Introduction

The innate arm of the immune system could critically shape the adaptive immune response against pathogens. Major efforts to understand these innate immune mechanisms against HIV-1 resulted in the identification of restriction factors such as TRIM5α [1] and APOBEC3G [2], but how these factors shape adaptive immune responses against HIV-1 is difficult to study in humans in vivo. In contrast, the interplay between innate and adaptive immunity has been studied extensively in the Friend retrovirus (FV) infection model [3–5]. FV is a complex of a replication-defective Spleen Focus Forming Virus (SFFV), that competent Friend Murine Leukemia Virus (F-MuLV) and a (FV) infection model [3–5]. FV is a complex of a replication-immunity has been studied extensively in the Friend retrovirus (FV) infection model [3–5], understanding the impact of Fv1 incompatibility as a retroviral vaccine paradigm in mice. Due to its susceptibility to disease that allows for pathogenic challenge studies, B-tropic FV infection of 129P2 mice may be a useful model to study the immunological pathways induced by retroviral capsid restriction.  

Abstract

Understanding the host genetics of the immune response in retrovirus infection models could provide insights for basic HIV vaccine discovery. In Friend retrovirus (FV) infection of mice, Fv1 differentially inhibits N-tropic versus B-tropic FV infection by mediating a capsid-dependent post-entry block, Fv2 susceptibility governs splenomegaly induction, and Rfv3 resistance primes a stronger neutralizing antibody response due to more potent Apobec3 activity. Apobec3 polymorphisms in inbred mouse strains correlate with Rfv3 resistance and susceptibility, with one unresolved exception. The 129OlaHsd (129P2) mouse strain is Fv2 and Rfv3 susceptible based on genotyping, but infection of 129P2 mice with B-tropic FV resulted in strong neutralizing antibody responses and no splenomegaly. Here we confirm that 129P2 mice are Fv1\textsuperscript{3/4},\textsuperscript{b/b}, explaining its resistance to B-tropic FV. Infection of 129P2 mice with NB-tropic FV, which can efficiently infect mice independent of Fv1 genotype, resulted in severe splenomegaly, high levels of viremia and weak neutralizing antibody responses regardless of Apobec3 status. Notably, high-dose B-tropic FV infection of 129P2 Apobec3-deficient mice induced significant adaptive immune responses and conferred high levels of protection following challenge with pathogenic NB-tropic FV. This immunological protection complemented previous studies that N-tropic FV can act as a live-attenuated vaccine in Fv1\textsuperscript{3/4},\textsuperscript{b/b} mice. Altogether, the results obtained in 129P2 mice strengthen the conclusion that Rfv3 is encoded by Apobec3, and highlight Fv1 incompatibility as a retroviral vaccine paradigm in mice. Due to its susceptibility to disease that allows for pathogenic challenge studies, B-tropic FV infection of 129P2 mice may be a useful model to study the immunological pathways induced by retroviral capsid restriction.
susceptible allele in vivo. However, it remains unknown whether the Rfv3 susceptible Apobec3 allele could promote recovery from FV viremia and NAb responses compared to Apobec3-deficient mice.

The Apobec3 polymorphisms in the inbred mouse strains used to define Rfv3 are highly concordant. However, the Apobec3/Rfv3 status of a more recently studied mouse strain in the FV infection model, 129/OlaHsd (129P2 [20]; cited previously as 129/Ola [7]), remains unclear. 129P2 is a substrain of a diverse family of inbred mice under the generic 129 background ([20–22]; Fig. 1A), and was used extensively for gene-targeting studies, including Apobec3 [7]. In a previous study, 129P2 mice were classified as Rfv3 resistant because high-dose infection with B-tropic FV resulted in viremia and NAb responses by 28 days post-infection (dpi), similar to B6 mice [7]. However, quantitative PCR data showing that 129P2 mice had relatively high Apobec3 mRNA levels were incorrect [12]. The Fv2 genotype of 129P2 mice also needed to be clarified. Fv2 is a dominant susceptibility gene that dictates splenomegaly induction and is encoded by the Stk gene (Stk) [24]. Fv2 interacts with the erythropoietin receptor in conjunction with the SFFV gp55 protein, resulting in the uncontrolled proliferation of erythroblast precursors that dictate splenomegaly induction and is encoded by the Stk gene ([23,24]). In Fv2 susceptible mice, a 3 nt (GGA) insertion in the Stk intron 10 of Fv2 susceptible strains results in an alternative promoter that drives the transcription of a short-form of the Stk kinase (sf-Stk) [24]. sf-Stk interacts with the erythropoietin receptor in conjunction with the SFFV gp55 protein, resulting in the uncontrolled proliferation of erythroblast precursors that lead to severe splenomegaly [25,26]. Since 129P2 mice did not develop splenomegaly following B-tropic FV infection, 129P2 mice were classified as Fv2 resistant [7]. However, a different 129 substrain, 129X1 (formerly 129/SvJ [20]), was genotyped as Fv2 susceptible [24]. Since 129 mice were long separated from the Fv2 resistant C57BL/6J lineage [20–22,27], it is unlikely that 129P2 mice are Fv2 resistant. However, due to the complex genealogy of the 129 lineage (Fig. 1A) [20–22,27], direct confirmation of the Fv2 genotype of 129P2 mice would be ideal. Recently, infection of 129P2 mice with an N-tropic MuLV strain, CasFrKP, resulted in high viremia levels [28]. These results contrasted from the lack of FV viremia following B-tropic FV infection of 129P2 mice [7]. Thus, the use of a B-tropic [7] versus an N-tropic [28] MuLV strain resulted in divergent infection outcomes in 129P2 mice.

