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Monocyte/Macrophage Dysfunctions Do Not Impair the Promotion of Myelofibrosis by High Levels of Thrombopoietin

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Several lines of evidence indicate that the megakaryocyte/platelet lineage is crucial in myelofibrosis induction. The demonstration that NOD/SCID mice with functionally deficient monocytes do not develop fibrotic changes when exposed to thrombopoietin (TPO) also suggests an important role for monocyte/macrophages. However, in this animal model, the development of myelofibrosis is dependent on the level of TPO. This study was conducted to investigate whether NOD/SCID mice exposed to high TPO levels mediated by a retroviral vector would be refractory to the development of bone marrow fibrosis. We show that TPO and TGF-β1 in plasma from NOD/SCID and SCID mice engrafted with TPO-overexpressing hemopoietic cells reach levels similar to the ones reached in immunocompetent mice, and all animals develop a myeloproliferative disease associated with a dense myelofibrosis at 8 wk posttransplantation. Monocytes in NOD/SCID mice are functionally deficient to secrete cytokines such as IL-1α in response to stimuli, even under TPO expression. Surprisingly, the plasma of these mice displays high levels of IL-1α which was demonstrated to originate from platelets. Together, these data suggest that completely functional monocytes are not required to develop myelofibrosis and that platelets are able, under TPO stimulation, to synthesize inflammatory cytokines, which may be involved in the pathogenesis of myelofibrosis and osteosclerosis.

Fibrosis is a prominent clinical complication in several disorders and occurs particularly in the lung, kidney, heart, or liver. In human hemopoietic tissues, myelofibrosis is less frequent but is typically associated with two distinct clinical entities: chronic idiopathic myelofibrosis (IM) with myeloid metaplasia and acute myelofibrosis with myelofibrosis (1). Bone marrow fibrosis occurs as a cytokine-mediated secondary response to a clonal malignant event originating in a multipotent hemopoietic stem cell (2, 3) and is characterized by excessive deposits of extracellular matrix proteins (4). In vivo and in vitro studies have involved several cytokines in the aberrant stromal reaction, with a strong emphasis on the pleiotropic cytokine TGF-β1. Through fibroblast stimulation, this cytokine is able to produce extracellular matrix, induce cell adhesion proteins, and enhance expression of proteases that inhibit enzymes involved in the degradation of the extracellular matrix. TGF-β1 is secreted by numerous cell lineages, but mainly by monocytes/macrophages and megakaryocytes (MKs)/platelets. Several lines of evidence obtained both from studies of patients with IM (5, 6) or murine models ending with myelofibrosis (7–9) are in favor of a crucial role of MK in myelofibrosis induction. However, other studies suggested a central role for the monocyte/macrophage lineage. This theory is based on two different observations: 1) Rameshwar et al. (10) reported that monocytes from patients with IM are spontaneously activated and secreted abnormally TGF-β1; and 2) Frey et al. (11) reported that thrombopoietin (TPO) overexpression in SCID mice led to myelofibrosis, but not in NOD/SCID mice. Because NOD/SCID mice differ from SCID mice by impaired mononuclear phagocyte functions (12, 13), the authors postulated that monocytes and macrophages must be required for promotion of myelofibrosis in TPO-overexpressing mice. However, these experiments were performed with a human TPO cDNA vectorized in an adenovirus construct that leads to a relatively low elevation of plasma TPO. In the present study, to ensure a high and sustained TPO level, we used a retroviral vector encoding a mouse TPO cDNA to overexpress TPO in NOD/SCID, SCID, and immunocompetent wild-type (WT) mice. All TPOhigh mice invariably developed a myeloproliferative syndrome associated with a myelofibrosis and osteosclerosis. At any time during the follow-up, TPO and TGF-β1 plasma levels were substantially and similarly elevated in the three groups of mice. Our data provide evidence that the monocyte/macrophage lineage does not play a crucial role in the development of the myelofibrosis induced by TPO. In addition, platelets and MKs when stimulated are able to synthesize IL-1α and thus to replace monocytes in the synthesis of inflammatory cytokines, which may be involved in the pathogenesis of myelofibrosis and osteosclerosis.
Materials and Methods

Mice

All procedures were approved by the local Institut Gustave Roussy ethics committee. WT C57BL/6j mice (Janvier) were maintained at the Institute Gustave Roussy animal facility under specific pathogen-free conditions. SCID mutant mice (C57BL/6j-scidscid) (14) (Charles River Laboratories) and double NOD/SCID mutant mice (15) (NOD/IL2s-cid/scid obtained from J. Dick (University Health Network, Toronto, Ontario, Canada)) were bred under sterile conditions and maintained in isolators. All mice used in this study have the same C57BL/6j genetic background.

