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Intrinsic Requirement for Zinc Finger Transcription Factor Gfi-1 in Neutrophil Differentiation

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Summary

We report essential roles of zinc finger transcription factor Gfi-1 in myeloid development. Gene-targeted Gfi-1$^{-/-}$ mice lack normal neutrophils and are highly susceptible to abscess formation by gram-positive bacteria. Arrested, morphologically atypical, Gr1^{+}Mac1^{+} myeloid cells expand with age in the bone marrow. RNAs encoding primary but not secondary or tertiary neutrophil (granulocyte) granule proteins are expressed. The atypical Gr1^{+}Mac1^{+} cell population shares characteristics of both the neutrophil and macrophage lineages and exhibits phagocytosis and respiratory burst activity. Reexpression of Gfi-1 in sorted Gfi-1$^{-/-}$ progenitors ex vivo rescues neutrophil differentiation in response to G-CSF. Thus, Gfi-1 not only promotes differentiation of neutrophils but also antagonizes traits of the alternate monocyte/macrophage program.

Introduction

Differentiation of blood cells from hematopoietic stem cells (HSCs) is controlled by lineage-restricted transcription factors (Orkin, 2000). Nonlymphoid hematopoietic lineages develop from a common myeloid progenitor (CMP), which later gives rise to more restricted progenitors, the granulocyte/monocyte (GMPs) and the megakaryocyte/erythrocyte (MEPs) progenitors (Akashi et al., 2000). In establishing cellular identity, lineage-determining factors serve dual roles: promoting lineage-specific transcriptional programs while simultaneously suppressing alternative lineage programs. How the identities of the two major myeloid lineages, neutrophils and monocyte/macrophages, are distinguished from one another is incompletely understood (Friedman, 2002). Although multiple transcription factors have been implicated in neutrophil development (Tenen et al., 1997), thus far only PU.1 (McKercher et al., 1996; Scott et al., 1994), C/EBPα (Zhang et al., 1997), and C/EBPϵ (Yamanaka et al., 1997a) have been shown to be essential. PU.1 loss leads to the absence of B cells, monocytes, eosinphils, and neutrophils (McKercher et al., 1996; Scott et al., 1994). C/EBP$\alpha^{-/-}$ mice lack neutrophils and eosinophils, though monocyte/macrophage development is intact (Zhang et al., 1997). C/EBPϵ disruption in mice causes failure of terminal differentiation of neutrophils and eosinophils (Yamanaka et al., 1997a).

Neutrophil development proceeds in a sequential fashion with defined features of morphology and gene expression (Borregaard et al., 2001; Friedman, 2002). At the myeloblast stage, neutrophil precursors become morphologically distinct. Progressively with maturation, precursors acquire three classes of granules distinguished by their protein constituents (Borregaard et al., 2001). At the promyelocyte stage, myeloperoxidase and neutrophil elastase are expressed and primary (azurophil, peroxidase-positive) granules form. With maturation to the myelocyte stage, transcription of these genes ceases and the production of secondary (peroxidase-negative, lactoferrin-positive) granules is initiated. Finally, tertiary (gelatinase-containing) granule production commences at the metamyelocyte stage (Borregaard et al., 2001).

Gfi-1 was first identified as a target gene for proviral activation conferring interleukin-2-independent growth to a rat thymoma cell line ex vivo (Gilks et al., 1993). The Gfi-1 gene corresponds to pal-1, a site of retroviral insertions that cooperate with c-*myc* in lymphomagenesis (van Lohuizen et al., 1991). Studies suggest that Gfi-1 inhibits apoptosis and cell cycle arrest induced by growth factor withdrawal (Grimes et al., 1996). Gfi-1 encodes a nuclear zinc finger transcriptional repressor (Grimes et al., 1996; Zweidler-Mckay et al., 1996). Gfi-1 shares nearly identical zinc fingers and a transcriptional repressor domain (SNAG) with a related protein, Gfi-1b (Grimes et al., 1996; Tong et al., 1998), which is essential for megakaryocytic and erythroid cell development (Saleque et al., 2002). Gfi-1 expression, initially described only in thymus, spleen, testis, and T cell lines (Gilks et al., 1993; Grimes et al., 1996a), was more recently detected in the bone marrow (Tong et al., 1998). We demonstrate here that Gfi-1 is essential for neutrophil differentiation and also antagonizes traits of the alternative monocyte/macrophage lineage.

Results

Gfi-1 Loss Results in Growth Retardation, Early Lethality, and Bacterial Infection

The expression of Gfi-1 in hematopoietic lineages was assessed (Figure 1A). T cells, B cells, and primary granulocytes expressed high levels of Gfi-1 RNA. Expression was not detected in erythroid, megakaryocytic, or macrophage cell lines, or primary macrophages.

The Gfi-1 gene was disrupted as shown in Figure 1B. Proper targeting was confirmed by Southern blot analysis using 5′ and 3′ probes (Figure 1C). The neomycin

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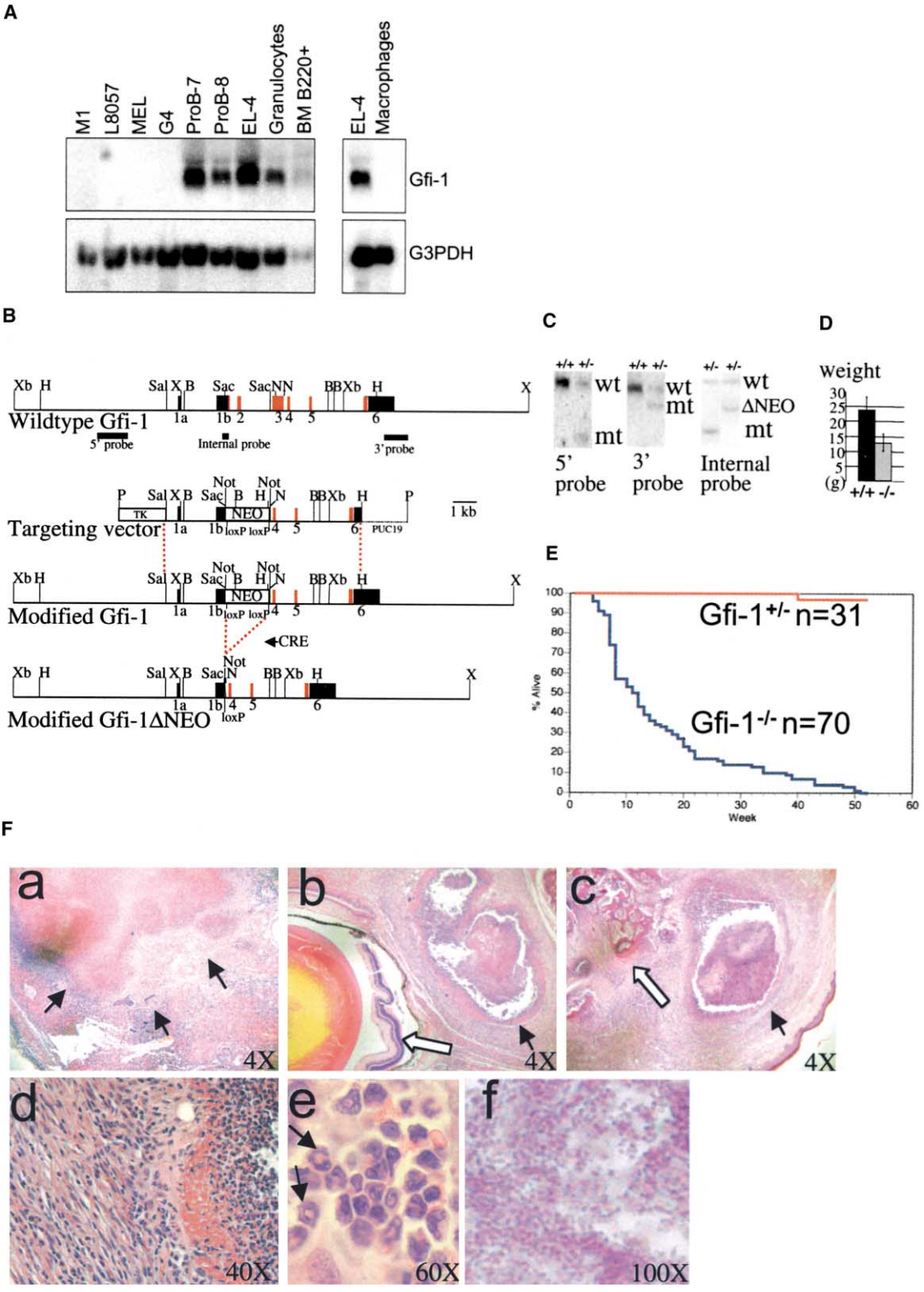

