of CD117 (36,700 ± 6500 vs. 8700 ± 1100 AFU/cell, respectively, $P < 0.05$) and FcεRI (two populations expressing either 600 ± 30 or 1870 ± 760 AFU/cell vs. one population with 120 ± 10 AFU/cell, respectively, $P < 0.05$) (Fig. 7) than the corresponding wild-type cells (Fig. 7).

By quantitative RT-PCR analyses, wild-type and Mpl^{null} serosal mast cells are also different. Wild-type serosal CD117^{pos} cells express high levels of c-Kit ($2^{-\Delta Ct} = 1.4 \pm 0.2 \times 10^{-2}$), high levels of Mitf and Gata2 ($2^{-\Delta Ct} \approx 1.4 \times 10^{-2}$ for both), low levels of Gata1 ($2^{-\Delta Ct} = 3.1 \pm 0.3 \times 10^{-4}$), high levels of MC-CPA ($2^{-\Delta Ct} = 9.9 \pm 1.0 \times 10^{-2}$) and MMCP-6 ($2^{-\Delta Ct} = 1.2 \pm 0.1$), and relatively low levels of MMCP-7 ($2^{-\Delta Ct} = 2.1 \pm 0.2 \times 10^{-2}$) (Fig. 8), a protease mainly produced by dermal mast cells [33]. On the other hand, serosal mast cells from Mpl^{null} mice express levels of c-Kit, Gata1, Gata2, Mitf, Bcl-2, and MMCPs 0.5–2-log, higher than those expressed by the corresponding wild-type cells (Fig. 8).

Decreases in blood platelet numbers alter the pattern of TPO expression in the extramedullary tissues of mice

It is currently accepted that TPO is mainly produced in the liver [34] and to a less extent in the bone marrow [25]. Recent evidences indicate, however, that TPO is expressed in a variety of other organs [35]. For this reason, we investigated the levels of TPO expressed in organs (stomach, intestine, spleen, and ear) rich in mast cells using quantitative RT-PCR and ELISA. As platelets exert multiple controls on the levels of TPO levels expressed in vivo [25,34–36], the amount of TPO expressed by tissues of mice in which the number of platelets has been reduced either by bleeding (as a model of acute thrombocytopenia) or by the Mpl^{null} mutation (a model of chronic thrombocytopenia) is also compared. The levels of TPO expressed by bone marrow and liver cells of the thrombocytopenic animals, as well as the levels of TPO protein present in plasma, is presented for comparison.

As expected [34,37], in mice, the major site of TPO mRNA production is the liver, and the expression of TPO in this organ is not affected by changes in the number of platelets in circulation (Table 1). The data presented in Table 1 also confirm that TPO mRNA is expressed in bone, kidney, and, to a lower extent, spleen of untreated wild-type mice. In addition, in these wild-type mice, TPO mRNA is found expressed by the intestine and stomach, although it is not detected in the ear. Mild bleeding significantly reduces

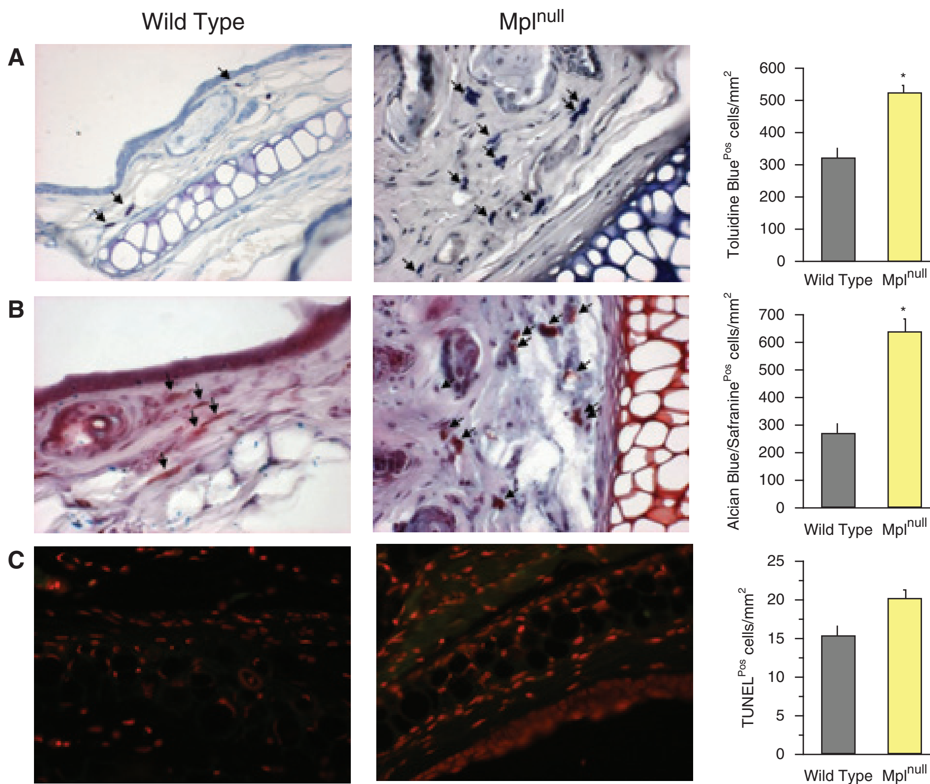

FIG. 3. The dermis from Mpl^{null} mice contains many immature (metachromatic^{neg} after Toluidine Blue) (**A**) and mature (Alcian Blue^{pos}/Safranine^{pos}) (**B**) mast cells. Ear sections were also stained using TUNEL, as presented in (**C**). Photograph of representative sections from the ears of wild-type and Mpl^{null} mice are presented on the left, while the mean (±SD) frequency of cells observed in sections from three separate animals per experimental point is presented on the right. Values statistically different ($P < 0.01$) from wild-type controls are indicated by asterisks. The values for wild-type mice used in these experiments are the same as those published in [27]. Magnification: 40×.

