

## Permissive role of thrombopoietin and granulocyte colony-stimulating factor receptors in hematopoietic cell fate decisions *in vivo*

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**ABSTRACT** The question of whether extracellular signals influence hematopoiesis by instructing stem cells to commit to a specific hematopoietic lineage (instructive model) or solely by permitting the survival and proliferation of predetermined progenitors (permissive model) has been controversial since the discovery of lineage-dominant hematopoietic cytokines. To study the potential role of cytokines and their receptors in hematopoietic cell fate decisions, we used homologous recombination to replace the thrombopoietin receptor gene (*mpl*) with a chimeric construct encoding the extracellular domain of *mpl* and the cytoplasmic domain of the granulocyte colony-stimulating factor receptor (G-CSFR). This chimeric receptor binds thrombopoietin but signals through the G-CSFR intracellular domain. We found that, despite the absence of a functional *mpl* signaling domain, homozygous knock-in mice had a normal platelet count, indicating that *in vivo* the cytoplasmic domain of G-CSFR can functionally replace *mpl* signaling to support normal megakaryopoiesis and platelet formation. This finding is compatible with the permissive model, according to which cytokine receptors provide a non-specific survival or proliferation signal, and argues against an instructive role of *mpl* or G-CSFR in hematopoietic cell fate decisions.

The mechanism of how hematopoietic cytokines exert lineage-dominant effects on hematopoiesis *in vivo* is not completely understood (1, 2). These effects are mediated by cell surface receptors, which belong to the cytokine receptor superfamily and signal by activating Janus kinases (Jak) and several downstream signaling pathways (3). The instructive model postulates that these signals force stem cells to commit to a particular hematopoietic lineage. This model would require that the signals generated by each receptor be specific and noninterchangeable. In support of the instructive model, experiments in immortalized hematopoietic cell lines demonstrated that the expression of lineage-specific markers can be induced by cytokine signaling (4–6). However, from other studies of immortalized cell lines (7, 8) or primary hematopoietic progenitors, researchers (9) reached the opposite conclusion and favored the permissive model, which predicts that hematopoietic cell fate decisions are independent of extracellular signals and the role of cytokines is to provide nonspecific survival and proliferation signals.

Thrombopoietin (TPO) and granulocyte colony-stimulating factor (G-CSF) are potent hematopoietic cytokines that exert lineage-dominant effects on hematopoiesis *in vivo* and selectively increase the production of platelets (10, 11) and granulocytes (12), respectively. Although both activate a similar set

of downstream signaling molecules *in vitro* (3), analysis of Jak2-deficient mice demonstrated that *in vivo* different pathways are essential, i.e., *mpl* function requires Jak2, whereas G-CSF receptor (G-CSFR) can signal in the absence of Jak2 (13, 14). *mpl* and G-CSFR are good candidates to play an instructive role, because both are expressed on early hematopoietic progenitor or stem cells (15, 16) and are able to induce lineage-specific differentiation of immortalized cell lines *in vitro* (5, 17).

Mice deficient for *mpl* are viable but display severe thrombocytopenia and a reduction in megakaryocytes and multipotential progenitors (16, 18–20). Thus, *mpl* deficient mice can be used as a genetic background to introduce mutant receptors and assess their capability to complement the platelet deficiency *in vivo*. Here we have used a knock-in (ki) approach to test whether the G-CSFR-signaling domain can rescue the *mpl* knock-out (ko) phenotype. We observed a complete rescue of the thrombocytopenia and a substantial rescue of the deficiency at the megakaryocyte progenitor level.

