Innate Retroviral Restriction by Apobec3 Promotes Antibody Affinity Maturation In Vivo

Mario L. Santiago, Robert L. Benitez, Mauricio Montano, Kim J. Hasenkrug and Warner C. Greene

*J Immunol* published online 21 June 2010
http://www.jimmunol.org/content/early/2010/06/21/jimmunol.1001143

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Innate Retroviral Restriction by Apobec3 Promotes Antibody Affinity Maturation In Vivo

Mario L. Santiago,*†‡ Robert L. Benitez,§ Mauricio Montano,§ Kim J. Hasenkrug,§ and Warner C. Greene§,§,#,**

Apobec3/Rfv3 is an innate immune factor that promotes the neutralizing Ab response against Friend retrovirus (FV) in infected mice. Based on its evolutionary relationship to activation-induced deaminase, Apobec3 might directly influence Ab class switching and affinity maturation independently of viral infection. Alternatively, the antiviral activity of Apobec3 may indirectly influence neutralizing Ab responses by reducing early FV-induced pathology in critical immune compartments. To distinguish between these possibilities, we immunized wild-type and Apobec3-deficient mice with (4-hydroxy-3-nitrophényl) acetyl (NP) hapten and evaluated the binding affinity of the resultant NP-specific Abs. These studies revealed similar affinity maturation of NP-specific IgG1 Abs between wild-type and Apobec3-deficient mice in the absence of FV infection. In contrast, hapten-specific Ab affinity maturation was significantly compromised in Apobec3-deficient mice infected with FV. In highly susceptible (B6 × A.BY) F1 mice, the B6 Apobec3 gene protected multiple cell types in the bone marrow and spleen from acute FV infection, including erythroid, B, T, and myeloid cells. In addition, B6 Apobec3 deficiency was associated with elevated Ig levels, but decreased induction of splenic germinal center B cells and plasmablasts during acute FV infection. These data suggest that Apobec3 indirectly influences FV-specific neutralizing Ab responses by reducing virus-induced immune dysfunction. These findings raise the possibility that enabling Apobec3 activity during acute infection with human pathogenic retroviruses, such as HIV-1, may similarly facilitate stronger virus-specific neutralizing Ab responses. The Journal of Immunology, 2010, 185: 000–000.

The biological outcome of Friend retrovirus (FV) infection of mice is dictated by multiple resistance and susceptibility genes (1, 2). FV infection of susceptible mouse strains, such as A.BY and BALB/c, results in the polyclonal activation of erythroblast precursors that leads to severe splenomegaly and erythroleukemia. In contrast, resistant mouse strains, such as C57BL/6 (B6), do not develop splenomegaly due to the absence of a dominant Fv2 susceptibility gene (3, 4). FV disease is also alleviated in certain mouse strains by the development of potent cell-mediated and humoral immune responses, both of which map to specific genetic loci. Cell-mediated immune responses are primarily controlled by the MHC (H-2 locus) (5), whereas the neutralizing Ab response against FV is significantly influenced by a single autosomal dominant gene known as Rfv3 (6). Rfv3-resistant strains, such as B6, produce stronger neutralizing Ab responses compared with Rfv3-susceptible strains (A.BY, BALB/c), and this phenotype maps to a 60-gene region on chromosome 15 based on three consecutive studies of recombinant inbred mice (7–9).

We recently provided evidence that Rfv3 is encoded by an innate restriction factor known as Apobec3 (10). This conclusion was based on our demonstration that genetic inactivation of Apobec3, which is located within the 60-gene region on chromosome 15, reproduced the phenotype of an Rfv3 susceptibility allele. Thus, (B6 Apobec3+/− × A.BY)F1 mice developed weaker neutralizing Ab responses than (B6 Apobec3+/+ × A.BY)F1 mice assessed at 28 d postinfection (dpi) with FV. At high virus doses, even Apobec3-deficient B6 mice, which are resistant to splenomegaly, developed weaker neutralizing Ab responses against FV at 28 dpi compared with the corresponding wild-type (WT) mice.

Apobec3 encodes a deoxyctydine deaminase that, when incorporated into budding retroviral particles, can render these virions noninfectious in the next target cell (as reviewed in Ref. 11). Apobec3 can physically impede reverse transcription by its enzymatic activity, resulting in lethal G-to-A hypermutation in the viral plus strand (14–17), and/or impair viral integration through the formation of aberrant cDNA ends (18, 19). Rfv3-susceptible mouse strains (A.BY, BALB/c) exhibit decreased Apobec3 mRNA expression (20, 21), aberrant mRNA splicing of exon 2 (10), reduced Apobec3 mRNA induction following infection (2), and destabilizing amino acid polymorphisms (21, 22) that altogether could compromise Apobec3 function. In addition, the alternatively spliced Apobec3 Δexon5 variant in the Rfv3-resistant B6 strain was also shown to have stronger antiviral activity in vitro than full-length Apobec3 (21, 22). Thus, a combination of these Apobec3 polymorphisms most likely explains why Apobec3 is more potent in Rfv3-
immune responses. These findings raise the possibility of an indirect mechanism of Apobec3 action, in which the development of neutralizing Ab responses is augmented as a result of Apobec3-mediated reduction in virus-induced pathology during the acute phase of infection.

