

APOBEC3 Proteins Expressed in Mammary Epithelial Cells Are Packaged into Retroviruses and Can Restrict Transmission of Milk-Borne Virions

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SUMMARY

Viruses, including retroviruses like human immunodeficiency virus (HIV) and mouse mammary tumor virus (MMTV), are transmitted from mother to infants through milk. Lymphoid cells and antibodies are thought to provide mammary gland and milk-borne immunity. In contrast, little is known about the role of mammary epithelial cells (MECs). The APOBEC3 family of retroviral restriction factors is highly expressed in macrophages and lymphoid and dendritic cells. We now show that APOBEC3 proteins are also expressed in mouse and human MECs. Lymphoid cell-expressed APOBEC3 restricts *in vivo* spread of MMTV to lymphoid and mammary tissue. In contrast, mammary gland-expressed APOBEC3 is packaged into MMTV virions and decreases the infectivity of milk-borne viruses. Moreover, APOBEC3G and other APOBEC3 genes are expressed in human mammary cells and have the potential to restrict viruses produced in this cell type. These data point to a role for APOBEC3 proteins in limiting infectivity of milk-transmitted viruses.

INTRODUCTION

A number of viruses are transmitted through milk from mother to infants, including cytomegalovirus (Meier et al., 2005), vaccinia virus (Abraham et al., 2009), papillomaviruses (Lindsey et al., 2009; Sarkola et al., 2008), and the retroviruses HIV, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), human T cell leukemia virus (HTLV) I/II, and MMTV (Allison and Hoover, 2003; Li et al., 2004; Nandi and McGrath, 1973; O'Neil et al., 1995; Rychert et al., 2006; Sellon et al., 1994; Seltzer and Benjamin, 1990; Ziegler et al., 1985). The best-studied milk-transmitted virus is the murine retrovirus, MMTV, which causes breast cancer in mice (Nandi and McGrath, 1973). MMTV *in vivo* infection occurs first in dendritic cells (DCs) of the gut, which then transfer the virus to lymphocytes (Ross, 2008). The

infected lymphocytes traffic to the mammary gland, resulting in the infection of mammary epithelial cells (MECs) during puberty and pregnancy. Lactating mice shed high levels of cell-free virus into milk, which is then acquired by nursing offspring during the first week of life (Ross, 2008).

In addition to being a source of virus that can be transmitted via milk, lymphoid cells in the mammary gland and milk provide passive immunity to the neonate against pathogenic infections (Walker, 2004). Indeed, strong neutralizing anti-MMTV humoral responses in mice result in antibody-coated milk-borne virions that are rendered noninfectious and are not transmitted to newborns (Case et al., 2005). Moreover, it has been suggested that anti-HIV antibodies in human milk block milk-borne transmission (Bouhhal et al., 2005) and that CD8⁺ T lymphocytes control SIV replication in breast milk lymphocytes (Permar et al., 2008), thereby potentially limiting milk-transmitted infection (John-Stewart et al., 2009). In contrast to the important role that lymphoid cells play in providing mammary gland and milk-borne immunity, little is known about how MECs participate in this process.

Recently, several cellular genes have been identified that function in addition to the innate and adaptive immune response to limit virus infection and spread. These include members of the APOBEC3 family of restriction factors, which encode DNA-editing enzymes characterized by the presence of at least one cytidine deaminase domain (LaRue et al., 2009). The number of APOBEC3 genes varies between species, from one gene in rodents to seven genes in humans (LaRue et al., 2009). APOBEC3G and APOBEC3F confer intrinsic immunity to HIV-1 lacking the *vif* gene (Chiu and Greene, 2008; Cullen, 2006; Malim, 2009). In *vif*-deficient-HIV-1 producer cells, APOBEC3G and -3F are packaged into progeny virions, while in cells infected with *vif*⁺ virus, Vif binds APOBEC3F and -3G and targets them for degradation in the proteasome, thereby preventing their packaging and overcoming the antiviral activity. Virion-packaged APOBEC3 proteins inhibit infection in target cells by deaminating deoxycytidine residues on the DNA minus strand following reverse transcription, inducing mutations in newly synthesized HIV-1 DNA; this leads to both degradation of reversed transcribed DNA prior to integration and to mutation of viral genes in the integrated provirus (Cullen, 2006; Malim, 2009). There is also evidence that APOBEC3 proteins can restrict

