Thrombopoietin Stimulates Colony-Forming Unit-Megakaryocyte Proliferation and Megakaryocyte Maturation Independently of Cytokines That Signal Through the gp130 Receptor Subunit

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Thrombopoietin (Tpo), the ligand for the c-Mpl receptor, is a major regulator of megakaryopoiesis. Treatment of mice with Tpo raises the platelet count fourfold within a few days. Conversely, c-mpl knock-out mice have platelet counts that are 15% that of normal. The subunit structure of the c-Mpl receptor is not fully understood. Some cytokines that stimulate megakaryopoiesis (IL-6, IL-11, leukemia inhibitory factor, and oncostatin M) bind to receptors that use gp130 as a signal transduction subunit. For these reasons, we determined whether gp130 function was required for Tpo-induced signal transduction. Murine marrow cells were cultured in semi-solid media in the presence of Tpo or IL-3, with or without a neutralizing anti-gp130 monoclonal antibody (RX187) or a soluble form of c-Mpl receptor (soluble Mpl) that blocks Tpo bioactivity, and the numbers of colony-forming unit-megakaryocyte (CFU-Meg) colonies were counted on day 5. Murine marrow cells were also cultured in suspension under serum-free conditions for 5 days, and megakaryocyte DNA content was measured by flow cytometry, as an index of nuclear maturation. The addition of RX187 did not block Tpo-induced CFU-Meg colony growth nor CFU-Meg nuclear maturation in suspension culture. However, IL-3-induced CFU-Meg colony growth and megakaryocyte nuclear maturation decreased in the presence of RX187. Soluble Mpl completely ablated Tpo-induced CFU-Meg growth, and partially blocked IL-3-stimulated CFU-Meg growth. Thus the effects of Tpo on megakaryopoiesis in vitro do not depend on cytokines that signal through gp130. Furthermore, it is unlikely that gp130 serves as a beta chain for the c-Mpl receptor, as Tpo signalling is unimpaired in the presence of RX187. In contrast, the effects of IL-3 on CFU-Meg growth are mediated in part through Tpo and through gp130-signalling cytokines.

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Materials and Methods

Megakaryocyte colony assays. B6D2F1 mice (Jackson Laboratories, Bar Harbor, ME), 8 to 10 weeks old, were killed by rapid
cervical dislocation, and marrow cells were obtained by flushing the femurs with Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Grand Island, NY) supplemented with 2% fetal calf serum (FCS; Hyclone, Logan, UT). The marrow cells were cultured in IMDM supplemented with 15% horse serum (Hyclone) and 5 x 10^{-2} molar β mercaptoethanol (Sigma Chemical Co, St Louis, MO) in 0.285% agar (DIFCO, Detroit, MI) in triplicate plates as previously described.8 Megakaryocyte colonies were counted after a 5-day incubation at 37°C in a humidified atmosphere containing 5% CO₂. The growth factors used included recombinant murine Tpo and recombinant murine IL-3, both expressed in BHK cells.16,17 Fifty units of Tpo or IL-3 activity were defined as that quantity that gave one-half maximal stimulation in the Baf3/Mpl proliferation assay, or in the murine marrow CFU-GM colony assay, respectively.18 In some experiments, recombinant human IL-11 (Genzyme, Boston, MA) was also used. A soluble form of c-Mpl receptor (soluble Mpl) was generated by truncation at the interface of the extracellular and transmembrane domains, was purified as previously described,24 and was provided by ZymoGenetics, Inc (Seattle, WA). Soluble Mpl is a specific competitive antagonist of Tpo activity.24

