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Humoral immunity in the Friend retrovirus infection model

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Abstract

Major conceptual roadblocks impede the development of an HIV-1 vaccine that can stimulate a potent neutralizing antibody response. Animal models that support HIV-1 replication and allow for host genetic manipulation would be an ideal platform for testing various immunological hypotheses, but progress on this research front has been slow and disappointing. In contrast, many valuable concepts emerged from more than 50 years of studying the Friend retrovirus model. This

was recently exemplified by the identification of an innate restriction gene, *Apobec3*, that could promote the retrovirus-specific neutralizing antibody response. Here we review both classical and recent data on humoral immunity against Friend retrovirus infection, and highlight the potential of this model for unraveling novel aspects of the retrovirus-specific antibody response that may guide HIV-1 vaccine development efforts.

Keywords

Friend retrovirus; HIV; APOBEC3; Rfv3; Neutralizing antibodies; Vaccines

Introduction

A vaccine against HIV-1 is a global health priority, but remains a difficult challenge. An HIV-1 vaccine should elicit potent adaptive immune responses that include both cell-mediated and neutralizing antibody (NAb) responses. However, the best means to elicit these responses remain unclear. Recent discoveries suggested that valuable insights may be gained by understanding the earliest events following infection, when the innate arm of the immune response is at the critical juncture of shaping adaptive immunity. However, studies on HIV-infected individuals are limited by difficulties in obtaining samples at the time of transmission and the inability to directly control specific biological and genetic factors. It is therefore not surprising that many concepts that guided HIV vaccine development can be traced to basic discoveries in virus infection models.

The current gold standard for AIDS pathogenesis and vaccine studies is the SIV/rhesus macaque model [1]. SIV and HIV-1 are phylogenetically distinct, but both are lentiviruses that cause CD4⁺ T cell depletion, resulting in opportunistic infections, a hallmark of AIDS. Rhesus macaques can be infected with defined molecular clones of SIV, and the development of immunity in the context of viral pathogenesis can be precisely monitored. However, several factors that include high cost, elaborate infrastructure, long duration of follow-up, lack of monkey-specific reagents and inability to genetically modify the host have limited its widespread utility.

Mouse models, on the other hand, allow access to inbred strains that can be genetically modified, not to mention a plethora of reagents and protocols to dissect immune responses. Major efforts were therefore made to develop mice that are susceptible to HIV-1. One approach is to genetically “knock-in” human genes in mice to promote HIV-1 replication, but this effort proved quite difficult with the existence of multiple restriction blocks [2]. Another approach is to engraft a functional human immune system in immunocompromised mice [3]. However, the cost, variability in engraftment, outbred genetics, immaturity of the adaptive immune response and inability to completely “knock-out” specific genes currently limit their full utility for immunological studies. Thus, retroviruses that cause pathology in “normal” mice may be the most suitable starting point for basic vaccine discovery and proof-of-concept immunological studies.

Friend retrovirus infection as a model system to study retrovirus-specific humoral immunity

Friend retrovirus (FV) infection is one of the most well-characterized models of pathogenic retrovirus infection in adult, immunocompetent mice [4–6]. FV is a complex of two retroviruses: (1) a replication-competent, but non-pathogenic, Friend murine leukemia virus (F-MuLV) and (2) a replication-defective, but pathogenic, spleen focus-forming virus (SFFV). SFFV cannot produce viral particles on its own and therefore relies on F-MuLV-encoded particles to carry its genome. In contrast to HIV-1, which is a complex retrovirus encoding multiple accessory genes in addition to *Gag*, *Pol* and *Env*, FV is a simple retrovirus and is a member of the gammaretrovirus family. Similarities and differences between FV and HIV-1 are highlighted in Table 1.

FV infection of susceptible mice results in chronic viremia, polycythemia and splenomegaly within 2 weeks and erythroleukemia and death starting 1–2 months post-infection [4–6]. Polycythemia and splenomegaly are caused by SFFV. SFFV encodes an altered form of Env known as gp55^P, a glycoprotein that associates and activates the erythropoietin receptor (EpoR) [7]. This process is dependent on *Fv2*, a dominant susceptibility gene that encodes a short form of the Stk kinase [8]. The signaling events orchestrated by gp55^P, EpoR and *Fv2* result in the rapid expansion of erythroblasts that are highly susceptible to FV infection [9, 10]. Subsequent erythroleukemia is linked to proviral integration events into the host genome near proto-oncogenes and tumor suppressor genes [11, 12].

