Resistance to Friend Virus-Induced Erythroleukemia in \( W/W^v \) Mice Is Caused by a Spleen-Specific Defect Which Results in a Severe Reduction in Target Cells and a Lack of \( Sf-Stk \) Expression

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The characteristic progression and specificity of Friend virus for the erythroid lineage have allowed for the identification of a number of host-encoded loci that are required for disease progression. Several of these loci, including the Friend virus susceptibility gene 2 (\( Fv2 \)), dominant white spotting gene (\( W \)), and Steel gene (\( Sl \)), regulate the initial polyclonal expansion of infected erythroid progenitor cells. \( W \) and \( Sl \) encode the Kit receptor tyrosine kinase and its ligand, stem cell factor, respectively. \( W \) mutant mice are severely anemic, and earlier work suggested that this defect in erythroid differentiation is the cause for the resistance to Friend virus-induced erythroleukemia. Here we show that in bone marrow, \( W/W^v \) mice have near normal numbers of target cells and the initial infection of bone marrow occurs normally in vivo. In contrast, spleen cells from \( W/W^v \) mice infected both in vitro and in vivo with Friend virus failed to give rise to erythropoietin-independent colonies at any time following Friend virus infection, suggesting that mutation of the Kit receptor specifically affects target cells in the spleen, rendering the mutant mice resistant to the development of Friend virus-induced erythro- leukemia. In addition, we show that the Kit receptor tyrosine kinase, while \( Sl \) encodes its ligand, stem cell factor, plays a role in erythroid differentiation by stimulating the proliferation of early erythroid progenitor cells (burst-forming unit-erythroid or BFU-E), leading to the development of late erythroid progenitors (CFU-erythroid or CFU-E) (22). \( W \) and \( Sl \) mutant mice exhibit multiple phenotypes that include severe macrocytic anemia and pigmentation defects (3, 31). The SCF/Kit signaling pathway plays a key role in erythroid differentiation by stimulating the proliferation of early erythroid progenitor cells (burst-forming unit-erythroid or BFU-E), leading to the development of late erythroid progenitors (CFU-erythroid or CFU-E) (22). \( W \) mutant mice exhibit near normal levels of early BFU-E but are severely deficient in CFU-E (25). Early work suggested that the target cell for Friend virus was a late BFU-E (14). The initial observation that \( W \) and \( Sl \) mice were resistant to Friend virus suggested that the defect that led to the anemia in these mice was responsible for the resistance (7, 32). These results underscored the idea that Friend virus required the normal erythroid differentiation machinery for the pathogenesis of erythroleukemia and suggested that the defect in proliferation of BFU-E in \( W \) and \( Sl \) mice could impair erythropoiesis to such an extent that the mutant mice were resistant to Friend virus-induced erythroleukemia (6, 23).

Friend virus induces acute erythroleukemia in adult mice. The disease proceeds through a two-stage progression (6, 23). The initial stage of the disease is characterized by the polyclonal expansion of infected cells in bone marrow and the spleen. Because of the interaction of the viral envelope glycoprotein, gp55, with the erythropoietin (Epo) receptor, the induction of leukemia by the virus is specific for the erythroid lineage (16). The later stage is characterized by the acquisition of new mutations, specifically, the mutation of the \( p53 \) gene and proviral insertion activation of \( Spi1 \), which leads to the emergence of a leukemic clone (20, 21). Because of its characteristic staged progression to leukemia and specificity for the erythroid lineage, several host genes that are required for the progression of Friend erythroleukemia have been identified (4, 34). One group of genes that are required for the initial polyclonal expansion of infected cells has been identified. This group includes the dominant white spotting (\( W \)), Steel (\( Sl \)), and Friend virus susceptibility gene 2 (\( Fv2 \)) loci.