Figure 1. Disruption of the Mouse Gfi-1 Locus

(A) Restricted expression of Gfi-1 in hematopoietic lineages assessed by Northern blot. M1, macrophage cell line; L8057, megakarycocytic cell line; MEL, G4, erythroleukemia cell lines; pro-B-7, pro-B-8, pro-B cell lines; EL4, T cell line; BM 220^{+}, B220 positive bone marrow cells. (B) Scheme of targeting strategy shows Gfi-1 locus with coding exons in red (top), targeting vector (second), and the locus before (third) and after (bottom) deletion of NEO. Restriction enzymes: XbaI (Xb), HindIII (H), SalI (Sal), XhoI (X), BamHI (B), SacII (Sac), NsiI (N), NotI (Not), PvuI (P). (C) Southern blot of ES cell DNA using 5′ probe (Digest: HindIII, 14.6 kb wild-type, 8.3 kb mutant) or 3′ probe (Digest: NotI/XhoI, 15.1 kb wild-type, 10.6 kb mutant). Cre-mediated deletion of NEO in the germline was detected using internal probe (wild-type 6.2 kb, mutant 4.5 kb, after

gene was removed in vivo by Cre-recombinase mediated excision (Figure 1C). The phenotype of Gfi-1$^{-/-}$ mice described below was independent of the neomycin-resistance gene (data not shown). Gfi-1$^{-/-}$ mice were small (Figure 1D) and had a median survival of 11 weeks (Figure 1E). Heterozygous animals appeared normal (Figure 1E). Gfi-1$^{-/-}$ mice exhibited a high incidence of infections. Many suffered from chronic eye infections. Nine Gfi-1$^{-/-}$ animals, found dead or euthanized in moribund condition, were subjected to pathological analysis. In six mice (6–22 weeks of age), massive, partially encapsulated abscesses were seen. These were chronic and eroded normal tissue (extremities, liver, lung, retroorbital space, and lymph node) (Figures 1Fa, 1Fb, and 1Fc). Abscesses, which were surrounded by thick-walled, organized capsules (Figure 1Fd), contained inflammatory cells, including granulocyte-like cells with ring-shaped nuclei (Figure 1Fe). Two animals had abscesses simultaneously at different locations. In all abscesses, gram-positive cocci were demonstrated by gram-staining (Figure 1Ff). In three animals, no abscesses were detected, yet pulmonary abnormalities (cellular infiltrates, edema) were found in the absence of documented infection. In all mice, hypotrophic thymic corteces and myeloid hyperplasia of the bone marrow and extramedullary erythropoiesis in the spleen were observed. In some animals, plasma cell hyperplasia was seen in lymphoid tissues.

Thymic T Cell Number Is Reduced in Gfi-1$^{-/-}$ Mice, but Mature T and B Cells Populate Lymphatic Tissues

At 4–6 weeks of age, the thymic cellularity of Gfi-1$^{-/-}$ mice was reduced to ~10% that of controls (Figure 2A). In normal thymus a small population of CD4$^-$CD8$^-$ cells gives rise to CD4$^+$CD8$^+$ cells that mature to single-positive T cells following selection (Figure 2Ba). Developmental progression of the earliest T cells (CD4$^-$CD8$^-$) is marked by sequential changes in CD44 and CD25 expression (CD44$^+$CD25$^-$→CD44$^+$CD25$^+$→CD44$^-$CD25$^+$→CD44$^-$CD25$^-$) (Godfrey et al., 1993). In Gfi-1$^{-/-}$ mice, CD44$^+$CD25$^+$ cells were increased and more mature CD44$^-$CD25$^+$ cells were sharply reduced (Figures 2Bc and 2Bd). This suggests an incomplete block in T cell development at this stage. In agreement with this, CD4$^+$CD8$^+$ T cells, which arise from the CD4$^-$CD8$^-$ CD44$^-$CD25$^+$ population, are also reduced. Mature single-positive CD8$^+$CD4$^-$ and CD4$^+$CD8$^-$ were found not only in the thymus, but also in the spleen, lymph nodes, and blood, albeit at reduced numbers (Figures 2Ca and 2Cb). We note that the ratio of CD4/CD8 was moderately but consistently reduced in Gfi-1$^{-/-}$ mice (Figures 2Ca and2Cb). Peripheral T cells displayed normal $\alpha\beta$- and $\gamma\delta$-T cell receptors (data not shown).

As described below, Gfi-1$^{-/-}$ mice develop progressive myeloid hyperplasia of the bone marrow, complicating assessment of B cell development. However, we note that B cell numbers in the bone marrow of Gfi-1$^{-/-}$ mice were 5-fold reduced even in young mice in which marrow erythropoiesis was largely preserved (Figure 2D and Figures 3Eh and 3Ei, below). Immunophenotyping of the B cell compartment revealed that the proportions of B cells at different maturational stages were not strikingly altered. Pro-B cell (B220$^+$S7$^+$) maturation appeared normal as assessed by acquisition of HSA and BP-1 antigens (BP-1$^-$HSA$^-$→BP-1$^-$HSA$^+$→BP-1$^+$HSA$^+$) (Figures 2Ea and 2Eb) (Hardy et al., 1991). Likewise, expansion of B cells from the pro-B to the pre-B cell stage was only moderately decreased in the mutant (Figures 2Ec and 2Ed). Therefore, immunophenotyping did not reveal a major developmental block in the B cell lineage. In contrast to the striking reduction of developing B cells, mature B cells (IgM$^+$ IgD^{bright}) were not drastically reduced in spleen, lymph nodes, and blood of Gfi-1$^{-/-}$ mice (Figure 2F).

Gfi-1 $^{-/-}$ Mice Lack Phenotypically Mature Neutrophils

In blood of 4- to 6-week-old Gfi-1$^{-/-}$ mice, white blood cell, hemoglobin, and platelet counts were similar to controls (Table 1A). In many older mice, total white blood cell counts were elevated to ~30,000/μl (data not shown). Monocytes (Figures 3Aa and 3Ab), lymphocytes (Figures 3Ac and 3Ad), and eosinophils (Figures 3Ae and 3Af) from the peripheral blood of Gfi-1$^{-/-}$ mice were morphologically normal and present in normal numbers. In contrast, normal neutrophils were absent (Table 1A and Figure 3A). Normal neutrophils display segmented ring-shaped nuclei, faint to invisible cytoplasm and subtle neutrophilic granulation (Figures 3Ah and 3Ai). Normal juvenile granulocytes, which are infrequent, have nonsegmented thin, ring-shaped nuclei and neutrophilic granulation (Figure 3Ag). Instead of neutrophils, Gfi-1$^{-/-}$ mice had atypical cells, distinguished by a bluer cytoplasm and pleiomorphic nuclei (Figures 3Aj–3Ar). Approximately twenty percent of these abnormal cells had ring-shaped nuclei, a hallmark of neutrophil differentiation (Table 1A and Figures 3Aj–3Al). Even in such cells, however, nuclei were less condensed and their cytoplasm was blue and lacked neutrophilic granulation, suggesting a maturation defect (Figures 3Aj–3Al). Other atypical cells resembled activated monocytes, but most did not display unequivocal characteristics of a single lineage (Figures 3Am–3Ar). FACS analysis revealed that many of the abnormal blood cells had the same immunophenotype (Gr1$^+$Mac1$^+$) as granulocytes (Figure 3B).

In normal bone marrow, juvenile neutrophils with thin ring-shaped nuclei and faint cytoplasm are the predominant cell type (Table 1B and Figure 3C). Remarkably, such cells were absent in the bone marrow of Gfi-1$^{-/-}$ mice (Table 1B and Figure 3C). Instead, atypical cells with ring-shaped nuclei were evident (Table 1B and Fig-

NEO deletion [ΔNEO] 2.6 kb).

(D) Body weight of mutant (n = 12) mice compared to wild-type (n = 10) at 4–6 weeks.

(E) Survival of Gfi-1$^{-/-}$ mice compared to heterozygous littermates.

(F) In Gfi-l$^{-/-}$ mice massive abscesses were found in the liver ([a] arrows), retroorbital space ([b] abscess, black arrow; retina, white arrow), and lower extremity ([c] abscess, black arrow; bone, white arrow). Abcesses are surrounded by thick organized walls (d). Inflammatory infiltrate contains ring-shaped cells with little segmentation ([e] arrows). Innumerable gram-positive cocci in abscess cavity (f).