Until 2008 [13], practically all FV studies were conducted with “classical” FV stocks containing lactate dehydrogenase elevating virus (LDV). LDV is an RNA virus endemic in wild mouse populations and belongs to the *Arteriviridae* family [14]. LDV does not significantly affect acute FV infection levels [13], but it could strongly stimulate Type I Interferon (IFN) responses [15, 16]. LDV has been shown to delay CD8⁺ T cell immunity [13], induce polyclonal activation in B cells and slow the development of NAb responses [17, 18]. Interestingly, LDV was reported in the FV stocks as early as the 1960s [19, 20], but was largely ignored. While there is growing interest to move the field toward “LDV-free” FV stocks, the results obtained with LDV-free FV should be viewed with caution if the intent is to build on results from historical data. To directly link new work with previous FV/LDV studies, the use of FV/LDV stocks are recommended initially, followed by infections with LDV-free stocks for comparison. In this review, recent studies performed with LDV-free FV stocks will be noted with a bulletpoint (•).

While HIV-1 and FV are clearly different retroviruses, the FV model continues to provide basic information on the retrovirus-specific cell-mediated immune response (reviewed in [21, 22]). Here we argue that FV infection is also a tractable model to dissect basic mechanisms underlying the retrovirus-specific humoral immune response. The humoral response to FV is relevant to HIV-1 for several reasons. First, both the HIV-1 and FV envelope (Env) glycoproteins are the primary targets of the NAb response in their respective hosts. Similar to the HIV-1 Env, the MLV Env gp85 glycoprotein protomer consists of a surface (SU; gp70) and transmembrane (TM; p15E) subunit, which form tripod-like trimers on the virus particle based on cryoelectron tomography studies [23, 24]. Second, both HIV-1

and FV propagate in immune organs and/or compartments, resulting in humoral immune dysfunction. B cell abnormalities that include polyclonal B cell activation, hypergammaglobulinemia and delayed NAb development were observed in both HIV and FV infection (Table 1). Finally, a single dominant resistance gene that promotes the FV-specific NAb response, *Recovery from Friend Virus 3* (*Rfv3*) [25], has a direct counterpart in humans that is counteracted by HIV-1. It took 30 years to decipher the molecular identity of *Rfv3* [26], but the result of these efforts has opened the door for exciting studies that compare the development of a protective versus a non-protective humoral response in congenic mice.

Recovery from Friend retrovirus involves the humoral arm of the immune response

Since the discovery of FV by Charlotte Friend in 1957 [27], it was already apparent that host genetic factors had a tremendous impact on FV resistance and susceptibility. This led to the identification of *Fv1* and *Fv2* by Frank Lilly and colleagues in the 1960s [28, 29], which correspond to nonimmunological genes that control N- and B-tropic FV tropism and susceptibility to splenomegaly, respectively. In addition, immunological genes that influenced recovery from viremia and disease were identified, notably *Recovery from Friend retrovirus 1 and 2* (*Rfv1* and *Rfv2*) which both map to the major histocompatibility complex (*H-2*) [30–32]. The *H-2* locus was identified as an important determinant for recovery when it was observed that mice with the *H-2^{b/b}* genotype recovered from splenomegaly at a higher frequency than mice with the *H-2^{b/d}* genotype in a backcross study between C57BL/6 (*H-2^{b/b}*) and DBA/2 (*H-2^{d/d}*) strains [30]. However, B₁ progeny from crosses between A.BY (*H-2^{b/b}*) and BALB/c (*H-2^{d/d}*) mice all failed to recover from FV infection. This led to the discovery of a non-*H-2* recovery gene, *Rfv3* [25]. *Rfv3* encoded a gene that suppressed viremia and lowered the frequencies of virus-producing cells via the production of NAbs [33]. An example of the genetic cross that revealed *Rfv3* is shown in Fig. 1a.

Rfv3* is encoded by *Apobec3—The first major clue to the molecular identity of *Rfv3* came when Kim Hasenkrug and colleagues mapped *Rfv3* to a 20-centimorgan region of mouse chromosome 15 in 1995 [34]. Four years later, the same group narrowed *Rfv3* to a 0.83-centimorgan region [35]. In 2005, Masaaki Miyazawa and colleagues further cut the *Rfv3* locus by half [36]. By this time, two major developments have occurred: (1) the near-complete sequencing of the mouse genome [37]; and (2) the identification of a potent antiretroviral restriction factor, *APOBEC3G* [38]. The sequencing of the mouse genome revealed that the *Rfv3* locus encompass just 61 genes. The identification of *APOBEC3G* as the factor counteracted by the HIV-1 protein Vif provided strong support that the murine homologue, mouse *Apobec3*, may encode *Rfv3*.

To directly test whether *Apobec3* encodes *Rfv3*, our group employed an F₁ transcomplementation approach [26] (Fig. 1b). B6 wild-type (WT) and *Apobec3* knockout (KO) mice were crossed to two *Rfv3* susceptible strains (A.BY and BALB/c) to generate *Fv2^{r/s}* (*Fv2* susceptible) F₁ strains that differ only in the presence or absence of the B6 *Apobec3* gene. If the B6 *Apobec3* gene is the *Rfv3* resistance gene, then removal of the B6 *Apobec3* gene should result in *Rfv3* susceptibility in the F₁ strains. On the other hand, if *Apobec3* is not *Rfv3*, then inactivation of the B6 *Apobec3* gene will not influence viremia

and NAb responses. Our results revealed that the B6 *Apobec3* gene indeed influenced NAb responses and recovery from viremia in F₁ mice, providing direct evidence that *Apobec3* encodes *Rfv3*. This was further strengthened with additional cohorts showing the strong influence of B6 *Apobec3* on survival, NAb responses and recovery from viremia, cellular infection and splenomegaly [39]. To date, the *Apobec3* association with NAb responses has been reproduced in pure B6, 129/OlaHsdP2 (129/Ola), (B6 × A.BY)F₁ and (B6 × BALB/c)F₁ [26, 39, 40] and (B6 × A/WySn)F₁ strains [41•].