Fv1 is the gene that dictates N- versus B-tropism [29]. Mouse strains classified as Fv1p+/p+ support the replication of N-tropic FV strains, whereas Fv1p−/p− mice support B-tropic FV replication [30,31]. Some FV strains can infect both Fv1p+/p+ and Fv1p−/p− mouse strains efficiently and are considered dual- or NB-tropic. Fv1 incompatibility, such as N-tropic FV infection of Fv1p+/p+ mice, results in a potent post-entry block [30,31] that map to amino acid differences in Fv1 and the F-MuLV capsid [32–35]. Interestingly, another 129 substrain, 129Sv (formerly 129/SvEv [20]) was genotyped as Fv1p+/p+, a variant of Fv1p−/p− [33,36]. Fv1p−/p− restricts B-tropic as well as some N-tropic strains. The Fv1 data on 129Sv [33,36] strongly suggest that 129P2 mice should also be Fv1p−/p−. However, the 129 sublines 129/Rf and 129/Rt that gave rise to 129P2 and 129Sv, respectively, were separated and independently maintained since 1948 (Fig. 1A). Thus, confirmation that 129P2 mice are Fv1p−/p− may be warranted. We therefore re-evaluated the Fv1, Fv2 and Rfv3 genotype status of 129P2 mice and confirmed these genotypes with in vivo infection with a dual-tropic FV strain. The results demonstrate that the previous classification of 129P2 mice as Fv2 and Rfv3 resistant based on phenotypic data [7] was confounded by Fv1 incompatibility. Importantly, this endeavor revealed new insights on the virological and immunological impact of Apobec3 and Fv1 restriction in 129P2 mice, a mouse genetic background that may be useful for basic retroviral vaccine studies.

### Materials and Methods

**Mice**

Wild-type (WT) 129P2 mice were purchased from Harlan Laboratories, Incorporated. Apobec3 knock-out (KO) mice were generated from the C57BL/6J embryonic stem cell line (BayGenomics) that was made in the 129P2 background. The chimeric mouse was backcrossed once to 129P2 to generate a 100% congenic 129P2 Apobec3 KO strain [7]. B6, A.BY and BALB/c mice were obtained from The Jackson Laboratory. Experimental protocols in mice specifically for this study were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver [Permit Number B-89709(10)E]. Infections were performed under isoflurane anesthesia. At indicated time-points, mice were euthanized using a double-procedure consisting of carbon dioxide inhalation followed by cervical dislocation. All efforts were made to minimize suffering.

**Cell culture**

*Mus dunni* and 293T cells were maintained in DMEM (Mediatech) with 2% penicillin/streptomycin/glutamine (Mediatech) and 10% fetal bovine serum (FBS; Gemini).

**Genotyping**

Tail DNA from 129P2, B6 (Fv1b/b Fv2r/r Rfv3r/r) and BALB/c (Fv1b/b Fv2+/r Rfv3r/r) mice were extracted using the DNAEasy Kit (Qiagen). To genotype Fv1, the full-length gene was amplified using a forward primer Fv1.F (5’-AAGCTTGGCCCGCCGGAATTTCG-CAAGGCGGTGTTGCT) and a reverse primer that was either Fv1p−/-specific, Fv1b.R (5’-GAATTCCTAGACTATTAACGTTGCTTGTGATGTTC), or Fv1p−/specific (5’-GAATTCCTAGACTATTAACGTTGCTTGTGATGTTC). PCR products were directly purified (Qiagen), sequenced, and compared to published Fv1 alleles.

