Differential Requirements of Cellular and Humoral Immune Responses for Fv2-Associated Resistance to Erythroleukemia and for Regulation of Retrovirus-Induced Myeloid Leukemia Development

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To assess the possible contribution of host immune responses to the exertion of Fv2-associated resistance to Friend virus (FV)-induced disease development, we inoculated C57BL/6 (B6) mice that lacked various subsets of lymphocytes with FV containing no lactate dehydrogenase-elevating virus. Fv2 B6 mice lacking CD4+ T cells developed early poly cycythemia, while B6 mice lacking CD8+ T cells remained resistant. Erythroid progenitor cells infected with spleen focus-forming virus (SFFV) were eliminated, and no poly cycythemia was observed in B cell-deficient B6 mice, but they later developed myeloid leukemia associated with oligoclonal integration of ecotropic Friend murine leukemia virus. Additional depletion of natural killer and/or CD8+ T cells from B cell-deficient B6 mice resulted in the expansion of SFFV proviruses and the development of poly cycythemia, indicating that SFFV-infected erythroid cells are not only restricted in their growth but are actively eliminated in Fv2 mice through cellular immune responses.

Detection of viral infection results in the activation of adaptive immune responses that clear the pathogen and provide protection against future reinflection with the same virus. The host immune system that exerts these adaptive immune responses is mainly comprised of B cells, which produce antivirus antibodies (Ab) and thus neutralize the extracellular virus, and cytotoxic T cells (CTLs), which lyse infected cells and thus restrain intracellular viral reservoirs. Many successful vaccines induce both cell- and Ab-mediated immune responses, which can last a lifetime. However, even these successful vaccines do not completely prevent infections, but rather they control viral replication upon infection and thus protect against virally induced disease development. Most HIV-infected individuals are able to mount anti-HIV immune responses, but these responses generally do not result in protection against virus replication and HIV-induced disease development (44). Therefore, additional factors are required to translate the viral detection into effective protection, as occurs in the cases of HIV elite controllers, who are able to naturally control the virus without the aid of antiretroviral drugs (13, 42). These resistant individuals have been employed to understand the mechanisms underlying protective immune responses against retrovirus infection.

We have used Friend leukemia virus (FV) infection as a model to assess the different roles of cell- and Ab-mediated immune responses in protection against retrovirus infection. Since Friend disease was first reported in 1957 (17), acute erythroleukemia induced by various strains of FV in different strains of mice has provided an excellent model to study multistage leukemogenesis, which is affected by several host factors (2, 9, 25). FV is a pathogenic retrovirus complex composed of replication-competent Friend murine leukemia virus (F-MuLV) and defective spleen focus-forming virus (SFFV). In the initial stage of FV-induced disease development, the product of the SFFV env gene, gp55, forms a complex with the erythropoietin receptor and the short form of the stem cell-specific receptor tyrosine kinase (Skt), and this interaction induces the growth and terminal differentiation of erythroid progenitor cells, causing increased hematocrit values and massive splenomegaly (37, 41). The late stage of Friend disease is marked by proviral integration into the Flil or PU.1 (Sfpi1) locus, resulting in a block of erythroid differentiation and the development of mono- or oligoclonal erythroleukemia (3, 39).

Several host genetic factors control FV replication and leukemogenesis, thus conferring resistance to FV-induced disease development (9, 37). These can be divided into a few categories based on the mechanism of resistance: the first group consists of two genes, Fv4 and Fv1, the products of which directly restrict target cell entry or proviral integration of F-MuLV and SFFV (5, 23). The second group consists of host genes that influence FV-induced disease more indirectly by affecting antiviral immune responses. These include major histocompatibility complex (MHC) genes (9, 36) and a non-MHC gene, Rfy3. The latter has been shown to influence the duration of viremia and the production of virus-neutralizing antibodies (11, 20, 26). Recently, it was demonstrated that polymorphisms in both the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3 (APOBEC3) and BAFF receptor (BAFF-R) are associated with the levels of viremia, and the C57BL strain of mice possesses resistance alleles at both of these gene loci (32, 52, 60, 61). The third group consists of genes that influence the progression of FV-induced disease.
disease. Mice of the C57BL background possess a mutation in the intron of the Stk gene and lack the expression of the short-form Stk (sf-Stk), by which they resist the development of SFFV-induced erythroid cell proliferation and the resultant massive splenomegaly (46). This host factor was first described as the Fv2 gene, with the resistance allele found in C57BL mice being designated as the recessive Fv2’ gene (33).

C57BL/6 (B6) mice potently resist FV-induced diseases due to their resistant genotypes at multiple loci, but the resistance is not absolute (14). Thymus-deprived B6 nu/nu mice develop FV-induced leukemia (28). In addition, treatment with a single dose of anti-Thy-1.2 Ab permitted the continued replication of FV in B6 mice (63). Further, B6 mice lacking either CD4+ or CD8+ T cells developed splenomegaly upon infection with FV containing lactate dehydrogenase-elevating virus (LDV) (19, 50). Therefore, T cell-mediated immune responses are essential for controlling FV replication and pathogenesis, even in the Fv2’ B6 background. However, it is not clear whether Ab-mediated immune responses are also required for the control of FV-induced leukemia development in B6 mice. We recently revealed that B6 mice lacking the resistance allele at the APOBEC3 or BAFF-R locus show a significant delay in the initiation of virus-neutralizing Ab production and harbor more than 100 times higher numbers of virus-producing cells than do the wild-type (WT) counterparts during acute infection with FV (61). However, these mice later recovered from FV infection and never developed leukemia, indicating that at least early production of virus-neutralizing Ab is not required for resistance to FV-induced leukemia development in B6 mice. In contrast, B cells, but not CD8+ T cells, were essential for protective immune responses against FV infection induced in highly susceptible Fv2’/C57BL/6 x BALB/cF1 (CB6F1) mice by immunization with a peptide containing a single CD4+ T cell epitope that was identified within the F-MuLV env gene product (27, 35).

To examine the possible roles of Ab-mediated immunity in controlling FV-induced leukemogenesis in the Fv2’ setting, we utilized B6 mice lacking various subsets of lymphocytes and followed the long-term consequences of lymphocyte deficiencies. The results revealed that antiretroviral Ab responses are essential for the long-term control of F-MuLV replication and for the prevention of leukemogenesis, but SFFV-infected erythroid cells are eliminated by cellular responses in the absence of B cells.

MATERIALS AND METHODS

Mice. C57BL/6NCrSlc and BALB/cCrSlc mice were purchased from Japan SLC, Inc., Hamamatsu, Japan. B6.129P2–β2mnu/nu/nu mice carrying homozygous disruption of the β2-microglobulin gene (β2m) were purchased from The Jackson Laboratory, Bar Harbor, ME. B6.129S2-Lphnuem1cre1/1 mice carrying homozygous disruption of the membrane exon of the Ig μ-chain gene (μ-chain membrane exon targeted; μMT/μMT) and thus lacking B lymphocytes (29) were also purchased from The Jackson Laboratory, and BALB/c-μMT/μMT mice were established in our animal facilities by backcross mating of BALB/c mice with the B6-μMT/μMT mice (27). CB6F1 mice, lacking B cells were produced by crossing the B6-μMT/μMT and BALB/c-μMT/μMT mice as previously described (27). C57BR/cd1 mice, which do not express membrane-bound CD4, have been described (40). They were backcrossed to B6 mice at least nine times. Both male and female mice at 7 to 8 weeks of age were infected and followed for 20 to 24 weeks. All animals were housed and bred in the Experimental Animal Facilities at Kinki University Faculty of Medicine under specific-pathogen-free conditions, and the experiments described here have been approved by Kinki University.