The \( Fv2 \) locus encodes a naturally occurring truncated form of the Mst1R (also called Stk) receptor tyrosine kinase (29). This form of the receptor referred to as short-form Stk or \( Sf-Stk \) interacts with gp55 and the Epo receptor in infected cells, and \( Sf-Stk \) kinase activity is required for the expansion of infected cells in bone marrow (11, 24). \( Sf-Stk \) is expressed from an internal promoter in the \( Mst1R \) gene. Mice that are homozygous for the resistant allele of \( Fv2 \), \( Fv2^r \), fail to express \( Sf-Stk \) because of a deletion in the \( Sf-Stk \) promoter. The \( W \) locus encodes the Kit receptor tyrosine kinase, while \( Sl \) encodes its ligand, stem cell factor (SCF) (9, 38, 39). \( W \) and \( Sl \) mutant mice exhibit multiple phenotypes that include severe macrocytic anemia and pigmentation defects (3, 31). The SCF/Kit signaling pathway plays a key role in erythroid differentiation by stimulating the proliferation of early erythroid progenitor cells (burst-forming unit-erythroid or BFU-E), leading to the development of late erythroid progenitors (CFU-erythroid or CFU-E) (22). \( W \) mutant mice exhibit near normal levels of early BFU-E but are severely deficient in CFU-E (25). Early work suggested that the target cell for Friend virus was a late BFU-E (14). The initial observation that \( W \) and \( Sl \) mice were resistant to Friend virus suggested that the defect that led to the anemia in these mice was responsible for the resistance (7, 32).
To investigate the role of the Kit/SCF signaling pathway in the pathogenesis of Friend virus-induced erythroleukemia, we took advantage of the observation that Friend virus-infected erythroid progenitor cells can form BFU-E and CFU-E colonies in the absence of erythropoietin. In this report we show that W mutant bone marrow cells are sensitive to Friend virus infection both in vitro and in vivo as measured by the formation of Epo-independent (Epoind) BFU-E colonies. Furthermore, the number of Friend virus-infected cells in bone marrow is similar to the number in control mice. However, the situation is different in the spleen. In both in vitro and in vivo assays, few Epoind BFU-E colonies were identified in the spleen, and in fact, few Friend virus-infected cells were present in the spleens of W/W mice. We also show that the Kit+ megakaryocyte-erythroid progenitor (MEP) in the spleen expresses Sf-Stk and is the target cell for Friend virus in vitro assays. We observed a marked decrease in the number of MEPs in the spleens of W/W mice, and they lack Sf-Stk expression. Bone marrow MEPs, however, do not form Epoind BFU-E following Friend virus infection. These data demonstrate that the pathogenic targets of Friend virus in the spleen are distinct from those in bone marrow and suggest that the Kit/SCF signaling pathway is required for development and expansion of Friend virus targets in the spleen. They also underscore the unique nature of the spleen microenvironment in its role in supporting erythropoiesis and the requirement of the spleen for the progression of acute leukemia in Friend virus-infected mice.

MATERIALS AND METHODS

Mice. Eight- to 12-week-old WBB6F1/J-Kit+/W(W/W) mice, WBB6F1 control mice, and BALB/cJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All research involving the use of mice were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Pennsylvania State University.

Analysis of Stk and Sf-Stk expression by RT-PCR. Expression of Stk and Sf-Stk in bone marrow and spleen cells was determined by reverse transcriptase PCR (RT-PCR). Total RNA was obtained from bone marrow and spleen cells using Trizol (Invitrogen, Carlsbad, CA). Primer sequences were as follows: Stk sense, 5'-AGCACTGAGAACCCCTTCCA-3'; Stk antisense, 5'-ATGCTTACCTCGAAGATGTC-3'; Sf-Stk sense, 5'-TCTGCGACTGTTCTGTCGTG-3'; and Sf-Stk antisense, 5'-CGACGAGGGGACCACGTGCC-3'. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. RT parameters were as follows: 70°C for 5 min, 23°C for 10 min, 42°C for 2 min, 42°C for 50 min, 70°C for 15 min, and 37°C for 20 min. RNase H was added prior to the last incubation time. PCR cycling parameters were as follows: 95°C for 5 min; five cycles of 95°C for 45 seconds, 60°C for 1 min, and 72°C for 2 min; 30 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 2 min, and 72°C for 10 min.