The RX187 rat antimonoclonal antibody (IgG2a) recognizes the extracellular domain of gp130, and neutralizes the bioactivity of IL-6 and IL-11 in vitro. To generate the RX187 antibody, a soluble form of the extracellular domain of murine gp130 was prepared by introducing a stop codon just above the transmembrane domain. Soluble gp130 was used to immunize a rat, and the resultant hybridomas were screened by flow cytometry for ability to recognize the native transmembrane form of murine gp130. The RX187 antibody was able to immunoprecipitate a 130 kD protein from murine megakaryocytes labeled with c-Mpl cDNA encoding murine gp130 had been introduced, but not from untransfected parental cells; control rat IgG2a did not immunoprecipitate the 130 kD protein. The RX187 antibody blocked the ability of the T1165 mouse plasmacytoma cell line to proliferate in the presence of IL-6 or IL-11.25 Further evidence of the neutralizing activity of the RX187 antibody is provided in the Results section. The control rat IgG2a antibody was obtained from Pharmingen, San Diego, CA.

To determine if the horse serum used for the megakaryocyte colony assays contained Tpo, a Tpo-responsive murine cell line, DA-1-Tpo26 was cultured in the presence of horse serum with or without soluble Mpl, and cell proliferation was quantitated. Parallel cultures of the DA-1-Tpo cells contained known quantities of Tpo. These experiments showed that the quantity of horse serum used for the Tpo-Meg colony assays would contribute approximately 2 U/mL of Tpo activity to the culture. Megakaryocyte colony assays containing 15% horse serum but no added growth factor had no CFU-Meg colonies, demonstrating that this quantity of Tpo alone was insufficient to support CFU-Meg colony growth.

Analysis of megakaryocyte ploidy. Murine marrow cells were cultured in serum-free suspension as previously described.8,16,24 Briefly, the marrow cells were cultured at a concentration of 1 x 10⁷ cells/mL in IMDM supplemented with 1% Nutridoma-SP (Boehringer Mannheim Corp, Indianapolis, IN), 5 x 10⁻³ mol/L β mercaptoethanol, and growth factors with or without RX187 antibody as described above. The cells were incubated at 37°C for 5 days. The DNA content of the megakaryocytes was analyzed by flow cytometry after labeling with the 4A5 monoclonal antibody and with propidium iodide as previously described.8,27 The 4A5 monoclonal antibody recognizes a 74 kD glycoprotein present on murine megakaryocytes and platelets,27,28 and was generously provided by Dr Sam Burstein (University of Oklahoma, Oklahoma City, OK). Briefly, the cells were labeled with the 4A5 monoclonal antibody (15 μg/mL) or with control rat IgG2a antibody in 0.15 mol/L NaCl (pH 7.4) containing 0.5% bovine serum albumin (Sigma) and 0.05% sodium azide (Sigma) for 30 minutes at 4°C, then incubated with FITC-conjugated goat antirat IgG (Jackson Immunoresearch Labs, Inc, West Grove, PA) for 1 hour at 4°C. The cells were then incubated with propidium iodide (50 μg/mL; Molecular Probes, Inc, Eugene, OR) in a hypotonic citrate solution, and analyzed in a Coulter Epics Elite flow cytometer.8

| Table 1. Effect of Blocking gp130 Activity on CFU-Meg Colony Growth |
|------------------|-----------------|-----------------|
| Experiment No    | Addition        | CFU-Meg Colonies/2 x 10⁶ Cells |
| 1                | Tpo             | 35.0 ± 4.3      |
|                  | Tpo + RX187     | 37.7 ± 2.4      |
|                  | Tpo + Sol Mpl   | 0               |
|                  | IL-3            | 22.7 ± 0.3      |
|                  | IL-3 + RX187    | 12.7 ± 0.3      |
|                  | IL-3 + Sol Mpl  | 10.0 ± 1.5      |
| 2                | Tpo             | 41.0 ± 2.2      |
|                  | Tpo + RX187     | 41.1 ± 4.3      |
|                  | Tpo + Sol Mpl   | 0               |
|                  | IL-3            | 33.0 ± 1.4      |
|                  | IL-3 + RX187    | 18.0 ± 1.2      |
|                  | IL-3 + Sol Mpl  | 15.3 ± 2.6      |
|                  | IL-3 + RX187 + Sol Mpl | 18.3 ± 3.9   |

