THROMBOPOIETIN MEDIATED REGULATION OF MURINE HEMATOPOIETIC PROGENITORS

BY

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ABSTRACT

A number of studies have suggested that primitive hematopoietic cells respond to thrombopoietin (TPO) and that this growth factor has a non-redundant role in regulating early stages of hematopoiesis. The present study was designed to investigate more precisely the effects of an absence of TPO-mediated signaling in vivo on the generation/maintenance of specific subsets of primitive hematopoietic cells and to evaluate their ability to generate derivative progenitors in response to various growth factors in vitro. Initial tests confirmed that colony-forming cell (CFC) frequencies in the marrow of both types of mutant mice were reduced (~2-fold) and showed that longterm culture-initiating cell (LTC-IC) numbers were even further reduced (~3-fold). The marrow of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice also contained fewer Sca-1^{+} (S^{+}) lineage marker-negative (lin^{-}) Hoechst 33342-stained side population (SP) and S^{+} c-kit^{+} L^{-} ($S^{+}K^{+}L^{-}$) cells (~2-fold). The CFC output per LTC-IC from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice was also reduced (~3-fold) as shown by both limiting dilution assays and single cell LTC-IC cultures. Similarly, 10-day suspension cultures of $S^{+}K^{+}L^{-}$ and $S^{+}L^{-}$ SP cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice produced 3- 5-fold fewer CFCs, regardless of the growth factors added. Single cell cultures showed this was due to a decreased number of mutant clones containing CFCs. Addition of either TPO or interleukin-11 to Steel factor (SF) and flt3-ligand (FL) in cultures of *tpo*$^{-/-}$ $S^{+}K^{+}L^{-}$ cells also failed to enhance the generation of LTC-IC activity in spite of an ability of IL-11 to elicit such a response. The most significant findings from these studies are: 1) in vivo TPO-mediated signaling is required for the generation of normal sized compartments of several stages of early hematopoietic progenitors with the most profound influence seen on the more primitive cells; and 2) in the presence of

SF and FL in vitro, TPO does not enhance the output of primitive progenitors from +/+ cells beyond that obtained by the activation of gp130.

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LIST OF ABBREVIATIONS

CFC	colony forming cell
FL	flt-3 ligand
HSC	hematopoietic stem cell
IL-6	Interleukin-6
IL-11	Interleukin-11
LTC-IC	long-term culture-initiating cell
pre-CFC	pre-colony forming cell (produces CFCs in 10 day cultures)
S+K+L-	Sca-1^{+}c-kit^{+}lin^{-} cells
S+L- SP	Sca-1^{+}Lin^{-} Hoechst side population
SF	Steel factor
TPO	thrombopoietin

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CHAPTER 1 INTRODUCTION

Throughout life the full complement of blood cells is maintained by the activation, proliferation and differentiation of a small pool of cells dedicated to blood formation, the hematopoietic stem cells (HSCs). Orchestration of the molecular events that regulate the activities of these cells and their immediate progeny is dictated by their interaction with numerous growth factors via specific cell surface receptors. In the last 2 decades many of these factors have been defined and their mechanisms of intracellular signaling partially characterized. Discovery of these factors and a growing understanding of their roles is essential to the development of improved methods for expanding and manipulating primitive hematopoietic cells ex vivo, as well as for furthering our knowledge of how blood cell production is normally regulated. Thrombopoietin (TPO) is one of the later hematopoietic growth factors to be identified and cloned. As its name suggests, it was initially isolated as a key regulator of megakaryocyte and platelet production. However it was soon found to have potent effects on hematopoietic progenitors of other lineages as well as more primitive pluripotent cells. These findings prompted further investigation of how TPO may regulate the earliest stages of hematopoiesis.

Hematopoiesis

Currently at least 10 types of mature blood cells are recognized. Most of these are incapable of further division and are short lived. Nevertheless, their numbers are normally maintained throughout life at levels appropriate to physiological needs. These cells must therefore be supplied by hematopoietic precursors with considerable

proliferative capacity. The current model of hematopoiesis suggests a continuous hierarchy of progenitors with decreasing proliferative potential as they differentiate. At the apex of this hierarchy is a small pool of HSCs believed to be ultimately responsible for the production of all the blood cells in the circulation. In mice, several lines of evidence support the existence of a hierarchy of primitive cell types which differ from one another in their capacities to sustain hematopoiesis of different lineages for various periods of time after transplanation into lethally irradiated or genetically defective recipients (Magli et al 1982; Ploemacher and Brons 1988; Jones et al 1989; Szilvassy et al 1989b). The most primitive of these are defined by their ability to permanently reconstitute the formation of all blood cell types in transplantation assays (Wu et al 1968; Harrison and Astle 1982; Dick et al 1985; Lemishcka et al 1986; Szilvassy et al 1989a). At least some of the murine HSCs defined in this way can individually and permanently reconstitute the entire hematopoietic system (Wu et al 1968, Abramson et al 1977).

Although less experimental data is available on the human hematopoietic system, in vitro findings (Sutherland et al 1990; Ploemacher et al 1989; Lemieux et al 1995; Hogge et al 1996) and studies of human hematopoietic cells able to repopulate immunodeficient mice and sheep as well as autologous and allogeneic patients (Turhan et al 1989, Cashman et al 1996; Nolta et al 1996) suggest many similarities with the murine system. As a result, the mouse has served as a useful experimental model that has yielded many findings relevant to the human system, often with clinical significance. For example, many human growth factors cross-react with murine progenitors and vice versa, similar conditions are effective for the culture of hematopoietic cells of both species, and progenitors with apparently similar functions can be isolated from each.

Further understanding of the processes that occur in the earliest stages of hematopoiesis and the factors that influence their development could facilitate the development of clinical therapies for diseases where hematopoiesis is defective. Such information should also offer valuable insights into the mechanisms of HSC deregulation that lead to leukemia and other malignancies of the blood-forming system.

Characterization and properties of hematopoietic progenitors

Co-ordinated changes in cellular properties are the hallmark of hematopoietic cell differentiation. These include changes in both phenotypic characteristics and functional capabilities and can be observed to occur over a number of cell generations (Killman et al 1962; Killman et al 1964; Warner et al 1964). Such changes serve as the basis for identifying and evaluating different types of hematopoietic progenitors. For example, murine cells with functionally defined HSC potential can now be purified to near homogeneity using fluorescence activated cell sorting (FACS) technology (Spangrude et al 1991; Wolf et al 1993; Morrison and Weissman 1994). These studies have exploited the use of carefully validated, specific assays for quantitating HSCs based on their ability to reconstitute both the lymphoid and myeloid compartments in irradiated recipients for at least four months when transplanted at limiting dilution (Szilvassy et al 1990; Rebel et al 1994). Multi-parameter cell sorting studies have also shown that the functional discrimination of progenitor populations with distinct differentiation abilities can be matched to changes in surface phenotype (Lansdorp et al 1990; Mayani et al 1993; Sauvageau et al 1994).

The intermediate amplifying stages of hematopoietic cell differentiation have been most extensively characterized based on the different functional properties these

cells exhibit. These studies were made possible by the discovery that certain hematopoietic cells could generate colonies of mature blood cells in semi-solid media and that the numbers and types of colonies obtained was determined in part by the culture conditions and in part by the particular cell types present (Nakahata and Ogawa 1982; Leary et al 1984; McNeice et al 1987). The in vitro conditions and growth factors necessary to reliably support the formation of colonies of all the myeloid lineages are now known (Guilbert and Iscove 1976; Fauser and Messner 1979; Mayani et al 1993). In vitro colony assays can thus now be used to quantify the frequency of progenitors in whole blood and bone marrow or purified cell samples that contain cells with colony-forming ability (so called colony-forming cells or CFCs) and to study changes in CFC numbers after various types of manipulations.

Culture conditions that permit the quantitation of a population of cells that are not directly able to form colonies in semi-solid media but can generate CFCs after being co-cultured with stromal cells in liquid cultures for at least 4 weeks have also been defined. These cells are called long-term culture-initiating cells (LTC-ICs) and they can be identified in both mouse (Ploemacher et al 1989; Lemieux et al 1995) and human samples (Gartner et al 1980; Coulombel et al 1983; Sutherland et al 1989; Sutherland et al 1990). As for CFCs, highly purified populations of LTC-ICs can be isolated from adult bone marrow based on their expression of a unique profile of cell surface markers. Limiting dilution techniques are used to quantify LTC-ICs in the initial sample (based on the detection or not of CFCs in the cells harvested from the 4-week co-cultures). LTC-ICs represent a much rarer cell type than any other in vitro defined progenitor yet identified. In the normal adult mouse LTC-ICs co-purify with long-term in vivo repopulating cells suggesting that they represent a subset of multi-potent primitive

precursors near the top of the putative hematopoietic hierarchy (Eaves et al 1999). Human cells currently defined as LTC-ICs are phenotypically heterogeneous (Sutherland et al 1989, Uchida et al 2001) and may also be biologically heterogeneous. In addition, in both species, detection of HSCs by in vivo repopulation assays and in vitro LTC-IC assays is differentially affected when the cells are actively proliferating (Szilvassy et al 1990).

Another progenitor type, possibly representing an intermediate between the LTC-IC and CFC, can be reliably identified in short term cultures. These progenitors, termed pre-CFC or pre-progenitor cells, are defined as cells which give rise to CFC after 10 to 14 day suspension culture with growth factors (Smith et al 1991, Metcalf 1991). This progenitor compartment can be detected in both human and murine bone marrow cultures, providing an assessment of another level of hematopoeitic activity.

The regulation of hematopoiesis

Although a basis for the hierarchical model of hematopoeisis has been broadly delineated and supported by the purification and culture of functionally distinct cell populations from both the mouse and human systems, knowledge of the molecular changes that underlie hematopoietic cell differentiation and its complex regulation remains poorly understood. There are a number of options facing multipotential hematopoietic progenitor cells including quiescence, apoptosis, proliferation, self-renewal and differentiation. Along most of these paths several secondary options exist, most notably in the differentiation of progenitors into the various hematopoietic lineages. Of perhaps more restricted scope but of equal importance is the ability of

HSCs to maintain or increase their numbers as necessary to provide for hematopoietic needs throughout life.

The pluripotential status of HSCs is regulated by a complex system of transcription factors that affect gene activation and repression and moderate molecular signaling programs in HSCs and their progeny (Orkin 1992; Hu et al 1997). Recent evidence suggests that these processes can, like the proliferative status of HSCs, be regulated by how these cells interact with extrinsic factors. The availability of purified recombinant growth factors has furthered the analysis of the actions of specific growth factors on progenitors and has defined many of their functions to reveal both synergies and redundancies in their effects. Among the first of the HSC regulatory factors to be identified was Steel factor (SF), also known c-kit ligand (Broudy 1997). SF can play a positive role in maintaining HSCs in vitro (Broxmeyer et al 1991) and the administration of SF to mice also expands the number of transplantable HSCs (Bodine et al 1993). However, SF alone can not maintain HSCs numbers in vitro. Two other cytokines which can act alone or in synergy with others to support or modestly increase primitive hematopoietic cells in vitro are flt-3 ligand (FL) and interleukin-11 (Il-11) (Ogawa 1993; Miller and Eaves 1997, Audet et al in press). Although neither SF nor FL alone or in combination can amplify HSC numbers in serum-free cultures (Yonemura et al 1997), the addition of Il-11 yields a net increase in the number of transplantable HSCs after 10 days (Rebel et al 1994). A net expansion of LTC-ICs can also be obtained under these conditions (Miller and Eaves 1997).