 Colony assay. For in vitro BFU-E formation, total bone marrow and spleen cells were harvested from W/W mice and control mice. Supernatant from FP63 cells expressing polyclimma-inducing Friend virus (a kind gift from Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada) or an equal volume of Dulbecco modified Eagle medium containing 10% fetal bovine serum (mock infection) was incubated with bone marrow or spleen cells (1 × 10^6 cells) on ice for 1 h (bone marrow) or 1 1/2 hours (spleen). For bone marrow assays, the mock-infected cells were plated in Methocult medium (Stem Cell Technologies, Vancouver, British Columbia, Canada). Bone marrow cells were plated in IL-3 with and without Epo at the concentrations indicated above. Spleen cells were plated in IL-3 with and without Epo or in IL-3 plus SCF with and without Epo at the concentrations indicated above. The cultures were scored for BFU-E as previously described (11).

 Flow cytometry. W/W and control mice were injected intravenously with Friend virus. On days 0, 4, 8, and 12 postinfection, bone marrow and spleen cells were isolated. Cells were stained with either monoclonal antibody MA834, which is specific for Friend murine leukemia virus glycosylated Gag protein and stains both Friend murine leukemia virus- and spleen focus-forming-virus-infected cells (10) (provided by K. Hasekrag, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases) alone or in combination with R-phycocyanin-conjugated rat anti-mouse TER-119 (Ly-76) (Pharmingen, San Diego, CA). MA834 was detected by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G2b (Caltag Laboratories, Burlingame, CA), which was preabsorbed on spleen cells of C57BL/6 mice prior to use as previously described (36). Expression of surface antigens was analyzed using a Beckman Coulter flow cytometer. Isolation of MEPs from bone marrow and spleen was done as described previously (2).

 Infection of mice with Friend virus. W/W and control mice were infected as previously described (28). Mice were sacrificed, and spleens were removed on the designated dates. The spleen was weighed with an analytical balance and measured in grams.

 Induction of Friend disease by transplantation of in vitro-infected bone marrow cells. Bone marrow cells isolated from W/W and control mice were infected in vitro with Friend virus as described above. The infected cells were repeatedly washed with phosphate-buffered saline (PBS) to remove surface-bound virus. Infected cells (4 × 10^6) were injected into the tail vein of one of either a WBB6F1 control recipient mouse or a W/W recipient mouse. Two weeks later, the mice were sacrificed and the spleens were isolated and weighed. In order to determine the contribution of donor infected cells to the splenomegaly observed in W/W recipients, total bone marrow cells were isolated from control mice, and red blood cells were lysed using ice-cold 0.16 M NH_4Cl. The cells were then infected with Friend virus in vitro. Surface-bound virus was washed off with repeated PBS washes. The cells were irradiated with 1,200 rads using a J.L. Shepherd Mark I irradiator (17). Irradiated cells (4 × 10^6) were resuspended in 200 μl PBS and intravenously injected into control and W/W recipient mice. The mice were sacrificed 15 days after transplantation and splenectomized. Spleens were weighed to determine splenomegaly.

RESULTS

W/W mice have normal numbers of Friend virus target cells in bone marrow. In vitro infection of bone marrow cells with Friend virus results in the development of Epoind BFU-E colonies. Although Friend virus can infect cells of other hematopoietic lineages, this assay identifies Friend virus target cells in the erythroid lineage, which represent the pathogenic target cells in vivo. Previously, we have shown that by assaying Epoind BFU-E forming followed by in vitro infection of bone marrow cells, we could faithfully recapitulate the in vivo resistance of Fv2rr mice (11). In order to characterize the mechanism by which W/W mice are resistant to Friend virus, we tested whether W/W bone marrow cells infected in vitro could form Epoind BFU-E. As shown in Fig. 1, the number of BFU-E present in the Epo-containing cultures from W/W mice is approximately the same as that of control cultures, which is in agreement with earlier work that showed that W/W mice have near normal numbers of BFU-E (25). Surprisingly, the numbers of Epoind BFU-E exhibited by cultures of W/W bone marrow cells infected with Friend virus were similar to those of cultures of control bone marrow cells infected with Friend virus. These results show that the number of Friend virus targets in bone marrow is not affected by mutation of genes encoding components of the Kit/SCF signaling pathway. Resistance to Friend virus in W/W mice, however, could be due to a role for this signaling pathway in the expansion of infected
cells during infection. To address this possibility, we extended this analysis by measuring the in vivo expansion of Epo\textsuperscript{ind} colony-forming cells during the first 6 days postinfection. \textit{W/W\textsuperscript{v}} and control mice were infected with Friend virus, and bone marrow cells were harvested days 0, 2, 4, and 6 postinfection and assayed for Epo\textsuperscript{ind} BFU-E. Similar to the in vitro study, the expansion of Epo\textsuperscript{ind} BFU-E in the \textit{W/W\textsuperscript{v}} mice was not significantly different in control animals (Fig. 2). These results demonstrate that the initial number of Friend virus target cells and the expansion of Friend virus-infected cells during infection in bone marrow do not require the Kit/SCF signaling pathway.

