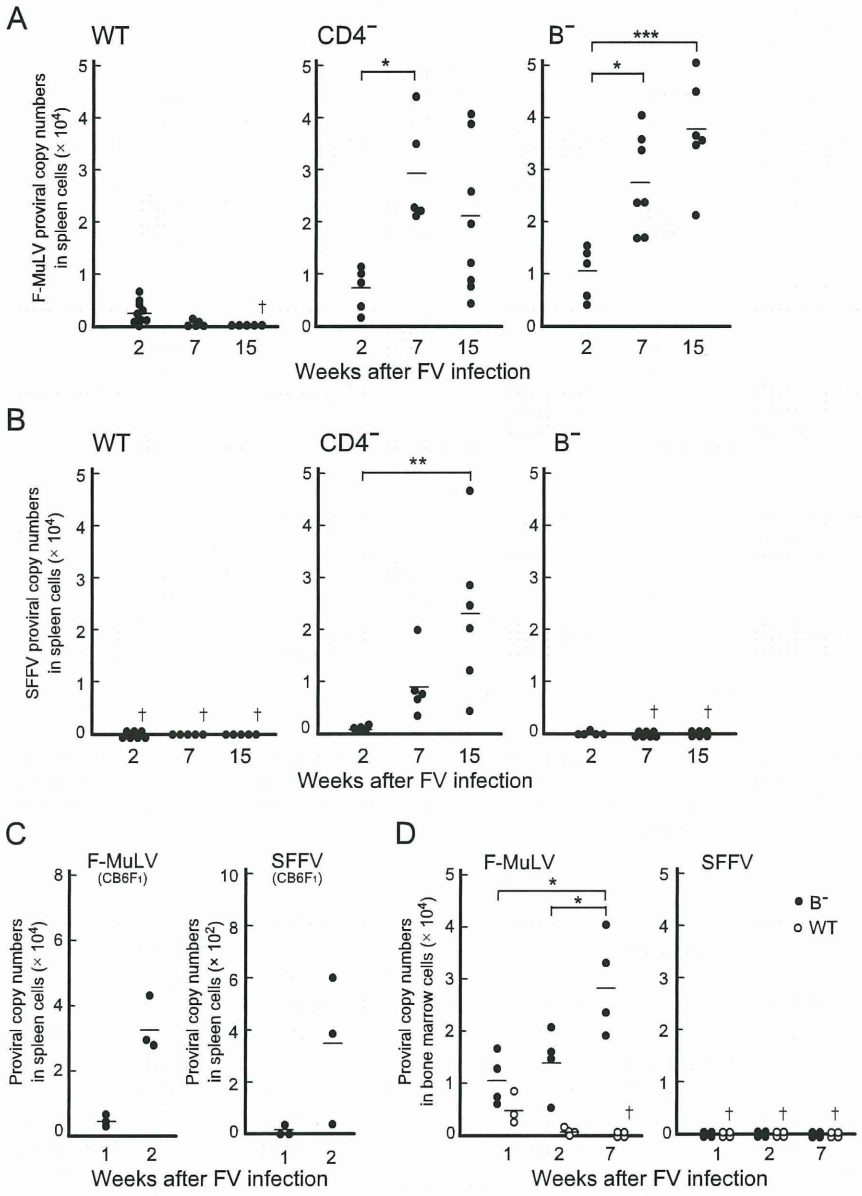

FIG 4 Kinetic changes in proviral copy numbers in FV-infected B6 or $CB6F_1$ mice. Each circle represents the actual proviral copy number detected from an individual mouse, and horizontal bars represent the mean values for each group. The FV stock inoculated contained about the same number of infectious 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; **, $P < 0.01$; ***, $P < 0.001$; †, undetectable in all animals examined. (C) B cell-deficient $CB6F_1$ 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.