Virus and inoculation. A stock of B-tropic FV complex without contamination of LDV has been described elsewhere (60). Previously used FV stocks were prepared as spleen homogenates contained LDV as an unintended consequence of repeated in vivo passage (37, 50). Coinfection with LDV strongly suppressed FV-specific CD8+ T cell responses, but FV-reactive CD4+ T cell and Ab responses were not affected (50). LDV contamination was also associated with potent type I interferon responses that were not attributable to FV (18) and also polyclonal activation of both T and NK cells (37). The FV stock used here was generated in vitro (50), passaged in BALB/c mice for 9 days to obtain a high enough titer, and confirmed to be free of LDV by PCR assays and by the lack of polyclonal immune stimulation (37). The SFFV titer was determined by enumerating 14-day spleen foci in BALB/c mice and the F-MuLV titer by infectious focus assays on Mus dunnii cells and with monoclonal Ab (MAb) 720 (35, 49). No infectious propagation of mink cell focus-inducing (MCF) viruses reactive with MAB 514 (8) was observed after inoculation of the FV stock onto Mus dunnii cells. Replication-competent helper virus of FV, F-MuLV, was purified from a culture supernatant of Mus dunnii cells persistently infected with an infectious molecular clone, FB29 (56). B6 mice were infected with 5,000 spleen focus-forming units (SFFU) of the FV stock or 5,000 focus-forming units of the FB29 stock, and FV-susceptible CB6F1 mice were infected with 150 SFFU of FV. Infected mice were observed daily, and their hematocrit values were measured every 2 weeks as described previously (61).

Cell harvest and flow cytometry. Flow cytometric analyses of cell surface markers were performed as described elsewhere (59, 61). Spleen and bone marrow tissues were dissociated in phosphate-buffered balanced salt solution (PBBS) containing 2% fetal bovine serum (FBS), and a single-cell suspension was prepared by passing each dissociated tissue through a nylon mesh. Cells were incubated with anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA) to prevent the test Ab from binding to Fc receptors and then stained with a combination of the following MAbS with appropriate fluorescence labeling (61): anti-mouse CD3, CD11b, CD11c, CD14, MHc class II I-E/I-A, Ly6G, and TER-119 (BD Biosciences). F-MuLV-infected cells were detected with biotinylated MAb 720 and fluorescence-conjugated streptavidin as described previously (49). The SFFV Env protein was detected with MAb 514, which also reacts with some recombinant polytropic viruses, including Friend MCF virus isolates (8), and endogenous and recombinant polytropic viruses were detected with MAb 24-6 (47), 7-Aminoactinomycin D (7-AAD) viability dye (Beckman Coulter, Inc., Brea, CA) was used to exclude dead cells. Data were acquired with a FACSort and were analyzed with CellQuest Pro software (BD Biosciences).

Quantitation of proviral copy numbers. Genomic DNA was purified from spleens or bone marrow cells by using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and treated with RNase I. Proviruses in 100 ng or 500 ng of genomic DNA were quantified by using Platinum quantitative PCR superMix-UDG with ROX (Life Technologies, Carlsbad, CA) and a Prism 7900HT real-time PCR system (Life Technologies). PCR primers and TaqMan probes for the differential detection of F-MuLV and SFFV were designed based on the env portion of each provirus with primers 5’-AAGTCTCTCCCCCCTCTCTA-3’ and 5’-AGTGCTCTGGTGAAGT CCGTGT-3’ and a 6-carboxyfluorescein (FAM)-labeled probe, 6-FAM– 5’-ACTCCTACATGTTAAGCGGCCC-3’ for the detection of F-MuLV and primers 5’-TCTAACCCTCAACCCCTTGGT-3’ and 5’-TTTGTAGGGCAG ATGGTATGTTAATAAA-3’ and a FAM-labeled probe, 6-FAM–5’- CCTAGTCTCGGCCCCCCTTATCGGG-3’ for the detection of SFFV (60). After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out at 95°C for 30 s and 58°C for 1 min. A TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagent (Life Technologies) was used as an internal control. Standard curves obtained by using plasmids containing the env gene of each virus as templates were linear over a range of 10 to 10^4 copies in the above reaction. The estimated melting temperature indicated that none of the above primers or probes cross-hybridized with the endoge-
nous retrovirus Env2 sequence (GenBank accession number AC158362, position 97057 to 105700) under the above reaction conditions.

**Inverse PCR and sequence analysis.** Clonality of and proviral integration sites in FV-infected cells were determined by inverse PCR followed by molecular cloning and DNA sequence analysis (58, 62). Genomic DNA (2 μg) was digested with BamHI enzyme for 2 h and self-ligated with T4 ligase (TaKaRa Bio, Inc., Shiga, Japan) at 16°C overnight. The resultant circular DNA was used in the primary PCR. Amplification of the virus-host junction was performed in 50 μl of PCR buffer containing 350 μM deoxynucleoside triphosphates, 3.75 units of Taq DNA polymerase (Expand Long Template PCR system; Roche Diagnostics, Mannheim, Germany), and 0.3 μm of each primer. The forward primer was designed to bind to the long terminal repeat (LTR) regions of both proviruses, and the reverse primers were specific for the env portion of each provirus, as follows: common forward primer, 5′-CCAAAGGACCTGAAATGACCCTG-3′; reverse primer specific for F-MuLV, 5′-GACTTGCGAGGTGGGT AGG-3′; reverse primer specific for SFV, 5′-GGAGAAGGTGCGGCGAT CTCCG-3′. PCR amplification was carried out in an iCycler thermal cycler (Bio-Rad Lab, Tokyo, Japan) under the following conditions: the first step was 15 cycles at 92°C for 10 s, 59°C for 30 s, and 68°C for 8 min, preceded by an initial denaturation at 92°C for 2 min; the second step was 20 cycles at 92°C for 10 s, 59°C for 30 s, and 68°C for 8 min, with a 20-s extension of the elongation step for each successive cycle, followed by a final extension at 68°C for 7 min. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. Separated DNA bands with a length of more than 25 bp were purified using a Wizard PCR Preps DNA purification system (Promega KK, Tokyo, Japan) and cloned into the pCR2.1-TOPO or pCRXL-TOPO (Life Technologies) plasmid. Sequence analysis was performed by using a primer located in the LTR, 5′-GAGCTCCAACCCCTCAGTCTC-3′. Database analysis of the obtained sequences was performed by using the BLASTN homology search program provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), and proviral integration sites were determined. When proviruses were integrated outside of a previously identified gene locus, the distances between the integrated provirus and the nearest gene were calculated. To determine proviral sequences within genomic DNA prepared from spleens of B cell-deficient B6 mice, the 3′ env-LTR portions of F-MuLV proviruses were amplified from 200 ng of genomic DNA by PCR using the following primers: forward primer specific for the env portion of F-MuLV, 5′-AAGTTCTCCCCGCCCTCCTCT-3′; reverse primer for the LTR region, 5′-AAGCAGGGTCTATTTACA GGTCT-3′. The amplified PCR products were separated by agarose gel electrophoresis, and their lengths were measured.