Given the history of intensive research on hematopoiesis, why have the mechanisms underlying the maintenance of the proliferative and multi-lineage potential of HSCs remained elusive? There are many possibilities including the relatively small

numbers of true HSCs present among the millions of cells that make up blood and bone marrow and the difficulty of identifying phenotypic markers that reproducibly allow the exclusive isolation of cells with functional HSC activity. In addition, it is quite possible that some key regulatory factors have not yet been found. Thus, it is of considerable interest when a new protein with demonstrable effects on primitive hematopoietic cells is discovered. One such hematopoietic factor is TPO.

TPO and its receptor, c-MPL

As early as the 1950s, it had been postulated by Yamamoto (1957) and Kelemen et al (1958) that a single hormone could selectively stimulate platelet production from megakaryocyte precursors. Although it was possible to detect thrombopoietic activity in certain fractions of plasma and serum, failure to purify the responsible substance left the existence of a "thrombopoietin" in doubt. Serious interest in a thrombopoietic factor was rekindled in 1990 when an orphan receptor of the hematopoietic growth factor receptor superfamily was discovered (Vigon et al 1992; Vigon et al 1993; Mignotte 1994). The expression of this receptor was restricted to megakaryocytes, platelets and hematopoietic progenitor cells and this led to speculation that its ligand might be the long sought thrombopoietic factor. This receptor, dubbed c-mpl, was isolated through its relationship to the transforming oncogene, v-mpl, a protein encoded by the murine myeloproliferative leukemia viral oncogene (Souryi et al 1990). One year later, a ligand for c-mpl was isolated by a number of groups. This ligand was shown to induce both proliferation and maturation of megakaryocytes leading to enhanced platelet formation (Kaushansky et al 1994; Kuter et al 1994; Wendling 1994). In 1994, several groups succeeded in cloning TPO and proved that it was the primary physiological regulator of

megakaryocytopoiesis and thrombopoiesis (de Sauvage et al 1994; Lok et al 1994; Gurney et al 1995; Rollinger-Holzinger 1998).

The possible clinical importance of TPO sparked a great deal of further investigation. Early studies showed potent enhancement of platelet production in vivo following the administration of TPO to both mice and humans (Neelis et al 1998; Harker et al 1999; Mouthon et al 1999). However, some of these studies also soon suggested that TPO had a broader role in hematopoiesis than initially suspected. For example, in addition to its thrombopoietic activity, the administration of TPO to normal animals was found to increase the number of progenitors of all blood cell lineages (Farese et al 1996; Kaushansky et al 1998). In order to study the function of TPO in vivo further, mice with targeted deletions of the genes for *tpo* and *c-mpl* were generated (Gurney et al 1994; de Sauvage et al 1996). These mice were found to have <15% of normal platelet levels and decreased numbers of megakaryocytes but levels of all other mature blood cells were normal. However, further investigation showed that the $tpo^{-/-}$ and $c\text{-}mpl^{-/-}$ mice had significant decreases in erythroid and myeloid progenitors (CFU-GMs and CFU-Es) (Carver-Moore et al 1996) as well as blast CFC (undifferentiated colonies representing the progeny of pre-progenitor cells, Kimura et al 1998). HSC activity, as assessed in a competitive long-term repopulating strategy was also reduced ~10-fold in the $c\text{-}mpl^{-/-}$ mice (Kimura et al 1998; Solar et al 1998).

TPO's broad hematopoietic progenitor cell-stimulating activity is also supported by its effects in vitro. For example, in vitro, TPO alone caused a small net increase in LTC-ICs in 10 day cultures of human marrow cells (Petzer et al 1996; Kobari et al 1998) and could also maintain and possibly moderately increase murine $S^+K^+L^-$ cells (Matsunaga et al 1998; Shimomira et al 2000). In the presence of other cytokines such

as SF and IL-3, TPO enhanced the formation of murine and human multi-lineage colonies (Kobayashi et al 1996; Ku et al 1996; Sitnicka et al 1996; Zandstra et al 1997; Shimomura et al 2000). This synergistic effect of TPO on the stimulation of primitive bone marrow progenitors has also been demonstrated with other early acting cytokines, including FL and IL-11 (Ramsfjell et al 1996; Kobari et al 1998, Luens et al 1998).

Although the potential of TPO for stimulating early progenitors in culture is clear, the mechanisms by which TPO may affect HSC activity are not well defined. Ku et al (1996) found that TPO could accelerate the entry of murine HSCs into cycle and thereby hasten the production of daughter progenitor cells. Other studies have shown that TPO can directly stimulate HSC division and the preservation of multilineage repopulating ability in their progeny (Matsunaga et al 1998; Ema et al 2000).

A role for TPO in regulating HSC activity is further supported by the finding that c-mpl is expressed on HSCs (de Sauvage et al 1994; Zeigler et al 1994; Solar et al 1998). However, forced over-expression of c-mpl confers an ability for multi-potent progenitors to be amplified but not HSCs (Goncalves et al 1997; Hong et al 1998; Yamada et al 1998). These findings and the results of in vivo and culture assays clearly suggest that TPO has the potential to act directly on HSCs although many of the effects on HSC activity may be explained by effects on their immediate progeny with more restricted self-renewal potentialities.

OBJECTIVES

Previous studies of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice have shown that the loss of TPO signaling decreases primitive hematopoietic progenitor detection. Data from in vitro experiments have indicated that in vitro TPO can directly stimulate HSCs and their

immediate progeny. However, the precise spectrum of biological responses that TPO can elicit from specific subsets of primitive hematopoietic cells has yet to be determined. Similarly, definitive information as to the specific progenitor compartments prior to CFCs that are compromised in *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice is lacking although available evidence suggests the possibility that more primitive progenitors may also be affected.

This study was designed first to measure directly how an absence of TPO signaling in vivo affects the regulation of the compartment size of other progenitors. Specifically the number of LTC-IC, pre-CFC and two stem cell candidate populations were measured in adult bone marrow cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice in comparison to +/+ cells. A second set of experiments was then undertaken to determine how TPO stimulation may affect the total cell, pre-CFC and LTC-IC activity of +/+ progenitor enriched cells. These assays were then used to assess the response of the same populations in *tpo*$^{-/-}$ and c-mpl$^{-/-}$ mice to several established growth factor combinations. Finally, the ability of TPO as a growth factor to "rescue" *tpo*$^{-/-}$ progenitors in culture was tested to obtain insights into the role of TPO signaling in maintaining primitive normal hematopoietic progenitor function.

CHAPTER 2 METHODS AND MATERIALS

Animals

Mice heterozygous for a null mutation of either the *c-mpl* or *tpo* gene (*c-mpl*$^{+/-}$ and *tpo*$^{+/-}$ mice) were obtained as a gift from from Genentech (South San Francisco, CA, USA) and were then bred and maintained at the Joint Animal Facility of the British Columbia Cancer Research Centre. The mutant mice were initially developed by targeted disruption of the given gene with a vector containing the neomycin resistance gene to create 129xC57Bl/6J embryos (Gurney et al 1994; de Sauvage et al 1996). These mice were then back crossed on a C57Bl/6J background to homogeneity. Tail snips were collected from newborn pups and DNA isolated for genotyping of individual pups as +/+, +/-, -/-. Primers for sequences at the 3' and 5' ends of the *tpo*, *c-mpl* and the neomycin resistance gene (Gurney et al 1994; de Sauvage et al 1996) were used to amplify the intervening regions of genomic DNA by PCR (see Table 1). Homozygous *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ as well as +/+ mice were identified after resolution of the DNA on gel electrophoresis. The identified mutant mice were compared to +/+ littermates or the descendents of these mice in all subsequent experiments.

Cells

Mice were killed by asphyxiation with CO_2 and tibiae and femora dissected out and the ends trimmed. Bone marrow cells were flushed out of these bones with Hank's balanced salt solution containing 2% fetal bovine serum (FBS, Stem Cell Technologies Inc, Vancouver, BC) (HF) using a syringe fitted with a 26-gauge needle. A suspension of single cells was obtained by passing the sample through an 18-gauge needle several

Table 1. Primers for sequences at the 3' and 5' ends of the TPO, *c-mpl* and the neomycin resistance gene (neo) (sequences written 5' to 3').

Gene	5' oligo	3' oligo
Neo	cgg ttc ttt ttg tca aga	atc ctc gcc gtc ggg cat gc
tpo	gtc gac cct ttg tct atc cct	ggt gaa tgt aac ctg gga taa
c-mpl	tcg atc tag agc ccc gtg cat gcc ccc tgt att	tcg aat cga tac cca cat cgt gaa aga cta

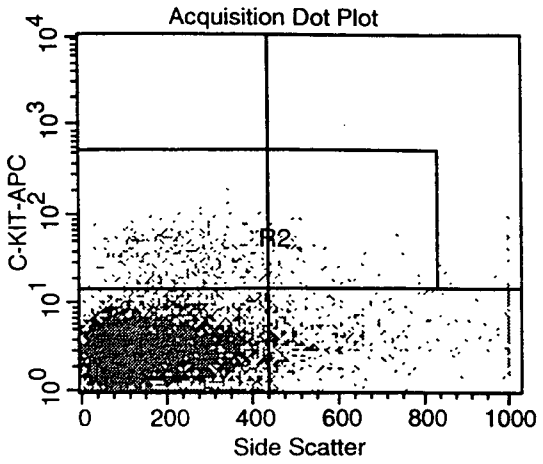
times. The cell concentration was then determined using a hemacytometer. Cells were kept at 0-4°C throughout all remaining procedures.

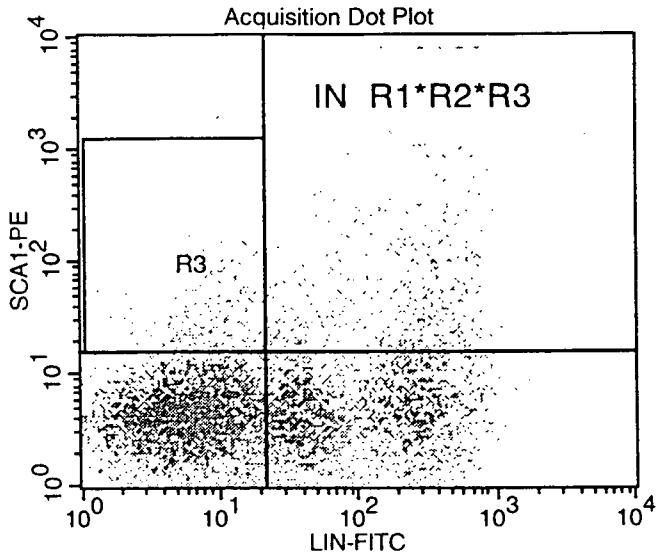
Bone marrow samples were prepared shortly after collection for FACS purification of one of two different HSC-enriched populations. Numerous studies have shown that normal adult mouse bone marrow cells that express stem cell antigen (Sca-1) and c-kit (the receptor for SF) and that lack several markers associated with differentiation along a specific blood cell lineage (i.e. B-lymphocytes, granulocytes, T-lymphocytes and macrophages) are highly enriched for HSCs (see Figure 1a) (Spangrude et al 1988; Okada et al 1992; Osawa et al 1996; Randall et al 1996; Morrison et al 1997). These cells are referred to as Sca-1^+c-kit^+ lin^- ($S^+K^+L^-$) cells and contain HSCs as defined by the competitive repopulation unit (CRU) assay at a frequency of 1/30 (Osawa et al 1996; Audet et al 2001). Another even more highly enriched HSC population can be isolated from normal adult mouse bone marrow after staining not only for a Sca-1 and lin- phenotype but also with the DNA dye, Hoechst 33342. The resultant fluorescence is then displayed simultaneously at two emission wavelengths. As illustrated in Figure 1b, this permits a small side population of cells (SP cells) to be identified and the isolation of S^+L^- SP cells by fluorescent-activated cell sorting (FACS) (Goodell et al 1996).