the levels of TPO mRNA expressed by spleen, bone, and stomach but increases the levels of TPO mRNA expressed by the bone marrow and, to a surprise, by the ear. On the other hand, the presence of the Mpl^{null} mutation increases the levels of TPO mRNA levels expressed by the spleen and decreases those expressed by the bone, bone marrow, and intestine (Table 1).

At protein levels, bleeding and the presence of the Mpl^{null} mutation significantly reduce the amount of TPO present in the blood (Table 2). This result is consistent with the observation that in humans the levels of TPO in the circulation are exquisitely sensitive to thrombocytopenia [36]. In addition, TPO protein is clearly detectable in washes from the marrow, liver, spleen, and peritoneal cavity (Table 2). Bleeding and the presence of the Mpl^{null} mutation increases and decreases, respectively, the levels of TPO protein present in spleen and liver washes.

These results indicate that TPO is expressed by a wide range of extramedullary sites, including those (intestine, gut, and skin) rich in mast cells and that the expression of TPO in these tissues is regulated by the number of platelets.

Discussion

We have suggested a model for the effects of TPO on mast cell differentiation in which TPO increases the number of mast cell precursors by favoring the expansion of the hematopoietic stem/progenitor cell compartment but decreases the total mast cell output by inducing apoptosis of the precursor cells that therefore cannot progress into maturation [27]. This model predicted that Mpl should be expressed on the surface of cells at all the stages of mastocytopoiesis and that targeted deletion of the Mpl gene should increase the number of mast cells found in the periphery. In this study, it is shown that this is indeed the case. In fact, robust levels of Mpl expression is detected using flow cytometry on the surface of MCP and mast cell precursors present in the marrow (Fig. 1). On the other hand, Mpl^{null} mice express decreased frequency of MCP and increased frequency of mast cell precursors in the marrow and spleen (Fig. 2). The Mpl^{null} phenotype also includes increased frequency of mast cells in the dermis, stomach, and peritoneal cavity (Figs. 3–8). Interruption of the Mpl–TPO interaction forced mast cell maturation as revealed by the more mature morphology of dermal (Fig. 4) and serosal (Fig. 8) mast cells from Mpl^{null} mice. In addition, serosal Mpl^{null} mast cells express high levels of MMCP-7 (Fig. 8), a protease characteristic of wild-type dermal mast cells [33]. This finding suggests that the Mpl^{null} mutation favors maturation and expansion of dermal-type mast cells and provides the first indication of the existence of factors, such as TPO, that may act as in vivo maturation switch between the different mast cell types.

The changes in the MMCP expression profile induced by the Mpl^{null} mutation are consistent with the current understanding of the molecular control of mastocytopoiesis: the high number of Mpl^{null} serosal mast cells reflecting that increased proliferation is associated with enhanced expression of Gata2, a gene that controls the proliferation of hematopoietic cells [18,38,39]. On the other hand, more mature features of the Mpl^{null} cells are reflected by increased expression of Gata1 and its target genes, MC-CPA [40] and FcεRI [41,42], and of Mitf and its target genes, MMCP-6 [43] and Bcl2 [44] (Fig. 8).

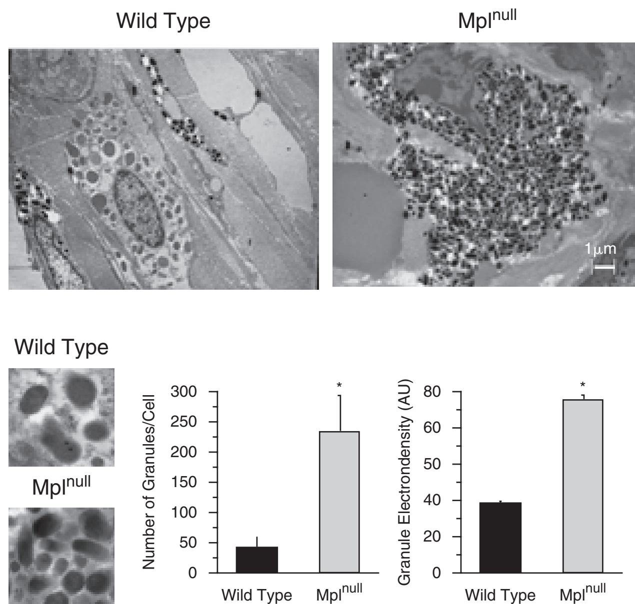

FIG. 4. By using electron microscopy, Mpl^{null} mast cells present more highly dense heterogenous cytoplasmic granules than wild-type mast cells. The number and electron density of granules present within the cytoplasm of the mast cells were quantified by analyzing with the Methamorph program at least 10 separate wild-type and Mpl^{null} cells. Values statistically different ($P < 0.01$) from those observed with wild-type controls are indicated by asterisks. Mast cells and granules are presented at 4400× and 30,000× magnification, respectively.