### MATERIALS AND METHODS

**DNA Constructs.** A chimeric *mpl*/G-CSFR cDNA was generated by recombinant PCR with the primers 5'-CTGG-GCCTACTGCTGCTATCCCACCGCCGACTCTG-3' and 5'-CAGAGTCCGGCGGTGGGATAGCAGCAGTAGGC-CCAG-3', subcloned into the expression vector pGD, and completely sequenced. Stably transfected BaF3 clones were selected in G418 and assayed for proliferation in response to TPO as previously described (21). To generate a targeting construct for the *mpl*/G-CSFR chimera, genomic clones for *mpl* were isolated from a  $\lambda$  FIX II Sv129 genomic DNA library (Stratagene). A 0.7-kb *Kpn*I fragment comprising exons 9 and 10 was subcloned into pGEM-7. A cDNA encoding the G-CSFR-signaling domain was fused in frame to *mpl* exon 10 encoding the transmembrane domain by recombinant PCR. The resulting amino acid sequence of the junction between *mpl* and G-CSFR sequences is LLGLLLL-*KRRGKT* (the G-CSFR sequence is in italic). A simian virus 40 polyadenylation signal and a PGK-neo gene flanked by lox sites were added, and the entire construct was subcloned into pGEM-3 as a *Kpn*I-*Xba*I fragment. A 3' homology fragment of 800 bp was generated by PCR with the primers 5'-TGCTCTAGAAAGGCCACGGT-TACCACCGAT-3' and 5'-TGCTCTAGAAACAGAACCA-GGAAG-3' and ligated into the unique *Xba*I site in pGEM-3. This construct was excised as a *Kpn*I-*Sal*I fragment and ligated

Abbreviations: TPO, thrombopoietin; *mpl*, TPO receptor (= the cellular homolog of the myeloproliferative leukemia virus oncogene); G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; ko, knockout; ki, knock-in; CFU-Meg, colony-forming unit-megakaryocytes; Jak, Janus kinase.

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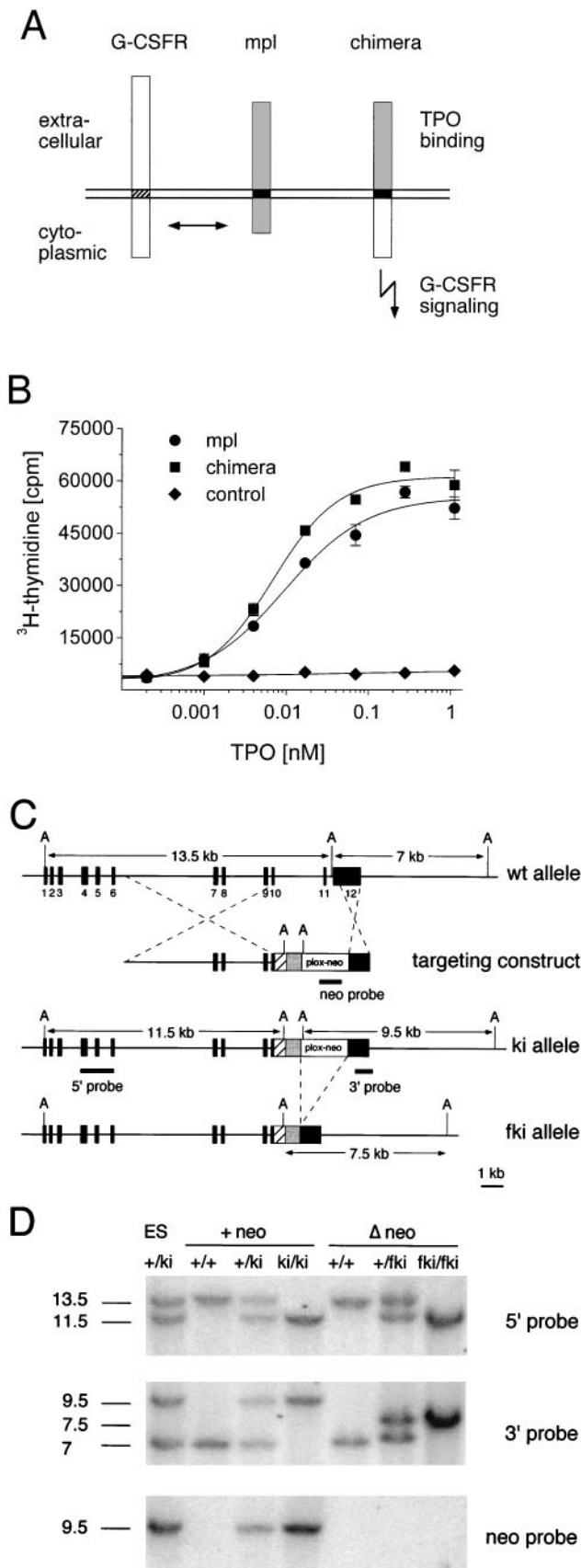


