Role of Gas6 in erythropoiesis and anemia in mice

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Many patients with anemia fail to respond to treatment with erythropoietin (Epo), a commonly used hormone that stimulates erythroid progenitor production and maturation by human BM or by murine spleen. The protein product of growth arrest–specific gene 6 (Gas6) is important for cell survival across several cell types, but its precise physiological role remains largely enigmatic. Here, we report that murine erythroblasts released Gas6 in response to Epo and that Gas6 enhanced Epo receptor signaling by activating the serine-threonine kinase Akt in these cells. In the absence of Gas6, erythroid progenitors and erythroblasts were hyporesponsive to the survival activity of Epo and failed to restore hematocrit levels in response to anemia. In addition, Gas6 may influence erythropoiesis via paracrine erythroblast-independent mechanisms involving macrophages. When mice with acute anemia were treated with Gas6, the protein normalized hematocrit levels without causing undesired erythrocytosis. In a transgenic mouse model of chronic anemia caused by insufficient Epo production, Gas6 synergized with Epo in restoring hematocrit levels. These findings may have implications for the treatment of patients with anemia who fail to adequately respond to Epo.

Introduction
Anemia results from insufficient production, excessive destruction, or loss of rbc (1). By limiting the capacity of the blood to carry oxygen, anemia causes tissue hypoxia and may lead to severe cardiovascular, neurological, and musculoskeletal malfunction. In response to anemia, new rbc are generated. This process of erythropoiesis relies on the commitment of hematopoietic stem cells to differentiate into erythroblasts, which subsequently mature into circulating rbc. While erythropoiesis is regulated by several cytokines, erythropoietin (Epo) is a key factor that induces the maturation and proliferation of burst-forming unit and CFU erythroid progenitors (BFU-Es and CFU-Es, respectively) and early erythroblasts (2). Epo is also an important survival factor for these cells, as deprivation of Epo leads to apoptosis (3). Besides regulating erythropoiesis in baseline conditions, Epo also induces a compensatory erythropoietic response to reduced tissue oxygen tension (hypoxia) and anemia. In humans, Epo promotes recovery from anemia by stimulating the generation of erythroid progenitors in the BM, while in the mouse, Epo enhances erythropoiesis primarily in the spleen (4).

Recombinant Epo is widely used to treat anemia associated with numerous disorders such as chronic renal failure, myelodysplastic syndromes, cancer, and rheumatoid arthritis (5–8). However, Epo is not always effective, with many individuals being refractory to even high doses (9, 10). Approximately 10% of patients with end-stage renal disease are hyporesponsive, over 50% of patients with cancer have an inadequate response to Epo, and only 20%–25% of patients with myelodysplastic syndromes respond favorably (6, 9, 10). Although inflammation, iron and vitamin deficiency, inadequate dialysis, aluminum toxicity, and hyperparathyroidism impair the response to Epo, the molecular mechanisms of the resistance to Epo remain largely obscure (9, 11).

We therefore explored whether other factors might stimulate erythropoiesis. We considered growth arrest–specific gene 6 (Gas6) − acting via its receptor tyrosine kinases, the Gas6 receptors (Gas6Rs) Tyr3, Axl, and Mertk − as a likely candidate, because each of its receptors is expressed in hematopoietic tissue, megakaryocytes, myelomonocytic precursors, and BM stromal cells (12–15). Gas6 is a secreted vitamin K–dependent protein that interacts with phospholipid membranes via its γ-carboxyglutamic acid–containing tail and binds its receptors via its carboxyterminal globular domain (16–19). This leads to further intracellular signaling, including activation of the PI3K and Akt pathways (20, 21). Gas6 has been implicated in reversible cell growth arrest, survival, proliferation, cell adhesion, and hemostasis (reviewed in ref. 22). Apart from its expression pattern, the biological role of Gas6 in hematopoiesis remains largely unknown. Conflicting lines of evidence suggest that it lacks mitogenic activity for hematopoietic progenitors or stromal fibroblasts (12), whereas in vitro overexpression of Gas6 in fibroblasts facilitates the generation of hematopoietic CFUs (23).
In this study, we identified what we believe to be a novel role for Gas6 in amplifying the erythropoietic response to Epo and characterized its therapeutic potential in mouse models of acute and chronic anemia. These findings warrant further investigation of the therapeutic potential of Gas6 for the treatment of patients who are hyporesponsive or resistant to Epo.

Results

Deficits in the erythroid lineage in Gas6⁻/⁻ embryos. We previously reported that adult Gas6⁻/⁻ mice have a platelet dysfunction but otherwise appear phenotypically normal (24). However, development of Gas6⁻/⁻ embryos was not previously investigated. Here we found that livers of Gas6⁻/⁻ embryos were smaller at E13.5 compared with WT embryos, while the other organs appeared normal. Because fetal liver is a site of embryonic erythropoiesis (25), we further assessed the role of Gas6 in this process. By flow cytometry, we found fetal livers to contain fewer erythroblasts in Gas6⁻/⁻ than WT embryos (Ter-119⁺ erythroblasts per liver, WT, 8.2 ± 0.11 × 10⁶; Gas6⁻/⁻, 3.9 ± 0.05 × 10⁶; n = 6; P < 0.01). When using in vitro clonogenic assays, fetal livers of Gas6⁻/⁻ embryos contained half the number of BFU-Es and CFU-Es as those present in WT embryos (Figure 1, A and B). Thus, absence of Gas6 leads to a deficit in embryonic erythroid precursor cells.

Defects in the erythroid lineage in adult Gas6⁻/⁻ mice. We next examined whether erythropoiesis was impaired in adult Gas6⁻/⁻ mice. Because of the decreased erythroid reserves in the embryo, we expected the defect to manifest in adult Gas6⁻/⁻ mice by lower hematocrit levels. Surprisingly, Gas6⁻/⁻ mice had normal hematocrit levels under baseline conditions (WT, 48.8% ± 1.0%; Gas6⁻/⁻, 49.1% ± 3.9%; n = 12; P = NS).

Indices and morphology of rbc were comparable in WT and Gas6⁻/⁻ mice. BM cytological examination showed that Gas6⁻/⁻ erythroid cells were present at all maturation stages and did not display signs of dysplasia (data not shown). However, reticulocyte counts were lower in adult Gas6⁻/⁻ than WT mice (WT, 2.5% ± 0.1%; Gas6⁻/⁻, 1.3% ± 0.1%; n = 8; P < 0.01). The reduced reticulocyte levels in adult Gas6⁻/⁻ mice were not attributable to a defect in reticulocyte maturation (Supplemental Note 1; supplemental material available online with this article; doi:10.1172/JCI30375DS1), but rather to a partial depletion of erythroid reserves in both BM and spleen. Indeed, flow cytometry of Ter-119⁺ cells revealed the presence of fewer erythroblasts in BM and spleen of Gas6⁻/⁻ mice (Figure 1, C and D). Fewer Ter-119⁺ erythroblasts were also detected by immunostaining in the spleen (data not shown). Furthermore, the spleens weighed approximately 20% less in Gas6⁻/⁻ mice (WT, 113 ± 3 mg; Gas6⁻/⁻, 91 ± 15 mg; n = 33; P < 0.01).