Several positive regulators of mast cell differentiation have been identified. In addition to SCF and IL-3, IL-4 [45], IL-9 [46], and IL-16 [47], all play important positive regulatory functions of these lineages [48]. With the exception of glucocorticoids [49,50], however, negative regulators for mast cell differentiation have not been reported so far. Therefore, the overall effects of TPO on mast cell differentiation revealed by ectopic treatment of mice and mast cell cultures with this growth factor [27] and by targeted deletion of its receptor (this study) identify the first in vivo inhibitor of mastocytopoiesis.

The results provided in this article, and data obtained from the literature, allow speculation on the physiological role of TPO as a regulator of mast cell function. TPO is one of the most important in vivo regulators of megakaryocytic differentiation [15] as both Mpl^{null} [28] and TPO-treated [51] mice have altered levels of megakaryocytes in their marrow and of platelets in the blood. TPO is regulated by a negative feedback exerted by the platelets [25]. This negative loop ensures that changes in platelet concentrations due to exogenous stimuli, that is, bleeding, would result in increased TPO production in the marrow with resultant megakaryocyte production. An increase in the megakaryocyte population requires an increase in progenitor cell pool. However, the effects of TPO on the progenitor cell compartments are not selective as TPO increases the production of progenitor cells of all types [28,52] including the MCP (Fig. 2). Increase of these progenitor cells explain why, although modest, the numbers of all the hematopoietic precursors increase in the blood after TPO treatments [15]. Owing to the high reactivity of mast cells to exogenous stimuli, the number of mast cells must not be increased in this setting. This is ensured by the apoptotic-inducing effects of TPO on mast cell precursors [27]. Although allergic reactions do not involve bleeding, parasitic infections are associated with blood losses. It has been reported that experimentally induced infections with the worm *Trichinella spiralis* promote mast cell differentiation in the marrow [32]. However, the factors that increase the numbers of these precursors and that selectively amplify the serosal mast cells are unknown. As it is conceivable that this infection is also associated with TPO production in the marrow, the selective sensitivity of dermal (more sensitive) and serosal (less sensitive) mast cells to the apoptotic inducing effect of TPO might represent the physiological signal to selectively increase the number of serosal mast cells during parasite infections.

A corollarium of this hypothesis is that TPO should be expressed in extramedullary sites rich in mast cells, and that this expression should be sensitive to blood losses. It is currently accepted that TPO constitutively expressed by the endothelial cells of the liver represents the major source of growth factor in the plasma [34,37]. Megakaryocytes and platelets, by binding TPO and by internalizing and degrading the growth factor/receptor complex, play a major role in tuning the concentration of TPO in the plasma [53]. Accordingly, in humans, thrombocytopenias characterized by reduced megakaryocyte production (reduced Mpl pool) or increased platelet destruction (accelerated destruction of the TPO/Mpl complex) are associated, respectively, with increased and reduced TPO concentration in the plasma [36]. It is recently emerging that, in addition to liver, TPO expression is also detectable in bone marrow, spleen, and kidney by in situ hybridization [35]. An alternative spliced form of TPO is expressed in the human amygdala and hippocampus [54]. Although the functions exerted by TPO in spleen and kidney are not known, the expression of this growth factor in brain and bone marrow plays a major role in the regulation of neurogenesis and stem cell biology, respectively. In the brain, TPO induces apoptosis of newly generated neurons [55]. In the bone marrow, TPO is expressed by the osteoblasts of the endosteum [26,56] and by the stromal cells of the medulla [57]. TPO expression by the osteoblasts is supposed to contribute to the chemical architecture of the stem cell niche [26,56]. Quiescent stem cells associated with the osteoblasts are supposed to be induced to proliferate by the synergistic action of TPO and SCF, both produced by the osteoblasts and to leave the niche to complete their cycle. If stimulated with TPO again, the stem cell progeny enters into quiescence, returns to the osteoblast niche, and retain stemcellness [26,56]. In contrast to the constitutive TPO expression of the liver, expression of TPO in the marrow is exquisitely regulated by the platelets that establish a negative regulatory loop on the ability of the osteoblasts to produce TPO [25]. In addition, the granules within the platelets contain both factors that stimulate (platelet-derived growth factor [PDGF] and fibroblast growth factor 2 [FGF2]) and those that inhibit (platelet factor 4, thrombospondin, and TGF-β) TPO production by the stromal cells of the medulla [58]. The data presented in Tables 1 and 2 complement this information by indicating that TPO is produced by numerous extramedullary sites, including