\textit{W/W\textsuperscript{v}} mice have a defect in Friend virus target cells in the spleen. In addition to bone marrow, the spleen is also a major site of Friend virus infection in wild-type mice. Indeed, the expansion of infected erythroblasts in the spleen leads to splenomegaly, one of the hallmarks of Friend virus infection (6, 23). In order to test whether \textit{W/W\textsuperscript{v}} mice had a defect in Friend virus target cells in the spleen, spleen cells isolated from \textit{W/W\textsuperscript{v}} and control mice were infected in vitro to determine whether they could form Epo\textsuperscript{ind} BFU-E. Control spleen cells exhibited very modest Epo\textsuperscript{ind} BFU-E formation when plated with IL-3 in the medium; however, when SCF was added (100 ng/ml), the number of Epo\textsuperscript{ind} BFU-E was approximately equal to the number of BFU-E observed in the cultures that contained Epo (Fig. 3A). In contrast, the \textit{W/W\textsuperscript{v}} cells exhibited a marked reduction in the number of BFU-E in the cultures containing Epo, which increased when SCF was included in the medium. However, the \textit{W/W\textsuperscript{v}} cells gave rise to very few Epo\textsuperscript{ind} BFU-E, and this number was not increased by the inclusion of SCF in the medium (Fig. 3B). Therefore, unlike bone marrow, the spleens of \textit{W/W\textsuperscript{v}} mice contain very few target cells that can be productively infected by Friend virus.

We next analyzed the expansion of infected cells in the spleens of \textit{W/W\textsuperscript{v}} and control mice. The mice were infected with Friend virus, and spleen cells were isolated at 0, 2, 4, and 6 days postinfection. The spleen cells were plated out in medium containing IL-3 with or without Epo. Bars represent the total number of colonies ± standard deviation (error bars) from one representative experiment of three independent experiments. Values that are significantly different from day 0 values are indicated by asterisks (*, P < 0.01; **, P < 0.05).
cells, no expansion of Epoind BFU-E was observed at any time point and the addition of Epo did not significantly increase the number of BFU-E (Fig. 4B). These results demonstrate that W/Wv mice have normal numbers of Friend virus targets in bone marrow that expand during the early course of infection similar to control mice. However, W/Wv mice have few targets in the spleen that do not expand during the early course of infection.

**W/Wv mice have few Friend virus-infected cells in the spleen.**

The lack of Friend virus target cells in the spleens of W/Wv mice suggests that either mutation of the Kit receptor in these mice impairs the Epoind expansion and differentiation mediated by Friend virus infection or they lack pathogenic target cells in the spleen. We analyzed the percentage of Friend virus-infected cells in bone marrow and the spleen during the course of infection by flow cytometry. We used MAb34, which recognizes the Gag protein of Friend virus and has been used previously to follow infected cells during infection (10, 36).