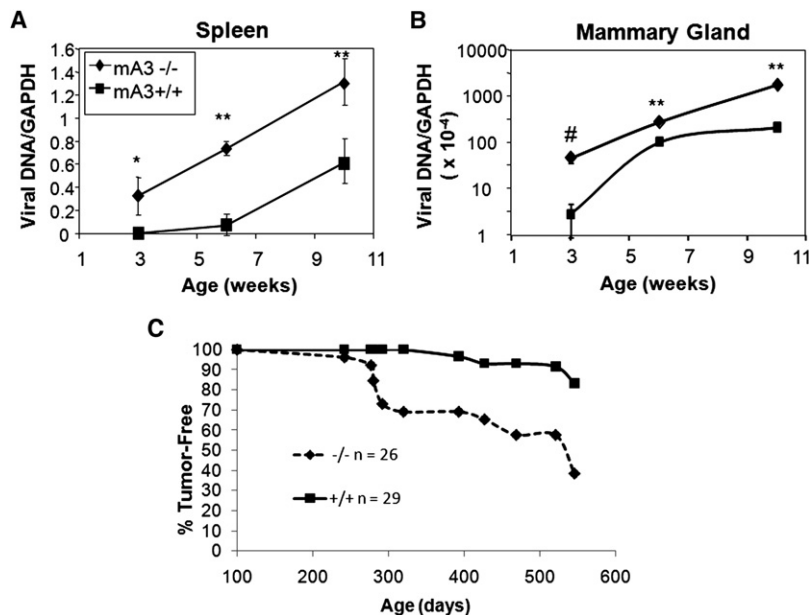


Figure 1. Infection of Tissues of *mA3^{+/+}* and *mA3^{-/-}* Mice after Milk-Borne Transmission of MMTV

(A and B) RT-qPCR analysis of DNA isolated from spleen and nonlactating mammary gland, respectively, using MMTV- and GAPDH-specific primers; shown are the MMTV values normalized to GAPDH. All error bars represent SD from the averages of the mice analyzed at each time point. From 6 to 19 mice were analyzed for each time point. * $p \leq 0.02$; ** $p \leq 0.001$; # $p \leq 0.01$; comparison is between *mA3^{+/+}* and *mA3^{-/-}* at each time point.

(C) Mammary tumor incidence in MMTV-infected *mA3^{-/-}* and *mA3^{+/+}* mice after milk-borne infection. Shown are Kaplan-Meier survival curves that were computed for age at tumor onset. The p value for the test that these two curves are significantly different (null hypothesis of equality) was <0.0001 . $N = 26$ *mA3^{-/-}* mice, 29 *mA3^{+/+}* mice.

HIV infection by non-CDA-dependent mechanisms (Malim, 2009).

APOBEC3 proteins also restrict infection by a number of mouse retroviruses. Mice with targeted deletion of the *Apobec3* gene are more susceptible to infection by MMTV and a number of murine leukemia viruses (MLVs), all of which target cells of hematopoietic origin in vivo (Langlois et al., 2009; Low et al., 2009; Okeoma et al., 2007; Santiago et al., 2008; Takeda et al., 2008). APOBEC3-mediated restriction of MMTV and Friend MLV, but not AK virus infection, is independent of cytidine deamination (Langlois et al., 2009; Okeoma et al., 2007; Takeda et al., 2008). Because mothers can transfer viruses to their neonates via milk, it is also possible that intrinsic immune factors like APOBEC3 proteins could limit milk-borne transmission. Here we demonstrate that APOBEC3 proteins are expressed in mouse and human MECs and mammary tissue. We also show that APOBEC3 protein made in MECs is packaged into retrovirus particles and affects viral infectivity. These data highlight the potential importance of MEC-expressed APOBEC3 on the transmission of milk-borne retroviruses.

RESULTS

APOBEC3 Knockout Mice Have Increased Mammary Gland Infection

APOBEC3 RNA can be detected in cultured murine MECs and primary mammary tissue (Okeoma et al., 2007; Pauklin et al., 2009). To determine if APOBEC3 in mammary tissue restricts in vivo virus infection, we foster-nursed APOBEC3 wild-type (*+/+*) and knockout (*-/-*) mice on the same MMTV-infected mothers and monitored infection of mammary gland from 3 to 10 weeks after birth. MMTV infection of spleens served as a positive control, since this tissue was more highly infected in MMTV-inoculated *mA3^{-/-}* mice than in *mA3^{+/+}* mice (Okeoma et al., 2007). In addition, a cohort of MMTV-infected *mA3^{+/+}* and *mA3^{-/-}* mice was monitored for mammary tumor induction.