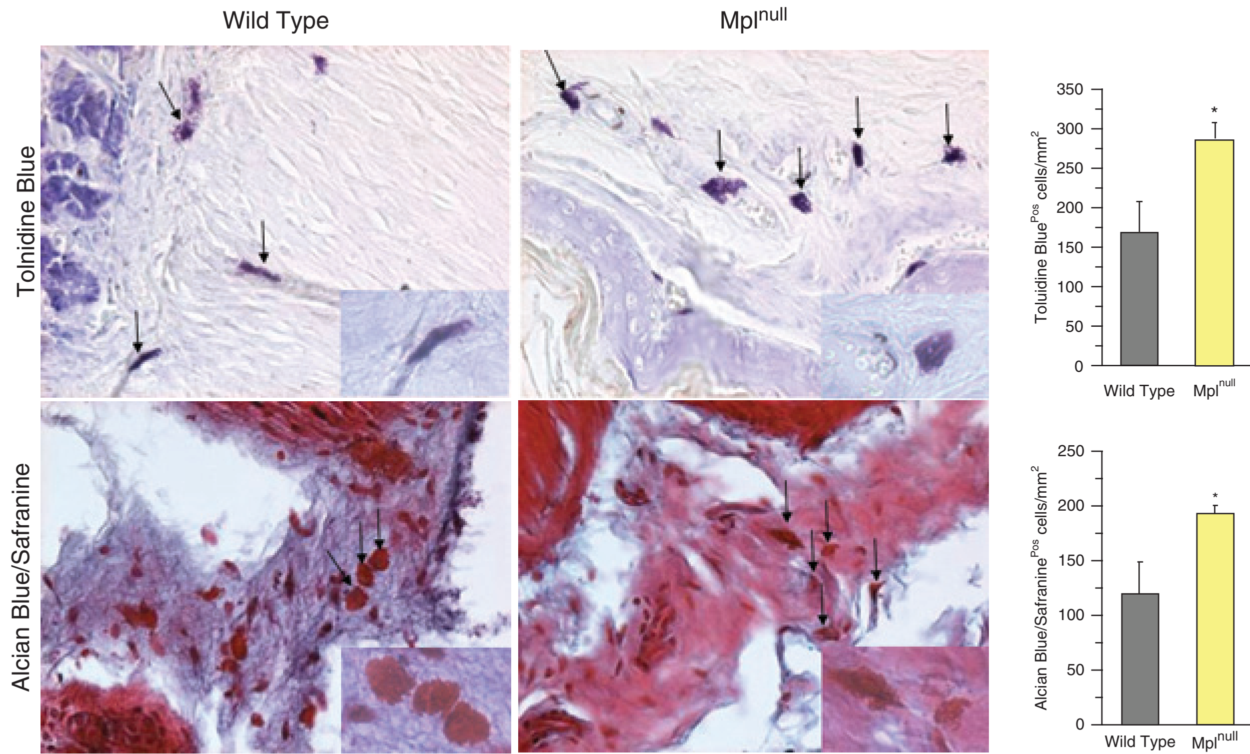

FIG. 5. The connective tissue from the stomach of Mpl^{null} mice contains numerous immature (metachromatic^{neg} after Toluidine Blue) and mature (Alcian Blue^{pos}/Safranine^{pos}) mast cells. The bar graphs on the right show the significant increase ($P < 0.01$) in the number of mast cells/mm^2 in the stomach from Mpl^{null} mice with respect to those found in wild-type mice. The results are presented as mean (±SD) of independent determinations with three different wild-type and Mpl^{null} mice. Mast cells are indicated by arrows. Magnification: 20× in the panels and 40× in the inserts.

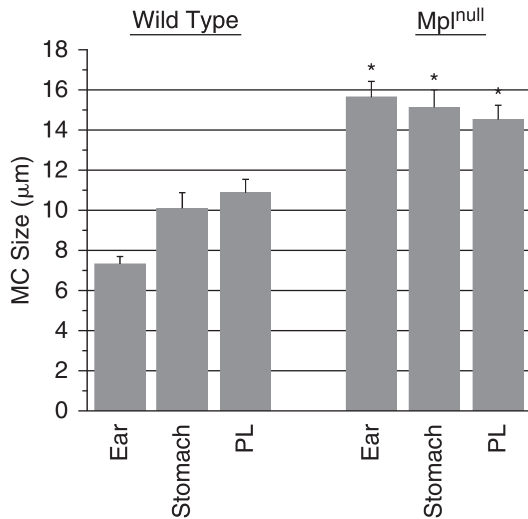

FIG. 6. Mast cells from Mpl^{null} mice are bigger than those from wild-type animals. Results are presented as mean (±SD) length of the larger diameter of 10 randomly selected mast cells identified in histological preparation of ears, stomachs, or peritoneal lavages (PL) of three separate wild-type and Mpl^{null} mice. Values statistically different from those observed in wild-type mice are indicated with an asterisk. The diameter was measured using the Methamorph program.

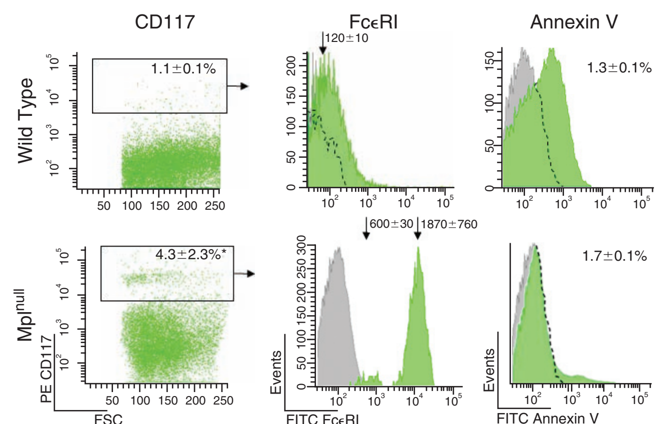

FIG. 7. The Mpl^{null} mutation increases the number of serosal mast cells. FACS analysis for CD117/FcεRI, CD117/Annexin-V, or CD117/irrelevant antibodies (negative controls, gray curves, and dotted lines) of cells from the peritoneal lavages of wild-type and Mpl^{null} littermates, as indicated. The mean (±SD) frequency of c-Kit^{pos} cells, the average (±SD) fluorescent intensity of the FcεRI signal and the frequency of Annexin^{pos} cells are also indicated. Magnification: 40×.