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 the bone marrow of *Fv2*^{r} B6 mice irrespective of B cell proficiency or deficiency. As SFFV proviruses were detected and increased 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

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*^{r} B6 mice. As SFFV expanded in *Fv2*^{r} 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 injection 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- versus 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 6×10^7 and 3×10^7 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 lacking $CD4^+$ T or B cells 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*^{s} 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

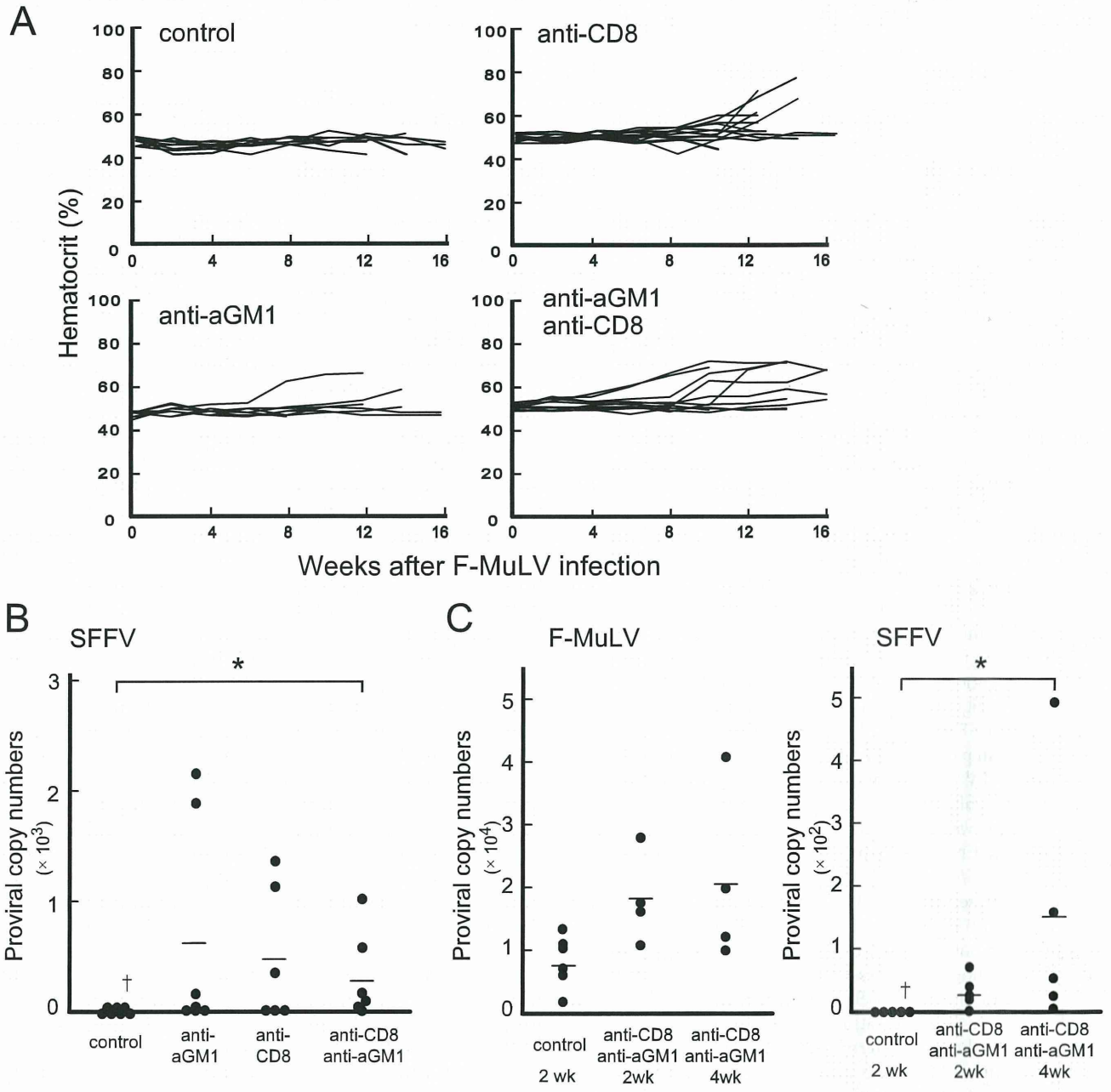

FIG 5 Roles of $CD8^+$ T and NK cells in the regulation of SFFV-infected cell expansion in B cell-deficient B6 mice. B cell-deficient B6 mice were injected with anti-mouse CD8 and/or anti-asialoGM1 (aGM1) Ab and inoculated with 5,000 SFFU of FV. Control mice were injected with a mixture of normal rabbit and normal rat sera before FV infection. (A) Changes in hematocrit values in B cell-deficient mice depleted of $CD8^+$ T ($n = 15$), NK ($n = 10$), or both types of cells ($n = 12$) or in those given the control sera ($n = 9$). Each line shows time-dependent changes in hematocrit values in each individual mouse. (B) Copy numbers of SFFV proviruses in spleen genomic DNA (100 ng) prepared from B cell-deficient B6 mice depleted of $CD8^+$ T, NK, or both types of cells. The mice were analyzed at 12 to 16 weeks