FIG. 1. The *mpl*/*G-CSFR* chimera. (A) Domain swap and the resulting chimeric protein. Hatched box, transmembrane domain of the *G-CSFR*; solid box, *mpl* transmembrane domain. (B) Proliferation of BaF3 cells transfected with expression vectors carrying the indicated cDNAs in response to increasing TPO concentrations. (C) Strategy used for gene targeting of the *mpl* locus. Solid boxes represent exons.

into a pGEM-11 vector containing the 5' homology region starting in intron 6, comprising exons 7 and 8, and ending with the *KpnI* site in intron 8. The final targeting vector was designated pMG-loxNEO.

**Embryonic Stem (ES) Cell Culture and Blastocysts Injection.** TC-1 ES cells (22) were transfected with pTG-loxNEO DNA linearized with *SaI* and selected in 200  $\mu\text{g}/\text{ml}$  G418 (Geneticin, GIBCO/BRL), starting after 48 h. G418-resistant colonies were picked on day 10, and pools of 12 clones were screened for homologous recombination by PCR. Single clones were derived from replica plates by PCR testing of single ES clones. Targeted clones were expanded, and homologous recombination was confirmed by Southern analysis. ES cells were injected into C57BL/6 blastocysts as described (23), and blastocysts were implanted into pseudopregnant mice. The chimeric progeny were identified by coat color chimerism. Several chimeric mice from one targeted ES clone transmitted the mutant allele through the germ line. Heterozygous +/*ki* mice were intercrossed to obtain homozygous *ki/ki* mice. To excise the lox-NEO gene from the *ki* locus, *ki/ki* mice were crossed with a cre recombinase-expressing deleter mouse strain (24).

**RNA Analysis.** Ribonuclease protection analysis was performed as described (25). Total RNA from mouse spleen was prepared by the acid phenol method (26), and 25  $\mu\text{g}$  was used for analysis. For the simultaneous detection of wild-type and chimeric transcripts, we generated a riboprobe by subcloning a PCR fragment of the chimeric cDNA amplified with the primers 5'-AAGGTGCCGTTTCACAGCTAC-3' (sense) and 5'-ACTGTGGGCTGGGTCTGGCA-3' (antisense) into pKS-Bluescript. This riboprobe protects a 479-nt fragment for the full-length chimeric *mpl*/*G-CSFR* transcripts and a 428-nt fragment for the wild-type *mpl* mRNA.