To genotype Fv2, we designed primers flanking a 3 nt (GGA) indel in the Stk intron 10 of Fv2 susceptible strains. This indel results in an alternative promoter that drives the transcription of a short-form of the Stk kinase (Stk) [24]. The primers were Stk.Fv2.F (5’-CAGTCCCCCTGATGTCCTCACAAT) and Stk.Fv2.R (5’-CCACGGTCATGTTCGTTACAG). DNA (100 ng) was subjected to PCR amplification in a 50 μl reaction that contained 1X Phusion HF buffer (Thermo Scientific), 0.2 μM primers, 20 μM dNTP (New England Biolabs), and 1 U of Phusion High-fidelity DNA polymerase (New England Biolabs). The cycling conditions

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**Table 1. FV genotype status of various inbred mouse strains.**

| Host strain     | Genotypeb | Fv1 | Fv2 | Rfv3 | References |
|-----------------|-----------|-----|-----|------|------------|
| C57BL/6J (B6)   | b/b       | r/r | r/r | r/r  | [7,12,13]  |
| C57BL/10 (B10)  | b/b       | r/r | r/r | r/r  | [13]       |
| BALB/c          | b/b       | s/s | s/s | s/s  | [7,12,13]  |
| A.BYb           | b/b       | s/s | s/s | s/s  | [7,12]     |
| A/WySnb         | b/b       | s/s | s/s | s/s  | [11,12,13] |
| 129/OlaHsd (129P2) | n/r | n/r | n/r | n/r  | This study |

Fv1 controls capsid-dependent tropism; Fv2 is a dominant susceptibility (s) gene governing splenomegaly; Rfv3 resistance (r) is associated with a stronger NAb response. A/WySn mice also have a defective B-cell activating receptor gene, BAFF-R, that maps near the Rfv3 locus [11,12], but this was not observed in the related A.BY mice [12].

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consisted of a 98°C initial denaturation step for 30 s followed by 36 cycles of 98°C for 10 s, 59°C for 15 s, and 72°C for 15 s, and a final extension step of 72°C for 7 min. The 450-bp PCR products were purified (Qiagen) and directly sequenced.

To genotype Rfv3, a primer pair flanking the X-MLV LTR insertion in the exon 2 splice site of Apobec3 was used for PCR [12,16]. PCR amplicons were sized by agarose gel electrophoresis.

Quantification of Apobec3 mRNA levels

Spleen RNA was extracted using the RNeasy kit and quantified by real-time PCR as described [12] and normalized against GAPDH. Apobec3 mRNA levels from spleens of 2 mice of each strain were quantified in triplicate. Quantitative real-time PCR was performed in a Biorad CFX6 machine.

Virus stocks and FV infection

B-tropic and NB-tropic FV stocks, previously obtained from Kim Hasenkugl, Leonard Evans and Bruce Chesebro at the Rocky Mountain Laboratories, National Institutes of Allergy and Infectious Diseases, were prepared and tiered in BALB/c mice [7,37]. Both stocks contain F-MuLV, SFFV and lactate-dehydrogenase elevating virus (LDV). LDV is an RNA virus endemic in wild mice [38] and was present in the classical FV stocks used to define Fv1, Fv2, H-2 and Rfv3 [39]. Since this study builds on historical FV studies that defined Fv1, Fv2, H-2 and Rfv3, FV/LDV stocks were required. Infections were performed by intravenous injection through the retro-orbital route of 140 to 7,500 Spulen Focus Forming Units (SFFU) of FV. Mock infections were performed using DMEM without FBS. Mice were sacrificed at indicated timepoints. In some experiments, 129P2 Apobec3 KO mice that were previously infected with B-tropic FV were challenged with an NB-tropic strain at 28 dpi.

Infectious viremia

Infectious viremia was determined using a focal infectivity assay involving incubations of infected Mus dunni cells with FV gp70-specific MAb 720 [7,40]. Briefly, serial dilutions of plasma were added into Mus dunni cells seeded the previous day with polybrene, fixed after 2–3 days with ethanol, then MAB 720 supernatant was added for 3 h at 37°C. The fixed cells were washed 3× with TNE buffer (10 mM Tris, 200 mM NaCl, 1 mM EDTA, pH = 7.4) with 0.25% Tween-20 (TBS-T), then 1:500 sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP; Amersham) was added for 3 h at 37°C. The fixed cells were washed 3× with TNE, then developed with aminoethylcarbazole (Sigma) substrate in the presence of H2O2 (Sigma). Foci were counted and expressed as focal infectivity units (FFU) per ml of plasma.