postinfection. †, undetectable in all animals examined; *, significantly different in copy numbers in comparison with the control group [$P = 0.0056 < \alpha_3(0.05) = 0.017$ by Mann-Whitney test for non-Gaussian distributions with Bonferroni's *post hoc* test for multiple comparisons]. (C) Copy numbers of F-MuLV and SFFV proviruses in spleen genomic DNA (100 ng) of B cell-deficient B6 mice depleted of both $CD8^+$ T and NK cells at 2 and 4 weeks after FV inoculation. Each circle represents the actual proviral copy number detected from each individual mouse, and horizontal bars indicate the mean values for each group. †, undetectable in all animals examined; *, significantly different in copy numbers in comparison with the control group [$P = 0.0075 < \alpha_3(0.05) = 0.017$ by Mann-Whitney test for non-Gaussian distributions with Bonferroni's *post hoc* test for multiple comparisons].

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 $CD4^+$ 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 B6 mice upon 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

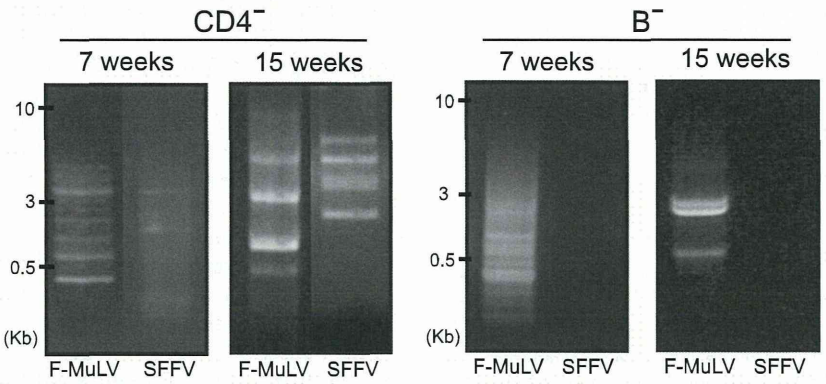

FIG 6 Changes in the clonality of virus-infected cells. Spleen cells were prepared from B6 mice lacking $CD4^+$ T cells ($CD4^-$) or B cells (B^-) at 7 weeks or at around 15 weeks after FV inoculation, and genomic DNA was purified. The host-virus junctional segments were amplified by the inverse PCR 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.

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-1* 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