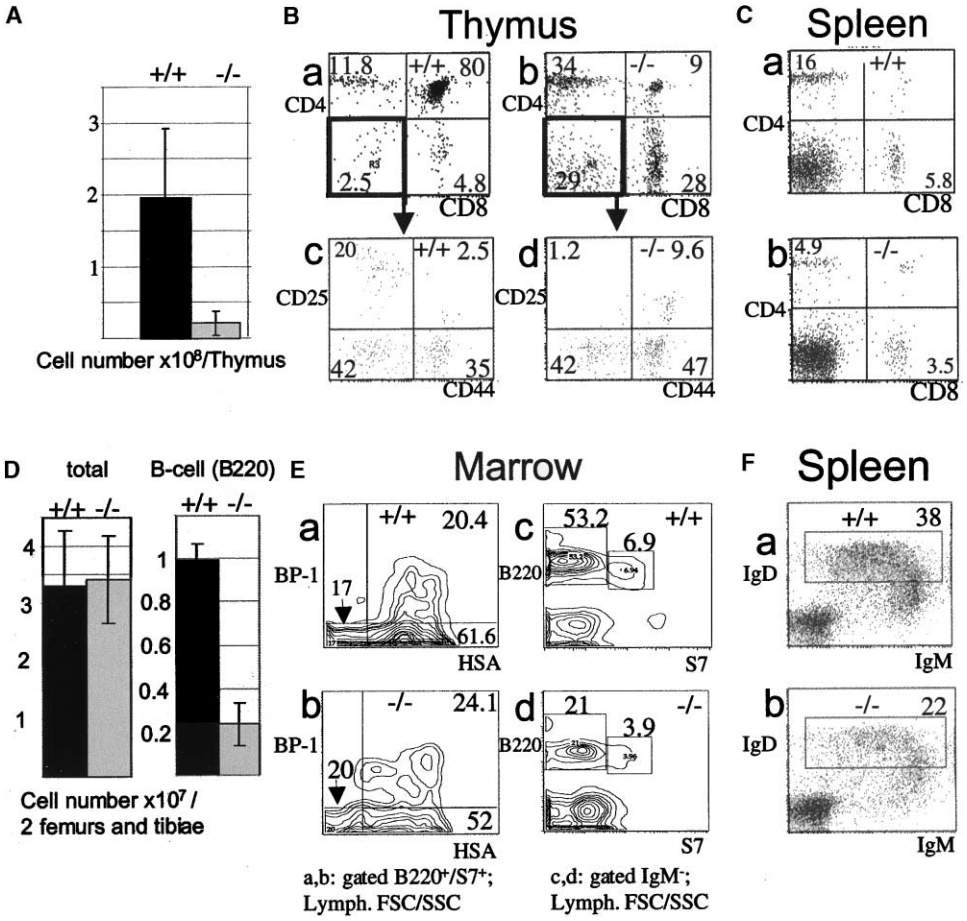

Figure 2. Lymphoid Phenotype Due to Gfi-1 Loss

(A) Cell numbers in the thymus of Gfi-1$^{-/-}$ mice (n = 16) compared to wild-type littermates (n = 11).

(B) Analysis of CD4 and CD8 expression in the thymus (a and b). CD4^{-}CD8^{-} thymocytes are staged by CD25 and CD44 expression ([c and d]; see Results).

(C) Mature CD4^{+} and CD8^{+} single-positive T cells (a and b) in the spleen.

(D) Numbers of total bone marrow cells and B220^{+} cells in mutant mice (n = 5) compared to littermates (n = 5).

(E) Analysis of pro-B cell stages (a and b) and comparison of pro-B cell (IgM^{-}, B220^{+}, S7^{+}) and pre-B (IgM^{-}, B220^{+}, S7^{-}) cell numbers.

(F) Mature B cells (IgM^{+}, IgD^{bright}) in the spleen of Gfi-1$^{-/-}$ (a and b).

ure 3C). Promyelocytes, the precursors of granulocytes, were present but contained fewer cytoplasmic granules than their wild-type counterparts (Table 1B, Figure 3C [insets], and data not shown). Similar to findings in blood, many cells in the bone marrow of Gfi-1$^{-/-}$ mice appeared atypical and could not be assigned readily to a distinct myeloid lineage by morphological criteria (Table 1B and Figure 3C).

An Abnormal Population of Gr1^{+}Mac1^{+} Cells, Sharing Characteristics of Immature Granulocyte and Macrophage Precursors, Accumulates in the Bone Marrow of Gfi-1$^{-/-}$ Mice

Abnormal myeloid cells in the bone marrow of Gfi-1$^{-/-}$ mice were characterized by FACS. Forward (FSC) and side light-scatter (SSC) analysis revealed a cell population of abnormal size and granularity (Figure 3D, filled arrow). FACS sorting of this population, followed by cytospin and inspection after May-Gruenwald-Giemsa staining, confirmed its identity with the atypical myeloid cells seen in the differential blood and bone marrow counts (data not shown). Gfi-1$^{-/-}$ bone marrow lacked a defined population with FSC/SSC characteristics of mature granulocytes (Figure 3D, open arrows), and cells with the characteristics of lymphocytes and normal precursors were sharply reduced (Figure 3D, arrowheads).

Atypical Gfi-1$^{-/-}$ cells failed to react with markers of B cells (B220, IgM, CD19), T cells (CD3, CD4, CD8), NK cells (NK1.1, DX5), hematopoietic stem cells (Sca1, c-kit), dendritic cells (MHC, class 2), and mature macrophages (F4/.80, CD14) (data not shown). In contrast, atypical cells stained brightly with antibodies to Gr1 and Mac1 (Figures 3Eb and 3Ec). Gr1 staining was slightly less intense than in wild-type granulocytes (Figure 3Ea). Gr1 is expressed on granulocytes in the bone marrow and its expression increases with maturation; immature macrophages also transiently express intermediate levels of Gr1 (Hestdal et al., 1991; Lagasse and Weissman, 1996). Thus, the Gr1/Mac1 staining pattern of atypical Gfi-1$^{-/-}$ cells in the marrow is compatible with either

Table 1. Differential Counts of Blood (A) and Bone Marrow (B) from 4- to 6-Week-Old Gfi-1$^{-/-}$ Mice and Wild-Type Littermates

A. Blood	Gfi-1$^{+/+}$, n = 3	Gfi-1$^{-/-}$, n = 3
White blood cells ($10^{-3}/\mu l$)	8.7 ± 0.9	8.2 ± 3.6
Hemoglobin (g/dl)	14.8 ± 2.4	13.4 ± 7
Hematocrit (%)	50.3 ± 5.5	48.9 ± 1.6
Platelet ($10^{-3}/\mu l$)	1192 ± 122	1397 ± 131
Differential WBC (%)		
Lymphocytes	90 ± 1.7	44.3 ± 20
Neutrophils	7 ± 2.0	0
Eosinophils	2 ± 0	1.3 ± 1.2
Monocytes	1 ± 1	1.7 ± 1.5
Atypical myeloid (ring nucleus)	0	10.7 ± 6.4
Atypical myeloid (other)	0	42 ± 13
B. Bone Marrow Differential (%)	**Gfi-1$^{+/+}$, n = 4**	**Gfi-1$^{-/-}$, n = 4**
Blasts	3.0 ± 1.4	0.75 ± 0.5
Neutrophils	29.5 ± 8.2	0
Eosinophils	2.2 ± 2.8	3.2 ± 1.9
Lymphocytes	21.0 ± 7.4	5 ±2.9
Promyelocytes	3.7 ± 0.5	4.5 ± 1.9
Myelocytes/Metamyelocytes	15.5 ± 3.8	0
Atypical myeloid (ring nucleus)	0	18.7 ± 2.2
Atypical myeloid (other)	0	48.7 ± 4.7
Erythrocytes	23.5 ± 7.3	16.5 ± 6.4
Monocytes	2.0 ± 1.8	2.5 ± 1.3

arrested granulocyte or macrophage differentiation. Surprisingly, further investigation of this atypical population revealed traits of both lineages. Gr1$^+$ Gfi-1$^{-/-}$ cells express Mac3 (Figures 3Ed and 3Ee) (Ho and Springer, 1983) and M-CSF receptor (Figures 3Ef and 3Eg) (Rothwell and Rohrschneider, 1987), suggesting macrophage differentiation. On the other hand, the majority of atypical Gfi-1$^{-/-}$ cells in the bone marrow were chloracetate-esterase (CAE) positive, considered specific for the granulocyte lineage (Yam et al., 1971) (Figure 3F). In summary, Gfi-1$^{-/-}$ Gr1$^+$/Mac1$^+$ cells exhibit a mixed granulocyte-macrophage lineage phenotype; hence, we refer to their "atypical myeloid" nature.

We note that the proportion of Gfi-1$^{-/-}$ Gr1$^+$/Mac1$^+$ cells in the bone marrow increased with age. In young Gfi-1$^{-/-}$ animals (4 weeks, Figure 3Eb and Table 1B), the proportion of Gr1$^+$ Mac$^+$ cells in bone marrow was consistently increased, but significant normal erythropoiesis was preserved (Figures 3Eh and 3Ei). In contrast, in animals >3–5 months old, Gr1$^-$Mac1$^-$ cells were reduced to <20% of bone marrow cells and Gr1$^+$Mac1$^+$ cells were highly increased (Figure 3Ec). Nevertheless, animals did not become markedly anemic or thrombocytopenic. Leukemic transformation was not seen.