Mice lacking APOBEC3 had higher levels of milk-borne MMTV infection of both splenic and mammary tissue at all times examined (Figures 1A and 1B). The difference in infection in the wild-type and knockout mice changed with age, from 130-fold to 2-fold and from 17-fold to 3-fold from 3 to 10 weeks in spleen and mammary tissue, respectively. The higher level of infection in *mA3^{-/-}* mice also resulted in increased tumor incidence and decreased time to tumor formation (Figure 1C); at the conclusion of the analysis, 16 of 26 *mA3^{-/-}* developed mammary tumors (average time to tumor = 382 days; median time to tumor = 307 days) in contrast to 4 of 29 *+/+* mice that developed tumors (average time to tumor = 476 days; median time to tumor = 483 days), while no uninfected *mA3^{+/+}* or *mA3^{-/-}* mice developed mammary tumors (data not shown). Thus, loss of APOBEC3-mediated restriction of MMTV infection resulted in higher levels of mammary gland infection and thus caused the virus to be more pathogenic in vivo.

Mammary Gland Infection Is Restricted by Lymphocyte but Not Mammary Cell APOBEC3

The higher level of mammary gland infection leading to increased mammary tumor formation in *mA3^{-/-}* mice could result from lack of APOBEC3-mediated restriction of MMTV spread within lymphocytes, MECs, or both. To determine in which compartment APOBEC3-mediated restriction occurred, we lethally irradiated *mA3^{-/-}* and *mA3^{+/+}* mice, reconstituted them with bone marrow from *mA3^{+/+}* mice, and then infected them with MMTV. Reconstitution was monitored by FACS analysis of peripheral blood mononuclear cells for CD8+ and CD4+ T cells, CD11c+ DCs, and B220+ B cells; in all cases, no differences in reconstitution were seen between the *mA3^{+/+}* and *mA3^{-/-}* mice (Figure 2A). In addition, RNA was isolated from the spleens, lymph nodes, and mammary tissue of the reconstituted animals and examined by reverse-transcribed PCR for APOBEC3. All lymphoid tissue, but not the mammary gland of the *mA3^{-/-}* mice, expressed APOBEC3 RNA at levels similar to that seen in the reconstituted *mA3^{+/+}* mice (Figure 2B).

Not surprisingly, given that the lymphoid tissues of the *mA3^{+/+}* and *mA3^{-/-}* mice were equally reconstituted with the *mA3^{+/+}*

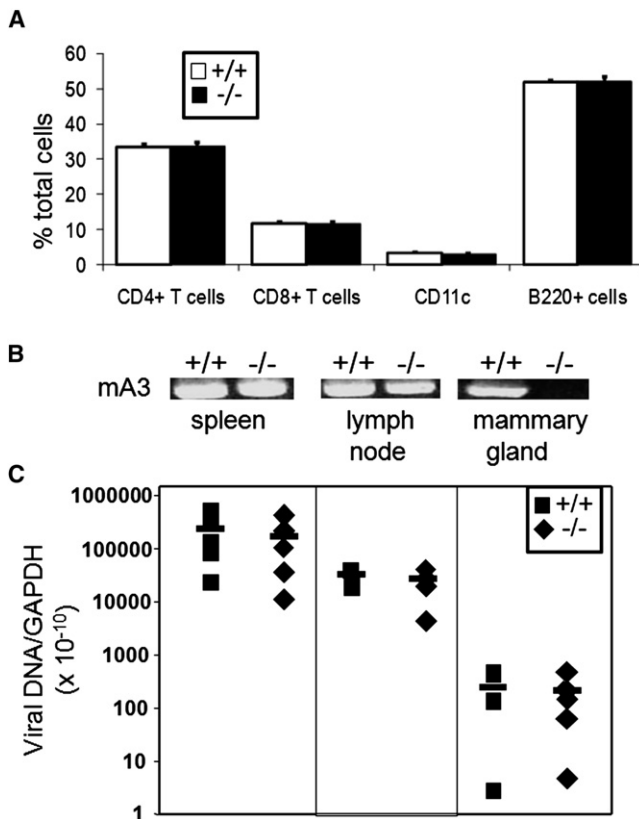


Figure 2. Adoptive Transfer of Lymphocytes into $mA3^{+/+}$ and $mA3^{-/-}$ Mice

Five mice of each genotype were lethally irradiated, reconstituted with bone marrow $mA3^{+/+}$ mice, infected with MMTV, and then sacrificed 9 weeks after irradiation.

(A) FACS analysis of reconstituted mice. PBLs were isolated from the individual mice at sacrifice and stained with the indicated antibodies. Error bars represent SD from the average for the five mice of each genotype.

(B) RNA was isolated from the spleen, lymph nodes (LN), and mammary gland (MG) and subjected to reverse-transcribed PCR with primers specific for APOBEC3.