Murine marrow cells were cultured in 0.286% agar in IMDM supplemented with 16% horse serum and 5 x 10⁻⁴ mol/L β mercaptoethanol and Tpo (2,000 U/mL) or IL-3 (150 U/mL). The RX187 antibody was added at a concentration of 1.5 μg/mL, and soluble Mpl was added at a concentration of 5 μg/mL. The number of CFU-Meg-derived colonies was counted on day 5. The data are presented as the mean ± SEM of triplicate plates. Five additional experiments gave similar results.

RESULTS

Megakaryocytic colony growth. We examined the ability of the neutralizing anti-gp130 monoclonal antibody RX187 to block growth of CFU-Meg colonies in the presence of Tpo or IL-3 (Table 1). In seven independent experiments, the addition of RX187 antibody did not affect the number of CFU-Meg colonies found in the presence of 2,000 U/mL of Tpo. In contrast to the results with Tpo, the addition of RX187 antibody blocked IL-3-stimulated CFU-Meg colony growth by approximately 45% to 45%. The addition of control IgG2a had no effect on CFU-Meg colony growth in the presence of Tpo or of IL-3 (data not shown). Soluble Mpl, which competes with cell surface c-Mpl for binding Tpo, completely inhibited Tpo-stimulated megakaryocyte colony growth, and inhibited IL-3-stimulated megakaryocyte colony growth by approximately 50% (Table 1). The horse serum used in the CFU-Meg colony assay contributed approximately 2 U/mL of Tpo activity to the culture; this may account in part for the ability of soluble Mpl to inhibit CFU-Meg colony growth in cultures containing IL-3.

To determine whether RX187 antibody would inhibit CFU-Meg colony growth in the presence of lower concentrations of Tpo, we cultured murine marrow cells with Tpo (0, 15, 60, 350, 1,000, or 2,000 U/mL) with or without RX187 antibody (Fig 1). The addition of RX187 antibody did not block Tpo-induced CFU-Meg colony growth in the presence...
enhanced CFU-Meg colony growth (Fig 3). The RX187 antibody was able to ablate the incremental effect of IL-11 on growth of CFU-Meg colonies (Fig 3), directly demonstrating the neutralizing activity of this antibody. Consistent with the data in Table 1 and Fig 1, the RX187 antibody did not diminish CFU-Meg colony growth below that seen with Tpo alone.

To further define the neutralizing activity of RX187 antibody, murine marrow cells were cultured in the presence of a range of concentrations of IL-11, with or without RX187 antibody or control IgG2a antibody (Fig 4). The addition of RX187 antibody blocked the effects of IL-11 on CFU-Meg colony growth. At high concentrations of IL-11, a modest number of CFU-Meg colonies was found even in the presence of RX187 antibody (Fig 4). This suggests that IL-11 and RX187 antibody compete for binding to the gp130 subunit of lower concentrations of Tpo (Fig 1). The combination of Tpo plus IL-3 is a very potent stimulant of megakaryocytic colony growth (Fig 2). The addition of RX187 antibody did not diminish CFU-Meg colony growth in the presence of Tpo plus IL-3 (Fig 2), suggesting that signal transduction via gp130 is not required for megakaryocytic colony growth under these conditions.

Although IL-11 exerts its predominant effects on megakaryocytic maturation rather than on CFU-Meg proliferation, IL-11 can synergize with Tpo to augment the growth of megakaryocytic colonies. The addition of IL-11 (10 to 100 ng/mL) to a submaximal concentration of Tpo (100 U/mL)
the receptor, and also demonstrates that the effects of RX187 antibody are not due to nonspecific toxicity. To ensure that appropriate concentrations of RX187 antibody were used for these experiments, an additional experiment examined the effects of varying amounts of the RX187 antibody on CFU-Meg colony growth in the presence of Tpo plus IL-11 (Table 2). The RX187 antibody at a concentration of 0.5 to 1.5 µg/mL completely blocked the incremental effect of IL-11 on CFU-Meg colony growth. The control IgG2a antibody had no effect (Table 2).