Recently, a linked gene that controls B cell survival, the B cell activating factor receptor (*BAFF-R*), was suggested to control the *Rfv3* susceptibility of A/WySn mice [41•]. However, a *BAFF-R* defect could not account for the *Rfv3* susceptible phenotype of BALB and A.BY strains, which have normal B cell levels [39]. Given that the initial description of *Rfv3* encompassed C57BL, A/WySn, A.BY and BALB strains [25], we concluded that *Apobec3* encodes *Rfv3*.

Apobec3 properties that correlate with Rfv3 resistance and susceptibility—

Significant progress has been made in determining which *Apobec3* property accounts for the *Rfv3* resistance and susceptible phenotype. The B6 *Apobec3* gene predominantly encodes a splice variant lacking exon 5 [26, 42–44] that appears to have more potent activity [42, 43] and higher resistance to the viral protease [42] than the full-length BALB *Apobec3* gene in vitro. Amino acid residues that differed between B6 and BALB strains were predicted to have undergone positive selection, suggesting a role in differential virus restriction [45]. Recently, minigene analyses revealed that the B6 $\Delta 5$ transcript was translated more efficiently than the full-length BALB transcript [46]. Thus, *Apobec3* $\Delta 5$ splicing is likely important in dictating the *Rfv3* phenotype.

Another striking difference between *Rfv3* resistant (B6) and susceptible (A.BY, BALB, A/WySn) strains is the steady state levels of total *Apobec3* transcripts, which could vary by up to 20-fold [39, 43, 47, 48]. In contrast to the antiviral properties of the *Apobec3* $\Delta 5$ variant that were studied in co-transfected cells, the difference in *Apobec3* mRNA expression was derived from primary cells. Thus, *Apobec3* mRNA expression is likely the fundamental property that distinguishes *Apobec3* between *Rfv3* resistant and susceptible strains. The only exception appears to be the 129/Ola strain, which was initially classified as *Rfv3* resistant [26] but upon re-analysis, had *Apobec3* mRNA levels similar to that of A.BY, BALB and A/WySn strains (D. Smith and M. Santiago, unpublished). 129/Ola mice were also classified as *Fv2* resistant due to high resistance to splenomegaly even with very high FV inoculum. However, the FV stock used for infections was B-tropic [26], and 129/Ola mice are in fact *Fv1^{nr}* [49, 50]. Thus, B-tropic FV likely encountered a potent *Fv1* post-entry block in 129/Ola mice and served as a “live-attenuated vaccine” that elicited a potent NAb response, resulting in its erroneous designation as *Rfv3* resistant (see Part III.D.). Infection of 129/Ola strains with dual-tropic (NB-tropic) FV resulted in splenomegaly, chronic viremia and weak NAb responses (K. Halemano, B. Barrett and M. Santiago, unpublished). Thus, 129/Ola mice should be classified as *Fv2^{s/s} Rfv3^{s/s} Fv1^{nr}*, linking the *Rfv3* susceptible status of 129/Ola mice with its low *Apobec3* mRNA expression levels.

The *mA3* polymorphism(s) responsible for the difference in *mA3* mRNA levels between *Rfv3* resistant and susceptible strains remains unknown. Sequencing of the *mA3* locus between *Rfv3* resistant and susceptible strains revealed a 530-bp xenotropic MLV long-terminal repeat (X-MLV LTR) insertion just after the exon 2 splice site in B6 but not BALB or A.BY mice [39, 45]. Interestingly, we detected *Apobec3* transcripts that lacked exon 2 in *Rfv3* susceptible A.BY and BALB mice [26]. These 2 transcripts represent only a minor fraction (<10 %) of the total *Apobec3* mRNA pool in the spleen [39, 47, 51], as observed for the human homologues *APOBEC3G* and *APOBEC3F* [52]. However, *Apobec3* 2 transcripts may represent the remnants of actively degraded transcripts due to the lack of an X-MLV LTR. This hypothesis is currently being tested.