To isolate $S^+K^+L^-$ cells the whole bone marrow cell suspension was diluted to 5 to 8 x 10^6 cells/ml in HF and incubated with 6 μg/ml of 2.4G2 (an anti-Fc receptor antibody) to reduce non-specific staining (Unkeless et al 1979) and then a mixture of fluorescein isothiocyanate (FITC) - labeled lineage-specific rat monoclonal antibodies (mAbs), phycoerythrin (PE)-labeled anti-Sca-1 (anti-Ly6A/E; E13-161.7, PharMingen,

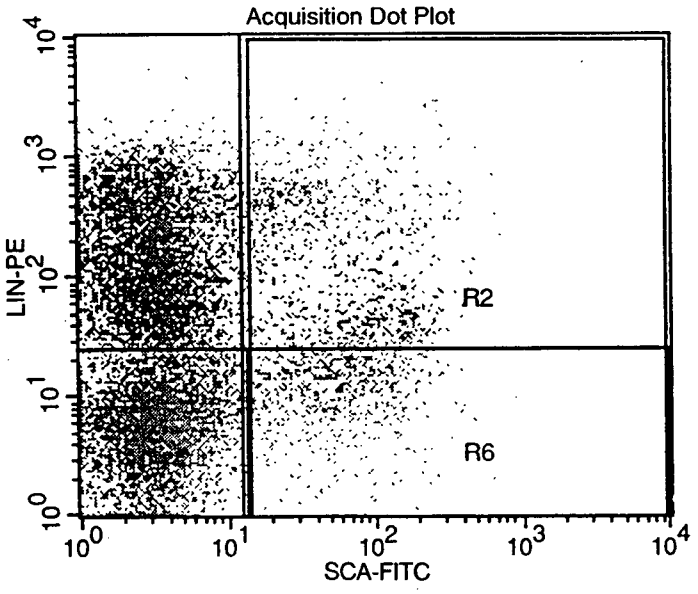
Figure 1a and b. Sca-1$^+$c-kit$^+$lin$^-$ ($S^+K^+L^-$) cells (a) and Sca-1$^+$lin$^-$ SP (S^+L^- SP) cells (b) are sorted based on gates set on fluoresence levels representing the desired cell populations after erythrocytes, debris and dead cells were excluded by their forward and side light scattering properties and propidium iodide staining.

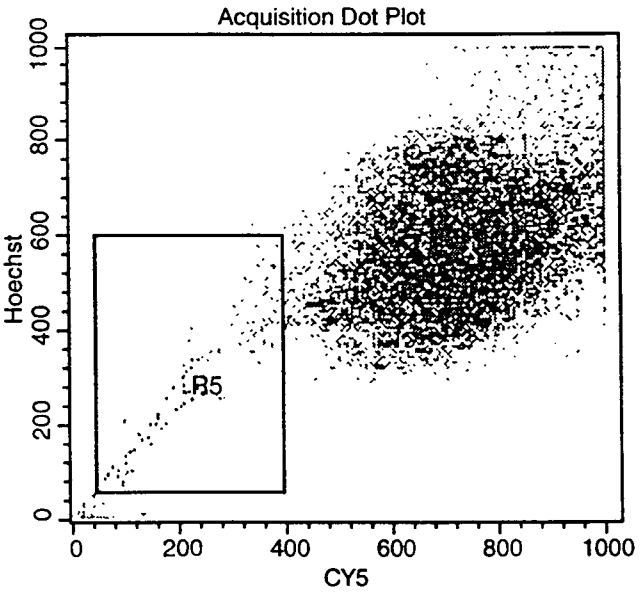
a.





b.





San Diego, CA) and allophycocyanin (APC)-labeled anti-c-kit (CD117; 2B8, Pharmingen) for 40-60 minutes. The lineage antibodies used were anti-B220 (RA3-6B2), anti-Gr-1 (RB6-8C5), anti-TB104 and anti-Mac-1 (M1/70). When S^+L^- SP cells were to be isolated, cells were suspended at 1 to 5 x 10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) pre-warmed to 37°C containing 2% FBS, penicillin and streptomycin and 5 μg/ml Hoechst 33342 (Sigma Chemical Co., St. Louis, MO). Samples were then incubated at 37°C for 90 minutes prior to being further stained with mAbs. Sort gates for the SP cells were set by examining the shift in fluorescence seen in a separate aliquot of cells also stained with 50 μM verapamil (Sigma). Cells were then washed in HF and stained with Sca-1 and lineage mAbs as above. Cells were then washed twice again in HF with 1 μg/ml propidium iodide (PI, Sigma) in the last wash to identify and exclude dead cells from the analyses and sorts. Cells were analyzed and sorted on a FACStar$^+$ (Becton Dickinson, San Jose, CA). Erythrocytes and debris were excluded by their forward and side light scattering properties. Other sort gates were set using control samples stained with an irrelevant antibody labeled with the same fluorochrome. Sorted cells were collected into HF and, except when single cells were sorted directly into multi-well plates, the total cell concentration was determined using a hemacytometer.

Serum-free suspension cultures

100-200 $S^+K^+L^-$ cells or 40-100 S^+L^- SP cells were cultured in 1 ml Iscove's medium supplemented with 20 ng/ml of bovine serum albumin (BSA), 10 μg/ml of insulin and 200 μg/ml of transferrin (BIT; Stem Cell Technologies), 40 μg/ml low-density lipoproteins (Sigma), 10^{-4} M 2-mercaptoethanol, 2mM glutamine (Sigma), 50 μg/ml

streptomycin, 50 units/ml penicillin and one or more of the following growth factors: 50 ng/ml murine SF (expressed in COS cells and purified in the Terry Fox Laboratory), 100 ng/ml human flt-3 ligand (FL, Immunex Corporation, Seattle, WA), 100 ng/ml human interleukin (IL)-11 (Genetics Institute, Wyeth-Ayerst Inc, Cambridge, UK), 100 ng/ml hIL-6 (Cangene, Missassauga, ON) and 50 ng/ml murine TPO (Genentech). Cultures were incubated for 10 or 11 days in 24-well plates (Nunc) at 33°C in an atmosphere of 5% CO_2 in air. At the end of the culture period, cells and media were collected and wells were rinsed several times with HF. If adherent cells were present, the culture plates were incubated for 5 minutes with 0.5 ml of trypsin in Hank's solution after removal of the media. This was followed by two rinses with HF to collect residual cells. In some cases, cells from groups of 3 to 6 wells were pooled. Collected cells were centrifuged at 350g for 7 minutes, re-suspended in HF and the cell concentration determined. CFC and LTC-IC assays were then performed on appropriate aliquots of these cells.

In several experiments, single cells were sorted directly into 0.1 ml volumes of serum-free media pre-aliquoted into 96-well plates. These were set up with different growth factor combinations as indicated. After 10 days of incubation at 33°C, the presence (≥2 refractile cells) and size of clones in each well was scored. In some of these experiments, the contents of each well were also assayed for their CFC content.

CFC assays

CFC assays were performed at the start of each experiment on whole bone marrow and sorted subpopulations to determine CFC frequencies by plating cells at appropriate concentrations (to give ≤200 colonies/1 ml culture) in prepared

methylcellulose medium supplemented with 10 ng/ml murine IL-3, 10 ng/ml human IL-6, 50 ng/ml murine SF and 3 units/ml human erythropoietin (HCC-3434; StemCell). These assays were repeated on cultured cells to measure the reported changes in CFC content. Briefly, 1 ml aliquots of the culture mixture were transferred to 35mm petri dishes (Stem Cell) and after 12-14 days of incubation at 37°C, the types and numbers of colonies present were scored. These included large erythroid colonies derived from burst forming units-erythroid (BFU-Es), colonies of granulocyte (G) and macrophages derived from colony-forming units (CFU-GMs) and large multi-lineage colonies derived from CFU-granulocyte/erythroid/megakaryocyte/macrophage (CFU-GEMMs).

pre-CFC assays

Pre-CFC are defined here as cells which give rise to CFC progeny after 10 days in serum-free suspension cultures. Serum-free suspension culture conditions are the same as those outlined above (see Serum-free Suspension Cultures section). Pre-CFC activity was measured by determining the number of CFC present in the harvested 10 day cultured cells. When single cells were assessed, purified $S^+K^+L^-$ cells from *tpo*$^{-/-}$ and +/+ mice were sorted directly into 96 well plates pre-loaded with serum-free medium plus specific growth factors and 10 days later the number of cells and CFCs present in each well was determined.

LTC-IC assays

The LTC-IC content of pre-culture cell suspensions was determined either by single cell or limiting dilution analysis of bulk LTC-IC assays. The latter were used to assess starting marrow and sorted populations. Limiting dilution assays were

performed on unseparated bone marrow cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice and single cell and limiting dilution assays were performed on sorted $S^+K^+L^-$ and S^+L^-SP populations obtained from these. In both cases, suitable numbers of cells were suspended in myeloid LTC medium (M5300; StemCell) to which 10^{-6} M hydrocortisone sodium hemisuccinate (Sigma) was added. Cells were cultured in plates containing a pre-established feeder layer of mouse marrow which had been irradiated with 1500cGy to curtail their further growth (Lemieux et al 1995). All LTCs were maintained at 33°C for 4 weeks with weekly replacement of half the medium with fresh LTC medium plus new hydrocortisone. At the end of the 4-week period, all cells were harvested from each well or plate and a single cell suspension prepared. CFC assays were performed according to the method described above. For limiting dilution analyses, several different doses of cells were plated in 8 to 16 replicate wells of 96-well culture plates or 4 to 8 replicates in 24-well plates. The entire contents of each well was plated and the LTC-IC frequency was calculated by the method of maximum likelihood based on the proportion of wells in which no CFCs were detected (i.e. negative wells). For single cell assays, cultures were scored directly as positive or neative and LTC-IC frequencies then derived from these ratios. For bulk LTC-IC assays of +/+ cells, media containing the non-adherent cells was first collected, the wells then rinsed with Hank's and the remaining adherent layer cells removed after a 10 to 15 minute incubation with trypsin. Plates were then rinsed with HF and all collected cells pooled and washed prior to plating in suitable aliquots in CFC assays. The frequency of LTC-ICs present in the original sample was then calculated from the total number of CFCs measured in a culture initiated with a known number of cells based on the assumption that each LTC-IC produces approximately 30 CFC as confirmed by previous limiting dilution analysis.

Statistical analysis

The mean, standard deviation and standard error of the mean were calculated from raw data for the measured endpoints in whole bone marrow and serum-free cultures using Microsoft Excel spread sheets. When values were compared, the significance of differences was determined using the student t-test to obtain the given p-values.

