JOURNAL OF VIROLOGY, Nov. 2008, p. 10998–11008
0022-538X/08/$08.00+0 doi:10.1128/JVI.01311-08

Vol. 82, No. 22

Mouse APOBEC3 Restricts Friend Leukemia Virus Infection and Pathogenesis In Vivo▽

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Received 24 June 2008/Accepted 3 September 2008

Several members of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like complex 3 (APOBEC3) family in primates act as potent inhibitors of retroviral replication. However, lentiviruses have evolved mechanisms to specifically evade host APOBEC3. Likewise, murine leukemia viruses (MuLV) exclude mouse APOBEC3 from the virions and cleave virion-incorporated APOBEC3. Although the betaretrovirus mouse mammary tumor virus has been shown to be susceptible to mouse APOBEC3, it is not known if APOBEC3 has a physiological role in restricting more widely distributed and long-coevolved mouse gammaretroviruses. The pathogenicity of Friend MuLV (F-MuLV) is influenced by several host genes: some directly restrict the cell entry or integration of the virus, while others influence the host immune responses. Among the latter, the *Rfv3* gene has been mapped to chromosome 15 in the vicinity of the *APOBEC3* locus. Here we have shown that polymorphisms at the mouse *APOBEC3* locus indeed influence F-MuLV replication and pathogenesis: the *APOBEC3* alleles of F-MuLV-resistant C57BL/6 and -susceptible BALB/c mice differ in their sequences and expression levels in the hematopoietic tissues and in their abilities to restrict F-MuLV replication both in vitro and in vivo. Furthermore, upon infection with the pathogenic Friend virus complex, (BALB/c × C57BL/6)F_1 mice displayed an exacerbated erythroid cell proliferation when the mice carried a targeted disruption of the C57BL/6-derived *APOBEC3* allele. These results indicate, for the first time, that mouse APOBEC3 is a physiologically functioning restriction factor to mouse gammaretroviruses.

The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3 (APOBEC3) proteins are cellular cytidine deaminases with potent antiretroviral activities (reviewed in reference 8). Thus, after the penetration of retroviral nucleocapsids into target cells of infection and the initiation of reverse transcription, APOBEC3 enzymes can induce the conversion of cytosine to uracil in the minus-sense single-strand viral DNA, leading to G-to-A hypermutations in the subsequent plus-strand viral DNA. The resultant detrimental levels of mutations in the proviral genome, along with a deamination-independent mechanism that works prior to the proviral integration (9), together exert efficient antiretroviral effects in infected target cells. However, retroviruses have evolved to evade their natural hosts' APOBEC3. Thus, human immunodeficiency virus (HIV) counters the action of human APOBEC3G (hAPOBEC3G) through its viral infectivity factor (Vif). The Vif protein expressed in virus-producing cells interacts with hAPOBEC3G to recruit ubiquitin ligase complex and thus mediates polyubiquitination of hAPOBEC3G and Vif, resulting in rapid degradation of hAPOBEC3G (11, 29, 49). Vif is also known to partially impair de novo synthesis of hAPOBEC3G (45). Therefore, hAPOBEC3G is neutralized by HIV Vif, as well as simian immunodeficiency virus (SIV) Vif from the chimpanzee, rhesus macaque, and sooty mangabey (28). However, the antiretroviral effects of human APOBEC3 (hAPOBEC3) proteins are not limited to Vif-deficient lentiviruses of the above-mentioned primate species but are readily exerted with other lentiviruses including SIV from the African green monkey, equine infectious anemia virus, and more distantly related retroviruses such as murine leukemia virus (MuLV), porcine endogenous retrovirus, and foamy viruses (12, 16, 21, 27). The hAPOBEC3G has also been shown to restrict retrotransposition of Alu elements (10), indicating a possible physiological role for APOBEC3 proteins in protecting cells from endogenous retroelements.

Similarly, mouse APOBEC3 (mAPOBEC3) restricts the replication of HIV type 1 (HIV-1) without being countered by Vif (28, 42), whereas mouse gammaretroviruses are relatively resistant to mAPOBEC3 (1, 4, 13). This resistance of mouse gammaretroviruses to the APOBEC3 protein of their natural host seems to be mediated through the exclusion of mAPO BEC3 from MuLV particles and cleavage of virion-incorporated mAPOBEC3 by the viral protease (1, 4, 13). Interestingly, however, mouse mammary tumor virus (MMTV), a betaretrovirus, is susceptible to mAPOBEC3 (39), and evidence has been shown that endogenous polytropic and modified polytropic retroviruses have been genetically modified through the action of mAPOBEC3 (19). Thus, increasing evidence indicates a possible physiological role for mAPOBEC3 in restricting the replication of gammaretroviruses, not just betaretroviruses, of cognate origin; however, direct demonstration of the protective effects exerted by mAPOBEC3 on pathogenic MuLV infection has been lacking (4).

Friend virus (FV) is the pathogenic retrovirus complex composed of replication-competent Friend MuLV (F-MuLV), a

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▽ Published ahead of print on 10 September 2008.

prototypic ecotropic gammaretrovirus, and defective spleen focus-forming virus (SFFV). When integrated into erythroid progenitor cells, the SFFV component induces rapid proliferation and differentiation of these target cells, causing increased hematocrit values (polycythemia) and massive splenomegaly within a few weeks after inoculation into a susceptible strain of mice (5, 32, 36). This increase in the number of erythroid progenitor cells leads to increasing copy numbers of F-MuLV and SFFV proviruses and ultimately causes the emergence of erythroleukemia through promoter insertion or silencing of a tumor suppressor gene (22). The pathogenicity of FV is, however, influenced by several host genes: some directly restrict the target cell entry or integration of F-MuLV and SFFV, and others interfere with the growth potentiation of SFFV-infected erythroid progenitor cells (5, 32, 36). Yet other host genes, however, influence the FV-induced pathogenesis more indirectly by affecting the host immune response to the viral antigens. These include the major histocompatibility complex class I and class II genes that regulate $CD8^+$ and $CD4^+$ T-cell recognition of viral epitopes (33, 34); a class Ib gene putatively influencing natural killer cell activities toward FV-infected cells (18, 35); and a non-major histocompatibility complex gene, *Rfv3*, that influences the duration of viremia (6, 17, 47) partly through its effects on the production of virus-neutralizing antibodies (Ab) (23). The *Rfv3* gene has been mapped to within a narrow segment of mouse chromosome 15, colocalizing with the *APOBEC3* locus (23, 36), indicating that the possible polymorphisms in the *APOBEC3* locus might constitute a physiological resistance factor to FV infection in mice. We demonstrate here that an allelic variant of mAPOBEC3 expressed in C57BL/6 mice does restrict F-MuLV replication as well as FV-induced pathogenesis in vivo.

MATERIALS AND METHODS

Mice and virus. C57BL/6, BALB/c, B10.A/SgSn, and (BALB/c × C57BL/6)F_1 ($CB6F_1$) mice were purchased from Japan SLC, Inc., Hamamatsu, Japan. A/WySnJ mice were purchased from the Jackson Laboratory, Bar Harbor, ME. The mAPOBEC3-deficient mice have been described previously (31). They were backcrossed to C57BL/6 mice at least seven times and mated with BALB/c mice. Both male and female mice, 6 to 10 weeks old, were used for virus inoculation. All animals were housed and bred in the experimental animal facilities at Kinki University School of Medicine under a specific-pathogen-free condition, and the experiments described here have been approved by Kinki University. Replication-competent helper virus of the FV complex, F-MuLV, was purified from the culture supernatant of *Mus dunni* cells persistently infected with an infectious molecular clone, FB29 (44), as described previously (33).

Vector constructions. Total RNA was extracted from tissues and cells by using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA synthesis was performed by using a SuperScriptIII first-strand synthesis system (Invitrogen). The primers described below were purchased from GeneDesign, Inc., Osaka, or Operon Biotechnologies, Tokyo, Japan. The full-length mAPOBEC3 cDNA were amplified by PCR using the oligonucleotide primers 5′-GGGGTACCGCCGCCACC ATGGGACCATTCTGTCTGGGATGCAGCCATCGC-3′ and 5′-GGTCTAG ACATCGGGGGTCCAAGCTGTAGGTTTCC-3′, with primary cDNA samples prepared from the spleens of C57BL/6 and BALB/c mice. The hAPOBEC3G and hAPOBEC3F cDNA were amplified by PCR using the primers 5′-GGGGTACCG CCGCCACCATGAAGCCTCACTTCAGAAACACAGTGGAGCG-3′ (for both hAPOBEC3G and hAPOBEC3F) and 5′-GGACCGGTGTTTTCCTGATCTGG AGAATGGCCCGC-3′ (for hAPOBEC3G) or 5′-GGACCGGTCTCGAGAATC TCCTGCAGCTTGCTGTCCAGG-3′ (for hAPOBEC3F), with templates prepared from peripheral blood mononuclear cells of a healthy individual. The above-described APOBEC3 cDNA were cloned into the SalI/EcoRI digest (for mAPOBEC3) or HindIII/KpnI digest (for hAPOBEC3) of the pFLAG-CMV2 vector (Sigma-Aldrich Corp., St. Louis, MO.). The control plasmid pFLAG-CMV2-GFP was constructed by amplifying the green fluorescent protein (GFP) gene from the pEGFP vector (Clontech Laboratories, Inc., Mountain View, CA) with the oligonucleotide primers 5′-GGAAGCTTATGGTGAGCAAGGGCGAGGAGC-3′ and 5′-TCAGTACTTGTACAGCTCGTCCATGCCG-3′ and inserting it into the HindIII/EcoRI digest of the pFLAG-CMV2 vector.

The catalytic site mutants were produced based on the mAPOBEC3 cDNA lacking the exon 5 cloned from C57BL/6 mice ($mA3^bΔ5$) by a quick-change site-directed mutagenesis using the pFLAG-CMV2-$mA3^bΔ5$ plasmid as the template. The following primers were used to introduce each mutation (underlined). Primers 5′-CAACATCCACGCTGCAATCTGCTTTTTATACTGGTTCCATG ACAAAGTACTGAAAGTGCTGTCTCCG-3′ and 5′-GTACTTTGTCATGG AACCAGTATAAAAAGCAGATTGCAGCGTGGATGTTGTCCTTGTTCT TAAAGACCCC-3′ were used for the generation of $mA3^bΔ5^{I73A}$, and primers 5′-AAAGGCAAACAGCATGCAGCAATCCTCTTCCTTGATAAG-3′ and 5′-TCTTATCAAGGAAGAGGATTGCTGCATGCTGTTTGCC-3′ were used for the generation of $mA3^bΔ5^{E257A}$. The double mutant $mA3^bΔ5^{E73A\ E257A}$ was generated on pFLAG-CMV2-$mA3^bΔ5^{E257A}$ by using the oligonucleotide pairs used for the generation of $mA3^bΔ5^{E73A}$. These mutants were verified by DNA sequencing. The plasmids expressing the chimera between $mA3^bΔ5$ and the mAPOBEC3 lacking the exon 5 cloned from BALB/c mice ($mA3^dΔ5$), pFLAG-CMV2-$mA3^dΔ5$/$mA3^bΔ5$ and the reciprocal, pFLAG-CMV2-$mA3^bΔ5$/$mA3^dΔ5$, were constructed by mutually exchanging the cytidine deaminase catalytic domain 2 (CDD2)-encoding fragments between the $mA3^bΔ5$ and the $mA3^dΔ5$ cDNA at the unique HindIII site.

The plasmid vectors used for the establishment of stably expressing cells, the pIRES-PURO-FLAG-mA3, pIRES-PURO-FLAG-hA3, pIRES-PURO-FLAG, and pIRES-PURO-FLAG-GFP plasmids, were constructed by inserting the SpeI-XbaI fragment from the pFLAG-CMV2-mA3, pFLAG-CMV2-hA3, pFLAG-CMV2, and pFLAG-CMV2-GFP plasmids, respectively, into the SpeI/NheI digest of the pIRES-PURO vector (Clontech Laboratories, Inc.). These constructs were verified by DNA sequencing.

Establishment of stably transfected cell lines. To determine the possible restricting effects of the mAPOBEC3 allelic variants in a focus formation assay that mimics physiological MuLV replication, we established BALB/3T3 cell lines that stably expressed different FLAG-tagged versions of mAPOBEC3, the short isoform of mAPOBEC3 derived from C57BL/6 mice ($mA3^bΔ5$) and the full-length ($mA3^d$) and the short ($mA3^dΔ5$) isoforms derived from BALB/c mice (Fig. 1). DNA transfection into BALB/3T3 cells was performed by using Lipofectamine 2000 reagent (Invitrogen). For the establishment of stable transfectants, cells expressing FLAG peptide (FLAG) and FLAG-fusion proteins (FLAG-proteins) were selected in the presence of 6 μg/ml puromycin (Sigma-Aldrich) and 200 μg/ml Geneticin (GIBCO Industries Inc., Los Angeles, CA), and colony-forming cells were picked into a well of 96-well culture plates. The expression of FLAG and FLAG-proteins was confirmed by immunoblotting analyses of the cell lysates and with immunofluorescence staining of the cells.