G-CSF Administration Fails to Induce Neutrophilia in Gfi-1$^{-/-}$ Mice but Increases Atypical Myeloid Cells

We investigated the in vivo hematological response to the major cytokine for neutrophil granulocytes, G-CSF (Figure 4). Gfi-1$^{-/-}$ mice and wild-type mice were injected with murine G-CSF for 10 days. Automated and manual differential blood counts of Gfi-1$^{-/-}$ and wild-type mice were obtained before and after treatment. In automated differential blood counts, a distinct population of neutrophils was detected before treatment in wild-type mice and is increased ~17-fold by G-CSF treatment (Figures 4A and 4B). In Gfi-1$^{-/-}$ mice, cells with neutrophil characteristics remained absent following G-CSF administration (Figures 4C and 4D). A small number of cells were registered in the neutrophil gate but exhibited less than normal peroxidase activity. In contrast, a distinct population of cells, large but low in peroxidase content (LUC), was seen in Gfi-1$^{-/-}$ mice before treatment (Figure 4C) and was increased ~4-fold after G-CSF treatment (Figure 4D). Manual differential blood counts confirmed both the absence of normal neutrophils and an increase in the abnormal cell population after G-CSF treatment. The increase in the abnormal cell population in vivo after G-CSF treatment demonstrates initial neutrophil lineage commitment of Gfi-1$^{-/-}$ myeloid precursors but an inability to complete neutrophil differentiation.

Atypical Myeloid Cells from Gfi-1$^{-/-}$ Mice Are Capable of Phagocytosis and Oxidative Burst

To assess functional properties of myeloid cells with respect to host defense, we examined phagocytosis and the respiratory burst. Both wild-type neutrophils and abnormal Gfi-1$^{-/-}$ blood cells incorporated fluorescein-labeled *E. coli* at 37°C but not at 4°C (Figure 5A). The oxidative burst in response to phorbol myristate acetate (PMA) stimulation was evaluated by monitoring the generation of intracellular H_2O_2 which is rate limiting in generating 2′,7′-dichlorofluorescein from a nonfluorescent precursor. The respiratory burst activities of wild-type granulocytes and Gfi-1$^{-/-}$ myeloid cells were similar (Figure 5B). Thus, both phagocytosis and respiratory burst activity appear intact in the atypical myeloid cells of Gfi-1$^{-/-}$ mice.

Gfi-1$^{-/-}$ Gr1$^+$Mac1$^+$ Cells Lack Granules and Transcripts for Secondary and Tertiary Granule Proteins

Ultrastructural examination of FACS-sorted Gr1$^+$Mac1$^+$ cells from bone marrow revealed a paucity of granules in Gfi-1$^{-/-}$ cells (Figure 5C). Nuclei of the Gfi-1$^{-/-}$ cells were pleiomorphic, including forms resembling neutrophil nuclei but with less pronounced segmentation and condensation. Even in cells with more condensed nuclei, cytoplasmic granules were reduced or absent. A small proportion of cells (<5%) with visible granules corresponded to eosinophils and promyelocytes that were observed upon May-Gruenwald-Giemsa staining of cytospun preparations of the sorted population.

To assess further neutrophil maturation, we analyzed RNA of the bone marrow cells for transcripts encoding marker proteins characteristic of primary, secondary, and tertiary granules (Borregaard et al., 2001). Transcripts for primary granule proteins (myeloperoxidase and neutrophil elastase) were increased in Gfi-1$^{-/-}$ bone marrow, while transcripts for secondary (lactoferrin) and tertiary (gelatinase B) granules were absent (Figure 5D). These data suggest that the abnormal myeloid cells in Gfi-1$^{-/-}$ mice lack features of cells beyond the promyelocyte stage and corroborate the morphological findings. The apparent increase in abundance of myeloperoxidase and neutrophil elastase transcripts most likely reflects the increased numbers of abnormal early myeloid forms in the bone marrow.

Consistent with preserved, albeit altered, respon-

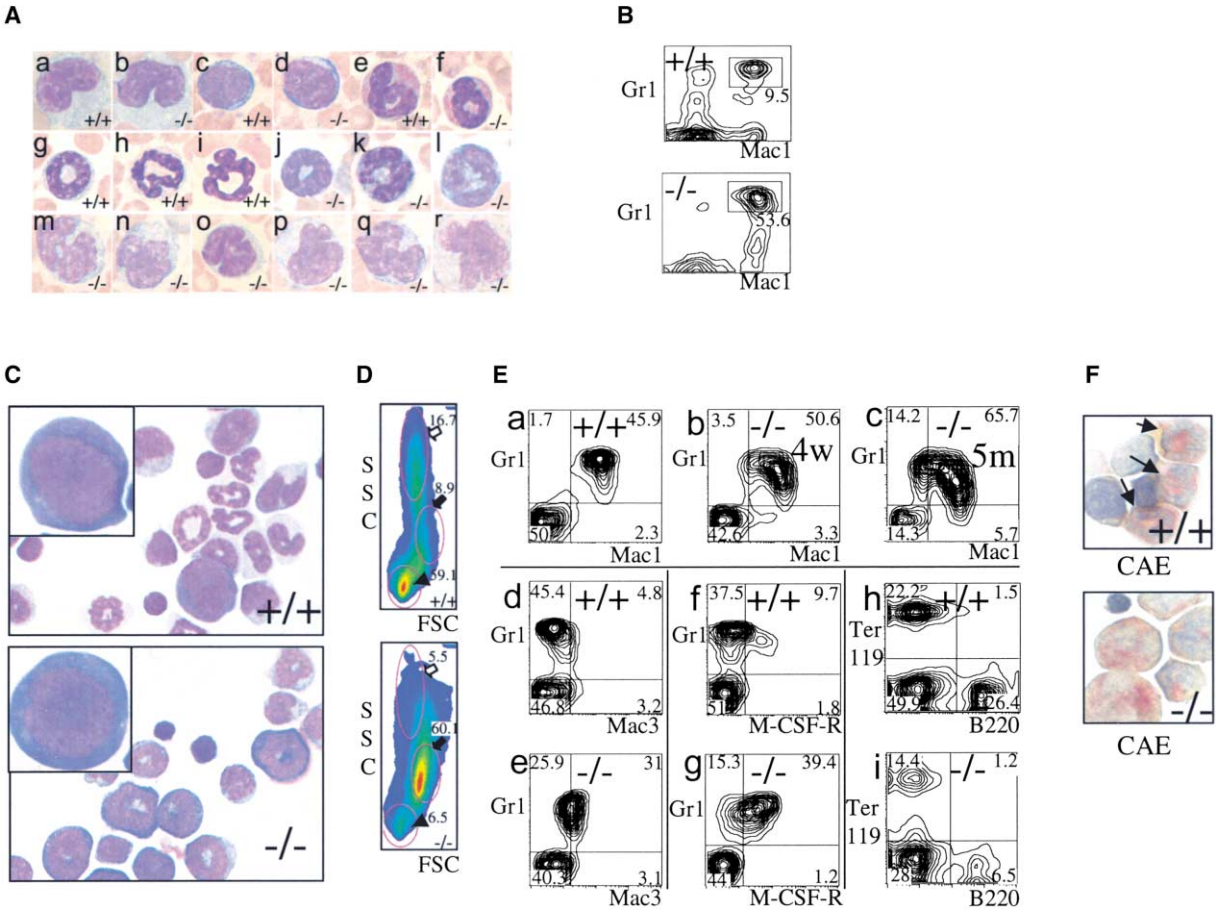

Figure 3. Phenotypic Analysis of Blood and Bone Marrow in Gfi-1$^{-/-}$ Mice (See Results for Details)

(A) Blood cells of mutant (−/−) and control (+/+) mice (original magnification, 100×). Normal monocytes (a and b), lymphocytes (c and d), and eosinophils (e and f) in normal and Gfi-1$^{-/-}$ blood. Normal juvenile (g) and mature neutrophils (h and i) wild-type blood but not Gfi-1$^{-/-}$ blood (j–r).

(B) Gr1^{+}Mac1^{+} blood cells in both mutant and wild-type mice.

(C) Neutrophils (note thin ring-shaped nuclei and clear cytoplasm) in the bone marrow of wild-type (+/+) mice but not in mutant (−/−) mice. Mutant promyelocytes display fewer granules (insets).