**Fractionation of Bone Marrow Cells and Flow Cytometry.** Bone marrow cells from femurs and tibiae were flushed with cold CATCH buffer (129 mM NaCl/8.6 mM  $\text{Na}_2\text{HPO}_4$ /1.6 mM  $\text{KH}_2\text{PO}_4$ /13.6 mM sodium citrate/11.1 mM glucose/1 mM adenosine/2 mM theophylline/2.3  $\mu\text{M}$  prostaglandin  $\text{E}_1$ /1% BSA). Megakaryocytes were enriched by centrifugation through a Percoll (Pharmacia) step gradient (27): approximately  $1 \times 10^8$  cells in 4 ml of CATCH medium were mixed with 3 ml of PBS/Percoll (density 1.02 g/ml) and layered over 4 ml of PBS/Percoll (density 1.05 g/ml). These gradients were centrifuged for 20 min at  $400 \times g$  at 4°C. The cell band was removed and washed once in CATCH buffer; the content of megakaryocytes was estimated by analyzing a Wright stained cytopsin preparation according to morphological criteria for megakaryocytes. A 10- to 20-fold enrichment of megakaryocytes compared with the unfractionated bone marrow was achieved. Approximately  $10^6$  cells for each genotype were incubated with previously optimized concentrations of fluorescein isothiocyanate-labeled rat anti-mouse CD41 (PharMingen) and/or biotinylated hamster monoclonal antibodies directed against the extracellular domain of mouse *mpl* (28). Streptavidin-PE (GIBCO) was used to detect expressing cells. The appropriate rat and hamster isotype controls were purchased from PharMingen. All antibody incubations were done in the presence of an Fc-blocking agent (PharMingen). Anal-

Exons 11 and 12 were replaced with a cDNA for the *G-CSFR*-signaling domain (hatched box), followed by a simian virus 40 polyadenylation signal (shaded box). Excision of the neo cassette by cre recombinase generates the floxed *ki* (fki) allele. A, *ApaI* recognition site. (D) Southern analysis of the *mpl* locus after *ApaI* digestion of DNA from the targeted ES clone (ES), compared with genomic DNA from mice before (+ neo), and after germ-line excision of the plox-neo gene ( $\Delta$  neo). Numbers indicate sizes of restriction fragments in kilobases. The same membrane was successively probed with the three hybridization probes that are depicted in C.

ysis was performed on a FACScan (Becton Dickinson) with the CellQuest software.

**Blood Counts.** Blood was obtained by cardiac puncture. Approximately 300  $\mu$ l of blood was immediately mixed with EDTA, and blood counts were performed with a Tecnicon H-1 counter (Bayer, Tarrytown, NY) calibrated for the mouse.

**Clonal Culture of Hematopoietic Progenitors.** Clonal cultures of hematopoietic cells were performed as described (29). Briefly, to assay for myeloid and granulocytic progenitors,  $5 \times 10^4$  bone marrow cells were placed in 0.3% agar in Iscove's modified Dulbecco's medium supplemented with 20% horse serum and 10 mg/ml BSA (Boehringer Mannheim) and stimulated with Hemostim M2100 (StemCell Technologies, Vancouver) at a final concentration of 2% and 100 ng/ml human G-CSF (Roche, Basel). These conditions resulted in optimal stimulation of colony-forming units for granulocytes (CFU-G), granulocytes-macrophages (CFU-GM), and macrophages (CFU-M). To assess the number of megakaryocytic progenitors (CFU-Meg),  $2 \times 10^5$  bone marrow cells were grown in 0.3% agar under serum-free conditions (29) and were stimulated with 2% Hemostim M2100 and 10% serum-free conditioned medium of a TPO-expressing NIH/3T3 cell line (21). The cultures were incubated for 7 days at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. CFU-M and CFU-G were scored by morphology, whereas CFU-Meg were identified by histochemical staining for acetylcholinesterase. Colonies containing at least three acetylcholinesterase-positive cells were scored as CFU-Meg (30).

## RESULTS

To test a potential instructive role of mpl in cell fate determination *in vivo*, we designed a chimeric receptor and exchanged the mpl-signaling domain with the corresponding G-CSFR domain (Fig. 1A). Because the signaling specificity of cytokine receptors is determined by the intracellular domain (8, 31), the resulting chimeric receptor will bind TPO but should elicit the G-CSFR signal. This was confirmed by transfection of a cDNA expression construct into a factor-dependent hematopoietic cell line (Fig. 1B). The mpl/G-CSFR chimera provided a dose-dependent proliferative signal in response to TPO (Fig. 1B) and activated the expected Jak and STAT isoforms (not shown). To study the effect of this chimeric receptor in a setting most closely resembling the physiologic situation *in vivo*, we used a knock-in (ki) approach (32) and replaced the signaling domain of mpl with the corresponding G-CSFR sequences by homologous recombination in ES cells (Fig. 1C). Because mpl exons 11 and 12, which encode the cytoplasmic signaling domain (33), were deleted during homologous recombination and an analogous deletion completely inactivated mpl signaling *in vitro* (34), no mpl-specific signal can be generated by the gene product of this targeted allele. Instead, a cDNA encoding the entire cytoplasmic domain of the G-CSFR was placed in frame with the mpl transmembrane domain, changing the signaling specificity to G-CSFR. We confirmed the correct structure of the targeted mpl ki allele by Southern analysis (Fig. 1D).