PCR analysis of mAPOBEC3 mRNA and genomic DNA. Endogenous mAPOBEC3 and GAPDH mRNA from tissues and cells were detected by reverse transcription-PCR (RT-PCR) using primers 5′-GGGGTACCGCCGCCAC CATGGGACCATTCTGTCTGGGATGCAGCCATCGC-3′ and 5′-GGTCTA GACATCGGGGGTCCAAGCTGTAGGTTTCC-3′ for the full-length mAPOBEC3; primers 5′-TTACAAATTTTAGATACCAGGATTCTAAGCTTCAGG AG-3′ and 5′-TTGGTTGTAAAACTGCGAGTAAAATTCCTCTTCAC-3′ for the mAPOBEC3 exon 5 region; and primers 5′-GCCAAGGCTATCCATGAC AACTTTGG-3′ and 5′-GCCTGCTTCACCACCTTCTTGATGTC-3′ for mouse GAPDH. Genomic allele analyses of APOBEC3-deficient mice were done by detecting the insertion of a neomycin resistance gene in the exon 3 (31) by using the oligonucleotide primers 5′-CCCCCCAAGGACAACATCCACGCTG-3′ and 5′-GGCGGCAGGCGATTCAGGCCTTGGAA-3′.

Quantitative real-time PCR analyses of mAPOBEC3 mRNA. Real-time PCR assays for quantitative comparisons of mAPOBEC3 mRNA expression levels were performed as described previously (36). The mAPOBEC3 fragment was amplified from 50 ng of total cDNA and quantified using Platinum Quantitative PCR SuperMix-UDG with 6-carboxyl-X-rhodamine (ROX) reference dye (Invitrogen) with an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers and 6-carboxyfluorescein (FAM) dye-labeled probe used for the quantification of mAPOBEC3 messages were as follows. Primers were 5′-GCGGCTCCACAGGATCAA-3′ and 5′-TCC AAGCTGTAGGTTTCCAAAGT-3′; and the probe was 5′-TCTGCAAGATT GGTGAAT-3′. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out for 15 s at 95°C, followed by 1 min at 60°C. TaqMan rodent GAPDH control reagent (Applied Biosystems) was used as an internal control.

Northern and Western blot analyses. Total RNA was prepared from mouse tissues by using TRIzol reagent. Two micrograms of the RNA was denatured

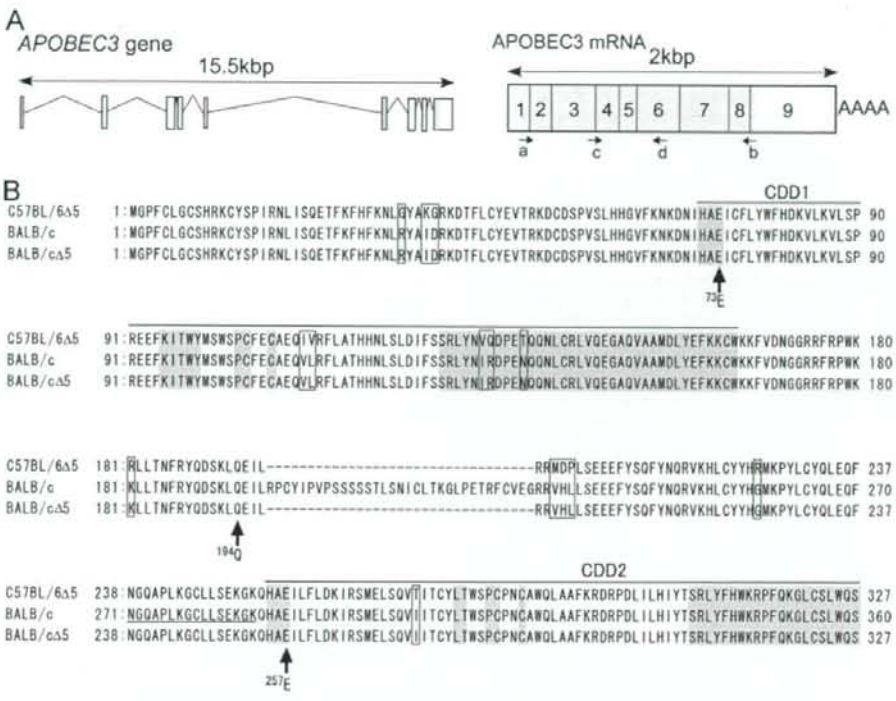

FIG. 1. Alleles and isoforms of mAPOBEC3 in FV-resistant C57BL/6 and -susceptible BALB/c mice. (A) The known genomic organization and splicing pattern of the mouse *APOBEC3* gene is shown with short arrows indicating the positions of the PCR primers used. (B) Alignment of the amino acid sequences of mAPOBEC3 for the C57BL/6-derived exon 5-lacking isoform ($mA3^{b}\Delta5$) (GenBank accession no. NM_030255), and BALB/c-derived full-length ($mA3^{d}$) [BC003314] and exon 5-lacking ($mA3^{d}\Delta5$) isoforms (GenBank accession no. EDL04624). Open boxes show different amino acid residues, shaded boxes show the regions necessary for CDD activities, and the long horizontal lines indicate two CDDs as described in previous reports (15, 37). B10.A/SgSn mice showed amAPOBEC3 sequence that was completely identical to that of C57BL/6 mice, while A/WySn mice shared the mAPOBEC3 sequence with BALB/c mice.

with RNA loading mixture (GenHunter Corporation, Nashville, TN), separated in a 1% formaldehyde-agarose gel, transferred to nitrocellulose membrane, and hybridized with ^{32}P-labeled probes that were prepared by random priming with the templates generated by RT-PCR (mAPOBEC3, 5′-CCCGTCTCCCTTCAC CATGGGG-3′ and 5′-GGGAGACCTTTTGTTAGACAGATATTTGACAGA GTGG-3′; and mouse β-actin, 5′-ATGGATGACGATATCGCTGCGCTGGT CGTCGACAACGGCTCCGGC-3′ and 5′-GGTCATCTTTTCACGGTTGGC CTTAGGGTTCAGGGGGGCC-3′). Specific hybridization was visualized using a BAS-MS imaging plate (Fujifilm Corp., Tokyo, Japan). Densitometric analyses of the detected bands were done by using Image Gauge software (Fujifilm Corp.), and the results were normalized with GAPDH for each sample. Anti-FLAG M2 (Sigma-Aldrich) and anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) Ab were purchased from the above-mentioned suppliers. Horseradish peroxidase-conjugated secondary Ab was purchased from Zymed Laboratories (San Francisco, CA). Detection by immunoblotting of F-MuLV gp70 and $p30^{gag}$ with monoclonal antibodies (MAb) 720 and R18-7 has been described previously (40).

F-MuLV infection in vitro and APOBEC3 packaging analysis. BALB/3T3 cells stably expressing FLAG-APOBEC3 were seeded at 3×10^{4} cells/well in 24-well plates and infected with purified F-MuLV at a multiplicity of infection of 2.0 in the presence of 1 μg/ml Polybrene (Sigma-Aldrich). After 2 h of incubation, the cells were washed, fed with fresh medium, and cultured for 2 days. For packaging analyses, the culture supernatants were centrifuged to remove cells, and viral particles were precipitated with polyethylene glycol and step purified into a 15%/85% sucrose interface (33). For flow cytometry analyses of the surface gp70 expression, F-MuLV-infected transfectants were detached from culture wells with a brief trypsin treatment and stained with biotinylated MAb 720 (40), followed by an incubation with allophycocyanin-conjugated streptavidin (eBioscience Inc., San Diego, CA), and were analyzed with a Becton-Dickinson (Franklin Lakes, NJ) FACScalibur system.

Sequence analysis of proviral DNA. F-MuLV in 1 ml of culture medium were inoculated onto a culture of *Mus dunni* cells and incubated for 18 h. After trypsinization and washing, the cells were treated with RNase I, and their DNA was isolated by using DNeasy (Qiagen, Hilden, Germany). A 1.2-kbp fragment of the F-MuLV proviral genome harboring the U3 and a part of the *gag* sequence was amplified by PCR using the primers 5′-CGGGATCCAAGGACCTGAAA TGACCCTG-3′ and 5′-GAAGAGAGAGGGGAGGTTTAGGG-3′. The amplified fragments were cloned into the pCR-Blunt vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen). Sequencing was performed by using the T7 and T3 primers.

PCR quantification of F-MuLV genomic RNA and integrated proviral DNA. F-MuLV viral RNA in culture medium was purified with a QIAamp viral RNA kit (Qiagen) and cDNA generated by RT with SuperScriptIII First-Strand synthesis system (Invitrogen) after a treatment with DNase I (Invitrogen). Genomic DNA was purified from F-MuLV-infected BALB/3T3 cells expressing FLAG or FLAG-protein or *Mus dunni* cells infected with F-MuLV as described above. Viral DNA was quantified using Platinum Quantitative PCR SuperMix-UDG with ROX and a 7900HT Fast Real-Time PCR system. Primers for the detection of the F-MuLV genome and the FAM-labeled probe were designed for the *env* region by using the following oligonucleotides. Primers were 5′-AAGTCTCCC

CCCGCC-3′ and 5′-AGTGCCTGGTAAGCTCCCTGT-3′; and the FAM-labeled probe was 5′-ACTCCACATTGATTTCCCCGTCC-3′. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out for 30 s at 95°C, followed by 1 min at 60°C. TaqMan rodent GAPDH control reagent was used as an internal control for genomic DNA.

Quantification of F-MuLV gp70 in culture supernatant. Ninety-six-well plates were coated with the gp70-specific MAb 48 (7) at 0.5 mg/well in 0.1 M $NaHCO_3$. Wells were blocked with 10% fetal bovine serum and incubated with a culture supernatant containing F-MuLV for 2 h at room temperature. After washing with PBS containing 0.05% Tween 20, 2 μg/well biotin-conjugated MAb 720 (40) was added and incubated for 1 h. After washing, the plates were incubated with a 1:30,000 dilution of horseradish peroxidase-conjugated streptavidin (Zymed Laboratories), and the chromogenic reaction was performed with 3,3′,5,5′-tetramethylbenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The assay signals were measured as optical density at 450 nm, and the gp70 concentration was determined by adjusting it to the standard curve set with purified F-MuLV particles.

F-MuLV infection in vivo. C57BL/6 mice were inoculated with 1×10^5 focus-forming units (FFU) of F-MuLV by injecting 0.5 ml of a dilution via the tail vein. The spleen and bone marrow were removed, and single-cell suspensions were prepared for infectious center assays. $CB6F_1$ mice were inoculated with 1×10^4 FFU of F-MuLV.

F-MuLV infectivity and infectious center assays. These assays were performed as described previously (24, 46). In brief, 1 ml of culture supernatant from F-MuLV-infected FLAG- or FLAG-protein-expressing BALB/3T3 cells was diluted serially and plated in duplicate with 1 μg/ml Polybrene on monolayers of *Mus dunni* cells. For infectious center assays, spleen or bone marrow cell suspensions were serially diluted and plated at concentrations between 1.0×10^3 and 1.0×10^6 cells/well onto monolayers of *Mus dunni* cells. After being washed and fixed with methanol on the second day of coculturing, F-MuLV-infected cell foci were visualized with MAb 720 as described previously (40).

FV complex and assessment of its pathogenicity in vivo. A B-tropic FV complex free of lactate dehydrogenase-elevating virus was kindly provided by K. J. Hasenkrug, Laboratory of Persistent Viral Diseases, NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT. Inoculation of $CB6F_1$ mice with FV complex, monitoring of hematocrit values, and flow cytometry analyses of bone marrow cells were performed as described previously (18, 24).

Statistics. One-way analysis of variance (ANOVA) for the comparison of multiple groups was performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., San Diego, CA), with an indicated posttest. When significant differences were pointed out by the ANOVA analyses, an individual level of significance was calculated for each pair of groups by two-tailed Student's or Welch's *t* test, depending on whether the variances were regarded as equal or not, respectively. Frequencies of mutations were evaluated by two-sided Fisher's exact test, between selected groups following an extended Fisher's exact test performed for the entire contingency table.

RESULTS

Allelic differences at the *APOBEC3* locus between FV-resistant and -susceptible strains of mice. The *Rfv3* gene that influences the duration of viremia after FV infection has been mapped to a segment of mouse chromosome 15 harboring the *APOBEC3* gene (17, 23, 36, 47); this led us to explore possible allelic differences in the expression of mAPOBEC3. We found that mAPOBEC3 mRNA expression levels in the hematopoietic tissues from naturally FV-resistant C57BL/6 and B10.A/SgSn mice were higher than those in susceptible BALB/c and A/WySnJ mice (Fig. 2A). Quantitative real-time PCR analyses confirmed that C57BL/6 and B10.A/SgSn mice expressed three- to fourfold higher levels of mAPOBEC3 mRNA than BALB/c and A/WySn mice did, both in the spleen and bone marrow (Fig. 2B). In addition, the mAPOBEC3 isoforms derived from C57BL/6 and BALB/c mice differed at several amino acid residues, five of which were located within the CDD1 (Fig. 1). Interestingly, A/WySnJ mice lacking the ability to control viremia (6) and to produce F-MuLV-neutralizing Ab (23) shared the mAPOBEC3 sequence with BALB/c mice.