(D) Forward (FSC) and side (SSC) light-scatter of bone marrow shows absence of a cell population with granulocyte characteristic (open arrow) and presence of an abnormal population in mutant (filled arrow) compared to control mice. Cells with FSC/SSC profile of lymphocytes and normal precursors (arrowheads) are reduced in the mutant.

(E) Increased Gr1^{+}Mac1^{+} cells in mutant (b) marrow compared to control (a). Gr1^{+}Mac1^{+} population expands as mice age (4 weeks, [b]; 5 months, [c]). Gr1^{+} cells of mutant but not wild-type mice coexpress Mac3 (d and e) and M-CSF receptor (f and g). Erythroid cells (Ter119^{+}) are preserved in young mutant mice, but B cells (B220^{+}) are drastically reduced (h and i).

(F) Chloracetate esterase-reacted wild-type and mutant bone marrow cells. Granulocytes (arrows) and the majority of atypical cells from mutant mice (−/−) show positive substrate reaction (red). Erythroid precursors and lymphocytes from wild-type (+/+) and Gfi-1$^{-/-}$ mice are negative.

siveness of Gfi-1$^{-/-}$ bone marrow cells to G-CSF, transcripts for G-CSF receptor were reduced yet detectable in Gfi-1$^{-/-}$ bone marrow. In accordance with immunofluorescence data, M-CSF receptor expression was strikingly elevated (Figure 5E). We examined expression of other essential transcription factors in granulocyte development. C/EBPα and PU.1 were increased in Gfi-1$^{-/-}$ bone marrow, and C/EBPϵ was somewhat reduced (Figure 5E). Thus, Gfi-1 is not essential in controlling expression of these regulators of granulopoiesis.

Neutrophil Differentiation of Gfi-1$^{-/-}$ Progenitors Is Perturbed Ex Vivo

Colony assays of bone marrow in semisolid medium supplemented with single cytokines revealed a significant increase in Gfi-1$^{-/-}$ progenitors responsive to IL-3, M-CSF, GM-CSF, and G-CSF but not IL-7 (Figures 6Aa and 6Ab). Analysis of colonies following 3–4 days culture in G-CSF (CFU-G) showed a striking difference in phenotype between wild-type and Gfi-1$^{-/-}$ cells. Wild-type colonies contain clusters of cells with ring-shaped nuclei and cytoplasmic granules, as well as individual cells representing all stages of granulocyte differentiation, including mature neutrophils with segmented mature nuclei, pale cytoplasm, and visible granules (Figure 6Ac). Clusters observed in cell preparations of Gfi-1$^{-/-}$ colonies contain slightly larger cells with fewer granules, blue cytoplasm, and more diverse nuclear morphologies, including some with ring-shaped nuclei or an appearance similar to macrophages; yet, the clusters lack

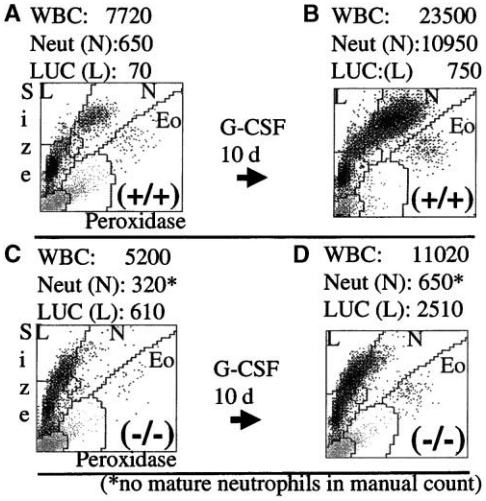

Figure 4. Automated Blood Counts of Wild-Type (+/+) and Mutant Mice (−/−) before (A and C) and after (B and D) Treatment with G-CSF In Vivo

Abbreviations: White blood cells (WBC), neutrophil granulocytes (Neut, N), large unclassified cells (LUC, L), eosinophil granulocytes (Eo). Numbers denote cells/μl of blood.

mature neutrophils (Figure 6Ac). Following GM-CSF stimulation, colonies contained a higher proportion of macrophages in the mutant compared to controls and lacked mature granulocytes (data not shown). Thus, the failure of neutrophil differentiation in Gfi-1$^{-/-}$ mice is cell autonomous and recapitulated ex vivo.

Transfer of Gfi-1 cDNA into Gfi-1$^{-/-}$ Bipotential Granulocyte/Monocyte Progenitors Rescues Neutrophil Differentiation

In wild-type bone marrow, CMPs (CD34^{+}, FcγR^{low}) can be distinguished from their more committed progeny MEPs (CD34^{-} FcγR^{low}) and GMPs (CD34^{+} FcγR^{high}) (Akashi et al., 2000). In Gfi-1$^{-/-}$ bone marrow, the overall staining pattern was preserved but the proportion of GMPs was increased 2- to 3-fold, in agreement with the increase in myeloid colonies seen with total Gfi-1$^{-/-}$ bone marrow (Figure 6B). When sorted and seeded in methylcellulose in the presence of G-CSF, wild-type GMPs undergo full granulocytic maturation after 3–4 days; in contrast, Gfi-1$^{-/-}$ colonies contain only cells arrested in differentiation (data not shown).

To determine if reintroduction of Gfi-1 rescues differentiation, we infected sorted Gfi-1$^{-/-}$ GMPs with retroviruses encoding Gfi-1 and GFP, or GFP alone and seeded infected cells into methylcellulose in the presence of G-CSF (Figure 6C). After 4 days, GFP-expressing colonies were harvested under direct fluorescence visualization and stained. As anticipated, Gfi-1$^{-/-}$ colonies infected with retroviruses encoding GFP alone consisted of abnormal myeloid cells and macrophage-like cells. In contrast, colonies from Gfi-1$^{-/-}$ marrow infected with Gfi-1-expressing retroviruses exhibited full granulocyte maturation, including the production of mature neutrophils Therefore, the defect in neutrophil differentiation resulting from loss of endogenous Gfi-1 is cell autonomous and corrected by reexpression of Gfi-1.

Discussion

Although the Gfi-1 loci (Gfi-1 and Gfi-1b) have been recognized as encoding zinc finger repressors and as sites of retroviral integrations associated with growth-factor independence and lymphomagenesis, their in vivo roles have been obscure until recently. Previously, we demonstrated that Gfi-1b is essential for the maturation of the erythroid and megakaryocytic hematopoietic lineages (Saleque et al., 2002). Here, we have explored requirements for Gfi-1 in hematopoiesis.

Role in Lymphocytes

It is provocative that the pool size of both developing T cells and B cells is strikingly reduced in Gfi-1$^{-/-}$ mice. To date, Gfi-1 has been primarily studied in T cells. However, we found equally high expression in pro-B cell lines and in B220^{+} sorted cells from the bone marrow. A role for Gfi-1 in both major lymphoid lineages is plausible in light of its capacity to contribute to lymphomagenesis of both T and B cells in vivo (van Lohuizen et al., 1991). The mechanism of the striking decrease in developing lymphocytes remains to be elucidated. In T cells absence of Gfi-1 results in a partial block in maturation of early, CD4^{-}CD8^{-} thymocytes, with an increase in CD44^{+}CD25^{+} and a sharp reduction in CD44^{-}CD25^{+} cells (Figure 2B). This pattern is complementary to findings reported with transgene-mediated overexpression of Gfi-1 (Schmidt et al., 1998). The progression from the CD44^{+}CD25^{+} to CD44^{-}CD25^{+} stage is associated with T cell receptor β chain rearrangement (Godfrey et al., 1993). However, since mature β chain^{+} T cells are present in peripheral lymphoid tissues of mutant mice, Gfi-1 is not essential for this process. Rather, Gfi-1 may enhance proliferation of T cells utilizing paracrine or autocrine growth factor stimulation after passing this developmental checkpoint. Gfi-1 may operate similarly in both lineages, because of the extensive overlap in utilization of cytokines by early B and T cells. Alternatively, it might be critical for the function of common lymphoid progenitors. The pool size of mature lymphocytes does not always reflect abnormalities in developing lymphocytes as only a small fraction of precursors is selected to join the mature pool (Freitas et al., 1986). Thus, the lack of major abnormalities in mature T and B cells is not in conflict with a critical, earlier role. IgM and IgG levels were not decreased in Gfi-1$^{-/-}$ mice (data not shown). In contrast to Gfi-1$^{-/-}$ mice, SCID or RAG mice, deficient of T and B lymphocytes, frequently develop *Pneumocystis carinii* pneumonia but rarely gram-positive abcesses when housed in our facility (data not shown). The lymphoid phenotype of Gfi-1$^{-/-}$ mice, therefore, does not explain their specific propensity to develop infections. Conditional gene targeting of Gfi-1 is needed to further assess the potential role of Gfi-1 in lymphopoiesis.