The erythropoietic defect in adult Gas6⁻/⁻ mice could be attributable to a defect in erythroid progenitor cells, as fewer BFU-E and CFU-E colonies developed after plating out equal numbers of BM cells (Figure 1, E and F). Supplementation of recombinant Gas6 (rGas6; 400 ng/ml) to the culture medium rescued the impaired formation of BFU-E colonies in Gas6⁻/⁻ mice to levels approximating those found in WT mice (BFU-E colonies per 10⁶ BM cells, WT, 139 ± 4; WT plus rGas6, 143 ± 6; Gas6⁻/⁻, 59 ± 3; Gas6⁻/⁻ plus rGas6, 125 ± 4; n = 10; P < 0.001). These data suggest that rGas6 regulates erythropoiesis, at least in part, by increasing the plating efficacy of Gas6⁻/⁻ erythroid progenitors (see below for effects of Gas6 on erythroblast adhesion). Thus, in the absence of Gas6, fewer erythroid progenitors and precursors were generated in the adult BM.

Reduced clearance rate of senescent rbc in Gas6⁻/⁻ mice. Our data demonstrate that an abnormal (accelerated) rate of reticulocyte maturation could not explain the normal hematocrit levels despite the reduced reticulocyte counts. We therefore analyzed whether rbc have a longer lifespan in Gas6⁻/⁻ mice. Indeed, the clearance rate of biotinylated rbc was lower in Gas6⁻/⁻ than WT mice, as evidenced by the higher percentage of residual biotinylated rbc at 30 days after injection (WT, 19% ± 3%; Gas6⁻/⁻, 31% ± 4%; n = 12; P < 0.05). The

Figure 1

Defects in the erythroid lineage of WT and Gas6⁻/⁻ mice. (A and B) Reduced number of erythroid progenitors in fetal liver of Gas6⁻/⁻ mice, analyzed as the number of BFU-Es (A) or CFU-Es (B). n = 6 per group. *P < 0.05. (C and D) Reduced percentage of Ter-119⁺ nucleated cells (erythroblasts) in adult BM (C; *P = 0.02) and spleen (D; *P = 0.02) in Gas6⁻/⁻ mice. The erythroblasts were quantified as the percent of all nucleated cells by flow cytometry. n = 9 per group. (E and F) Reduced number of erythroid progenitors in the BM of adult Gas6⁻/⁻ mice, analyzed as the number of BFU-Es (E) or CFU-Es (F), n = 8 per group. *P < 0.05. Values are mean ± SEM.
Subpopulation of rbc labeled with both biotin and annexin V was considered senescent rbc (26). We found that a higher number of senescent rbc circulated in Gas6−/− than WT mice. The percentage of biotinylated rbc that were annexin V+ after 30 days was 0.11% ± 0.03% in WT mice versus 0.31% ± 0.11% in Gas6−/− mice (n = 12; P < 0.05). These data indicate that rbc in Gas6−/− mice had a longer lifespan, because senescent rbc were not efficiently cleared.

Gas6 has been previously documented to form a bridge between phosphatidylserine (exposed on apoptotic or senescent cells) and Gas6Rs on macrophages, which then engulf and clear these cells (27). To investigate whether Gas6−/− macrophages similarly engulf senescent rbc less efficiently, we treated rbc with phenylhydrazine (PHZ) to expose phosphatidylserine on their surface (28) and incubated them with adherent macrophages. The number of macrophages that engulfed 3 or more rbc was reduced by more than 50% in Gas6−/− mice (Figure 2). Similar findings were obtained when using Gas6−/− mice on mixed 50% 129/Sv × 50% Swiss background (Supplemental Figure 2). As in the PHZ model, hematocrit levels returned to normal by day 6; in contrast, hematocrit levels in Gas6−/− mice failed to recover until day 10 after PHZ, indicating that erythropoietic response was impaired and delayed in the absence of Gas6. Similar findings were obtained when using Gas6−/− mice on mixed 50% C57BL/6 × 50% Swiss (Figure 3A) and 100% C57BL/6 genetic backgrounds (Supplemental Figure 1).

Because erythropoiesis in response to anemia occurs primarily in the spleen in mice (4), we measured the spleen weight as an indirect parameter of erythropoiesis. By day 3 after PHZ injection, the spleen was already enlarged more in WT than Gas6−/− mice (before and 3 days after PHZ, WT, 113 ± 3 and 270 ± 7 mg; Gas6−/−, 91 ± 15 and 177 ± 12 mg; n = 10–21; P < 0.01). By day 6, the difference was more apparent (WT, 620 ± 45 mg; Gas6−/−, 410 ± 36 mg; P < 0.01); spleen weight increased 5.5-fold in WT mice but only 4.5-fold in Gas6−/− mice. In addition, the reticulocyte response was delayed in Gas6−/− mice, as evidenced by the 2-fold lower reticulocyte counts in Gas6−/− than WT mice at day 6 after PHZ (Figure 3B). Thus, the absence of Gas6 did not cause anemia spontaneously, but impaired the erythropoietic recovery in conditions of acute anemia.

In the second model, immune hemolytic anemia was induced by administration of the anti-mouse rbc antibody 34-3C (29). This antibody caused a comparable degree of hemolysis in both genotypes (Supplemental Note 2). As in the PHZ model, hematocrit levels and reticulocyte counts recovered more slowly in Gas6−/− than WT mice (Figure 3, C and D). When analyzing the number of erythroblasts in the spleen by flow cytometry, we found that Gas6−/− mice also contained fewer Ter-119+ cells at day 4 after induction of anemia (WT, 45% ± 7%; Gas6−/−, 26% ± 10%; n = 10; P < 0.05). Thus, also in this model, after induction of acute anemia, the Gas6−/− mice were not able to achieve a normal hematocrit as rapidly as WT mice.

**Role of Gas6 in BM erythropoiesis.** In humans, erythropoiesis in response to anemia is induced in the spleen and in the BM. This is in contrast to mice, where erythropoiesis under similar conditions occurs primarily in the spleen, although the BM can also contribute when splenic function is compromised (4). To assess how relevant our genetic findings in the mouse might be for humans, we studied whether Gas6 also regulated erythropoiesis in the BM in response to anemia after removal of the spleen. As expected, splenectomy itself retarded the erythropoietic recovery in WT mice, and hematocrits only approximated normal levels by day 10 after PHZ injections on day 0 and 1 (Figure 3E). Notably, hematocrit levels were consistently lower in splenectomized Gas6−/− mice and were still not completely restored by day 10 after PHZ (Figure 3E). This is also in contrast to the full recovery in hematocrit achieved at day 6 and sustained through day 10 by PHZ-treated nonsplenectomized Gas6−/− mice (Figure 3A).

From these findings, we conclude that Gas6 regulates the erythropoietic response to acute anemia both in spleen and in BM.
Impaired survival of Gas6−/− mice in acute anemia. Anemia may be life-threatening when hematocrit levels acutely drop to low levels, particularly in clinical settings where cardiac reserves are jeopardized (30). To further assess the possible preclinical relevance of the erythropoietic defect in the Gas6−/− mice, we challenged mice with a more severe form of acute anemia by injecting PHZ on 3 consecutive days (days 0, 1, and 2). In both genotypes, hematocrit levels dropped below 20% by day 3 (Figure 3F). All WT mice were capable of restoring their hematocrit levels by day 9 and survived for the entire period. In contrast, up to two-thirds of the Gas6−/− mice died from day 3 onward; we sampled blood whenever possible from preterminal mice and found that all had extremely diminished hematocrit levels (mean, 11%). Thus, Gas6−/− mice remained significantly more anemic than did WT mice (Figure 3F). Even those few mice that survived the challenge throughout the period of observation remained anemic. Thus, Gas6 confers erythropoietic protection against life-threatening acute anemia.