To distinguish between these possibilities, we performed immunization studies in mice using a model hapten, (4-hydroxy-3-nitrophenyl) acetyl (NP). The NP-hapten immunization system has been used extensively to evaluate Ab affinity maturation (27–29). Thus, we monitored the affinity maturation of the resulting NP-specific Abs in the presence or absence of FV infection. In addition, we characterized various immune compartments in the presence and absence of FV infection, looking for evidence of humoral immune dysregulation. Taken together, our data strongly support an indirect mechanism of Apobec3 action. These results raise the possibility that enabling Apobec3 function during acute infection may augment neutralizing Ab responses against a range of pathogenic human retroviruses, including HIV-1.

Materials and Methods

Mouse strains

C57BL/6 (B6) (H-2b/d Fv2r/r Rfv3r/r), BALB/c (H-2d/d Fv2s/s Rfv3s/s), and A.BY (H-2b/d Fv2s/s Fy3s/s) strains were purchased from The Jackson Laboratory (Bar Harbor, ME). Apobec3-deficient mice, constructed using the XN450 gene-trap embryonic cell line (BayGenomics, San Francisco, CA), were backcrossed for eight generations in the B6 background (10). The mouse studies were performed in full accordance with University of California, San Francisco and University of Colorado Denver institutional policies regarding animal care and use.

real-time PCR

Erythroid, B, T, myeloid, and dendritic cells were purified from B6 spleenocytes and bone marrow cells using magnetic beads linked to Abs against Ter119, CD19, CD90.2 (Thy1.2), and CD11c, respectively (Miltenyi Biotec, Auburn, CA). RNA from these cell subpopulations was extracted using the RNAEasy kit (Qiagen, Valencia, CA) and subjected to quantitative TaqMan real-time RT-PCR. The primers mA3.F (5′-CTGCCATGGA-CTTACGAA) and mA3.R (5′-TCTGGAAAGCTT AGAATCCTGGT) are located in exons 3 and 4 of Apobec3 and amplify a 124-bp fragment. These primers, including the TaqMan probe mA3.P (5′-FAM-CCAAGGCCT-CTGCCATGGAC-3′-TAMRA-3′), are conserved between Rfy3-resistant (B6) and Rfy3-susceptible (A.BY, BALB/c) strains. Total RNA (10 ng) was reverse transcribed using the mA3.F primer and the RT2 reverse-transcription kit (SA Biosciences, Frederick, MD). cDNA samples were

![FIGURE 1](image1.png) Expression of Apobec3 mRNA in bone marrow and spleen cell subpopulations. B cells (CD19+), T cells (CD90.2+), erythroblasts (Ter119+), and DCs (CD11c+) were magnetically purified from bone marrow or spleen of B6 mice and subjected to quantitative RT-PCR. MEFs were derived from B6 mice. Note that all of these cell subpopulations express readily detectable Apobec3 mRNA, with the highest levels of expression found in B cells. DCs, dendritic cells; MEFs, mouse embryonic fibroblasts.

FIGURE 2. Apobec3 does not influence NP-specific IgG1 affinity maturation. A, Immunization schedule. Mice were primed and boosted with 100 μg NP3-CGG, and plasma samples were collected at 2 and 4 wk after priming and boosting (*). Time points following the NP boost were designated as weeks 0′, 2′, and 4′. The rest period corresponds to the time interval between the bleed 4 wk after NP priming and the time point of NP boosting. B, Kinetics of NP-specific Ab affinity maturation in B6 mice. Plasma samples from B6 Apobec3+/+ (n = 7) and Apobec3−/− mice (n = 7) were subjected to a differential NP-binding ELISA. The relative binding affinity of NP-specific Abs was expressed as the ratio of the mean 50% binding titers to low (NP3)– versus high (NP33)-molar hapten reactivities multiplied by 100. SDs were indicated as vertical lines. The rest period for this cohort was 2 wk. C, Kinetics of affinity maturation in (B6 × BALB/c)F1 mice. Plasma samples from (B6 Apobec3+/+ × BALB/c)F1 (n = 7) and (B6 Apobec3−/− × BALB/c)F1 (n = 7) were subjected to a differential NP-ELISA, and relative binding affinities are shown. The rest period for this cohort was 2 wk.
subjected to quantitative PCR by mixing 2 μl cDNA with 20 nM mA3-F and mA3-R, 20 nM mA3.P, and 1X of the TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) in a 22 μl reaction. The reaction conditions consisted of a hot-start activation step at 95˚C for 15 min, followed by 40 cycles of 94˚C for 15 s and 60˚C for 60 s. β-actin levels were quantified using 10 nM mouse β-actin primers (SA Biosciences; catalogue PPM029454A), followed by quantitative PCR using SYBR Green. Relative Apobec3 mRNA levels were computed using a power equation using the cycle threshold versus log quantity, \(2^{-\Delta\Delta Cq}\) based on an in-plate standard curve generated from serial dilutions of an Apobec3 expression plasmid with known copy number, and values were normalized against input β-actin levels.

