micromagnetic beads as described [58]. Cells were then stained with anti-CD44-FITC, and $CD44^{lo}$ cells were sorted using FACSVantage cell sorter with DIVA enhancement software (BD Bioscience). A total of $1–2\times10^7$ cells were transferred into recipient $B6AF_1$ mice infected with either FV or FV-OVA 42 days prior to the cell transfer.

Real-Time PCR

Total RNA was purified from the spleen, BM, and thymus of FV-infected mice using RNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and cDNA synthesis was performed by using PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). The viral DNA fragments were amplified from 0.5 μg or 1 μg of total cDNA and were quantified 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 cDNAs were designed on the *env* portion of each provirus: primers 5′-AAGTCTCCCCCGCCTCTA-3′ and 5′-AGTGCCTGGTAAGCTCCCTGT-3′, and a FAM-labeled probe 5′-ACTCCCACATTGATTTCCCCGTCC-3′ for the detection of F-MuLV, and primers 5′-TCTAACCTCAC-CAACCCTGAT-3′ and 5′-TTTTAGGGCAATGGTATGAT-TAAAATAA-3′, and a FAM-labeled probe 5′-CCTAGTGT-CTGGACCCCCCTATTACGAGG-3′ for the detection of SFFV [59]. 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 sec and at 58°C for 1 min. A TaqMan rodent 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 $10–10^6$ copies in the above reaction.

Statistical analysis

Statistical analyses were performed using Prism software (GraphPad Software, Inc., San Diego, CA). Methods of comparison and corrections for multiple comparisons are indicated in each relevant figure legend.

Accession numbers

Friend murine leukemia virus FB29 complete genome (accession number: Z11128).

Supporting Information

Figure S1 Virus-specific $CD8^+$ T cells in mice chronically infected with FV are non-responsive to the viral antigen. (A) FV-infected (6–8 weeks post infection) and age-matched uninfected mice were injected s.c. with FBL3 tumor cells (5×10^6). (B) At day 42–56 after infection (Before tumor injection) cells purified from the BM (upper panels) and spleen (lower panels) were stained with the indicated Abs and F-MuLV $gag_{75–83}/D^b$ tetramer. Shown are representative staining patterns for CD8 and the tetramer of $CD8^+$ T cells, and PD-1, CD69, LAG-3 and Tim-3 on $tetramer^+$ cells. (C) At day14 after FBL3 injection, splenocytes were isolated and stained with the indicated Abs and F-MuLV $gag_{75–83}/D^b$ tetramer. Shown are representative staining patterns for CD8 and the tetramer of $CD8^+$ T cells. (D) Fractions of spleen cells were stimulated with the $gag_{75–83}$ peptide or cultured without stimulation (Medium). The intracellular expression of IFN-γ and the surface expression of CD107a were measured by flow cytometry. Shown are representative staining patterns for intracellular IFN-γ and surface CD107a expression of stimulated and unstimulated $CD8^+$ T cells. Tumor sizes (E) and host survival (F) are shown for uninfected (upper panels) and FV-infected (lower panels) animals ($n=6–11$). Note that lines with tumor size zero in panel E include multiple individuals. Survival curves were compared between the uninfected and FV-infected groups by Mantel-Cox log-rank test: *, $p=0.0001$.
(DOC)

Figure S2 Viral antigen expression in each cell population in the thymus after FV or F-MuLV infection. $B6AF_1$ or B6 mice were infected with various doses of FV or F-MuLV. At day 14 after infection, cells in the thymus were isolated and stained with the indicated Abs. Shown are representative staining patterns and gating protocols of thymocytes. Data are representative of two independent experiments with essentially similar results.
(DOC)

Figure S3 Immunohistochemistry of the thymuses from FV-infected mice. Experiments were performed as described for Figure 3C. Arrowheads indicate cells doubly positive for the indicated cell surface marker and the viral antigen. Representative view-fields from those shown here are presented in Figure 3C.
(DOC)

Figure S4 Spleen weights and gp70 expression on B cells and erythroblasts after thymic transplantation. Experiments were performed as described for Figure 5. (A) Spleen weights were measured at day 14 after transplantation. (B) At day 14 after transplantation, splenocytes were isolated and stained with the indicated antibodies. Shown are representative staining patterns for gp70 on $CD19^+$ and $Ter119^+$ cells.
(DOC)