**Cell morphology.** Single-cell suspensions were prepared from the spleens as described above. Cytospin preparations of the spleen cells and peripheral blood smear preparations were stained with May-Grunwald Giemsa stain.

**Infectious center assays.** Infectious center assays were performed as described previously (27, 35). Briefly, spleen and bone marrow cell suspensions prepared from mice challenged with F-MuLV were serially diluted with PBBS containing 2% PBS, plated in triplicate at concentrations between 10^3 and 10^5 cells per well of 24-well plates onto monolayers of Mus dunni cells that had been seeded at 1 x 10^4 cells per well on the previous day, and cocultured for 2 days. To ensure the lack of detectable virus-producing cells, 6 x 10^5 spleen cells and 3 x 10^5 bone marrow cells from each mouse were plated in 20 and 10 wells of 24-well plates, respectively, at a concentration of 3 x 10^5 cells per well. After fixation with methanol, cocultured Mus dunni cells were stained with biotinylated MAb 720, 514, 24-6, or 24-8 (8, 47, 49), and foci were visualized by using the avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA) and counted under a magnifier.

**Cell depletion in vivo.** NK cells were depleted as described previously (24, 43). Briefly, rabbit antiserum specific for mouse asialo-ganglio-N-tetraosylceramide (asialoGM1) and control normal rabbit serum were purchased from Wako Pure Chemicals (Osaka, Japan), and the IgG fraction was concentrated by precipitation with 45% (final concentration) ammonium sulfate. Mice were injected intravenously with 60 μg/dose of the anti-asialoGM1 Ab 1 day prior to FV inoculation and 2, 5, 8, 11, 14, 21, and 28 days after virus infection. CD8⁺ T cells were depleted by injecting anti-mouse CD8 (Lyt-2.2) MAb. The rat anti-mouse CD8 MAb was purified from the culture supernatant of hybridoma 2.43 cells (American Type Culture Collection, Manassas, VA) as described previously (51). Mice were intravenously given a 200 μg/dose of the purified anti-CD8 MAb at 7, 3, and 1 day prior to FV inoculation and 1, 2, and 3 weeks after virus infection. Rat IgG was given to the control group.

**Statistical analyses.** One-way or two-way analyses of variance (ANOVA) and Mantel-Cox tests of survival curves were performed using Prism software (GraphPad Software, Inc., San Diego, CA) with indicated posttests. The Mann-Whitney test was used for non-Gaussian distributions, with Bonferroni’s post hoc test for multiple comparisons.

**RESULTS**

**Development of distinctive fatal pathologies upon FV infection in Fv2² B6 mice lacking CD4⁺ T or B Lymphocytes.** To investigate the possible involvement of various immune cell populations in controlling FV-induced leukemogenesis in Fv2² mice, B6 mice lacking a specific subset of immune cells were utilized: cell membrane CD4⁻/defective mutant mice lack CD4⁺ T cells, cell membrane IgM-deficient mutant mice lack CD4⁺ T cells, cell membrane IgM-deficient mutant mice lack CD4⁺ T cells, and B2m knockout mice, which are deficient in peripheral CD8⁺ T cells (29, 30, 40). It has been shown that B6 mice lacking either CD8⁺ or CD4⁺ T cells develop splenomegaly within 6 to 10 weeks after FV infection, while most B cell-deficient B6 mice do not develop splenomegaly (19). However, the above experiment was performed by using an FV stock that contained LDV, which caused severe immune dysfunction that rendered B6 mice less resistant to FV infection (50). Further, the development of FV-induced disease takes multiple steps (25, 37, 38), which may require a longer observation period to fully assess the role of immune cell subsets, especially in highly resistant B6 mice. Therefore, we used LDV-free FV and performed more detailed cellular and molecular analyses in a long-term setting.

To study the kinetics of FV-induced disease development, we first analyzed the changes in hematocrit values and survival rates after FV inoculation in B6 mice lacking CD4⁺ or CD8⁺ T cells or B lymphocytes. All of the WT B6 mice survived for 20 weeks after FV inoculation, confirming that B6 mice are highly resistant to FV infection (Fig. 1). However, 80 to 95% of B6 mice deficient for either CD4⁺ or B cells died within 8 to 20 weeks after FV infection, with significantly shorter survival periods than infected WT mice (P = 0.01 and P = 0.0003, respectively). In contrast, only a small fraction of mice lacking CD8⁺ T cells died upon FV inoculation, and their survival rate was not different from that of WT mice (P > 0.48). As for the hematocrit values, none of the WT B6 mice showed polycythemia upon FV infection, whereas only one mouse in the CD8⁺ T cell-deficient group showed a decrease in hematocrit values just before its death, and none showed polycythemia (Fig. 2A). On the other hand, most of the CD4⁺ T cell-deficient mice developed progressive polycythemia starting around 5 weeks postinfection or earlier. Most of these mice showed a sharp decline in hematocrit values just before death. Spleen sizes at the time of death in CD4⁺ T cell-deficient mice were significantly larger than those in the infected WT mice (Fig. 2B). Intriguingly, the hematocrit values of B cell-deficient mice were slightly decreased but never increased upon FV infection (Fig. 2A). Nevertheless, in a long-term follow-up experiment, B
cell-deficient B6 mice died more rapidly than the CD4\(^+\) T cell-deficient mice that showed massive polycythemia in the earlier stage (Fig. 1; \(P = 0.001\)). Consistent with the lack of increases in their hematocrit values, spleen weights of B cell-deficient B6 mice at around the day of their death were only slightly higher than those of the infected WT mice (Fig. 2B). These results indicate that CD4\(^+\) T cells are indispensable for the control of FV-induced erythroid cell proliferation and the resultant development of fatal leukemia, while CD8\(^+\) T cells are not required, contrary to the previous results obtained with the LDV-containing FV (19).

FIG 1 Survival of immunodeficient B6 mice upon FV infection. B6 mice lacking CD4\(^+\) T cells (CD4\(^-\), \(n = 13\)), CD8\(^+\) T cells (CD8\(^-\), \(n = 12\)), or B cells (B\(^-\), \(n = 17\)) and wild-type B6 mice (\(n = 11\)) were inoculated with 5,000 SFFU of FV, and their survival was monitored for 20 weeks. *, significantly different from the survival curve of the WT mice based on the Mantel-Cox test. The entire set of experiments was repeated with >10 mice for each group, and essentially the same results were obtained.

FIG 2 Changes in hematocrit values and spleen weights in FV-infected B6 mice. B6 mice lacking CD4\(^+\) T cells (CD4\(^-\), \(n = 13\)), CD8\(^+\) T cells (CD8\(^-\), \(n = 12\)), or B cells (B\(^-\), \(n = 17\)) and WT B6 mice (\(n = 11\)) were inoculated with 5,000 SFFU of FV. (A) Hematocrit values were measured every 2 weeks after FV inoculation. Each line shows the time-dependent changes in hematocrit values in each individual mouse. (B) Spleen weights were measured at around the day of death or at 20 weeks postinfection for surviving mice. Each circle shows the actual weight of the spleen of an individual mouse, and horizontal bars represent the mean values for each group. Note that some mice died before the measurement of spleen weight, and thus not all individuals in panel A are included. ***, \(P < 0.001\) by one-way analysis of variance with Tukey’s post hoc test for multiple comparisons. Similar data were obtained in two separate experiments.
ther, B cells are not required to control FV-induced erythroid cell proliferation, but FV induces a fatal disease in the absence of B cells without causing massive splenomegaly.