**Hapten immunization**

B6 Apobec3+/+ and Apobec3−/− mice (>4 mo old) and (B6 Apobec3+/+ × BALB/c)F1 and (B6 Apobec3−/− × BALB/c)F1 (>6–8 wk old) mice were immunized i.p. with 100 μg NP conjugated to chicken γ-globulin (NP35-CGG; Biosense Technologies, Novato, CA) in Inject alum (Pierce, Rockford, IL). Plasma samples were harvested 2 and 4 wk after priming and boosting. In some experiments, the NP boost was preceded by infection with 7500 spleen focus-forming units (SFFU) FV stock. The plasma samples were subjected to an ELISA using BSA conjugated to low (NP3 or NP4) or high levels (NP3+ or NP3+) of nitrophenyl hapten per molecule (Biosense Technologies). Total NP-specific Abs (both low and high affinity) were expected to bind CGG heavily substituted with NP, whereas only high-affinity NP-specific Abs are expected to bind to CGG containing low-level NP substitution. Thus, the ratio of the binding titers for the low versus the high NP-substituted CGG (or the NP reactivity ratio) provides a reliable measure of binding affinity. This approach was previously validated using a panel of NP-specific mAbs, which revealed a strong positive correlation between the NP reactivity ratio and the binding affinity of the mAbs to NP as measured by competitive RIA (30), isothermal titration calorimetry (31), and surface plasmon resonance (32). Importantly, an increase in NP reactivity ratio following NP boosting is accompanied by increased somatic hypermutation of the VH186.2 locus from NP-specific B cells in germinal centers (GC) (27–29). Briefly, 100 ng NP-BSA was coated into 96-well Immulon-4 plates overnight, followed by blocking for >2 h with PBS containing 0.05% Tween 20 (PBS-T). Serial 10-fold dilutions of plasma in PBS were incubated at 37˚C for 2 h and washed three times with PBS-T, and then 1/50,000–1/250,000 dilution of goat anti-IgG1 conjugated to HRP (Bethyl Laboratories, Montgomery, TX) was incubated for 1 h at 37˚C. After three washes with PBS-T, the samples were developed using tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO) and stopped using 2 N H2SO4. Absorbances were read at 450 nm and used to construct binding curves. The 50% binding concentrations were estimated graphically using Microsoft Excel (Redmond, WA). Relative binding affinities were estimated by dividing the 50% binding concentration against the historic phenotype (6). Mice (BALB/c)F1 and (B6 Apobec3+/+ × BALB/c)F1 (6–8 wk old) were immunized i.p. with 7500 spleen focus-forming units (SFFU) FV stock. The plasma samples were harvested 2 and 4 wk after priming and analyzed using a FACSCalibur II (BD Biosciences) flow cytometer with collection of 80,000–120,000 events per sample. Cell subpopulations were analyzed using FlowJo (Tree Star, Ashland, OR).

**Measurement of Ig levels**

The levels of total IgA, IgG, IgM, IgG1, IgG2b, and IgG3 were measured using a commercial ELISA kit (ICL, Newberg, OR). The levels of these Iggs were computed using an in-plate standard curve.

**Results**

**Relative expression of Apobec3 in immune cell subpopulations**

Previous studies have shown that Apobec3 mRNA is highly expressed in bone marrow and spleen, the primary sites of FV infection in vivo (38). However, Apobec3 mRNA expression in specific cell subpopulations within these tissues has not been carefully analyzed. In particular, for Apobec3 to directly alter anti-FV somatic hypermutation and Ab affinity, Apobec3 must be expressed in B cells. Alternatively, an indirect mechanism of action requires expression of Apobec3 in at least one cell type targeted by FV. Of note, erythroid (Ter119+) cells are primary targets of FV infection and may be principally responsible for generating the resultant viremia (39). We therefore measured Apobec3 mRNA levels from magnetic bead-purified Ter119+ erythroid, CD19+ B cells, CD3+ T cells, and CD11c+ dendritic cells using quantitative real-time PCR. As shown in Fig. 3, each of these cell types expressed Apobec3 mRNA. B cells present in bone marrow and splenic tissue exhibited the highest levels of expression, whereas erythroid cells expressed 2-fold more.
Apopec3 than total bone marrow cells. This pattern of expression of Apobec3 did not obviously favor either a direct or indirect mechanism of Apobec3/Rfv3 action.