Spleen infectious center assay

A day before harvesting spleens, Mus dunni cells were seeded at 8,000 cells per well in 24-well plates and 32,000 cells per well in 6-well plates in media with polybrene. The next day, spleens were preweighed, and no more than 400 mg were disaggregated and diluted to 10 ml with complete media. For the 6-well plates, 1 ml of spleen suspension was added, while for 24-well plates, the splenocytes were serially diluted 10-fold, adding 100 μl of cell suspension. Infectious centers were detected using the same procedure for the infectious viremia assay described above, using MAB 720 supernatant as the detecting antibody. Foci were counted, multiplied by the dilution factor for the entire spleen, and expressed as spleen infectious centers per spleen.

Plasma viral load

Viral RNA copy numbers were quantified from 10 μl plasma by quantitative real-time PCR [12,41]. T7-transcribed RNA was used as a standard for absolute quantification. The assay has a limit of detection of 107 copies/ml and >95% efficiency.

Endpoint FV-specific IgG titer

Endpoint ELISAs were performed as described [41]. Native FV virions were coated (500 ng/well) into Immulon-4 plates overnight at 4°C, then blocked for 2 h with Superblock (Pierce). Serial 2-fold dilutions of plasma were added onto the plate, incubated at 37°C,
for 1 h, then washed 6× with PBS with 0.25% Tween-20 (PBS-T). Biotinylated goat anti-mouse IgG (Southern Biotechnology) was added at 1:4000 and incubated at 37°C for 1 h then washed 6× with PBS-T. Streptavidin conjugated to HRP was added at 1:4000 and incubated at 37°C for 30 min then washed 6× with PBS-T, followed by the addition of 3,3,5,5-tetramethylbenzidine (TMB) substrate (BioFX). Reactions were stopped with 0.3N H₂SO₄, then read in a Victor X5 (Perkin Elmer) plate reader at 405 nm. Endpoint titers were calculated by interpolating 2× the average background per plate from a best-fit nonlinear regression curve calculated using Prism 5.0 software (GraphPad).

NAb assay

NAb titers were determined by incubating serial dilutions of plasma with 150 FU of N-tropic F-MuLV for 1 h at 37°C and adding a third of the mixture onto plated Mus musculus cells. FV infection foci were counted [7], and 80% inhibitory concentrations were calculated by interpolation from a best-fit nonlinear regression curve using Prism 5.0 (GraphPad).

Flow cytometry

To assess cell-mediated immune responses, splenocytes (10⁶ cells) were stimulated for 3 h with PMA (25 ng/ml) and ionomycin (0.7 μg/ml) (Sigma) at 37°C and simultaneously stained with anti-CD138-PE (281-2), GL7-FITC (GL7) (BD Biosciences) and IgD-CD11c-PE-Cy7 (N418) (eBioscience). To monitor B cell subpopulations, the cells were washed then fixed in 1% paraformaldehyde in PBS. Isotype controls were used for gate construction. Stainings, the cells were washed then fixed in 1% paraformaldehyde in PBS. Isotype controls were used for gate construction. To assess FV restriction, 129P2 mice were infected with NB-tropic FV, which should infect mice regardless of Fv genotype [37]. The mice were sacrificed at 28 dpi (Fig. 2A), because recovery from viremia and NAb responses could be reproducibly detected by this time point [9,10].

Infection of 129P2 WT and Apobec3 KO mice with B-tropic versus NB-tropic FV

High-dose (7500 SFFU) infection of 129P2 mice with B-tropic FV did not induce splenomegaly and resulted in potent NAb responses, prompting the earlier designation of 129P2 mice as Fv1nr and Rfv3nt [7]. However, these results were likely due to Fv1 incompatibility, since 129P2 mice are Fv1ntB/B (Table 1). To overcome a potential Fv1 restriction block, 129P2 mice were infected with NB-tropic FV, which should infect mice regardless of Fv1 genotype [37]. The mice were sacrificed at 28 dpi (Fig. 2A), because recovery from viremia and NAb responses could be reproducibly detected by this time point [9,10]. Consistent with previous data [7], high-dose B-tropic FV infection did not elicit severe splenomegaly in 129P2 mice (median spleen mass = 2.1 g) at 28 dpi. In sharp contrast, NB-tropic FV infection resulted in 10-fold higher splenomegaly (median spleen mass = 2.2 g), despite a 15-fold lower inoculum dose compared to B-tropic FV infection (Fig. 2B). This result confirms that 129P2 mice are Fv1ntB/B.