**SFFV becomes undetectable in B cell-deficient B6 mice after FV infection.** In order to address why FV-induced polycythemia was observed in CD4<sup>+</sup> T cell-deficient, but not in B cell-deficient, B6 mice, we first analyzed the expansion and differentiation of virus-infected erythroblasts and copy numbers of F-MuLV and SFFV proviruses in the spleens of B6 mice deficient for each type of the immune cells. MAb 720 exclusively detects the polytropic virus-derived portion of the SFFV and recombinant MCF virus Env proteins, but not with ecotropic, xenotropic, or amphotropic viruses (8, 49). In the spleens of CD4<sup>+</sup> T cell-deficient mice, TER-119<sup>+</sup> terminally differentiating erythroid cells massively expanded with time after FV inoculation. These cells also expressed both F-MuLV Env gp70 and SFFV Env gp55 (Fig. 3). Consistent with these observations, the copy numbers of both proviruses markedly increased in the spleens of CD4<sup>+</sup> T cell-deficient animals (Fig. 4A and B). On the contrary, in B cell-deficient B6 mice, 514<sup>+</sup> SFFV-infected cells were hardly detectable by flow cytometry, and the percentages of erythroid cells were only slightly increased at 7 weeks after FV infection, while F-MuLV gp70<sup>+</sup> cells were readily detected and increased with time (Fig. 3). Note that, due to the lack of B cells, percentages of TER-119<sup>+</sup> erythroid cells in µMT/µMT mice were higher than those in WT mice even before FV infection. Similarly, in the quantitative analysis of proviral copy numbers, SFFV provirus was barely detectable in the B cell-deficient mice at 2 weeks after FV inoculation and became undetectable by 7 weeks postinfection, whereas the copy numbers of F-MuLV provirus increased over time (Fig. 4A and B). As expected, small numbers of F-MuLV proviruses were detectable at 2 weeks postinfection, but both proviruses became undetectable in the WT B6 mice by 15 weeks after FV inoculation.

As SFFV proviruses and cells reactive with MAb 514 were both barely detectable despite a high dose of FV inoculation, we asked if SFFV fails to replicate or if SFFV-infected cells are selectively eliminated from the spleens of B cell-deficient B6 mice. One possible explanation for the lack of SFFV provirus detection in B cell-deficient B6 mice is that B cells are required for the propagation of SFFV. To examine this possibility, CB6F1 mice without Fv2<sup>+/−</sup> or Fv2<sup>−/−</sup> and lacking B cells were infected with a low dose of FV complex. In B cell-deficient CB6F1 mice, SFFV proviruses were detectable in the spleen of 1 of the 3 animals tested as early as 1 week after FV inoculation, and significantly high copy numbers were detected 2 weeks postinfection in all tested animals despite the low inoculum dose (Fig. 4C), indicating that SFFV can replicate in the absence of B cells. In fact, B cell-deficient CB6F1 mice showed moderate splenomegaly at 2 weeks after FV infection (average spleen weight, 0.20 ± 0.03 g [mean ± standard error of the mean; n = 10]), compared to 0.07 to 0.10 g in uninfected µMT/µMT mice of the same background), and their spleens became significantly larger (0.76 ± 0.16 g; n = 8) as early as 3 weeks after infection.

It has been shown that FV initially infects target cells in the bone marrow, and infectious center cells then migrate to the

**FIG 3** Changes in the proportions of F-MuLV- and SFFV-infected erythroid cells in the spleens of CD4<sup>+</sup> T cell- or B cell-deficient B6 mice. Spleen cells were prepared from B6 mice lacking CD4<sup>+</sup> T cells (CD4<sup>−</sup>) or B cells (B<sup>−</sup>) and from WT B6 mice before or at 7 or 15 weeks after FV inoculation and were stained with MAb 514 or 720 as well as the MAb specific for TER-119, a marker for late erythroid lineage cells. Representative dot plots are shown. The value in each quadrant indicates the percentage of cells that expressed the indicated marker.
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spleen and induce stress erythropoiesis, ultimately causing splenomegaly (57). Thus, we next examined if SFFV proviruses were detectable in the bone marrow in B6 mice. As expected, F-MuLV proviruses were transiently detected in the bone marrow of WT B6 mice but quickly became nearly undetectable by 2 weeks after infection (Fig. 4D). On the other hand, F-MuLV proviruses were detectable by 1 week at higher levels and increased copy numbers through 2 to 7 weeks after infection in B cell-deficient B6 mice. Nevertheless, SFFV proviruses were not detectable even in B6 mice in the absence of CD4+ T cells (Fig. 4B), these data collectively indicate the possibility that SFFV is commonly eliminated in WT and B cell-deficient B6 mice as rapidly as 2 weeks after infection.

![Image of Figure 4](http://jvi.asm.org/)

**FIG 4** Kinetic changes in proviral copy numbers in FV-infected B6 or CB6F1 mice. Each circle represents the actual proviral copy number detected from an individual mouse, and horizontal bars represent the mean values for each group. The FV stock inoculated contained about the same number of infectious units of F-MuLV and SFFV. (A and B) Spleen cells were prepared from WT, CD4− T cell-deficient (CD4−), or B cell-deficient (B−) B6 mice at 2, 7, and 15 weeks after FV inoculation, and the genomic DNA was obtained from the spleens. Copy numbers of either F-MuLV (A) or SFFV (B) proviruses integrated into 100 ng of spleen genomic DNA (equal to about 1.7 × 10^4 cells) were determined by the real-time-PCR method. *, significant increase in comparison with relevant copy numbers at 2 weeks after infection (P < 0.05) based on a one-way analysis with Dunnett’s post hoc test for multiple comparisons; **, P < 0.01; †, undetectable in all animals examined. (C) B cell-deficient CB6F1 mice were inoculated with 150 SFFU of FV, and their spleen cells were prepared at 1 or 2 weeks postinfection. The copy numbers of each provirus integrated into spleen genomic DNA (100 ng) were analyzed. (D) Bone marrow cells were prepared from B cell-deficient (○) or WT (●) B6 mice at 1, 2, and 7 weeks after FV inoculation, and the proviral copy numbers in 500 ng of the bone marrow genomic DNA (equal to about 8.5 × 10^4 cells) were analyzed. *, significant changes in copy numbers were observed between the indicated time points (P < 0.05), based on a one-way analysis of variance with Tukey’s post hoc test for multiple comparisons; †, undetectable in all animals examined.
FV infection, but B6 mice lacking CD4+ T cells fail to eliminate SFFV and as a result develop massive splenomegaly and polycythemia.