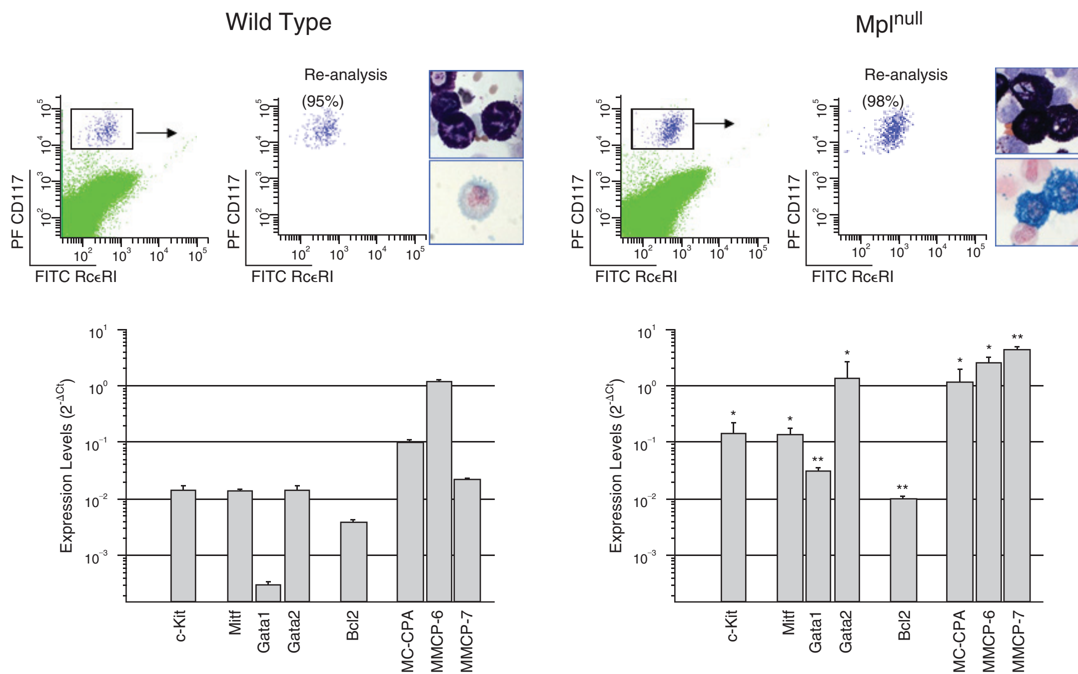

FIG. 8. The Mpl^{null} mutation alters the differentiation state of serosal mast cells. Expression profiling by quantitative RT-PCR of CD117^{high}FcεRI^{pos} cells purified from wild-type and Mpl^{null} littermates. Gates used for purification and reanalysis of sorted cells for purity (>95%) are presented on the top. May-Grunwald (*top panels*) and Alcian Blue (*bottom panels*) staining of purified cells are also present as control. Magnification: 40×. Mean (±SD) of three separate experiments performed in triplicate. *($P < 0.05$), **($P < 0.01$), statistically different from wild-type controls.

those (peritoneum, gut, and intestine) rich in mast cells. Platelet numbers not only regulates TPO concentration in plasma (both bled and Mpl^{null} mice express TPO blood levels lower than normal) but also exert a variegated effect on the levels of TPO expressed by cells of the bone, bone marrow, and extramedullary sites. In particular, both bleeding and the presence of the Mpl^{null} mutation reduce the levels of TPO expressed by bones, while bleeding increases the levels of TPO expressed by the medulla and to a surprise, those expressed in ears (Table 1).

In conclusion, these data confirm TPO as an important physiological negative regulator of mastocytopoiesis and identifies this growth factor as a possible effector of lineage switch between dermal and serosal mast cells.

TABLE 1. TPO EXPRESSION IN TISSUES OF UNTREATED AND BLED WILD-TYPE MICE AND OF MPL^{null} MICE

Mice	*Wild type*	*Bled wild type*	*Mpl^{null}*
Blood values	*Plt (×10^6)/μL: 0.9(±0.03)* *Hct (%): 47.8(±0.3)*	*Plt (×10^6)/μL: 0.7(±0.03)** *Hct (%): 43.1(±1.1)**	*Plt (×10^6)/μL: 0.03 (±0.004)** *Hct (%): 42.3 (±1.3)**
	mRNA (×10^{-3} $2^{-\Delta Ct}$)	*mRNA (×10^{-3} $2^{-\Delta Ct}$)*	*mRNA (×10^{-3} $2^{-\Delta Ct}$)*
Liver	39.3 ± 7	41.3 ± 4.3	35.5 ± 10.5
Spleen	1.3 ± 0.1	0.5 ± 0.2*	73.0 ± 18.0*
Bone marrow**	0.1 ± 0.006	3 ± 1*	0.01 ± 0.002*
Bone***	1.9 ± 0.5	0.5 ± 0.1*	b.d.
Ear	b.d.	1.2 ± 0.2*	b.d.
Kidney	6.2 ± 0.001	7.6 ± 0.9	8.9 ± 2.1
Stomach	0.7 ± 0.1	0.3 ± 0.03*	1.0 ± 0.1
Intestine	4.0 ± 0.2	8.2 ± 2.0	2.2 ± 0.1*

Results are presented as mean (±SD) of independent determinations with three mice per experimental group.