Additional variability between mouse strains was also observed in part for the relative amounts of the two splice isoforms of mAPOBEC3. The mAPOBEC3 cDNA obtained by RT-PCR amplification from C57BL/6 mice was slightly smaller than that from BALB/c mice (Fig. 2C), and this was due to the lack of a 99-bp stretch corresponding to the exon 5 (Fig. 1). The use of primers placed in exons 4 and 6 showed that the predominant mAPOBEC3 mRNA isoform expressed in C57BL/6 mice lacked exon 5, while BALB/c mice expressed full-length mAPOBEC3 with very low levels of the short isoform (Fig. 2C). $CB6F_1$ mice expressed readily detectable full-length and truncated isoforms, with the latter in excess. Although the observed differences in the intensities of the PCR product bands might reflect different efficiencies in the amplification of the short and long fragments, the observed high intensity of the short-fragment band in C57BL/6 mice and the low intensity of the long-fragment band in BALB/c mice (Fig. 2C) are in agreement with the results of the Northern blotting (Fig. 2A) and real-time PCR (Fig. 2B) analyses. Thus, it is reasonable to conclude that BALB/c mice express a low level of full-length messages and C57BL/6 mice a high level of truncated mAPOBEC3 messages.

In vitro restriction of F-MuLV replication with C57BL/6-derived mAPOBEC3. It has been shown that mAPOBEC3 lacking the exon 5 (mAPOBEC3Δ5) can be packaged into MuLV particles more efficiently than the full-length mAPO BEC3 protein and, thus, can exert partial restriction of MuLV integration (1), although a recent report (4) has indicated similarly efficient incorporation of the full-length and the exon 5-lacking mAPOBEC3 into MuLV virions. However, the previous reports of the possible restricting effects of mAPOBEC3 on MuLV integration employed acute transfection of mAPO BEC3-expressing vector into MuLV packaging cells and examined a single-round integration of the MuLV vector and resultant expression of an inserted indicator gene. We intended to examine the possible restricting effects of mAPOBEC3 isoforms on more physiological replication cycles of infectious MuLV. Thus, we established BALB/3T3 cell lines that stably expressed different FLAG-tagged versions of mAPOBEC3 and measured the infectivity of F-MuLV produced from the transfectants in a focus formation assay that mimics physiological MuLV replication. BALB/3T3 cells stably expressing hAPOBE C3G, hAPOBEC3F, or GFP were also established as controls. These transfectants expressed comparable levels of APOBEC3 mRNA and produced APOBEC3 proteins of the expected sizes (Fig. 2D and F). Acute infection of the BALB/3T3 lines with an infectious molecular clone of F-MuLV resulted in a similar range of the envelope glycoprotein gp70 detected in the lysates, regardless of the APOBEC proteins expressed (Fig. 2F). The levels of F-MuLV infectivity and gp70 expression in the transfected lines were also confirmed to be similar by flow cytometric analyses (Fig. 2E). However, when we measured the infectivities of progeny viruses produced from the stable transfectants by focus formation assays on fully permissive *Mus dunni* cells, we found wide differences depending on the particular APOBEC3 protein expressed (Fig. 2H). Thus, the infectivity of F-MuLV produced from the $mA3^b\Delta5$-expressing cells was drastically reduced to a level similar to that obtained with F-MuLV derived from the hAPOBEC3G-expressing cells, whereas some 2.0×10^4 FFU/ml of infectious particles were

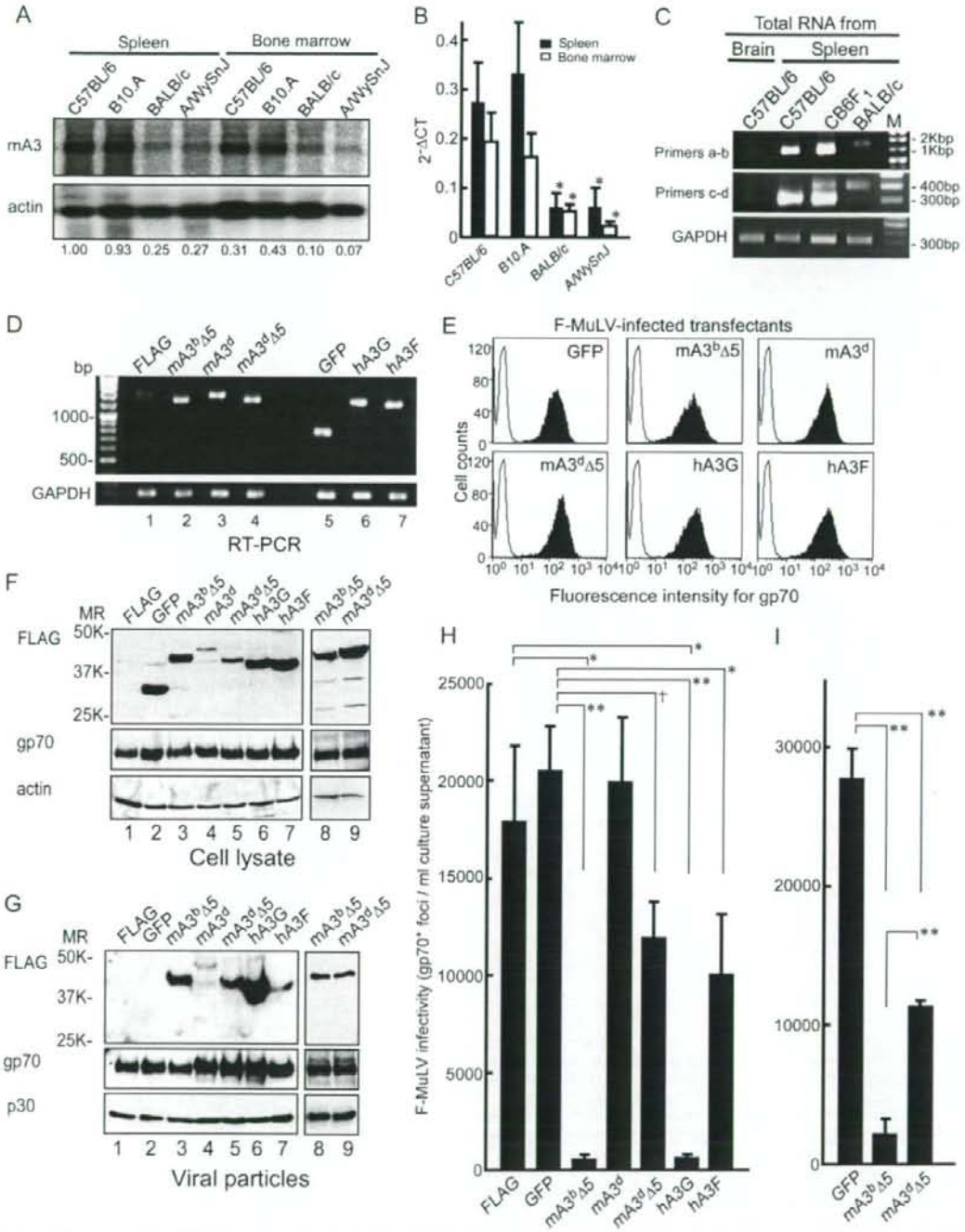

FIG. 2. Expression of the different alleles and isoforms of mAPOBEC3 in FV-resistant and -susceptible mice and infectivities of F-MuLV virions produced from mAPOBEC3-expressing cells. (A) Comparisons of mAPOBEC3 mRNA expression levels between mouse strains, by Northern blotting. Female mice, 7 to 8 weeks old, were analyzed for endogenous mAPOBEC3 mRNA expression. mAPOBEC3 mRNA was detected in 5 μg total RNA extracted from the spleen and bone marrow of the indicated strains of mice. β-Actin was used as an internal control. The numbers shown below each lane indicate densitometric ratios of expression levels between mAPOBEC3 and β-actin messages, normalized to that in the spleen of C57BL/6 mice. (B) Levels of expression of mAPOBEC3 mRNA relative to GAPDH quantified by real-time PCR are shown. Means of three samples each are shown with bars indicating standard errors of the means. *, statistically significant differences from the expression

TABLE 1. Number of F-MuLV provirus, infectious particles, and provirus in cells infected with progeny virus

Stable transfectant	Proviral copy no. (mean ± SEM) in producer cells (10^4)[a]	Concn (μg/ml) of gp70 (mean ± SEM) in culture supernatant[b]	Viral genomic copy no. (mean ± SEM) in culture supernatant (10^6)[c]	Proviral copy no. (mean ± SEM) in indicator cells (10^4)[e]
FLAG	99.47 ± 21.23	87.61 ± 4.76	5.43 ± 0.47	14.38 ± 4.79
GFP	156.80 ± 31.84	70.97 ± 11.17	4.06 ± 0.29	18.89 ± 4.00
mA3^{b}Δ5	140.19 ± 23.25	73.01 ± 7.63	3.17 ± 0.40	0.57 ± 0.05[f]
mA3^d	94.57 ± 24.01	70.71 ± 15.41	5.05 ± 0.71	16.45 ± 1.74
mA3^{d}Δ5	128.18 ± 23.60	72.57 ± 13.27	4.18 ± 0.31	4.59 ± 1.25[f]
hAPOBEC3G	53.78 ± 4.43	53.35 ± 8.44	2.82 ± 0.33[d]	0.35 ± 0.05[f]
hAPOBEC3F	99.80 ± 2.99	65.95 ± 6.05	3.54 ± 0.78	2.55 ± 0.40

[a] F-MuLV proviral DNA was quantified in the genomic DNA of the infected cells by quantitative real-time PCR using an F-MuLV-specific probe and primers. Data shown are means ± standard errors of the means (SEM) of copies per 50 ng of cellular genomic DNA, calculated from three repeated experiments. One-way ANOVA with Tukey's posttest for multiple comparisons indicated a significant difference only between GFP and hAPOBEC3G, but this was not significant ($P = 0.08$) by individual analysis using Welch's t test.

[b] Concentration of F-MuLV structural protein gp70 in culture supernatant from the acutely infected stable transfectants was analyzed by captured enzyme-linked immunosorbent assay using anti-gp70 MAb. Data shown here are means ± SEM calculated from three repeated experiments. No significant differences between the groups were found by one-way ANOVA with Tukey's posttest.

[c] Viral genomic RNA in the culture supernatant from the acutely infected stable transfectants was quantified by quantitative RT-PCR. Data shown here are means ± SEM of copies per 1 ml culture supernatant calculated from 3 repeated experiments. One-way ANOVA with Tukey's posttest indicated a significant group-wise difference only between the FLAG and hA3G.

[d] Student's t test for individual level of significance gave a P value of 0.011.

[e] F-MuLV proviral DNA was quantified from infected *Mus dunni* cells as described in the text. One-way ANOVA with Tukey's posttest for multiple comparisons indicated significant group-wise differences, which were then individually analyzed by t test.

[f] $P < 0.05$, in comparison with that of GFP.

detected when the supernatants from the BALB/3T3 cells expressing FLAG alone or control GFP were tested (Fig. 2H). In contrast, only a marginal reduction in F-MuLV infectivity was observed for the supernatant of mA3^{d}Δ5-expressing cells. Enforcing higher levels of expression of the mA3^d cDNA in the BALB/3T3 cells, compared with a low expression level of the endogenous *mA3^d* allele (Fig. 2D, lanes 1 and 3), did not result in any significant decrease in the infectivity of the virus produced (Fig. 2H), although the detectable amounts of mA3^d protein were apparently lower than those of the exon 5-lacking isoforms in several tested clones, which might have caused inefficient incorporation of the full-length protein into the virions (Fig. 2G). Nevertheless, these results indicate strain-dependent differences in F-MuLV-restricting activities of mAPOBEC3, with the C57BL/6-derived short isoform restricting F-MuLV with an efficacy similar to that shown by heterologous hAPOBEC3G.