**Cytotoxic cells are required for early elimination of SFFV in Fv2+ B6 mice.** As SFFV expanded in Fv2+ B6 mice in the absence of CD4+ T cells but SFFV-infected cells and SFFV proviruses were undetectable or barely detectable at 2 weeks after FV infection in both WT and B cell-deficient B6 mice, it is possible that cellular, but not humoral, immune responses under the control of CD4+ T cells may be required for early elimination of SFFV-infected cells. In fact, it has been shown that NK cells are required for immune control of FV-induced disease development that is brought about by priming CD4+ T cells with a peptide vaccine (24), and NK cells directly recognize and control the expansion of FV-infected erythroid cells (43). Therefore, to explore the presumable roles of cytotoxic effector cells on the elimination of SFFV-infected cells, we administered Ab specific to asialoGM1, CD8, or both to B cell-deficient B6 mice and depleted the numbers of NK and/or CD8+ cells. In B cell-deficient B6 mice depleted of either NK or CD8+ cells or both NK and CD8+ cells, polycythemia was observed after FV infection (Fig. 5A). More importantly, the analyses of proviral copy numbers in the late phase of FV infection also showed that SFFV proviruses became detectable in the spleens of B cell-deficient B6 mice upon depletion of CD8+ and/or NK cells (Fig. 5B), although the copy numbers of SFFV provirus were lower than in CD4+ T cell-deficient B6 mice (Fig. 4B). Furthermore, at earlier time points after FV infection, SFFV proviruses also became detectable in B cell-deficient B6 mice after infection with both anti-CD8 and anti-asialoGM1 Ab, while F-MuLV copy numbers were not significantly affected by Ab injections (Fig. 5C), indicating that SFFV-infected cells are indeed selectively eliminated, at least in part, by CD8+ T cells and/or NK cells in the very early phase of FV infection in B6 mice lacking B cells. Taken together, these results indicate that, in FV-infected B cell-deficient B6 mice, SFFV was not detectable because SFFV-infected cells were eliminated by cellular immune responses exerted at least partly by NK and/or CD8+ T cells.

**F-MuLV alone, but not SFFV, is responsible for the deaths of B cell-deficient B6 mice.** As B cell-deficient B6 mice died without signs of either polycythemia or massive splenomegaly, we next asked if the pathogenic process after FV infection is different between CD4+ T cell- and B cell-deficient B6 mice. To this end, we first detected proviral integration sites in the spleen by using the inverse PCR method and determined the clonality of cells harboring the proviruses. At 7 weeks postinfection, numerous different integration sites for F-MuLV proviruses were found in the spleens of both CD4+ T cell- and B cell-deficient B6 mice, reflecting the polyclonal nature of FV-infected cells (Fig. 6). Consistent with the results of flow cytometry and real-time PCR analyses, SFFV proviral integration into the spleen cell genome was not detected in B cell-deficient mice. In contrast, limited numbers of FV integration sites were observed at 15 weeks postinfection in both strains of mice, indicating the emergence of transformed leukemic clones. Again, oligoclonal integration of F-MuLV alone was observed in B cell-deficient B6 mice. In addition, to analyze proviral sequences in the genomic DNA purified from B cell-deficient B6 mice, the 3’ env-LTR fragments of F-MuLV provirus were directly amplified in PCR assays. The sizes of amplified DNA fragments were all identical to that of F-MuLV provirus (data not shown), ruling out the possible insertion of endogenous retroviral sequences into the amplified segment. Taken together, these results suggest that the FV-induced mortality observed in B cell-deficient B6 mice might be caused by leukemia associated with persistent replication of F-MuLV.

**Inoculation of F-MuLV alone is sufficient to induce fatal disease in adult B6 mice when B cells are lacking.** F-MuLV free of SFFV has been shown to induce leukemia when inoculated into newborn mice of susceptible strains (10, 54, 55). However, our results, described in the above sections, strongly indicated that F-MuLV alone in the absence of SFFV replication induced a fatal disease with oligoclonal expansion of largely TER-119+ cells in B cell-deficient adult B6 mice. Thus, we next asked if inoculation of F-MuLV free of SFFV into adult B6 mice induced a similar pathology in the absence of B cells. In fact, most of the B cell-deficient B6 mice died after inoculation of F-MuLV alone, while their hematocrit values were not increased until death (Fig. 7). This indicated that F-MuLV in the absence of SFFV is sufficient to induce the fatal pathology in B cell-deficient B6 mice. Therefore, we next investigated whether the fatal pathology observed in B cell-deficient B6 mice was caused by the persistent infection with F-MuLV itself or through the generation of recombinant MCF viruses and their insertion into the host cell genome. To detect the possible presence of recombinant MCF viruses, we performed infectious center assays with three MAbs that reacted with known recombinant MCF viruses (8, 47). In the spleen and bone marrow prepared from B cell-deficient B6 mice between 14 and 17 weeks after F-MuLV infection, no recombinant MCF viruses were detectable by any of these MAbs (Fig. 8), while F-MuLV was readily detected. Even when as many as 6 × 10⁷ and 3 × 10⁷ cells prepared from the spleen and bone marrow, respectively, were seeded as infectious centers, no foci were detectable with the above MCF-reactive MAbs. Further, in fluorescence-activated cell sorter analyses performed at 7 and 15 weeks after infection, spleen cells in FV-infected, B cell-deficient B6 mice were not only negative for MAb 514 (Fig. 3), but also were negative for cell surface binding of MAb 24-6, which is known to react with representative FV MCF isolates (data not shown) (47). Thus, the fatal pathology was most likely induced by persistent infection by F-MuLV itself, and not through the emergence of infectious MCF viruses in B6 mice lacking B cells.

**Development of myeloid leukemia in Fv- or F-MuLV-infected B6 mice lacking B cells.** In the analyses of provirus integration sites by use of the inverse PCR method, oligoclonal expansion of F-MuLV-infected cells was observed in the spleens of B cell-deficient B6 mice in the late phase of FV infection (Fig. 6), suggesting presumable leukemia development in these mice as the cause of the observed fatality (Fig. 1). To explore whether the fatal outcome observed in B6 mice lacking B cells was caused by the development of leukemia after persistent F-MuLV infection, we next compared cellular phenotypes in the spleen as well as peripheral blood of B6 mice and B cell-deficient B6 mice under pathological conditions after FV infection. In most of the CD4+ T cell-deficient B6 mice under pathological conditions after FV infection, expansion of various stages of erythroblast-like cells was observed in their spleens, with the appearance of immature erythroid progenitor cells in the peripheral blood (Fig. 9A), similar to those changes observed in FV-susceptible mice possessing the Fv2+ allele after FV infection. The low- to medium-level expression of TER-119 on the gp70+ cells expanded in the spleen was demonstrated by flow cytometry in cells from the majority of CD4+ T cell-deficient B6
mice under FV-induced pathology (Fig. 9B), confirming the erythroid nature of leukemic cells. The expansion of lymphoid cells expressing CD3 was observed in 1 of the 10 infected CD4<sup>+</sup> T cell-deficient mice examined (data not shown), indicating occasional development of lymphoid leukemia. On the other hand, large myeloid-like cells with cleaved nuclei expanded in the spleens of B cell-deficient mice after FV infection associated with vastly increased numbers of granulocytes with hypersegmented nuclei in the peripheral blood (Fig. 9A). Further, we found the expression of CD11b, Ly6G, and F-MuLV gp70, but not anti-CD8 anti-aGM1, and anti-aGM1 anti-CD8.
TER-119, CD3, CD11c, CD14, NK1.1, or MHC class II, on the surfaces of larger cells expanding in the spleens of FV-infected B cell-deficient mice (Fig. 9B), indicating the development of myeloid leukemia. Similarly, cells with morphological characteristics consistent with myeloid lineage with large cleaved nuclei dominated the spleens of B cell-deficient B6 mice in the pathological condition after F-MuLV inoculation and their peripheral blood contained large blastic cells with convoluted nuclei and granulocytes with hypersegmented nuclei (Fig. 9C). They also possessed in the spleens large cells with higher side scatters that were positive for F-MuLV gp70, CD11b, and Ly6G, but lacking the expression of TER-119, CD3, NK1.1, or CD14 (Fig. 9D). Thus, cellular phenotypes of large blastic cells in the spleen were essentially identical between FV-infected and F-MuLV-infected B cell-deficient B6 mice in their pathological conditions. Again, the expression of polytropic viral envelope antigens reactive with MAb 24-6 or 514 were not detectable by flow cytometric analyses of the spleen cells (data not shown). Taken together, these results indicate that persistent infection with F-MuLV induces the development of myeloid leukemia in B cell-deficient adult B6 mice, whereas persistent infection with SFFV plus F-MuLV induces predominantly erythroleukemia in adult B6 mice in the absence of CD4 T cells.