*Results statistically different ($P < 0.01$) from those observed in untreated wild-type mice. b.d. = below detectable levels.

**Bone marrow indicates mRNA prepared from cells harvested from the medullar cavity.

***Bone indicates mRNA extracted from the frozen femurs grounded under liquid nitrogen vapors.

Table 2. Levels of TPO Protein Present in Plasma, in Organ Washes and in Peritoneal Lavages of Untreated and Bled Wild-Type Mice and of Mpl^{null} Mice

	*Wild-type mice***	*Bled wild-type mice*	*Mpl^{null} mice*
	Protein (pg/mL)	*Protein (pg/mL)*	*Protein (pg/mL)*
Plasma	1287 ± 205	451 ± 11*	351 ± 31*
Bone marrow washes	98 ± 13	96 ± 14	47 ± 13
Spleen washes	99 ± 15	140 ± 4*	119 ± 7
Liver washes	289 ± 28	179 ± 6*	271 ± 74
Peritoneum lavages	93 ± 2	88 ± 3	97 ± 15

*Results statistically different ($P < 0.01$) from those observed in untreated wild-type mice.
**The same mice analyzed in Table 1. Results are presented as mean (±SD) of independent determinations with three mice per experimental group.

Acknowledgments

This study was supported by a grant from the National Cancer Institute (P01-CA108671). Murine TPO and the Mpl-specific monoclonal AMM2 antibody were provided by Kirin (MTA of March 29, 2002; Gunma, Japan). Recombinant rat SCF was provided by Amgen (MTA No. 19982634–005; Thousand Oaks, CA). This work was partially presented at the Hematopoietic Stem Cell VI meeting (September 14–16, 2006), Tübingen, Germany.

Author Disclosure Statement

One of the authors (M.N.) was employed by a company (Kirin) whose potential products (Thrombopoietin and the AMM2 anti-Mpl antibody) were used in the present work. None of the other authors have potential conflict of interest.

References

1. Galli SJ and M Tsai. (2008). Mast cells: versatile regulators of inflammation, tissue remodeling, host defense and homeostasis. J Dermatol Sci 49:7–19.
2. Metcalfe DD. (2008). Mast cells and mastocytosis. Blood 112:946–956.
3. Kitamura Y, M Yokoyama, H Matsuda, T Ohno and KJ Mori. (1981). Spleen colony-forming cell as common precursor for tissue mast cells and granulocytes. Nature 291:159–160.
4. Gurish MF and KF Austen. (2001). The diverse roles of mast cells. J Exp Med 194:F1–F5.
5. Galli SJ, S Nakae and M Tsai. (2005). Mast cells in the development of adaptive immune responses. Nat Immunol 6:135–142.
6. Rodewald HR, M Dessing, AM Dvorak and SJ Galli. (1996). Identification of a committed precursor for the mast cell lineage. Science 271:818–822.
7. Chen CC, MA Grimbaldeston, M Tsai, IL Weissman and SJ Galli. (2005). Identification of mast cell progenitors in adult mice. Proc Natl Acad Sci USA 102:11408–11413.
8. Kitamura Y and A Ito. (2005). Mast-cell committed progenitors. Proc Natl Acad Sci USA 102:11129–11130.
9. Arinobu Y, H Iwasaki, MF Gurish, S Mizuno, H Shigematsu, H Ozawa, DG Tenen, KF Austen and K Akashi. (2005). Developmental checkpoints of the basophil/mast cell lineages in adult murine hematopoiesis. Proc Natl Acad Sci USA 102:18105–18110.
10. Jamur MC, AC Grodzki, EH Berenstein, MM Hamawy, RP Siraganian and C Oliver. (2005). Identification and characterization of undifferentiated mast cells in mouse bone marrow. Blood 105:4282–4289.
11. Pejler G, M Abrink, M Ringvall and S Wernersson. (2007). Mast cell proteases. Adv Immunol 95:167–255.
12. Takemoto CM, YN Lee, AG Jegga, D Zablocki, S Brandal, A Shahlee, S Huang, Y Ye, S Gowrisankar, J Hunyh and MA McDevitt. (2008). Mast cell transcriptional networks. Blood Cells Mol Dis 41(1):82–90.
13. Reynolds DS, RL Stevens, WS Lane, MH Carr, KF Austen and WE Serafin. (1990). Different mouse mast cell populations express various combinations of at least six distinct mast cell serine proteases. Proc Natl Acad Sci USA 87:3230–3234.
14. Yamada Y, N Itano, K Hata, M Ueda and K Kimata. (2004). Differential regulation by IL-1beta and EGF of expression of three different hyaluronan synthases in oral mucosal epithelial cells and fibroblasts and dermal fibroblasts: quantitative analysis using real-time RT-PCR. J Invest Dermatol 122: 631–639.
15. Kaushansky K and JG Drachman. (2002). The molecular and cellular biology of thrombopoietin: the primary regulator of platelet production. Oncogene 21:3359–3367.
16. Migliaccio AR, RA Rana, M Sanchez, R Lorenzini, L Centurione, L Bianchi, AM Vannucchi, G Migliaccio and SH Orkin. (2003). GATA-1 as a regulator of mast cell differentiation revealed by the phenotype of the GATA-1^{low} mouse mutant. J Exp Med 197:281–296.
17. Shivdasani RA, Y Fujiwara, MA McDevitt and Orkin SH. (1997). A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J 16:3965–3973.
18. Harigae H, S Takahashi, N Suwabe, H Ohtsu, L Gu, Z Yang, FY Tsai, Y Kitamura, JD Engel and M Yamamoto (1998). Differential roles of GATA-1 and GATA-2 in growth and differentiation of mast cells. Genes Cells 3:39–50.
19. Vannucchi AM, L Bianchi, F Paoletti, A Pancrazi, E Torre, M Nishikawa, M Zingariello, A Di Baldassarre, RA Rana, R Lorenzini, E Alfani, G Migliaccio and AR Migliaccio. (2005). A pathobiologic pathway linking thrombopoietin, GATA-1, and TGF-β1 in the development of myelofibrosis. Blood 105:3493–3501.
20. Migliaccio AR, RA Rana, AM Vannucchi and FA Manzoli. (2007). Role of Thrombopoietin in mast cell differentiation. Ann N Y Acad Sci 1106:152–174.
21. Sawai N, K Koike, HH Mwamtemi, T Kinoshita, Y. Kurokawa, K Sakashita, T Higuchi, K Takeuchi, M Shiohara, T Kamijo, S Ito, T Kato, H Miyazaki, T Yamashita and A Komiyama. (1999).