Erythroblasts express the Gas6Rs Tyro3, Axl, and Meretk. We examined the mechanisms by which Gas6 mediates an erythropoietic response. For erythroblasts to respond directly to Gas6, they should express 1 or more of the Gas6Rs: Tyro3, Axl, and Meretk. We therefore analyzed whether Gas6Rs were expressed by splenic erythroblasts under steady-state conditions and 6 days after PHZ. Ter-119+ splenic erythroblasts from WT and Gas6−/− mice were isolated, and quantitative RT-PCR analysis was used to measure transcript mRNA levels of each receptor, normalized to β-actin transcript levels. Transcripts of all the Gas6Rs — Tyro3, Axl, and Meretk — were expressed by erythroblasts of WT and Gas6−/− mice. Accumulation of each Gas6R mRNA did not differ, however, between the 2 genotypes under steady-state conditions (Table 1), nor did the specific mRNA levels increase in response to administration of PHZ 6 days earlier (data not shown).

To provide more direct evidence that erythroblasts express the Gas6Rs at the protein level, we immunoblotted lysates of isolated Ter-119+ splenic erythroblasts. SDS gels were loaded with equivalent amounts of protein. As expected, both Axl and Meretk were indeed detectable in splenic erythroblasts, but not in Axl−/− or Meretk−/− splenic erythroblasts, respectively (Figure 4A). Expression levels of Axl and Meretk did not increase in erythroblasts that were isolated 6 days after PHZ treatment of WT mice (data not shown). Available anti-Tyro3 antibodies were not able to immunodetect denatured Tyro3 by Western blotting. As an alternative, we used a sensitive ELISA and found measurable levels of Tyro3 protein in

Table 1
Gene expression of Gas6Rs Tyro3, Axl, and Meretk in spleen erythroblasts

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. copies per 1,000 β-actin copies</th>
<th>WT</th>
<th>Gas6−/−</th>
</tr>
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<tbody>
<tr>
<td>Tyro3</td>
<td>49.5 ± 9.5</td>
<td>57.8 ± 7.2</td>
<td></td>
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<tr>
<td>Axl</td>
<td>51.5 ± 14.5</td>
<td>53.3 ± 6.5</td>
<td></td>
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<tr>
<td>Meretk</td>
<td>15.3 ± 1.0</td>
<td>15.3 ± 2.5</td>
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Erythroblasts were isolated from spleen under steady-state conditions using MACS Ter-119 MicroBeads. Gene expression of Tyro3, Axl, and Meretk was quantified by real-time PCR relative to the expression of β-actin. Values are mean ± SEM.
WT splenic erythroblast extracts (0.87 ± 0.12 ng/mg erythroblast extracts, n = 6), but none in Tyro3–/– splenic erythroblast extracts, as expected. Thus, all 3 Gas6Rs are expressed by splenic erythroblasts and are candidates to mediate the erythropoietic activity of Gas6.

**Axl mediates the erythropoietic response of Gas6.** To provide direct evidence for a functional role of Gas6Rs in the erythropoietic response to acute anemia, we challenged Gas6R-deficient mice with PHZ to induce hemolytic anemia. Under baseline conditions, Tyro3–/–, Axl–/–, and Mertk–/– mice had normal hematocrit levels. Induction of hemolytic anemia in mice with Gas6R gene inactivations and their WT counterparts was achieved by injections of PHZ on days 0 and 1. After administration of PHZ, all WT and Mertk–/– mice recovered from the acute anemic stress with comparable hematocrit levels (Figure 4B). Most Tyro3–/– mice were also capable of recovering from anemia with hematocrit levels similar to those of WT mice (Figure 4B); only 17% of Tyro3–/– mice died because of low hematocrit levels. In sharp contrast, hematocrit dropped to extremely low levels (11%) in Axl–/– mice. In fact, 56% of the Axl–/– mice failed to restore their hematocrit levels and died 3 or more days after the second PHZ injection (Figure 4B). Surviving Axl–/– mice also had lower hematocrit levels and were not able to fully recover from anemia (hematocrit nadir, WT, 25% ± 1.4%; Axl–/–, 19.5% ± 1.6%). Consistent herewith, reticulocyte counts at day 6 were comparable in WT, Tyro3–/–, and Mertk–/– mice (data not shown), but lower in Axl–/– mice (WT, 83% ± 8%; Axl–/–, 47% ± 6%; n = 4; P < 0.05).

The data indicates that in the response to hemolytic anemia, Axl is the more important Gas6R. To better clarify the relative contribution of the different Gas6Rs, we assessed the sensitivity of Gas6R-deficient mice to rescue with rGas6. Therefore, the effect of rGas6 in the acute hemolytic anemia model in Axl–/– mice was analyzed. Because our previous experiment showed that PHZ at the usual dosage was lethal for Axl–/– mice, we reduced the PHZ dose by 50% on days 0 and 1. Mice received 2 μg/d of rGas6 or vehicle (0.9% NaCl). Consistent with a model whereby Axl would mediate the response to Gas6, rGas6 did not significantly improve the erythropoietic response in Axl–/– mice (Figure 4C). These findings suggest that the residual expression of Tyro3 and Mertk cannot compensate for the absence of Axl and that Axl is more important than the other 2 Gas6Rs.

Our data also showed that Axl–/– mice were more sensitive to PHZ than Gas6–/– mice. However, because the genetic background of the Gas6–/– mice and mice deficient in any one of the Gas6Rs is not identical, firm conclusions about the relative importance, when directly comparing these mutant mice, cannot be drawn. However, additional experiments revealed that the response to PHZ was comparable in Gas6–/– mice on mixed 50% 129/Sv × 50% Swiss (Figure 3A) and 100% C57BL/6 genetic backgrounds (Supplemental Figure 1), indicating that differences in genetic backgrounds do not seem to determine, at least not in a major way, the magnitude of the erythropoietic response.

**Release of Gas6 from erythroblasts in response to Epo.** To assess whether the erythropoietic defect in Gas6–/– mice is cell autonomous, we analyzed whether erythroblasts produce and release Gas6 in response to Epo. By quantitative real-time RT-PCR, we detected Gas6 mRNA transcript levels (normalized for GAPDH levels), but these remained unchanged in unfraccionated spleens of WT mice 0 and 3 days after PHZ injection (0.89 ± 0.24 and 0.20 ± 0.11 copies/10^5 copies GAPDH, respectively; n = 3; P > 0.5). However, from day 3 to day 6, with the generation of more splenic erythroblasts, Gas6 mRNA levels increased significantly to 5.81 ± 3.48 copies/10^5 copies GAPDH (n = 3; P = 0.04). Furthermore, ELISA revealed that upon stimulation with Epo (10 IU/ml), isolated Ter-119+ splenic erythroblasts from WT mice released Gas6 into culture medium (6 ± 0.3 ng/ml/24 h with Epo versus undetectable levels without Epo, n = 6), whereas Gas6 was undetectable in the conditioned culture medium of Epo-stimulated Gas6–/– erythroblasts. Western blotting confirmed detection of Gas6 protein in lysates of unstimulated UT7 cells (a human erythroid cell line; ref. 31) and that both the intracellular Gas6 content and the amount of secreted Gas6 were dose-dependently increased by Epo (Figure 5).