CHAPTER 3 RESULTS

Decreases in functionally and phenotypically defined subsets of primitive hematopoietic cells in adult *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice

Previous studies have shown that CFC numbers and in vivo repopulating activity are both reduced in *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice. To establish baseline levels for our breeding stock of these two mouse genotypes, the numbers of CFCs in unseparated bone marrow cells were measured and compared with simultaneously assayed cells from +/+ mice. In addition, pre-CFCs and LTC-IC numbers and the CFC outputs of both of these progenitors were measured to determine the extent to which the previously observed defect in HSC activity might be explained, at least in part, by a defect in the size of these related progenitor compartments, or in the ability of these primitive cells to generate CFCs. The proportion of bone marrow cells with a phenotype characteristic of HSC candidates was also compared between the mutant and +/+ mice.

A significantly reduced frequency of CFCs in both *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice was confirmed (45% $\pm$ 4% and 59% $\pm$ 3%, respectively, of the frequencies measured in their +/+ counterparts, see Table 2). This decrease was the same for all 3 types of CFCs measured (i.e., CFU-GMs, BFU-Es and CFU-GEMMs). There was also no obvious difference in the sizes of colonies produced by the *tpo*$^{-/-}$ or *c-mpl*$^{-/-}$ CFCs as compared to those produced by their +/+ counterparts.

The results of the LTC-IC assays are shown in Table 3. The frequencies of LTC-ICs in unseparated bone marrow cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice were similarly decreased

Table 2. CFC frequencies in the bone marrow of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice are reduced as compared to +/+ mice.

Genotype	CFU-GM	BFU-E	CFU-GEMM	Total	(n)
tpo$^{-/-}$	270 ± 15	20 ± 3	3 ± 1	290 ± 20	(11)
c-mpl$^{-/-}$	340 ± 30	20 ± 3	3 ± 1	360 ± 30	(12)
+/+	550 ± 20	43 ± 6	10 ± 2	610 ± 20	(18)

Results represent the mean ± SEM of CFC numbers per 10^5 unseparated bone marrow cells obtained from *tpo*$^{-/-}$, *c-mpl*$^{-/-}$ and +/+ mice assayed individually. n = no. of experiments.

Table 3. The frequency and CFC output of LTC-ICs in the bone marrow (BM) of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice are reduced.

Genotype	LTC-IC Frequency		CFC/LTC-IC[1]	
	Unsep BM[2]	$S^+K^+L^-$ (Single Cells)[3]	Unsep BM	$S^+K^+L^-$ (Single cells)
tpo$^{-/-}$	[4]1.0 ± 0.2 (n=6)	17, 20 %	11	4.0 ± 0.4
c-mpl$^{-/-}$	[4]1.2 ± 0.2 (n=7)	24, 25 %	11	4.1 ± 0.4
+/+	[4]3.0 ± 0.4 (n=3)	26, 22 %	34	11 ± 3

[1] Calculated by dividing the total number of CFC detected by the total number of LTC-IC from which these were derived (i.e. the starting frequency x the number of cells assayed to produce the CFC detected).
[2] Results represent the mean ± SEM LTC-IC per 10^5 unseparated bone marrow cells calculated by limiting dilution assay and n = the number of experiments performed.
[3] Data from 2 separate experiments are shown separately and are expressed as a percent of the total $S^+K^+L^-$ population.
[4] Values are significantly different from the +/+ value ($p<0.001$).

(~3-fold by comparison to the value for +/+ mice) and the CFC output by the mutant LTC-ICs was also reduced (~3-fold). Thus there was an overall reduction of almost 10-fold in the CFC-generating activity in the LTC-IC assays of the mutant bone marrow cells.

Assessment of the total cellularity of the marrow of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice showed this was not different ($p>0.05$) in either case from +/+ mice, as previously reported (Gurney et al 1994; de Sauvage et al 1996; Carver-Moore et al 1996). Therefore the reduced frequencies of CFCs and LTC-ICs documented here are indicative of corresponding reductions in the absolute numbers of these progenitors in both mutant genotypes. Taken together, these findings support earlier evidence suggesting that the elimination of TPO-mediated responses in vivo has an even greater effect on the generation of primitive progenitors than progenitors detectable as CFCs.

As a complementary approach, two phenotypically defined fractions of bone marrow cells that in +/+ mice are highly enriched in their content of HSCs were also quantified in bone marrow suspensions prepared from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice. One of these is the $S^+K^+L^-$ population (Okada et al 1992; Osawa et al 1996}. The other is the S^+L^- SP population which has a higher frequency of transplantable HSCs than the $S^+K^+L^-$ population in normal mice (Goodell et al 1996; Uchida et al 2001). Both populations could be identified in the marrow cells from the two mutant genotypes and examples of the FACS profiles obtained for each are illustrated in Figure 1. A comparison of the representation of these two populations in the bone marrow of mutant and +/+ mice is given in Table 4. These analyses showed the frequency of cells with an S^+L^- SP phenotype was decreased in both *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice (~2 to 4-fold)

Table 4. The frequency of phenotypically defined subsets of primitive cells in the bone marrow (BM) of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice is reduced.

Genotype	No. per 10^5 BM cells		% CFC	
	$S^+K^+L^-$	S^+L^-SP	$S^+K^+L^-$	S^+L^-SP
tpo$^{-/-}$	7,700 ± 2,500	1,700 ± 300	12 ± 1	11 ± 1
c-mpl$^{-/-}$	11,000 ± 4,000	4,300 ± 1,400	11 ± 1	12 ± 2
+/+	15,000 ± 2,500	9,500 ± 4,300	20 ± 2	28 ± 1

with a slightly greater effect seen in this comparison in the *tpo*$^{-/-}$ mice. Although a slight decrease was also seen in the $S^+K^+L^-$ populations from the mutant mice, only that seen in the in the *tpo*$^{-/-}$ mice was significant ($p<0.05$). Again, because the total cellularity of the marrow is unchanged in both mutant genotypes, these frequency values can be used to infer absolute reductions in the $S^+K^+L^-$ and S^+L^- SP populations present.

The $S^+K^+L^-$ cells were also assessed functionally for their content of LTC-ICs in single cell assays. The results of these experiments are given in Table 3 together with the results of the assays performed on unseparated bone marrow. They show first, that the *frequency* of LTC-ICs in the $S^+K^+L^-$ population was not markedly decreased, as predicted by the fact that the frequency of $S^+K^+L^-$ cells (that would be expected to contain all the LTC-ICs) was already reduced. In addition, the single cell assays revealed the same ~3-fold reduction in CFC output per *tpo*$^{-/-}$ or *c-mpl*$^{-/-}$ LTC-IC as had been found in the assays of unseparated cells.

The frequency of pre-CFCs in the $S^+K^+L^-$ population was also assayed under two different growth factor combinations, SFand FL with either IL-11 or TPO in two separate experiments in which $S^+K^+L^-$ cells were assayed in single cell cultures. In both experiments, the $S^+K^+L^-$ cells from *tpo*$^{-/-}$ mice showed an initially normal mitogenic response to either combination of growth factors used (SL + FL + TPO or IL-11), as indicated by the proportion of wells that contained >2 cells (Table 5). However, this response was not sustained, as indicated by the reduced number of wells of *tpo*$^{-/-}$ cells that contained >200 cells or CFCs. This reduction in pre-CFC is similar to the reduction in frequency of CFCs within either the $S^+K^+L^-$ or S^+L^- SP populations (Table 4) and differs from the frequency of LTC-ICs within the $S^+K^+L^-$ population which was generally the same in both +/+ and *tpo*$^{-/-}$ subsets (Table 3).

Table 5. $S^+K^+L^-$ BM cells from tpo-/- mice show an initially normal mitogenic response to either SF+FL+IL-11 or SF+FL+TPO but this is neither sustained nor accompanied by a normal output of CFC.

			No. of Positive Clones (per 96 single cell cultures)	
Growth factors present	**Endpoint**	**Gentotype (TPO)**	**Exp 1**	**Exp 2**
SF11	≥2 cells	+/+	72	73
		-/-	56	76
	>200 cells	+/+	32	35
		-/-	7	13
	>1 CFC	+/+	16	20
		-/-	2	6
SFT	≥2 cells	+/+	69	73
		-/-	54	70
	>200 cells	+/+	31	21
		-/-	7	15
	>1 CFC	+/+	22	18
		-/-	6	12

Single $S^+K^+L^-$ BM cells were cultured in serum-free medium for 10 days prior to assessment. S = 50 ng/ml of SF, F = 100 ng/ml FL, 11 = 100 ng/ml of IL-11, T = 50 ng/ml of TPO.

Altered response of *tpo*$^{-/-}$ cells to TPO

A second series of experiments was then undertaken to investigate the effects of TPO (either alone or in combination with several other hematopoietic growth factors) on progenitor amplification/generation in vitro and to determine how these might be altered in populations obtained from *tpo*$^{-/-}$ or *c-mpl*$^{-/-}$ mice.

The protocol adopted was to assess total cell, CFC and LTC-IC maintenance/generation in 10-day serum-free suspension cultures initiated with $S^+K^+L^-$ or S^+L^- SP cells. All data were expressed in terms of the number of cells or CFCs produced per input cell.

An initial series of experiments was carried out using +/+ cells. The results are summarised in Figure 2. It can be seen that in the presence of 50 ng/ml of TPO alone, neither the survival nor the proliferation of +/+ CFCs (Figure 2b) or LTC-ICs (Figure 2c) was supported, although TPO alone did stimulate a small increase (6- and 14-fold change in $S^+K^+L^-$ and S^+L^- SP cultures, respectively) in the total number of cells present after 10 days (Figure 2a). In contrast, the same concentration of TPO synergized with SF and FL to increase the cell output, the CFC content and the LTC-IC activity of the cultures by at least a factor of 2 (relative to cultures containing SF plus FL only), although in most cases these increases were not significant due to interexperimental variability. Because previous studies have shown that IL-11 also synergizes with SF and FL to enhance the production of CFCs and stem cells in such cultures (Audet et al 2001; Audet et al in press), a comparison of the effects of additionally added TPO versus IL-11 (or IL-6) was also evaluated. As also shown in Figure 2, the synergy obtained by adding TPO to the combination of SF and FL in promoting the generation

Figure 2a. The addition of TPO at 50 ng/ml slightly increased the total numbers of cells produced in 10-day cultures of $S^+K^+L^-$ or S^+L^- SP cells also containing SF and FL. When TPO was added alone or to combinations including IL-11 or IL-6, it had no further effect in enhancing cell output. S = SF at 50 ng/ml, F= FL at 100 ng/ml, 11= IL-11 at 100 ng/ml, 6= IL-6 at 50 ng/ml. (* = significant difference from the growth factor combination without TPO, $p<0.05$)