**Megakaryocyte nuclear endoreduplication.** We examined the DNA content of the megakaryocytes generated in serum-free suspension cultures in the presence of Tpo or IL-3, with or without RX187 antibody, to assess the effects of these cytokines on nuclear endoreduplication. Some megakaryocytes were produced even in the absence of exogenous growth factor, and the modal ploidy of these megakaryocytes was 16N (Fig 5). When RX187 antibody was added to the cultures containing no exogenous growth factor, megakaryocyte ploidy decreased (data not shown), suggesting that endogenous production of cytokines that require gp130 as a signal transduction subunit is contributing to megakaryocyte endoreduplication in these cultures. IL-6 and IL-11, which are produced by stromal cells, are likely candidate cytokines; IL-6 can also be produced by megakaryocytes.

The addition of Tpo profoundly increased megakaryocyte nuclear endoreduplication over that seen with no added growth factor (Fig 5). In the presence of Tpo, the majority of megakaryocytes were of 32-64N ploidy (Fig 5). The addition of RX187 did not diminish the proportion of 32N, 64N, and 128N megakaryocytes produced in the presence of Tpo (Fig 5). A relatively small proportion of the megakaryocytes generated in the presence of IL-3 exhibited 32N, 64N, or 128N DNA content (Fig 6), in contrast to megakaryocytes produced in the presence of Tpo. Neutralizing gp130 activity decreased megakaryocyte nuclear endoreduplication in the presence of IL-3, and virtually eliminated the production of 64N megakaryocytes (Fig 6). We interpret these results to mean that the modest effect of IL-3 to stimulate megakaryocyte nuclear endoreduplication is dependent on the presence of endogenous IL-6 or IL-11 in these cultures.

### Table 2. Effect of RX187 Antibody on CFU-Meg Colony Growth

<table>
<thead>
<tr>
<th>Addition</th>
<th>CFU-Meg Colonies/2 × 10⁶ Cells</th>
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<tbody>
<tr>
<td>Tpo (100 U/mL)</td>
<td>41.0 ± 2.2</td>
</tr>
<tr>
<td>Tpo + IL-11 (25 ng/mL)</td>
<td>59.0 ± 5.1</td>
</tr>
<tr>
<td>Tpo + IL-11 + RX187 (0.05 µg/mL)</td>
<td>63.7 ± 7.8</td>
</tr>
<tr>
<td>Tpo + IL-11 + RX187 (0.15 µg/mL)</td>
<td>55.7 ± 0.3</td>
</tr>
<tr>
<td>Tpo + IL-11 + RX187 (0.5 µg/mL)</td>
<td>41.0 ± 1.4</td>
</tr>
<tr>
<td>Tpo + IL-11 + RX187 (1.5 µg/mL)</td>
<td>43.0 ± 0.6</td>
</tr>
<tr>
<td>Tpo + IL-11 + IgG2a (0.15 µg/mL)</td>
<td>58.0 ± 1.2</td>
</tr>
<tr>
<td>Tpo + IL-11 + IgG2a (0.5 µg/mL)</td>
<td>62.0 ± 2.2</td>
</tr>
<tr>
<td>Tpo + IL-11 + IgG2a (1.5 µg/mL)</td>
<td>56.7 ± 2.3</td>
</tr>
</tbody>
</table>

Murine marrow cells were cultured as described in Table 1 in the presence or absence of RX187 antibody (0.05 to 1.5 µg/mL). The data represent the mean ± SEM of triplicate plates from one experiment.