Moreover, in WT mice, Gas6 plasma levels significantly increased during the erythropoietic response to hemolytic anemia (baseline, 5.5 ± 1.3 ng/ml; day 6 following PHZ, 9.1 ± 1.3 ng/ml; n = 10, P = 0.49 (n = 18); Axl–/–, P = 0.007 (n = 9); Mertk–/–, P = 0.97 (n = 5). WT and Axl–/– mice were subjected to PHZ-induced hemolytic anemia (injections of a half dose of PHZ on days 0 and 1) and treated with saline or rGas6 (2 μg daily intraperitoneally), P < 0.05, Axl–/– and Axl–/– plus rGas6 versus WT; P > 0.05, Axl–/– versus Axl–/– plus rGas6.
Gas6 enhances the survival response of erythroblasts to Epo. We next sought to determine the mechanisms by which Gas6 regulates erythropoiesis. We first excluded the possibility that the impaired erythropoietic response in anemic Gas6−/− mice was attributable to a deficiency in iron or Epo production (Supplemental Notes 3 and 4). We then evaluated whether the erythropoietic defect in Gas6−/− mice was caused by an impaired response of their erythroblasts to the survival activity of Epo. Because Gas6 reduces apoptosis of fibroblasts (20), diminished survival of erythroblasts in Gas6−/− mice might indeed account for the suboptimal erythropoietic response in these mice. This hypothesis was further suggested by experiments indicating that the red pulp of spleens contained comparably low TUNEL+ apoptotic cells in both genotypes under baseline conditions, but more apoptotic cells in Gas6−/− than WT mice at 3 (Figure 6, A and B) and 6 (data not shown) days after PHZ-induced hemolysis.

To analyze directly whether survival of Gas6−/− erythroblasts in response to Epo is impaired, we isolated spleen erythroblasts at day 6 after PHZ injection. When cultured for 16 hours in the absence of Epo, approximately 50% of the erythroblasts of either genotype were apoptotic (Figure 6C). However, the survival response to exogenous Epo was different in WT versus Gas6−/− mice. Indeed, a low concentration of Epo sufficed to lower the number of WT apoptotic cells, from 52% ± 0.7% in controls to 25% ± 1.1% in cells treated with 0.6 IU/ml Epo (Figure 6C). In contrast, a similar concentration of Epo decreased the number of apoptotic Gas6−/− cells from 51% ± 1.1% in controls to 36% ± 2.1% in cells treated with 0.6 IU/ml Epo (Figure 6C). Maximal protection of Gas6−/− erythroblasts from apoptosis was achieved with a minimum of 1.5 IU/ml

Figure 6
Impaired Epo survival response of Gas6−/− erythroblasts. (A and B) TUNEL staining of spleen 3 days after induction of hemolytic anemia by PHZ. Black staining with TUNEL denotes localized apoptotic cells in WT (A) and Gas6−/− (B) spleen. F, follicle; RP, red pulp. Scale bars: 100 μm. (C) TUNEL assay of WT and Gas6−/− erythroblasts isolated 6 days after PHZ injection. Cells were cultured for 16 h in the presence or absence of recombinant human Epo at the indicated concentrations. The TUNEL assay was quantified by flow cytometry (n = 4); results are expressed as the percentage of apoptotic erythroblasts, cells double-stained for TUNEL and Ter-119. (D) Dead cell count of splenic erythroblasts (Trypan blue− cells) collected on day 6 after PHZ injection and maintained in culture for 24 h in the presence or absence of Epo (1.5 IU/ml) or Epo plus human rGas6 (400 ng/ml). n = 8 per group. Values are mean ± SEM. *P < 0.05 versus WT.

Figure 7
Gas6 is required for adhesion of erythroblasts. (A) Adherence of WT versus Gas6−/− erythroblasts to fibronectin (n = 6), a mechanism essential for proliferation, survival, and expansion of these cells. The adherence defect of Gas6−/− erythroblasts was corrected by the addition of rGas6. (B) Survival response to Epo of WT and Gas6−/− erythroblasts adherent to fibronectin (n = 3). Apoptotic adherent erythroblasts stained by DAPI were counted as cells with nuclear fragmentation. (C) Expression of VLA4 and Ter-119 in WT (n = 9) and Gas6−/− (n = 8) spleen determined by flow cytometry. VLA4 expression is proportional to Ter-119 expression. (D) Adherence of WT versus Gas6−/− erythroblasts to fibronectin in the presence of 0.9% NaCl (vehicle), LY294002, or anti-VLA4 antibody (2 μg/10⁶ cells) (n = 6). Values are mean ± SEM. *P < 0.05. Data were reproduced in 100% C57BL/6 background (not shown).
Epo, and this was still not sufficient to suppress apoptosis to the levels observed with WT erythroblasts (Figure 6C).

These findings were further confirmed and extended by using an independent method: counting dead erythroblasts after staining them with Trypan blue dye. At a concentration of 1.5 IU/ml, Epo induced a maximal survival response in WT erythroblasts ($P < 0.001$); the same dose of Epo also exhibited a survival response in Gas6–/– erythroblasts ($P = 0.02$), but the response was significantly less prominent than with the WT erythroblasts, indicating that Gas6 was required for a maximal response to Epo (Figure 6D). When tested alone, rGas6 significantly enhanced erythroblasts survival in both genotypes ($P < 0.001$ versus corresponding baseline controls). Although the protection against apoptosis by rGas6 was not as effective as Epo alone in WT cells, Gas6 itself provided substantial survival signals for erythroblasts. Indeed, addition of rGas6 together with Epo completely restored the survival response of Gas6–/– erythroblasts to Epo ($P < 0.001$; Figure 6D). Thus, Gas6 is a survival factor for erythroblasts by acting alone and by synergizing with Epo.

Gas6 is required for adhesion of erythroblasts. Adhesion to fibronectin is essential for the proliferation, survival, and expansion of BM-derived erythroid progenitors as well as for the terminal stages of erythroid differentiation (33–35). We therefore investigated whether Gas6 regulates the adhesion of Ter-119+ erythroblasts to fibronectin. When erythroblasts were placed on a fibronectin-coated surface, we found that fewer Gas6–/– than WT erythroblasts adhered to fibronectin (Figure 7A). Notably, this adhesion defect of Gas6–/– erythroblasts was completely corrected by the addition of rGas6. Thus, Gas6 regulates the adhesion of erythroblasts, a critical step in erythropoiesis.
In addition, we compared the survival response to Epo of WT-and Gas6−/−-adherent erythroblasts. More WT- than Gas6−/−-adherent erythroblasts survived in response to Epo (reduction of apoptotic cells, WT, 50%; Gas6−/−, 30%; Figure 7B). Similar findings were obtained when using Gas6−/− mice on mixed 50% 129/Sv × 50% Swiss and 100% C57BL/6 genetic backgrounds (data not shown). However, the magnitude of the genotypic difference in survival response was not more notable when culturing the cells on fibronectin (Figure 7B) than in suspension (Figure 6C).

We then studied in more detail why adhesion of erythroblasts to fibronectin was impaired in the absence of Gas6. We observed that Gas6−/− and WT erythroblasts expressed comparable levels of VLA4/α4β1 integrin (Figure 7C), raising the question of whether downstream signaling (which promotes VLA4 activation) was impaired. Indeed, adhesion in the presence of anti-VLA4 antibodies was reduced in WT but not in Gas6−/− erythroblasts, suggesting that absence of Gas6 impaired VLA4 activation (Figure 7D). In addition, the PI3K inhibitor LY294002 impaired the adhesion of WT and Gas6−/− erythroblasts to the same extent as the anti-VLA4 antibody (Figure 7D). Thus, Gas6 favors PI3K and VLA4 activation.

Gas6, alone and together with Epo, activates the Akt survival pathway. The finding that Gas6 regulated the response to Epo suggested a possible role of Gas6 in the signaling induced by Epo. By immunoblotting, we found that the expression levels of Epo receptor (EpoR) in splenic erythroblasts, isolated at day 6 after PHZ treatment, were comparable in WT and Gas6−/− mice (Figure 8A). Tyrosine phosphorylation of EpoR was also not different between genotypes (Figure 8A), indicating that absence of Gas6 did not impair the activation of EpoR in erythroblasts and thus suggesting that the defect may affect more downstream signaling steps. As signal transduction of Epo in erythroblasts and of Gas6 in other cell types is known to involve the activation of PI3K and its downstream target, Akt (20, 21, 36–39), we assessed whether absence of Gas6 affected the activation of Akt in erythroblasts.