F-MuLV proviruses are not preferentially integrated at the FlI-I and PU.1 loci in B cell-deficient B6 mice. Our results clearly demonstrate that FV-induced leukemia cell types were different between CD4 T cell- and B cell-deficient B6 mice. Thus, we next

**FIG 6** Changes in the clonality of virus-infected cells. Spleen cells were prepared from B6 mice lacking CD4 T cells (CD4⁻) or B cells (B⁻) at 7 weeks or at around 15 weeks after FV inoculation, and genomic DNA was purified. The host-virus junctional segments were amplified by the inverse PCR method and specific primers for F-MuLV or SFFV, and the PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. Data shown are representative of those obtained from more than 10 individuals.

**FIG 7** Survival rates and hematocrit values in B cell-deficient B6 mice upon infection with F-MuLV free of SFFV. B cell-deficient (B⁻, n = 10) or WT B6 mice (n = 6) were inoculated with 5,000 focus-forming units of F-MuLV, and their survival rates and hematocrit values were analyzed. The entire set of experiments was performed twice with essentially the same results. *, significantly different from the survival curve of the WT mice (P = 0.0040) by Mantel-Cox test.
FIG 8 MCF viruses were undetectable in B6 mice lacking B cells upon F-MuLV infection. Bone marrow and spleen cells were prepared from B cell-deficient B6 mice between 14 and 17 weeks after F-MuLV inoculation and then incubated on monolayers of Mus dunni cells for 2 days. Foci of virus-infected cells were stained with MAb 720, which is specific for the env gene product of F-MuLV, Mab 514, which reacts with SFFV and some MCF viruses, and MCF virus-reactive MAbs 24-6 and 24-8 (8, 47, 49). Each circle represents the actual number of infectious centers detected from an individual mouse.

Investigated the locations of the proviruses integrated into the mouse genome in leukemic mice. The host-provirus junctional segments were amplified from spleen genomic DNA by the inverse PCR method as described above (Fig. 6), and the amplified DNA segments were sequenced. In CD4<sup>+</sup> T cell-deficient B6 mice, SFFV proviruses were integrated within or near the loci of Fli1 (in 3 out of 10 mice tested) and PU.1 (in 4 out of the 10 mice) (Table 1), which were reported previously as major targets for integration of F-MuLV or SFFV proviruses in erythroleukemia cell lines established from FV-susceptible mice (3, 39). In addition, SFFV proviruses were integrated within the Erg gene locus 2 of the 10 CD4<sup>+</sup> T cell-deficient B6 mice examined. The provirus integrations into these three gene loci were repeatedly observed, but other integration sites were each identified in only one mouse. Unexpectedly, none of the F-MuLV proviruses were integrated into Fli1 gene locus in these CD4<sup>+</sup> T cell-deficient mice, although Fli1 gene has been first identified as a frequent target for F-MuLV provirus integration in the FV-induced erythroleukemia cells established from FV-susceptible mice (3, 39). In B cell-deficient B6 mice, none of F-MuLV proviruses detected were integrated into the Fli1 or PU.1 locus (Table 2). Instead, the actual integration sites of F-MuLV proviruses were various, and none were found repeatedly in the 10 leukemic mice examined. However, as some of the genes within or near the location where the provirus integration was observed function as transcription factors, intracellular signal transduction molecules, or regulators for cell growth/differentiation, these may be related to the leukemogenesis induced by F-MuLV infection in B cell-deficient B6 mice.

DISCUSSION

In the present study, we have shown that Fv2<sup>+</sup> B6 mice nevertheless develop polycythemia and splenomegaly followed by fatal leukemia when CD4<sup>+</sup> T cells are lacking. The expansion of TER-119<sup>+</sup> erythroid progenitor cells in the spleen with high level expression of F-MuLV gp70 and SFFV gp55, the progressive increase in the numbers of F-MuLV and SFFV proviral integrations in the spleen cell genome, and oligoclonal integration of both F-MuLV and SFFV proviruses in the late stage of infection all agree with the previously described cellular and molecular characteristics of FV-induced erythroleukemia that develops in Fv2<sup>+</sup>-possessing susceptible mice (2, 38), although the temps of disease development were much slower in CD4<sup>+</sup> T cell-deficient B6 mice than in Fv2<sup>+</sup>-possessing mice. In fact, Fv2<sup>+</sup> CB6F<sub>1</sub> mice develop significant splenomegaly by 2 weeks after FV infection (27, 43) and severe polycythemia at as early as 3 weeks post FV infection (60), while spleen weights of CD4<sup>+</sup> T cell-deficient B6 mice at 2 weeks after FV infection were not different from those of infected WT B6 mice (data not shown). Nevertheless, the frequent involvement of the Ets-family PU.1 and Fli1 genes as SFFV integration sites indicates that the fatal leukemia observed in CD4<sup>+</sup> T cell-deficient B6 mice, despite the lack of the Fv2<sup>+</sup> allele, resembles authentic FV-induced erythroleukemia that emerges through the insertional activation of the transcription factors in proliferating erythroid cells (3, 39, 45). Further, another Ets-family gene encoding the transcription factor Erg was shown to be involved in SFFV proviral integration in the present study. The Erg locus was identified in a previous report as a proviral integration site in B6 mice injected as neonates with F-MuLV in association with the development of myeloid leukemia (65). Further, Erg has recently been shown to induce erythro-megakaryocytic leukemia upon hematopoietic cell-specific overexpression (7). Collectively, these data indicate that the erythroleukemia induced with FV in CD4<sup>+</sup> T cell-deficient B6 mice is indeed similar to the classical Friend disease in its molecular pathogenesis.

On the other hand, the fatal pathology that developed in B cell-deficient B6 mice differs from previously described Friend disease as the pathology developed without preceding polycythemia and splenomegaly, and neither the expression of SFFV gp55 on erythroid cells nor oligoclonal integration of SFFV proviruses was observed. The disease rather resembles leukemia that develops after a long latency period upon neonatal inoculation of F-MuLV, as both diseases develop independently of SFFV. In fact, neonatal inoculation of C57BL/10 or B6 mice with F-MuLV has been shown to induce differentiated (granulocytic) or undifferentiated myeloid leukemia (10, 55). It has been generally accepted that during ongoing ecotropic murine leukemia virus infection the emergence of recombinant MCF viruses that can utilize a different receptor and show an altered tissue tropism markedly increase the chances of insertional gene activation, ultimately resulting in the development of oligoclonal leukemia (16, 64). In the case of B cell-deficient B6 mice, however, leukemia developed through oligoclonal integration of F-MuLV itself without the emergence of infectious MCF viruses detectable with currently used MAbs. Interestingly, a similar lack of the generation of MCF viruses has also been reported in C57BL mice neonatally infected with F-MuLV (10, 55). Although we cannot exclude the possibility that recombinant viruses not detectable with the MAbs we used were generated, it is possible that in the absence of B cells and thus a lack of virus-neutralizing Ab, rapid and uncontrolled replication of ecotropic F-MuLV alone can result in a leukemogenic promotor insertion.