An Essential Regulator of Neutrophil Differentiation

Our data reveal an unexpected and stringent requirement for Gfi-1 in the differentiation of neutrophils. In its absence, mature neutrophils are absent, as are transcripts for neutrophil-specific secondary and tertiary granule proteins (lactoferrin and gelatinase B). Retroviral

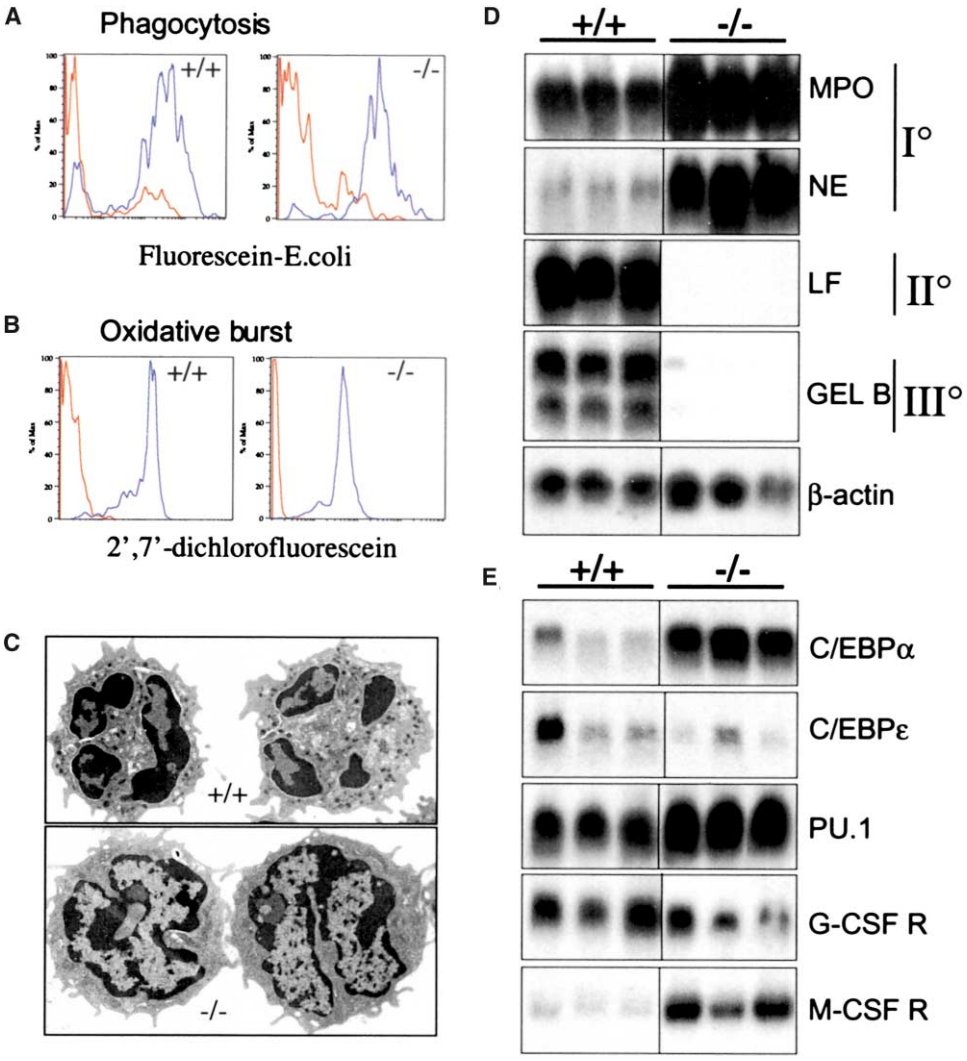

Figure 5. Myeloid Cell Function and Gene Expression in the Absence of Gfi-1

(A) Ingestion of fluorescein-labeled *E. coli* by wild-type granulocytes (+/+) and mutant atypical myeloid cells (−/−) at 4°C (red) and 37°C (blue).

(B) Flow cytometric analysis of oxidant production and subsequent 2′,7′-dichlorofluorescein generation in wild-type granulocytes (+/+) and mutant atypical myeloid cells (−/−) before (red) and after (blue) PMA stimulation.

(C) Electron microscopy of mutant (−/−) and control (+/+) $Gr1^+Mac1^+$ bone marrow cells.

(D and E) Northern blot of total RNA bone marrow from three wild-type and three Gfi-$1^{-/-}$ mice. β-actin loading controls were performed on all blots; one representative example is shown. Abbreviations: myeloperoxidase (MPO), neutrophil elastase (NE), lactoferrin (LF), gelatinase B (GEL B).

expression of Gfi-1 in sorted Gfi-$1^{-/-}$ progenitors rescues their potential to give rise to mature neutrophils ex vivo. Taken together, our findings demonstrate a direct and cell autonomous role for Gfi-1 in neutrophil development from myeloid progenitors.

Gene targeting experiments have shown three other transcription factors to be essential for the development of neutrophils in vivo: PU.1 (McKercher et al., 1996; Scott et al., 1994), C/EBPα (Zhang et al., 1997), and C/EBPε (Yamanaka et al., 1997a). PU.1 and C/EBPα exert essential functions upstream of Gfi-1 in granulocyte development. PU.$1^{-/-}$ mice are impaired in both granulopoiesis and macrophage development. In the absence of PU.1, early myeloid progenitors are formed and respond efficiently to IL-3 but, in contrast to Gfi-$1^{-/-}$ progenitors, not to G-CSF, M-CSF, or GM-CSF (Anderson et al., 1998; DeKoter et al., 1998). PU.$1^{-/-}$ progenitors can be induced to produce primary granules and their products ex vivo (Anderson et al., 1998; DeKoter et al., 1998), but transcripts for primary granule proteins are not expressed in fetal liver hematopoietic tissue in vivo (Iwama et al., 1998). Disruption of C/EBPα ablates granulocyte formation in fetal liver hematopoiesis (Zhang et al., 1997). Immature C/EBP$\alpha^{-/-}$ myeloid progenitors are generated but, unlike Gfi-$1^{-/-}$ progenitors, do not form colonies in G-CSF (Zhang et al., 1997, 1998). Expression of C/EBPε and the G-CSF receptor is absent in fetal liver tissue from PU.$1^{-/-}$ and C/EBP$\alpha^{-/-}$ embryos (Iwama et al., 1998) but preserved in Gfi-$1^{-/-}$ bone marrow. Consistent with a role upstream of Gfi-1, both PU.1 and

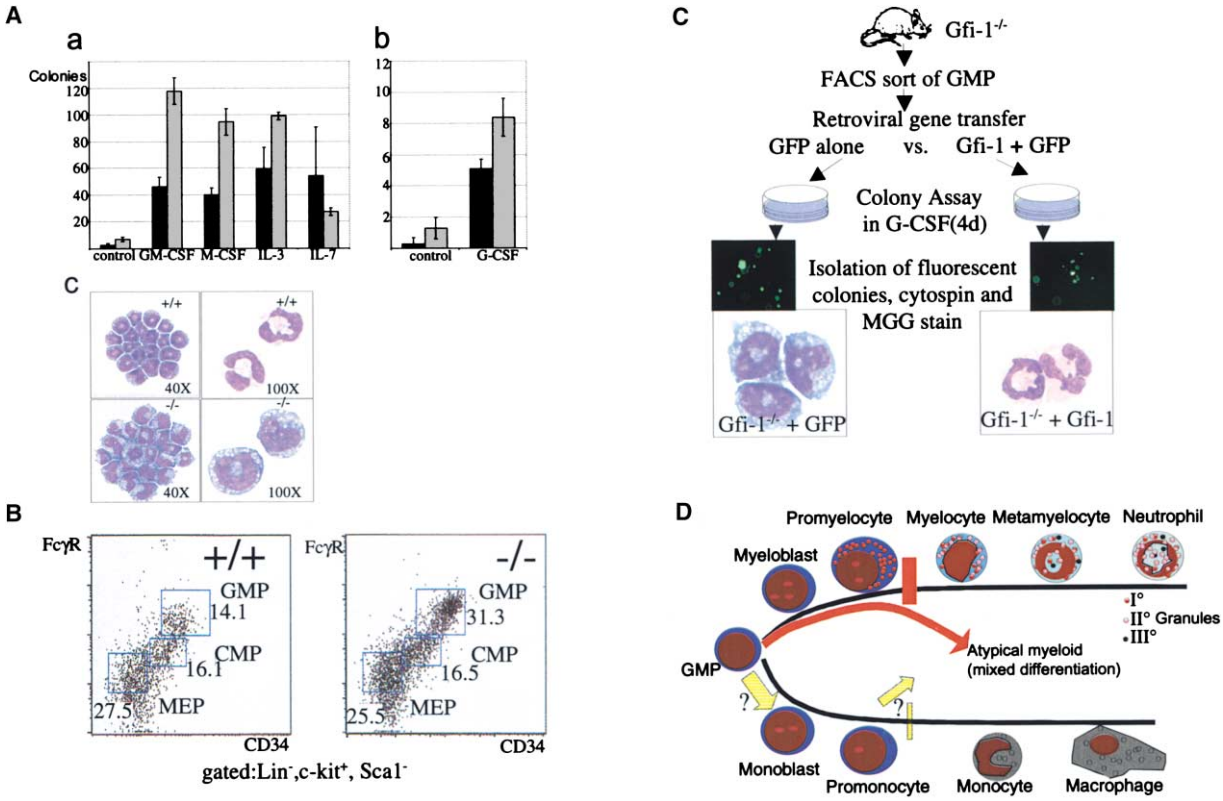

Figure 6. Rescue of Neutrophil Differentiation

(A) Progenitor assays from wild-type (black bars) and mutant (gray bars) bone marrow in response to individual cytokines (a and b). Mean and standard deviation of colony numbers from assays done in triplicate per 10^5 bone marrow cells are plotted (a and b). Morphology of wild-type (+/+) and mutant (−/−) colonies in response to G-CSF (c).