As shown in Figure 8B, treatment of WT splenic erythroblasts with rGas6 alone (400 ng/ml) slightly stimulated phosphorylation of Akt, indicating that Gas6 itself was capable of initiating this survival pathway. A comparable, although smaller, effect was detected upon treating Gas6−/− erythroblasts with rGas6. We therefore tested whether a higher dose of rGas6 restored Akt activation in Gas6−/− cells. Increasing the dose of rGas6 did not increase Akt phosphorylation, indicating that maximal activation was reached with a rGas6 concentration ranging 200–400 ng/ml. This suggests that other signaling pathways are defective in Gas6−/− erythroblasts.

Cotreatment of WT erythroblasts with Epo and rGas6 together also resulted in a strong Akt phosphorylation signal, which was comparable to the signal induced by Epo alone. The lack of a synergistic effect of rGas6 and Epo in WT cells is likely attributable to the fact that WT erythroblasts already release saturating levels of Gas6. In contrast, cotreatment of Gas6−/− erythroblasts with Epo and rGas6 together induced a much stronger Akt phosphorylation signal than treatment with Epo or rGas6 alone. In fact, Akt phosphorylation in Gas6−/− erythroblasts after cotreatment with Epo plus rGas6 was comparable to the levels observed in WT erythroblasts treated with Epo alone. Thus, apart from stimulating the Akt survival pathway itself, Gas6 also synergized with EpoR signaling to activate the Akt survival pathway.

Gas6 dampens the release of erythroid-inhibitory factors by macrophages. Gas6 and its cognate receptors Tyro3, Axl, and Mertk are also expressed by macrophages (40). Because erythroblasts proliferate, differentiate, and enucleate within the erythroblastic island, which includes a central macrophage (reviewed in ref. 41), we also sought to determine whether Gas6, via a paracrine effect, regulates the release of erythroid-inhibitory factors from macrophages. We therefore analyzed the expression of various cytokines in the plasma and supernatant of BM-derived macrophages of Gas6−/− mice using a mouse protein cytokine array. This analysis revealed that loss of Gas6 increased the expression of several cytokines known to inhibit erythropoiesis (i.e., IL-10, IL-13, IL-1α, IL-1β, IL-6, and TNF-α; Figure 9) (42). Thus, in addition to its autocrine activity on the erythroblast (reinforcing Epo signaling), Gas6 also seemed to regulate erythropoiesis indirectly in a paracrine loop by regulating the release of erythroid-inhibitory factors from macrophages in the erythroblastic island.

Treatment of mouse models of acute and chronic anemia with rGas6. Prompted by the finding that Gas6 regulated the erythropoietic response to anemia, we evaluated whether administration of rGas6 to WT mice would improve the erythropoietic response to anemia.
A day after the last bleeding. Thus, unlike the prevention trial in the PHZ model, we used the bleeding model to examine whether rGas6 could prevent the development of PHZ-induced hemolytic anemia by delivering these compounds from day 0, when PHZ injections were initiated (prevention trial). Upon administration of Epo or rGas6 to WT mice, hematocrit levels failed to drop after induction of hemolysis, indicating that both Epo and rGas6 protected these mice against the development of anemia (Figure 10A). Unlike Epo, rGas6 did not increase hematocrit levels to supraphysiologic levels from day 6 onward (Figure 10A). Compared with the control, the reticulocyte counts at 3 and 6 days after PHZ were increased by Epo (control, 9% ± 1% and 30% ± 2%; Epo, 31% ± 4% and 50% ± 4%; n = 6; P < 0.001) as well as by rGas6 (21% ± 2% and 35 ± 3%; n = 6; P < 0.05, calculated by repeated measurements statistics). Epo stimulated the reticulocytosis to higher levels than rGas6 (P < 0.05).

The therapeutic potential of rGas6 to stimulate erythropoiesis in WT mice in response to acute anemia was not restricted to the PHZ model, but was also detected in a bleeding model of acute anemia. In these experiments, we administered Epo or rGas6 from day 2, 1 day after the last bleeding. Thus, unlike the prevention trial in the PHZ model, we used the bleeding model to examine whether we could restore the hematocrit levels once the mice were already anemic (regression trial). Administration of Epo restored hematocrit levels by day 6 and significantly increased the hematocrit levels even to supraphysiologic levels (Supplemental Figure 2). Delivery of rGas6 also accelerated the erythropoietic recovery, although less rapidly than Epo, and not to supraphysiologic levels (Supplemental Figure 2). Taken together, rGas6 induced a therapeutic effect in both a prevention and a regression trial.

We also analyzed whether delivery of rGas6 was capable of increasing the hematocrit levels in a mouse model of chronic anemia. As very few mouse models of chronic anemia exist and a model of chronic anemia caused by a relative resistance to Epo was unavailable, we used a transgenic mouse strain (134.3 LC, Epo-TAg43) that has low Epo levels and develops chronic anemia (hematocrit levels of 30%, compared with 50% in WT) (32). This model has been previously validated for testing of erythropoietic therapies (43). Administration of 5 or 10 IU Epo every other day for 4 days increased relative hematocrit levels by 28% and 36%, respectively (P < 0.001; Figure 10C). Thus, although Epo was capable of stimulating erythropoiesis in this model of chronic anemia, its erythropoietic activity was less potent than in the acute anemia models. Consistent herewith, rGas6 also induced more modest effects. A daily dose of 2 μg rGas6 was ineffective, while a dose of 4 μg rGas6 increased the relative hematocrit levels by 12%, a statistically significant effect (Figure 10C). Thus, in 3 different models of anemia, delivery of rGas6 induced an erythropoietic effect.

Treatment of Epo-resistant anemia with rGas6. The above findings suggested that Epo requires Gas6 for an optimal response. Considering the growing medical need to develop alternative strategies to treat patients with relative Epo resistance, we assessed whether rGas6 amplifies the therapeutic potential of Epo. To try to mimic the clinical situation, we used 2 models characterized either by impaired Epo responsiveness (Gas6+/− mice) or by low Epo levels (134.3 LC, Epo-TAg43 mice).

After PHZ, and compared with untreated controls, Gas6+/− mice had a similar and significant erythropoietic response to rGas6 or Epo alone (Figure 10B). These responses, when compared with the corresponding and more prominent rises in identically treated WT mice (Figure 10A), indicated that Gas6+/− mice were relatively resistant to administration of Epo or rGas6 (Figure 10B). For example, in WT mice, Epo yielded an absolute rise in hematocrit of 21% and 23% at 3 and 4 days after PHZ, respectively, whereas increases in Gas6+/− mice were 16% and 13%. Similarly, rGas6 caused an absolute increase in hematocrit in WT mice of 23% and 17% at days 3 and 4, respectively, compared with 9% and 7% increases in Gas6+/− mice. The dampened response to Epo in Gas6+/− mice provides further evidence that Gas6 enhances the activity of Epo, while the
suboptimal response to rGas6 probably reflects the depleted stores of endogenous Gas6 in Gas6−/− mice that could not be restored by systemic delivery of the protein.

