

# The Integrin $\alpha 9\beta 1$ Contributes to Granulopoiesis by Enhancing Granulocyte Colony-Stimulating Factor Receptor Signaling

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## Summary

The integrin  $\alpha 9\beta 1$  is widely expressed on neutrophils, smooth muscle, hepatocytes, endothelia, and some epithelia. We now show that mice lacking this integrin have a dramatic defect in neutrophil development, with decreased numbers of granulocyte precursors in bone marrow and impaired differentiation of bone marrow cells into granulocytes. In response to granulocyte colony-stimulating factor (G-CSF),  $\alpha 9$ -deficient bone marrow cells or human bone marrow cells incubated with  $\alpha 9\beta 1$ -blocking antibody demonstrated decreased phosphorylation of signal transducer and activator of transcription 3 and extracellular signal-regulated protein kinase. These effects depended on the  $\alpha 9$  subunit cytoplasmic domain, which was required for formation of a physical complex between  $\alpha 9\beta 1$  and ligated G-CSF receptor. Integrin  $\alpha 9\beta 1$  was required for granulopoiesis and played a permissive role in the G-CSF-signaling pathway, suggesting that this integrin could play an important role in disorders of granulocyte development and other conditions characterized by defective G-CSF signaling.

## Introduction

Integrins are transmembrane heterodimers that participate in the translation of spatially fixed extracellular signals into a wide variety of changes in cell behavior (Hynes et al., 1999). Members of this family share common functions (e.g., cell attachment, spreading, and survival) and utilize a number of common signaling intermediates (e.g., the focal adhesion kinase and src family kinases) (Clark and Brugge, 1995; Hynes, 1992). However, the diverse and largely nonoverlapping phenotypes of mice expressing null alleles of individual integrin subunit genes underscores the biological importance of integrin specificity (Hynes and Bader, 1997; Sheppard, 2000).

The phenotype of  $\alpha 9$ -deficient mice provided strong evidence that  $\alpha 9\beta 1$  plays a unique and nonredundant role in vivo. These mice survive embryonic development normally, but all die by 12 days of age from respiratory failure (Huang et al., 2000). The presence of bilateral chy-

lothorax suggested a defect in lymphatic development in these animals.

The integrin  $\alpha 9\beta 1$  is widely expressed in airway epithelium; smooth, skeletal, and cardiac muscle cells; hepatocytes; and neutrophils (Palmer et al., 1993). Multiple ligands have been identified for this integrin, including the inducible endothelial counter-receptor, vascular cell adhesion molecule-1 (VCAM-1) (Taooka et al., 1999), the extracellular matrix proteins tenascin C (Yokosaki et al., 1994) and osteopontin (Smith et al., 1996; Yokosaki et al., 1999), some members of ADAMs family (a disintegrin and metalloproteases) (Bridges et al., 2004; Eto et al., 2002; Tomczuk et al., 2003), coagulation factor XIII, and von Willebrand factor (Takahashi et al., 2000).

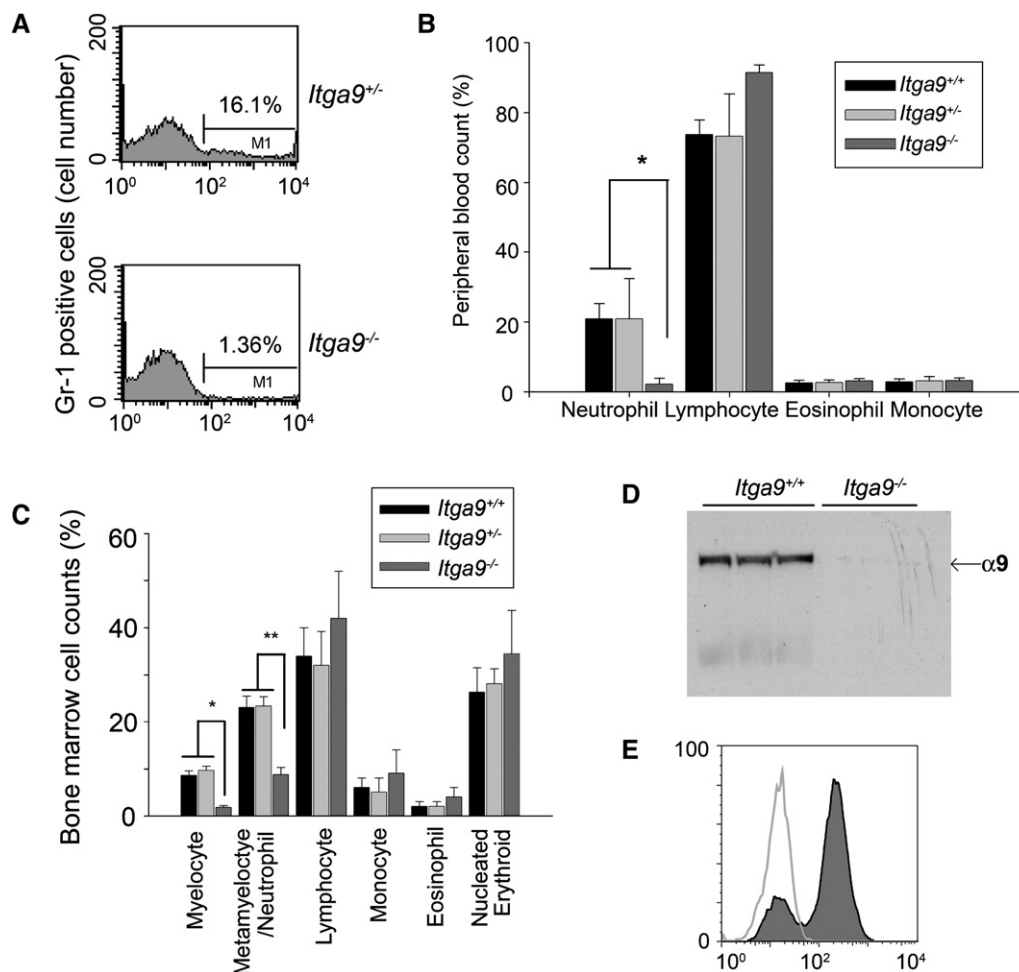
$\alpha 9\beta 1$  is highly expressed on human neutrophils and is critical for neutrophil migration on VCAM-1 and tenascin-C (Taooka et al., 1999). However, the in vivo function of  $\alpha 9\beta 1$  on neutrophils is not clear. Because of the early postnatal mortality in  $\alpha 9$ -deficient mice, neither we nor others have previously characterized the effects of loss of this integrin on neutrophil function. By evaluating peripheral blood and bone marrow from juvenile  $\alpha 9$ -deficient mice, we now describe a dramatic defect in granulocyte development in these animals. Characterization of this defect led us to the identification of a pathway by which integrin  $\alpha 9\beta 1$  enhanced G-CSF-receptor signaling and facilitated the effects of G-CSF on neutrophil development. These findings suggest a potentially important role for the  $\alpha 9\beta 1$  integrin in diseases characterized by defects in neutrophil development or other abnormalities in G-CSF signaling.

## Results

### Integrin $\alpha 9$ -Deficient Mice Have Defective Granulopoiesis

Peripheral blood from  $\alpha 9$ -deficient mice and littermate controls was stained for the presence of the neutrophil cell-surface marker Gr-1 (Figure 1A). The percentage of neutrophils was dramatically decreased in *Itga9*<sup>-/-</sup> mice (Figure 1B). There were no major differences in total white blood cells, platelets, lymphocytes, or eosinophils between *Itga9*<sup>-/-</sup>, *Itga9*<sup>+/-</sup>, and *Itga9*<sup>+/+</sup> mice (Figure 1B). The percentage of relatively mature myeloid cells (metamyelocytes and granulocytes) and myelocytes in bone marrow was also decreased in *Itga9*<sup>-/-</sup> mice. There were no differences in any other cell types (Figure 1C). Integrin  $\alpha 9\beta 1$  is expressed on granulocytes in human peripheral blood (Taooka et al., 1999). We confirmed (by immunoblotting) that  $\alpha 9\beta 1$  is also expressed on murine bone marrow cells (Figure 1D). Expression of  $\alpha 9\beta 1$  was also detected by flow cytometry on a subset of human bone marrow cells (Figure 1E). Integrin  $\alpha 9\beta 1$  is known contribute to cell adhesion and migration in vitro (Taooka et al., 1999; Young et al., 2001). It was thus possible that the decreased number of neutrophils could be due to redistribution. We therefore examined neutrophil and metamyelocyte numbers in the spleen. Neutrophils were 2% and metamyelocytes were 1% of total spleen cells. There was no difference between integrin  $\alpha 9$  null

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**Figure 1.  $\alpha$ 9-Deficient Mice Are Neutropenic**

(A) Flow cytometry analysis of neutrophils in peripheral blood. Peripheral blood was collected from wild-type, *Itga9*<sup>+/+</sup>, and *Itga9*<sup>-/-</sup> mice, stained with PE-conjugated anti-Gr-1, and analyzed by flow cytometry.