The link between *Apobec3* mRNA expression levels and *Rfv3* could have important implications, especially since B6 *Apobec3* restriction is saturable in vivo [40]. In the recombinant CasFrKP MLV strain, an alternatively translated FV-derived gene product, Glyco-Gag, appears to function as an antagonist of *Apobec3* [49], analogous to HIV-1 Vif antagonizing human APOBEC3G. Thus, *Apobec3* overexpression may “swamp out” the antagonist and unleash the antiviral activity of *Apobec3* in vivo. Interestingly, higher *APOBEC3G* mRNA expression levels correlated with HIV-1 resistance in humans and nonhuman primates [53, 54]. Thus, in addition to the discovery of compounds that can block the HIV-1 Vif–APOBEC3G interaction, upregulating *APOBEC3G* levels may also be a viable therapeutic strategy.

Mechanism for how *Apobec3* promotes the NAb response—The identification of *Rfv3* as *Apobec3* immediately raised the issue of how *Apobec3* promotes NAb responses. We formulated two hypotheses [26]: a direct and indirect mechanism. The direct mechanism proposes that *Apobec3* may directly affect antigen-specific antibody development even in the absence of FV infection. This derives from the fact that *Apobec3* is highly expressed in B cells [41, 55] and is evolutionarily related to activation-induced cytidine deaminase [56], an enzyme that catalyzes antibody affinity maturation and class switching [57]. However, B6 *Apobec3* deficiency was also found to have a significant impact on infectious viremia during acute infection. This was observed not only in FV infection [26], but also in an earlier study with mouse mammary tumor virus [58], as well as Moloney MLV [59] and the CasFrKP MLV [49]. Thus, *Apobec3* may enhance NAb responses through an indirect mechanism that involves limiting virus-induced humoral immune dysfunction.

To delineate which mechanism is more relevant, we utilized an immunization approach that allows for quantitative assessments of antibody affinity based on reactivity to low- versus high-molar conjugation of nitrophenyl (NP) hapten. B6 *Apobec3* KO mice produced antibodies of similar affinity to NP compared with B6 WT mice. Only in the context of FV infection did the production of high-affinity antibodies to NP-hapten become impaired in B6 *Apobec3* KO mice [55]. Miyazawa and colleagues also found no impact of B6 *Apobec3* on antibody responses to dinitrophenyl (DNP)-Ficoll and DNP-Ovalbumin [41]. Together, these results suggest that *Apobec3* modulates antibody production via an indirect mechanism.

Apobec3 may promote the NAb response by limiting FV-induced immune dysfunction. B6 *Apobec3* WT F₁ mice have significantly lower FV⁺ erythroid, myeloid, B and T cells compared with B6 *Apobec3* KO F₁ mice [55], suggesting that protection of these cell subsets from FV infection may limit immune dysfunction. Mice infected with FV have decreased abilities to produce antibodies against sheep red blood cells, and this suppression was dependent on the loss of IFN- γ and the increased IL-10 production [60]. Whether IL-10 dependent immunosuppression is enhanced in B6 *Apobec3* KO mice remains untested. Interestingly, B6 *Apobec3* WT mice displayed polyclonal B cell activation [41•] and a significant delay in hypergammaglobulinemia [55]. Thus, FV-induced humoral immune dysfunction may be directly linked to perturbations in B cell function. So far, B cell immunophenotyping studies at 3 days post-infection (dpi) revealed no *Apobec3*-dependent perturbations in early B cell subsets in the bone marrow (K. Halemano and M. Santiago, unpublished). In contrast, at 7 dpi, we observed significantly increased induction of germinal center (GC) B cells and plasmablasts in B6 *Apobec3* deficient mice [55]. In fact, the kinetics of FV-specific IgG and NAb development was enhanced if the mice encoded the B6 *Apobec3* gene [26, 41•]. Thus, in addition to preventing FV-induced immune dysfunction, *Apobec3* may also drive virus-specific antibody development.

Maintenance of antibody responses is highly dependent on antigen levels [61–63]. Since most restriction factors inhibit viruses in the infected cell and decrease antigen levels, these factors may not be optimal for driving B cell responses. In contrast, *Apobec3* appears to have evolved to maintain antigen levels during acute infection. In cell culture, the APOBEC3 proteins do not inhibit virus production, but inhibit the infectivity of the resulting virions [38]. This was thought to occur by incorporation of *Apobec3* into nascent virions, after which, *Apobec3* inhibits reverse transcription in the *next* target cell [64] (Fig. 2). Investigating 7 dpi plasma samples from B6 *Apobec3* WT and KO F₁ mice, we provided the first evidence that this phenomenon occurs in vivo [40]. Similar viral RNA loads were found in plasma of B6 WT and *Apobec3*-deficient mice, while the infectivity of these virions differed markedly [40]. Thus, B6 *Apobec3* activity was associated with the release of noninfectious particles during acute FV infection, accounting for up to 80 % of the total virion pool relative to B6 *Apobec3* KO mice. These noninfectious particles are fusion-competent and could serve as immunogens to prime the B cell response. Priming the B cell response provides a strong rationale for why mammalian hosts evolved a restriction factor that inhibits retroviruses in the next target cell (Fig. 2).