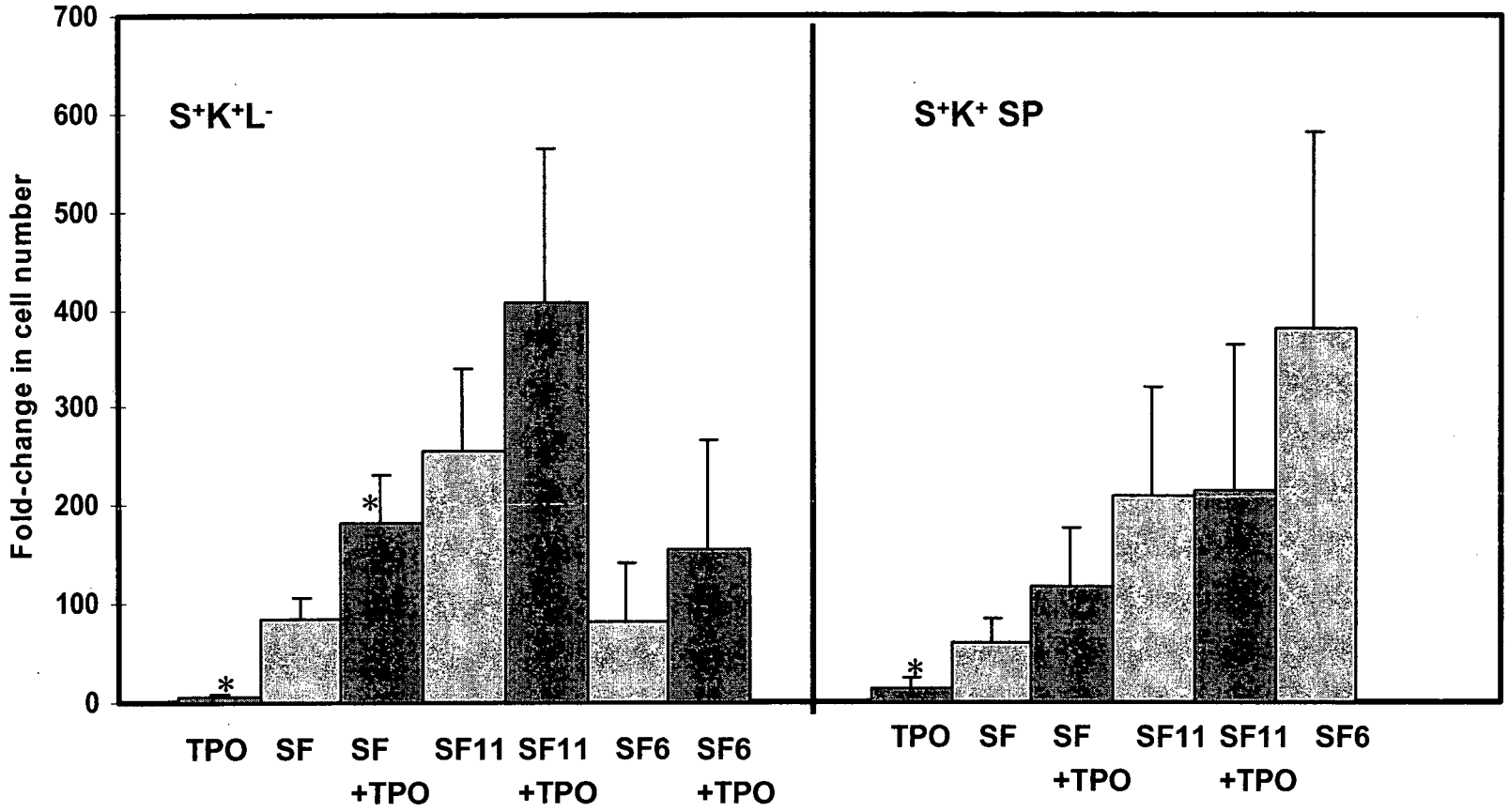


Figure 2b. The addition of TPO at 50 ng/ml enhanced the mean number of CFCs produced per sorted $S^+K^+L^-$ or S^+L^- SP cell after 10 days of culture when added to SF and FL. TPO alone supported the production of very few CFC and when added to combinations including IL-11 or IL-6, there was no enhancing effect on CFC output. Abbreviations and growth factor concentrations are as in Figure 2a. (* = significant difference from results without TPO, $p<0.05$)

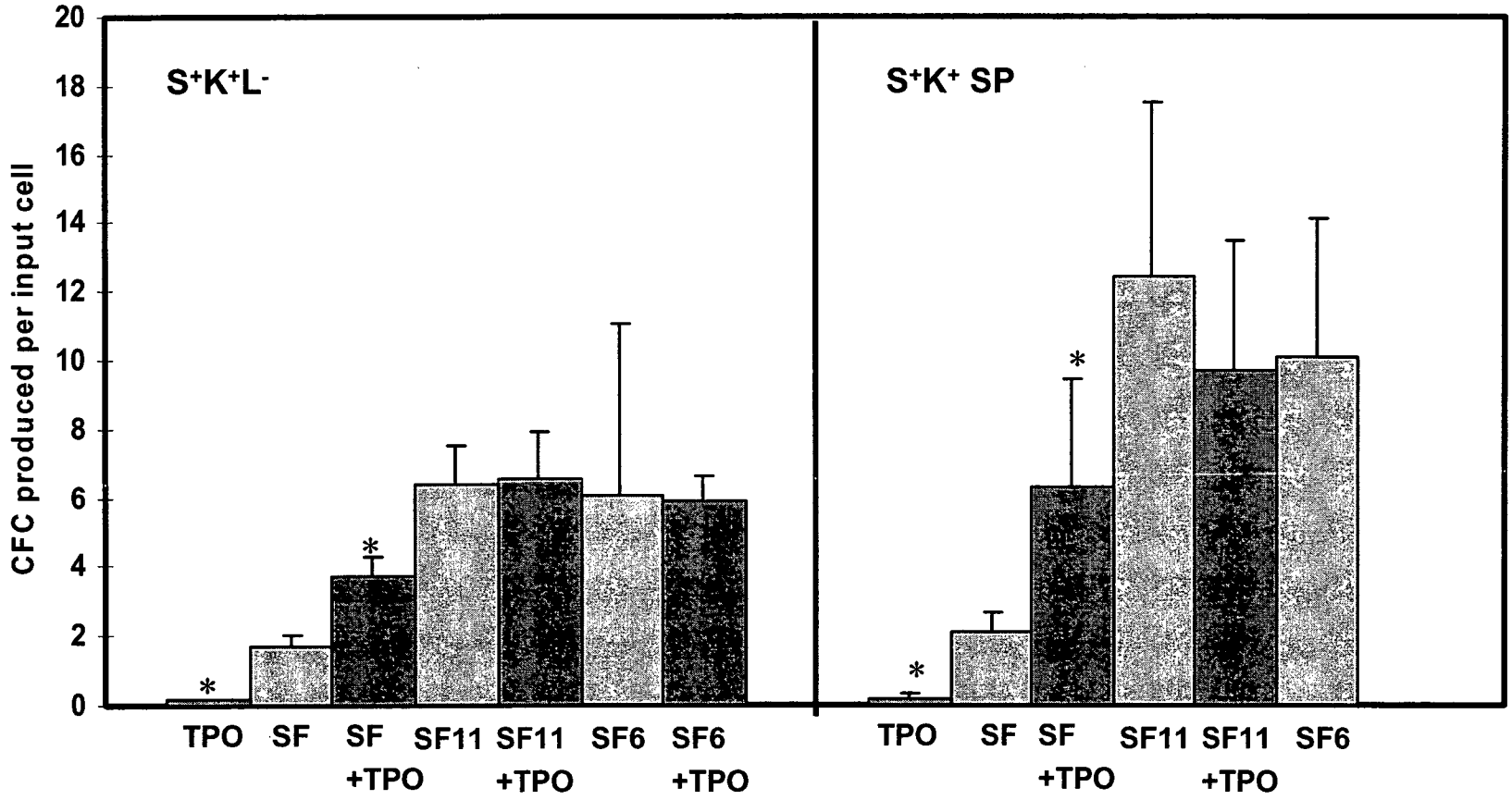
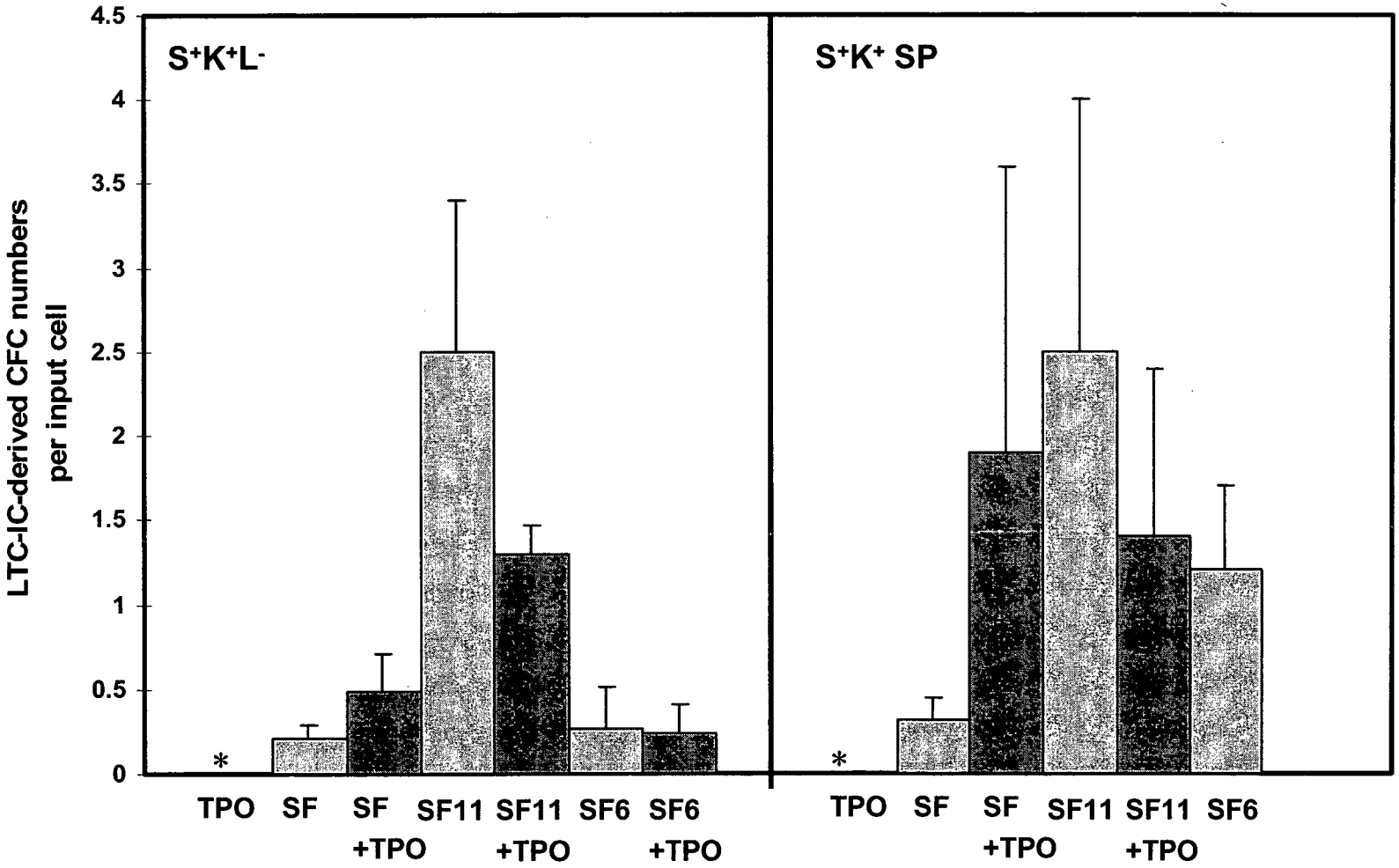


Figure 2c. The addition of TPO to SF and FL slightly increased the LTC-IC activity detected in 10-day cultures of $S^+K^+L^-$ or S^+L^- SP cells but did not further enhance the LTC-IC activity obtained when other growth factors were present. Data shown represent the number of CFCs obtained per input cell in LTC-IC assays of cells harvested from the 10-day cultures. Abbreviations and growth factor concentrations are as in Figure 2a. (* = significant difference from results without TPO, $p<0.05$)



of CFCs was similar to that obtained from gp130 activation (either by the addition of IL-11 or IL-6) but was not as strong in promoting the maintenance/expansion of LTC-ICs. In addition, when TPO was present in cultures that contained either IL-11 or IL-6 as well as SF and FL, no further enhancement of CFC or LTC-IC production was seen. These findings indicate that TPO alone has little effect in stimulating either the amplification of LTC-ICs or the steps that intervene between LTC-ICs and CFCs. However, TPO can synergize with SF and FL to positively affect all of these developmental stages, but only as an alternative to growth factors that activate gp130.