Normal Ab responses in Apobec3-deficient mice are significantly compromised in the context of FV infection

To evaluate whether Apobec3 directly influenced Ab responses by supplementing AID function in vivo, we performed immunization studies against a well-documented model immunogen, NP (Fig. 2A). NP consistently elicits a T cell-dependent IgG1 response that maps to the V_{H}186.2 locus (27). This model system can be used to evaluate Ag-specific B cell development. For example, it has been shown that AID^+/− mice do not mount a NP-specific IgG1 response (24). Furthermore, the binding affinity of NP-specific Abs can be assessed using a well-established differential ELISA employing BSA conjugated to low- or high-molar levels of hapten (30). As expected, boosting NP-immunized mice with the NP hapten resulted in an increase in the relative binding affinity of NP-specific Abs in B6 Apobec3^+/+ mice (Fig. 2B). However, an essentially overlapping affinity maturation response was observed in B6 Apobec3^+/− mice (Fig. 2B). Similar data were also observed for (B6 Apobec3^+/+ × BALB/c)F_{1} and (B6 Apobec3^+/− × BALB/c)F_{1} mice (Fig. 2C). These findings suggested that Apobec3 did not directly influence NP-specific IgG1 Ab affinity maturation in vivo, and thus argue against a direct model for Apobec3/Rfv3 action.

We next investigated whether Apobec3 influenced the NP-specific Ab response in the context of FV infection. NP-primed B6 mice (WT and Apobec3 deficient) were infected with FV 3 d prior to the NP boost (Fig. 3A). The binding affinity of NP-specific Abs was estimated based on the ratio of 50% binding titers obtained with low (NP_{2})- versus high-molar (NP_{23}) hapten. Similar to results in Fig. 2B, the binding affinity of NP-specific Abs was similar between WT and Apobec3-deficient mice 2 and 4 wk following NP priming (data not shown). However, the binding affinity of NP-specific Abs in Apobec3-deficient mice was significantly lower 4 wk following FV infection and NP boosting than observed in B6 mice expressing Apobec3.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** B6 Apobec3 protects specific subpopulations of bone marrow cells from acute FV infection. *A,* Gating strategy for assessing FV infection. Live bone marrow cells were gated based on forward and side scatter, and the percentage of specific cell subpopulations (in this case Ter119+ erythroblasts) was assessed within this live cell population. A Glyco-Gag–specific mAb (mAb34), coupled with allophycocyanin-conjugated secondary Ab, was used to detect FV-infected cells. Uninfected cells had <1% reactivity with mAb34. Using these gates, the percentages of FV+ cells were estimated. Representative panels from B6 Apobec3^+/+ and deficient F_{1} mice are highlighted. *B,* Bone marrow cell subpopulations in FV-infected mice. The proportion of erythroid (Ter119+), B (CD19+), T (CD3+), and myeloid (CD11b+) cells in live bone marrow cells was quantified. There was no significant difference in the proportion of these cell subpopulations found in B6 Apobec3^+/+ (n = 8) and B6 Apobec3-deficient (n = 8) F_{1} mice. Error bars depict SEM. *C,* Higher levels of FV-infected cells in multiple bone marrow subpopulations were detected in mice lacking B6 Apobec3. FV+ erythroid, B, T, and myeloid cells were quantified by flow cytometry. Statistical analyses were performed using two-tailed Student t test: *p < 0.05. The data correspond to cohort 2 as outlined in Table I.
Apobec3+/+ experiments were performed in the (B6 x B.ABY)F1 background that encode or do not encode the B6 Fv2 susceptibility gene, (B6 x B.ABY)F1 mice (Fig. 3A,B). Our prior data indicate that the presence of Apobec3 results in lower plasma viremia in FV-infected animals examined at 7 dpi (10). Direct viral infection in various cell subsets could result in altered adaptive immune response, leading in turn to a lack of Ab affinity maturation. We next investigated potential alterations in B cell subpopulations in (B6 Apobec3+/− x A.BY)F1 versus (B6 Apobec3+/− x A.BY)F1 mice at 7 dpi. Ag-specific B cell development initiates in the bone marrow where initial Ig gene rearrangements and maturation occur. IgM and IgD can be used as markers of B cell maturation, whereas syndecan-1 (CD138) can be used to mark B cells that have become Ab-secreting cells (Fig. 5A). FV infection resulted in a significant (~30%) decrease in the proportion of B cells in the bone marrow of both (B6 Apobec3+/− x A.BY)F1 and (B6 Apobec3+/− x A.BY)F1 mice at 7 dpi (Fig. 5B, upper right panel). Similar phenomena were also observed for both IgM+ and IgD+ B cells at 7 dpi (Fig. 5B, lower right panel). Thus, we found no obvious defects in early B cell proportions associated with B6 Apobec3 deficiency at 7 dpi. Interestingly, there was a significant increase in CD138+ plasmablasts that had migrated to the bone marrow of FV-infected (B6 Apobec3+/− x A.BY)F1, but not (B6 Apobec3+/− x A.BY)F1 mice at 7 dpi (Fig. 5B, lower right panel). This result suggested possible differences in B cell subpopulations in secondary lymphoid organs.