Innate immune recognition of retrovirus particles—*Apobec3*-restricted virions may be potent inducers of humoral immunity because they maintain critical immunogenic properties of infectious virions that include size, multivalency and uptake kinetics [65], but without promoting cellular dysfunction due to virus replication. In addition, virion nucleic acids have the capacity to stimulate pattern recognition receptors that may directly activate B cells. Browne et al. [66•] demonstrated that the NAb response against FV was completely abrogated in B6 *MyD88* KO mice, suggesting an essential role for Toll-like receptor (TLR) signaling. B cell intrinsic *MyD88*, as opposed to dendritic cell intrinsic *MyD88*, was found to be crucial to the antibody response to FV [67•]. TLR7 was also shown to be essential to the production of NABs and the induction of GCs [67•, 68]. Collectively, these data suggest

that B cell intrinsic TLR7 signaling is essential for NAb development against retroviruses. Because TLR7 recognizes single-stranded (ss) RNA in endosomes, it is possible that when a B cell that recognizes FV Env binds to virions, endocytosis of the virion particle exposes TLR7 to the viral ssRNA, leading to a GC response (Fig. 2). In this way, Apobec3-restricted virions may be acting like “virus-like particle (VLP)” vaccines. Interestingly, we recently found that *Apobec3* is the primary direct effector of IFN- α treatment against FV infection in vivo (M. Harper, B. Barrett and M. Santiago, unpublished). Thus, the TLR7-Myd88 dyad may directly activate *Apobec3* through Type I IFN induction, providing a potential amplification loop. However, the Type I IFN receptor does not appear to be critical for NAb induction [68], implying the existence of alternative pathways for intrinsic priming of the B cell response.

The protective antibody response against Friend retrovirus

A global HIV-1 vaccine should elicit NAbs that can broadly neutralize different HIV-1 strains. Progress in this area is rapid and exciting, and revealed several broadly neutralizing Abs (bNAbs) and their respective epitopes in the HIV-1 Env trimer (for example, see [69]). However, designing an immunogen to elicit these bNAbs remains a major challenge and would likely require basic knowledge on how to activate B cells with a specific germline configuration, focus the GC reaction to strongly disfavored Env trimer epitopes and induce high-affinity antibodies through extensive somatic hypermutation.

In 2009, the first evidence of protection from HIV-1 acquisition was observed in the RV144 trial in Thailand [70]. Surprisingly, protection did not correlate with strong NAbs or CD8⁺ T cell responses. Instead, the moderate 31.2 % protection correlated with HIV-1 Env-specific binding antibodies that appear to have Fc effector functions. In addition to direct neutralization, antibodies have the capacity to counteract infected cells through (1) complement-mediated lysis, (2) opsonization for macrophage phagocytosis and (3) antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells. These understudied properties of antibodies have now become a major focus of HIV/AIDS research [71, 72].

Lessons from FV-specific monoclonal antibodies—While the threat of the HIV-1/AIDS pandemic was unraveling in the early 1980 s, the first attempt to characterize the antibody response against a pathogenic retrovirus using monoclonal antibodies (MAbs) was being undertaken. Bruce Chesebro and colleagues produced hundreds of hybridomas from FV-infected *Rfv3* resistant mice, 17 of which were reactive to FV⁺ erythroleukemia cells [73]. The viral protein targets of 11 of these MAbs were identified: 8 reacted to gp70, while 3 reacted to p15E and Glyco-Gag. None of these FV-reactive MAbs neutralized F-MuLV in vitro. However, in the presence of guinea pig complement, 7 out of 8 FV gp70-reactive MAbs lysed FV virions and 4 out of 6 FV-reactive MAbs lysed infected cells in vitro [73, 74]. These four cytotoxic MAbs included MAbs 48 and 350 (IgG2a), MAb 34 (IgG2b) and MAb 307 (IgM). However, only the IgG2a MAbs conferred significant reduction in spleen infectious centers (ICs) following passive immunization in vivo [74].

The most potent MAb from this initial panel, MAb 48, was further used in in vivo studies. In contrast to the in vitro data, MAb 48 administration did not lead to clearance of FV-infected cells. Instead, MAb 48 appeared to inhibit the expansion of virus-producing cells [74]. Subsequent studies demonstrated that cell-mediated immune responses were essential to the in vivo effectiveness of MAb 48 [75, 76]. Interestingly, MAb 34, a nonneutralizing MAb specific to Glyco-Gag, enhanced the antiviral activity of MAb 48 [76].

In 1992, another MAb, MAb A9D41, was demonstrated to lyse Friend leukemia cells in vitro in a complement-dependent manner [77]. Removal of the Fc portion of MAb A9D41 completely abrogated its ability to inhibit the replication of Friend leukemia cells injected into DBA/2 mice. Moreover, passive protection mediated by MAb A9D41 was significantly reduced in NK-deficient *bg/bg* mice compared with *bg/+* mice, suggesting a role for NK-mediated ADCC in the in vivo potency of MAb A9D41.