W/Wv and control mice were infected with Friend virus, and bone marrow and spleen cells were isolated and stained with MAb34. In the control mice, the percentage of MAb34+ cells in bone marrow starts to increase on day 4, peaks at day 8, and by day 12 has decreased. In the W/Wv mice, the infection of cells in bone marrow proceeds in a manner similar to control mice; however, the percentage of infected cells is slightly less than control mice at day 8 and decreases further on day 12 (Fig. 5A). In the spleens of control mice, the infection starts to increase on day 4 but continues to increase so that by day 12, >90% of the spleen cells are MAb34+. The most striking difference in the W/Wv mice is in the spleen where few cells are infected and the Friend virus-infected cells do not increase over time (Fig. 5B). Staining with MAb34 identifies all cells infected with Friend virus; in order to track infected cells of the erythroid lineage, we repeated the flow cytometry analysis except this time we double stained the cells with MAb34 and an anti-body against TER119 that recognizes late erythroid progenitors (13). We observed in control bone marrow cells that about 45% of the MAb34+ cells are TER119+ in the bone marrow (C) and spleens (D) of control and W/Wv mice infected with Friend virus for the indicated days. The results from one representative experiment of two independent experiments are shown.
>90% of the MAb34+ cells also TER119+. These results were reflected in the splenomegaly observed in the control mice (Fig. 5D and E). In contrast to the spleens of control mice, we observed that very few of the MAb34+ cells were TER119+ cells in the spleens of W/V mice, which correlated with the complete lack of splenomegaly in these mice (Fig. 5D and E). These results demonstrate that spleen cells in W/V mice are not infected by Friend virus and coupled with the colony assay results suggests that Kit/SCF signaling is required for the development or maintenance of Friend virus targets in the spleen.

**W/V** bone marrow cells infected in vitro with Friend virus can induce splenomegaly when transplanted into control mice. During the course of infection, Friend virus infects targets in both the bone marrow and spleen. Although in vitro, both target cell populations can form Epo<sup>+</sup>BFU-E colonies, the relationship between the infection of bone marrow cells and spleen cells is not well understood. Early work in the field has demonstrated that bone marrow cells infected in vitro when transplanted into susceptible mice can home to the spleen and establish an infectious center (IC). It has been shown that these IC-forming cells infect spleen cells, which then expand and form a macroscopic spleen colony (33, 35). This observation suggests that a component of the pathogenesis of Friend virus may include the migration of infected bone marrow cells into the spleen, the establishment of ICs, and the subsequent infection of spleen cells. Our data demonstrate that W/V mice lack Friend virus targets in the spleen; however, other work has suggested that the Kit receptor is required for homing and lodgement of cells in the spleen during the response to acute hemolytic anemia (8). We wanted to determine whether W/V bone marrow cells infected in vitro with Friend virus could induce splenomegaly when transplanted into control recipient mice. Bone marrow cells from W/V or control mice were infected with Friend virus in vitro and transplanted into either W/V or control recipients. Two weeks later, the mice were sacrificed, and the spleens were removed and weighed to test for splenomegaly. Figure 6A shows that control mice that received infected W/V cells developed splenomegaly, which demonstrated that W/V bone marrow cells are capable of homing to the spleen and initiating infection of spleen Friend virus targets. However, when infected W/V cells were transplanted into W/V recipients, no increase in spleen size was observed. As expected, control infected bone marrow cells induced splenomegaly when transplanted into control recipients. However, when control bone marrow cells were transplanted into W/V recipients, we observed a mild but significant splenomegaly. This minor splenomegaly could be caused by the infection and expansion of a few target cells in the spleens of W/V mice or the splenomegaly could be due to the expansion of infected donor bone marrow cells in the spleen. To test these possibilities, we infected control bone marrow cells with Friend virus and irradiated the infected cells to inhibit their proliferation. Figure 6B shows that irradiated infected control bone marrow cells transplanted into W/V mice failed to induce any increase in spleen size, but when transplanted into control recipients, they induced splenomegaly. These data support the model where W/V mice lack pathogenic targets in the spleen; however, the defect in the ability of in vitro-infected bone marrow cells to induce splenomegaly when transplanted into W/V mice could also be due to a defect in the spleen microenvironment that impairs the homing of bone marrow cells to the spleen. To test this possibility, we transplanted bone marrow cells from the C57BL/6 congenic strain, B6.SJL-Ptprca Pep3b/H11022, which carries the CD45.1 allele, into W/V and control mice which carry the CD45.2 allele. After 24 h we measured the percentage of donor cells present in the spleen by flow cytometry. An equal percentage of donor cells homed to the W/V spleen compared to the control spleen (data not shown). These data show that the defect in the W/V mice is in the number of target cells in their spleens, not in their ability to migrate to the spleen or in the ability of the spleen microenvironment to retain cells. Furthermore, the expansion of infected donor bone marrow cells in the spleen requires Kit/SCF signaling because W/V donor cells failed to induce any increase in spleen size when trans-
planted in $W/W^v$ mice, which is in contrast to the mild splenomegaly observed when control infected cells were transplanted into $W/W^v$ recipients.