(B) Percentage of neutrophils in peripheral blood from wild-type, *Itga9*<sup>+/+</sup>, and *Itga9*<sup>-/-</sup> mice (determined by Wright-Giemsa staining). Values are means from at least six mice from each group. \*p = 0.003 (*Itga9*<sup>+/+</sup> versus *Itga9*<sup>-/-</sup>).

(C) Bone marrow analysis. Cell counts and differential counts were performed on cells recovered from six sex-matched littermates of each genotype. \*p = 0.002, \*\*p = 1.1 × 10<sup>-4</sup> (*Itga9*<sup>+/+</sup> versus *Itga9*<sup>-/-</sup>).

(D) Expression of integrin  $\alpha$ 9 $\beta$ 1 in mouse bone marrow cells. Lysates of bone marrow cells were blotted by  $\alpha$ 9 cytoplasmic domain antiserum 1057 (Palmer et al., 1993).

(E) Expression of integrin  $\alpha$ 9 $\beta$ 1 in human bone marrow cells. Cells from human donor bone marrow were stained with anti-integrin  $\alpha$ 9 $\beta$ 1, Y9A2. Open peaks represent fluorescence (FL) of mouse IgG1 control-stained cells, and shaded peaks represent fluorescence of CHO cells stained with Y9A2. Error bars denote the mean  $\pm$  SD.

mice and controls, suggesting that the decrease in neutrophils in peripheral blood and bone marrow of  $\alpha$ 9 null mice is not due to redistribution.

#### Integrin $\alpha$ 9-Deficient Mice Have a Defect in Granulocyte Differentiation

To determine whether the neutropenia in  $\alpha$ 9-deficient mice could be a consequence of cytokine-signaling defects in hematopoietic progenitors, we tested the in vitro responses of bone marrow to a range of hematopoietic growth factors. Responses to granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-3, IL-6, and stem cell factor (SCF) were the same for bone marrow harvested from *Itga9*<sup>-/-</sup> and *Itga9*<sup>+/+</sup> mice. However, in

response to granulocyte colony-stimulating factor (G-CSF), there was a selective decrease in both the number of colonies formed (Figure 2A) and the number of cells in each colony in *Itga9*<sup>-/-</sup> cells (Figure 2B). Integrin  $\alpha$ 9 null bone marrow cells had decreased number of colonies in response to range of concentration of G-CSF (Figure 2C). Both wild-type and integrin  $\alpha$ 9 null colonies contained 40%–50% mature neutrophils, suggesting that loss of  $\alpha$ 9 does not lead to maturation arrest (Figure 2D). This defect was partially rescued by restoring the expression of  $\alpha$ 9 to bone marrow from *Itga9*<sup>-/-</sup> mice (Figure 2E).

To determine the significance of this pathway for human bone marrow cells and to exclude the possibility that the abnormal response of  $\alpha$ 9-deficient mouse