Thrombopoietin augments stem cell factor dependent growth of human mast cells from bone marrow multipotential hematopoietic progenitors. Blood 93:3703–3712.
22. Kirshenbaum AS, C Akin, JP Goff and DD Metcalfe. (2005). Thrombopoietin alone or in the presence of stem cell factor supports the growth of KIT(CD117)low/ MPL(CD110)+ human mast cells from hematopoietic progenitor cells. Exp Hematol 33:413–421
23. Yoshikubo T, T Inoue, M Noguchi and H Okabe. (2006). Differentiation and maintenance of mast cells from CD34(+) human cord blood cells. Exp Hematol 34:320–329.
24. Kaushansky K. (2003). Etiology of the myeloproliferative disorders: the role of thrombopoietin. Semin Hematol 40:6–9.
25. McIntosh B and K Kaushansky. (2008). Transcriptional regulation by bone marrow thrombopoietin by platelet proteins. Exp Hematol 36(7):799–806.
26. Yoshihara H, F Arai, K Hosokawa, T Hagiwara, K Takubo, Y Nakamura, Y Gomei, H Iwasaki, S Matsuoka, K Mihamoto, H Miyasaki, T Takahashi and T Suda. (2007). Trombopoietin/ MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 1(6): 685–696.
27. Martelli F, B Ghinassi, R Lorenzini, AM Vannucchi, RA Rana, M Nishikawa, S Partamian and AR Migliaccio. (2008). Thrombopoietin Inhibits Murine Mast Cell Differentiation. Stem Cells 26(4):912–919.
28. Alexander WS, AW Roberts, NA Nicola, R Li and D Metcalfe. (1996) Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. Blood 87:2162–2170.
29. Ghinassi B, M Sanchez, F Martelli, G Amabile, AM Vannucchi, G Migliaccio, SH Orkin and AR Migliaccio. (2007). The hypomorphic Gata1^{low} mutation alters the proliferation/differentiation potential of the common megakaryocytic-erythroid progenitor. Blood 109:1460–1471.
30. Migliaccio AR, F Martelli, M Verrucci, G Migliaccio, AM Vannucchi, H Ni, M Xu, Y Jiang, B Nakamoto, T Papayannopoulou and R Hoffman. (2008). Altered SDF-1/CXCR4 axis in patients with primary myelofibrosis and in the Gata1 low mouse model of the disease. Exp Hematol 36:158–171.
31. Luc S, K Anderson, S Kharazi, N Buza-Vidas, C Böiers, CT Jensen, Z Ma, L Wittmann and SEW Jacobsen. (2008) Down-regulation of Mpl marks the transition to lymphoid-primed multipotent progenitors with gradual loss of granulocyte-monocyte potential. Blood 111:3424–3434.
32. Pennock JL and RK Grencis. (2004). In vivo exit of c-kit+/ CD49d(hi)/beta7+ mucosal mast cell precursors from the bone marrow following infection with the intestinal nematode Trichinella spiralis. Blood 103:2655–2660.
33. Stevens RL, DS Friend, HP McNeil, V Shiller, N Ghildyal and KF Austen. (1994). Strain-specific and tissue-specific expression of mouse mast cell secretory granule proteases. Proc Natl Acad Sci USA 91:128–132.
34. Qian S, F Fu, W Li, Q Chen and FJ de Sauvage. (1998). Primary role of the liver in thrombopoietin production shown by tissue-specific knockout. Blood 92:2189–2191.
35. Sungaran R, OT Chisholm, Markovic B, LM Khachigian, Y Tanaka and BH Chong. (2000). The role of platelet alpha-granular proteins in the regulation of thrombopoietin messenger RNA expression in human bone marrow stromal cells. Blood 95:3094–3101.
36. Emmons RV, DM Reid, RL Cohen, G Meng, NS Young, CE Dunbar and NR Shulman. (1996). Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood 87:4068–4071.
37. Cardier JE and J Dempsey. (1998). Thrombopoietin and its receptor, c-mpl, are constitutively expressed by mouse liver endothelial cells: evidence of thrombopoietin as a growth factor for liver endothelial cells. Blood 91:923–929.
38. Tsai FY and SH Orkin. (1997). Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood 89:3636–3643.
39. Jippo T, H Mizuno, Z Xu, S Nomura, M Yamamoto and Y Kitamura. (1996). Abundant expression of transcription factor GATA-2 in proliferating but not in differentiated mast cells in tissues of mice: demonstration by in situ hybridization. Blood 87:993–998.