(B) Myeloid progenitor profile in wild-type (+/+) and mutant (−/−).

(C) Rescue of neutrophil differentiation from Gfi-$1^{-/-}$ GMPs by retroviral gene transfer of Gfi-1.

(D) Model for myeloid phenotype in Gfi-$1^{-/-}$ mice. The mixed phenotype of Gfi-$1^{-/-}$ mutant myeloid cells is most consistent with a block to development after the promyelocyte stage with failure to suppress monocyte/macrophage properties (shown by the red bar and arrow). Less likely are effects indicated in yellow (arrows and bar with question marks).

C/EBPα are highly expressed in Gfi-$1^{-/-}$ bone marrow cells (Figure 5E).

Like Gfi-1, C/EBPϵ is critical later in granulocyte development (Lekstrom-Himes 2001). C/EBPϵ is not required for G-CSF receptor expression or the generation of colonies in response to G-CSF (Lekstrom-Himes, 2001; Yamanaka et al., 1997a), but colonies are arrested at the promyelocyte stage in its absence (Lekstrom-Himes, 2001). In fact, C/EBPϵ has been implicated as a critical target of G-CSF stimulation (Nakajima and Ihle, 2001). C/EBP$\epsilon^{-/-}$ and Gfi-$1^{-/-}$ mice contain promyelocytes in the bone marrow. Accordingly, myeloperoxidase and neutrophil elastase, are abundant in CEBP$\epsilon^{-/-}$ and Gfi-$1^{-/-}$ marrow cells (Lekstrom-Himes and Xanthopoulos, 1999). Neither Gfi-$1^{-/-}$ nor C/EBP$\epsilon^{-/-}$ mice generate precursors with normal morphology beyond the promyelocyte stage. Transcripts for secondary and tertiary granule proteins are absent in C/EBP$\epsilon^{-/-}$ and Gfi-$1^{-/-}$ bone marrow (Lekstrom-Himes and Xanthopoulos, 1999). In spite of these similarities, the phenotypes of Gfi-$1^{-/-}$ and C/EBP$\epsilon^{-/-}$ mice significantly differ. (1) Oxidative burst and phagocytosis are impaired in C/EBP$\epsilon^{-/-}$ mice (Lekstrom-Himes and Xanthopoulos, 1999; Yamanaka et al., 1997a) but are unaffected by Gfi-1 loss (Figures 5A and 5B). (2) C/EBP$\epsilon^{-/-}$ mice are susceptible to a wide spectrum of infections (Yamanaka et al., 1997a) whereas Gfi-$1^{-/-}$ mice are susceptible specifically to gram-positive infections. (3) Eosinophils are absent in C/EBP$\epsilon^{-/-}$ but not in Gfi-$1^{-/-}$ mice (Yamanaka et al., 1997a) (Figure 3). (4) Atypical cells in C/EBP$\epsilon^{-/-}$ mice retain closer morphologic resemblance to granulocytes (Lekstrom-Himes, 2001; Yamanaka et al., 1997a). (5) C/EBP$\epsilon^{-/-}$ colonies grown in G-CSF contain mostly promyelocytes (Lekstrom-Himes, 2001) whereas Gfi-$1^{-/-}$ colonies cultured in G-CSF contain atypical cells and macrophages (Figure 6Ac).

Role of Secondary Granule Proteins in Host Defense

In spite of the absence of mature granulocytes, Gfi-$1^{-/-}$ mice survive up to a year without antibiotic treatment and are susceptible primarily to infections by gram-positive cocci. Protection against other bacteria, for which neutrophils are essential, is relatively preserved in Gfi-$1^{-/-}$ mice, consistent with the activity of Gfi-$1^{-/-}$ atypical myeloid cells in phagocytosis and oxidative burst assays ex vivo (Figures 5A and 5B). Susceptibility of Gfi-$1^{-/-}$ mice to infection is likely related to the lack of granule proteins and their antibacterial activity. Intrigu-

ingly, humans with neutrophil-specific granule deficiency (SGD) suffer from recurrent infections with *Staphylococcus aureus* and *Staphylococcus epidermidis*, both gram-positive cocci (Lekstrom-Himes, 2001). Mutations in C/EBPε have been identified in two patients with specific granule deficiency (Gombart et al., 2001; Lekstrom-Himes, 2001), but other patients lack such mutations (Lekstrom-Himes, 2001). We consider Gfi-1 an attractive candidate gene for cases of SGD in which no mutations in C/EBPε are found.

Gfi-1 Antagonizes Traits of the Monocyte/Macrophage Lineage

Reduced levels of Gr1 in the presence of Mac1 have been observed in immature granulocytes (Hestdal et al., 1991), in arrested CEBPε$^{-/-}$ granulocytes (Lekstrom-Himes and Xanthopoulos, 1999), and in immature macrophages (Lagasse and Weissman, 1996). The expression of the M-CSF receptor and Mac3 (Figures 3Eg and 5) provides direct evidence for upregulation of the macrophage lineage program (Ho and Springer, 1983; Rothwell and Rohrschneider, 1987) in Gfi-1$^{-/-}$ bone marrow. We propose that Gfi-1 not only is required for neutrophil maturation but also prevents expression of monocyte/macrophage lineage traits. In its absence, therefore, arrested neutrophil precursors acquire a mixed lineage phenotype (Figure 6D, red arrow). Such a dual role of Gfi-1 in the granulocyte lineage is consistent with its expression in neutrophils but not macrophages and the lack of a parallel massive accumulation of normal mature monocytes/macrophages (Table 1 and Figure 3) and F4/80^{+} peritoneal macrophages (data not shown) in Gfi-1$^{-/-}$ mice. A dual role for Gfi-1 in the control of myeloid development parallels the postulated dual roles of GATA-1 and PU.1 in regulation of erythroid/megakaryocytic versus monocyte/neutrophil axes (Orkin, 2000).

Formally, we cannot dismiss an alternative model in which a proportion of Gfi-1$^{-/-}$ Gr1^{+}Mac1^{+} cells represent arrested monocyte/macrophage precursors resulting from an independent defect in maturation of these precursors (see Figure 6D, yellow arrows). A regulatory role for Gfi-1 at the early progenitor level, in concert with an additional role in macrophage development, might lead to an accumulation of arrested monocytes/macrophages without a concomitant major increase in more mature macrophages. We think this possibility is unlikely given that: (1) macrophages do not express Gfi-1 (Figure 1A), (2) Gfi-1$^{-/-}$ colonies formed in response to M-CSF ex vivo contain normal-appearing macrophages, and (3) Gfi-1 is downregulated upon macrophage differentiation of bipotential HL-60 cells (Zweidler-Mckay et al., 1996).

Our data exclude the possibility that commitment of early progenitors toward the macrophage lineage (Figure 6D, yellow arrows) is the sole basis for the lack of mature neutrophils and the presence of atypical Gr1^{+}Mac^{+} cells in Gfi-1$^{-/-}$ mice. Evidence for incomplete differentiation of the granulocyte lineage includes: (1) preserved colony formation in response to G-CSF ex vivo, (2) an increase in atypical cells in response to G-CSF in vivo, (3) presence of promyelocytes and cells with nuclei resembling those of mature granulocytes in Gfi-1$^{-/-}$ bone marrow and blood, and (4) preserved expression of the G-CSF receptor, neutrophil elastase, chloracetate-esterase (Yam et al., 1971), C/EBPα (Radomska et al., 1998), and C/EBPε (Yamanaka et al., 1997b), which are all exclusively or preferentially expressed in granulocytes.