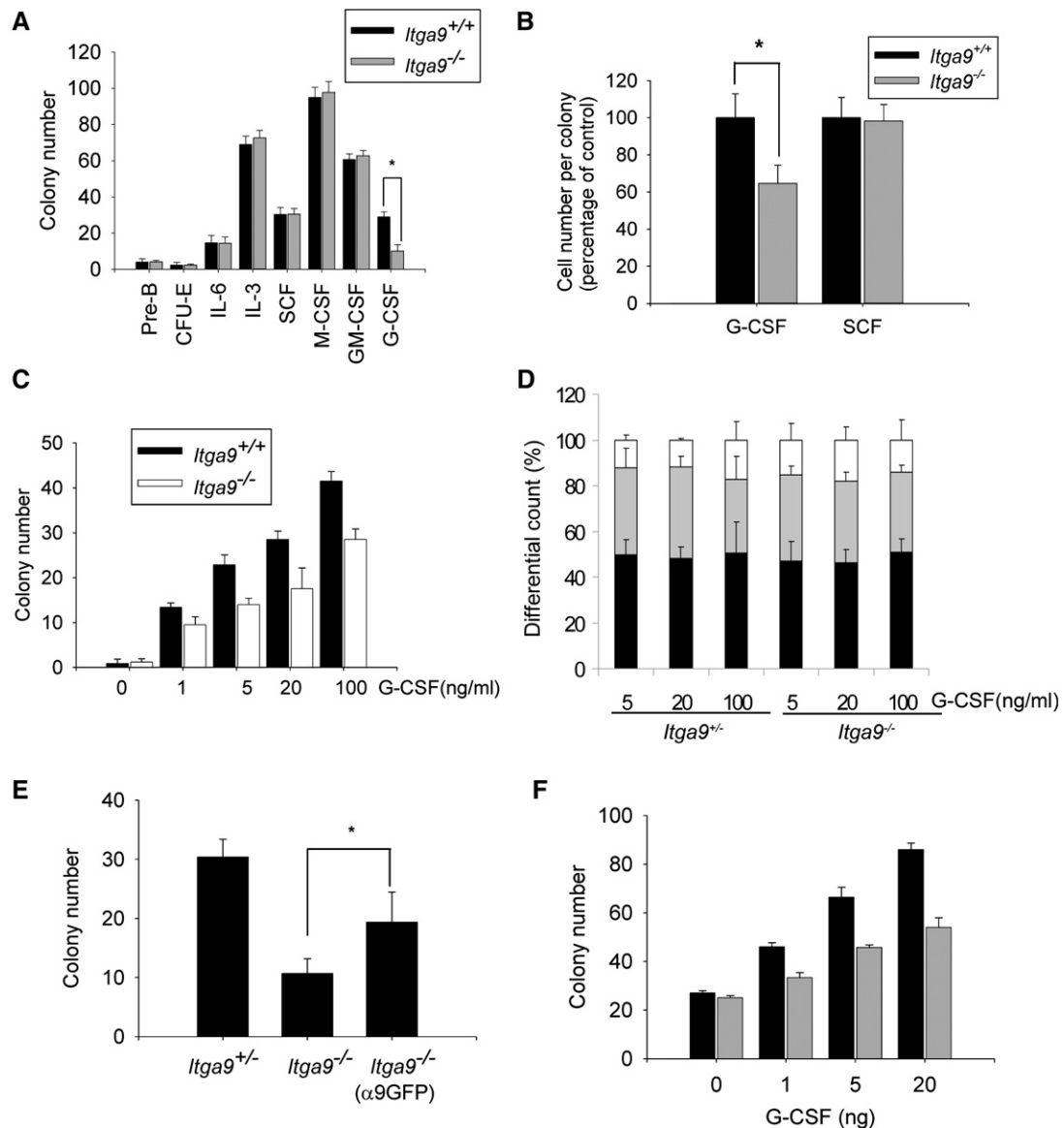


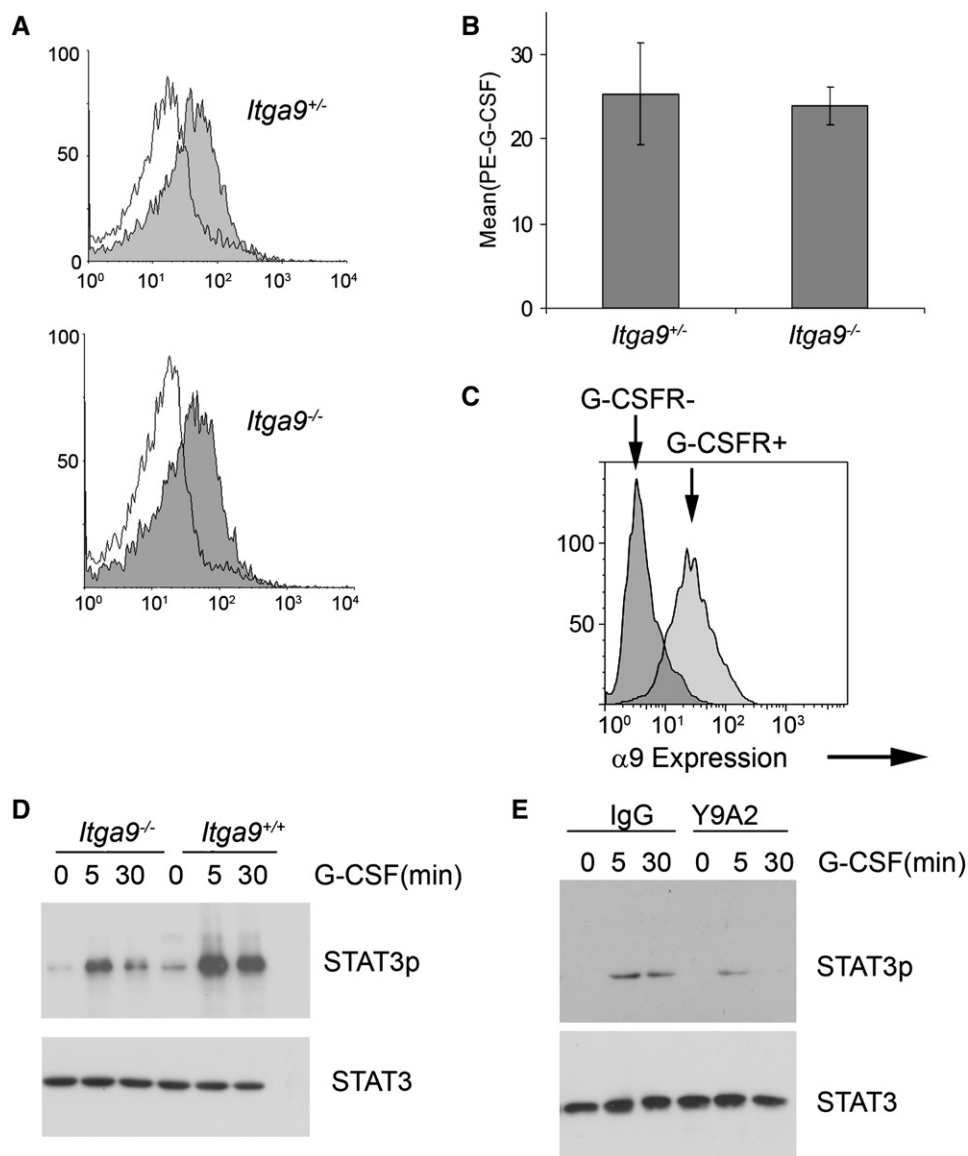
Figure 2.  $\alpha 9$ -Deficient Bone Marrow Is Hyporesponsive to G-CSF

(A) Bone marrow cells were plated in methylcellulose-containing media supplemented with the indicated cytokine. Hematopoietic colonies containing more than 30 cells were scored after 7–8 days.  $n = 7$  for each genotype.  $*p = 5.6 \times 10^{-4}$ .  
 (B) Cellular content of  $\alpha 9$ -deficient colonies relative to controls. 10–80 consecutive colonies from parallel cultures of bone marrow cells were picked, pooled, and counted. Mean  $\pm$  SD of results from four to six mice per genotype.  $*p = 1.5 \times 10^{-3}$ .  
 (C) Colony-forming assay for mouse bone marrow cells stimulated by G-CSF (0–100 ng/ml).  
 (D) Differential counts of bone marrow cells. Cells were collected from colony-forming assay. Black bars, neutrophils and bands; gray bars, myelocytes and metamyelocytes; white bars, other cells.  
 (E) Mouse bone marrow cells were infected with a retrovirus expressing full-length, mature  $\alpha 9$  ( $\alpha 9$ ) or with empty vector (vector). Colony-forming assays were performed with 20 ng/ml G-CSF.  $*p = 5 \times 10^{-3}$ .  
 (F) Colony-forming assays with human bone marrow cells stimulated by a range of concentrations of G-CSF (0–20 ng/ml) in the presence of integrin  $\alpha 9 \beta 1$ -blocking antibody Y9A2 (gray bars) or control normal mouse IgG (black bars). Error bars denote the mean  $\pm$  SD.

bone marrow to G-CSF was simply due to a decreased number of granulocyte precursors, we examined the effects of a blocking antibody to  $\alpha 9 \beta 1$  on in vitro colony formation with normal human bone marrow. Responses to G-CSF were substantially attenuated in these cells by treatment with the  $\alpha 9 \beta 1$ -blocking antibody Y9A2 (Figure 2F). Together, these results suggest that  $\alpha 9 \beta 1$  contributes to neutrophil development by specifically facilitating neutrophil differentiation in response to G-CSF.

#### Integrin $\alpha 9$ -Deficient Bone Marrow Cells Exhibit Attenuated Signal Transducer and Activator of Transcription 3 Activation in Response to G-CSF

To determine whether the reduced responsiveness to G-CSF could be due to decreased expression of G-CSF receptor in bone marrow cells from integrin  $\alpha 9$  null mice, we examined the surface expression of G-CSF receptor on mouse bone marrow cells by incubation with labeled G-CSF. There was no difference in receptor expression



**Figure 3. G-CSF-Induced STAT3 Phosphorylation Is Attenuated by Loss or Blockade of Integrin  $\alpha 9\beta 1$**   
**(A)** Flow cytometry of G-CSF receptor expression on mouse bone marrow cells. Cells were incubated with PE-conjugated G-CSF (shaded peak) or streptavidin-PE (open peak).  
**(B)** Mean fluorescence intensity for G-CSF receptor expression ( $n = 3$  for each genotype).  
**(C)** Flow cytometry analysis of expression of G-CSF receptor and integrin  $\alpha 9$  in human bone marrow mononuclear cells. Cells were incubated with PE-conjugated anti-G-CSF receptor and biotinylated anti- $\alpha 9\beta 1$  (Y9A2) or biotin mouse IgG1 control followed by incubation with FITC-conjugated streptavidin. G-CSF receptor-positive and -negative cells were gated as light-shaded and dark-shaded peaks.  
**(D)** STAT3 phosphorylation in mouse bone marrow cells. Bone marrow cells from  $\alpha 9$ -deficient or wild-type mice were treated with G-CSF for 5 or 10 min and analyzed by immunoblotting with anti-Phospho-STAT3 (Tyr705) or anti-total STAT3.  
**(E)** STAT3 phosphorylation of human bone marrow cells incubated with  $\alpha 9\beta 1$ -blocking antibody (Y9A2) or control antibody for 30 min followed by G-CSF treatment. Error bars denote the mean  $\pm$  SD.

between integrin  $\alpha 9$  null mice and controls (Figures 3A and 3B).

To determine whether the impaired responsiveness to G-CSF could be explained by interaction between  $\alpha 9\beta 1$  and the G-CSF-signaling pathway, we first examined whether  $\alpha 9\beta 1$  and the G-CSF receptor were expressed in the same cells. Flow cytometry showed that the majority of human bone marrow cells that express the G-CSF receptor also express  $\alpha 9\beta 1$  (Figure 3C). To assess the ability of the  $\alpha 9$ -deficient bone marrow cells

to produce the signals immediately downstream of the G-CSF receptor, we stimulated bone marrow with G-CSF and evaluated activation of the proximal signaling intermediate, STAT3 (signal transducer and activator of transcription 3). As expected, STAT3 phosphorylation was readily observed 5 min and 30 min after G-CSF stimulation of  $Itga9^{+/+}$  bone marrow cells. However, this effect was markedly reduced in  $\alpha 9$ -deficient bone marrow cells (Figure 3D). Similarly, STAT3 phosphorylation was decreased in human bone marrow treated with