In a previous study, CasFrKP MuLV that lacks the putative Apobec3 antagonist, Glyco-Gag, replicated to higher levels in 129P2 Apobec3 KO versus WT mice [26]. These results, coupled with data showing that 129P2 Apobec3 can significantly inhibit F-MuLV in vitro [7], suggested that 129P2 Apobec3 is a functional antiretroviral restriction factor. However, the virological and immunological impact of 129P2 Apobec3 in pathogenic FV infection remains unknown. We therefore infected 129P2 WT and Apobec3 KO mice with NB-tropic FV and evaluated infection parameters at 28 dpi. There was no significant difference in splenomegaly between 129P2 WT and Apobec3 KO mice (Fig. 2B). Importantly, plasma viral load (Fig. 2C), infectious viremia (Fig. 2D) and NAb titer (Fig. 2E) at 28 dpi were not significantly different between 129P2 WT and Apobec3 KO mice. Thus, the 129P2 Apobec3 allele did not promote recovery from viremia and NAb responses during pathogenic FV infection, consistent with the genotype status of 129P2 mice as Rfv3nt/B.
tropic FV infection of Apobec3-deficient 129P2 mice resulted in substantial protection from pathogenic B-tropic FV challenge [43–46] (Table 2). N-tropic FV induced potent cellular and humoral immune responses that can be adaptively transferred into naive hosts [45,47]. Thus, N-tropic FV functioned as a live-attenuated vaccine in an Apobec3-incompatible host. However, the reverse scenario with B-tropic FV had not previously been tested.

We therefore investigated if B-tropic FV can function as a live-attenuated vaccine in 129P2 mice. We utilized 129P2 Apobec3 KO mice to ensure that Apobec3 will have no impact on the results. Although the 129P2 Apobec3 allele did not influence NAb responses and recovery from pathogenic FV infection (Fig. 2), 129P2 Apobec3 may still restrict less pathogenic or attenuated MuLV strains [28]. 129P2 Apobec3 KO mice were infected with either mock (DMEM) or high-dose (7500 SFFU) B-tropic FV. At 28 dpi, the mice were evaluated for FV infection and immune responses (Fig. 3A). As expected, B-tropic FV infection of 129P2 Apobec3 KO mice did not induce severe splenomegaly (Fig. 3B) and infectious titers were below the limit of detection (≤600 FFU/ml) in the plasma (Fig. 3C). In contrast, infection of BALB/c mice with 53-fold lower dose (140 SFFU) of the same B-tropic FV stock induced 11-fold higher splenomegaly (Fig. 3B). Moreover, assuming that the infectious titer in B-tropic FV infected 129P2 mice is the limit of detection (600 FFU/ml), the same virus stock induced at least an 900-fold higher infectious viremia in BALB/c mice (Fig. 3C). The relatively mild splenomegaly induced by B-tropic FV infection of 129P2 Apobec3 KO mice (Fig. 3B) suggested that B-tropic FV replicated below the detection limit of the plasma infectious viremia assay (Fig. 3C). We therefore measured FV infection levels using the more sensitive spleen infectious center assay, which involves co-incubating splenocytes with Mus dunni cells. As expected, this assay revealed very high (>10⁷) spleen infectious centers in BALB/c mice, whereas only 2 of 5 129P2 Apobec3 KO mice had detectable signals (Fig. 3D). We also evaluated 129P2 wild-type mice infected with B-tropic FV at 28 dpi and found only 2 of 4 infected mice had detectable spleen infectious centers (data not shown). The mild splenomegaly, undetectable infectious viremia and sporadic detection of spleen infectious centers suggested that B-tropic FV replicated at very low levels in 129P2 Apobec3 KO mice by 28 dpi, and was therefore ‘live-attenuated’.

We next evaluated the induction of immune responses by flow cytometry (Fig. 4A). Compared to uninfected controls, significant induction of IFN-γ in stimulated CD4+ T cells, CD8+ T cells and DX5+NK cells were observed (Fig. 4B). Significant induction of CD107a, a degranulation marker, was observed in CD8+ T cells but not in CD4+ T cells and DX5+NK cells (Fig. 4C). In addition, splenic germinal center (GL7+ IgD+) B cells were significantly induced in mice infected with B-tropic FV (Fig. 4D). Using 28 dpi plasma from 129P2 Apobec3 KO mice infected with pathogenic NB-tropic FV as a reference, we observed similar FV-specific endpoint IgG titers from 28 dpi plasma of B-tropic FV infected 129P2 Apobec3 KO mice (Fig. 4E). However, significantly higher NAb responses (Fig. 4F) were observed in B-tropic FV infected 129P2 Apobec3 KO mice. These findings demonstrate that B-tropic FV infection of 129P2 Apobec3 KO mice induced significant cell-mediated and humoral immune responses.