Figure S5 Construction of F-MuLV-OVA. (A) Schematic representation of the F-MuLV-OVA construct. A synthetic oligonucleotide encoding the SIINFEKL epitope was inserted in-frame at the 3′ end of the *env* gene (B) Detailed strategy for the generation of F-MuLV-OVA. Oligonucleotide primers harboring the OVA epitope sequence and hybridizing with the F-MuLV genome at the end of the *env* gene were used for PCR-based mutagenesis with the permutated molecular clone of F-MuLV as the template. F-MuLV genome sequence and base numbers shown are according to the database information (Z11128). The vertical arrow indicates the site of cleavage that generates fusogenic TM protein and R peptide [60]. (C) Splenocytes from naïve $B6AF_1$ mice were infected in vitro with either F-MuLV or F-MuLV-OVA. Cells were then cocultured with $CD8^+$ T cells purified from (OT-1-Thy1.1× A/WySnJ)F_1 mice (OT-1 cell). Shown are representative histograms for CD69 expression on OT-1 cells.
(DOC)

Figure S6 FACS profiles of cells from FTOC. Experiments were performed as described for Figure 6. Either tumor cells (A) or thymic cell populations purified from FV-OVA-infected mice (B) were used as the third population. Shown are representative dot plots of positive control settings (A) and experimental settings (B).
(DOC)

Figure S7 Post-thymic maturation of $CD8^+$ RTEs in mice chronically infected with FV. (*Rag1*-GFP×A.WySnJ)F_1 mice were infected with 1,000 SFFU of FV. Splenocytes were isolated at the indicated time-points and stained with the indicated Abs. Shown are dot plots for GFP expression among $CD4^+$ and $CD8^+$ T cells at day 42 after infection (A), and actual numbers of GFP^+CD8^+ T cells in either FV-infected or age-matched naïve

animals (B). No significant difference was observed between the groups. (C–D) Shown are representative staining patterns for PD-1, CD69 and CD44 of GFP^+CD8^+ T cells or GFP^-CD8^+ T cells (C), and for CD24, Qa2 and CD127 of GFP^-, GFP^{lo} or GFP^{hi} cells (D). (E–F) Splenocytes were stimulated *in vitro* with anti-CD3 Ab. The intracellular expression of IFN-γ and IL-2 were then measured by flow cytometry. Shown are representative staining patterns for IFN-γ and CD107a of GFP^+CD8^+ T cells (E), and frequencies of IFN-γ^+ cells and IL-2^+ cells among GFP^+CD8^+ T cells (F). Each symbol represents an individual mouse. Average percentages were compared between uninfected and FV-infected groups by two-way ANOVA with Bonferroni's corrections for multiple comparisons, and no significant difference was detected. Data are representative of two independent experiments with essentially equivalent results.
(DOC)

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Author Contributions

Conceived and designed the experiments: ST. Performed the experiments: ST EK STK TM MF MK TC YK SK. Analyzed the data: ST MM. Contributed reagents/materials/analysis tools: ST EK MI NS MM. Wrote the paper: ST MM.

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Elimination of Friend Retrovirus in the Absence of CD8$^+$ T Cells

Sachiyo Tsuji-Kawahara, Masaaki Miyazawa

Department of Immunology, Kinki University Faculty of Medicine, Osaka-Sayama, Osaka, Japan

Friend retrovirus complex (FV) induces acute erythroid cell hyperplasia and massive splenomegaly followed by the emergence of fatal erythroleukemia upon inoculation into adult mice of susceptible strains (1–3). Because the disease can progress in the presence of host immune responses, FV has served as a useful model to study how retroviruses evade immune control (1, 3, 4). Depending on genotypes at several host loci, some strains of mice can eliminate virus-producing cells and recover from splenomegaly, while others progress rapidly to fatal pathology (1, 3, 5). Results from several research groups largely agree on the role of virus-neutralizing antibodies and CD4$^+$ T cells in immune control of FV infection (6–15). Natural killer cells also contribute to FV elimination and are essential for vaccine-induced protection of highly susceptible mice (8, 16). However, there are conflicting views on the role of CD8$^+$ T cells in FV control.

Earlier studies associated major histocompatibility complex class I (MHC-I) alleles with spontaneous recovery from FV-induced splenomegaly, and FV-specific, CD8$^+$ cytotoxic T cells were detected (1, 5). Further, the recovery in *H2*^{b} mice was abrogated when CD8$^+$ T cells were depleted (6). On the other hand, by using FV-encoded epitopes recognized by CD4$^+$ T cells as peptide vaccines, we have shown that highly susceptible (BALB/c × C57BL/6)F$_1$ mice can still be protected from FV challenge and eliminate virus-producing cells in the absence of CD8$^+$ T cells (9). Interestingly, MHC-I genotypes influenced cytokine production from CD4$^+$ T cells upon FV infection (17, 18), indicating the possible indirect role of CD8$^+$ T cells.