Infection procedure

Six- to 8-wk-old WT, SCID, or NOD/SCID male mice were used as bone marrow donors. Seven- to 10-wk-old WT, SCID, or NOD/SCID female mice were recipients. The infection procedure was performed as described previously (7, 16). Briefly, 4 days after 5-fluorouracil treatment (one injection of 150 mg/kg administered i.p.), total bone marrow cells were cocultured with 1 × 10⁶ MPZenTPO virus-producing GP-E-86 cells in DMEM supplemented with antibiotics, 20% FBS and murine IL-3 (100 U/ml), murine IL-6 (20 ng/ml), and murine stem cell factor (20 ng/ml). All cytokines were purchased from R&D Systems. After 4 days, nonadherent cells were harvested. An aliquot was used immediately in clonogenic progenitor assays to determine the percentage of infected colony-forming cells (CFC) as described previously (7, 16). The remaining cells were inoculated i.v. via the retro-orbital sinus into irradiated hosts (9.5 Gy for WT mice or 2.5 Gy for SCID and NOD/SCID mice, x-ray apparatus, single dose) in a ratio of two donors per one recipient. Fifty-one recipients were studied.

Determination of chimerism

Two approaches were used in parallel. First, a PCR analysis was performed on myeloid colonies derived from the bone marrow of sacrificed animals at week 8 after transplantation, with primer sets corresponding to specific sequences on the Y chromosome and with actin primers as control (16). The second approach was a fluorescent in situ hybridization (FISH) analysis performed on bone marrow cells from mice sacrificed at week 8 after transplantation. Briefly, 1 × 10⁶ bone marrow cells were cytospun and fixed with a 4% formaldehyde solution. Slides were successively incubated for 5 min in 0.1 M HCl and three times in a permeabilization solution containing 0.5% saponin and 0.5% Triton X-100 (Sigma-Aldrich), and then washed in a solution containing 20% glycerol. After three freezing/thawing steps, slides were incubated in 20% glycerol, dehydrated with ethanol, and incubated in a 70% formamide solution at 75°C, and dehydrated again. A denatured rhodamine-labeled Y probe (purchased by QBiogene) was hybridized overnight at 37°C in a humidified atmosphere. The slides were incubated in a 60% formamide solution at 43°C, washed in SSC 0.1% Tween 20, and counterstained with Vectorshield and 4′,6-diamidino-2-phenylindole (Vector Laboratories). Numeration of Y-labeled cells was performed on 500 cells for each slide using a fluorescence microscope (Nikon Eclipse 600).

Hematologic evaluation and histopathology

Blood from the orbital plexus was collected in citrated tubes at indicated times. Nucleated blood cells, hematocrit level, and platelet counts were determined using an automated blood counter calibrated for mouse blood (MS9; Schleisong Melet). Differential cell counts were performed after May–Grunwald-Giemsa staining. Platelet-poor plasma was used for determination of TPO, IL-1α, IL-1β, and TGF-β1 levels. Eight weeks after transplantation, mice in each group were killed. Bones were excised and cleaned of soft tissue. One femur and one tibia were fixed in formaldehyde, decalcified, and paraffin embedded. Spleen, liver, and pulmonary samples were fixed and embedded in the same manner. Sections (4.5 μm) were stained with H&E, periodic acid-Schiff, and Giemsa for overall cytology analysis. Reticulin fibers were revealed by silver staining according to Gordon Sweet method. In parallel, 10³ bone marrow, spleen, and blood cells were grown in semisolid medium for CFC analysis as described previously (16, 17).

TUNEL analysis

To detect and quantify MK apoptosis, we performed in situ TUNEL analysis on histological sections of bone marrow and spleen from control mice (WT, SCID, and NOD/SCID) or TPO⁶⁶ mice (WT, SCID, and NOD/SCID). DNA strand breaks generated during apoptosis were identified using In Situ Cell Death Detection Kit (POD; Roche) according to the manufacturer’s instructions. Samples were analyzed under a light microscopy, and quantification of apoptotic MKs was performed. Results were expressed as the percentage of apoptotic MKs among the total MK population.

Production of bone marrow-derived monocyes

Bone marrow cells were harvested from femurs and tibia of age- and sex-matched control or TPO⁶⁶ mice (WT and NOD/SCID). Cells were cultured (1.6 × 10⁶ cells/ml), as described previously (18), in DMEM supplemented with antibiotics, 10% FBS, and 10% CSF-1-conditioned medium (provided by M. Lebastic, Unité Immunophysiologie et Parasitisme Intracellulaire, Institut Pasteur, Paris, France) with and without 10 U/ml IFN-γ. Bone marrow cells were cultured at 37°C in a humidified incubator (5% CO₂) with an addition of fresh medium at day 3. After 5 days in culture, nonadherent cells were removed and centrifuged for 10 min at 400 × g. Pellets were suspended in DMEM with antibiotics and 10% FBS by flushing through, successively, 25-, 27-, and 30-gauge needles, and single-cell suspensions were produced. Cells (2 × 10⁶ cells/ml) were seeded in fresh medium alone (DMEM supplemented with antibiotics, 10% FBS, and 10% CSF-1-conditioned medium) or in fresh medium containing Escherichia coli LPS at 10 μg/ml. After a 24-h incubation period, culture supernatants were harvested and used for determination of IL-1α level.