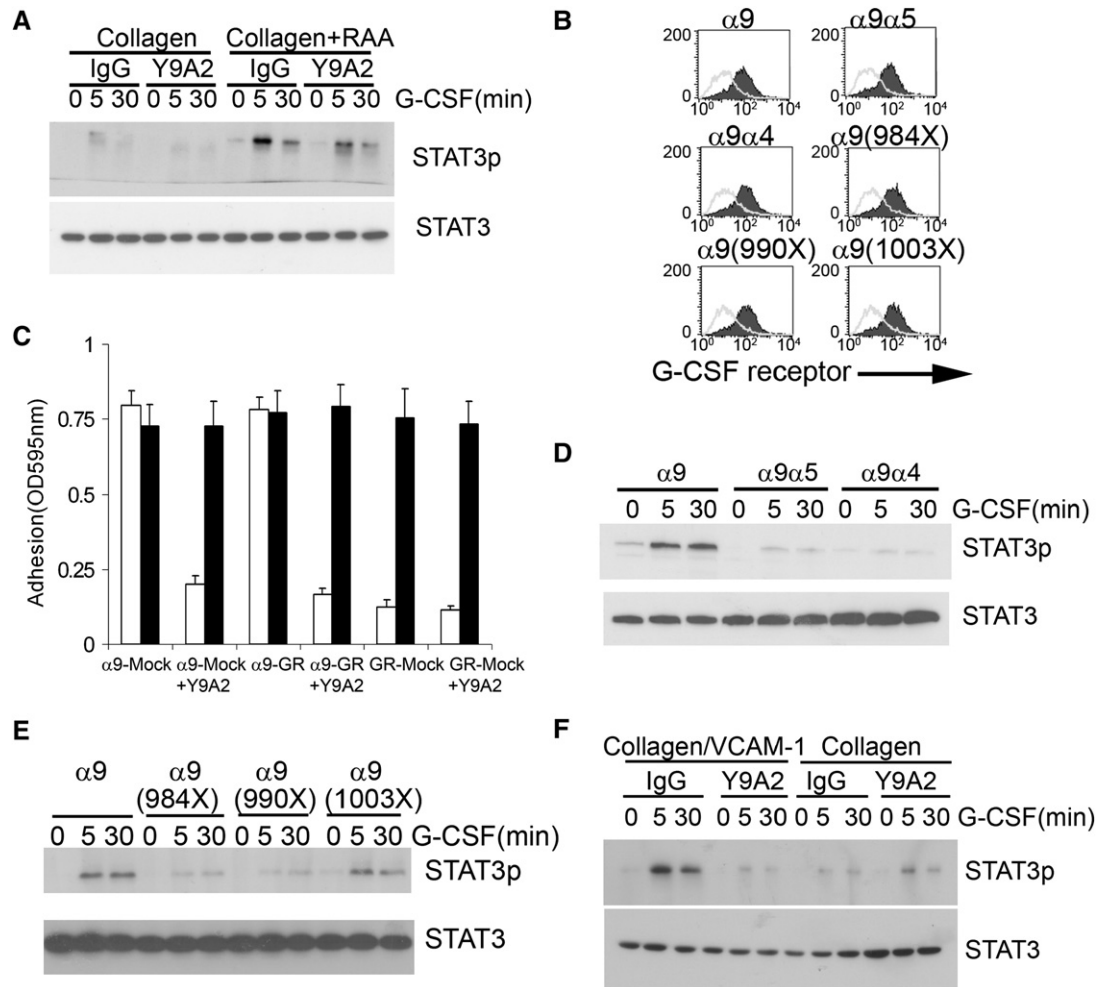


Figure 4. G-CSF-Induced STAT3 Phosphorylation Depends on Specific Sequences in the  $\alpha 9$  Cytoplasmic Domain

(A) Cells were treated with  $\alpha 9 \beta 1$ -blocking antibody, Y9A2, or control IgG and plated on either an irrelevant ligand (collagen) or on collagen and an  $\alpha 9 \beta 1$ -specific ligand (Tnfn3RAA) and were either untreated or treated with G-CSF for 5 or 30 min. Lysates were evaluated by immunoblotting with anti-Phospho-STAT3 (Tyr705) or anti-total STAT3.

(B) Flow cytometry of G-CSF receptor expression on transfected CHO cells. Open peaks represent fluorescence (FL) of unstained CHO cells, and shaded peaks represent fluorescence of CHO cells stained with the anti-G-CSF receptor.

(C) CHO cells expressing either G-CSF receptor alone, integrin  $\alpha 9 \beta 1$  alone, or both G-CSF receptor and integrin  $\alpha 9 \beta 1$  were plated on either 5  $\mu$ g/ml Tnfn3RAA or fibronectin after incubation with or without  $\alpha 9 \beta 1$  mAb, Y9A2. Adherent cells were stained with crystal violet and quantified by measurement of absorbance at 595 nm (Young et al., 2001).

(D) STAT3 phosphorylation in response to G-CSF in CHO cells expressing G-CSF receptor and wild-type integrin  $\alpha 9$  or  $\alpha 9$  chimeras.

(E) STAT3 phosphorylation in response to G-CSF in CHO cells expressing the G-CSF receptor and full-length integrin  $\alpha 9$  or  $\alpha 9$  deletion mutants.

(F) STAT3 phosphorylation in response to G-CSF in CHO cells expressing G-CSF receptor and integrin  $\alpha 9$ . Cells were plated on collagen or collagen and human recombinant VCAM-1-coated dishes. Error bars denote the mean  $\pm$  SD.

integrin  $\alpha 9 \beta 1$ -blocking antibody (Figure 3E). These results suggest that expression of  $\alpha 9 \beta 1$  does not affect G-CSF receptor expression, but enhances downstream STAT3 signaling in response to receptor ligation.

#### The Cytoplasmic Domain of Integrin $\alpha 9$ Is Critical for Enhancement of G-CSF Signaling

To further elucidate the role of the integrin  $\alpha 9$  subunit in G-CSF signaling, we stably expressed the G-CSF receptor and the integrin  $\alpha 9$  subunit (and thus  $\alpha 9 \beta 1$ ) in CHO cells. Cotransfected CHO cells were stimulated with G-CSF while plated on collagen (an adhesive substrate that is not a ligand for  $\alpha 9 \beta 1$ ) or on both collagen and Tnfn3RAA (an integrin  $\alpha 9 \beta 1$ -specific ligand) (Yokosaki et al., 1998). G-CSF-induced STAT3 phosphorylation

was markedly reduced in cells plated on collagen alone, suggesting that ligation of integrin  $\alpha 9 \beta 1$  is required for enhancement of G-CSF signaling (Figure 4A). G-CSF-induced phosphorylation of STAT3 was also markedly inhibited by  $\alpha 9 \beta 1$ -blocking antibody (Y9A2), as we saw in human bone marrow cells (Figure 4A).

To determine whether the cytoplasmic domain of the  $\alpha 9$  subunit specifically enhances G-CSF signaling, chimeric  $\alpha$  subunits composed of the extracellular and transmembrane domain of  $\alpha 9$  fused to the cytoplasmic domain of either the integrin  $\alpha 5$  or  $\alpha 4$  subunit were used (Young et al., 2001). CHO cell lines expressing equal amounts of each  $\alpha 9$  construct were transfected to express the G-CSF receptor, and similar G-CSF receptor expression was confirmed by flow cytometry