Together, these studies on MAbs 48 and A9D41 suggested the involvement of complement, T cells, NK cells and nonneutralizing Abs in inhibiting FV in vivo. However, these MAbs represent only a small sampling of the entire repertoire of FV-specific antibodies. Thus, these MAbs may not recapitulate the dominant mechanism(s) governing the *Rfv3*-dependent, FV-specific NAb response.

Studies on the FV-specific polyclonal antibody (pAb) response—Passive immunization with anti-FV serum into susceptible FV-infected mice increased survival rates [78] and dramatically lowered infection levels in splenocytes [33, 66, 79]. The combined activity of antibodies in these “bulk” antisera may better capture the most critical mechanisms governing retrovirus antibody neutralization. However, such mechanisms have yet to be studied in-depth.

The identification of *Rfv3* as *Apobec3* provided a unique opportunity to investigate the characteristics of a protective versus a nonprotective pAb response against retroviruses in congenic mice with or without the B6 *Apobec3* gene. We are in the process of analyzing 28 dpi plasma samples from (B6 × BALB/c)F₁ and (B6 × A.BY)F₁ mice for isotype and IgG subclass distribution, avidity to various FV virion preparations and ultimately, effector functions through passive immunization in susceptible mice. A simple approach is to treat purified FV virions with 1 % detergent, which disrupts native trimers of HIV-1 Env [80, 81]. Using this approach, we found that 28 dpi IgM and IgG endpoint binding titers to detergent-treated FV Env were equivalent between B6 *Apobec3* WT and KO F₁ mice. In contrast, the IgG endpoint titers to native FV preparations were significantly higher in F₁ mice with the B6 *Apobec3* gene [40]. Thus, B6 *Apobec3* activity (or *Rfv3* resistance) is associated with the development of IgG antibodies specific to B cell epitopes on native virions, for example, functional trimers (Fig. 2).

Our ongoing analyses reveal that the protective *Apobec3*-dependent IgG response may be biased to the IgG2 subclass (K. Heilman and M. Santiago, unpublished). Since subclass designations are dependent on the Fc portion of IgG that interacts with Fcγ receptors, we hypothesize that antibody neutralization in vivo will have a significant Fc-mediated effector component. To test this hypothesis, we performed passive immunization studies with 28 dpi

plasma from B6 *Apobec3* WT and KO F₁ mice with FV into susceptible BALB/c mice. Our results revealed significant inhibition of FV infection in the bone marrow with B6 *Apobec3* WT F₁ plasma but not B6 *Apobec3* KO F₁ plasma (K. Halemano, B. Barrett and M. Santiago, unpublished). Thus, an in vivo neutralization assay recapitulated the *Rfv3* phenotype, and should allow for future studies on antibody effector mechanisms.

Vaccine modalities to elicit FV-specific NAb

Most vaccines work through the induction of NAb, and FV is no exception. Thus, the FV model has been used to investigate the ability of various retroviral vaccine preparations to elicit NAb-based protection. Many of these NAb-based studies pre-dated large HIV-1 vaccine trials, while some have been designed after HIV-1 or SIV vaccine studies, particularly in the context of developing T cell vaccines. We review here some of these studies, highlighting the efficacy of various vaccine configurations and their ability to elicit NAb.

Inactivated virus vaccines—Using formalin-fixed serum preparations, Charlotte Friend was the first to demonstrate that mice can be protected from FV infection with a vaccine [78]. However, it eventually became clear that formalin-fixed, heat-inactivated, or UV-inactivated virus were poor immunogens. Using inactivated virus without an adjuvant offers moderate protection against FV-induced splenomegaly in *H-2^{b/b}* mice while offering no protection to *H-2^{a/a}* mice, which coincided with a lack of NAb [82, 83]. Poor protection with inactivated F-MuLV may be due to the lack of TLR stimulation, as implicated in the unfortunate formalin-inactivated respiratory syncytial virus vaccine trial in 1966 [84]. It is possible that treatments that render the virus inactive also denature or destroy TLR agonists. Supporting this contention, the addition of adjuvants to inactivated F-MuLV improved protection levels [82, 85, 86]. However, even though these immunized mice were able to produce FV Env-specific IgG, they still did not produce NAb [82]. A pair of mutations within the “immunosuppression domain” of FV *Env* renders UV-inactivated virions more immunogenic, resulting in increased TM- and Gag-specific IgG and activated FV-specific T cells [87]. However, it was not shown whether these mutations resulted in a more potent NAb response.