**B-tropic FV infection of Apobec3-deficient 129P2 mice protects from pathogenic NB-tropic FV challenge**

We next evaluated whether B-tropic FV infection of 129P2 Apobec3 KO mice (Fig. 5) will protect from subsequent infection with pathogenic NB-tropic FV. Mice previously inoculated with either DMEM or B-tropic FV were challenged with pathogenic NB-tropic FV at 28 dpi (Fig. 5A). FV infection levels were determined 7 days later by flow cytometry (Fig. 5B). Significantly lower cellular FV infection in BM cells as well as major BM target cells that include Ter119+ erythroblasts and CD11b+ myeloid cells were observed in B-tropic FV ‘vaccinated’ mice (Fig. 5C). Significant protection was also observed in the spleen, including erythroblasts and B cells (Fig. 5D). Mice previously inoculated with B-tropic FV had undetectable infectious plasma viremia at 7 days post-challenge with NB-tropic FV, whereas mock-infected mice showed high levels of infectious plasma viremia (Fig. 5E). More sensitive plasma viral load assays showed that mice previously inoculated with B-tropic FV had >2500 fold lower viral RNA load in the plasma compared to mock (Fig. 5F). Thus, B-tropic FV acted as a live-attenuated vaccine in Fv1-incompatible 129P2 mice.
Discussion

The Friend retrovirus (FV) infection model was instrumental in the identification of host genes that can influence the retrovirus-specific immune response. One classical gene, Rfv3, influences recovery from FV viremia by modulating the NAb response, and was recently found to be encoded by the innate restriction factor Apobec3. However, the correlation between Apobec3 polymorphisms and Rfv3 genotype is challenged by uncertainties in the Apobec3/Rfv3 genotype of an inbred strain known as 129P2. We therefore resolved the FV resistance genotype and phenotype of 129P2 mice. The results revealed that 129P2 are resistant to B-tropic FV (Fv1nr/nr), are susceptible to splenomegaly (Fv2s/s), and encode an Rfv3-susceptible allele of Apobec3 (Table 1). The latter result provided a unique opportunity to evaluate the Rfv3-susceptible allele of Apobec3. Most studies on Apobec3/Rfv3 have focused on the B6 Apobec3 allele, which exhibit properties such as higher levels of expression that make it more potent in vivo [11-16]. Here we show that the 129P2 Apobec3 allele did not promote recovery from viremia and NAb responses relative to Apobec3-null mice.

Table 2. Retrovirus vaccine studies based on Fv1 incompatibility.

| Year | Host strain | Fv1 | Fv2 | H2 | Rfv3 | Vaccine strainb | Challenge Strainb | Results | Ref. |
|------|-------------|-----|-----|----|------|-----------------|-------------------|---------|-----|
| 1986 | (B10.A × A.BY)F1 | b/b | r/s | a/b | r/s | N-tropic FV complex | B-tropic FV complex | Protection from challenge independent of H2 | [43] |
| 1986 | (B10.A × A/WySnF1)F1 | b/b | r/s | a/a | r/s | N-tropic FV complex | B-tropic FV complex | Protection from challenge independent of H2 | [43] |
| 1998 | (B10.A × A/WySnF1)F1 | b/b | r/s | a/a | r/s | N-tropic F-MuLV helper | B-tropic FV complex | Protection due to immunity, not receptor interference | [44] |
| 1999 | (B10.A × A.BY)F1 | b/b | r/s | a/b | r/s | N-tropic F-MuLV helper | B-tropic FV complex | T-cell protection transferrable to naıve host | [45,46] |
| 2004 | B6 (adoptive transfer) | b/b | r/r | b/b | r/r | N-tropic F-MuLV helper | B-tropic FV complex | Virus-specific antibodies are critical for protection | [47] |
| 2013 | 129P2 Apobec3 KO | nr/nr | s/s | b/b | null | B-tropic FV complex | NB-tropic FV complex | Protection from challenge independent of Apobec3 | This study |

*aH2 dictates cell-mediated immune responses, with the b haplotype being more protective than a. bEntries designated as FV complex correspond to the classical FV stocks that contain F-MuLV helper virus, SFFV and LDV. cWithout SFFV, F-MuLV is nonpathogenic and does not cause splenomegaly.

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![Figure 3. B-tropic FV replication in 129P2 Apobec3 KO versus BALB/c mice.](image-url)
consistent with \textit{Rfv3} susceptibility. Thus, the results resolve issues on the concordance between \textit{Apobec3} polymorphisms and \textit{Rfv3} genotype status with respect to the 129P2 strain, and further strengthen the case for \textit{Apobec3} as the gene encoded by \textit{Rfv3}.