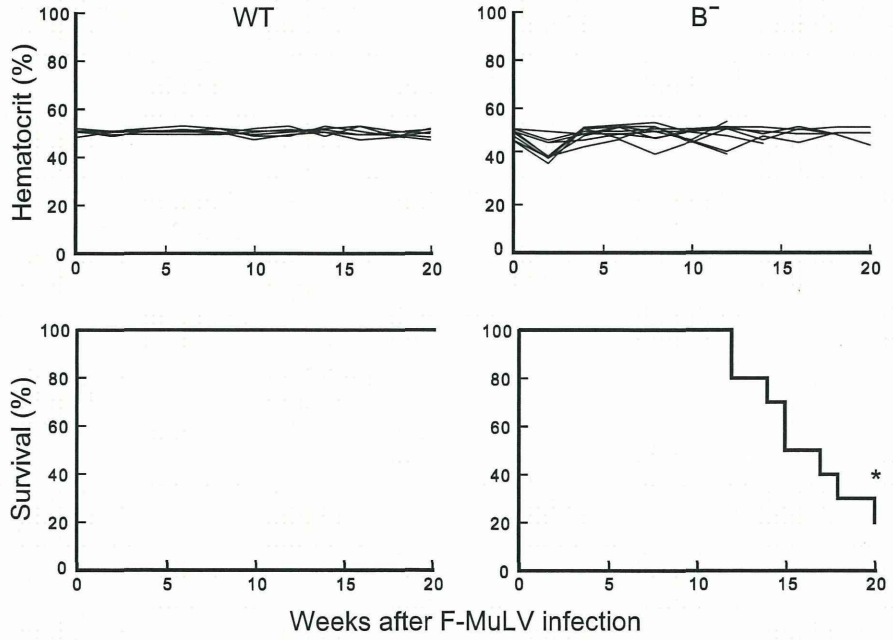

FIG 7 Survival rates and hematocrit values in B cell-deficient B6 mice upon infection with F-MuLV free of SFFV. B cell-deficient (B^-, $n = 10$) or WT B6 mice ($n = 6$) were inoculated with 5,000 focus-forming units of F-MuLV, and their survival rates and hematocrit values were analyzed. The entire set 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.

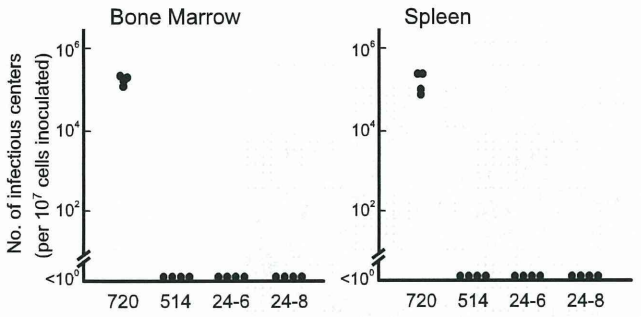

FIG 8 MCF viruses were undetectable in B6 mice lacking B cells upon F-MuLV infection. Bone marrow and spleen cells were prepared from B cell-deficient B6 mice between 14 and 17 weeks after F-MuLV inoculation and then incubated on monolayers of *Mus dunni* cells for 2 days. Foci of virus-infected cells 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 $CD4^+$ 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 in 2 of the 10 $CD4^+$ 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 $CD4^+$ 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). 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*^{r} B6 mice nevertheless develop polycythemia and splenomegaly followed by fatal leukemia when $CD4^+$ T cells are lacking. The expansion of TER-119^+ 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*^{s}-possessing susceptible mice (2, 38), although the tempos of disease development were much slower in $CD4^+$ T cell-deficient B6 mice than in *Fv2*^{s}-possessing mice. In fact, *Fv2*$^{r/s}$ $CB6F_1$ 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 $CD4^+$ 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 $CD4^+$ T cell-deficient B6 mice, despite the lack of the *Fv2*^{s} 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 $CD4^+$ 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 MAb. 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 MAb 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 promoter 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