**DISCUSSION**

Hematopoiesis in vivo is intricately controlled to maintain the numbers of circulating erythrocytes, leukocytes, and platelets with a narrow range under normal circumstances. Much is understood about the physiology of the key growth factors that regulate the terminal stages of erythropoiesis and myelopoiesis, and several of these cytokines have been in clinical use for approximately 10 years. In contrast, the cytokine that predominantly controls megakaryopoiesis had remained elusive. The cloning of the c-mpl proto-oncogene, and the subsequent use of c-Mpl receptor affinity chromatography to isolate its ligand in parallel to a functional cloning strategy, and other purification approaches, yielded Tpo. Contrary to predictions from experimental models of thrombocytopenia, and previous studies with partially purified molecules, recombinant Tpo was found to promote both the proliferation of CFU-Meg and the maturation of their progeny.

The network of hematopoietic cytokines is characterized...
cells were cultured as described in Fig 2 in the presence of no added growth factor (A), IL-3 (100 U/mL, B), or IL-3 plus RX187 (C). After 5 days of culture, the DNA content of the WNS was analyzed as described in Fig 2. The data from one of four similar experiments are shown.

Some of the members of the hematopoietic growth factor receptor superfamily share a common β chain, which plays a critical role in signal transduction. In this study, we used a neutralizing anti-gp130 monoclonal antibody to determine if gp130 was a component of the c-Mpl receptor complex, and to explore the extent to which the IL-6 family of cytokines contribute to Tpo-stimulated and to IL-3–stimulated megakaryopoiesis in vitro.

Ablation of signal transduction through gp130 did not alter the ability of Tpo to stimulate the cytoplasmic maturation of megakaryocytes or the proliferation of CFU-Meg or the nuclear endoreduplication of megakaryocytes. These results demonstrate that gp130 is not a component of the functional receptor complex for Tpo. Expression of a chimeric receptor consisting of the extracellular and transmembrane domains of the IL-4 receptor with the cytoplasmic domain of c-mpl is sufficient for signal transduction. Subsequent studies have dissected the regions of the c-mpl cytoplasmic domain that are required for activation of specific JAK and STAT proteins. However, because of the ubiquitous expression of gp130, these studies do not rule out its participation in Tpo signal transduction. Furthermore, the present results with the anti-gp130 monoclonal antibody RX187 show that the activity of IL-11 is not required for the full spectrum of Tpo biological effects to be realized in vitro. These data confirm and extend a report that a polyclonal antibody to IL-11 did not impair the ability of Tpo to stimulate CFU-Meg growth.

In contrast to the results with Tpo, the effects of IL-3 on megakaryopoiesis do require the cooperation of cytokines that signal through the gp130 receptor subunit. A monoclonal antibody that neutralizes the effects of IL-6 blocked the ability of IL-3 to enhance murine megakaryopoiesis. IL-3 can also induce megakaryocytes to produce IL-6, which can then stimulate megakaryocyte cytoplasmic maturation and nuclear endoreduplication via an autocrine loop mechanism. Studies with soluble Mpl have demonstrated that full megakaryocyte cytoplasmic maturation is dependent in part by Tpo and by the IL-6 family of cytokines.

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**REFERENCES**

7. Wallace PM, MacMaster JF, Rilema JR, Peng J, Burstein SA,
Shoyab M: Thrombocytopoietic properties of oncostatin M. Blood 86:1310, 1995


11. Patterson PH: Leukemia inhibitory factor, a cytokine at the interface between neurobiology and immunology. Proc Natl Acad Sci USA 91:7833, 1994


37. Kuter DJ, Reeder DL, Rosenberg RD: The purification of


42. Gurney AL, Wong SC, Henzel WI, de Sauvage FI: Distinct regions of c-Mpl cytoplasmic domain are coupled to the JAK-STAT signal transduction pathway and Shc phosphorylation. Proc Natl Acad Sci USA 92:5292, 1995


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