The chimera is under the control of the mpl regulatory elements and is expected to be expressed in the same tissues and lineages as mpl. By a ribonuclease protection assay, we found that expression of the chimeric mRNA paralleled expression of endogenous mpl mRNA in tissues from heterozygous +/ki mice (Fig. 2A and B). Unexpectedly, the expression levels of the chimeric mRNA were lower than the wild-type mpl mRNA. The reason for this is not clear. We found no rearrangements in the targeted allele (Fig. 1D), and we can also exclude inhibitory effects of the neo cassette on gene expression, because germ-line excision of the neo cassette by mating to a transgenic mouse expressing the cre recombinase had no effect on chimeric mRNA levels (Fig. 2C). We con-

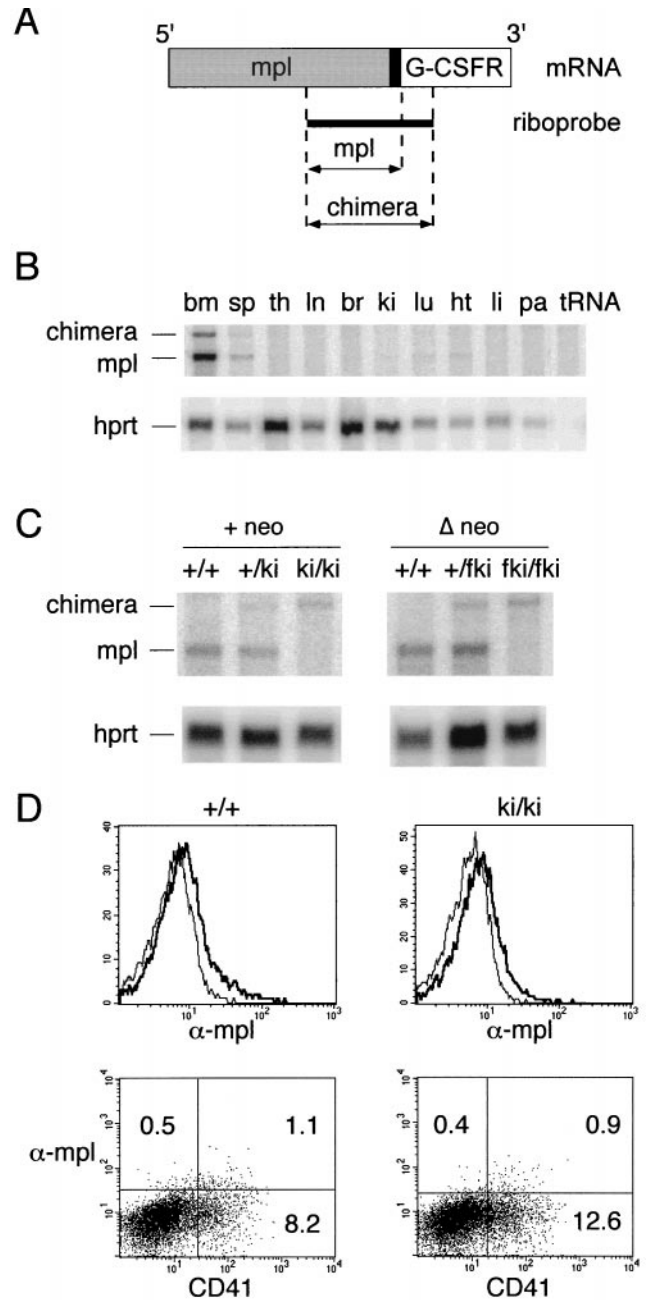


FIG. 2. Expression of chimeric mpl/G-CSFR. (A) Ribonuclease protection assay. Position of riboprobe used to distinguish wild-type from chimeric mpl/G-CSFR mRNA. Solid box, transmembrane domain. Arrows, length of protected fragments for the wild-type mpl and chimeric transcripts. (B) Expression of wild-type and chimeric mRNA in tissues of heterozygous +/ki mice: bm, bone marrow; sp, spleen; th, thymus; ln, mesenteric lymph node; br, brain; ki, kidney; lu, lung; ht, heart; li, liver; pa, pancreas. A riboprobe for mouse hypoxanthine phosphoribosyltransferase (hprt) was used as an internal control for RNA loading. (C) Expression of chimeric mRNA in spleens of ki and wild-type mice. On the left is the original mouse strain containing the neo cassette (+ neo); on the right is the mouse strain after germ-line excision of the plox-neo gene ( $\Delta$  neo); fki, the floxed ki allele. (D) Flow cytometric analysis of Percoll-fractionated bone marrow cells. In the top row is shown the biotinylated anti-mpl with streptavidin-PE (thick line) versus isotype control (thin line). In the bottom row is two-color staining with anti-mpl/streptavidin-PE and fluorescein isothiocyanate-labeled anti-CD41. Numbers indicate percentages of cells in each quadrant.

firmed the correct composition of the chimeric mRNA by sequencing the full-length reverse transcription-PCR product from homozygous ki mice (ki/ki; not shown). To follow the