Platelets and leukocytes preparations

Mice were anesthetized and bled by cardiac puncture. Blood from age- and sex-matched control or TPO⁶⁶ mice (WT and NOD/SCID) was collected onto 3.8% Na citrate (9 vol blood/1 vol citrate). Whole blood (500 μl) was mixed with 500 μl of buffer saline glucose citrate (BSGC) (129 mM NaCl, 13.6 mM Na₂ citrate, 11.1 mM D-glucose, 1.6 mM K₂PO₄, and 8.6 mM NaH₂PO₄) and spun at 400 × g. Platelet-rich plasma was layered over a 10-ml Sepharose B column (Pharmacia), and pellets were used for leukocyte RNA analysis after red cell lysis. To minimize leukocyte contamination, only the upper (9 of 10) platelet-rich plasma was used for gel filtration. Platelets were collected, adjusted at a concentration of 50 × 10⁶/ml, and pelleted by centrifugation. Pellets were used for protein or RNA analysis. For protein analysis, platelets were lysed by three successive freezing/thawings, suspended in 1 ml of BSGC, and centrifuged to remove platelet debris. Supernatants were used to determine IL-1α level.

ELISA

Plasma TPO levels and IL-1α and IL-1β levels in plasma or supernatant were determined with the appropriate murine Quantikine Kits from R&D Systems, according to manufacturer’s instructions. Sensitivity limits of the assays were 62.5, 4.69, and 7.8 pg/ml, respectively, for TPO, IL-1α, and IL-1β. Human TGF-β1 immunoassay, which detects only active forms of TGF-β1, was used for circulating TGF-β1 level determination (Quantikine Kit; R&D Systems). Samples were assayed after acidification (active and latent forms) according to manufacturer’s instructions. The sensitivity of the assay was 31.2 pg/ml active TGF-β1.

Real-time PCR for cytokine quantification

Total RNA extraction from circulating platelets and leukocytes of age- and sex-matched control or TPO⁶⁶ WT mice was performed using either TRIzol reagent (Invitrogen Life Technologies) or the RNacys Mini Kit (Qiagen), following the manufacturer’s recommendations. Total RNA was reverse transcribed in first-strand cDNA using random hexamers (Roche Diagnostics) and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen Life Technologies).

PCR amplifications were conducted on the LightCycler (Roche Diagnostics) in duplicate or triplicate capillaries in a final volume of 11 μl. Two microliters of cDNA along with primers (500 nM; Roche Diagnostics) and SYBG Green containing mix (QuantiTect SYBR Green; Qiagen) were used per reaction. CD45-, CD11b-, IL-1α-, IL-1β-, and TGF-β1-specific primers were obtained from published literature or designed by the LightCycler Probe Design software (version 1.0; Idaho Technology). Identical thermal cycling conditions were used for all targets, allowing efficient synthesis of all amplicons (Table I). Crossing Points were determined with the FitPoint method (LightCycler software, version 3.3) at a fluorescence threshold that was 10-fold the background level in the early stage of the exponential phase of the reaction. Using the tool Gene Expression Macro from Bio-Rad, relative expression values were calculated for each sample compared with a sample calibrator (a pool of platelets from control WT mice). The underlying principles and calculations are described in studies by VandeSompele et al. (19). Briefly, the Bio-Rad tool calculates a gene expression normalization factor for each sample based on the geometric mean of a user-defined number of housekeeping genes. To allow a better normalization between samples within this study, three housekeeping genes were used, namely β-actin, GAPDH.
Table I. Primers and amplicons for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession no.</th>
<th>Source</th>
<th>Primer Sequences (5'-3')</th>
<th>Tm</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NM_001001303</td>
<td>Lean et al.</td>
<td>FW CggATTTggCCgTATTgg</td>
<td>83.4</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(21)</td>
<td>RV ggTCTCgCTCCTgAAgATg</td>
<td>84.8</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FW ggACTCCTAgTgAgCGTACgg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_007393</td>
<td>Colle et al.</td>
<td>FW AgTTCCTCgCTCAgCAT</td>
<td>82.5</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20)</td>
<td>RV CTTgACTTgCgTTAggg</td>
<td>80.7</td>
<td>235</td>
</tr>
<tr>
<td>αMI/CD41</td>
<td>NM_010575</td>
<td>Probe Design</td>
<td>FW gTATgCCTACCTgTCg</td>
<td>81.4</td>
<td>167</td>
</tr>
<tr>
<td>IL-1α</td>
<td>NM_010554</td>
<td>Probe Design</td>
<td>FW gTgCAATTAAACgCTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>NM_008361</td>
<td>Probe Design</td>
<td>RV AggCAggCAgTAAGC</td>
<td>82</td>
<td>243</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>NM_011577</td>
<td>Probe Design</td>
<td>FW CACACAgAgAGTTgACAC</td>
<td>82.8</td>
<td>223</td>
</tr>
<tr>
<td>CD11b</td>
<td>NM_008401</td>
<td>Probe Design</td>
<td>FW gCggACTACATgCgTAAGA</td>
<td>79.2</td>
<td>122</td>
</tr>
<tr>
<td>CD45</td>
<td>NM_011210</td>
<td>Probe Design</td>
<td>FW TAsgACATCgCgCCAcg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RV CTggAgAgACAggTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Accession nos. of housekeeping and target genes along with characteristics of primers and amplification products are depicted. The LightCycler run protocol used in this study included a denaturation and Taq polymerase activation program (95°C for 15 min), an amplification program repeated 40 times with a temperature transition rate of 20°C/s (denaturation step, 95°C for 10 s; annealing step, 54°C for 30 s; elongation step, 72°C for 25 s with a single fluorescent measurement), a melting curve program (72–95°C with a heating rate of 0.1°C/s and a continuous fluorescent measurement), and finally a cooling step to 35°C. FW, Forward primer; RV, reverse primer; Tm, melting temperature is the temperature, where 50% of the DNA is single stranded.