Notably, and providing additional support for the cooperative function of Gas6 and Epo in acute anemia, combined administration of rGas6 and Epo synergistically stimulated erythropoiesis in the PHZ-induced anemic Gas6−/− mice. Indeed, compared with Epo and rGas6 treatment alone, hematocrit levels were higher after cotreatment with Epo and rGas6 (Figure 10B). Reticulocyte counts in Gas6−/− mice were also higher after combined treatment with Epo and rGas6 together than after administration of each factor alone (reticulocyte counts at days 3 and 6 after PHZ, rGas6 plus Epo, 35% ± 4% and 55% ± 7%; rGas6, 16% ± 1% and 16% ± 2%; Epo, 10% ± 1% and 19% ± 3%; n = 6; P < 0.001). Similar findings were obtained when using Gas6−/− mice on mixed 50% 129/Sv × 50% Swiss and 100% C57BL/6 genetic backgrounds (data not shown).

Thus, rGas6 amplified the therapeutic effect of Epo. When using the 134.3 LC Epo-TAg−/− mouse, a model of chronic anemia caused by reduced Epo levels, delivery of Epo alone (10 IU) increased relative hematocrit levels by 36% (see above). However, when rGas6 and Epo were coadministered, hematocrit levels increased by 78%, much higher than those obtained with administration of either drug alone (Figure 10C). Thus, in preclinical models of both Epo resistance and Epo insufficiency, rGas6 amplified the therapeutic activity of Epo.

Discussion
The most important finding of our study is that Gas6 plays a critical role in the generation of erythroid progenitors and erythroblasts, in part by enhancing their survival response to Epo. As a result, absence of Gas6 impairs the erythropoietic response and hematocrit recovery upon induction of acute anemia. Conversely, treatment with rGas6 stimulates hematocrit recovery and protects WT mice against the development of anemia, induced by hemolysis or in part by blood loss. Moreover, in preclinical models of Epo resistance (Gas6−/− mice) or Epo insufficiency (134.3 LC Epo-TAg−/− mice), Gas6 amplified the therapeutic effect of Epo to increase hematocrit levels in conditions of acute and chronic anemia. These genetic, pharmacologic, and molecular insights warrant further investigation of the therapeutic potential of Gas6 for the treatment of anemia, particularly in the setting of Epo resistance.

To the best of our knowledge, a role for Gas6 in erythropoiesis has not been reported previously. Gas6 and its receptors were known to be expressed in the BM (12–15), but only conflicting effects on hematopoietic progenitors were documented thus far (12, 23). Here, we identified a role for Gas6 in the generation of sufficient numbers of erythroid progenitors, i.e., in the absence of Gas6, adult mice had fewer erythroblasts. The isolated erythroblast culture experiments revealed that Gas6 stimulates the survival of these cells, not only on its own but also by amplifying the response to Epo. Similar findings were obtained when we studied the survival Akt pathway. PI3K/Akt signaling is well known to mediate the survival activity of various growth factors (44) and was already previously shown to mediate the survival response to Gas6 in nonerythroblast cell types (20). We now show that Gas6 also activated Akt, alone and in combination with Epo, in erythroblasts. Furthermore, administration of rGas6 alone to Gas6−/− mice also provided some protection against the development of PHZ-induced anemia. Thus, our physiological, cellular, and molecular studies consistently revealed the involvement of Gas6 in erythroblast survival. These findings do not establish Akt signaling as the sole mechanism of action of Gas6. Indeed, we also observed that Gas6 allowed erythroblasts to adhere to fibronectin. Adhesion to the extracellular matrix is essential for the proliferation, survival, and expansion of erythroid progenitors and precursors as well as for the terminal stages of erythroid differentiation (33–35). In the present study, we found that this adhesion process was mediated, at least in part, by PI3K and VLA4 activation. Thus, Gas6, through activation of its receptors, appears to affect PI3K and integrin function in erythroblasts (Figure 11A), as we have previously documented for platelets (45).

Gas6 might also influence erythropoiesis via alternative mechanisms, independently of erythroblasts. For instance, we observed that Gas6−/− rbc had a longer lifespan than did WT rbc and that the number of senescent rbc increased in Gas6−/− mice compared with WT mice, implicating Gas6 in the clearance of senescent rbc by macrophages (Figure 11B). A similar role for Gas6 has been documented for apoptotic cells (46). When cells become apoptotic or senescent, phosphatidylserine is exposed on their surface; by binding to phosphatidylserine on one side and to the Gas6R on the other side, Gas6 forms a bridge between dying cells and macrophages, expressing Gas6Rs, which then engulf and clear these dying cells.

Moreover, Gas6 is known to downregulate the expression of inflammatory cytokines such as TNF-α by macrophages (reviewed in ref. 40). Because apoptotic depletion of BM progenitors by TNF-α has been implicated in the development of anemia (47), Gas6 might also play a beneficial role in erythropoiesis by reducing TNF-α production by macrophages (Figure 11C). Here we found that loss of Gas6 in BM-derived macrophages resulted in an increase, under steady-state conditions, in the production of several cytokines known to inhibit erythropoiesis (i.e., IL-10, IL-13, IL-1β, IL-6, and TNF-α).

The role of Gas6 in erythropoiesis appears to be cell autonomous: erythroblasts produce their own Gas6, they release Gas6 upon activation by Epo, and the released Gas6 then binds to Axl on the same or neighboring erythroblasts and amplifies the survival activity of Epo (Figure 11A). In addition, Gas6 favors erythroblast adhesion via PI3K and VLA4. Gas6 has a similar intrinsic role in blood platelets, which release their Gas6 stores upon activation by platelet agonists and thereby amplify the platelet aggregation response to these agonists (24). The advantage of such a system is that the effector cell itself (i.e., erythroblast or platelet) is capable of enhancing its own sensitivity to the primary stimulus (i.e., Epo or platelet agonists), thereby amplifying the biological response. Obviously, apart from an autocrine role for Gas6 in erythroblasts, Gas6 may also be released by neighboring cells in hematopoietic tissues. For example, Gas6 secreted by macrophages in the spleen or BM may play a paracrine role (Figure 11, B and C). All 3 Gas6Rs were expressed on isolated erythroblasts and were capable of binding Gas6, but Axl played a prominent role in erythropoiesis. It remains to be determined why Tyro3 and MerTK play apparently negligible roles and whether this relates to the lack of an appropriate intracellular signaling machinery for these receptors in erythroblasts.

One might expect that the survival defect of the erythroid progenitors would cause reduced hematocrit levels under baseline conditions in Gas6−/− mice. Two reasons might explain why this did not occur: (a) the increased lifespan of the mature rbc compensated for this defect; and (b) the defect was too subtle to affect the low level of erythropoiesis in baseline conditions, but sufficient
to impair the intense erythropoietic response to anemia. Indeed, regardless of the stimulus (i.e., blood loss or hemolysis), hematocrit levels were lower and reticulocytosis was impaired, requiring a longer recovery time to restore the hematocrit levels in Gas6−/− mice. The defect was even more dramatic in Axl−/− mice, resulting in life-threatening reductions in hematocrit levels (i.e., below 11%) and causing demise of more than half of the mutant mice. Moreover, rGas6 failed to improve the erythropoietic response in these mice, while WT mice displayed an improvement under similar conditions. These data indicate that the residual expression of Tyro3 and Mertk cannot compensate for the absence of Axl and that Axl is more important than the other 2 Gas6Rs in erythroblasts. The more predominant role of Axl compared with Tyro3 and Mertk might relate to their relative affinities for Gas6, which vary more than 100-fold, with Axl having a higher affinity than Tyro3 and both in turn higher than Mertk (17).