expression of the chimeric protein we performed flow cytometry with two biotinylated monoclonal anti-mpl antibodies directed against the extracellular domain of mouse mpl (28). Because cells of the megakaryocytic lineage normally account for less than 1% of bone marrow cells, only a very small population of mpl-positive cells was detectable in +/+ and ki/ki bone marrow (not shown). To more reliably detect mpl, we prepared an approximately 20-fold enriched population of megakaryocytes by a Percoll step gradient centrifugation (27). A reproducible shift was observed in both genotypes when compared with a hamster isotype control (Fig. 2D, upper row). The same result was observed with the second anti-mpl monoclonal (not shown). The low intensity of fluorescence is not surprising, because cytokine receptors are expressed in low numbers on the cell surface. Consistent with the higher expression of mpl mRNA (Fig. 2C), the +/+ mice showed a slightly higher maximal intensity of fluorescence. To confirm lineage-restricted expression of the chimeric protein, we performed a double stain with fluorescein isothiocyanate-labeled anti-CD41 versus a mixture of the two biotin-streptavidin-PE-labeled anti-mpl monoclonal antibodies (Fig. 2D, lower row). We found in both +/+ and ki/ki that approximately 70% of anti-mpl-positive cells were also positive for the megakaryocyte marker CD41, indicating that the majority of cells expressing the mpl extracellular domain belongs to the megakaryocytic lineage. Thus, the mpl/G-CSFR is expressed in the same lineage-restricted fashion as wild-type mpl.

To assess the function of the chimeric receptor, we performed blood counts and compared ki/ki mice with ko/ko and wild-type littermates (Fig. 3A). As has been described (18), mpl ko/ko mice had a reduction of platelets to approximately 20% of wild-type levels ( $P < 10^{-6}$ ). In contrast, ki/ki mice had platelet counts not significantly different from wild type, indicating that the G-CSFR-signaling domain of the chimeric receptor can rescue the ko/ko phenotype and promote platelet formation *in vivo*. The platelets of ki/ki mice showed normal morphology, and the mice had no bleeding problems. Thus, the G-CSFR-signaling domain can fully promote platelet formation *in vivo*.