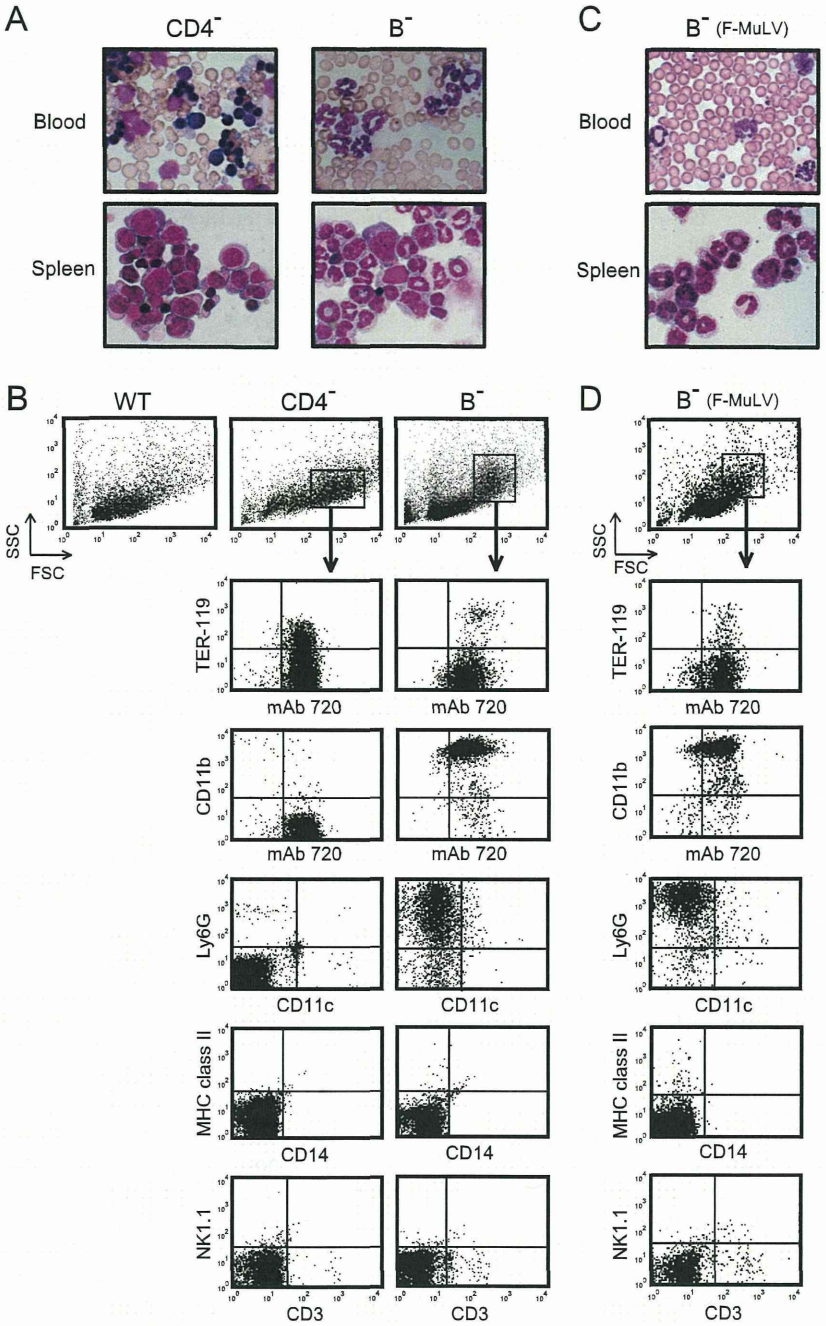

FIG 9 Expansion of myeloid cells in B cell-deficient B6 mice upon FV or F-MuLV infection. (A) Representative peripheral blood smear specimens (upper panels) and cytospin preparations of the spleen cells (lower panels) from $CD4^+$ T cell- or B cell-deficient B6 mice prepared between 12 and 17 weeks after FV infection and stained with May-Grunwald Giemsa stain (all photographs were taken at the same magnification). (B) Cell populations expanded in the spleen of each infected mouse were gated as indicated and analyzed for the expression of the surface molecules by flow cytometry. Similar data were obtained in the analyses of more than 5 individuals per group. (C) Representative peripheral blood smear and cytospin preparation of the spleens from B cell-deficient B6 mice between 14 and 17 weeks after F-MuLV inoculation (May-Grunwald Giemsa stain). Note the large myeloid cells of different differentiation stages, similar to those observed in FV-infected B cell-deficient B6 mice in the spleen, and the appearance of immature myeloid cells with convoluted nuclei and granulocytes with hypersegmented nuclei in the peripheral blood. (D) Flow cytometric analyses of cells expanded in the spleens of F-MuLV-infected, B cell-deficient B6 mice. Representative dot plots from an individual mouse are shown. Similar data were obtained from 6 individuals.