$W/W^v$ mice do not express Sf-Stk in the spleen. The expression of Sf-Stk is required for the expansion of Friend virus-infected cells. Mice that are homozygous for the Fv2-resistant (Fv2$^r$) allele fail to express Sf-Stk and are resistant to Friend virus infection (29). The background strain for the $W/W^v$ mice and controls is WBB6F1. Genotyping these mice at the Fv2 locus showed that WBB6F1 mice are heterozygous (Fv2$^r$). We tested WBB6F1 control mice and WBB6F1 $W/W^v$ mice for the expression of Sf-Stk and full-length Stk by RT-PCR. Figure 7 shows that $W/W^v$ and control mice express similar levels of Sf-Stk mRNA in bone marrow; however, in the spleen, $W/W^v$ mice did not express Sf-Stk. Full-length Stk was expressed at comparable levels in the bone marrow and spleens of both $W/W^v$ and control mice. The absence of Sf-Stk expression further supports the idea that $W/W^v$ mice lack Friend virus targets in the spleen and the inability of Friend virus to infect cells in the spleen results in resistance to Friend virus-induced erythrolemacu.

Kit$^+$ megakaryocyte-erythroid progenitors are Friend virus target cells in the spleen, but not in bone marrow. Given that mutations in the Kit receptor severely decrease the number of Friend virus target cells in the spleen, we next analyzed Kit$^+$ subpopulations of spleen cells for Friend virus target cells using the in vitro infection assay. Analysis of erythroid progenitors in the bone marrow showed that two distinct Kit$^+$ subpopulations, the common myeloid progenitor and the megakaryocyte-erythroid progenitor give rise to BFU-E in culture (2). We have shown that only MEPs are present in the spleen (15). We tested whether MEPs isolated from the spleens of control mice could form Epo$^{ind}$ BFU-E colonies following Friend virus infection in vitro. Figure 8A shows that spleen MEPs efficiently form Epo$^{ind}$ BFU-E following infection with Friend virus. Interestingly, bone marrow MEPs fail to form Epo$^{ind}$ BFU-E colonies in this assay. These results demonstrate that MEPs, a population of Kit$^+$ erythroid progenitors in the spleen, are the pathogenic targets for Friend virus. Furthermore, this target cell population in the spleen is distinct from the bone marrow target cell population.

Taken together, all the data we have presented suggests that $W/W^v$ mice lack target cells in the spleen. However, it is possible that the defect in $W/W^v$ mice is not in the number of targets but in the ability of these cells to respond to infection. To differentiate between these possibilities, we compared the numbers of MEPs in the spleens of $W/W^v$ and control mice by flow cytometry. In four independent experiments, the percentage of MEPs (Lin$^-$ Sca1$^-$ IL-7Ra$^-$ CD34$^-$ Kit$^+$ FcyR$^<$low$>$) in the spleen was approximately 10-fold lower than in the controls (Fig. 8B). Calculation of the absolute number of MEPs per spleen showed a consistent 4.5- to 5.5-fold decrease in the number of MEPs in the $W/W^v$ spleens. $W/W^v$ mice have slightly enlarged spleens compared to control mice. In addition, we measured the expression of Sf-Stk mRNA in MEP populations sorted from $W/W^v$ and control mice (Fig. 8C). Control MEPs express Sf-Stk, which correlates with their ability to form Epo$^{ind}$ BFU-E following Friend virus infection. $W/W^v$ MEPs express little if any Sf-Stk mRNA. Taken together, these data demonstrate that $W/W^v$ mice contain few MEP target cells in the spleen, which fail to express Sf-Stk, and that Kit/SCF signaling is required for the expansion of infected cells in the spleen.