Next, we determined the numbers of committed progenitors by performing *in vitro* colony assays from bone marrow cells of ki/ki mice and controls (Fig. 3B). The megakaryocyte pro-

genitors (CFU-Meg) were clearly higher in ki/ki mice than in ko/ko mice, indicating that the chimeric receptor can, to a large extent, rescue the defect at the progenitor level. The slight reduction of CFU-Meg in ki/ki mice, as compared with wild type, might be because of the reduced expression of the chimeric receptor (Fig. 2). In support of this interpretation, we found that further reducing the gene dosage and thereby the expression of the chimeric receptor by generating compound heterozygous (ki/ko) mice resulted in platelet levels between ki/ki and ko/ko (Fig. 3A). Unfortunately, it is not possible to directly assess expression of the chimeric protein on committed megakaryocytic progenitors. In addition to rescuing the megakaryocytic lineage, we found that the chimeric receptor also normalized the neutrophils (Fig. 3A), as well as CFU-G and CFU-GM (Fig. 3B), which were previously reported to be reduced in ko/ko mice (19, 20).

DISCUSSION

Our results contradict the expectations of the instructive model (Fig. 4A). This model predicts that mpl provides specific signals that force the stem cells and multipotent progenitors to

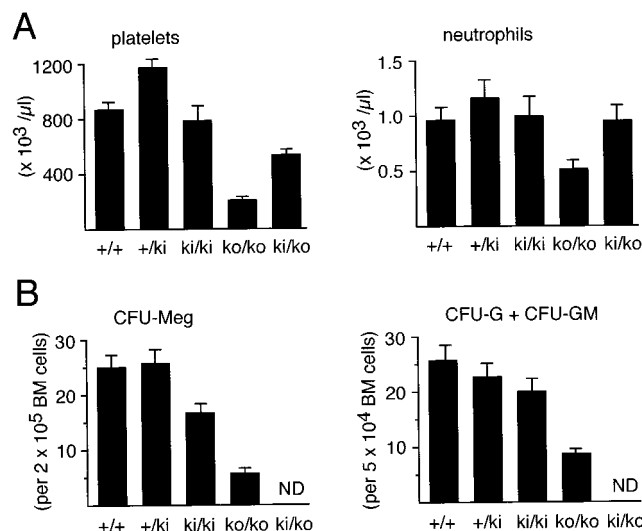


FIG. 3. Blood counts and bone marrow progenitor numbers. (A) Analysis of platelets and neutrophil granulocytes. Results represent the means  $\pm$  SEM of nine mice for each genotype except for ki/ko mice, for which the means  $\pm$  SEM of four mice are given. (B) Analysis of megakaryocyte progenitors (CFU-Meg), granulocytic progenitors (CFU-G), and granulocyte-macrophage progenitors (CFU-GM). Results represent the means  $\pm$  SEM of three mice. ND, not determined.