It has recently been shown that in B6 mice with a range of immunodeficiencies commonly affecting Ab-producing functions, spontaneous activation of a replication-defective, endogenous ecotropic murine leukemia retrovirus, Emv2, results in the emergence of infectious recombinant viruses, and the resultant viremia leads to the development of lymphocytic leukemia/lymphoma in aged animals (66, 67). However, the spontaneous tumor development started to be observed after 6 months of age in Rag1-deficient B6 mice (66), and mortality started after 40 weeks of age.

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in Tlr3, Tlr7, Tlr9 triple-knockout B6 mice (67). On the other hand, we inoculated CD4^+ T cell- or B cell-deficient B6 mice with FV or F-MuLV at 7–8 weeks, and the infected animals started to show mortality at around 10 weeks after infection (≤18 weeks in age) and most died by 20 weeks after infection (≥28 weeks of age), well before the reported onset of spontaneous lymphoma/lymphoid leukemia. Further, the tumor cells growing in the immuno-deficient B6 mice with Env2-related viremia are reported to be CD3^+ and strongly express terminal deoxynucleotidyl transferase (TdT) (67), while the cells expanding in FV- or F-MuLV-infected,
were detected with MAb 83A25 (15) which exhibits a range of functions.


that harbor a C-terminal segment of gp70 derived from ecotropic F-MuLV gp70 detectable with MAb 720, which is known to react with CD3+ T cells. We observed in B cell-deficient B6 mice also expressed flow cytometric characteristics of myeloid cells. The large-sized B cell-deficient B6 mice were CD3+/H11002 and exhibited cytological and flow cytometric characteristics of myeloid cells. The large-sized blasts cells we observed in B cell-deficient B6 mice also expressed F-MuLV gp70 detectable with MAb 720, which is known to react only with F-MuLV and a limited range of recombinant viruses that harbor a C-terminal segment of gp70 derived from ecotropic F-MuLV (49). It should be noted that Env2-related infectious retroviruses emerged spontaneously in immunodeficient B6 mice were detected with MAb 83A25 (15) which exhibits a range of reactivities reciprocal to that of MAb 720. Thus, MAb 83A25 detects all known mouse retroviruses except F-MuLV and its recombinants that harbor the C-terminal segment of F-MuLV gp70 (15, 49), and MAb 720 is unlikely to react with Env2-derived recombinant viruses. Further, if Env2-related infectious ecotropic viruses were produced in the spleens of B cell-deficient B6 mice we examined, such viruses likely would have been detected with MAB 24-8 in infectious center assays. Therefore, the observed development of erythroleukemia and myeloid malignancy in CD4+ T cells and B cell-deficient B6 mice, respectively, is most likely FV-induced and independent of the reported spontaneous emergence of Env2-related retrovirus.

It is of particular interest that when NK and/or CD8+ T cells were depleted, some B cell-deficient B6 mice developed polycythemia with a progressive increase in SFFV proviral integration. It is possible that in Fv2- B6 mice SFFV-infected cells are not only restricted in their growth due to the lack of sf-Stk, but are actively eliminated in the very early phase of FV infection through cellular, but not humoral, immune responses. This is consistent with the previous findings that Fv2- associated resistance to FV infection did not operate in the absence of T-lymphocytes (19, 28, 63). However, the simple absence of CD8+ T cells alone did not result in the development of polycythemia and did not largely affect the resistance to the disease development in B6 mice as we have shown in the present study with LDV-free FV. Thus, in CD8+ T cell-deficient mice other effector mechanisms must be compensating for the lacking CTLs in eliminating SFFV-infected cells. It should be noted that CD4+ T cell responses are operational in CD8+ T cell-deficient mice, and the priming of CD4+ T cells with an FV-derived epitope can protect highly susceptible CB6F1 mice from FV-induced leukemia development in the absence of CD8+ T cells (27), indicating the actual presence and effectiveness of an FV-eliminating effector mechanism other than CD8+ T cells that is under the control of CD4+ T cells. In this regard, it is also noteworthy that the FV-specific priming and reactivation of CD4+ T cells were not severely affected in B cell-deficient mice (27). Further, activation of both CD8+ effector T cells and NK cells upon pathogen invasion are known to depend on CD4+ T cells, as CD4+ T cells are required for the induction of CD8+ effector cells (1, 4, 48, 53) and NK cell activation upon pathogen infection is shown to be induced in the presence of CD4+ effector T cells (6, 22). In addition, CD4+ T cells themselves may act as cytotoxic effector cells in eliminating FV-infected target cells (24). Thus, it is conceivable that NK cells and CD8+ effector T cells, as well as CD4+ cytotoxic cells, are eliminating SFFV-infected cells in WT and B cell-deficient B6 mice, while these are not activated in CD4+ T cell-deficient mice. In CD8+ T cell-deficient mice, the SFFV eliminating function is probably exerted by NK and CD4+ effector T cells.

It has been widely accepted that the interaction of SFFV gp55 with erythropoietin (Epo) receptor is required for Epo-independent differentiation of erythroid progenitor cells and the resultant polycythemia, while sf-Stk-associated signaling is required for

### Table 1: Integration sites of F-MuLV and SFFV in CD4+ T cell-deficient B6 mice

<table>
<thead>
<tr>
<th>Provirus Location</th>
<th>Locus</th>
<th>Chr</th>
<th>Gene ID</th>
<th>Definition or function</th>
<th>Provirus location</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-MuLV</td>
<td>Bcl11a</td>
<td>11</td>
<td>14025</td>
<td>Zinc finger</td>
<td>Intron 2</td>
</tr>
<tr>
<td></td>
<td>Zfat</td>
<td>15</td>
<td>380993</td>
<td>Zinc finger</td>
<td>20 kb down</td>
</tr>
<tr>
<td></td>
<td>Rabgap11</td>
<td>1</td>
<td>29809</td>
<td>GTPase</td>
<td>Intron 20</td>
</tr>
<tr>
<td></td>
<td>Grb2</td>
<td>11</td>
<td>14784</td>
<td>Signal</td>
<td>15 kb up</td>
</tr>
<tr>
<td></td>
<td>Ikaros</td>
<td>11</td>
<td>22778</td>
<td>Unknown</td>
<td>35 kb up</td>
</tr>
<tr>
<td></td>
<td>Athl</td>
<td>7</td>
<td>212974</td>
<td>Trehalase</td>
<td>Intron 3</td>
</tr>
<tr>
<td></td>
<td>Ubp51, Snx31</td>
<td>15</td>
<td>18458, 66696</td>
<td>Poly(A) binding, lipid binding</td>
<td>11 kb down, 27 kb up, respectively</td>
</tr>
<tr>
<td></td>
<td>Cdc63</td>
<td>7</td>
<td>75338</td>
<td>DNA binding</td>
<td>Intron 6</td>
</tr>
</tbody>
</table>

a Integration sites of F-MuLV or SFFV in the spleen cell genome were determined in 10 leukemic mice by inverse PCR (Fig. 6) and sequencing of each amplified DNA band.