Differences in genetic background between Gas6−/− and Axl−/− mice (see Methods) did not determine in a major way the magnitude of the erythropoietic response because responses to PHZ were comparable in Gas6−/− mice on pure 100% C57BL/6 and mixed 50% 129/Sv × 50% Swiss backgrounds. One possible explanation, therefore, why loss of Axl resulted in a more severe phenotype than loss of Gas6, could relate to the fact that Axl may bind another ligand than Gas6. Protein S might be such a candidate, as it has previously been proposed to bind to Axl (reviewed in ref. 48). Unfortunately, to the best of our knowledge, mice lacking protein S do not exist to allow us to test the in vivo role of protein S in erythropoiesis in response to PHZ. In addition, Axl has been proposed to induce biological responses, independently of Gas6, via mechanisms involving homophilic interactions of Axl-expressing cells (49).

How relevant a player is Gas6 in erythropoiesis? At first sight, one might argue that the fact that loss of Epo, but not of Gas6, results in embryonic lethality (24, 25) would imply that Gas6 is redundant. When compared side by side, in absolute numbers, Epo induced a stronger erythropoietic response than did Gas6 in WT mice after PHZ and in 134.3LC Epo-TAg+ mice as well as stronger activation of Akt in isolated WT erythroblasts. Nonetheless, Gas6 entirely normalized the hematocrit levels in WT mice and was equipotent to Epo in Gas6−/− mice after PHZ; moreover, Gas6 directly promoted the survival of isolated erythroblasts. Perhaps even more relevant is the finding that Gas6 amplified the response to Epo. This was most evident when analyzing the hematocrit levels induced in response to acute anemia in Gas6−/− mice (a model of Epo resistance) and in the 134.3LC Epo-TAg+ mice (a model of chronic anemia due to Epo insufficiency). Furthermore, whereas Epo regulates embryonic and adult erythropoiesis, Gas6 is more important for switching on erythropoiesis in pathological conditions in adulthood and thus may also be an attractive therapeutic target (see below). Considering that the role of Gas6 might be more important in disease than in normal health, it will be interesting to examine whether abnormal Gas6 signaling contributes to the development of anemia associated with renal disease, cancer, arthritis, and other common disorders. Conversely, our findings, in combination with overexpression data of Gas6Rs in myeloproliferative disorders (15), raise the additional intriguing question as to whether activation of the Gas6 pathway also contributes to disease phenotypes characterized by too many circulating rbc (i.e., the myeloproliferative syndrome polycythemia vera).

What are the medical implications of our findings? Anemia is a common complication of many diseases, and, while Epo has been the drug of choice in the past (7, 8), resistance to Epo is becoming an increasing problem for numerous patients. In addition, optimal treatment with Epo is inconvenient in that it is generally administered several times per week, must be given intravenously or subcutaneously, and requires careful dose management to avoid polycythemia leading to thrombosis (11). The present findings that Gas6, alone or in combination with Epo, increased hematocrit levels in 3 models of acute anemia and 1 model of chronic anemia therefore warrant further study of its therapeutic potential. Overall, these findings provide insight into the molecular mechanisms by which Gas6 promotes erythropoiesis and highlight its potential clinical importance in the treatment of anemia.

**Methods**

**Mice.** Gas6−/− and WT mice were progeny of the original colony, with a genetic background of 50% 129/Sv × 50% Swiss (24), and backcrossed onto C57BL/6 background for more than 10 generations. Tyro3−/−, Axl−/−, and Mertk−/− mice were progeny of the original colony on a 50% 129/Sv × 50% C57BL/6 background (50); control WT mice had the same genetic background. The 134.3 LC, Epo-TAg+ heterozygous mice were progeny of the original colony on a pure C57BL/6 background (134). Animal experiments were approved by the Ethical Committee for Animal Experiments of the Medical Faculty of the University of Geneva, the Veterinary Service of Canton de Vaud, and the Institutional Animal Care and Research Committee of the University of Leuven.

**Immunohistochemistry.** Spleen and liver paraffin sections were stained for Ter-119 (BD Biosciences—Pharimgen). Apoptosis was evaluated by TUNEL labeling. ABC Vectastain kit (Vector) with DAB-cobalt-nickel staining was used to localize TUNEL+ cells.

**Flow cytometry.** Single-cell suspensions were obtained from BM, spleen, or E13.5 embryo liver. Ammonium chloride lysis of rbc was performed. BM and spleen cells (106) were incubated with rat anti-mouse CD16/CD32 to block nonspecific binding to Fe receptors. Cell suspensions were then stained with PE-conjugated anti-Ter119 antibodies and/or FITC-conjugated anti-FLA4 antibodies (BD Biosciences—Pharimgen) For FACS- TUNEL, cells were fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100, and incubated with Terminal Transferase and FITC-dUTP (Roche Diagnostics) according to the manufacturer’s instructions. Cell surface expression was analyzed using a Beckton Dickinson FACScan instrument and CellQuest software.

**Colony formation assays.** Single-cell suspensions of adult BM or E13.5 embryo liver were mixed with MethoCult M3434 (StemCell Technologies), plated in 25-mm dishes, and cultured at 37°C, 5% CO2. Colonies including BFU-E were scored at day 7. For CFU-E assay, cells were cultured in MethoCult 3230 containing murine Epo (R&D Systems), and colonies were scored at day 3.

**Hematocrit and reticulocyte count.** Blood was collected from the inferior vena cava or the retrobulbar plexus. Hematocrit was measured on blood drawn into a capillary tube. Reticulocyte counts were performed manually on blood smears stained with New Methylen Blue (Sigma-Aldrich). The reticulocyte index, assuming a normal hematocrit of 45%, equals reticulocyte count (% × (hematocrit/45).

**In vivo biotinylation of rbc and annexin V binding.** rbc were biotinylated in vivo by an intravenous infusion of N-hydroxysuccimide biotin (NHS-biotin, Boehringer Mannheim) as previously described (51). In vivo biotinylation procedure resulted in essentially all the rbc being biotinylated, as judged by staining with streptavidin Red 670 (Invitrogen) 24 h after NHS-biotin infusion. Sessent rbc were detected by flow cytometry 30 d after NHS-biotin infusion as cells labelled with both biotin (detected by streptavidin Red 670) and FITC-conjugated annexin V (which binds phosphatidylserine; ref. 26).
Engulfment of rbc by BM-derived macrophages. Macrophages were derived from BM precursors in the presence of M-CSF for 1 week. We added 30% of supernatant of the L929 cell line to cell culture medium (IMDM; Invitrogen) as a source of M-CSF. rbc isolated from peripheral blood were exposed to 3 mM PHZ in PBS for 1 h at 37°C, because PHZ induces lipid peroxidation and thereby phosphatidylserine translocation from the inner to the outer membrane surface (28). Cells were then washed in PBS, incubated with serum for 30 min, and resuspended in IMDM to reach hematocrit of 50%. This rbc suspension was incubated with adherent macrophages for 3 h at 37°C. Adherent macrophages were washed with PBS, and noninternalized rbc were lysed by incubation in distilled water for 20 s. Macrophages were covered with IMDM and examined in phase-contrast illumination.

Human rGas6. rGas6 was produced in the HEK293 line stably transfected with the pSec/TagHygro expression vector encoding the human Gas6 cDNA. rGas6 was purified from conditioned medium with a modified calcium affinity chromatographic procedure similar to that reported for other γ-carboxyglutamic containing proteins (52).

Models of hemolytic anemia. Anemia was induced in mice by PHZ as described previously (53). We injected 2 or 3 doses of 0.5 mg/g body wt intraperitoneally as indicated (first dose on day 0 in the evening, second dose on day 1 in the morning, third dose on day 3 in the morning). For rescue experiments, mice received rGas6 or recombinant human Epo (Eprex; Janssen-Cilag) or a combination of both at the indicated concentrations. In a second model, autoimmune hemolytic anemia was induced by a single intraperitoneal injection of 200 μg purified anti-rbc 34-3C IgG2a derived from unmanipulated NZB mice (29).