**DISCUSSION**

Friend virus induces erythrolemacu by co-opting the erythroid differentiation machinery. The Kit/SCF signaling pathway regulates the expansion of erythroid progenitors and is thought to function during the transition from BFU-E to CFU-E (25). Because of the severe anemia exhibited by the W and Sf mutant mice, it was proposed that this defect in erythropoiesis was the cause of the resistance to Friend virus in these mice (6). In this report we show that the role of the Kit/SCF signaling pathway in Friend virus pathogenesis is more complex. Our data show that the resistance of $W/W^v$ mice is caused by a defect specific to the spleen. These mice exhibit very few Friend virus targets in the spleen, which lack Sf-Stk expression, while the number of target cells in the bone marrow is similar to control mice.

More than 40 years ago, work from Mirand and colleagues showed that the spleen provided a microenvironment that favored the expansion of Friend virus-infected cells (19). Splenectomized mice exhibit a pronounced delay in the development of polycythemia and leukocytosis after infection despite the observation that the splenectomized mice exhibited significant expansion of erythroblasts in the bone marrow. These results demonstrated that the spleen, not the bone marrow, is the primary organ for Friend virus-induced expansion of erythroid progenitors. In the absence of the spleen, mice will eventually develop polycythemia and leukemia after long la-
tency; however, the liver becomes the primary site for Friend virus pathogenesis in these mice (18).

It is not surprising that the spleen plays a key role in the expansion of Friend virus-infected cells. The spleen provides a supportive microenvironment for erythropoiesis. Similar to fetal liver stromal cells, mouse spleen stromal cells are able to support the expansion of early erythroid progenitor cells, whereas bone marrow stromal cells cannot (26, 27, 37). The spleen is also the site of expansive erythropoiesis associated with the recovery from acute anemia or hypoxia. We have shown that acute anemia induces the rapid expansion of a novel stress erythroid progenitor, the stress BFU-E, which requires the BMP4 signaling pathway (15). In addition, our work showed that spleen MEPs are the BMP4-responsive cells in the spleen. Early work in the field showed that W and SI mice are slow to recover from phenylhydrazine (PHZ)-induced acute hemolytic anemia (12), and we have observed defects in the expansion of stress BFU-E in W/ W mice during the recovery from PHZ-induced acute anemia (J. Perry and R. F. Paulson, unpublished observations). The observation that both
Friend virus and the stress erythroid response to acute anemia utilize the same progenitor/target cells, spleen MEPs, explaining the early observation that PHZ treatment makes mice more sensitive to Friend virus. Indeed, we have observed that control mice treated with PHZ have an increased number of spleen target cells 36 h after treatment, but \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice treated with PHZ do not exhibit this increase (A. Subramanian and R. F. Paulson, unpublished observations). Interestingly, the number of bone marrow target cells do not increase with PHZ treatment, which further underscores the distinct nature of the two target cell populations.

Our data demonstrate that bone marrow MEPs are not targets of Friend virus, but if these cells are not target cells, then which cells are the bone marrow targets for Friend virus? Work from Baumann et al. has identified a bone marrow population that is distinct from MEPs and common myeloid progenitors, which has erythroid colony-forming activity (5). These cells are \( \text{CD}31^+ \text{Kit}^+ \text{Sca}1^- \text{Lin}^- \). Preliminary experiments suggest that these cells are able to form Epo\textsuperscript{ind} BFU-E following Friend virus infection in vitro. However, analysis of this population from the spleen suggests that spleen \( \text{CD}31^+ \text{Kit}^+ \text{Sca}1^- \text{Lin}^- \) are not targets for Friend virus (Subramanian and Paulson, unpublished). Further characterization of this population will establish the distinct nature of the bone marrow and spleen cell populations that are the pathogenic targets of Friend virus.