C57BL/6 (B6) mice lack the expression of a short form of hematopoietic cell-specific receptor tyrosine kinase, Stk, and do not develop FV-induced erythroid cell proliferation (19). Some reports have indicated that CD8$^+$ T cells are essential in controlling FV infection in B6 mice, as infectious centers at an early time point after FV infection increased upon depletion of CD8$^+$ T cells (20–22). However, infectious centers were detected in the above-described reports with monoclonal antibody 720 (23) that reacts only with the helper component of FV, Friend murine leukemia virus (F-MuLV), but not with the pathogenic component, the spleen focus-forming virus (SFFV). In our recent work (24), SFFV was eliminated from B6 mice by 2 weeks after infection, and CD8$^+$ T cell-deficient B6 mice remained resistant to FV-induced disease development. Thus, the increase of F-MuLV infectious centers after CD8$^+$ T cell depletion, albeit statistically significant, may not reflect pathologically significant changes in SFFV load.

Here, we examined changes in SFFV copy numbers in CD8$^+$ T cell-deficient B6 mice after FV infection. CD8$^+$ T cell-deficient B6 mice nevertheless eliminated both F-MuLV and SFFV proviruses, though more slowly than the wild-type B6 mice did, as shown in Fig. 1. Thus, while CD8$^+$ T cells do contribute to control FV infection, they are not essential for the elimination of FV in B6 mice.

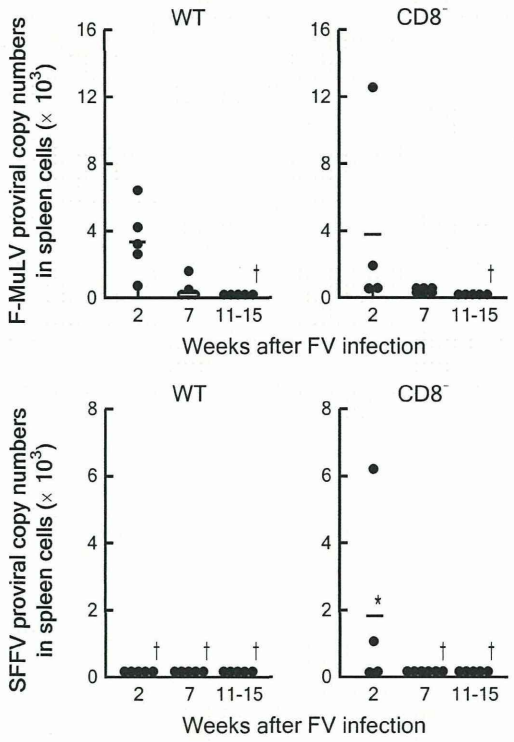

FIG 1 Changes in proviral copy numbers in the spleens of wild-type (WT) or CD8$^+$ T cell-deficient (CD8$^-$) B6 mice after inoculation of 5,000 spleen focus-forming units of FV. Wild-type B6 and CD8$^+$ T cell-deficient B6.129P2-β_2 m^{tm1Unc}/J mice carrying homozygous disruption of the β_2 microglobulin gene are those described in reference 24. Genomic DNA extraction and real-time PCR quantification of F-MuLV and SFFV proviruses were performed as described previously (24). Each closed circle represents an absolute copy number of F-MuLV or SFFV provirus in 100 ng of genomic DNA (equal to about 1.7 × 10^4 cells) detected from an individual mouse. Bars indicate averages for each genetic group and time point. *, significantly higher copy numbers than those in the WT animals [$P = 0.0159 < \alpha_3(0.05) = 0.0170$ by Mann-Whitney test for non-Gaussian distributions with Bonferroni's *post hoc* test for multiple comparisons]. †, undetectable in all animals examined.

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Address correspondence to masaaki@med.kindai.ac.jp.