Measurement of Epo, Gas6 and Tyro3 levels. Epo concentrations were measured by ELISA (Quantikine IVD; R&D Systems). Cross-reaction of the kit was allowed by mouse Epo. The corrected absolute values of mouse Epo were determined as previously reported (43). Gas6 concentrations were measured by ELISA. Goat polyclonal antibody against Gas6 (AF986; R&D Systems) was coated overnight at 4°C at 0.5 μg/ml. Microtiter plates were then washed and blocked for 2 h with assay buffer (PBS plus 10% FCS). Murine rGas6 (986-GS; R&D Systems) and samples were added after dilution in assay buffer. Plates were incubated overnight at 4°C. After washing, the secondary biotinylated antibody (BAF986; R&D Systems) was incubated for 45 min. After washing, avidin peroxidase (BD Biosciences — Pharmingen) was added for 30 min at 37°C. Sigma Fast OPD (Sigma-Aldrich) was used as a substrate. Color development was stopped with addition of 3 M H2SO4, and absorbance was measured at 492 nm. The standard curve was linear between 0.2 and 1 ng/ml with a detection limit of 0.1 ng/ml. Supernatants were diluted 1:10 and plasma samples 1:10 and 1:20. Gas6 levels were below the detection limit in Gas6−/− samples. Tyro3 concentration in erythroblast extracts was measured by ELISA (mouse D tk ELISA; R&D Systems).

Cell isolation, purification, and culture. Single-cell suspensions were obtained from spleen (6 days after PHZ injection) or BM. Ammonium chloride lysis of rbc was performed. Primary erythroblasts were isolated using MACS Ter-119 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. Cells were stimulated with rGas6, recombinant human Epo (Eprex), or both at the indicated concentrations. For phospho-Akt and total Akt detection, the experiment was terminated by diluting the cells with ice-cold PBS containing 50 μM NaVO₄. UT7 cells were maintained in DMEM containing 10% fetal bovine serum and 1 IU/ml Epo. Before each experiment, cells were starved in DMEM with 0.1% BSA overnight. Starved cells were then stimulated with the indicated concentrations of Epo. Stimulation was terminated by diluting the cells with ice-cold PBS containing 50 μM NaVO₄ (37). Conditioned medium of UT7 cells was concentrated using Amicon Ultra (Millipore).

Immunoprecipitations and Western blotting. For immunoprecipitation, erythroblasts were lysed in the presence of the following protease inhibitors: NaVO₄, 1 μM; NaF, 1 mM; complete inhibitors (Roche Diagnostics). For detection of EpoR phosphotyrosine residues, the resulting supernatants were immunoprecipitated with an antibody directed against EpoR. Western blot analysis was performed using the following antibodies: anti-Rs (Tyro3), anti-Axl, anti-Mertk (Santa Cruz Biotechnology), anti-phosphotyrosine (clone 4G10; Upstate) and anti-murine EpoR (Upstate and R&D Systems). For analysis of phospho-Akt and total Akt (PhosphoPlus Akt [Ser473] kit; Cell Signaling Technology), 100 μg of lysate was resolved by SDS-PAGE and subjected to immunoblotting.

Adhesion of erythroblasts to fibronectin. Microtiter plates were coated with fibronectin (Sigma-Aldrich) and then blocked for 2 h at room temperature with BSA. Ter-119+ erythroblasts (10⁵ in RPMI supplemented with BSA (2 mg/ml) were added per well. Unattached cells were removed after 1 h, and wells containing attached cells were washed twice with PBS. Control plates containing both unattached and attached cells were centrifuged, and supernatant was removed. Attached cells and controls (both attached and unattached cells) were stained with 0.2% crystal violet in 10% ethanol. The dye was eluted with 33% acetic acid, and the absorbance was measured at 570 nm. The percent of adhesion to fibronectin was calculated by the optical density of attached erythroblasts divided by the optical density of controls (attached plus unattached erythroblasts). When indicated, adhesion experiments were performed in the presence of recombinant human Epo, LY294002 (Sigma-Aldrich), or anti-VLA4 antibody (clone PS2/2, 2 μg/10⁶ cells) (54). Apoptotic adhering erythroblasts were identified by the fragmentation of their nucleus stained by DAPI (55).

Quantitative real-time RT-PCR. Gene expression of Gas6, Tyro3, Axl, and Mertk was quantified by real-time RT-PCR, relative to the expression of GAPDH (Gas6) or β-actin (Tyro3, Axl, and Mertk), using an ABI PRISM 7000 (Applied Biosystems) real-time cycler and the following primers: GAPDH forward, 5′-CAACCGGGAAGCCCATCAC-3′; reverse, 5′-CGCGCTTCACCCCATTTG-3′; Gas6 forward, 5′-CGAGTCCTTCTTCACACTGTCTT-3′; reverse, 5′-GACCTTTGATATCGTGATGATAAAATAC-3′; Tyro3 probe, 5′-CCTGGCCACCTCCGGACGAC-3′; forward, 5′-GTGAAGCGCCCAAATAAAAG-3′; reverse, 5′-GTGGCTTGGAAGGGCAACATT-3′; Axl probe, 5′-AAGGGAGTCACCACTTCCTCCGGAC-3′; forward, 5′-TCTATGGAAGCCCACTACATC-3′; reverse, 5′-GGAGCACTGTGATGTTGGTCT-3′; Mertk probe, 5′-GGGCACTGTGATGTTGGTCT-3′; forward, 5′-GGCCCAAGCGGCGT-3′; reverse, 5′-TCCTGGCCACGAGGCT-3′. For Gas6, each reaction contained 4 μl SYBR Green PCR Master Mix (Applied Biosystems), 50–500 nM primer pair, 5 μl cDNA, and water to a final volume of 20 μl. PCR parameters were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of 15 s at 60°C. Gas6 mRNA transcript levels were normalized to GAPDH levels. For Tyro3, Axl, and Mer, Taqman quantitative real-time PCR was performed with the Taqman Universal PCR master mix kit (Applied Biosystems). Gas6R mRNA transcript levels were shown as the number of copies of receptor per 1,000 copies of β-actin.

Cytokine antibody array. Expression of various cytokines in plasma and macrophage supernatant was investigated by using a mouse protein cytokine array (62 cytokines; RayBiotech). Array membranes were processed according to the manufacturer’s instructions. We individually added 1 ml of a plasma pool (8 mice) or supernatant to the membrane and incubated at room temperature for 2 h. The results with immunoreactivity were assessed and quantified by using a VersaDoc Imaging System (Bio-Rad).

Statistics. Data are represented as mean ± SEM of n determinations. Standard t tests were used to calculate significance levels between groups. When analyzing experiments performed at multiple time points, ANOVA for repeated measurement statistics were used to calculate the overall significance and average difference between WT and Gas6−/− mice, followed
by standard t tests to calculate differences at the individual time points. Because repeated measurement statistics cannot handle missing values, we assigned the lowest hematocrit level (i.e., 11%) to mice that died in the course of the experiment. To compare multiple genotypes (e.g., Tyr+–, Axl–/–, or Mertk−/−) against the WT control group, we applied a Dunnett’s correction for multiple testing, using the WT group as reference. To compare the different treatments with each other (e.g., Gas6, Epo, or the combination of both), we applied Bonferroni corrections to correct for multiple testing. We used SPSS (Statistical Software Package version 11.0 for Mac OS X) for all these calculations. A P value less than 0.05 was considered significant.

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