Results

Hematopoietic chimerism

The different x-ray irradiation protocols used for SCID and NOD/SCID mice compared with WT may lead to different levels of chimerism and subsequently different pathological processes in hematopoietic tissues. We analyzed the host chimerism in sex-mismatched transplantations using two approaches: PCR on Y chromosome (donors were males, recipients were females) on bone marrow-derived myeloid colonies, and FISH on Y chromosome analysis on whole nucleated bone marrow cells. Chimerism levels were similar in TPOhigh WT, CD45, and NOD/SCID mice when studied on myeloid colonies 8 wk posttransplantation (69 ± 18% (n = 3 mice), 62 ± 15% (n = 4 mice), and 67 ± 16% (n = 5 mice), respectively). Moreover, studies by FISH on marrow cells gave identical results as those from PCR.

Retroviral transfer efficiency

To obtain a highly elevated and long-lasting overexpression of a mouse TPO molecule in immunodeficient SCID and NOD/SCID mice, we used a previously described retroviral gene transfer protocol in which lethally irradiated hosts are hematologically repopulated with transduced stem cells (7). Because SCID and NOD/SCID mice could not be heavily irradiated before bone marrow transplantation (23), and to overcome a possible risk of a low chimerism, 10 times more cells (6–8 × 10⁶ cells) were engrafted into 2.5 Gy-irradiated SCID and NOD/SCID mice as compared with 6 × 10⁵ cells injected in 9.5 Gy-irradiated WT mice. Transduction efficiencies were determined at the end of the infection protocol before transplantation in myeloid progenitor-derived colonies. For each mouse genotype, two independent infection experiments were performed with a total of 51 hosts engrafted. As shown from PCR analysis, the percentage of colonies demonstrating the integrated TPO cDNA was comparable in the three groups of mice (Table II). Plasma levels of TPO were determined in the three groups of engrafted mice over time using an ELISA. Six weeks postengraftment, TPO concentration in plasma was 1,000- to 10,000-fold higher than normal. Thereafter, the magnitude of the increase was comparable in the three groups (Fig. 1).

Hematological parameters are similar in all TPOhigh mice

Platelet numbers in TPOhigh WT mice increased over 8 wk, achieving values 4-fold higher than control WT mice. TPOhigh SCID mice present the same features. In TPOhigh NOD/SCID mice, platelet numbers increased less significantly with barely a 2-fold increment over controls (Fig. 2A). Macroplatelets were observed in all mice for which blood smears were performed. As previously reported, no correlation between TPO levels and platelet numbers was observed (17). Nucleated blood cells were increased in all groups of mice (Fig. 2B) due to a striking increment in mature polymorphonuclear neutrophils in association with immature myeloid precursor cells (data not shown). Leukocyte numbers were more elevated in TPOhigh WT and SCID mice than in TPOhigh NOD/SCID mice, without significant difference. TPOhigh WT and NOD/SCID mice became progressively anemic; however, TPOhigh SCID mice spontaneously corrected this anemia over time (Fig. 2C).