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

Sachiyo Tsuji-Kawahara, Hiroyuki Kawabata,* Hideaki Matsukuma,* Saori Kinoshita, Tomomi Chikaishi, Mayumi Sakamoto, Yuri Kawasaki, Masaaki Miyazawa

Department of Immunology, Kinki University Faculty of Medicine, Osaka-Sayama, Osaka, Japan

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*^{r} B6 mice lacking CD4$^+$ T cells developed early polycythemia and fatal erythroleukemia, while B6 mice lacking CD8$^+$ T cells remained resistant. Erythroid progenitor cells infected with spleen focus-forming virus (SFFV) were eliminated, and no polycythemia 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 polycythemia, indicating that SFFV-infected erythroid cells are not only restricted in their growth but are actively eliminated in *Fv2*^{r} 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 reinfection 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 (Stk), 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 *Fli1* 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, *Rfv3*. 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

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Address correspondence to Sachiyo Tsuji-Kawahara, skawa@med.kindai.ac.jp, or Masaaki Miyazawa, masaaki@med.kindai.ac.jp.
* Present address: Hiroyuki Kawabata, Department of Internal Medicine, Saiseikai Matsusaka General Hospital, Mie, Japan; Hideaki Matsukuma, Department of Acupuncture, Faculty of Health Sciences, Morinomiya University of Medical Sciences, Suminoe, Osaka, Japan.

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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*^{r} 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*^{r} 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*$^{r/s}$ (C57BL/6 × BALB/c)F$_1$ (CB6F$_1$) 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*^{r} 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-$\beta 2m^{tm1Unc}$/J mice carrying homozygous disruption of the β_2-microglobulin gene ($\beta 2m$) were purchased from The Jackson Laboratory, Bar Harbor, ME. B6.129S2-*Ighm*^{tm1Cgm}/J 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). CB6F$_1$ mice lacking B cells were produced by crossing the B6-*μMT*/*μMT* and BALB/c-*μMT*/*μMT* mice as previously described (27). C57BR/cdJ 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 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 dunni* 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 dunni* cells. Replication-competent helper virus of FV, F-MuLV, was purified from a culture supernatant of *Mus dunni* 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 CB6F$_1$ 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 FACSCalibur 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′-AAGTCTCCCCCCGCCTCTA-3′ and 5′-AGTGCCTGGTAAGCT CCCTGT-3′ and a 6-carboxyfluorescein (FAM)-labeled probe, 6-FAM–5′-ACTCCCACATTGATTTCCCCGTCC-3′, for the detection of F-MuLV and primers 5′-TCTAACCTCACCAACCCTGAT-3′ and 5′-TTTTAGGGCA ATGGTATGATTAAAATAA-3′ and a FAM-labeled probe, 6-FAM–5′-CCTAGTGTCTGGACCCCCCTATTACGAGG-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 at 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 10^6 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 Emv2 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 μmol 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′-CCAAGGACCTGAAATGACCCTG-3′; reverse primer specific for F-MuLV, 5′-GACTTGGCAGGTTTGGGT AGG-3′; reverse primer specific for SFFV, 5′-GAGGAGGTGGGGCAGT CTCG-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 subcloned into the pCR2.1-TOPO or pCRXL-TOPO (Life Technologies) plasmid. Sequence analysis was performed by using a primer located in the LTR, 5′-GAGCTCACAACCCCTCACTC-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′-AAGTCTCCCCCCGCCTCTA-3′; reverse primer for the LTR region, 5′-AAGGCACAGGGTCATTTCA GGTC-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 specimens 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% FBS, plated in triplicate at concentrations between 10^3 and 10^6 cells per well of 24-well plates onto monolayers of *Mus dunni* cells that had been seeded at 1×10^4 cells per well on the previous day, and cocultured for 2 days. To ensure the lack of detectable virus-producing cells, 6×10^7 spleen cells and 3×10^7 bone marrow cells from each mouse were plated in 20 and 10 wells of 24-well plates, respectively, at a concentration of 3×10^6 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 inoculation. 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 by 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 *Fv2*^{r} B6 mice lacking $CD4^+$ T or B lymphocytes. To investigate the possible involvement of various immune cell populations in controlling FV-induced leukemogenesis in *Fv2*^{r} 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 μ*MT*/μ*MT* mice, which have no peripheral mature B cells, and β*2m* 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 $CD4^+$ T 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