Upon encountering Ag, B cells proliferate in secondary lymphoid organs and undergo affinity maturation in structures known as GC (27). Subsequently, selected Ag-specific B cells develop into CD138+ plasmablasts that migrate back to the bone marrow to become Ab-secreting plasma cells (40). Following the detection of higher levels of CD138+ plasmablasts in the bone marrow of FV-infected (B6 Apobec3+/− x A.BY)F1 mice, we sought to quantify less efficient induction of GC B cells in Apobec3-deficient mice.

Apobec3 protects multiple immune cell types from acute FV infection

Our prior data indicate that the presence of Apobec3 results in lower plasma viremia in FV-infected animals examined at 7 dpi (10). Because restriction of virus levels occurred prior to the peak of adaptive immune responses, it was possible that Apobec3-mediated innate immune activity restricted FV replication in specific cell types in the bone marrow and spleen. Because infection of immune cells could impair their development and function, restriction of such infection by Apobec3 could potentially improve NP-specific Ab affinity maturation and virus-specific neutralizing Ab response. To analyze infection of specific cell subsets, flow cytometry was used to detect the expression of a surface Ag of FV, GlycoGag, in conjunction with cellular subset-specific Ags. FV infection experiments were performed in the (B6 x A.BY)F1 background that encode or do not encode the B6 Apobec3 gene. Due to the expression of the dominant Fv2 susceptibility gene, (B6 x A.BY) F1 mice are more susceptible to FV infection and develop splenomegaly (10). Cells from both bone marrow (Fig. 4A) and spleen (data not shown) were analyzed at 7 dpi. No significant perturbations in the proportions of erythroid cells, B cells, T cells, and myeloid cells were observed in either tissue (Fig. 4B and data not shown). However, higher levels of viral infection were observed in each of these cell subsets in (B6 Apobec3+/− x A.BY)F1 versus (B6 Apobec3+/− x A.BY)F1 mice (Fig. 4C, Table I).

Table I. FV infection of (B6 x A.BY)F1 mice at 7 dpi

<table>
<thead>
<tr>
<th>Cell Subpopulation</th>
<th>Cohort</th>
<th>Marker</th>
<th>WT</th>
<th>KO</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythroid</td>
<td>1</td>
<td>Ter119+</td>
<td>3.83 ± 1.33</td>
<td>33.37 ± 4.43</td>
<td>0.0004</td>
</tr>
<tr>
<td>2</td>
<td>Ter119+</td>
<td>2.20 ± 0.45</td>
<td>25.65 ± 6.82</td>
<td>0.0108</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ter119+</td>
<td>2.37 ± 0.81</td>
<td>25.65 ± 2.70</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td>1</td>
<td>B220+</td>
<td>1.43 ± 0.31</td>
<td>9.93 ± 1.88</td>
<td>0.0037</td>
</tr>
<tr>
<td>2</td>
<td>CD19+</td>
<td>2.34 ± 0.23</td>
<td>17.50 ± 4.57</td>
<td>0.0128</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CD19+</td>
<td>2.11 ± 0.22</td>
<td>8.24 ± 0.99</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td>Myeloid</td>
<td>1</td>
<td>CD11b+</td>
<td>0.53 ± 0.11</td>
<td>2.52 ± 0.76</td>
<td>0.0335</td>
</tr>
<tr>
<td>3</td>
<td>CD11b+</td>
<td>0.37 ± 0.12</td>
<td>2.94 ± 0.57</td>
<td>0.0058</td>
<td></td>
</tr>
<tr>
<td>Granulocytes</td>
<td>1</td>
<td>Gr-1+</td>
<td>1.03 ± 0.20</td>
<td>4.51 ± 1.00</td>
<td>0.0128</td>
</tr>
<tr>
<td>2</td>
<td>Gr-1+</td>
<td>0.64 ± 0.12</td>
<td>3.00 ± 0.88</td>
<td>0.0321</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Gr-1+</td>
<td>0.17 ± 0.04</td>
<td>1.54 ± 0.25</td>
<td>0.0024</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>2</td>
<td>CD3+</td>
<td>1.84 ± 0.19</td>
<td>7.15 ± 1.66</td>
<td>0.0150</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythroid</td>
<td>1</td>
<td>Ter119+</td>
<td>12.01 ± 3.69</td>
<td>26.93 ± 5.13</td>
<td>0.0379</td>
</tr>
<tr>
<td>3</td>
<td>Ter119+</td>
<td>13.87 ± 3.47</td>
<td>33.47 ± 1.72</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ter119+</td>
<td>4.26 ± 0.95</td>
<td>18.59 ± 4.19</td>
<td>0.0178</td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td>1</td>
<td>CD19+</td>
<td>4.16 ± 0.78</td>
<td>6.52 ± 1.47</td>
<td>0.1896</td>
</tr>
<tr>
<td>3</td>
<td>CD19+</td>
<td>5.06 ± 1.12</td>
<td>12.32 ± 1.09</td>
<td>0.0009</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>4</td>
<td>B220+</td>
<td>1.87 ± 0.50</td>
<td>7.33 ± 1.26</td>
<td>0.0057</td>
</tr>
<tr>
<td>3</td>
<td>CD3+</td>
<td>2.70 ± 0.58</td>
<td>5.85 ± 0.79</td>
<td>0.0084</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CD3+</td>
<td>2.81 ± 0.46</td>
<td>6.80 ± 1.00</td>
<td>0.0084</td>
<td></td>
</tr>
<tr>
<td>Myeloid</td>
<td>1</td>
<td>CD11b+</td>
<td>3.46 ± 0.85</td>
<td>7.81 ± 1.24</td>
<td>0.0149</td>
</tr>
<tr>
<td>3</td>
<td>CD11b+</td>
<td>4.56 ± 0.98</td>
<td>10.09 ± 0.93</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CD11b+</td>
<td>1.99 ± 0.40</td>
<td>8.73 ± 1.25</td>
<td>0.0021</td>
<td></td>
</tr>
</tbody>
</table>