This difference in the target cell populations in bone marrow and spleen is clearly evident in our analysis of \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice. We observed that MEPs from the spleen express Sf-Stk and form Epo\textsuperscript{ind} BFU-E when infected with Friend virus in vitro, while bone marrow MEPs fail to respond to Friend virus infection. \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice exhibit near normal numbers of Friend virus targets in the bone marrow, which express normal levels of Sf-Stk. However, the spleen MEPs are markedly decreased in \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice and they do not express Sf-Stk. Consequently, \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice exhibit few pathogenic target cells in the spleen in both in vitro and in vivo assays.

On the basis of our observations, the Kit/SCF signaling pathway may play two roles in the pathogenesis of Friend virus. The \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice exhibit a clear deficit in target cells in the spleen as measured by in vitro and in vivo assays for Epo\textsuperscript{ind} BFU-E and flow cytometry analysis of MEPs and infected cells. These results suggest that the Kit/SCF pathway is required for the development or expansion of MEPs in the spleens of healthy mice. In addition to this role, Kit may also be involved in the expansion of Friend virus-infected cells. We observed that inclusion of SCF in the medium greatly facilitated the development of Epo\textsuperscript{ind} BFU-E in control spleen cells infected in vitro with Friend virus. These results are similar to those reported by Quang and coworkers using a chicken erythroblast system. They showed that Kit/SCF-dependent signaling induced extensive proliferation in cells that expressed an activated Epo receptor and Spi-1, which suggested that Kit signaling cooperates with the genetic events induced by Friend virus to promote the expansion of infected cells (30). These studies showed that Spi-1 expression was required for the Kit/SCF-dependent increase in proliferation, but this conclusion is complicated by the observation that overexpression of Spi-1 due to proviral insertion is a relatively late event in the pathogenesis of Friend erythroleukemia. Recent work that demonstrated that gp55-dependent signaling activates the expression of Spi-1 even at early times during infection removes this complication (1). Thus, early during infection, Friend virus-infected cells signaling through the gp55/Epo receptor/Sf-Stk complex express Spi-1 and are potential targets for the mitogenic activity of the SCF/Kit signaling pathway. Furthermore, when \( \textit{W}^\text{v} \textit{W}^\text{v} \) donor bone marrow cells, infected in vitro, were transplanted into \( \textit{W}^\text{v} \textit{W}^\text{v} \) recipients, no increase in spleen size was observed, in contrast to the mild, but significant splenomegaly observed when control infected bone marrow cells were transplanted into \( \textit{W}^\text{v} \textit{W}^\text{v} \) recipients. These results demonstrate that the Kit/SCF signaling pathway can drive the expansion of infected bone marrow cells in the spleen microenvironment. We also show that \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice fail to express Sf-Stk in spleen MEPs. This observation suggests that either defects in Kit receptor signaling, directly or indirectly, affect the expression of Sf-Stk or the reduction in spleen MEPs is severe enough to prevent detection of Sf-Stk expression by RT-PCR. Further analysis will be required to address this question.

In summary, we show that the pathogenesis of Friend virus-induced erythroleukemia requires the Kit/SCF signaling pathway in the spleen. Infection of \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice with Friend virus results in near normal infection of bone marrow cells, which expand similar to control mice. The situation is dramatically different in the spleen. Few cells are infected by Friend virus, and the Epo\textsuperscript{ind} BFU-E do not expand during the course of infection. These results demonstrate that the resistance to Friend virus-induced leukemia in \( \textit{W}^\text{v} \textit{W}^\text{v} \) mice is caused by a marked reduction of target cells in the spleen and a failure of these cells to express Sf-Stk. We have also identified a Kit\textsuperscript{+} population of erythroid progenitors, MEPs that are the target cells for Friend virus in the spleen. This same population of cells in the bone marrow is not a target for Friend virus, which clearly demonstrates that the target cells for Friend virus in bone marrow are distinct from the target cells in the spleen. Taken together, these results provide a clearer picture of the pathogenesis of Friend virus-induced erythroleukemia.

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