When F-MuLV particles were purified from the culture supernatant of acutely infected transfectants, virion-incorporated mAPOBEC3 lacking the exon 5, but not the full-length mAPOBEC3, was readily detectable along with viral gp70 and p30^{gag}, regardless of their strains of origin (Fig. 2G). Interestingly, although mA3^{d}Δ5 derived from BALB/c mice was incorporated into F-MuLV as efficiently as C57BL/6-derived mA3^{b}Δ5 (Fig. 2G), only mA3^{b}Δ5 inhibited F-MuLV replication as strongly as hAPOBEC3G did in vitro (Fig. 2H). To exclude the possibility that the difference observed for the effects of mA3^{b}Δ5 and mA3^{d}Δ5 in restricting F-MuLV replication in vitro was caused by the slightly smaller amount of mA3^{d}Δ5 than mA3^{b}Δ5 detected in the transfectants used (Fig. 2F), we examined separate pairs of the stable transfectants. As shown in lanes 8 and 9 in Fig. 2 F and G, a higher level of mA3^{d}Δ5 was detected in the separate clone of stable transfectant, and the detected levels of virion-incorporated mAPOBEC3Δ5 proteins were similar. Nevertheless, the progeny virus produced from the mA3^{d}Δ5-expressing cells showed only about 60% reduc-

levels in B10.A/SgSn mice, indicated by one-way ANOVA with Dunnett's posttest for multiple comparisons ($P < 0.05$). (C) Splicing variants of the *APOBEC3* gene expressed in C57BL/6 and BALB/c mice. The known genomic organization and splicing pattern of the *APOBEC3* gene along with the positions of the primers used are shown in Fig. 1. The primers a and b amplified the entire mAPOBEC3-coding region, while primers c and d encompassed exons 4 and 6. GAPDH was used as an internal control. (D) Expression of APOBEC3 mRNA in the BALB/3T3 cells stably transfected with each *APOBEC3* gene was analyzed by RT-PCR. The same primers for mAPOBEC3 were used for samples in lanes 1 to 4. Samples in lanes 5 to 8 were amplified with each specific primer set. GAPDH was used as an internal control. Note the faint band of endogenous mA3^d cDNA in lane 1. (E) Flow cytometric analyses of the cell surface expression of F-MuLV gp70 on acutely infected stable transfectants are shown. Cells expressing the indicated genes were infected with F-MuLV at a multiplicity of infection of 2.0 and analyzed for surface gp70 expression with MAb 720 2 days later. (F and G) Proteins detected in cell lysate (F) and virus particles in the culture supernatant (G) from the infected BALB/3T3 cells expressing FLAG and FLAG-proteins are shown. Immunoblot detection was performed with the anti-FLAG, anti-gp70, anti-p30 or anti-actin Ab. (H and I) Infectivities of progeny F-MuLV produced from APOBEC3-expressing BALB/3T3 cells. *Mus dunni* cells were infected with the progeny virus produced from the indicated transfectants, and foci of infected cells were stained with anti-gp70 MAb for enumeration. The vertical axis in panel I shows F-MuLV infectivity as in panel H. The infectivities are shown as an equivalent of infectious virus per 1 ml of culture supernatant ($n = 3$, mean ± standard deviation; *, $P < 0.05$; †, $P < 0.01$; **, $P < 0.005$). The F-MuLV infectivity detected in the supernatant of hAPOBEC3G-expressing cells was drastically reduced, while only a moderate reduction in F-MuLV infectivity was observed when the indicator cells were inoculated with the supernatant from the hAPOBEC3F-expressing cells, consistent with the previous reports (1, 4, 13). All the experiments shown in panels C to I were performed with at least two representative clones of stable transfectants for each gene, and the results obtained with the independent clones were in agreement with the data shown.

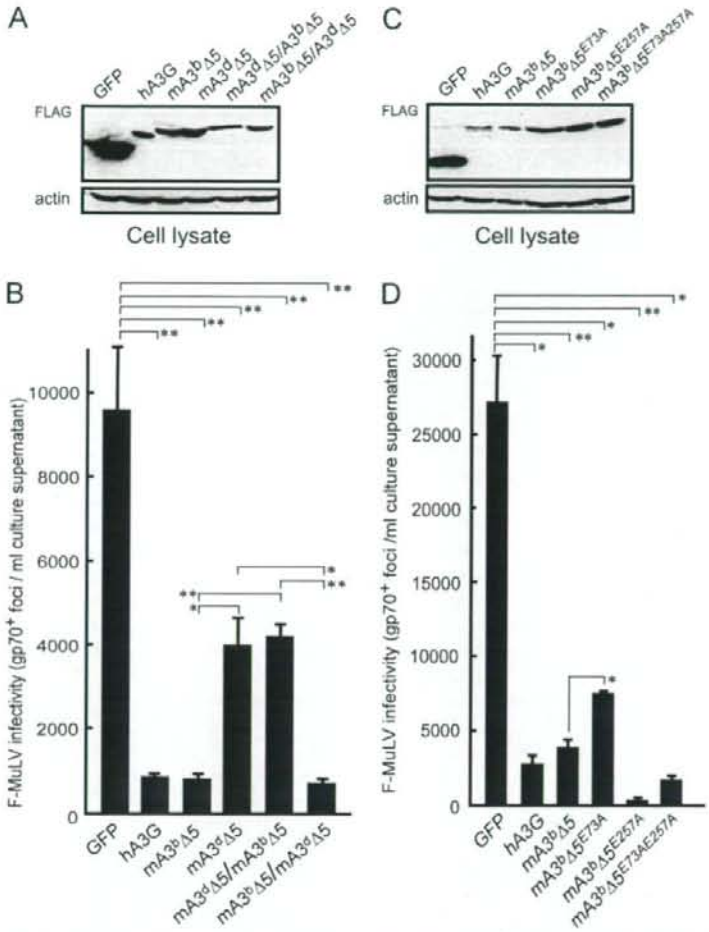

FIG. 3. Infectivities of F-MuLV virions produced from BALB/3T3 cells expressing mA3^{b}Δ5 mutants. (A and C) Cell lysates from the acutely infected transfectants were analyzed for the presence of APOBEC3 proteins. Immunoblot detection was performed with anti-FLAG and anti-actin Ab. (B and D) *Mus dunni* cells were infected with virus produced from the mAPOBEC3Δ5-expressing BALB/3T3 cells, and foci of gp70$^+$-infected cells were enumerated (n = 3, mean ± standard deviation; *, $P < 0.05$; **, $P < 0.005$). All the experiments shown were performed with at least two representative clones of stable transfectants for each gene, and the results obtained with the independent clones were in agreement with the data shown here.

tion in F-MuLV infectivity, while the progeny virus produced from the mA3^{b}Δ5-expressing cells showed vastly reduced infectivity, which was significantly lower than that shown by the mA3^{d}Δ5-containing F-MuLV.

The effectiveness of virion-incorporated mAPOBEC3 in restricting F-MuLV replication was further confirmed by quantitative analyses of viral copy numbers. Whereas neither the numbers of F-MuLV proviruses within the acutely infected stable transfectants nor the amounts of viral gp70 and genomic RNA in the supernatants were significantly different, regardless of the APOBEC3 or control genes expressed by the transfectants (Table 1), with the exception of a slight reduction in viral RNA in supernatants from the hAPOBEC3G-expressing cells, the number of F-MuLV proviral copies detected in infected *Mus dunni* indicator cells was reduced to less than 1/20 of the number detected in the cells infected with the control preparations when progeny virions were produced from mA3^{b}Δ5- or hAPOBEC3G-expressing cells. The number of proviral copies in the indicator cells was only moderately reduced when infected with progeny viruses produced from the mA3^{d}Δ5-expressing cells, and no reduction in the proviral copy numbers was observed for the cells infected with the virus produced from cells expressing the full-length mA3^d (Table 1). Thus, in agreement with the results of the infectious focus formation assays (Fig. 2H and I), mA3^{b}Δ5 restricts F-MuLV proviral integration more efficiently than does mA3^{d}Δ5.

C57BL/6-derived mAPOBEC3 restricts F-MuLV replication in the absence of the deaminase catalytic site. The above-

TABLE 2. Sequence variations of F-MuLV proviral genome observed for infected cells

Stable transfectant	Total no. of nucleotides analyzed[a]	No. of nucleotide exchanges[b]		
		G/A	C/T	Other mutations
FLAG	94,429	0	3	6
GFP	101,856	0	2	6
mA3^{b}Δ5	96,551	3	2	9
mA3^d	94,429	5[c]	0	5
mA3^{d}Δ5	90,185	3	2	5
hAPOBEC3G	95,490	55[d]	5	11
hAPOBEC3F	97,612	2	0	6

[a] A 1,061-nucleotide fragment between the U3 and *gag* sequences from the F-MuLV proviral DNA was cloned, and its sequence was analyzed for at least 90 clones.

[b] The entire contingency table was analyzed for the possible presence of group-wise difference, which indicated a P value of <0.001. An individual group-wise difference was then analyzed by Fisher's exact test.

[c] $P = 0.026$ in comparison with that of GFP but was not significant in comparison with that of FLAG.

[d] $P = 5.77 \times 10^{-17}$ in comparison with the data for FLAG, 4.55×10^{-18}, in comparison with that of GFP.

described results indicate that the major difference between the efficacies of the C57BL/6- and BALB/c-derived mAPOBEC3 proteins in restricting F-MuLV infection in vitro likely stems from the differences in their amino acid sequences (Fig. 1). To analyze this, we constructed reciprocal chimeras between mA3^{b}Δ5 and mA3^{d}Δ5 by mutually exchanging the N-terminal portion at ^{194}Q (Fig. 1) and established BALB/3T3 lines stably expressing the chimeric mAPOBEC3 (Fig. 3A). The progeny virus produced from the cells expressing the chimeric mA3^{d}Δ5/mA3^{b}Δ5 protein, with its N-terminal portion encoded by the BALB/c-derived *mA3^d*, failed to fully restrict F-MuLV replication, while the reciprocal mA3^{b}Δ5/mA3^{d}Δ5 construct restricted F-MuLV replication as efficiently as mA3^{b}Δ5 did (Fig. 3B). These results clearly localized the strain-dependent functional difference of mAPOBEC3Δ5 to the N-terminal portion harboring the CDD1.

It is not clear to what extent APOBEC3 proteins restrict retroviral integration through their deaminase activity as opposed to through a deaminase-independent mechanism (1, 4, 38, 39, 43). Sequencing of the multiple proviral genomes revealed a significant increase in G-to-A mutations in the *Mus dunni* cells infected with F-MuLV produced from the hAPOBEC3G-expressing cells (Table 2). Surprisingly, the proviruses cloned from the indicator cells infected with F-MuLV produced from the mA3^{b}Δ5-expressing cells did not show such a significant increase in G-to-A mutations in comparison with those observed for the cells infected with the control viruses. To further determine if the deaminase activity is required for the observed restriction of F-MuLV replication by mA3^{b}Δ5, mutations were introduced into its catalytic sites. mAPOBEC3, as well as hAPOBEC3G and hAPOBEC3F, harbors two CDD, of which only CDD2 is catalytically active in hAPOBEC3G (37, 38), while CDD1 is active in mAPOBEC3 (15). Mutation of the glutamic acid to alanine at position 73 (E73A) within CDD1, but not the equivalent mutation (E257A) within CDD2, abrogates mAPOBEC3 deaminase activity, as well as antiviral restriction, against *vif*-deficient HIV (15). We introduced these point mutations either individually or in combination into mA3^{b}Δ5 and generated stable transfectants in BALB/3T3 cells (Fig. 3C). The F-MuLV progeny viruses produced from all the mutant mA3^{b}Δ5 transfectants had reduced replicative activities in *Mus dunni* cells (Fig. 3D) in comparison with the virus produced from the control cells, regardless of the introduced mutation(s) into mAPOBEC3, although the E73A mutant showed a slightly reduced repressive activity, implicating some role for this catalytic site. These results, along with the lack of an evident increase in G-to-A substitutions in the integrated proviral genome (Table 2), indicate that mA3^{b}Δ5 may restrict F-MuLV replication in a deaminase-independent fashion.

C57BL/6-derived mAPOBEC3 restricts F-MuLV replication in vivo. The reduced infectivity of the progeny F-MuLV produced from the stably transfected cell lines might have resulted from an excessive amount of mAPOBEC3 protein that was forcibly incorporated into the virion. To examine directly the possible physiological effect of the putative resistant allele, *mA3^b*, on F-MuLV infection in vivo, we introduced the targeted disruption of the *APOBEC3* gene (31) into C57BL/6 mice by backcrossing. The progenies of heterozygous breeding pairs were genotyped (Fig. 4A), and the expression or lack of expression of the *mA3^b* allele in the spleen and bone marrow was confirmed by RT-PCR (Fig. 4B). Infectious center assays revealed that C57BL/6 mice deficient in mAPOBEC3 possessed nearly 100-fold higher numbers of F-MuLV-producing cells at postinfection day (PID) 6, both in the spleen and bone marrow, in comparison with that of the wild-type or heterozygous counterparts (Fig. 4C). To further determine if the allelic difference in the *APOBEC3* locus influences resistance to F-MuLV infection, heterozygous C57BL/6 *mA3$^{b/-}$* mice were mated with BALB/c mice, and the resultant CB6F$_1$ mice were genotyped and infected with F-MuLV. As expected, CB6F$_1$ mice of the *mA3$^{d/-}$* genotype expressed low levels of the full-length, as well as the short mAPOBEC3, mRNA in the bone marrow, while the *mA3$^{d/b}$* mice expressed a higher level of the mA3^{b}Δ5 message along with a low level of the full-length mA3^d mRNA (Fig. 4D). Importantly, *mA3$^{d/-}$* mice deficient in the C57BL/6-derived APOBEC3 protein harbored more than 100-times-larger numbers of F-MuLV-producing cells in their bone marrow than the wild-type *mA3$^{d/b}$* mice at PID 6, despite the expression of the BALB/c-derived *mA3^d* allele (Fig. 4E). Thus, the C57BL/6-derived *mA3^b* allele dominantly confers resistance to F-MuLV infection in the presence of the *mA3^d* allele.