*p values were computed using two-tailed Student t test.

KO, knockout.

8FV infections were performed on four separate cohorts of (B6 Apobec3+/− x A.BY) (WT) and (B6 Apobec3+/− x A.BY) (KO) F1 mice. The sample sizes are as follows: cohort 1, WT = 7, KO = 7; cohort 2, WT = 8, KO = 8; cohort 3, WT = 6, KO = 6; cohort 4, WT = 7, KO = 6.

9Entries correspond to the mean FV+ cells ± SEM of WT and KO F1 mice, respectively.
the frequencies of GC B cells and plasmablasts in the spleen (Fig. 6A). GC B cells can be preferentially marked with peanut agglutinin, which reacts with O-linked glycans, or GL7, which correspond to α2,6-linked sialic acid present on lactosamine chains (41).

High levels of foreign Ags associated with virus infection are thought to drive GC formation and Ag-specific B cell development. Consistent with this notion, acute FV infection of (B6 Apobec3+/+ x A.BY)F1 mice resulted in a 2.0- and 2.3-fold increase in the proportion of splenic GC B cells (GL7+) and CD138+ plasmablasts, respectively, relative to uninfected (B6 Apobec3+/+ x A.BY)F1 mice. Of note, (B6 Apobec3−/− x A.BY)F1 strains exhibited significantly lower levels of induction of these cell subpopulations (Fig. 6B), despite the fact that these mice strains display higher levels of viremia (Table I). In a separate cohort of mice, we detected higher frequencies of peanut agglutininhigh GC B cells in infected (B6 Apobec3+/+ x A.BY)F1 mice compared with infected (B6 Apobec3−/− x A.BY)F1 mice (data not shown). Thus, the presence of B6 Apobec3 is associated with greater induction of GC B cell and plasmablast cell subpopulations in the spleen during acute FV infection.

Apobec3 influences Ig levels during acute FV infection

Suppressed induction of GC B cells and plasmablasts in FV-infected B6 Apobec3-deficient F1 mice despite higher viral Ag loads suggested that B cell function in these mice was either blocked or dysregulated. To investigate potential humoral immune dysregulation, we assessed whether FV-infected B6 Apobec3−/− mice exhibited hypergammaglobulinemia, a hallmark of aberrant polyclonal B cell activation (42, 43).

Our results revealed that uninfected B6 Apobec3+/+ and B6 Apobec3−/− mice display similar levels of total IgM and IgG in
that these B6 mice lack detectable levels of IgG2a (44)]. The titers of these IgG subclasses did not vary significantly between uninfected WT and Apobec3-deficient mice. However, upon FV infection, there was a statistically significant increase in IgG2b and IgG3 titers in the Apobec3-deficient compared with the WT mice (Fig. 7B). There was also a trend for increased IgG1 and IgA expression in Apobec3-deficient mice (Fig. 7B). However, by 28 dpi, the total IgG titers in WT mice increased to the same elevated levels as Apobec3-deficient mice (data not shown). These findings revealed that Apobec3 activity delayed the onset of hypergammaglobulinemia during FV infection.

**Discussion**

The recent identification of Apobec3 as Rfv3 (10), a gene that influences neutralizing Ab responses against FV infection, immediately raised the issue of how the innate Apobec3 enzyme achieves this effect on the humoral immune response. Our studies indicate that Apobec3 does not act directly in B cells by promoting somatic hypermutation and Ab affinity maturation like the AID cytidine deaminase. Rather, Apobec3 appears to act in a more indirect manner by protecting key immune cells needed for optimal neutralizing Ab responses during FV infection. These results strengthen an emerging concept that the early host response occurring during pathogenic virus infection can have a profound impact on disease recovery. This could be due to the ability of innate immune responses to dampen early pathological events that may otherwise compromise the integrity and efficacy of subsequent adaptive immune responses. This concept may be particularly relevant for viruses that directly infect immune cells of the host, which, in the case of FV infection, were protected by Apobec3.