The responses of purified $S^+K^+L^-$ or S^+L^- SP cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice to SF and FL (with or without either Il-11 or Il-6) were then assessed in the same 10-day serum-free suspension cultures. The results are shown in Figure 3. Interestingly, cultures initiated with equal numbers of $S^+K^+L^-$ or S^+L^- SP cells from either *tpo*$^{-/-}$ or *c-mpl*$^{-/-}$ mice generally produced fewer total cells (Figure 3a), CFCs (Figure 3b) and less LTC-IC activity (Figure 3c) than cultures initiated with +/+ cells of the same phenotype, regardless of the growth factors added. Thus SF plus FL was less stimulatory on the *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ cells than on the corresponding +/+ cells in terms of total cell and CFC production and LTC-IC activity and the enhancing effect of activating gp130 was also reduced in the cultures initiated with the mutant cells. Although, results in the cultures of *tpo*$^{-/-}$ and c-mpl$^{-/-}$ cells were more variable, in general, the addition of IL-11 or IL-6 had relatively little enhancing effect.

Figure 3a. Total cell production by $S^+K^+L^-$ or S^+L^- SP cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice in 10-day serum-free cultures is reduced. S = SF at 50 ng/ml, F = FL at 100 ng/ml, 11 = IL-11 at 100 ng/ml, 6 = IL-6 at 50 ng/ml. ND = no data. +/+ values are the same as in Figure 2a. (* = significant difference from +/+ results, $p<0.05$)

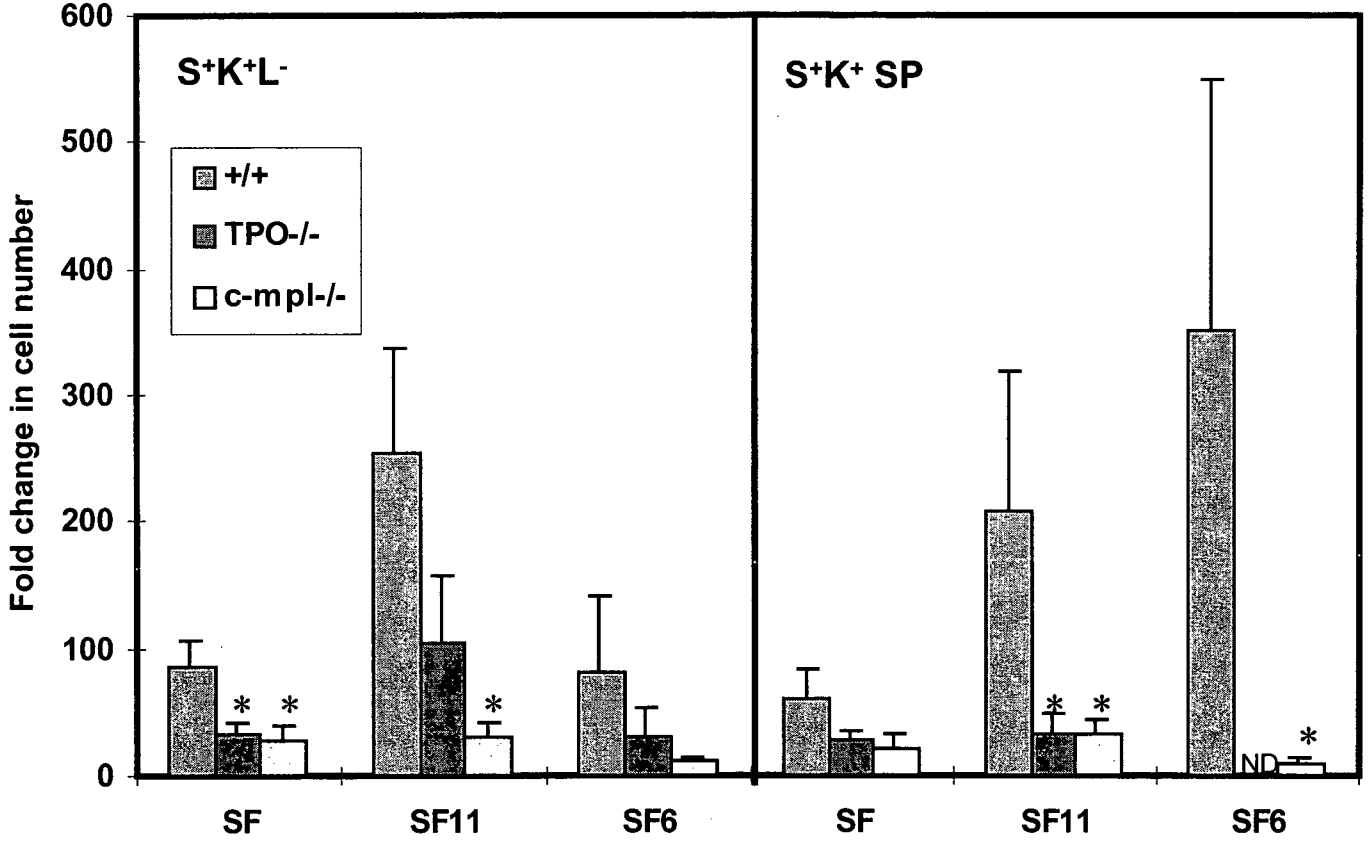


Figure 3b. CFC production in 10-day serum-free cultures of $S^+K^+L^-$ or S^+L^- SP cells from *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ is reduced. Abbreviations and growth factor concentrations as in Figure 3a. ND =not done. +/+ values are the same as in Figure 2b. (* = significant difference from +/+ results, $p<0.05$)

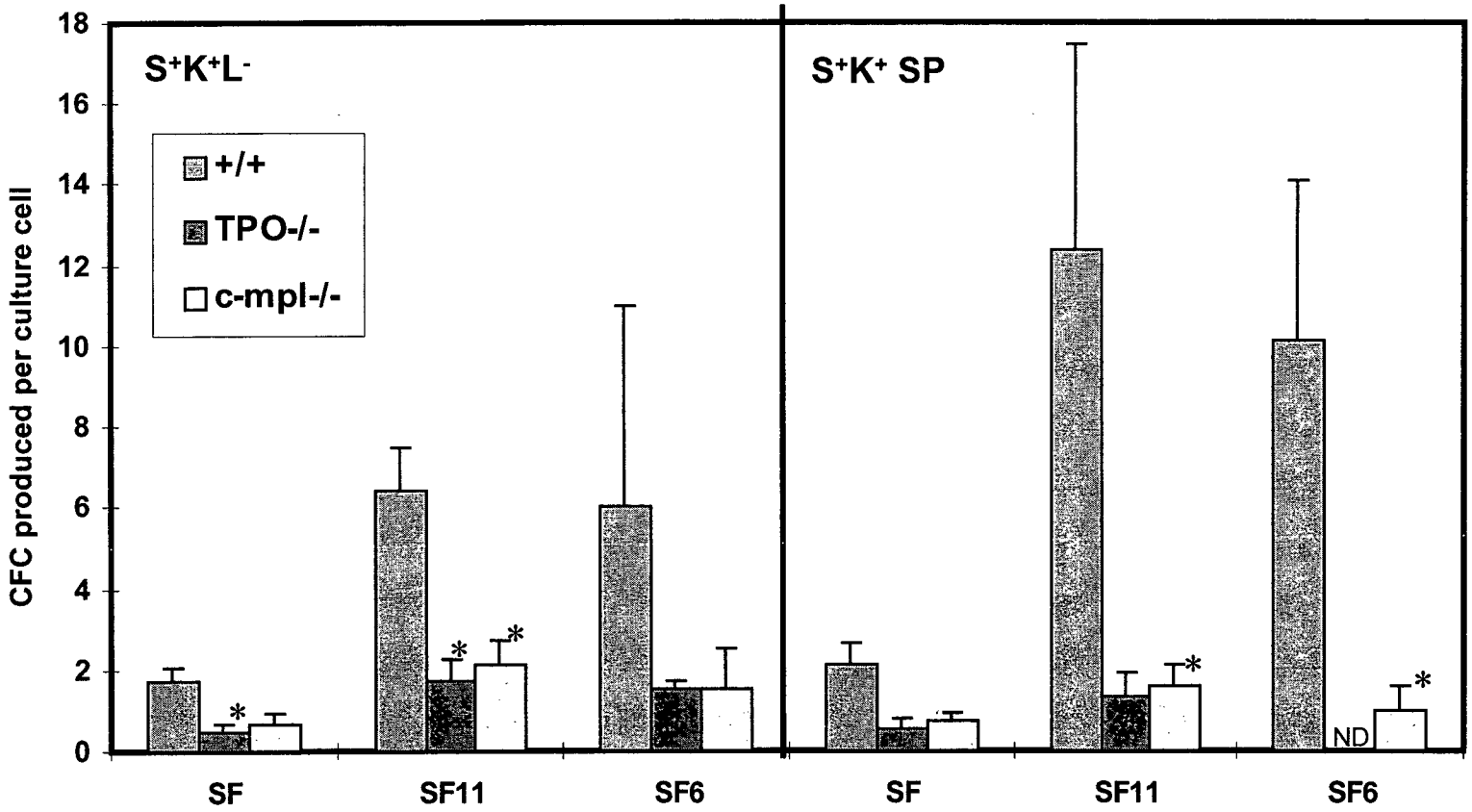
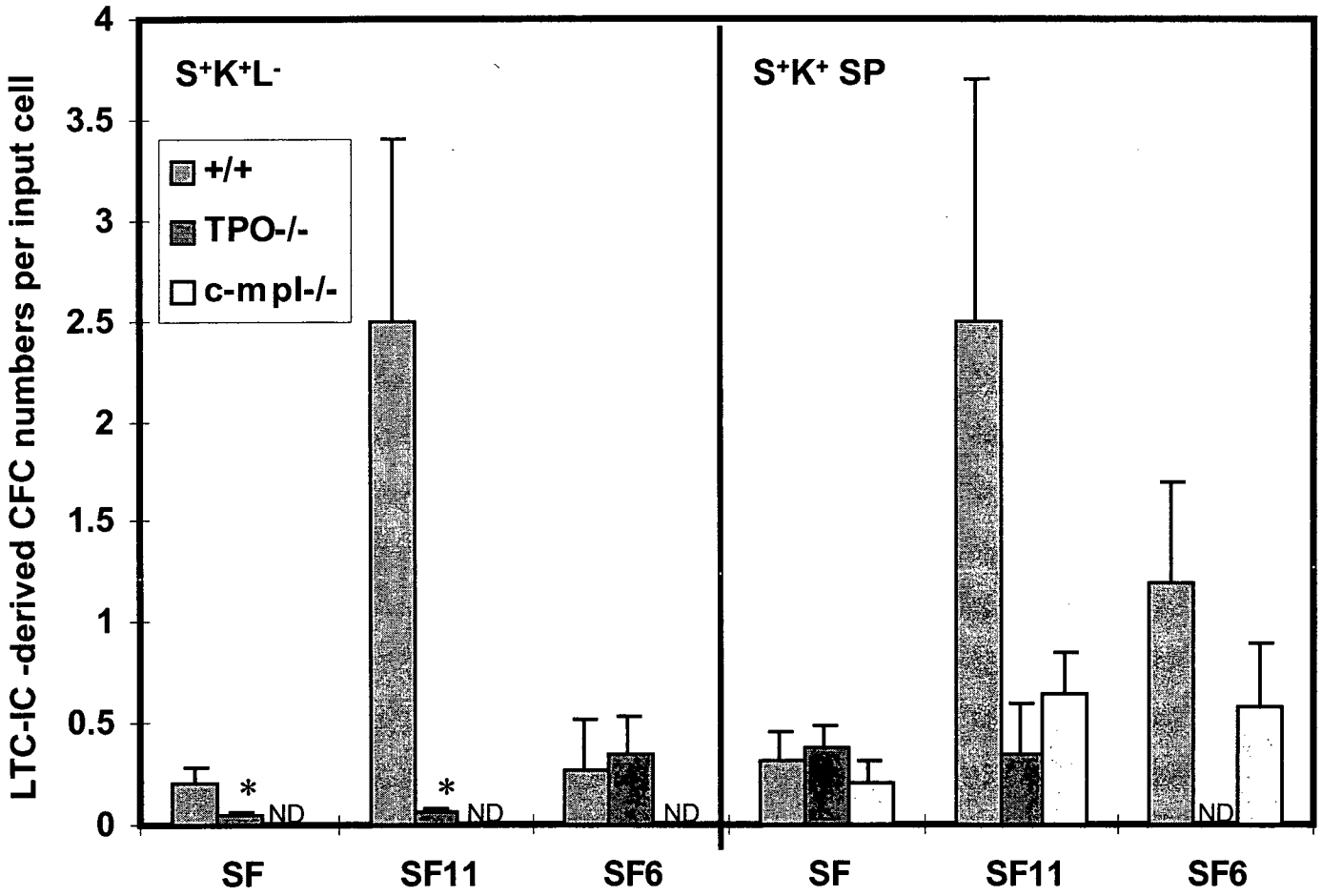