C57BL/6-derived mAPOBEC3 restricts erythroid cell proliferation in mice infected with the pathogenic FV complex. To further determine if the *mA3^b* allele physiologically functions in conferring resistance to FV-induced pathogenesis, FV-susceptible CB6F$_1$ mice possessing or lacking the *mA3^b* allele were infected with FV. CB6F$_1$ *mA3$^{d/b}$* mice possessed on average 13.1% ± 9.2% gp70-positive (gp70$^+$) cells in their bone marrow at PID 7 (Fig. 4F), a large majority of which belonged to the TER119$^+$ erythroblast population (25). On the other hand, when CB6F$_1$ mice lacking the *mA3^b* allele were infected with FV, significantly increased numbers (35.6% ± 11.9%, $P = 0.00015$) of bone marrow cells became positive for gp70, and these included more immature TER-119 cells. Reflecting the above differences, CB6F$_1$ *mA3$^{d/-}$* mice uniformly possessed extremely high hematocrit values at PID 21, while hematocrit

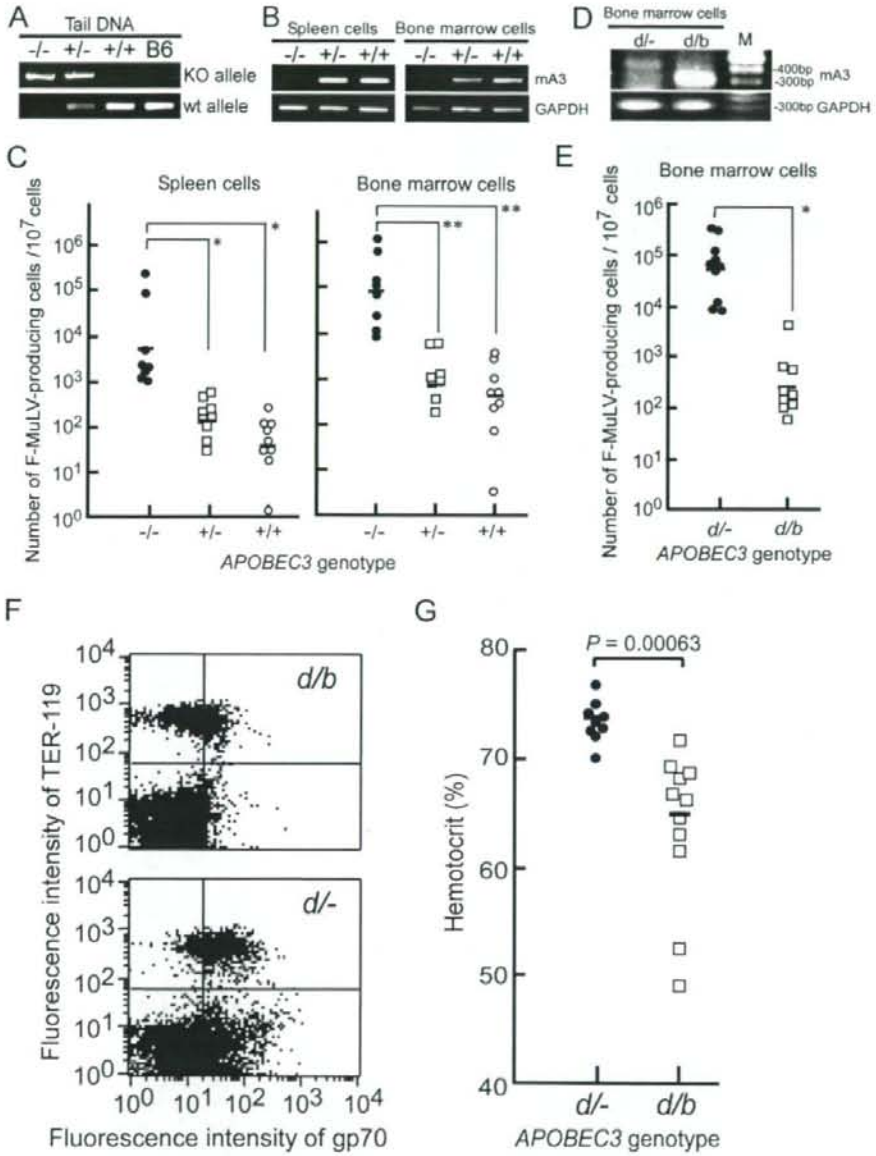

FIG. 4. Replication of F-MuLV in mAPOBEC3-deficient mice and the development of FV-induced disease in vivo. (A) Representative results for genotyping of the *APOBEC3* alleles by PCR. (B) Expression of mAPOBEC3 mRNA in the bone marrow and spleen of the APOBEC3-deficient C57BL/6 mice analyzed by RT-PCR is shown. (C) C57BL/6 mice possessing either the *APOBEC3*$^{-/-}$ (−/−) (n = 8), +/− (n = 10), or +/+ (n = 9) alleles were inoculated intravenously with 10^5 FFU of purified F-MuLV. F-MuLV infectious centers were enumerated on *Mus dunni* cells with anti-gp70 MAb on PID 6. Short horizontal bars indicate the means. *, $P < 0.02$; **, $P < 0.002$. (D) Expression of APOBEC3 mRNA in mAPOBEC3-deficient CB6F$_1$ mice analyzed by RT-PCR. (E) CB6F$_1$ mice possessing either the *APOBEC3*$^{d/-}$ (d/−) (n = 10) or d/b (n = 10) alleles were inoculated intravenously with 10^4 FFU of purified F-MuLV. F-MuLV infectious centers in the bone marrow were enumerated by coculturing *Mus dunni* cells and staining with anti-gp70 MAb on PID 6. Short horizontal bars indicate the means. The difference between the groups was analyzed by Student's t test: *, $P < 1 \times 10^{-7}$. (F and G) CB6F$_1$ mice possessing either the *APOBEC3*$^{d/-}$ (d/−) or d/b alleles were inoculated intravenously with 150 spleen FFU of FV complex. On PID 7, the cells prepared from the bone marrow were stained for TER119 and gp70 and were analyzed by flow cytometry (F). Five mice of each genotype were examined to calculate the means described in the text, and the dot graphs shown are of representative animals. On PID 21, hematocrit values were determined in the peripheral blood (G).

values for the $mA3^{d/b}$ mice were significantly lower (Fig. 4G). Thus, the $mA3^{b}$ allele does confer resistance to FV-induced erythroid pathology.

DISCUSSION

It has been generally accepted that retroviruses have evolved to evade the antiviral activities of their natural host's APOBEC proteins. In fact, primate lentiviruses counter the action of primate A3G with their Vif protein, while Vif is unable to counteract mAPOBEC3 and HIV-1 is highly susceptible to restriction by mAPOBEC3 (28, 42). The primate APOBEC3G gene predates HIV-related lentiviruses and has been under strong selective pressure for its conserved functionality (41). Thus, physiological targets of primate APOBEC3G might be endogenous retroviruses and nonautonomous retroelements (10, 14). Similarly, MuLV has also evolved mechanisms to block mAPOBEC3. These include the competitive exclusion of mAPOBEC3 from MuLV particles (1, 13), cleavage of virion-incorporated mAPOBEC3 by viral protease (1), and inhibition of the deaminase activity (4). However, possible inhibitory roles of APOBEC3 proteins on exogenous retroviral infection in vivo have become evident through the study of human lentiviruses. Thus, the patterns of hypermutation of HIV from infected individuals have indicated that the hAPOBEC3 proteins fulfill an inhibitory role (2, 26), and the levels of hAPOBE C3G expression have been associated with suppression of HIV-1 viremia and HIV-1-exposed but -uninfected status (3, 20). Regarding the mouse, however, the only previously demonstrated target for mAPOBEC3 in vivo was the betaretrovirus MMTV, with mAPOBEC3-deficient mice giving increased viral replication (39). Although putatively residual activities exerted by mAPOBEC3Δ5 in inhibiting MuLV integration have been reported (1, 4), these might have been caused by an excessive amount of mAPOBEC3 protein forcibly incorporated into the virion, especially when the experiments were performed by transfecting MuLV packaging cells with an mAPOBEC3-expressing plasmid vector.

Based on our previous demonstration that the FV resistance gene *Rfv3* colocalized with the *APOBEC3* locus (23, 36), we have shown here that the mouse gammaretrovirus F-MuLV is a target for mAPOBEC3 and, further, that mAPOBEC3 acts to restrict viral pathogenesis in vivo (Fig. 4). Gammaretroviruses have coevolved with their natural hosts (48), with MuLV and related endogenous retroviruses distributed more widely than MMTV among murine strains and species. Nevertheless, C57BL/6 and closely related C57BL/10 (B10) mice possess multiple host factors that make these strains resistant to FV-induced disease development (5, 22, 32, 36). We have shown in the present paper that differences in the sequence of mAPO BEC3 (Fig. 1), along with different expression levels in the hematopoietic tissues (Fig. 2A to C) account for part of this polymorphism. Further, we have also localized the functional difference between F-MuLV-restricting $mA3^{b}\Delta5$ and less-restricting $mA3^{d}\Delta5$ to the N-terminal portion other than the deaminase catalytic site (Fig. 3). Thus, F-MuLV infection in mice may not only provide a tractable model for the study of the in vivo mechanism of APOBEC3-mediated retroviral restriction, it may also provide insight into mechanisms of virus-host coevolution.

Finally, whether or not the *Rfv3* locus is identical to the *APOBEC3* locus must be discussed. The *Rfv3* gene was first described by comparing the persistence of viremia after FV infection between the prototypic FV-resistant B10.A/SgSn and the susceptible A/WySn mice that share the same $H\text{-}2^{a}$ haplotype (6). A/WySn mice remained viremic at more than 30 days after FV infection, while B10.A/SgSn mice had cleared viremia by PID 30. Since F_1 crosses between these two strains were not viremic and about half of the (B10.A × A/WySn) × A/WySn backcross mice showed viremia at PID 30, the presence of a recessive host gene in A/WySn mice was postulated in association with the persistence of viremia and was designated the $Rfv3^{s}$ allele. Thus, B10.A/SgSn mice possess a dominant allele, the $Rfv3^{r}$, conferring the early clearance of viremia. The *Rfv3* locus was later mapped to within chromosome 15 (17, 47). As we have shown here (Fig. 1), B10.A/SgSn mice share the *APOBEC3* sequence with C57BL/6 and A/WySn with BALB/c. Therefore, it is conceivable that the FV-restricting $mA3^{b}$ allele in B10.A/SgSn mice functioned to limit the replication of FV and thus contributed to the observed earlier clearance of viremia. However, for the clearance of viremia in FV-infected mice, the host immune responses are also required. In fact, B-cell-deficient C57BL/6 mice possessed higher levels of viremia than their wild-type counterparts at PID 7 (30), and FV-producing cells in the bone marrow and spleen could not be eliminated, even after effective priming of T cells with the viral antigens, in the absence of Ab-producing cells (24, 30). Thus, although the $mA3^{b}$ allele does contribute to the reduction in the number of virus-producing cells in the early stage of FV infection (Fig. 4), it must influence the immune responses, either directly or indirectly, to explain the phenotypes influenced by the *Rfv3* gene. In this regard, (B10.A × A/WySn)F_1 mice do produce F-MuLV-neutralizing Ab earlier than A/WySn mice do (23). The less massive expansion of FV-infected erythroid cells in the $mA3^{b}$-possessing mice than in those lacking this resistant genotype (Fig. 4) might result in the possible preservation of the stromal architecture that is required for cell-to-cell interactions involved in lymphocyte priming and B-cell activation. Further studies are required to clarify the presumable identity of the mouse *APOBEC3* gene as the *Rfv3* gene.

ACKNOWLEDGMENTS

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, including the High-Tech Research Center project, and grants from the Ministry of Health, Labor and Welfare of Japan, the Japan Health Sciences Foundation, and the Naito Foundation.

We thank J. B. Dowell for critical readings and corrections of the manuscript.