Our findings demonstrate that the presence of the B6Apobec3 gene promotes more vigorous induction or development of GC B cells and plasmablasts that participate in the production of high-affinity Abs. In addition, Apobec3 delays the onset of hypergammaglobulinemia, a hallmark of aberrant B cell activation (42, 43). Together, these findings suggest that Apobec3 preserves B cell function during acute FV infection and facilitates Ab affinity maturation. These results provide a working model (Fig. 8) to further dissect the sequence of events that underlie the weaker neutralizing Ab response occurring in mice with defective Apobec3 function.

**FIGURE 8.** Working model for how Apobec3 influences the FV-specific neutralizing Ab response. Acute FV infection of multiple immune cells (erythroid, B, T, and myeloid) that express Apobec3 (upper row) results in the release of noninfectious particles containing Apobec3 that in next round of infection display reduced infectivity for target cells. At the same time, these Apobec3+ viral particles could prime virus-specific Ab responses by presenting native envelope trimers. Reduced pathology in the presence of viral Ag results in the induction of GC B cells and plasmablasts that eventually translate to the development of high-affinity virus-specific Abs. In Apobec3-deficient/Rfv3-susceptible mice (lower row), uncontrolled FV replication in B cells and possibly other immune cells results in aberrant polyclonal B cell activation as highlighted by hypergammaglobulinemia and suppressed B cell responses. This translates to delayed affinity maturation of virus-specific Abs. In this model, we hypothesize that high-affinity envelope-specific Abs contribute significantly to a potent neutralizing Ab response.
Notably, B6 Apobec3-deficient mice at 7 dpi exhibited lower levels of splenic GC B cells, but higher levels of total IgG in plasma. We previously reported that at 14 dpi, FV-infected B6 Apobec3-deficient mice had lower levels of FV-specific IgG Abs compared with mice encoding B6 Apobec3 (10). Thus, the induced IgGs in B6 Apobec3-deficient mice are most likely not virus specific, and probably developed in an extrafollicular manner (45). Although it is known that CD4+ T cells (42), viral proteins (46), and the LDV component of the original FV stock (47) can directly participate in hypergammaglobulinemia induction, the impact of Apobec3 on these parameters requires further study. Finally, it remains unknown whether the Rfv3 phenotype is solely due to the ability of Apobec3 to protect B cells from FV infection, or whether protection of other cell types also plays a role. Uncontrolled FV replication in erythroblasts, myeloid cells, and T cells could result in displacement of other cell types and/or the aberrant release of cytokines that could misdirect the B cell response. FV infection of dendritic cells (48) and follicular CD4+ T cells (49), in particular, may have a dramatic impact on subsequent Ab responses. Dissecting the contribution of individual cell types in the Apobec3-mediated influence on humoral immunity will require adoptive transfer studies or FV infection of transgenic mice where Apobec3 is preferentially expressed only in specific immune cell subpopulations.

Our results suggest that decreased B cell pathology during acute infection contributes to the ability of Apobec3 to promote an effective FV-specific neutralizing Ab response (the hallmark of the Rfv3 phenotype). Whereas this would suggest that antiviral interventions during the acute phase of FV infection may also strengthen Ab responses, we hypothesize that the unique biology of Apobec3 may particularly promote this process. Apobec3 activity results in the release of noninfectious particles that present the envelope glycoprotein, the major target of neutralizing Abs in FV infection (34, 50), as functional, fusion-competent trimers. Thus, in contrast to other innate intervention mechanisms, Apobec3 activity not only could reduce FV-induced pathology, but it may also present viral envelope Ags in a manner that is highly suitable to elicit an effective Ab response. The possibility that Apobec3+ viral particles serve as endogenous vaccines in the FV model system is currently being explored. Notably, presenting functional envelope trimers for eliciting neutralizing Ab responses has been used to justify the development of HIV-1 vaccines based on noninfectious virus-like particles (51, 52).