Subunit vaccines—Purification of the FV Env in the 1970s allowed for testing a subunit vaccine approach. Early studies in STU mice demonstrated that immunization with FV Env gp70 (SU) or gp85 (SU + TM) in either complete or incomplete Freund’s adjuvant could significantly lower plasma viremia, splenomegaly and mortality in a dose-dependent manner [88, 89]. Immunization with gp85 conferred better protection against FV-induced mortality than gp70, which induced better protection than p15E (TM) [89]. Gag proteins (i.e., matrix, capsid and nucleocapsid) offered no protection from splenomegaly [82]. Hunsmann et al. [89] also demonstrated that gp70 coupled to keyhole limpet hemocyanin effectively protected mice from low-dose FV challenge. Although vaccinations with gp70 were able to produce FV-specific IgG binding titers, they were not able to produce neutralizing titers prior to challenge [82]. It remains unknown whether Fc effector mechanisms were responsible for the moderate protection levels observed with these subunit FV vaccines. The recent RV144 HIV-1 vaccine trials used a gp120 subunit boost that resulted in moderate

protection levels [70], and it is currently thought that Fc effector mechanisms may have contributed to protection [71, 72].

Vaccinia and adenovirus vectors—Current HIV/SIV vaccine strategies focus on the use of vaccinia and adenovirus (Ad) vectors as delivery systems for antigens. While the intent was to elicit CD8⁺ cytotoxic T cell (CTL) immunity, priming of CD4⁺ T cell immunity may also enhance NAb responses through these vaccine vectors.

Immunization with recombinant vaccinia expressing FV *Env* gp85 resulted in rapid recovery from splenomegaly and spleen ICs in *H-2^{a/b}* mice but conferred very little protection in *H-2^{a/a}* mice [85, 90, 91]. Interestingly, immunization with vaccinia-FV *Env* seemed to protect *H-2^{b/b}* mice by T lymphocyte priming, which led to a more rapid NAb response and potent CTL response [85]. Removal of CTL epitopes from vaccinia-FV *Env* or depletion of CD8⁺ T lymphocytes from mice vaccinated with complete vaccinia-FV *Env* still resulted in recovery from splenomegaly for *H-2^{a/b}* mice, suggesting that rapid induction of the IgG NAb response can compensate for a missing CTL response [92]. Vaccinia-FV *Gag* also conferred some protection, but the recovery from splenomegaly and induction of the NAb response was slower than in mice immunized with vaccinia-FV *Env* [93]. Thus, the importance of the retroviral *Env* gene product in a vaccine was already highlighted in early FV studies, a concept that was unfortunately dismissed in the failed human Phase IIb STEP trials that did not include the HIV-1 *Env* [94].

Replication-defective adenovirus vectors were also tested in the FV model. In addition to Ad5-based vectors expressing FV *Env* and *Gag*, fiber chimeric vectors, in which the fiber knob and shaft domains of Ad5 were replaced with those of Ad35 (Ad5F35), were produced [95]. Heterologous prime-boost vaccination with Ad5 and Ad5F35 resulted in reduced plasma viremia and splenomegaly compared with homologous prime-boost, and this protection correlated with a significantly increased NAb response [95]. This finding agrees with assessments in the SIV model that prior immunity to Ad vectors could significantly inhibit vaccine potency [96, 97]. More recently, the addition of IFN- α subtype 4 to the Ad vaccine increased CD4⁺ T cell activation and NAb titers to the point that CD8⁺ T cells can be depleted with no increases to plasma viremia or spleen size [98]. This recent study suggests that Type I IFN could be an effective adjuvant to augment NAb-based vaccine efficacy.

Live-“attenuated” vaccines—By far, the best vaccination outcome in the FV model involved the administration of live N-tropic F-MuLV (FV-N) in *FvI^{b/b}* mice. *FvI* encodes an endogenous retroviral *Gag* gene that determines tropism by interfering with the proviral integration of certain FV strains [99]. FV-N viruses replicate in *FvI^{n/n}* NIH Swiss mice while FV-B viruses replicate in *FvI^{b/b}* BALB or B6 strains. However, when an FV-N virus is used to infect an *FvI^{b/b}* mouse, the virus acts as a live-“attenuated” vaccine and induces NAb, FV-specific CTL activity and FV-specific T cell proliferation prior to challenge [85]. These parameters correlate with transient levels of spleen ICs at 12 days post-challenge and complete protection from splenomegaly [76, 83, 85].

Consistent with the success of live-attenuated vaccines in the FV model, the best vaccination outcome observed in the SIV model involved SIV with a deletion in the accessory protein Nef [100]. SIV Nef viruses replicate at low levels in the host, likely driving a potent CD8⁺ T cell response. These attenuated viruses should also somewhat preserve the CD4⁺ T cell response and thereby improve SIV antibody development. Interestingly, the SIV Vif protein, which antagonizes the respective simian APO-BEC3 proteins, can be mutated to generate a live-attenuated virus [101]. Studies are ongoing to determine if SIV Nef and SIV Vif-attenuated viruses can elicit potent neutralizing or blocking antibodies.

Virus-like particles—While highly potent, live-attenuated vaccines for HIV-1 may not be clinically feasible because of serious safety concerns. Our current working model (Fig. 2) on Apobec3-restricted noninfectious particles driving the B cell response suggests that VLP configurations may be a suitable alternative vaccine to elicit NABs. These VLPs should ideally preserve the functional, fusion-competent Env trimer and contain nucleic acids that can prime the B cell response through TLR signaling. HIV-1 pseudovirions expressing Gag and Env have the potential to express epitopes of the native virion trimer [102]. VLP-based vaccine regimens offer advantages to other approaches since it could circumvent the need for adjuvants and will not be subject to concerns on prior immunity to the vaccine vector and potential reversion from live attenuation. We anticipate that the FV model will play an important role in dissecting the induction of humoral immunity using the VLP vaccine platform.