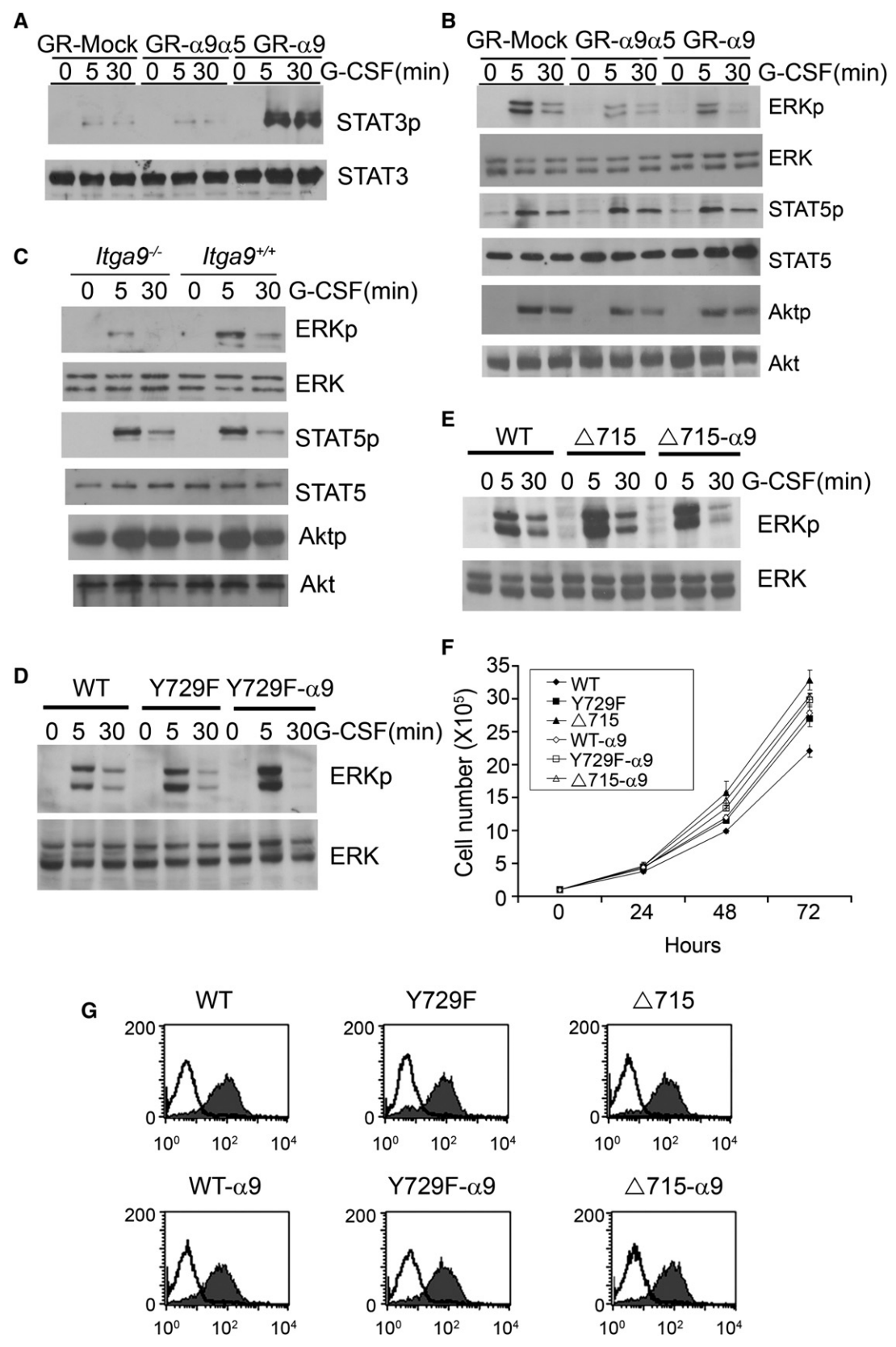


Figure 5. Integrin  $\alpha 9 \beta 1$  Enhances the Activation of STAT3 and ERK in Response to G-CSF in BaF3 Cells

(A) G-CSF-stimulated STAT3 phosphorylation of BaF3 cells expressing human G-CSF receptor and integrin  $\alpha 9 \beta 1$ .

(B) G-CSF-stimulated ERK, STAT5, and Akt phosphorylation.

(C) G-CSF-stimulated STAT3, ERK, STAT5, and Akt phosphorylation of mouse bone marrow cells from wild-type and  $\alpha 9$ -deficient mice.



(Figure 4B). We also examined adhesion of these transfected cell lines on Tnfn3RAA and the irrelevant ligand, plasma fibronectin. As expected, integrin  $\alpha 9\beta 1$ -expressing cells, but not mock transfectants, adhered to Tnfn3AA, and all cell lines adhered equally well to fibronectin (Figure 4C). There were no differences in morphology or cell proliferation between these cell lines (data not shown). G-CSF-induced phosphorylation of STAT3 was dramatically decreased in cell lines expressing either chimeric  $\alpha 9$  subunit (Figure 4D).

To identify the cytoplasmic sequences critical for enhancing G-CSF signaling, a series of  $\alpha 9$  cytoplasmic domain deletion mutants were used (translation stop codon was introduced into  $\alpha 9$  cDNA to generate different lengths of mutated  $\alpha 9$  protein: 984 aa, 990 aa, and 1003 aa; the full length of  $\alpha 9$  is 1006 aa). These deletion mutants were stably coexpressed with G-CSF receptor in CHO cells, and the expression of G-CSF receptor was similar in each line (Figure 4B). These mutants were chosen, in part, to evaluate the potential significance of two cytoplasmic proteins, spermine spermidine acetyl transferase (SSAT) and paxillin, that we have previously found to bind to the  $\alpha 9$  cytoplasmic domain and influence  $\alpha 9\beta 1$ -dependent effects on cell behavior (Chen et al., 2004; Liu et al., 2001; Young et al., 2001). Both of these functional effects are lost for  $\alpha 9(984X)$  but preserved for  $\alpha 9(990X)$ , and interaction of both paxillin and SSAT is preserved for  $\alpha 9(990X)$ . However, both of these mutants demonstrated markedly reduced G-CSF-induced STAT3 phosphorylation (Figure 4E). Only the C-terminal three amino acids of the  $\alpha 9$  cytoplasmic domain could be deleted without affecting this response (Figure 4E). Thus, sequences within the  $\alpha 9$  cytoplasmic domain are critical for enhancement of G-CSF signaling, but this response is not simply due to association with paxillin or SSAT. These results suggest that sequences within the  $\alpha 9$  cytoplasmic domain are critical for enhancement of G-CSF signaling, but that this response is not simply due to association with paxillin or SSAT.

#### Integrin $\alpha 9\beta 1$ Enhances G-CSF Signaling in Response to Naturally Occurring Ligand

Tnfn3RAA used in the above experiments is a recombinant form of the third fibronectin type III repeat of chicken tenascin-C containing an RGD to RAA mutation. To verify that a natural ligand for integrin  $\alpha 9\beta 1$  has the same function, we plated CHO cells on plates coated with collagen alone or collagen and human recombinant VCAM-1. Phosphorylation of STAT3 was enhanced in CHO cells from VCAM-1-coated plates and was inhibited by antibody against integrin  $\alpha 9\beta 1$  (Figure 4F).

#### Integrin $\alpha 9\beta 1$ Enhances G-CSF Signaling in Hematopoietic Cells

CHO cells expressing integrin  $\alpha 9\beta 1$  and the G-CSF receptor were convenient for examining the crosstalk between them in a simple system. However, CHO cells

are nonhematopoietic cells. To confirm the relevance of our findings to hematopoietic cells, we used a pre-B cell line BaF3 expressing the G-CSF receptor. We could not detect expression of integrin  $\alpha 9$  in BaF3 cells by reverse-transcript PCR or immunoblot (data not shown). We thus infected BaF3 expressing the G-CSF receptor with a retrovirus expressing either integrin  $\alpha 9$  or  $\alpha 9\alpha 5$ . Cells expressing both the wild-type integrin  $\alpha 9\beta 1$  and the G-CSF receptor demonstrated increased phosphorylation of STAT3 in response to G-CSF, whereas cells expressing the  $\alpha 9\alpha 5$  chimera were no different than cells expressing the G-CSF receptor alone (Figure 5A).

STAT3 has been reported as important for G-CSF-induced proliferation and differentiation (McLemore et al., 2001); however, observations in mice with a conditional knockout of STAT3 suggested that STAT3 could be a negative regulator of granulopoiesis (Lee et al., 2002). Some studies suggest that other signaling molecules might be more important in G-CSF-induced proliferation and differentiation, such as ERK (extracellular signal-regulated protein kinase) (Kamezaki et al., 2005), Akt, and STAT5 (signal transducer and activator of transcription 5) (Dong et al., 1998; Ilaria et al., 1999; Zhu et al., 2004). We therefore studied the activation of ERK, Akt, and STAT5 in integrin  $\alpha 9\beta 1$ -expressing BaF3 cells. Both the magnitude and duration of ERK phosphorylation were increased in  $\alpha 9\beta 1$ -expressing cells (Figure 5B). However, phosphorylation of Akt and STAT5 were unaffected by expression of  $\alpha 9\beta 1$ . To determine the relevance of these findings to primary bone marrow cells, we performed similar experiments with bone marrow from wild-type and  $\alpha 9$  null mice. ERK phosphorylation was enhanced in cells expressing  $\alpha 9\beta 1$ , whereas Akt and STAT5 phosphorylation was unaffected by the presence of the integrin (Figure 5C).

Suppressor of cytokine signaling 3 (SOCS3) is an important negative regulator of G-CSF signaling. SOCS3 downregulates signaling by binding the phosphorylated tyrosine 729 of the cytoplasmic domain of the G-CSF receptor (Zhuang et al., 2005). To determine whether  $\alpha 9\beta 1$  affects G-CSF signaling by modulating interactions with SOCS3, we examined ERK activation in BaF3 cells expressing the integrin and a mutant G-CSF receptor in which tyrosine 729 was changed to phenylalanine (Y729F). This mutation has been shown to prevent the interaction of the G-CSF receptor with SOCS3 (Hortner et al., 2002; Zhuang et al., 2005). As has been previously reported, the Y729F mutation enhanced G-CSF-induced ERK phosphorylation, but this effect was still further enhanced in cells expressing integrin  $\alpha 9\beta 1$  (Figure 5D). Thus, the effects of  $\alpha 9\beta 1$  ligation of G-CSF-receptor signaling are not likely due to inhibition of interactions with SOCS3.

In approximately 20% of patients suffering from severe congenital neutropenia (SCN), C terminus truncated mutations are found in the G-CSF receptor (Dong et al., 1995, 1997). These patients have an increased

(D and E) G-CSF-stimulated ERK phosphorylation in BaF3 cells expressing G-CSF receptor mutants (Y729F and  $\Delta 715$ ).

(F) Proliferation assay of BaF3 cells expressing wild-type or mutant G-CSF receptor. BaF3 cells were cultured in G-CSF (100 ng/ml) containing medium. Viable cell number was determined by counting and trypan blue exclusion.