Figure 3c. LTC-IC activity in 10-day serum-free cultures of $S^+K^+L^-$ or S^+L^- SP *tpo*$^{-/-}$ or *c-mpl*$^{-/-}$ cells is reduced although the difference is only significant in $S^+K^+L^-$ TPO-/- cells cultured with SF and FL or SF, FL and IL-11. Abbreviations and growth factor concentrations are as in Figure 3a. ND = not done. (* = significant difference from +/+ results, $p<0.05$)



Evidence of an abnormal response of primitive *tpo*$^{-/-}$ cells to TPO

The observation that the number of cells able to produce CFC in vitro – either in LTCs (Table 3), or in pre-CFC suspension assay cultures in the absence of stromal cells (Table 5) -are reduced in *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice implies that TPO is a non-redundant growth factor in vivo in maintaining normal levels of both these early compartments in normal adult mice. Accordingly, it was of interest to examine the effects of exposing primitive *tpo*$^{-/-}$ cells to TPO in vitro. Therefore, to determine whether TPO naïve cells have a normal or defective response to TPO, the following experiments were undertaken. $S^+K^+L^-$ cells from *tpo*$^{-/-}$ and +/+ mice were isolated and used to initiate serum-free cultures containing SF and FL with or without TPO or IL-11. Additional cultures containing TPO only were also set up. At the end of 10 days, the cells were harvested and CFC and LTC-IC assays performed. As shown in Figure 4, the addition of TPO to SF plus FL improved the output of *tpo*$^{-/-}$ CFC as seen with the addition of IL-11 (Figure 4a) but failed to improve *tpo-/-* LTC-IC activity (Figure 4b) as also seen when IL-11 was added.

Figure 4a. The addition of TPO to SF plus FL and/or IL-11 enhanced the CFC output of *tpo*$^{-/-}$ and +/+ $S^+K^+L^-$ cells after 10 days of culture to a similar degree although the effect was not significant ($p>0.05$) and absolute yields of CFC from the mutant cells were consistently lower. S = SF at 50 ng/ml, F = FL at 100 ng/ml FL, 11 = IL-11 at 100 ng/ml, TPO at 50 ng/ml. +/+ data is redrawn from Fig. 2b for the purpose of comparison. (* = significant difference from the growth factor combination without TPO, $p<0.05$)

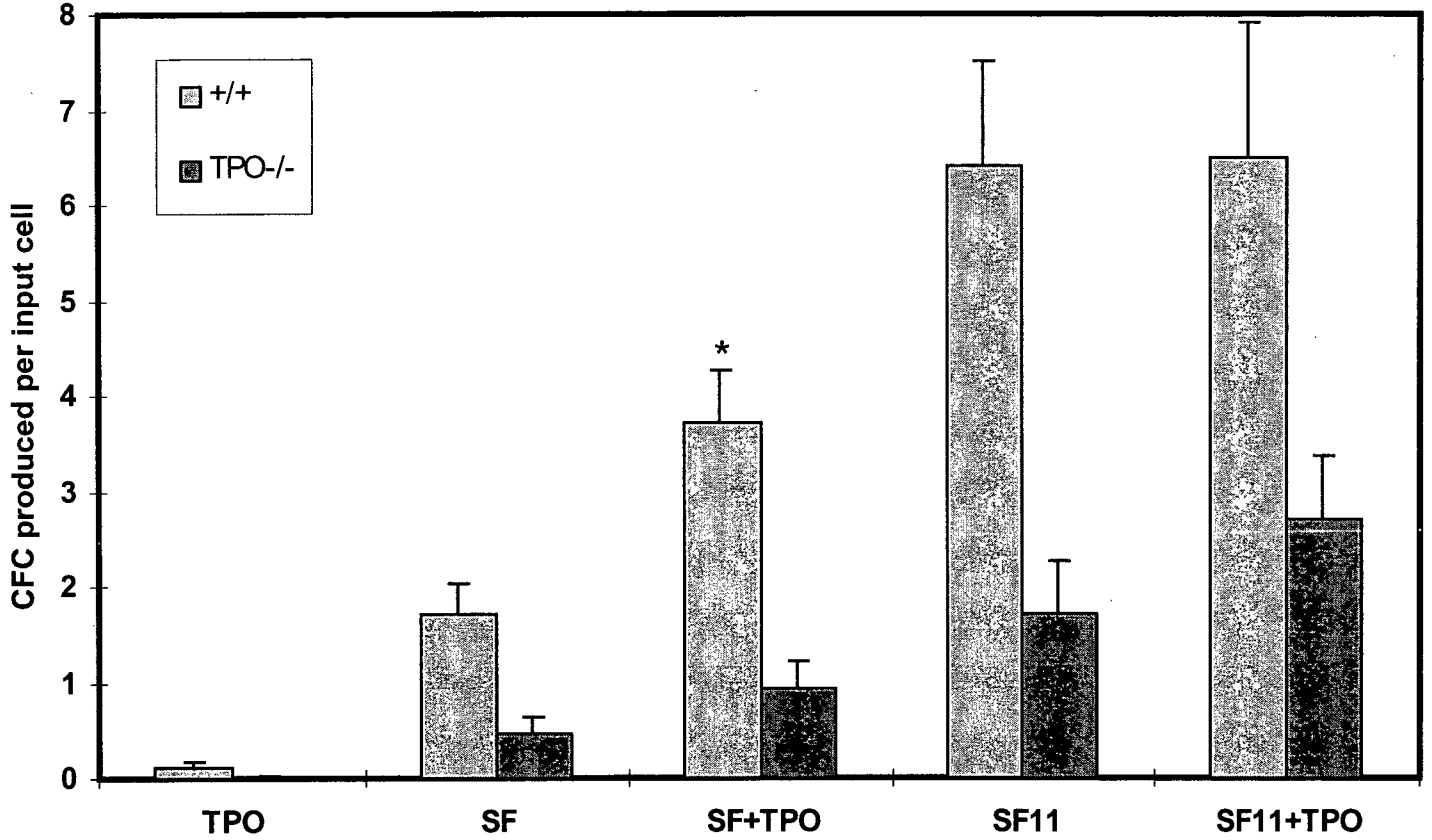
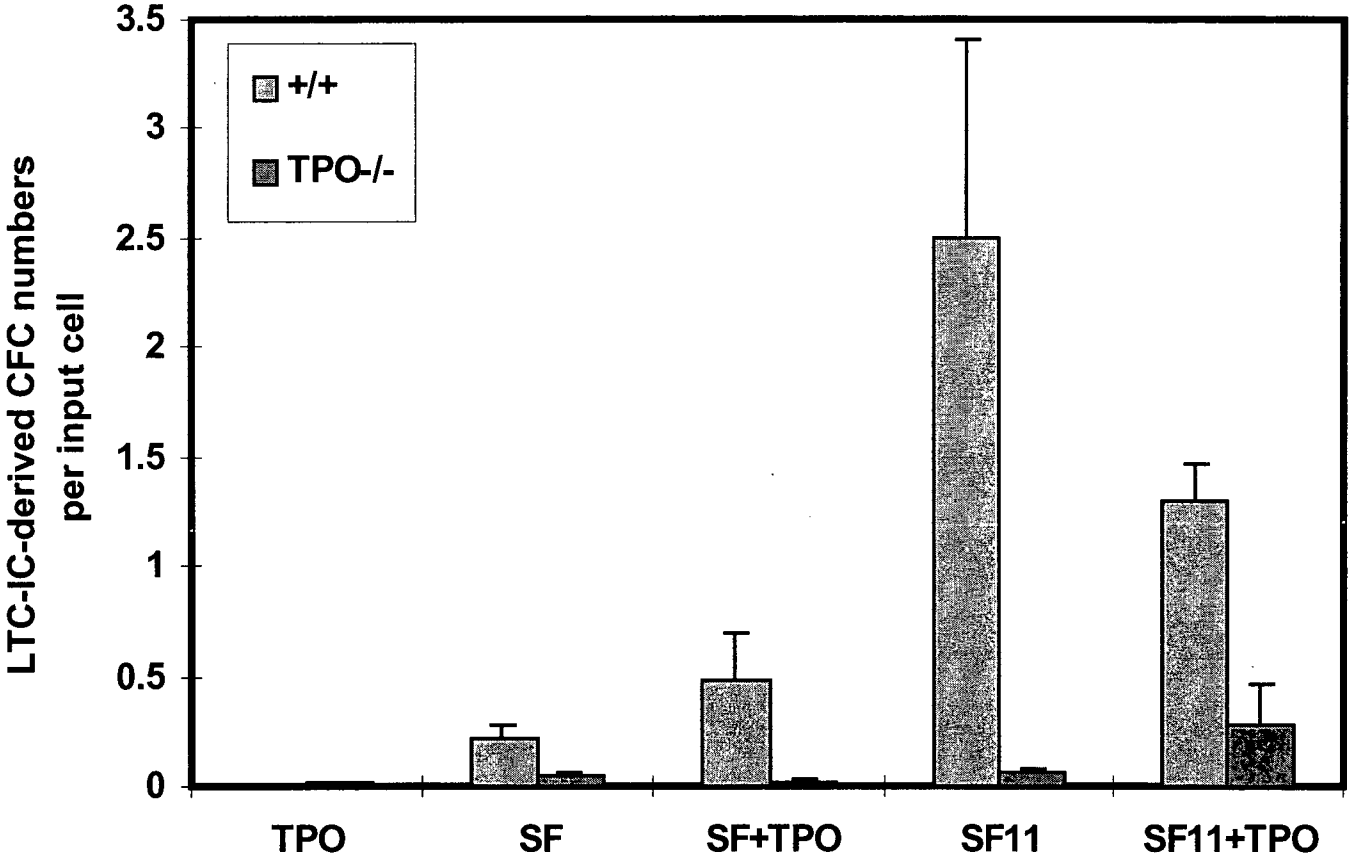


Figure 4b. The addition of TPO to SF, FL and IL-11 but not SF and FL alone slightly enhanced the LTC-IC activity generated in 10-day cultures of *tpo*$^{-/-}$ $S^+K^+L^-$ cells although this increase was less relative to that seen in cultures of +/+ cells and was not significant ($p<0.05$). +/+ data is redrawn from Fig. 2c for the purpose of comparison. S = SF at 50 ng/ml,F= FL at 100 ng/ml, 11 = IL-11 at 100 ng/ml, TPO at 50 ng/ml.