b The three gene loci were found in more than two mice out of 10 leukemic individuals examined, whereas the involvement of other genes was found in only a single mouse.

c The chromosome locus, gene ID, and putative function of the potential target genes are shown. Gene IDs are those given at the National Center for Biotechnology Information (NCBI) Gene site (http://www.ncbi.nlm.nih.gov/gene/).

b Proviruses were located within the indicated intron or near the potential target genes. To determine the location of proviruses integrated outside of previously identified gene loci, upstream distances from the transcription start site (up) or downstream distances from the polyadenylation signal (down) of the neighboring genes were calculated.
TABLE 2 Integration sites of F-MuLV in B cell-deficient B6 mice

<table>
<thead>
<tr>
<th>Provirus</th>
<th>Locus^b</th>
<th>Chr^b</th>
<th>Gene ID^b</th>
<th>Definition or function^b</th>
<th>Provirus location^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-MuLV</td>
<td>Ski</td>
<td>4</td>
<td>20481</td>
<td>Chromatin binding, enzyme regulator enzyme</td>
<td>Intron 1</td>
</tr>
<tr>
<td></td>
<td>Sex-4</td>
<td>13</td>
<td>20677</td>
<td>Transcription</td>
<td>Intron 1</td>
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<tr>
<td></td>
<td>Nf1u</td>
<td>6</td>
<td>56748</td>
<td>Iron ion binding</td>
<td>Intron 1</td>
</tr>
<tr>
<td></td>
<td>Ah1</td>
<td>10</td>
<td>52906</td>
<td>Protein binding</td>
<td>Intron 19</td>
</tr>
<tr>
<td></td>
<td>Hexokinase II</td>
<td>6</td>
<td>15277</td>
<td>Catalytic activity</td>
<td>Intron 1</td>
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<tr>
<td></td>
<td>GATA-1</td>
<td>x</td>
<td>14460</td>
<td>Transcription</td>
<td>Intron 1</td>
</tr>
<tr>
<td></td>
<td>Gpr142</td>
<td>11</td>
<td>217302</td>
<td>G-protein-coupled receptor activity</td>
<td>8 kb down</td>
</tr>
<tr>
<td></td>
<td>Cdk-like 4</td>
<td>17</td>
<td>381113</td>
<td>Cell cycle</td>
<td>10 kb down</td>
</tr>
<tr>
<td></td>
<td>Arpc5-like</td>
<td>2</td>
<td>74192</td>
<td>Actin binding</td>
<td>Intron 1</td>
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<tr>
<td></td>
<td>Zbp1</td>
<td>2</td>
<td>58203</td>
<td>DNA binding</td>
<td>Intron 1</td>
</tr>
<tr>
<td></td>
<td>Dnahc6</td>
<td>6</td>
<td>330355</td>
<td>ATP binding</td>
<td>4 kb down</td>
</tr>
<tr>
<td></td>
<td>Ginap5</td>
<td>6</td>
<td>317757</td>
<td>GTPase</td>
<td>Intron 1</td>
</tr>
<tr>
<td></td>
<td>Ntn1, STX8</td>
<td>11</td>
<td>18208, 5594</td>
<td>Protein binding, ubiquitin protein ligase binding</td>
<td>5 kb down</td>
</tr>
<tr>
<td></td>
<td>Mc2r</td>
<td>18</td>
<td>17200</td>
<td>Corticotrophin receptor</td>
<td>40 kb up</td>
</tr>
<tr>
<td></td>
<td>Cdh23</td>
<td>10</td>
<td>22295</td>
<td>Calcium ion binding</td>
<td>Intron 3</td>
</tr>
<tr>
<td></td>
<td>Egfl7, Notch1</td>
<td>2</td>
<td>335156, 18128</td>
<td>Calcium ion binding, chromatin DNA binding</td>
<td>28 kb up, 48 kb up, respectively</td>
</tr>
<tr>
<td></td>
<td>Lrcc25, Sshbp4</td>
<td>8</td>
<td>211228, 76900</td>
<td>Unknown, DNA binding</td>
<td>1 kb up, 9 kb up, respectively</td>
</tr>
<tr>
<td></td>
<td>Prkar2a</td>
<td>9</td>
<td>19087</td>
<td>cAMP binding</td>
<td>Intron 1</td>
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<tr>
<td></td>
<td>Fchsd2</td>
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<td>207278</td>
<td>Unknown</td>
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</tr>
<tr>
<td></td>
<td>Sfrs3</td>
<td>17</td>
<td>20383</td>
<td>Splicing factor</td>
<td>35 kb down</td>
</tr>
<tr>
<td></td>
<td>Igf1r</td>
<td>7</td>
<td>16001</td>
<td>Receptor</td>
<td>Intron 1</td>
</tr>
<tr>
<td></td>
<td>Smad 3</td>
<td>9</td>
<td>17127</td>
<td>Transcription</td>
<td>28 kb up</td>
</tr>
</tbody>
</table>

^a Integration sites of F-MuLV were determined in 10 leukemic mice by inverse PCR (Fig. 6) and sequencing of each amplified DNA band. All of the target genes were found in only a single mouse analyzed.

^b Chromosome locus, gene ID, and putative function of the potential target genes are shown. Gene IDs are those given at the NCBI Gene site (http://www.ncbi.nlm.nih.gov/gene).

^c Proviruses were located within the indicated intron or near the potential target genes. To determine the location of proviruses integrated outside of previously identified gene loci, upstream distances from the transcription start site (up) or downstream distances from the polyadenylation signal (down) of the neighboring genes were calculated.

Epo-independent proliferation (12, 68). As FV-induced erythroleukemia is suggested to develop through 2 stages, which are promotion of erythroid cell proliferation and the blockade of their differentiation (2, 12, 21, 38, 57, 68), one would wonder what caused erythroid cell proliferation prior to the putative secondary step of PU.1 or Fli1 activation in CD4^-^ T cell-deficient B6 mice in the absence of sf-Stk. However, a slow development of erythroleukemia similar to what we observed in CD4^-^ T cell-deficient B6 mice has been observed in Fv2^-^ mice with a mutant FV that encodes an SFFV env gene product, gp42, with a deletion in the membrane-proximal domain (31, 34). As intracellular signaling pathways are largely overlapping between gp55-Epo-receptor and gp55-sf-Stk interactions (12), FV infection of Fv2^-^ mice may induce sf-Stk-independent growth of erythroid cells that is slow but enough to function as the first stage of leukemogenesis.

Altogether, the present study has provided several new insights into the pathogenesis of FV-induced leukemia and the Fv2^-^-associated resistance to it. The Fv2^-^ allele operates through the lack of expression of sf-Stk (46), but does not provide complete resistance to SFFV-induced erythroid cell proliferation as previously pointed out (19, 28, 31, 34, 63). Rather, SFFV-infected cells are eliminated by cellular immune responses to which CD4^-^ T cells are indispensable. In the absence of B cells, the SFFV component of FV is eliminated as quickly as in WT mice in the presence of the immune responses control the emergence of SFFV-induced leukemia stem cells (21, 43, 57) and Ab responses restrict the emergence of F-MuLV-induced ones.

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REFERENCES


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