(G) Flow cytometry of G-CSF receptor expression (shaded peaks) in BaF3 cells expressing wild-type, Y729F, or  $\Delta 715$  G-CSF receptor. Error bars denote the mean  $\pm$  SD.

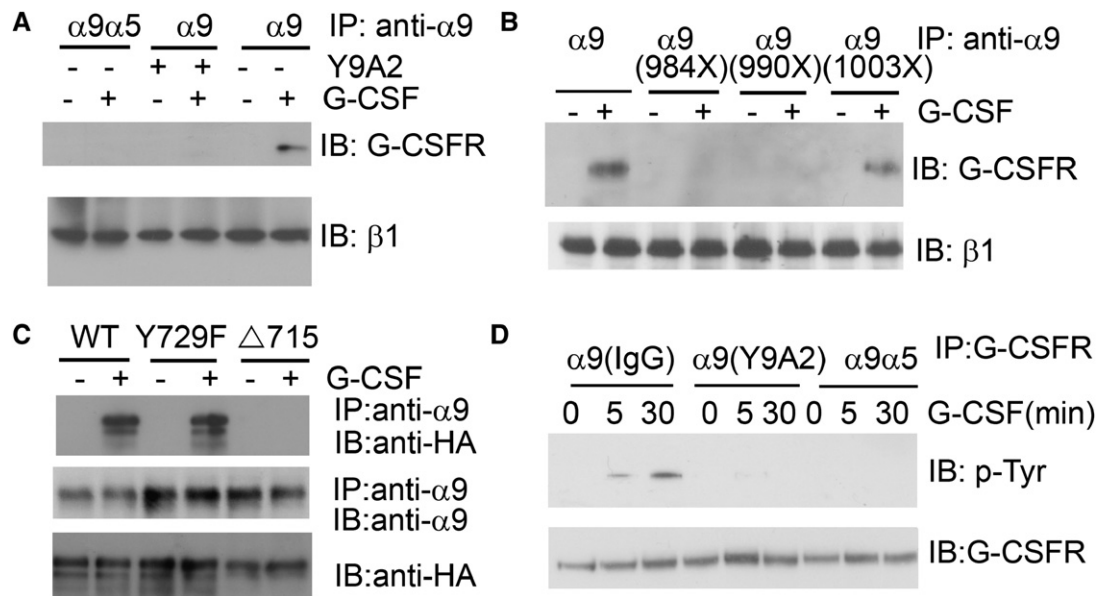


Figure 6. G-CSF-Induced G-CSF Receptor Phosphorylation and Association with Integrin  $\alpha 9\beta 1$

(A) CHO cells expressing G-CSF receptor and wild-type  $\alpha 9$  or an  $\alpha 9\alpha 5$  chimera were treated with  $\alpha 9$ -blocking antibody (Y9A2) or control IgG for 30 min, plated on Tnfn3RAA, and treated with G-CSF (20 ng/ml). Lysates were immunoprecipitated with  $\alpha 9\beta 1$  antibody A9A1, and precipitated protein was detected with anti-G-CSF receptor or integrin  $\beta 1$  cytoplasmic domain antiserum (to monitor equal capture of  $\alpha 9\beta 1$ ).

(B) CHO cells expressing G-CSF receptor and full-length integrin  $\alpha 9$  or  $\alpha 9$  cytoplasmic domain deletion mutants were incubated on Tnfn3RAA in the presence or absence of G-CSF, and lysates were analyzed as above.

(C) BaF3 cells expressing wild-type, Y729F mutant, or  $\Delta 715$  mutant G-CSF receptor and full-length integrin  $\alpha 9$  were incubated on Tnfn3RAA in the presence or absence of G-CSF. Lysates immunoprecipitated with  $\alpha 9\beta 1$  antibody A9A1 and probed with anti-HA or anti-integrin  $\alpha 9$  cytoplasmic domain (to monitor equal capture of  $\alpha 9\beta 1$ ).

(D) CHO cells expressing G-CSF receptor and wild-type integrin  $\alpha 9$  or an  $\alpha 9\alpha 5$  chimera were treated by anti- $\alpha 9\beta 1$  Y9A2 or control (IgG) and G-CSF as described above. Cells were lysed and immunoprecipitated by G-CSF receptor antibody. Precipitated protein was probed with anti-phosphotyrosine or anti-G-CSF receptor (to monitor equal capture of G-CSF receptor).

risk of developing acute myeloid leukemia (AML) (Dong et al., 1997). G-CSF receptor truncated at 715 mutant ( $\Delta 715$ ) shows a hyperproliferative response to G-CSF in vitro and increased phosphorylation of ERK (Ward et al., 1999; Zhu et al., 2004). To determine whether  $\alpha 9\beta 1$  ligation could modulate signaling mediated by the membrane-proximal region of the G-CSF receptor, we coexpressed the truncated  $\Delta 715$  mutant and  $\alpha 9\beta 1$  in BaF3 cells and examined ERK phosphorylation and proliferation in response to G-CSF. As previously reported, cells expressing  $\Delta 715$  had increases in G-CSF-induced ERK phosphorylation (Figure 5E) and increased rates of proliferation (Figure 5F), even in the absence of integrin  $\alpha 9\beta 1$ . However, expression of the integrin did not further enhance ERK phosphorylation or G-CSF-induced proliferation in these cells (Figures 5E and 5F). Expression of  $\alpha 9\beta 1$  increased G-CSF-induced proliferation in cells expressing either the wild-type or Y729F mutant. Flow cytometry demonstrated similar amounts of expression of wild-type, Y729F, and  $\Delta 715$  G-CSF receptors in both wild-type and  $\alpha 9\beta 1$ -expressing BaF3 cells (Figure 5G) and similar amount of  $\alpha 9\beta 1$  in all of the  $\alpha 9\beta 1$ -expressing cell lines (data not shown). Taken together, these results suggest that  $\alpha 9\beta 1$  modulates signaling through sequences in the distal region of the G-CSF receptor cytoplasmic domain to enhance ERK and STAT3 activity without affected signals mediated by STAT5, SOCS3, or Akt.

#### In Response to G-CSF, Ligated Integrin $\alpha 9\beta 1$ Physically Interacts with the G-CSF Receptor and Enhances Its Phosphorylation

To determine whether  $\alpha 9\beta 1$  could physically interact with the G-CSF receptor, we plated CHO cells expressing the G-CSF receptor and wild-type  $\alpha 9$  or an  $\alpha 9\alpha 5$  chimera on the  $\alpha 9\beta 1$ -specific ligand Tn3fnRAA and immunoprecipitated  $\alpha 9$ -containing complexes with the nonblocking  $\alpha 9\beta 1$  antibody, A9A1. In cells treated with G-CSF, but not in untreated cells, the G-CSF receptor was detectable in immunoprecipitates of wild-type  $\alpha 9\beta 1$ , but not in immunoprecipitates containing the chimeric  $\alpha 9\alpha 5$  subunit, despite equal capture of both integrins as determined by immunoblot for the associated  $\beta 1$  subunit (Figure 6A). Formation of complexes containing the G-CSF receptor and wild-type  $\alpha 9\beta 1$  clearly depended on ligation of the integrin, since complex formation was completely inhibited by the  $\alpha 9\beta 1$ -blocking antibody, Y9A2.

Two integrin  $\alpha 9$  cytoplasmic domain deletion mutations  $\alpha 9$ (984X) and  $\alpha 9$ (990X), which cannot enhance G-CSF signaling, were also evaluated for ability to physically associate with the G-CSF receptor by coimmunoprecipitation. Neither mutant appeared to associate with the G-CSF receptor (Figure 6B). In contrast, the one deletion mutant that preserved enhancement of G-CSF signaling ( $\alpha 9$ (1003X)) responded to G-CSF stimulation by associating with the G-CSF receptor to the same extent as seen with full-length  $\alpha 9$ . To confirm that physical



association was an important determinant of enhanced signaling, we also performed coimmunoprecipitation experiments in lysates from untreated or G-CSF-stimulated BaF3 cells expressing wild-type G-CSF receptor, the Y729F mutant (for which  $\alpha 9 \beta 1$  also enhances signaling) or the  $\Delta 715$  mutant (for which  $\alpha 9 \beta 1$  does not enhance signaling). As predicted, G-CSF treatment induced coassociation of the integrin with both the wild-type and Y729F mutants but not with the  $\Delta 715$  mutant (Figure 6C).