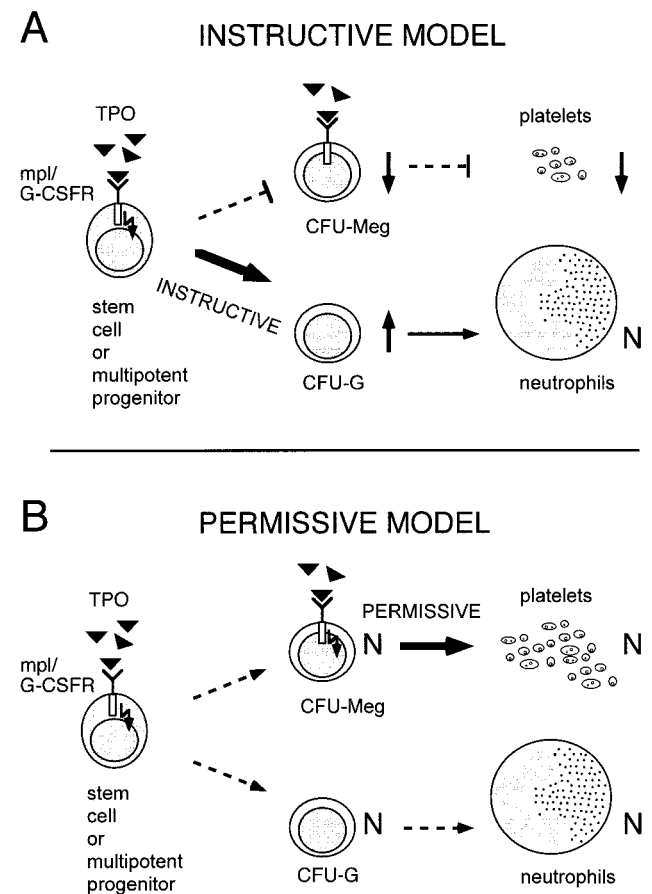


FIG. 4. Effects of the chimeric mpl/G-CSFR on megakaryopoiesis and granulopoiesis in ki/ki mice. (A) Predictions of the instructive model. Signals generated by the G-CSFR part of the chimeric receptor (open box) instruct the stem cell or early progenitor to commit to the granulocytic lineage (thick arrow). This results in increased numbers of granulocytic progenitors (CFU-G). Because the mpl signaling domain is absent in ki/ki mice, commitment to the megakaryocytic lineage is reduced (dashed lines). This results in a reduction of megakaryocyte progenitors (CFU-Meg) and platelets similar to the mpl ko/ko mice. N, normal. (B) Predictions of the permissive model. Commitment of stem cells and early progenitors is independent of cytokine signaling (dashed arrows). G-CSFR signals generated by the chimera can substitute for the absence of mpl signaling resulting in a normal megakaryopoiesis.

commit to the megakaryocytic lineage. In *ki/ki* mice the exons encoding the *mpl*-signaling domain have been deleted by homologous recombination (Fig. 1C). Therefore, these mice cannot generate the *mpl*-specific signals and should display an *mpl ko* phenotype, because the G-CSFR signaling domain is predicted to be specific for the granulocytic lineage and unable to substitute for the absence of *mpl*-specific signals (Fig. 4A). Furthermore, because of a surplus of G-CSF signals in early progenitors, an increase in CFU-G/CFU-GM might be expected (Fig. 4A). However, we did not observe *ko* levels of CFU-Meg and platelets or an increase in granulocytic progenitors (Fig. 3).

In contrast, the permissive model predicts that commitment is independent of extracellular signals and that cytokine receptors merely provide a nonspecific survival and/or proliferation signal in predetermined progenitors (Fig. 4B). Lineage specificity is obtained through lineage-restricted expression of the receptor and the high affinity and specificity between ligand and receptor. In agreement with this model, we found that, in a setting closely resembling the physiologic situation *in vivo*, the signaling domains of *mpl* and G-CSFR were functionally interchangeable. Although, our results do not exclude the possibility that other classes of ligands and their receptors might have instructive effects on hematopoietic lineage commitment, they strongly argue for a permissive role of *mpl* and G-CSFR in hematopoiesis.

We believe that the lower than physiological levels of expression of the chimeric receptor mRNA and possibly also protein do not preclude us from reaching these conclusions for the following reasons. Despite the lower expression levels, we observed a complete rescue of the platelet deficiency and a substantial rescue of the CFU-Meg (Fig. 3). This result indicates that the chimeric receptor is functional and that the G-CSFR-signaling domain can promote platelet formation *in vivo*. A rescue would not be observed if *mpl* signals were necessary for megakaryocytic lineage commitment or differentiation. Thus, an instructive *mpl*-specific signal is not required for normal megakaryopoiesis. It remains to be shown whether this holds true for other members of the cytokine receptor superfamily.

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