40. Zon LI, MF Gurish, RL Stevens, C Mather, DS Reynolds, KF Austen and SH Orkin. (1991). GATA-binding transcription factors in mast cells regulate the promoter of the mast cell carboxypeptidase A gene. J Biol Chem 266:22948–22953.
41. Nishiyama C, T Yokota, K Okumura and C Ra. (1999). The transcription factors Elf-1 and GATA-1 bind to cell-specific enhancer elements of human high-affinity IgE receptor alpha-chain gene. J Immunol 163:623–630.
42. Maeda K, C Nishiyama, T Tokura, Y Akizawa, M Nishiyama, H Ogawa, K Okumura and C Ra. (2003). Regulation of cell type-specific mouse FceRI beta-chain gene expression by GATA-1 via four GATA motifs in the promoter. J Immunol 170:334–340.
43. Morii E, T Jippo, T Tsujimura, K Hashimoto, DK Kim, YM Lee, H Ogihara, K Tsujino, HM Kim and Y Kitamura. (1997). Abnormal expression of mouse mast cell protease 5 gene in cultured mast cells derived from mutant mi/mi mice. Blood 90:3057–3066.
44. Adams JM and S Cory. (1998). The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326.
45. Lorentz A, D Schuppan, A Gebert, MP Manns and SC Bischoff. (2002). Regulatory effects of stem cell factor and interleukin-4 on adhesion of human mast cells to extracellular matrix proteins. Blood 99:966–972.
46. Townsend JM, GP Fallon, JD Matthews, P Smith, EH Jolin and NA Mckenzie. (2002). IL-9 deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. Immunity 13:573–583.
47. Qi JC, J Wang, S Mandadi, K Tanaka, BD Roufogalis, MC Madigan, K Lai, F Yan, BH Chong, RL Stevens and A Steven. (2006) Human and mouse mast cells use the tetraspanin CD9 as an alternate interleukin-16 receptor. Blood 107:135–142.
48. Shiohara M and K Koike. (2005) Regulation of mast cell development. Chem Immunol Allergy 87:1–21.
49. Finotto S, YA Mekori and DD Metcalfe. (1997). Glucocorticoids decrease tissue mast cell number by reducing the production of the c-kit ligand, stem cell factor, by resident cells: in vitro and in vivo evidence in murine systems. J Clin Invest 99:1721–1728.
50. Yamaguchi M, K Hirai, A Komiya, M Miyamasu, Y Furumoto, R Teshima, K Ohta, Y Morita, SJ Galli, C Ra and K Yamamoto. (2001). Regulation of mouse mast cell surface Fc epsilon RI expression by dexamethasone. Int Immunol 13:843–851.
51. Levin J, FC Levin, DF Hull 3rd and DG Penington. (1982). The effects of thrombopoietin on megakaryocytes-cfc, megakaryocytes, and thrombopoiesis, with studies of ploidy and platelet size. Blood 60:989–998.
52. Fox N, G Priestly, T Papayannopoulou and K Kaushansky. (2002). Thrombopoietin expands hematopoietic stem cells after transplantation. J Clin Invest 110:389–394.
53. Fielder PJ, P Hass, M Nagel, E Stefanich, R Widmer, GL Bennet, G-A Keller, FJ de Sauvage and D Eaton. (1997). Human platelets as a model for the binding and degradation of thrombopoietin. Blood 89:2782–2788.
54. Li B, H Pan, JC Winkelmann and W Dai. (1996). Thrombopoietin and its alternatively spliced form are expressed in human amygdala and hippocampus. Blood 87:5382–5384.
55. Ehrenreich H, M Hasselblatt, F Knerlich, N von Ahsen, S Jacob, S Sperling, H Woldt, K Vehmeyer, KA Nave and AL Sirén. (2005). A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. Proc Natl Acad Sci USA 102:862–867.

56. Qian H, N Buza-Vidas, CD Hyland, CT Jensen, J Antonchuk, R Månsson, LA Thoren, M Ekblom, WS Alexander and SEW Jacobsen. (2007). Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 1:671–684.
57. Guerriero A, L Worford, HK Holland, GR Guo, K Sheehan and EK Waller. (1997). Thrombopoietin is synthesized by bone marrow stromal cells. Blood 90:3444–3455.
58. Sungaran R, B Markovic and BH Chong. (1997). Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. Blood 89:101–107.

Address correspondence to:
Dr. Anna Rita Migliaccio
Department of Medicine
Mount Sinai School of Medicine
New York, NY 10029

E-mail: annarita.migliaccio@mssm.edu

Received for publication April 25, 2008
Accepted after revision November 21, 2008
Prepublished on Liebert Instant Online November 21, 2008