Table II. Analysis of transduction efficiency in progenitor cells injected to WT, SCID, and NOD/SCID mice

<table>
<thead>
<tr>
<th>Bone Marrow Cells</th>
<th>TPO/Actin</th>
<th>Transduced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>20/23</td>
<td>87</td>
</tr>
<tr>
<td>SCID</td>
<td>65/73</td>
<td>89</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>66/74</td>
<td>89</td>
</tr>
</tbody>
</table>

* WT, SCID, and NOD/SCID pooled bone marrow cells were transduced and used to reconstitute 10 WT, 19 SCID, and 22 NOD/SCID. Each pool was plated at the end of the infection protocol, and CFC-derived colonies were picked from methylcellulose. Samples were analysed by PCR with specific primers for the viral TPO gene and with actin primers to ascertain the presence of material. No significant differences were observed between the three groups of mice with more than 80% transduced progenitor cells.
The number of progenitor cells in bone marrow, spleen, and blood was studied at week 8 posttransplantation in the three groups of mice. Bone marrow progenitor numbers were 3- to 5-fold decreased in all TPO\textsuperscript{high} mice in comparison to controls, parallel to decrease of total bone marrow cell number. All TPO\textsuperscript{high} mice developed a splenomegaly. Probably due to the myeloproliferative disorder, spleen CFC increased achieving values 22-fold higher than control in TPO\textsuperscript{high} WT mice, 17-fold higher than control in TPO\textsuperscript{high} SCID mice, but only 2-fold higher than control in TPO\textsuperscript{high} NOD/SCID mice. Finally, blood-circulating CFC were 30- to 1,500-fold increased at 8 wk posttransplantation (Table III).

These data indicate that overproduction of TPO in hosts repopulated with WT, SCID, and NOD/SCID hemopoietic cells resulted in a similar myeloproliferative syndrome. A similar feature was observed at the histological examination. However, some hematological differences were observed. A lower increase in all cell types was observed in TPO\textsuperscript{high} NOD/SCID mice compared with TPO\textsuperscript{high} WT and SCID mice.

**Fibrosis on femur and spleen sections is similar in all TPO\textsuperscript{high} mice**

Histological sections revealed a massive hyperplasia of maturing granulocytic cells and dysmorphic MKs found in large clusters. Erythroblasts were rare. Increased reticulin fibers were noted in bone marrow samples at 8 wk postengraftment. No differences were observed between the TPO\textsuperscript{high} WT, SCID, or NOD/SCID mice (Fig. 3). Osseous cortex was a bit thicker in TPO\textsuperscript{high} NOD/SCID than in TPO\textsuperscript{high} SCID or WT, but some bone trabeculae were seen in the marrow cavity in the three lineages. In spleen samples, hyperplasia of the red pulp was observed without white pulp in TPO\textsuperscript{high} SCID and NOD/SCID. MKs were extremely abundant as well as neutrophils. Numerous MKs were observed in large sheets in TPO\textsuperscript{high} WT in the red pulp and in TPO\textsuperscript{high} NOD/SCID (more frequently than in TPO\textsuperscript{high} SCID mice). Apoptotic figures of MKs were often seen in TPO\textsuperscript{high} WT and SCID mice. Erythroid cells were observed. Similar reticulin deposits were seen 8 wk posttransplantation in all TPO\textsuperscript{high} mice analyzed.

**MK apoptosis is increased in TPO\textsuperscript{high} mice**

To study whether myelofibrosis could be the consequence of a dysmegakaryopoiesis, we studied whether apoptotic MKs were increased in TPO\textsuperscript{high} mice. Apoptotic MKs were detected using TUNEL analysis on histological sections of both bone marrow and spleen from control and TPO\textsuperscript{high} mice (WT, SCID, and NOD/SCID). Percentages of apoptotic MKs are illustrated in Table IV. Apoptosis of bone marrow and spleen MKs was increased in all TPO\textsuperscript{high} mice in comparison to controls. Representative sections of spleen from WT control (Fig. 3G) and TPO\textsuperscript{high} WT (Fig. 3H) mice are shown in Fig. 3.

**Increased plasma TGF-β1 level**

In this TPO\textsuperscript{high} mouse model, fibrosis development has been reported to be a direct consequence of high TGF-β1 level in blood and bone marrow fluids (17). TGF-β1 level was thus measured in the three groups of TPO\textsuperscript{high} mice at different times posttransplantation. As reported before in an immunocompetent background (16), TGF-β1 levels were invariably increased in plasma as soon as 8 wk posttransplantation (Fig. 1). Results are presented as the mean ± SEM of four to 10 animals per experimental group. Values in control WT, SCID, and NOD/SCID were 0.78 ± 0.04, 0.56 ± 0.07, and 1.05 ± 0.08 ng/ml, respectively. Results of statistical analysis with the two-tailed Student’s t test are as follows: SCID and NOD/SCID mice vs WT mice; *, p < 0.05 and **, p < 0.001.
as 3 wk after transplantation (data not shown). These levels increased with time, leading to a 2- to 4-fold higher level than in control mice after 6 wk (Fig. 2D). Such an increase in TGF-β1 level was also noticed in TPO\textsuperscript{high} SCID mice but was detected later in the TPO\textsuperscript{high} NOD/SCID mice background (2-fold higher level than normal at 8 wk only).