Concluding remarks

The FV infection model was initially used to understand basic mechanisms involved in oncogenesis. However, concepts derived from more than 5 decades of work in the FV model are now proving to be quite relevant to HIV-1 infection. This was most recently shown by the discovery of pathways that connect the innate and humoral arms of the immune response against retroviruses. These studies have set the stage for the modern use of the FV model in investigating basic mechanisms underlying the retroviral antibody response in the context of natural infection and vaccination.

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Fig. 1.

Rfv3 is encoded by *Apobec3*. **a** *Rfv3* genetics. A.BY mice are susceptible to FV-induced splenomegaly due to a dominant *Fv2* susceptibility gene. Crossing A.BY mice to C57BL strains (B6 or B10) results in an F₁ strain that should still be susceptible to splenomegaly. However, these F₁ mice recover from viremia about 1 month post-infection. Recovery from viremia correlated with NAb responses and segregated as a Mendelian trait in subsequent backcrosses with A.BY mice. B6 and A.BY mice are *H-2^{b/b}*, suggesting that a single autosomal dominant, non-*H-2* gene controls recovery from viremia and NAb responses. This gene was referred to as *Rfv3*. **b** F₁ transcomplementation to test if *Rfv3* is *Apobec3*. B6 *Apobec3* KO mice were crossed to A.BY mice to obtain F₁ strains similar to panel A. If *Rfv3* = *Apobec3*, then the *Rfv3* phenotypes of recovery from viremia and NAb responses should be similar with that of (B6 x A.BY)F₁ mice (panel A). However, the opposite result was observed: (B6 *Apobec3* KO x A.BY)F₁ strains had high levels of viremia, did not recover from splenomegaly and had weak NAb responses. Thus, the B6 *Apobec3* gene was critical for conferring *Rfv3* resistance. This was taken as direct proof that *Rfv3* is encoded by *Apobec3*.

Fig. 2.

Current model for the *Apobec3*-dependent NAb response. (*Left*) In *Rfv3* resistant mice, higher levels of the B6 *Apobec3* gene results in the release of noninfectious particles that stimulate GC B cells via the TLR7-Myd88 pathway, resulting in a strong NAb response. (*Right*) In *Rfv3* susceptible mice, released infectious particles result in virus replication and immune dysfunction, resulting in a weaker NAb response. Note that during acute infection, similar numbers of FV particles are released independent of B6 *Apobec3* activity. Not shown are the infection of other cell types, particularly erythroblasts, myeloid cells and T cells which could all contribute to humoral immune dysfunction in *Apobec3/Rfv3* susceptible mice

Table 1

Comparison of FV and HIV-1

Parameter	FV complex	HIV-1
Retrovirus family	Gammaretrovirus	Lentivirus
Target host	Mice	Humans, chimpanzees
Major infection route	Intravenous	Mucosal, intravenous
Target compartments	Bone marrow, lymphoid organs (spleen, lymph nodes)	Lymphoid organs (lymph nodes, gut), CNS
Target cells	Erythroblasts, myeloid cells, B cells, (T cells)	CD4 ⁺ T cells, macrophages
Target cell receptor	Mouse cationic amino acid transporter 1	CD4, CCR5 or CXCR4
Viral entry protein	Env gp85: SU, gp70; TM, p15E	Env gp160: SU, gp120, TM, gp41
Quaternary structure	Trimer, covalently linked SU and TM	Trimer, noncovalently linked SU and TM
Env glycosylation	7 N-linked, plus 1–2 O-linked sites	>24 N-linked glycans
Cause of pathology	Erythroblast proliferation, oncogenesis	CD4 ⁺ T cell depletion
Immunosuppression	Regulatory T cells	CD4 ⁺ T cell depletion
Disease	Splenomegaly, erythroleukemia	AIDS
Innate restriction		
Post-entry restriction	Fv1 (capsid-dependent)	TRIM5-alpha (capsid-dependent), SAMHD1
Virion release	Tetherin	Tetherin
Virion infectivity	Apobec3	APOBEC3B, D, G, F and H
Apobec3 antagonist	Glyco-gag (putative)	Vif
Humoral immunity		
Genetic control	<i>Rfv3</i>	Unknown
Polyclonal B cell activation	Yes	Yes
Hypergammaglobulinemia	Yes	Yes
NAb response	Delayed, effective in <i>Rfv3</i> resistant mice	Delayed, ineffective but could drive virus evolution
Memory B cell contraction	Unknown	Yes
B cell maturation	Decreased IgM ⁺ B cells in the bone marrow	Increased CD10 ⁺ B cells in the periphery