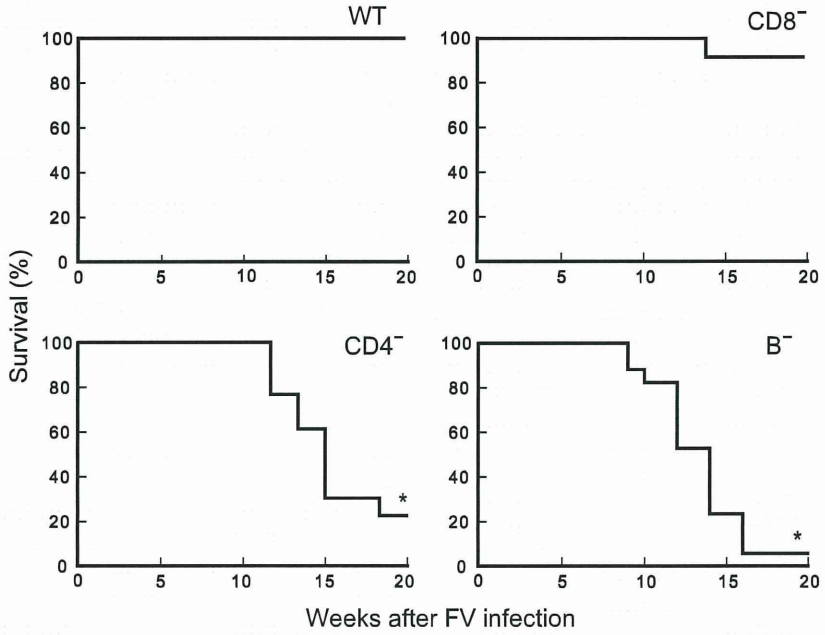

FIG 1 Survival of immunodeficient B6 mice upon FV infection. B6 mice lacking $CD4^+$ T cells ($CD4^-$, $n = 13$), $CD8^+$ T cells ($CD8^-$, $n = 12$), or B cells (B^-, $n = 17$) and wild-type B6 mice ($n = 11$) were inoculated with 5,000 SFFU of FV, and their survival was monitored for 20 weeks. *, significantly 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.

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). Fur-

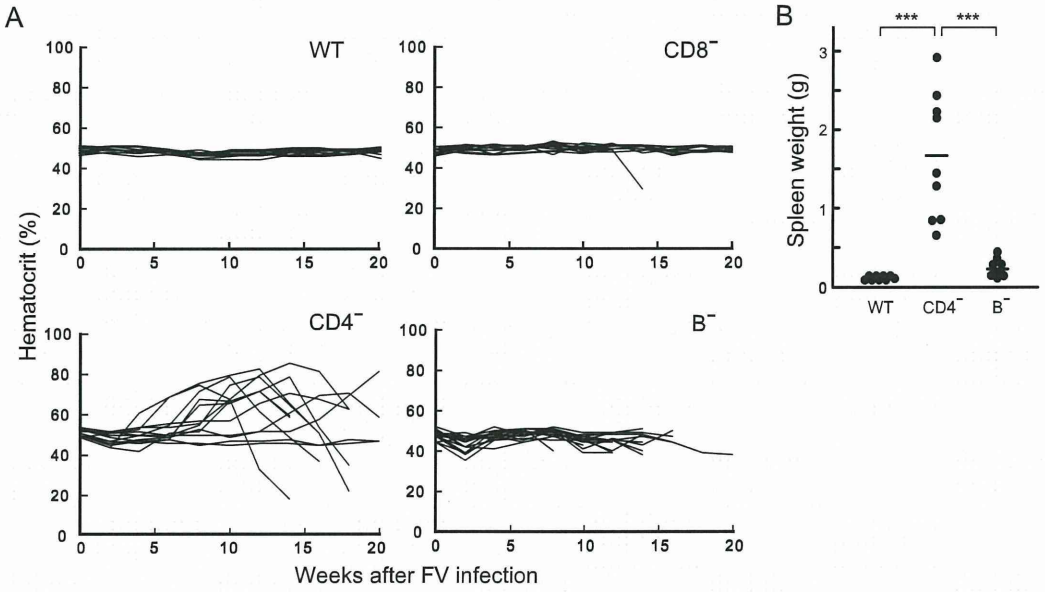

FIG 2 Changes in hematocrit values and spleen weights in FV-infected B6 mice. B6 mice lacking $CD4^+$ T cells ($CD4^-$, $n = 13$), $CD8^+$ T cells ($CD8^-$, $n = 12$), or B cells (B^-, $n = 17$) and WT B6 mice ($n = 11$) were inoculated with 5,000 SFFU of FV. (A) Hematocrit values were measured every 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.