The nature of a protective neutralizing Ab response against pathogenic retroviruses remains unclear. High-affinity envelope-specific Abs, high levels of low-affinity virus-specific Abs that confer high-avidity binding, Abs that bind to epitopes critical for envelope-mediated fusion, and specific IgG subclasses that mediate potent Ab-dependent cellular cytoxicity could all be important features of a potent neutralizing Ab response. The results of our hapten immunization studies may offer additional clues. One month following FV infection/hapten boosting, the total NP-specific binding titers were similar between WT and Apobec3-deficient mice, but the binding affinities were significantly lower for mice lacking Apobec3 (Fig. 3). Of note, we previously found that total virus-specific binding Abs at 28 dpi were not significantly different between mice with or without the B6 Apobec3 gene, but neutralizing Ab responses were significantly higher in B6 Apobec3+ mice (10) (M.L. Santiago, unpublished results). We hypothesize that a potent virus-specific neutralizing Ab response includes a significant proportion of virus-specific Abs that exhibit high affinity to the envelope glycoprotein (Fig. 8). Thus, a potent virus-specific neutralizing Ab response may be dictated as much by the quality as the quantity of virus-specific Abs. Profiling the FV-specific Ab repertoire in infected B6 Apobec3+ and B6 Apobec3-deficient mice may help dissect the molecular characteristics of a protective versus a nonprotective Ab response against this pathogenic retrovirus infection.

The importance of Apobec3 in human retrovirus infections has been highlighted by the identification of specific viral mechanisms that subvert this response. HIV-1 encodes a protein known as Vif that specifically antagonizes two human Apobec3 homologs, Apobec3G and Apobec3F (53, 54). Vif binds to Apobec3G and Apobec3F and links these deaminases to components of an E3–ubiquitin ligase complex that promotes polyubiquitination and subsequent proteasome-mediated degradation of these enzymes (55, 55–58). Thus, the function of two critical human Apobec3 proteins in HIV-1–infected individuals is impaired, analogous to Rfv3-susceptible mouse strains. Notably, HIV-1–infected individuals rarely mount a strong HIV-1–specific neutralizing Ab response. Whereas the heterogeneity and structural features of the HIV-1 envelope glycoprotein are considered the primary evasive mechanisms, extensive humoral immune deficiency during acute HIV-1 infection, such as hypergammaglobulinemia and aberrant polyclonal B cell activation (59), loss in memory B cell subsets (60), and GC destruction in the gastrointestinal tract (61), is gaining attention as a contributing force for poor neutralizing Ab responses. B cell defects also appear to underlie the poor virus- and hapten-specific Ab responses occurring in FV-infected mice lacking Apobec3 (Fig. 8).

By analogy, our studies raise the possibility that augmenting Apobec3G/Apobec3F function during acute HIV-1 infection may help strengthen the neutralizing Ab response by dampening virus-induced pathology, while facilitating the release of noninfectious particles. This possibility may be experimentally tested in pathogenic SIV infection models in rhesus macaques, where recently, mutations in SIV Vif that compromise its interplay with the E3 ligase component Elongin C resulted in the rescue of CD4+ T cells and virus-specific Ab responses (62). If true, manipulating Apobec3 activity to improve HIV-1–specific neutralizing Ab responses could significantly impact future HIV-1 vaccine development strategies.

Note added in proof. Following the submission of this manuscript, a study performed by Tsuji-Kawahara et al. (63) was published online. This study confirmed our initial findings that Apobec3 influences the FV-specific neutralizing Ab response (10), the distinguishing hallmark of Rfv3 (6, 33). Complementing the results we present in this work, Tsuji-Kawahara et al. also provided evidence favoring an indirect mechanism for how Apobec3 influences neutralizing Ab responses by demonstrating the following: 1) no difference in Ab-binding titers following immunization with DNP-Ficoll and DNP-OVA in uninfected mice with or without the B6 Apobec3 gene; 2) increased FV infection of spleen Tert119+ and B cells in the absence of B6 Apobec3; and 3) increased B cell activation and defects in B cell maturation in the spleen of B6 Apobec3-deficient mice at 10 dpi. However, unlike the data we present in this work, Tsuji-Kawahara et al. did not present evidence that the B6 Apobec3 gene promotes Ab affinity maturation, GC B cell induction, and plasmablast development during acute infection (7 dpi) by protecting multiple Apobec3+ cellular subsets in both the bone marrow and spleen from FV infection. Finally, Tsuji-Kawahara et al. suggested that a recessive B cell activating factor receptor (BAFF-R) deficiency in the A/WySn strain contributes more to the Rfv3 phenotype than Apobec3. This conclusion is based on the authors’ comparison of 28 dpi viremia for (B6 × A/WySn)F1 and (B6,Apobec3+ → A/WySn)+F1 mice. Surprisingly, no data were presented for congenic (B6 BAFF-R+/− × A/WySn)+F1 mice, making it currently impossible to ascertain the relative contributions of BAFF-R and Apobec3 in the recovery of (B6 × A/WySn)+F1 mice. Importantly, in our study, all the strains used [B6, (B6 × A.BY)+F1, and (B6 × BALB/c)+F1] express the dominant
WT ABAF-R gene from the B6 background. Thus, ABAF-R deficiency does not impact any of our presented results.

Acknowledgments

We thank Richard Locksley (University of California) for advice on the hapten immunization studies and Roberta Pelanda and Raul Torres (University of Colorado Denver) for discussion.

Disclosures

The authors have no financial conflicts of interest.

References