Comparison with Findings of Others

During the final stages of our work, others reported analysis of Gfi-1 knockout mice (Karsunky et al., 2002). The phenotype of their mice appears similar to ours, including premature death, reduced size, reduction of thymic T cells, the accumulation of Gr1^{+}Mac1^{+} cells (interpreted as immature monocytes) in the bone marrow and "neutropenia," i.e., reduced numbers of neutrophils. The emphasis of their analysis and overall conclusions, however, are quite different from ours. Karsunsky et al. demonstrate that Gfi-1 can be expressed in macrophages after LPS stimulation and describe an intrinsic propensity of Gfi-1$^{-/-}$ macrophages to generate elevated cytokine levels (TNFα, IL1β, IL-10) after stimulation by lipopolysaccharide. The authors attribute premature death of Gfi-1$^{-/-}$ mice in part to exaggerated inflammatory reactions and do not document severe infections. They describe a failure to generate mature granulocytes in G-CSF colonies ex vivo, but the data are equivocal as to whether neutrophils are reduced or absent in vivo and whether the defect is indicative of a cell autonomous role for Gfi-1 in neutrophil development.

Additional Implications of This Work

Our data should stimulate efforts to identify target genes for Gfi-1 in myeloid cells, to uncover its interaction partners, and to further delineate its role in governing myeloid development in concert with other transcription factors. Recently, somatic mutations in C/EBPα and PU.1 have been observed in human leukemias (Mueller et al., 2002; Pabst et al., 2001). Although no leukemia developed in Gfi-1$^{-/-}$ mice, it is possible that loss of function contributes to myeloid leukemia, particularly myelomonocytic subtypes (such as AML, FAB M4, M5; CMML; JMML), which feature both arrested myeloid differentiation and deregulation of cells with monocytoid phenotype.

Experimental Procedures

Northern Blot Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) from the following cell lines and primary cells: MEL; G4; L8057; M1; EL4 (ATCC); pro-B-7 and pro-B-8, IL-7- and stroma cell-dependent pro-B cell lines derived from Whitlock-Witte cultures; granulocytes, from mouse bone marrow identified by FSC/SSC profile; B220^{+} cells from bone marrow; bone marrow-derived macrophages, prepared by culture of density gradient purified mononuclear cell in M-CSF (50 ng/mL; R&D) for 4 days and removal of nonadherent cells. The following probes were used: murine Gfi-1, fragment encoding residues 1–257; β-actin; G3PDH (Clontech); neutrophil elastase; myeloperoxidase; lactoferrin; gelatinase B; G-CSF receptor; PU.1, M-CSF receptor, C/EBPα (Iwama et al., 1998); murine C/EBPε fragment encoding residues 3–90.

Disruption of the Gfi-1 Gene

Genomic clones were isolated from a mouse strain 129 λFixII library (Stratagene). A 9 kb fragment (Sal1-HindIII in Figure 1B) was transferred into PUC 19. A 2.6 kb SacII-NsiI fragment, containing the ATG

start codon in exon 1, exon 2, and most of exon 3, was replaced with a synthetic linker containing a Not1 site. A loxP-flanked neomycin-resistance gene was cloned into the NotI site and a thymidine kinase gene was cloned into the Sal1 site of PUC 19 to generate the final targeting vector (Figure 2B). A targeted CJ7 ES-cell clone was injected into C57Bl/6 blastocysts to generate chimeras for germline transmission. In some mice, the loxP-neomycin resistance gene was deleted by crossing with Gata1-Cre mice which were of CD1/Swiss-Webster background. Mice were housed in a specific pathogen-free animal facility.

Histology and Cytology

Tissues were fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin-eosin stain or Gram stain. Blood smears and cytocentrifuge preparationswere stained in May-Gruenwald-Giemsa for morphological assessment. Chloracetate-esterase activity was detected using a commercially available kit (Sigma).

Flow Cytometry

Cells were stained and analyzed with FACScalibur or Mo-Flo flow cytometers. Where appropriate, red cells were lysed, and nonspecific binding was reduced by preincubation with unconjugated antibody to FcγII/III (2.4G2). Dead cells were excluded by propodium iodide (1 μg/ml; Molecular Probes) or by FSC/SSC profile. Antibody conjugates and matched isotype controls were obtained from Pharmigen or eBiosciences, unless otherwise indicated: CD4 (RM4-5, PE), CD8 (53-6, PerCP), CD44 (IM7, FITC), CD25 (PC61, APC) CD16/32 IgM (R6-60, PE), IgD (11-26, FITC), CD115 (M-CSF-R, AFS98, PE), Mac3 (M3/84, FITC), Ter119 (PE), B220 (RA3-6B2, APC), Gr-1 (RB6-8C5, APC), Mac-1 (M1/70, FITC), F4/80 (PE, Serotec), DX-5 (PE), NK1.1 (PK136, PE), MHC class 2 (M5/114, PE), CD14 (rm C5-3, FITC). Staining and sorting of myeloid progenitors was preformed as described (Akashi et al., 2000) with a modified panel of conjugated antibodies: CD4 (RM4-5, Cy-Chrome), CD8 (53-6, Cy-Chrome), CD3ε (145-2C11, Cy-Chrome), B220 (RA3-6B2, Cy-Crome), Gr1 (RB6-8C5, PE-Cy5), CD19 (6D5, PE-Cy5), CD117 (2B8, APC), CD34 (RAM34, FITC), FcγII/III (2.4G2, PE), Sca-1 (E13-161, Biotin), Avidin-APC-Cy7 (Caltag).

G-CSF Treatment In Vivo and Automated Blood Counts

Gfi-1$^{-/-}$ mice (n = 2) and controls (n = 2) were injected intraperitoneally twice daily with 600 ng of murine G-CSF (R&D) for 10 days. Blood samples (250 μl) were analyzed using an ADVIA 120 hematology system (Bayer).

Phagocytosis and Oxidative Burst Assays

Phagocytosis was assayed with opsonized, fluorescein-labeled *E. coli* (Phagotest, Orpegen, Heidelberg). Heparinized blood was mixed with labeled *E. coli* either at 37°C or at 4°C (control) for 10 min. For analysis by flow cytometry, forward and side light-scatter gates were used to identify wild-type granulocytes and Gfi-1$^{-/-}$ atypical myeloid cells. Oxidative burst in response to PMA was measured by visualizing the intracellular conversion of dichlorofluorescein diacetate (DCFH-DA) to 2′7′-dichlorofluorescein by flow cytometry (Cellprobe/ Beckman Coulter). Bone marrow cells were incubated with or without PMA and DCFH-DA for 5 min at 37°C. Intracellular 2′7′ dichloroflourescein content was determined in wild-type granulocytes or atypical Gfi-1$^{-/-}$ myeloid cells, identified by FSC/SSC profile.

Electron Microscopy

Gr1^{+}Mac1^{+} cells were sorted from the bone marrow of Gfi-1$^{-/-}$ or wild-type mice and fixed in Karnovsky's solution. Sections (600 nm) were visualized using a Phillips EM208S electron microscope.

Colony Assays

Colony assays were performed using methocult M3234 (Stem Cell Technologies). 5×10^4 (for IL-3, GM-CSF, M-CSF) or 4×10^5 (for G-CSF, IL-7) bone marrow cells from wild-type or Gfi-1$^{-/-}$ mice were seeded into 35 mm^2 petri dishes in triplicate and incubated in 5% CO_2 at 37°C. Cultures were supplemented with human G-CSF (1 ng/ml), mouse IL-7 (10 ng/ml), mouse IL-3 (10 ng/ml), mouse GM-CSF (5 ng/ml), or mouse M-CSF (10 ng/ml), purchased from R&D.

Retroviral Gene Transfer

Murine Gfi-1 coding sequences were cloned into the retroviral vector MMP (Klein et al., 2000). An IRES-GFP cassette was cloned immediately 3′ to Gfi-1. Concentrated supernatants were generated as described (Klein et al., 2000). Sorted Gfi-1$^{-/-}$ GMPs were resuspended in medium (DMEM, FCS 20%, penicillin 1%, streptomycin 1%, glutamine 1%, nonessential amino acids 1%, 2-mercaptoethanol 50 μM) containing stem cell factor (murine-SCF, 50 μg/ml, R&D) and interleukin-3 (murine IL-3, 10 μg/ml, R&D). Cells (~10^4 GMPs in 250 μl) were incubated with 50 μl concentrated viral supernatant (MMP-Gfi-1iresGFP or GFP control; titers ~10^9) in the presence of 8 μg/ml polybrene for 1 hr at 0°C, followed by 12 hr at 37°C in 5% CO_2. Subsequently, cells were washed twice in medium without cytokines and distributed in dishes containing methylcellulose and G-CSF (~500 cells/dish). After 4 days, fluorescent colonies were harvested, cyto-centrifuged, and assessed for morphology.

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