The lack of replication of B-tropic FV in \textit{Fv1}^{nr/nr} 129P2 mice mirrored earlier studies involving N-tropic FV infection of \textit{Fv1}^{b/b} mice (Table 2). These studies showed that N-tropic FV could function as a live-attenuated vaccine in an \textit{Fv1}-incompatible host, resulting in the best immunological protection so far against FV infection [5]. Thus, \textit{Fv1} incompatibility may be an important mechanism to augment retrovirus-specific adaptive immune responses. However, these prior \textit{Fv1}-restricted vaccine studies utilized mice that encode the B6 \textit{Apobec3} allele and are therefore \textit{Rfv3} resistant (Table 2). In other words, the resulting protective adaptive immune response may not be exclusively due to \textit{Fv1} restriction, as B6 \textit{Apobec3} is known to prime a stronger NAb response [7,11,12]. In addition, the reverse scenario involving B-tropic FV ‘vaccination’ of an \textit{Fv1}^{nr/nr} host had not yet been

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**Figure 4.** \textit{Fv1} incompatibility induces significant cell-mediated and humoral immune responses. (A–D) \textit{Apobec3}-deficient 129P2 mice were infected with 7500 SFFU B-tropic FV (FV-B) or DMEM (mock). Mouse splenocytes were analyzed by flow cytometry at 28 dpi. (A–C) Cell mediated immune responses. Splenocytes were stimulated with PMA/ionomycin for 5 h prior to flow cytometric analysis. (A) Representative pseudo-color plots and histograms. Flow cytometry plots show the gating strategy for CD4+ and CD8+ lymphocytes. (B) Intracellular IFN-γ and (C) Surface CD107a in lymphocyte subsets. (D) Splenic germinal center (GL7+ IgD-) B cells. (E-F) Analysis of FV-specific antibody responses in 28 dpi plasma of 129P2 \textit{Apobec3} KO mice following infection with B-tropic FV (7500 SFFU) or NB-tropic FV (500 SFFU). (E) FV-specific IgG endpoint titers as measured by ELISA. (F) NAb titers. For panels B to F, the black bars represent the mean, and each dot corresponds to an individual mouse. Data were analyzed using a 2-tailed Student’s \(t\) test with \(p<0.05\) considered as significant. Data are representative of two independent experiments.

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Figure S. B-tropic FV protected 129P2 Apobec3 KO mice from pathogenic NB-tropic FV challenge. (A) Infection schedule. 129P2 Apobec3 KO mice were infected with B-tropic FV (FV-B) or mock (DMEM). At 28 dpi, the mice were challenged with NB-tropic FV (FV-NB) and evaluated for infection levels by flow cytometry 7 days later. (B) Gating strategy for FV+ BM cells. Ter119+ erythroblasts, CD11b+ myeloid cells and/or CD19+ B cells were gated from the live population. Plasma samples were analyzed for (E) Infectious viremia based on a focal infectivity assay in Mus dunni cells and (F) plasma viral load based on viral RNA copies detected by quantitative RT-PCR. Black bars represent the mean, and each dot corresponds to an individual mouse. Flow cytometry and plasma viral load data were analyzed using a 2-tailed Student’s t test. Infectious viremia data were analyzed using a 2-tailed Mann-Whitney U test. Data were considered statistically significant with \( p < 0.05 \). Data are representative of two independent experiments. doi:10.1371/journal.pone.0060500.g005

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We therefore performed a vaccination study to test if B-tropic FV can function as a live-attenuated vaccine. We utilized 129P2 Apobec3 KO mice to ensure that 129P2 Apobec3 will not contribute to the vaccine-elicted immune response. Even though the 129P2 Apobec3 allele did not promote NAb responses and recovery from FV infection, these phenotypes were observed in the context of pathogenic FV infection. Apobec3 restriction is saturable in vivo [41], and by 28 dpi, high replication levels may have overwhelmed a less potent 129P2 Apobec3 restriction phenotype [20]. Thus, 129P2 Apobec3 KO mice were ‘vaccinated’ with B-tropic FV and after 28 days, challenged with pathogenic, NB-tropic FV. Our results revealed that B-tropic FV replicated at near-undetectable levels by 28 dpi, induced significant cell-mediated and humoral immune responses and protected mice from pathogenic NB-tropic FV infection independent of Apobec3. Thus, Fv1 restriction was sufficient to induce immunological protection, strengthening the case for Fv1 incompatibility as a retrovirus vaccine paradigm in mice. Since Fv1 restriction prevented splenomegaly and promoted NAb responses in Fv2 and B6 susceptible mice, the data also demonstrate that in 129P2 mice, Fv1 restriction is dominant over Fv2 resistance and Apobec3 restriction. This finding is consistent with a proposed ‘restriction factor hierarchy’ [37], whereby dominant restriction mechanisms may mask the impact of other restriction mechanisms in vivo.