### Increased plasma IL-1α level

Because it has been reported that TGF-β1 is regulated by IL-1 in human IM (24), and that NOD/SCID monocytes have a major defect in the synthesis of IL-1 (12), we investigated the IL-1α and IL-1β levels in these mice. IL-1α and IL-1β were both quantified in plasma with specific ELISA. Surprisingly, we found a dramatic increase (up to 8-fold the normal value) in IL-1α levels in all TPO\textsuperscript{high} mice analyzed at 6 and 8 wk posttransplantation (Fig. 4A). However, IL-1β was detected only 8 wk posttransplantation (data not shown).

### TPO overexpression does not correct the monocyte defect of NOD/SCID mice

To test whether TPO overexpression could correct the IL-1 synthesis defect of NOD/SCID monocytes, monocytes were cultured from control WT, control NOD/SCID, TPO\textsuperscript{high} WT, and TPO\textsuperscript{high} NOD/SCID bone marrow isolated cells in the presence of CSF-1 and IFN-γ. After 5 days, LPS was added (10 μg/ml), and 24 h later, the culture supernatants were harvested and assayed for IL-1α level. Monocytes from NOD/SCID mice secreted less IL-1α in response to IFN-γ stimulation compared with WT monocytes as described previously (12). TPO overexpression did not improve IL-1α secretion, indicating that monocyte dysfunction in NOD/SCID mice was not corrected by TPO overexpression (Fig. 5).

### Platelets are a major source of IL-1α in TPO\textsuperscript{high} mice

Lindeman et al. (25) have reported that human platelets synthesize and secrete IL-1β in response to activation stimuli. Because we observed an increase in IL-1α level in TPO\textsuperscript{high} NOD/SCID mice that have deficient monocytes, we hypothesized that platelets from these mice could also synthesize high levels of IL-1α in response to TPO.

Platelets from control WT and NOD/SCID mice or from TPO\textsuperscript{high} WT and NOD/SCID mice were collected and lysed. Lysates (50 × 10⁶ platelets/ml) were harvested and assayed for IL-1α concentration. Levels of IL-1α in platelets from control WT mice were low and below the threshold of detection in control NOD/SCID mice (Fig. 4B). However, when platelets were collected from TPO\textsuperscript{high} WT and NOD/SCID mice, a marked elevation in IL-1α content in platelets was detected, indicating that TPO could

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### Table III. CFC analysis of TPO\textsuperscript{high} mice 8 wk postengraftment with MPZen-TPO-bone marrow cells

<table>
<thead>
<tr>
<th>Mice</th>
<th>Bone marrow</th>
<th>Spleen</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells × 10⁶/femur</td>
<td>CFU × 10⁷/femur</td>
<td>Weight, mg</td>
</tr>
<tr>
<td>Control WT</td>
<td>14.8 ± 2.4</td>
<td>21.7 ± 3.4</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>Control SCID</td>
<td>12.2 ± 2.0</td>
<td>14.9 ± 0.8</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>Control NOD/SCID</td>
<td>10.3 ± 1.0</td>
<td>17.9 ± 2.7</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>TPO\textsuperscript{high} WT</td>
<td>3.1 ± 1.0</td>
<td>4.4 ± 0.8</td>
<td>894 ± 141</td>
</tr>
<tr>
<td>TPO\textsuperscript{high} SCID</td>
<td>1.1 ± 0.1</td>
<td>3.5 ± 1.2</td>
<td>384 ± 63</td>
</tr>
<tr>
<td>TPO\textsuperscript{high} NOD/SCID</td>
<td>5.1 ± 0.9</td>
<td>5.2 ± 0.3</td>
<td>366 ± 52</td>
</tr>
</tbody>
</table>

*Values are means ± SEM of data from three controls and three TPO\textsuperscript{high} mice in each group. Progenitor cells numbers (CFC) were calculated from the number of colonies obtained from 10⁵ cells grown in semisolid medium.