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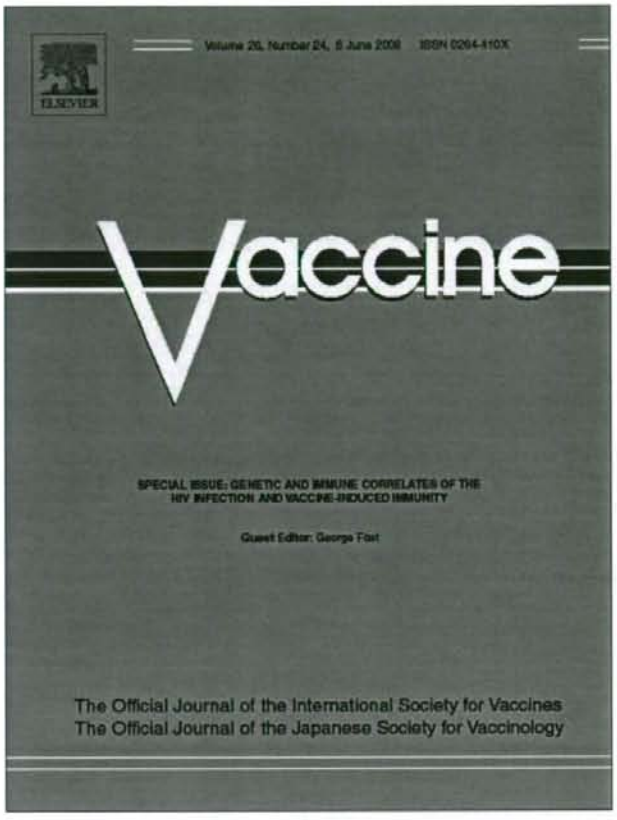

Vaccine (2008) 26, 2981–2996

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Host genetic factors that control immune responses to retrovirus infections

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Received 3 November 2007; received in revised form 1 January 2008; accepted 4 January 2008
Available online 22 January 2008

KEYWORDS
Host gene;
Immune response;
Retrovirus

Summary Several host genes control retroviral replication and pathogenesis. These include genes that directly affect the replication of retroviruses in target cells and those that control the host immune responses to the viral antigens. Host genetic factors that affect retroviral replication and immune responses to the viral antigens have been best studied in mouse models of Friend leukemia virus (FV) infection. Several genes located within the major histocompatibility complex (MHC), along with a separate gene not linked to the MHC, influence the host immune responses to FV antigens. The latter, the *Rfv3*, regulates the production of virus-neutralizing antibodies, and thus affects the duration of viremia. T-cell responses to the viral epitopes are controlled by MHC class I and class II genotypes, and both $CD8^+$ and $CD4^+$ T-cells are required for spontaneous immune resistance to FV infection. When $CD4^+$ T-helper cells are efficiently primed with a viral epitope, however, $CD8^+$ T-cells are not required for immune protection against FV infection, while B cells are absolutely required. There are individuals who possess human immunodeficiency virus type 1 (HIV-1)-reactive IgA antibodies in their mucosal secretions and show strong T-cell responses to HIV-1 antigens, even though they are negative for HIV-1 genome and HIV-1-reactive serum IgG. These HIV-1-exposed but uninfected individuals rarely possess resistance-associated alleles at known AIDS-restricting loci such as *CCR5Δ32*. Recent genetic analyses have indicated that a large proportion of such exposed but uninfected individuals may share a common genetic background.

Introduction

An estimated 38.6 million people worldwide were living with human immunodeficiency virus (HIV) and 4.1 million had become newly infected in the year 2005 [1]. More than two-third of the people living with HIV in Asia are in India, the world's second-most populous country. Approximately 650,000 people are reported to have been living with HIV in China by 2005, but an early projection predicted 10 million cases of HIV infection by 2010 in the absence of effective control measures [2]. Despite the effectiveness of highly active antiretroviral therapy (HAART) in reducing annual deaths from acquired immunodeficiency syndrome (AIDS) in

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doi:10.1016/j.vaccine.2008.01.004

Western countries, the high cost of the medicine remains the major obstacle in adopting HAART in developing countries. Some other obstacles include inconsistent adherence to the treatment regimens, the persistence of viral replication, and the resultant emergence of drug-resistant mutations. Only about one in six people who need antiretroviral therapy in Asia are actually receiving it, and the coverage still remains below 10% in India, which has more than 70% of the region's treatment needs [1]. With these difficulties, the possible immune prevention of HIV acquisition remains the best hope for stopping the spread of HIV infection.

The understanding of the mechanisms of natural protection from or spontaneous resistance to viral infections may contribute to the development of effective preventative measures. Several host factors have been associated with naturally occurring resistance against HIV acquisition or delayed development of AIDS after HIV seroconversion. These include genes encoding chemokine receptors that serve as coreceptors for the entry of HIV into target cells, chemokine ligands, cytokines, receptors expressed on antigen-presenting or natural killer cells including C-type lectin receptors, and those mapped within the major histocompatibility complex (MHC) (reviewed in [3–5]). Host factors that restrict mammalian retroviral infections, however, had been studied for decades prior to the first descriptions of AIDS and HIV [6]. In particular, the mouse model of Friend retrovirus infection has provided by far the deepest insights into the molecular mechanisms of action of the retrovirus-restricting factors (reviewed in [7–10]). This is because infection with Friend leukemia virus (FV) complex results in a rapid replication of the pathogenic retrovirus in immunocompetent adult mice, and various mouse strains exhibit different susceptibilities to FV-induced disease development. The availability of inbred, recombinant, mutant, transgenic, and gene-targeted strains, along with monoclonal antibodies (Ab) specific for various cell-surface markers and viral proteins, made this model an ideal system to dissect at the molecular level the role of each different host factor in conferring resistance to retroviral infections. In this review, we first summarize the knowledge we have obtained through the analyses of the above Friend virus model and proceed to the relevance and extension of this mouse model to HIV infection in humans.

Friend murine leukemia virus and host genes that modulate its replication and pathogenicity

Host mechanisms that affect retroviral replication and pathogenicities have been most extensively studied in the mouse model of FV infection [7,8]. FV is composed of replication-competent Friend murine leukemia helper virus (F-MuLV) and defective spleen focus-forming virus (SFFV), the latter of which induces rapid growth and terminal differentiation of infected erythroid progenitor cells. FV is known to induce fatal erythroleukemia associated with severe immunosuppression when inoculated into immunocompetent adult mice of susceptible strains. An advantage of the FV system in analyzing host factors that affect retroviral replication and pathogenesis is that FV causes the rapid development of readily detectable splenomegaly associated with severe polycythemia, and ultimately induces the development of fatal leukemia within a few months after inoculation into adult mice. Most other retroviruses cause neoplastic and/or neurological diseases only when they are inoculated into neonatal mice, and it usually takes more than 6 months for the virus-induced disease to develop. Inoculation of neonatal mice with an exogenous retrovirus causes immunological tolerance, and this induction of immune unresponsiveness and the resultant persistence of viremia are crucial for the development of the above pathologies. On the contrary, FV can cause splenomegaly and fatal leukemia even when the virus is inoculated into immunocompetent adult mice, making the analyses of host immune responses and their modifications by preventative and therapeutic interventions possible. In addition, the presence of inbred strains has made the genetic analyses of host factors with the FV model much easier than those performed with other retroviruses infecting non-mouse species. In fact, the FV system has led the genetic and molecular analyses of host factors in retroviral infections for decades, and the knowledge obtained through the analyses of the FV system has recently been applied and expanded to human retroviral infections [9,10].

Cellular factors that directly interfere with retroviral replication

Infection with a retrovirus starts with the attachment of virions to the surfaces of target cells, followed by the fusion of the viral envelope with the cellular membrane. The process of viral entry into target cells is mediated by the interactions between the viral envelope glycoprotein and cellular receptors. The presence of an appropriate receptor that can interact with the incoming viral envelope glycoprotein is required for efficient entry of infectious retrovirus particles into target cells. There are four major groups of mouse retroviruses, which differ in their ranges of host specificities: ecotropic viruses infect only mouse and rat cells, xenotropic viruses infect non-mouse cells, amphotropic viruses infect both mouse and non-mouse cells, and polytropic (or mink cell focus-forming, MCF) viruses show variable infectivities to mouse and non-mouse cells (reviewed in [11]). Infectious polytropic viruses are recombinants between infectious ecotropic and endogenous polytropic viruses, which carry varying segments from the proviral genome of an endogenous polytropic virus including the *env* gene [12]. Cellular proteins involved in the entry of murine leukemia virus (MuLV) particles exhibiting each of the above different host ranges have been identified (reviewed in [13]): ecotropic MuLV use a cationic amino acid transporter (CAT1) [14–16], xenotropic and polytropic viruses share a common receptor, XPR1, which shows orthomorphic variations in its amino acid sequence between species [17–20], and amphotropic MuLV utilize the sodium-dependent phosphate transporter, Pit2 [21,22]. A closely related type III phosphate receptor, Pit1, is utilized as the entry receptor by some recombinant MuLV [23]. CAT1 receptor orthologues expressed on the surfaces of cells from species other than mice or rats do not mediate the entry of ecotropic MuLV, due to the changes in critical amino acid residues in an extracellular loop. MuLV that utilize XPR1

as the entry receptor show wide variations in their capabilities to infect different cell lines. They can be grouped into three categories: xenotropic viruses that cannot utilize mouse XPR1, polytropic viruses that utilize both mouse and non-mouse XPR1, and SL3-2 MuLV that can utilize mouse XPR1 alone [24]. Viral envelope proteins of retroviruses that belong to the same host range group interfere with each other: thus, cells productively infected with an ecotropic virus cannot be superinfected with another ecotropic virus, because of the receptor blockade. Similarly, initial infection of cells with a xenotropic MuLV results in a complete resistance to subsequent infection with xenotropic as well as polytropic MuLV isolates. In reciprocal experiments, however, initial infection of cells with a polytropic MuLV only results in reduced titers of xenotropic MuLV, indicating a non-reciprocal pattern of interference. This non-reciprocal interference is accounted for by the presence of two distinct entry determinants on XPR1, one that can be utilized by both xenotropic and polytropic MuLV and the other that can be utilized only by xenotropic MuLV [25]. Similar non-reciprocal interference has been observed between amphotropic MuLV and the 10A1 isolate: amphotropic MuLV utilize Pit2 as the entry receptor, while the 10A1 utilizes both Pit1 and Pit2. Thus, the 10A1 envelope blocks the entry of amphotropic MuLV, but the envelope glycoprotein of an amphotropic virus cannot block infection with the 10A1 [26]. One of the host genes that restrict the replication of FV, the resistant allele of the *Fv4* locus, is an endogenous retroviral *env* gene closely related to an MuLV found in Asian wild mice [27,28]. The product of the $Fv4^r$ allele directly interacts with mouse CAT1 and blocks the entry of incoming ecotropic viruses.

Once the virion surface proteins interact with an appropriate cellular receptor and the viral envelope fuses with the cell membrane, the processes of uncoating and reverse transcription start immediately through the changes of pH and Mg^{++} or Mn^{++} concentrations and the resultant activation of the viral RNA-dependent DNA polymerase. Reverse transcription is believed to take place in the cytoplasm after the derivation of viral core from the infecting virions [29,30], although there is evidence of limited DNA synthesis within virions prior to infection [31–33]. At this stage, cellular cytidine deaminases that are members of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) family show broad antiretroviral activities. Thus, after the penetration of a retroviral nucleocapsid into target cells and the initiation of reverse transcription of the RNA genome into DNA, APOBEC enzymes can induce the conversion of cytosine to uracil in the minus strand viral cDNA, leading to a failure in reverse transcription and to a high number of G-to-A mutations in the integrated proviral genome, which greatly reduces viral efficiency in its replication [34,35]. Human APOBEC3G and APOBEC3F are shown to restrict the replication of *vif*-deficient human immunodeficiency virus type-1 (HIV-1) as well as mouse retroviruses [34–36]. HIV-1 Vif protein counteracts the activity of APOBEC3G by forming a complex with it in the cytoplasm and by impeding its packaging into virions, thus preventing editing mutations upon infection of the newly generated viral particles. The interaction with Vif also stimulates APOBEC3G degradation by the ubiquitine–proteasome pathway, resulting in the increase in viral replication [37–39].

Supposedly, mouse retroviruses have evolved to evade the natural host's APOBEC3, by the exclusion of mouse APOBEC3 from the virions and by the cleavage of incorporated mouse APOBEC3 with viral protease after virion maturation [40,41]. An isoform of mouse APOBEC3 that lacks the exon 5, however, has been shown to be incorporated into MuLV particles more efficiently than the full-length one [41]. Further, mouse APOBEC3 inhibits the replication of mouse mammary tumor virus, a betaretrovirus, both *in vitro* and *in vivo* [42]. Our recent experiments (Takeda E, Tsuji-Kawahara S, Miyazawa M, in preparation) have indicated that certain strains of mice that are naturally resistant to FV-induced disease development preferentially express the exon 5-lacking isoform of mouse APOBEC3.

After reverse transcription, the process of the integration of double-stranded viral DNA into cellular chromosomes is mediated by a complex of the double-stranded viral DNA and viral core proteins (reviewed in [29,30]). The preintegration complex (PIC) composed of the viral DNA, *gag*- and *pol*-encoded viral proteins, as well as some cellular proteins, is transported into the nucleus, and viral integrase mediates the nick formation in the chromosomal DNA and subsequent proviral integration [29,30,43]. The product of the host resistance gene *Fv1* interferes with the process of proviral integration through interaction with the PIC, presumably by retaining the PIC within the cytoplasmic compartment [44]. The *Fv1* is the *gag* gene of a polymorphic endogenous retrovirus that belongs to a new member of mouse endogenous viruses (MuERV) possessing similarities to a group of human endogenous retroviruses, HERV-L [44,45]. Ecotropic mouse retroviruses are divided into two major groups depending on their infectivity to cells of two different strain groups: one represented by cells derived from NIH Swiss mice (N-type) and the other represented by cells of BALB/c origin (B-type). N-tropic mouse retroviruses infect N-type cells more efficiently, while B-tropic viruses infect B-type cells more efficiently. The resistance is dominant, thus (BALB/c × NIH Swiss)F_1 mice are resistant to both N-tropic and B-tropic viruses. Some tissue culture-adapted viruses can infect both N-type and B-type cells with comparable efficiencies, and are called NB-tropic. As expected, the two alleles at the *Fv1* locus, $Fv1^n$ and $Fv1^b$, which determine the N- and B-types of cellular permissiveness, respectively, have been identified. They differ by a few mutations: amino acid substitutions at residues 358 and 399 and the different sequences in the C-terminus due to a deletion in the $Fv1^n$ allele [44,46]. A single amino acid within the *gag*-encoded CA protein is mainly responsible for determining this cell tropism [47], although other residues also modulate the *Fv1* restriction [48,49].