The mechanism for how Fv1 incompatibility elicited potent adaptive immunity could readily be explained by live-attenuation: FV replicated at low levels, thereby inducing a protective immune response [43–47]. However, the quality of the immunological response induced by Fv1 incompatibility raise the question of whether signaling pathways that potentiate and/or amplify the adaptive immune response were also induced. Recently, HIV-1 capsid restriction by TRIM5a induced downstream AP-1 and NF-κB regulated genes that may orchestrate the adaptive immune response [48]. TRIM5a exhibited all the attributes of a pattern-recognition protein or innate sensor [48]. Nanotube reconstitution studies revealed that Fv1 recognizes MLV capsids in a lattice arrangement [49], analogous to TRIM5a. Thus, we speculate that Fv1 incompatibility may also trigger a similar cascade of immunity genes. A vaccine model involving B-tropic FV infection of 129P2 mice could prove useful in testing the importance of specific immunological pathways induced by capsid-dependent restriction in vivo. 129P2 mice offer advantages that include disease susceptibility that allows for straightforward pathogenic retrovirus challenge studies, and a genetic background that is extensively used in KO/transgenic technologies. It is conceivable that these Fv1 restriction studies in 129P2 mice may help direct approaches for harnessing TRIM5a biology for human retroviral vaccines.
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References

1. Stremlau M, Owens CM, Perron MJ, Kiesling M, Antiserus P, et al. (2004) The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427: 806-853.

2. Sheehy AM, Gaido NC, Choi JO, Malim MH (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418: 666-650.

3. Friend C (1957) Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J Exp Med 105: 307-318.

4. Hasenkruk KJ, Chesebro B (1997) Immunity to retroviral infection: the Friend virus model. Proc Natl Acad Sci U S A 94: 7611-7616.

5. Halemano K, Harper MS, Guo K, Li SX, Heilman KJ, et al. (2012) Humoral immunity in the Friend retrovirus infection model. Immune Rev 55: 249-260.

6. Lahm J (2007) Cyclophilin A, TRIM5, and resistance to human immunodeficency virus type 1 infection. J Virol 81: 1054-1061.

7. Tsuji-Kawahara S, Chikaishi T, Takeda E, Kato M, Kinoshita S, et al. (2010) Persistence of viruria and production of neutralizing antibodies differentially regulated by polymorphic APOBEC3 and BAFF-R loci in Friend virus-infected mice. J Virol 84: 6802-6905.

8. Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE, et al. (1993) Genealogy of the laboratory mouse. Nucleic Acids Res 40: D881-886.

9. Eppig JT, Blake JA, Bult CJ, Kadin JA, Richardson JE (2012) The Mouse Genome Database (MGD): comprehensive resource for genetics and genomics of the laboratory mouse. Nucleic Acids Res 40: D1081-1086.

10. Pierson DA, Paulson RF, Loyd MR, Herley MT, Bodner SM, et al. (1999) Fv2 encodes a truncated form of the Fk receptor tyrosine kinase. Nat Genet 23: 159-165.

11. Thompson D, Ruscetti F, Nishigaki K (2005) Friend spleen focus-forming virus transforms rodent fibroblasts in cooperation with a short form of the receptor tyrosine kinase Fsk. Proc Natl Acad Sci U S A 102: 15488-15493.

12. Nishigaki K, Hanson C, Jelacic T, Thompson D, Ruscetti F (2005) The tyrosine kinase s-Stat and its downstream signals are required for maintenance of Friend spleen focus-forming-virus-induced fibroblast transformation. J Virol 82: 410-427.

13. Beck JA, Lloyd S, Hafizparast M, Lemon-Pierce M, Eppig JT, et al. (2000) Genealogies of mouse inbred strains. Nat Genet 24: 23-25.

14. Okeoma CM, Petersen J, Ross SR (2009) Expression of murine interferon-gamma proteins in different mouse strains and their effect on mouse mammary tumor virus infection. J Virol 83: 360-365.

15. Santiago ML, Benitez RL, Montano M, Greene WC (2010) Retroviral restriction by Apobec3 enzymes in different mouse strains and their effect on mouse mammary tumor virus infection. J Virol 84: 10933-10936.

Author Contributions

Conceived and designed the experiments: MLS KH. Performed the experiments: KH BSB SXL MSH DSS KJH MLS. Analyzed the data: KH BSB SXL MSH DSS KJH MLS. Wrote the paper: MLS KH.