Chapter 4 DISCUSSION

TPO acts as a non-redundant growth factor in vivo at multiple levels of early hematopoiesis

The experiments presented here made use of *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice to further our understanding of the effect that the absence of TPO signaling in vivo can have on the generation of hematopoietic progenitors at multiple early levels of differentiation. In a first series of investigations the number of CFCs and LTC-ICs in the bone marrow of these mutant genotypes was determined. Flow cytometric analyses provided an independent measurement of the number of cells with a phenotype characteristic of primitive progenitors in +/+ bone marrow and functional assays of these cells made possible direct assessment of altered LTC-IC and pre-CFC numbers and their proliferative potentialities. As summarized in Table 6, all primitive cell types assessed, from LTC-ICs to CFCs were present in the mutant mice at approximately 2-3 fold lower concentrations than normal. These findings are consistent with those previously reported using CFC and CFU-blast assays (Gurney et al 1994; de Sauvage et al 1996; Carver-Moore et al 1996; Kimura et al 1998).

Kimura et al (1998) and Solar et al (1998) examined long-term repopulating activity in *c-mpl*$^{-/-}$ mice and found that the repopulating HSC activity in these mice was reduced by comparison to +/+ controls. This suggested that TPO might be essential for the maintenance of a normal sized HSC compartment in the adult, in addition to playing a requisite role in supporting the proliferation and expansion of their early differentiating progeny (down to the CFC level). However, the HSC assessments reported were not

Table 6. Multiple primitive populations are reduced in *tpo*$^{-/-}$ and *c-mpl*$^{-/-}$ mice.

Progenitor Type	Genotype	Relative Frequency[1]
LTC-IC	*tpo*$^{-/-}$	.35 ± .02
	c-mpl$^{-/-}$	.42 ± .03
pre-CFC	*tpo*$^{-/-}$	.34
SP cells	*tpo*$^{-/-}$	.18 ± .03
	c-mpl$^{-/-}$	.45 ± .04
SLK cells	*tpo*$^{-/-}$	.51 ± .07
	c-mpl$^{-/-}$	.73 ± .14
CFC	*tpo*$^{-/-}$	.45 ± .04
	c-mpl$^{-/-}$	.59 ± .03

[1] Expressed as a proportion of the frequency in +/+ bone marrow

performed in a manner that could distinguish between a reduction in the frequency of *c-mpl*$^{-/-}$ HSCs and a reduction in their ability to generate mature progeny in vivo. Since TPO is clearly required for early hematopoiesis under normal homeostatic conditions in the adult mouse, it seemed likely that TPO might also regulate the detection of HSCs post-transplant.

The present studies provide support for the view that TPO acts as an essential regulator at a very early stage of hematopoiesis, as indicated by the reductions in LTC-ICs, pre-CFCs, $S^+K^+L^-$ and S^+L^- SP populations, all of which represent cells that are precursor to the majority of cells identified as CFCs (Johnson and Nicola 1984). It is possible that LTC-IC detection is also dependent in part on TPO signaling, as the factors that allow stromal cells to stimulate the sustained production of CFC from LTC-ICs are not currently known. However, the finding that *c-mpl*$^{-/-}$ cells respond similarly to *tpo*$^{-/-}$ cells suggests this may not be an important factor. In addition, such a criticism could not pertain to the pre-CFC results since these measured cells that produced CFCs in response to stimulation by SF, FL and IL-11, nor to the results from quantitating the $S^+K^+L^-$ and S^+L^- SP populations which are cells that are measured directly. It thus seems likely that TPO plays a non-redundant role at a very early level in the hematopoietic hierarchy.

It might then be argued that the reduction seen in CFC numbers is due exclusively to a decrease in compartment size already introduced at a much earlier stage in HSC differentiation and is not corrected by a later compensatory mechanism. The argument against this possibility is that events downstream of pre-CFC/ $S^+K^+L^-$/ S^+L^- SP cells are TPO-responsive, at least in vitro. Here this was shown by the strong synergistic response (as measured by CFC output) obtained when normal $S^+K^+L^-$ or

S^+L^- SP pre-CFCs were co-stimulated by TPO, SF, and FL (in comparison to the CFC outputs obtained when either TPO alone or SF plus FL only were present, Figure 2). A similar ability of c-mpl activation to enhance the production of CFCs from more primitive cells has been reported by Kaushansky et al (1995) and others (Goncalves et al 1997; Mitjavila et al 1998) and c-mpl has been shown to be expressed by a broader spectrum of early cells than those with HSC activity (Vigon et al 1992; Vigon et al 1993; Rollinger-Holzinger et al 1998). However, the decreased output of CFCs seen in bulk cultures of $S^+K^+L^-$ or S^+L^- SP cells from *tpo-/-* or *c-mpl-/-* mice could be largely explained by decreased frequency of input pre-CFC supporting the concept that the effects seen in vivo might also be largely due to poorly compensated effects of lack of TPO signaling at the earlier stages of HSC differentiation.

Thus, prevailing data would favor the hypothesis that TPO acts as a non-redundant regulator in vivo at multiple early stages of hematopoiesis in the normal adult and possibly during recovery of the system post-transplant. To address this question in a more definitive fashion would now require comparative assessments of the in vivo CFC generating potential of individual HSCs from *c-mpl*$^{-/-}$ and +/+ mice and of +/+ HSCs transplanted into *tpo*$^{-/-}$ and +/+ hosts. Such experiments are important because of the evidence that in vitro activation of gp130 can substitute for activation of c-mpl to obtain the same output of CFCs from +/+ pre-CFCs also stimulated by SF and FL and that co-stimulation of pre-CFCs with TPO as well as IL-11 or IL-6 (as activators of gp130) plus SF and FL gave no further enhancement in CFC production beyond that achieved just with SF, FL and IL-11 or IL-6 (Figure 2).

It is interesting to note that the defects reported here and by others in the generation of primitive hematopoietic cells in adult *c-mpl*$^{-/-}$ and *tpo*$^{-/-}$ mice have not been

seen at early stages of the ontogeny of hematopoiesis. Alexander et al (1996) examined day 14 fetal liver progenitors in *c-mpl*$^{-/-}$ mice and found no difference in their numbers compared to +/+ littermates. However, by the time of birth, reductions in hematopoietic progenitors could already be seen (Alexander et al 1996; Solar et al 1998). This suggests that TPO either does not play a significant role as a regulator of early progenitor production in the fetus or it is redundant with other growth factors whose effects can compensate for a loss of TPO signaling. Other examples of changes in the growth factor responses of primitive hematopoietic cells between fetal and adult life have been described previously (Zandstra et al 1997; Rebel et al 1995) and may be part of a larger programmatic change that occurs in the HSC compartment during ontogeny and affects cell cycle duration and gene expression (Nicolini et al 1999; Oh et al 2000).

tpo$^{-/-}$ and *c-mpl*$^{-/-}$ progenitors show defective responses to other growth factors

While the results of the present series of experiments as well as the work of previous investigators establish TPO as an important factor in the production of multiple hematopoietic lineages, the nature of its role in this process is not clear. Is the mechanism of its effect to alter survival of primitive progenitors, stimulate proliferation of primitive progenitors or more committed progenitors, or to induce multi-potent progenitors to differentiate? Studies support all three roles for TPO under different conditions. HSCs have the potential to be influenced by TPO as evidenced by the fact that repopulating capacity segregates with *c-mpl* expression (Solar et al 1998). Several studies have shown that TPO, acting in concert with SF or other growth factors, speeds the entry of progenitors into cell cycle (Ku et al 1996) and decreases the time to the first

cell division of purified HSCs in single cell cultures (Sitnicka et al 1996; Ema et al 2000). The latter studies also showed that TPO supported HSC self-renewal, with at least one of the daughter cells having preserved HSC activity. Yagi et al (1999) found that the addition of TPO to murine LTCs could enhance the HSCs output of these cultures. Piacibello et al (1997) also found TPO could enhance the proliferation of human progenitors in certain culture conditions. Other investigations have found that TPO alone in culture permits the survival of $S^+K^+L^-$ cells (Shimomura et al 2000) and maintains but does not expand HSC activity (Matsunaga et al 1998). While TPO can act to expand or maintain primitive progenitors, in more committed progenitors, it may support differentiation. Sitnicka et al (1996) found that a more differentiated subset of cells did not proliferate but formed colonies in the presence of TPO and Ramsfjell et al (1996) showed that TPO alone or with SF and FL increased the number and size of multilineage clones produced by S^+L^- cells.

The investigation reported here confirmed that both +/+ and *tpo-/-* cells with pre-CFC activity were TPO-responsive (Figures 2b and 3b). As TPO clearly exerts a positive effect on +/+ progenitors in culture, it was of interest to determine what the effect of TPO would be on pre-CFCs and pre-LTC-ICs from mice whose cells had never been stimulated by TPO. These experiments indicated that the response of *tpo*$^{-/-}$ pre-CFCs to TPO ws similar to that of +/+ cells, but blunted in absolute numbers of CFCs generated because of the reduced frequency of pre-CFCs in the *tpo*$^{-/-}$ mice.

The findings of previous in vivo studies showing that injection of TPO alleviates the hematopoietic deficiencies in *tpo*$^{-/-}$ mice (Kimura et al 1998) shows however that TPO can have some restorative action. For example, injection of TPO to *tpo*$^{-/-}$ mice completely restores normal levels of platelets, megakaryocytic progenitors and CFCs of

other lineages. This response could reflect the ability of TPO to exert a greater effect in vivo due to other factors not examined here. TPO may also induce a greater corrective effect if introduced at an earlier stage of hematopoiesis. Thus in vivo, re-introduction of the previously lacking factor to *tpo*$^{-/-}$ mice could act on more primitive hematopoietic cells which are then capable of producing normal LTC-IC and CFC levels.

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