Another cell-autonomous factor that operates prior to the above process of reverse transcription is TRIM5α, which seems to interfere with the process of uncoating (reviewed in [43]). Human TRIM5α restricts the replication of N-tropic, but not B-tropic, MuLV. Various primate TRIM5α's restrict a wide range of retroviruses, but lack activity against the viruses normally found in the same species. The major determinant for MuLV susceptibility to human TRIM5α has been identified to be the same as that for Fv1, although other CA residues differentially govern the resistance to Fv1 and human TRIM5α [43,49].

Molecular mechanisms of Friend virus-induced disease development

Upon inoculation of FV complex, the virus replicates first in vascular endothelial cells and then reaches the hematopoietic cells of the bone marrow [50]. The product of the SFFV *env* gene, gp55, forms a complex with erythropoietin receptor (EpoR), and this interaction induces the growth and terminal differentiation of erythroid progenitor cells (Fig. 1). EpoR is expressed in erythroid cells at the stages later than burst-forming unit of erythroid (BFU-e) and is mainly effective in the growth regulation of the colony-forming unit of erythroid (CFU-e) [7]. Thus, the initial targets of FV infection are CFU-e in the bone marrow, and infected CFU-e migrate into the spleen, continue to divide and differentiate, and are sequestered there. This process is responsible for the development of initial splenomegaly and an increased hematocrit value (polycythemia). The transduction of growth signals from the gp55-EpoR complex also requires the involvement of another molecule, sf-STK, a short form of a kinase-type hematopoietic growth factor receptor, STK [51,52]. Due to mutations in the intron of the *Stk* gene, mice of C57BL background lack the expression of sf-STK, although they express the full-length STK receptor. This lack of sf-STK renders C57BL/6 (B6) and C57BL/10 (B10) mice resistant to the development of massive splenomegaly otherwise induced soon after FV inoculation. This genetic factor found in C57BL mice was first described as an FV-resistance locus, *Fv2* [53]. The recessive resistant allele $Fv2^r$ is actually the above-mutated *Stk* that results in the lack of sf-STK.

It has been widely accepted that SFFV-induced erythroid cell proliferation is crucial for the development of FV-induced erythroleukemia [7,8]. Upon inoculation of susceptible strains of mice with FV, SFFV gp55, the product of the *env* gene, induces rapid proliferation of infected erythroid progenitor cells and resultant splenomegaly. Since the stimulation of erythroid progenitor cells through EpoR induces not only their growth but terminal differentiation, the process of erythroid cell expansion is self-limited, and progenies of SFFV-infected erythroid cells ultimately stop dividing and mature into red blood cells, causing a high hematocrit value. Thus, even continuous rounds of infection of erythroid progenitor cells with FV cannot result in the development of leukemia. For the development of malignant transformation, changes in the expression of host genes due to proviral integration into specific chromosomal locations are required. Rapid expansion in the number of erythroid progenitor cells is believed to provide a large enough pool of target cells for repeated integration of SFFV and F-MuLV proviruses, and resultantly increases the chances for the proviruses to "hit" cellular genes by the promoter insertion adjacent to an oncogene or by the disruption and silencing of a tumor suppressor gene [54,55]. However, the proposed relationships between SFFV-induced early expansion of erythroid progenitor cells and malignant transformation due to proviral integration still remain circumstantial: for instance, it has not been proven that the SFFV-induced proliferation of erythroid cells is a prerequisite for allowing repeated proviral integration and resultant disease-inducing "hits." In this regard, it has been shown that even $Fv2^{r/r}$ B6 mice can develop FV-induced leukemia when T-lymphocytes are absent [56]. There are, therefore, definitely interactions between host immune responses to FV-infected cells and the progression from the SFFV-induced erythroid cell proliferation to malignant transformation of virus-infected erythroid cells.

Host genes that control immune responses to FV infection

Even when FV is inoculated to mouse strains that share the same susceptible genotypes at the above host loci regulating FV replication and SFFV-induced cell growth, the rate and tempo of the development of splenomegaly and fatal leukemia can change drastically depending on the host's genotypes at other genetic loci, which regulate immune

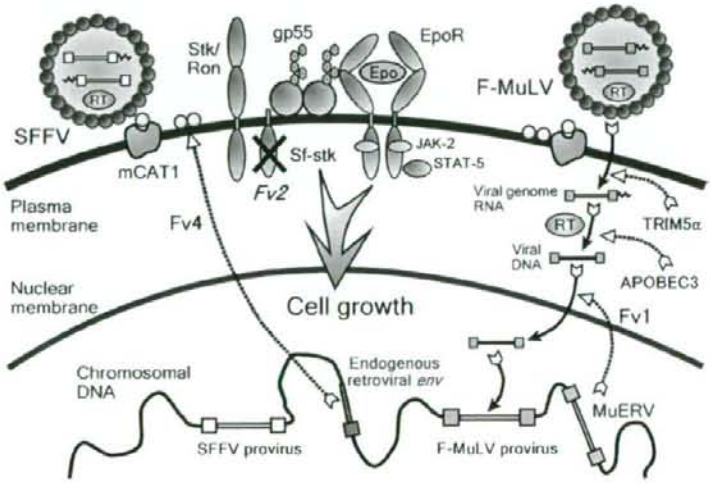

Figure 1 A schematic summary of the mechanisms of FV-induced erythroid cell growth potentiation and cellular factors that interfere with FV infection. Figure modified from that which appeared in [10].

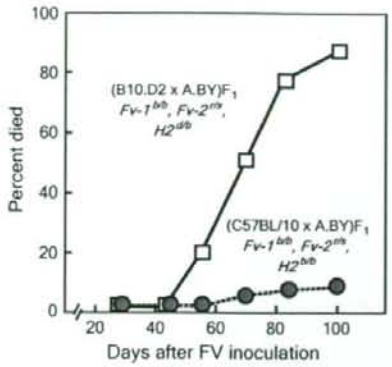

Figure 2 Differences in the tempos of leukemia development after FV infection between two strains of mice sharing the same *Fv1*, *Fv2*, and *Fv4* genotypes. Mice were infected with 3000 SFFU FV. The experiments were performed as described previously [8,59].

responses to FV antigens. For example, (B10 × A.BY)F_1 and (B10.D2 × A.BY)F_1 mice share the same (B10 × A)F_1 background, and thus share the same susceptible $Fv1^{b/b}$, $Fv2^{r/s}$, and $Fv4^{s/s}$ genotypes regarding FV infectivity and SFFV-induced erythroid cell proliferation. (B10 × A.BY)F_1 mice are, nevertheless, highly resistant to FV infection, and only <10% die within 100 days after inoculation of a high dose of FV in the range of 1,500 to 30,000 spleen focus-forming units (SFFU), while >80% of (B10.D2 × A.BY)F_1 mice die within 100 days after FV inoculation (Fig. 2). These two strains differ only at the major histocompatibility complex (MHC) loci: (B10 × A.BY)F_1 mice possess the homozygous $H2^{b/b}$ haplotype, while (B10.D2 × A.BY)F_1 mice possess $H2^{d/b}$. Genes within mouse MHC that influence the incidence and tempo of the development of FV-induced splenomegaly and fatal leukemia have been mapped by utilizing *H2* congenic, recombinant, and mutant strains [8,57–60], and their effects are summarized in Table 1. The presence of a resistant allele both at the class I and class II loci are required for full resistance against FV-induced disease development [58]. The requirement of both CD4$^+$ and CD8$^+$ T-cell subsets for the spontaneous immune resistance of $H2^{b/b}$ mice to FV-induced disease development has been further substantiated by the results of Ab-mediated T-cell depletions [61]: the otherwise resistant mice, depleted of CD8$^+$ T-cells, developed rapid splenomegaly, while the same strain of mice, depleted of CD4$^+$ T-cells, showed initial recovery from splenomegaly, but ultimately developed leukemia and died at a high frequency. Further, administration of monoclonal Ab reactive to the class II E molecule, but not Ab reactive to the A molecule, abrogated the spontaneous resistance against FV infection [62], indicating a critical role of E^b-restricted, rather than A^b-restricted, T-helper cell responses in immune resistance against FV infection. Interestingly, mice lacking the resistance-associated *b* allele at the class II loci, as well as those depleted of CD4$^+$ T-cells, nevertheless showed spontaneous recovery from the initial development of splenomegaly [58,61]. These results suggest that effector functions of CD8$^+$ T-cells are required in the earlier stages and those associated with CD4$^+$ T-cells in the later stages of the FV-induced disease development, together exerting the full immune resistance against FV infection.

Epitopes recognized by CD4$^+$ and CD8$^+$ T-cells have been identified in the *env* and *gag* gene products of F-MuLV [63–70]. Among these, an *env*-encoded, class II $E^{b/d}$-restricted peptide, VYSQFEKSYRHKR, and a *gag*-encoded class II-restricted peptide, IVTWEAIAVDPPP, identified within the matrix protein (MA), were effective in inducing protective immunity against FV challenge when given as a vaccine to $H2^{a/b}$ or $H2^{d/b}$ strains of mice [68,71–73]. In the cases of both of these single-epitope peptides, virus-neutralizing Ab were not detectable before FV challenge, although non-neutralizing anti-MA Ab were detectable in mice immunized with the gag peptide. The production and IgM-to-IgG class-switching of virus-neutralizing Ab were observed earlier in peptide-immunized than in unimmunized control mice after FV challenge, indicating an advantage of CD4$^+$ T cell-priming vaccines in facilitating virus-reactive Ab production. The production of virus-neutralizing Ab is crucial for the effectiveness of the above peptide-induced immune protections, and in fact, mice lacking B cells were not protected, despite the efficient priming of CD4$^+$ T-cells with the peptide and the activation of both CD4$^+$ and CD8$^+$ T-cells upon FV challenge [73].

Effects of coinfection with lactate dehydrogenase-elevating virus on host immune responses to FV infection

The stocks of FV complex used for the analyses of host immune responses by us and other groups had been prepared

Table 1 Host genes that influence immune responses to FV infection

Gene locus	Chromosomal location	Resistant allele	Susceptible allele	Phenotype influenced
Rfv3	15	$Rfv3^r$ (C57BL)	$Rfv3^s$ (A/WySn)	Recovery from viremia, kinetics of neutralizing Ab production
Rfv1	17, *H2D*	D^b	D^d, D^k, D^q, D^{dm14}	CD8$^+$ T-cell recognition of viral epitopes
Rfv2	17, *Q/TL*	$Qa1^a$	$Qa1^b$	NK susceptibility
H2A	17	A^b	A^d, A^k, A^{bm12}	T helper cell responses to viral epitopes
H2E	17	E^b (hybrid)	E^k, E^d	T helper cell responses to viral epitopes

as spleen homogenates from FV-inoculated BALB/c mice. This *in vivo* passage was required to obtain a high enough titer of SFFV infectivity (measured as SFFU), which can induce initial splenomegaly and fatal leukemia in mice of the above (B10 × A)F_1 background. The repeated *in vivo* passage of FV preparations, however, made them vulnerable to possible contamination with other viruses of insidious nature. In fact, FV complexes that had been used by several groups were shown to be contaminated with lactate dehydrogenase-elevating virus (LDV) [74,75]. LDV is a small, enveloped RNA virus belonging to the order *Nidovirales* (reviewed in [76]). Soon after infection, the virus establishes a highly productive and lytic infection of a subpopulation of macrophages. It has been postulated that LDV infection of the permissive macrophage subpopulation involves receptor-mediated endocytosis. Plasma LDV titers rapidly increase to up to 10^{10} of 50% infectious dose (ID_{50}) per ml even at 1 day post-infection. Plasma viral load decreases in the following weeks, but despite the continuous production of anti-LDV Ab and transient activation of cytotoxic T-lymphocytes (CTL), LDV establishes persistent infection, resulting in life-long viremia and sustained formation of immune complexes. Although the infection with LDV is lytic, as mentioned above, persistent infection is maintained by the replication of LDV in new macrophages continuously regenerated from non-permissive progenitor cells. The destruction of macrophages that normally clear lactate dehydrogenase from systemic circulation is responsible for the elevated levels of this enzyme in the serum [76].

The effects of the rapid replication of LDV and the destruction of the macrophage subpopulation on host immune systems are profound: concanavalin A- and anti-CD3 Ab-induced T-cell proliferation and interleukin (IL)-2 production are almost completely suppressed at 2–4 days after LDV infection [76]. Conversely, higher levels of interferon (IFN)-γ production and polyclonal activation of IgG2a-producing B cells are induced in the first week of LDV infection [77,78]. In our recent confirmation, FV preparations that were contaminated with LDV induced readily detectable activation of both $CD3^+$ T and NK cells at as early as 18 h after inoculation, while such early activation of the immune cells was not detected when the same strains of mice were infected with an LDV-free stock of FV (Fig. 3, Table 2). LDV also causes rapid production of type-I IFN [74], which are not attributable to FV. Since the T-cell responsiveness to the signal through CD3 is suppressed, LDV contamination of FV preparations significantly modifies the rate and tempo of the disease development. In fact, it was shown even nearly four decades ago that inoculation of LDV 2 days prior to FV drastically enhanced the SFFV-induced spleen focus formation [79]. A recent report [75] further demonstrated that coinfection with LDV suppressed the proliferation of and IFN-γ production from FV-reactive $CD8^+$ T-cells at 7 days after FV inoculation, and thereby delayed and prolonged the expansion of FV-producing cells in the spleen of (B10 × A.BY)F_1 mice. Interestingly, however, the kinetics of the production of virus-neutralizing Ab and $CD4^+$ T-cell responses were not affected.