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### Discussion

IM is a myeloproliferative disease known as a stem cell disorder with involvement of both the myeloid and the lymphoid lineage (3), whereas the associated stromal reaction in the bone marrow environment is considered reactive and cytokine mediated (2). The current pathogenetic hypothesis is that clonal proliferation of MKs or monocytes or both leads to collagen fibrosis and new bone formation called osteosclerosis. Development of mouse models has provided important insights into the pathophysiological mechanisms of myelofibrosis with special regard to the role of MK. The two most important mouse models are characterized by either systemic overexpression of TPO (7, 11, 26–28) (TPO\textsuperscript{high} mice) or MK lineage restricted under expression of the transcriptional factor GATA-1 (8) (GATA-1\textsuperscript{high} mice). These models display hyperplasia of MK, a result of TPO-driven proliferation in the TPO\textsuperscript{high} mice (29), and impaired MK proliferation and maturation in the GATA-1\textsuperscript{high} mice (30). However, the observation that TPO overexpression in SCID mice (T and B cell deficient) (14) led to myelofibrosis, but not in NOD/SCID mice (T and B cell as well as
multiple other defects in innate immunity including NK and monocyte/macrophage function (12, 13), suggested an important role for the monocyte/macrophage lineage (11). We hypothesized that insufficiently high TPO levels, and not lack of a specific cell population, was the reason why NOD/SCID mice failed to develop myelofibrosis.

In this study, we demonstrated, using a retroviral vector, that high TPO levels induce myelofibrosis in the NOD/SCID mice similar to the one developed by WT or SCID mice. All of these mice developed a very similar disease, but, strikingly, SCID mice had only a mild anemia. This demonstrates that T and B cells (deficient in SCID and NOD/SCID mice) as well as monocyte/macrophage (deficient in NOD/SCID mice) are probably not directly involved in the development of myelofibrosis in this murine model. In fact, both level and site of TPO overexpression appear to influence myelofibrosis development (31). For example, first-described transgenic mice overexpressing TPO (32) using a liver-specific apolipoprotein E promoter did not exhibit myelofibrosis or osteosclerosis, whereas recently described TPO transgenic mice driven by the IgH promoter (33) developed myelofibrosis and osteosclerosis with the same plasma TPO level as the former model.

The TPOhigh and GATA-1low mice develop a myeloproliferative disorder featuring numerous aspects of the human disease including dysmegakaryopoiesis (5). Histological analyses at the time of myelofibrosis occurrence showed very large-sized platelets with empty granules and dysmorphic MK with pycnotic nuclei and increased emperipolesis (34). Recently, it has been demonstrated that a unique and acquired mutation of JAK2 (JAK2V617F) is associated with BCR/ABL-negative myeloproliferative disorders including IM (35–38). When the JAK2V617F is expressed in mice, it leads to the development of a polycythemia vera, which progresses to myelofibrosis (C. Lacout, D. F. Pisani, M. Tulliez, F. Moreau-Gachelin, W. Vainchenker, and J. L. Villeval, submitted for publication). The fact that a constitutively active JAK2 may be associated with IM in human suggests that alteration in the TPO/c-mpl signaling may be at the origin of this dysmegakaryopoiesis. It is possible that in the TPOhigh mice, a long-lasting stimulation leads to a Mpl traffic defect and an altered signaling (S. Constantinescu,
unpublished observation). This may secondarily imply transcription factors such as GATA-1, which are implicated in MK survival, proliferation, and differentiation. In favor of this hypothesis, it has been shown by Vannucchi et al. (39) that in TPOhigh and GATA-1low mice, TPO, GATA-1, and TGF-β1 are linked in an upstream-downstream relationship.

However, how a high level of TPO can induce myelofibrosis development remains unknown. TPO could regulate synthesis and release of cytokines or induce a dysmegakaryopoiesis. In this study, we show that TPO overexpression directly or indirectly induces apoptosis of MKs in bone marrow and spleen from TPOhigh mice using TUNEL technology, regardless of genetic background. This result suggests that the main mechanism of TPO-induced myelofibrosis is related to a dysmegakaryopoiesis.

In this study, we were able to demonstrate that TPO induced an increased synthesis of IL-1α by platelets. Indeed, numerous cytokines are involved in myelofibrosis and osteosclerosis, including TGF (TGF-β1), platelet-derived growth factor, basic fibroblastic growth factor, and IL-1. Experiments in TPOhigh mice have established the pivotal role of the hemopoietic cell derived-TGF-β1 in the promotion of myelofibrosis (17) and the role of the stromal osteoprotegerin produced by the microenvironment in the promotion of osteosclerosis (16). Accordingly, we found elevated TGF-β1 plasma levels in all TPOhigh mice analyzed and a slight increase of the TGF-β1 transcript in platelets. Thus, TGF-β1 appears to originate from platelets. The slight increase of its transcript could be explained by the 2- to 4-fold higher thrombocytosis of both bone marrow and spleen from control and TPOhigh mice. Results are presented as number of apoptotic stained MKs per number of total MKs (percentage of apoptotic MK).