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 immunodeficient B6 mice with Emv2-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,

TABLE 1 Integration sites of F-MuLV and SFFV in $CD4^+$ T cell-deficient B6 mice[a]

Provirus	Locus[c]	Chr[c]	Gene ID[c]	Definition or function[c]	Provirus location[d]
SFFV	*Fli1*[b]	9	14247	Transcription	0.7–2 kb up
	PU.1 (*Sfpi1*)[b]	2	20375	Transcription	Intron 1 or 12 kb up
	Erg[b]	16	13876	Transcriptional regulator	Intron 3
	Myo6, *Impg1*	9	17920, 63859	Myosin, Receptor	2 kb down, 0.1 kb down, respectively
	Ddx3	x	13205	Helicase	40 kb up
	Lbp1	19	16825	Transcriptional regulator	Intron 1
	Myb	10	17863	Transcription	23 kb up
	ADARB2	13	94191	RNA-editing deaminase	Intron 3
	Tecr	8	106529	Oxidoreductase	5 kb up
	Spns3	11	77577	Lipid transporter	2 kb up
	Top3b	16	21976	Topoisomerase	8 kb up
	Gorasp2	2	70231	Golgi complex organization	35 kb up
	Zfp219	14	69890	DNA binding	2 kb up
F-MuLV	*Bcl11a*	11	14025	Zinc finger	Intron 2
	Zfat	15	380993	Zinc finger	20 kb down
	Rabgap1l	1	29809	GTPase	Intron 20
	Grb2	11	14784	Signal	15 kb up
	Ikzf1 (*Ikaros*)	11	22778	Transcription	Intron 3
	Ath1	7	212974	Trehalase	0.1 kb down
	Pabpc1, *Snx31*	15	18458, 66696	Poly(A) binding, lipid binding	11 kb down, 27 kb up, respectively
	Ccdc83	7	75338	Unknown	Intron 6

[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 2 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/).
[d] 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.

B cell-deficient B6 mice were $CD3^-$ and exhibited cytological and flow cytometric characteristics of myeloid cells. The large-sized blastic 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 Emv2-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 Emv2-derived recombinant viruses. Further, if Emv2-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 cell- and B cell-deficient B6 mice, respectively, is most likely FV-induced and independent of the reported spontaneous emergence of Emv2-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. This indicates that in *Fv2*^{r} 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*^{r}-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 $CB6F_1$ 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 $CD8^+$ effecter 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 2 Integration sites of F-MuLV in B cell-deficient B6 mice[a]

Provirus	Locus[b]	Chr[b]	Gene ID[b]	Definition or function[b]	Provirus location[c]
F-MuLV	*Ski*	4	20481	Chromatin binding, enzyme regulator enzyme	Intron 1
	Sox-4	13	20677	Transcription	Intron 1
	Nfu1	6	56748	Iron ion binding	Intron 1
	Ahi1	10	52906	Protein binding	Intron 19
	Hexokinase II	6	15277	Catalytic activity	Intron 1
	GATA-1	x	14460	Transcription	Intron 1
	Gpr142	11	217302	G-protein-coupled receptor activity	8 kb down
	Cdk-like 4	17	381113	Cell cycle	10 kb down
	Arpc5-like	2	74192	Actin binding	Intron 1
	Zbp1	2	58203	DNA binding	Intron 1
	Dnahc6	6	330355	ATP binding	4 kb down
	Gimap5	6	317757	GTPase	Intron 1
	Ntn1, STX8	11	18208, 55943	Protein binding, ubiqutin protein ligase binding	5 kb down
	Mc2r	18	17200	Corticotrophin receptor	40 kb up
	Cdh23	10	22295	Calcium ion binding	Intron 3
	Egfl7, Notch1	2	353156, 18128	Calcium ion binding, chromatin DNA binding	28 kb up, 48 kb up, respectively
	Lrrc25, Ssbp4	8	211228, 76900	Unknown, DNA binding	1 kb up, 9 kb up, respectively
	Prkar2a	9	19087	cAMP binding	Intron 1
	Fchsd2	7	207278	Unknown	43 kb up
	Sfrs3	17	20383	Splicing factor	35 kb down
	Igf1r	7	16001	Receptor	Intron 1
	Smad 3	9	17127	Transcription	28 kb up

[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 $Fv2^r$ 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 $Fv2^r$ 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 $Fv2^r$ 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 homozygous $FV2^r$ allele, but the remaining F-MuLV alone can induce fatal myeloid leukemia possibly without requiring the emergence of recombinant MCF viruses. These new observations may also contribute to the understanding of quantitative and kinetic aspects of retrovirus-induced multistep leukemogenesis, as future studies may pinpoint critical steps at which cellular 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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