Tyrosine phosphorylation of the G-CSF receptor is an important early step in G-CSF signaling. Phosphorylation of the G-CSF receptor was evaluated by immunoprecipitation of the receptor followed by immunoblotting with a phosphotyrosine antibody. In response to G-CSF, the G-CSF receptor was phosphorylated in cells expressing wild-type, full-length  $\alpha 9$  but not in cells expressing the  $\alpha 9a5$  chimera. Phosphorylation of the G-CSF receptor was also inhibited by blocking antibody to  $\alpha 9 \beta 1$  (Figure 6D). Thus,  $\alpha 9 \beta 1$  is recruited to a protein complex containing the ligated G-CSF receptor, and the presence of  $\alpha 9 \beta 1$  in this complex enhances phosphorylation of the G-CSF receptor in response to G-CSF.

## Discussion

In this report, we describe a specific and dramatic defect in development of neutrophils in mice lacking the integrin  $\alpha 9$  subunit. This phenotype appears to be explained by a role for the  $\alpha 9 \beta 1$  integrin in enhancing signaling through the G-CSF receptor, an effect that we show depends on physical association of  $\alpha 9 \beta 1$  with the G-CSF receptor. Both enhancement of signaling and receptor coassociation depend on specific sequences within the  $\alpha 9$  subunit cytoplasmic domain.

The observation that an integrin can physically associate with and specifically enhance signaling through another cell surface receptor is not novel. Several integrins have been shown to enhance signaling through growth factor receptors, including receptors for platelet-derived growth factor (Baron et al., 2002; Schneller et al., 1997), vascular endothelial growth factor (Soldi et al., 1999), and epithelial growth factor (EGF) (Moro et al., 1998, 2002). A similar interaction has been described between a  $\beta 1$  integrin and the interleukin-3 receptor (Defilippi et al., 2005). In most of these cases, coligation of the integrin and the growth factor receptor leads to both enhancement in growth factor receptor signaling and to physical coassociation of the two receptors. At least one example has been described where ligation of an integrin,  $\alpha 5 \beta 1$ , induces tyrosine phosphorylation of a growth factor receptor (the EGF receptor) that appears to be independent of growth factor receptor ligation (Moro et al., 1998). Our data suggest that cooperative interaction between ligated G-CSF receptors and  $\alpha 9 \beta 1$  occurs at a step quite proximal to the initiation of G-CSF signaling, since three early steps in this pathway, phosphorylation of STAT3, ERK, and phosphorylation of the G-CSF receptor itself, are clearly enhanced by  $\alpha 9 \beta 1$ . However, in this case it does not appear that  $\alpha 9 \beta 1$  signaling is itself sufficient to initiate G-CSF signaling, since in the four cell systems we studied (human and murine bone marrow and transfected

CHO and BaF3 cells), neither enhanced STAT3 phosphorylation nor G-CSF receptor phosphorylation was detected in the absence of exogenous G-CSF. Based on our findings that coligation of the integrin and the G-CSF receptor led to physical association of both receptors, and on our observations that each of three  $\alpha 9$  subunit mutants that failed to promote coassociation also failed to enhance G-CSF signaling, we speculate that physical coassociation is critical for enhancement of signaling.

The precise molecular mechanism(s) by which physical coassociation of integrins and other receptors promote enhanced signaling have not been determined. Since G-CSF receptor phosphorylation is required for association with STAT3 and its subsequent phosphorylation by activated Janus kinases (JAKs), it is likely that enhanced phosphorylation of the G-CSF receptor is a proximal step in enhancement of G-CSF signaling by  $\alpha 9 \beta 1$ . Because the amount of G-CSF receptor phosphorylation is thought to be determined by the balance of phosphorylation by activated JAKs and dephosphorylation by receptor-associated phosphatases, we speculate that the activity or physical association of one or more of these kinases or phosphatases is regulated through  $\alpha 9 \beta 1$ .

Our data clearly demonstrate that the short  $\alpha 9$  subunit cytoplasmic domain is critical for both enhanced G-CSF signaling and physical association between ligated G-CSF receptor and  $\alpha 9 \beta 1$ . Integrin cytoplasmic domains are highly divergent, but even the most closely related  $\alpha 4$  subunit cytoplasmic domain cannot substitute for  $\alpha 9$ . These findings raise the possibility that one or more cytoplasmic proteins could bind to the  $\alpha 9$  cytoplasmic domain and mediate receptor coassociation and enhancement of G-CSF receptor signaling. We and others have previously identified two proteins that interact with the  $\alpha 9$  cytoplasmic domain, SSAT (Chen et al., 2004) and paxillin (Liu et al., 2001; Young et al., 2001). Both interactions are biologically important, since binding to paxillin appears to be critical for prevention of cell spreading in cells expressing  $\alpha 9 \beta 1$  (Young et al., 2001) and SSAT appears to be critical for  $\alpha 9 \beta 1$ -mediated enhancement of cell migration (Chen et al., 2004). One of the deletion mutants examined in the current study, 990X, retains the ability to interact with both proteins and to mediate both inhibition of cell spreading and enhancement of cell migration. However, this mutant was unable to mediate either enhanced G-CSF signaling or receptor coassociation, suggesting that interactions with paxillin and/or SSAT are not sufficient to explain these effects. While we cannot exclude participation of either or both of these proteins, it is clear that interactions with more C-terminal sequences within the  $\alpha 9$  cytoplasmic domain are also required.

Previous studies have identified residues in the G-CSF receptor cytoplasmic domain involved in activation or repression of downstream signaling intermediates. The  $\alpha 9$  cytoplasmic domain could modulate responses to G-CSF by direct or indirect interactions with any of these. For example, the membrane-proximal region of the G-CSF receptor (proximal to amino acid 715) has been shown to activate Akt and ERK and to enhance proliferation in response to G-CSF (Zhu et al., 2004). Our results, demonstrating no enhancement of Akt

phosphorylation in cells expressing the wild-type G-CSF receptor and no enhancement of ERK phosphorylation or proliferation by  $\alpha 9\beta 1$  in cells expressing the  $\Delta 715$  truncation mutant G-CSF receptor, suggest that  $\alpha 9\beta 1$  ligation does not modulate responses mediated by the membrane-proximal region of the receptor. Our data also suggest that  $\alpha 9\beta 1$  does not increase G-CSF-receptor signaling by inhibiting interactions with SOCS3, since  $\alpha 9\beta 1$  still enhanced both ERK phosphorylation and G-CSF-induced proliferation in cells expressing the Y729F mutant, which cannot bind to SOCS3. Together, these results suggest that  $\alpha 9\beta 1$  most likely enhances STAT3 and ERK activation and neutrophil development through interactions with regions of the distal G-CSF receptor cytoplasmic domain other than the Y729 SOCS3 binding site.

G-CSF has been reported to inhibit neutrophil apoptosis and therefore enhance neutrophil survival (Colotta et al., 1992; Martin et al., 1995; Rex et al., 1995). One recent report suggests that ligation of  $\alpha 9\beta 1$  also prevents apoptosis and enhances survival (Ross et al., 2006). It is thus conceivable that the effects we describe could be explained by enhancement of neutrophil survival. However, we have been unable to demonstrate any effects of  $\alpha 9\beta 1$  on apoptosis or survival with bone marrow from  $\alpha 9$ -deficient mice or human bone marrow treated with  $\alpha 9\beta 1$ -blocking antibody (data not shown). We therefore favor the hypothesis that  $\alpha 9\beta 1$  contributes to increased circulating neutrophil numbers and increased bone marrow colonies through enhancement of effects on G-CSF-mediated proliferation, differentiation, or both.

Our studies in CHO cells and human bone marrow suggest that the effects of  $\alpha 9\beta 1$  on enhancing G-CSF signaling might depend on integrin ligation. Multiple  $\alpha 9\beta 1$  ligands have been identified, including VCAM-1 (Taooka et al., 1999), tenascin C (Yokosaki et al., 1994), osteopontin (Smith et al., 1996; Yokosaki et al., 1999), ADAMs proteases (Bridges et al., 2004; Eto et al., 2002; Tomczuk et al., 2003), coagulation factor XIII, and von Willebrand factor (Takahashi et al., 2000). There are a number of extracellular matrix proteins expressed by bone marrow stem cells and stromal cells in bone marrow. VCAM-1 is expressed on stromal cells and mediates cell adhesion of hematopoietic progenitor cells (Simmons et al., 1992). Osteopontin and tenascin C are also expressed in bone marrow (Seiffert et al., 1998; Yamate et al., 1997). Osteopontin is a component of the hematopoietic stem cell (HSC) niche, which plays an important role in HSC migration and proliferation (Nilsson et al., 2005). So far there are no reports that inactivating any of these  $\alpha 9\beta 1$  ligands leads to defects in granulopoiesis, which may be a consequence of redundancy given the large number of known ligands. In our studies, it appeared likely that bone marrow cells generate their own  $\alpha 9\beta 1$  ligands in vitro, since an antibody that interferes with ligand binding had the same effect as genetic absence or deletion of the integrin. Furthermore, addition of exogenous  $\alpha 9\beta 1$  ligands to cultured bone marrow cells did not further enhance the effects on G-CSF receptor signaling caused by expression of the integrin (data not shown).