In addition, there is strong evidence that IL-1 is implicated in the regulation of the bone mass. Notably, IL-1 is able to induce osteoprotegerin synthesis (42) by stromal cells, inhibiting the development of osteoclasts and consequently inducing the development

<table>
<thead>
<tr>
<th>Mice</th>
<th>Bone Marrow (%)</th>
<th>Spleen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control WT</td>
<td>3/164 (1.8)</td>
<td>0/27 (0)</td>
</tr>
<tr>
<td>Control SCID</td>
<td>6/226 (2.7)</td>
<td>6/315 (1.9)</td>
</tr>
<tr>
<td>Control NOD/SCID</td>
<td>7/486 (1.4)</td>
<td>3/85 (3.5)</td>
</tr>
<tr>
<td>TPOhigh WT</td>
<td>32/992 (3.2)</td>
<td>52/1478 (3.5)</td>
</tr>
<tr>
<td>TPOhigh SCID</td>
<td>42/716 (5.9)</td>
<td>67/783 (8.6)</td>
</tr>
<tr>
<td>TPOhigh NOD/SCID</td>
<td>63/981 (6.4)</td>
<td>57/862 (6.6)</td>
</tr>
</tbody>
</table>

*Apoptosis of MK was detected using TUNEL analysis on histological sections of both bone marrow and spleen from control and TPOhigh mice. Results are presented as the mean ± SEM of four to 10 animals per experimental group. Values in control WT, SCID, and NOD/SCID were 73 ± 8, 68 ± 9, and 29 ± 4 pm/ml, respectively. Results of statistical analysis with the two-tailed Student t test are as follows: SCID and NOD/SCID mice vs WT mice: *p < 0.05 and **p < 0.001. B, IL-1α quantification in platelets. Platelets from control WT and NOD/SCID mice or from TPOhigh WT and NOD/SCID mice were collected and lysed by three freezing/thawing. The lysates (50 × 10^6 platelets/ml) were harvested and assayed for IL-1α levels. Results are presented as the mean ± SEM of three animals per experimental group. IL-1α platelet levels were low for WT mice and under detection threshold for NOD/SCID mice. However, TPOhigh mice, particularly NOD/SCID, showed marked elevation in IL-1α platelet levels. Results of statistical analysis with the two-tailed Student t test are as follows: TPOhigh mice vs control mice.

### FIGURE 4. IL-1α quantifications. A, IL-1α quantification in plasma. Results are presented as the mean ± SEM of four to 10 animals per experimental group. Values in control WT, SCID, and NOD/SCID were 73 ± 8, 68 ± 9, and 29 ± 4 pm/ml, respectively. Results of statistical analysis with the two-tailed Student t test are as follows: SCID and NOD/SCID mice vs WT mice: *p < 0.05 and **p < 0.001. B, IL-1α quantification in platelets. Platelets from control WT and NOD/SCID mice or from TPOhigh WT and NOD/SCID mice were collected and lysed by three freezing/thawing. The lysates (50 × 10^6 platelets/ml) were harvested and assayed for IL-1α levels. Results are presented as the mean ± SEM of three animals per experimental group. IL-1α platelet levels were low for WT mice and under detection threshold for NOD/SCID mice. However, TPOhigh mice, particularly NOD/SCID, showed marked elevation in IL-1α platelet levels. Results of statistical analysis with the two-tailed Student t test are as follows: TPOhigh mice vs control mice.

### FIGURE 5. IL-1α secretion by bone marrow-derived monocytes in response to IFN-γ and LPS. Monocytes from control WT and NOD/SCID mice or from TPOhigh WT and NOD/SCID mice were cultivated in CSF-1 with or without IFN-γ. After 5 days, the culture medium was replaced with fresh medium containing LPS (10 µg/ml). After an additional 24-h incubation period, the cultured supernatants were harvested and assayed for IL-1α levels. Monocytes from NOD/SCID mice secreted less IL-1α in response to IFN-γ stimulation compared with WT monocytes as described previously (12). TPO overexpression does not improve IL-1α secretion. Results are presented as the mean ± SEM of three animals per experimental group. Bone marrow cells from each mouse were assayed separately in duplicate culture. Results of statistical analysis with the two-tailed Student t test are as follows: CSF-1 with IFN-γ vs CSF-1 alone.
of osteosclerosis. Thus, in the future, TPO overexpression in double IL-1α/IL-1β knockout mice (43) may be important in understanding the precise role of IL-1 in the promotion of both myelofibrosis and osteosclerosis. In addition, platelets are able to synthesize several other inflammatory molecules such as CD40-L and inflammatory signaling by regulated interleukin 1β synthesis. Journal of Cellular and Molecular Medicine 15:497–507.