To evaluate the effect of LDV contamination into FV stocks on the results of our immune protection experiments, we re-performed similar experiments with an LDV-free preparation of FV. As expected from the observed minimal activation of T cells at 18 h after inoculation of a low titer of FV (Fig. 3), the incidences of initial polycythemia and splenomegaly were the same as those observed previously when highly susceptible CB6F_1 mice were inoculated with a low dose of FV (Fig. 4A). Further, a single injection of the peptide vaccine that harbored the E^b-restricted T-helper cell epitope protected the immunized animals from the development of polycythemia and massive splenomagaly, reproducing the previous results [72,73]. On the other hand, when (B6 × A/WySn)F_1 mice were infected with a high dose

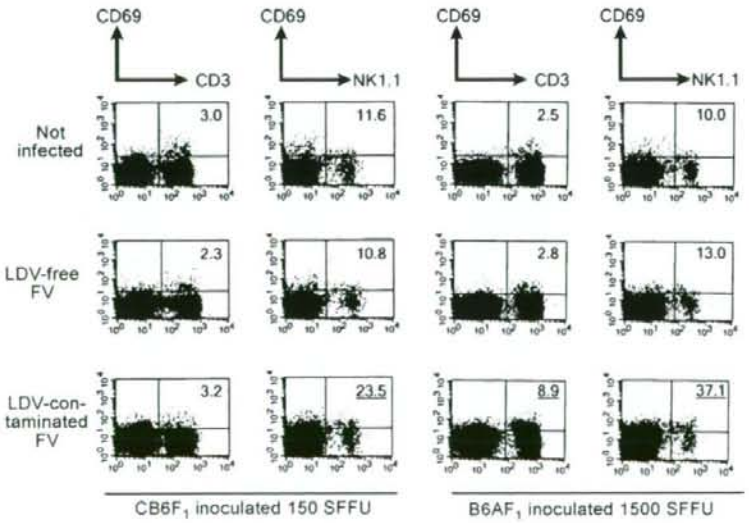

Figure 3 Activation of $CD3^+$ T and NK cells at 18 h after inoculation with FV or an FV preparation containing LDV. Percentages of the cells expressing the early activation marker CD69 among $CD3^+$ T or NK cells are shown in each upper right quadrant. FV inoculation, spleen cell preparation, and flow cytometric analyses were done as described previously [72,73].

Table 2 Percentages of CD69$^+$ activated cells in the spleen at 18 h after FV infection[a]

Mouse strain	FV stocks inoculated[b] (dose)			Uninfected control
	FV stock Saya-9.3	FV stock 45-12B	LDV-free FV	
$CB6F_1$ ($n=5$ each)	3.34 ± 1.02(150 SFFU)	$14.3 \pm 1.98^*$(70 SFFU)	1.46 ± 0.08(150 SFFU)	1.52 ± 0.12
$(C57BL/6 \times A/WySn)F_1$ ($n=3$ each)	8.93 ± 4.01 (1500 SFFU)	$33.1 \pm 8.50^*$ (700 SFFU)	1.23 ± 0.15 (1500 SFFU)	1.43 ± 0.09

[a] Lymphocyte fractions expressing the early activation marker CD69 were determined by flow cytometry as described previously [73].
[b] Stock 45-12B was prepared from the spleens of BALB/c mice infected with an FV stock prepared at the Rocky Mountain Laboratories in 1986. Stock Saya-9.3 was prepared by inoculating BALB/c mice with the stock 45-12B in 2004. A tissue culture-derived FV stock free of LDV contamination was kindly provided by K. Hasenkrug, NIH, NIAID, Laboratory of Persistent Viral Diseases, and was passed once in BALB/c mice. The stock used here was confirmed to be LDV-free by the lack of detectable LDV genomic sequence by polymerase chain reaction.
* Significantly higher in comparison with the percentages of CD69$^+$ cells in mice infected with the LDV-free FV: $p < 0.05$ by Welch's t-test.

of LDV-free FV, hematocrit values were variable between the infected animals, and most mice did not show significant enlargement of the spleen at 5 weeks after FV inoculation (Fig. 4B). FV-induced polycythemia and splenomegly were only observed in less than half of the unimmunized $(B6 \times A/WySn)F_1$ mice; however, the development of these pathologies was prevented to a significant degree by immunizing another group with the gag-derived peptide. The former observations are different from the much higher rate of splenomegaly observed previously by inoculating an LDV-contaminated FV [68]. In fact, it has been repeatedly described that mice of the $H2^{a/b}$ $(B10.A \times A.BY)F_1$ hybrid are "susceptible" to a high dose of FV and develop leukemia [8,59,61,80,81]. These experiments were performed by using LDV-contaminated FV ([75] and Hasenkrug K, Chesebro B, personal communication). It should be noted, however, that spontaneous recovery of $(B10.A \times A.BY)F_1$ or $(B10 \times A/WySn)F_1$ mice from even a high dose of FV inoculation, and variability in hematocrit values and spleen weights in these strains of mice after FV infection, were noted [71]. Thus, it is highly conceivable that the above $H^{a/b}$ strains are capable of spontaneously recovering from initial splenomegaly at a high rate, even after inoculation with a high dose of FV, but their spontaneous resistance was hindered, at least partially, by unintentional coinfection with LDV in the previous experiments. The revealed resistance of the $H2^{a/b}$ mice further substantiates the increased susceptibility of the $H2^{d/b}$ mice (Fig. 2), which was attributed to the difference in the class Ib *Qa1* alleles between the two H2 haplotypes [59]. Nevertheless, the results of the immuno-

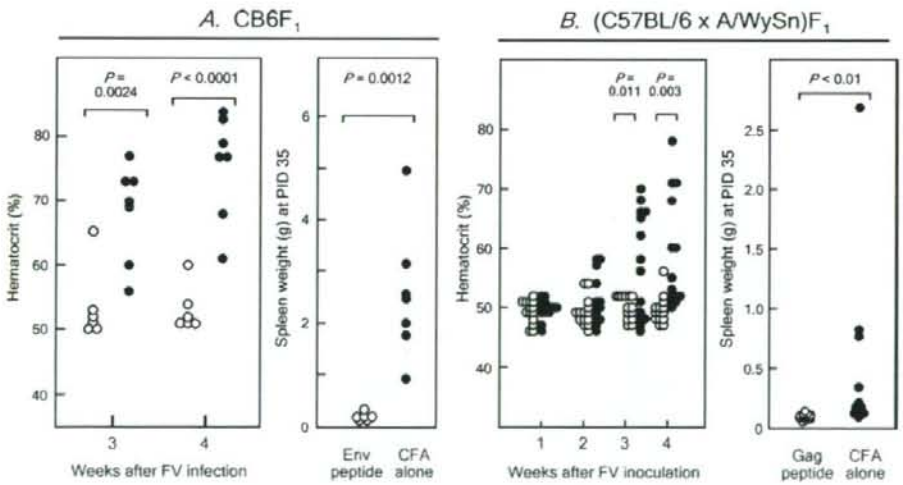

Figure 4 Development of polycythemia and splenomegaly in FV-infected $CB6F_1$ and $(B6 \times A/WySn)F_1$ mice and their prevention by immunization with peptide vaccines harboring a single T-helper cell epitope. (A) Highly susceptible $CB6F_1$ mice were either immunized with the *env*-encoded T-helper cell epitope (○) or given an adjuvant alone (●). Four weeks later, they were challenged with 300 SFFU FV. (B) $(B6 \times A/WySn)F_1$ mice were either immunized with the *gag*-encoded T-helper cell epitope (○) or given an adjuvant alone (●). Four weeks later, they were challenged with 3000 SFFU FV. Peptide immunization, FV challenge, and evaluations of the disease development were performed as described previously [68,71–73].

genetic experiments performed by using susceptible $H2^{a/a}$ and resistant $H2^{a/b}$ strains of mice and a low dose of FV [58] need not be changed, and the analyses of antigenic and epitope specificities of T-cell responses performed by using recombinant vaccinia viruses and synthetic peptides [57,64–70] are not affected at all.

Are cytotoxic T-cells required for vaccine-induced antiretroviral protection?

It has been postulated that CD8$^+$ cytotoxic T-lymphocytes (CTL) are crucial for the elimination of retrovirus-infected cells, and a large majority of HIV vaccine candidates are developed by aiming at efficient induction of CTL responses (reviewed in [82]). In fact, HLA class I genotypes are associated with resistance to the development of AIDS [3,5,83–85], and a successful confinement of simian immunodeficiency virus (SIV) replication in a preclinical vaccine trial has been observed in macaques possessing a particular MHC haplotype [86]. The emergence of a functionally defective mutation under a selective pressure exerted by CTL has been postulated [86]. It has been unclear, however, if cytotoxic effector functions of CD8$^+$ T-cells are directly required for the observed protection, or if other effector mechanisms activated through the production of cytokines/chemokines from primed CD8$^+$ T-cells are more important. Further, MHC class I molecules not only present viral epitopes to CD8$^+$ CTL, but they also regulate natural killer (NK) cell functions through interactions with inhibitory and activating NK receptors (reviewed in [87,88]). A particular allele in human killer immunoglobulin-like receptor (*KIR*) loci has been associated with delayed development of AIDS after HIV-1 infection [84]. Likewise, the *Rfv2* locus that is associated with the susceptibility of $H2^{d/b}$ (B10.D2 × A.BY)F_1 mice to FV infection (Fig. 2) has been mapped to *Qa1* [59], which encode a class Ib molecule that regulates NK cell activation [87,88].

In this regard, it has been enigmatic that the homozygosity at the class I locus for the responder D^b allele is required for spontaneous resistance to a high dose FV [60,89]. If the class I D^b molecule functions solely as a restriction element for the presentation of an FV epitope, a single allele should be enough for the immune responsiveness. When a low dose of FV was utilized, this was the case, and heterozygous $H2^{a/b}$ mice recovered spontaneously from the initial development of splenomegaly [58,59]. When inoculated with a high dose of FV, however, only the mice that possessed two D^b alleles showed recovery from initial splenomegaly [8,59,60]. This

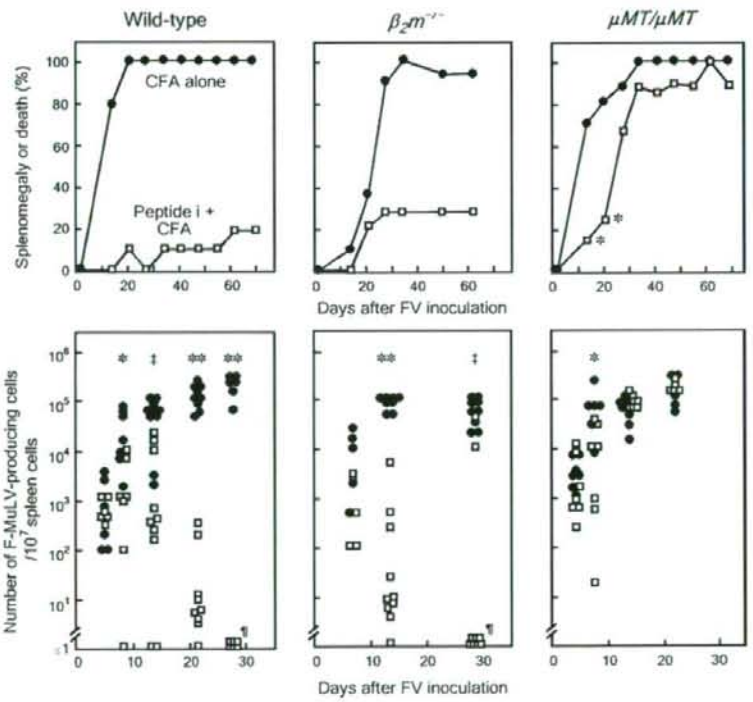

Figure 5 Development of splenomegaly or fatal leukemia (upper panels) and changes in the numbers of FV-producing cells in the spleen (lower panels) in wild-type, β_2-microglobulin-deficient ($\beta_2m^{-/-}$) and B cell-deficient ($\mu MT/\mu MT$) $CB6F_1$ mice. Mice were either immunized with peptide i that harbored the *env*-encoded T-helper cell epitope (□), or were given complete Freund's adjuvant (CFA) alone (■). See [73] for details. In the upper right panel, (*) significant differences in the frequency of splenomegaly between the immunized and control groups ($p < 0.001$). The cells prepared from the entire spleen (>10^8) at PID 28 were inoculated as infectious centers to ensure the lack of detectable virus-producing cells. Infectious centers were undetectable from any of the tested animals indicated with ¶at PID 28. Statistical significance of the difference between the immunized and control groups at each time-point was examined: *$p < 0.04$; **$0.001 < p < 0.01$; ‡$0.0002 < p < 0.001$.