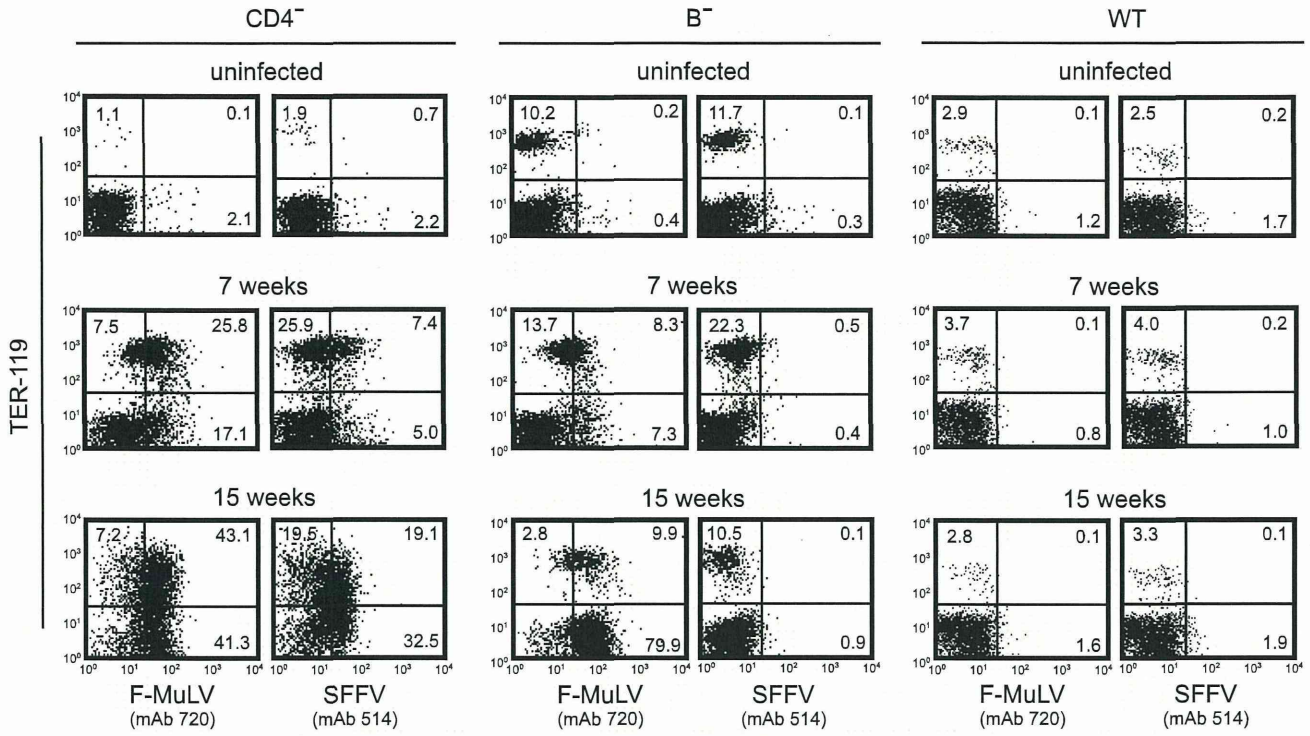

FIG 3 Changes in the proportions of F-MuLV- and SFFV-infected erythroid cells in the spleens of $CD4^+$ T cell- or B cell-deficient B6 mice. Spleen cells were prepared from B6 mice lacking $CD4^+$ T cells ($CD4^-$) or B cells (B^-) and from WT B6 mice before or at 7 or 15 weeks after FV inoculation and were stained with MAb 720 or 514 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.

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 $CD4^+$ 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 *env* gene product of F-MuLV, whereas MAb 514 reacts with 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 $CD4^+$ T cell-deficient mice, TER-119^+ 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 $CD4^+$ T cell-deficient animals (Fig. 4A and B). On the contrary, in B cell-deficient B6 mice, 514^+ 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 $gp70^+$ cells were readily detected and increased with time (Fig. 3). Note that, due to the lack of B cells, percentages of TER-119^+ erythroid cells in $\mu MT/\mu MT$ 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, $CB6F_1$ mice without *Fv2*-associated resistance ($Fv2^{r/s}$) and lacking B cells were infected with a low dose of FV complex. In B cell-deficient $CB6F_1$ 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 $CB6F_1$ mice showed moderate splenomegaly at 2 weeks after FV infection (average spleen weight, 0.20 ± 0.03 g [mean $\pm$ standard error of the mean; $n = 10$], compared to 0.07 to 0.10 g in uninfected $\mu MT/\mu MT$ 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