Recent evidence suggests that G-CSF signals important effects on a number of cell types other than

granulocytes. For example, the G-CSF receptor is expressed on endothelial cells, where expression of the adhesion receptors, E-selectin, vascular endothelial cell adhesion molecule-1, and intracellular adhesion molecule-1 at the cell surface are increased in response to G-CSF (Fuste et al., 2004). The G-CSF receptor is also expressed on neurons, and G-CSF inhibits apoptosis of mature neurons. G-CSF has been reported to inhibit acute neuronal degeneration and to contribute to long-term plasticity after cerebral ischemia (Schneider et al., 2005). Although we have not examined effects in other cell types,  $\alpha 9\beta 1$  is also widely expressed and we speculate that  $\alpha 9\beta 1$ -mediated enhancement of G-CSF signaling could contribute to effects of G-CSF at one or more of these other sites.

In summary, we have identified a pathway by which the integrin  $\alpha 9\beta 1$  dramatically enhances the response of bone marrow precursors to G-CSF signaling and thereby contributes to normal granulocyte development. This pathway involves physical association of  $\alpha 9\beta 1$  with the ligated G-CSF receptor and enhancement of the G-CSF-signaling pathway, beginning at a step proximal to receptor phosphorylation. Defects in this pathway could contribute to the development of congenital neutropenia and might also be important at other sites of G-CSF signaling.

## Experimental Procedures

### Cytokines, Reagents, and Antibodies

PE-conjugated anti-Gr1 and anti-G-CSF receptor were from BD Bioscience (San Jose, CA). Antibody against phospho-STAT3 (Tyr705), STAT3, phospho-ERK (Thr202/Ser204), ERK, phospho-STAT5 (Tyr694), STAT5, phospho-Akt (Ser473), Akt, HA, and phosphotyrosine mouse mAb were from Cell Signaling (Beverly, MA). The  $\alpha 9\beta 1$ -specific ligand Tnfn3RAA (Prieto et al., 1993; Yokosaki et al., 1994, 1998),  $\alpha 9$  mouse monoclonal antibody Y9A2 (Wang et al., 1996), A9A1 (Vlahakis et al., 2005), and rabbit polyclonal antibody 1057 (Palmer et al., 1993) were prepared in our laboratory. Purified normal IgG was from Jackson ImmunoResearch (West Grove, PA). Purified mouse IgG1 and biotinylated mouse IgG1 control were from eBioscience (San Diego, CA). Collagen was from Sigma-Aldrich (St. Louis, MO). Goat polyclonal anti-G-CSF receptor was from R&D Systems (Minneapolis, MN). Human G-CSF and polyclonal antibody against integrin  $\beta 1$  was from Chemicon International (Temecula, CA). Mouse cytokines G-CSF, GM-CSF, IL-3, IL-6, and SCF were from Stem Cell Technologies (Vancouver, Canada).

### Animal Husbandry

Mice maintained on a C57/BL6 background were housed at the animal care facility at the University of California, San Francisco, and all studies were approved by the institutional review board. Integrin  $\alpha 9\beta 1$  null mice were used for analysis between 6 and 7 days after birth.

### Murine Bone Marrow Analysis

Marrow was removed from femurs and tibias by flushing with PBS buffer. The cell pellet was incubated with red blood cell lysis buffer, resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, and captured on glass slides by cyto-spin. Slides were stained with Wright-Giemsa (Hema 3, Fisher Scientific, Tustin, CA), and 500 cells were counted per slide.

### Flow Cytometry

Murine bone marrow or peripheral blood cells were collected at autopsy and suspended in phosphate-buffered saline (PBS). Human bone marrow cells were obtained from Allicells (Berkeley, CA). Cells were incubated with primary antibody for 30 min at 4°C, followed by a secondary goat anti-mouse antibody conjugated with phycoerythrin. The expression of G-CSF receptor on mouse bone marrow

cell surface was assayed by G-CSF phycoerythrin fluorokine kit from R&D Systems.

#### Hematopoietic Progenitor Cell Assays

$2 \times 10^4$  bone marrow cells were plated in either 1 ml of methylcellulose media MethoCult M3231 (Stem Cell Technologies) for mouse bone marrow cells or MethoCult M4230 for human bone marrow cells, supplemented with the indicated cytokines. Colonies were counted on days 7–8. Recombinant cytokines were used at the following concentrations: G-CSF, 20 ng/ml; GM-CSF, 10 ng/ml; IL-3, 10 ng/ml; IL-6, 500 ng/ml; and stem cell factor, 100 ng/ml. To examine cell morphology and cell number, entire methylcellulose cultures were harvested, washed extensively, counted, and evaluated by Wright-Giemsa staining.

#### Retrovirus Infection

Retroviruses (pBABE-puro $\alpha 9$  [Young et al., 2001] or empty pBABE-puro) were generated by transfection into the Phoenix-E virus packaging cell line. Bone marrow cells were infected by incubation with supernatants from infected Phoenix E cells.

#### Immunoblot Analysis and Coimmunoprecipitation

For immunoblotting, bone marrow cells were pelleted by centrifugation, resuspended in PBS, and treated for 10 min at 0°C with 2 mM diisopropyl fluorophosphate (DFP), a cell-permeable serine protease inhibitor, as described previously [Selsted et al., 1992]. Cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM sodium ortho-vanadate, 50 mM NaF, 1% Triton X-100, lysates were separated by SDS-PAGE, transferred to immobilon, incubated with primary antibodies, and detected by ECL (Amersham Pharmacia Biotech, Piscataway, NJ). CHO cell lines were serum starved overnight, plated on Tnfr3RAA or collagen-coated plates, stimulated with human G-CSF (20 ng/ml) for 5 and 30 min, and lysed as described above. BaF3 cells were grown in RPMI medium with 10% fetal calf serum and 2 ng/ml murine recombinant interleukin 3. Cells were serum starved overnight, plated on Tnfr3RAA and fibronectin-coated plates, and stimulated with human G-CSF (100 ng/ml) for 5 and 30 min at 37°C. Cells were lysed in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM  $\text{Na}_3\text{VO}_4$ , 1 tablet per 10 ml of solution of Complete Mini Protease Inhibitor Cocktail tablets (Roche).

For coimmunoprecipitation, CHO or BaF3 cell lines were lysed on ice for 30 min in an immunoprecipitation buffer: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM sodium ortho-vanadate, 50 mM NaF, 1% CHAPS, 1 tablet per 10 ml of solution of Complete Mini Protease Inhibitor Cocktail tablets (Roche). Lysates were clarified by centrifugation at 16,000  $\times$  g and incubated with protein G-Sepharose coated with the  $\alpha 9\beta 1$  antibody, A9A1 at 4°C overnight. Beads were washed five times, and precipitated polypeptides were extracted in Laemmli sample buffer, separated by SDS-PAGE under reducing conditions, probed with a G-CSF receptor monoclonal antibody (for CHO cells) or anti-HA (for BaF3 cells), and detected by ECL.

#### Generation of Stable Cell Lines

Integrin  $\alpha 9$ -,  $\alpha 9\alpha 4$ -,  $\alpha 9\alpha 5$ - or  $\alpha 9(984\text{X})$ -,  $\alpha 9(990\text{X})$ -, and  $\alpha 9(1003\text{X})$ -expressing CHO cells were transfected with human G-CSF receptor cDNA (IMAGE: 5217305 clone from ATCC) in pSPORT6 vector at a 5:1 ratio with pcDNA3.1.Hygro to induce hygromycin resistance. Stable clones were obtained by hygromycin selection and cell sorting by G-CSF receptor expression. BaF3 cells were infected by incubation with  $\alpha 9$ -expressing retroviruses. Hemagglutinin (HA)-tagged G-CSF receptor and mutated receptors were described previously [Zhu et al., 2004]. Integrin  $\alpha 9$ -expressing BaF3 cell were transfected by electroporation. Stable cell lines were established by selection with 500  $\mu\text{g}/\text{mL}$  of G-418.

#### Cell Proliferation in Response to G-CSF

BaF3 cells proliferation was assayed as previously described [Zhu et al., 2004]. In brief, BaF3 cells were plated in Tnfr3RAA-coated plates at a density of  $1 \times 10^5$  cells/ml with 100 ng/ml human G-CSF in 10% serum medium. Cell numbers were counted daily and assessed for viability by trypan blue exclusion.

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