(C) DNA was isolated from the indicated tissue and subjected to RT-qPCR specific for exogenous MMTV(RIII) sequences. Values were normalized to GAPDH. This experiment was repeated two additional times with similar results.

bone marrow, the level of MMTV infection in spleen and lymph node was not significantly different between the two sets of mice (Figure 2C). However, although no APOBEC3 RNA was detected in the mammary tissue of the reconstituted $mA3^{-/-}$ mice, infection levels were equal to that seen in the reconstituted $mA3^{+/+}$ mice, which did express APOBEC3 (Figure 2C). These data strongly suggested that MMTV infection of MECs was determined by lymphocyte virus load and that restriction by APOBEC3 occurred in lymphocytes but not epithelial cells.

APOBEC3 Expressed in Mammary Tissue Is Packaged into Milk-Borne Virions and Restricts Infection

MECs are the only source of cell-free MMTV *in vivo*, which is believed to be the major form of milk-transmitted virus (Nandi and McGrath, 1973). If APOBEC3 made in MECs was packaged

into MMTV virions, it would limit virus transmission of milk-borne virus. We next tested the infectivity of virus produced in MMTV-infected $mA3^{-/-}$ and $mA3^{+/+}$ mammary glands; in both strains, MMTV was transmitted through milk for five generations or more. Virions were purified from the milk of the $mA3^{+/+}$ and $mA3^{-/-}$ mice, and RNA isolated from these virions was quantified by reverse transcribed RT-qPCR. There was about 10-fold more virus in the milk produced by $mA3^{-/-}$ mice compared to $mA3^{+/+}$ mice (Figure 3A), which reflects the higher infection levels in the former (Figure 1B).

To show that APOBEC3 was packaged into virions made in mammary tissue, virus was isolated from the mammary tumors of $mA3^{+/+}$ and $mA3^{-/-}$ mice; mammary tumor tissue has long been used as a source of MMTV (Golovkina et al., 1994). We also analyzed splenic extracts from $mA3^{+/+}$ and $mA3^{-/-}$ mice, as well as 293T cells transduced with plasmids encoding HA-tagged BL/6 and BALB/c APOBEC3 proteins (Takeda et al., 2008). Because of allelic differences that cause differential splicing of the RNA in BL/6 versus BALB/c mice, the predominant APOBEC3 RNA produced in C57BL/6 mice lacks exon 5 and is ~3.6 kDa smaller than the full-length BALB/c 51 kDa protein (Okeoma et al., 2009; Takeda et al., 2008). No APOBEC3 protein was found in either the spleen or virus from $mA3^{-/-}$ mice. In contrast, delta exon 5 APOBEC3 protein was detected in both the spleen and virions isolated from $mA3^{+/+}$ mammary glands (Figure 3B).

To examine the infectivity of the milk-borne $mA3^{+/+}$ and $mA3^{-/-}$ virus, serial dilutions were used to infect 293T-mTfR1 cells (293T cells stably expressing the MMTV entry receptor, transferrin receptor 1), and DNA was isolated from the infected cells and subjected to RT-qPCR to determine the level of infection. Because of the difference in the amount of virus produced in the two strains of mice, the infection levels are presented after normalization to virion RNA levels. Even after normalization, we found that the virus from $mA3^{-/-}$ mice infected cells at higher levels than that isolated from $mA3^{+/+}$ mice (Figure 3C). Taken together with the protein analysis in Figure 3B, these data indicate that murine APOBEC3 is packaged into milk-borne virions and limits infectivity.

APOBEC3G Is Expressed in Human Mammary Epithelial Cells

We next examined human MECs, to determine whether any of the human APOBEC3 genes were also expressed these cells. We performed reverse-transcribed RT-qPCR with APOBEC3G-, APOBEC3F-, APOBEC3A-, APOBEC3B-, and APOBEC3H-specific primers with RNA isolated from four different cultured MEC lines (MCF7, MCF10, HS578T, T47D) and primary human mammary tissue. APOBEC3G RNA was detected in HS578T and MCF10 cells, as well as all the primary tissue RNAs (Figure 4A) at approximately 10-fold lower levels than the H9 T cell line (see Figure S1 available online). In contrast, APOBEC3G RNA was almost undetectable in MCF7 and T47D cells. Very low levels of APOBEC3A, -3F, and -3H RNA were found in all four cell lines, only MCF7 cells produced APOBEC3A RNA and T47D, and HS578T and MCF7 but not MCF10 cells contained 3B RNA (Figure S1 and data not shown). All of the tested APOBEC3 RNAs were detected in primary mammary tissue, with the exception of APOBEC3H (Figure S2).

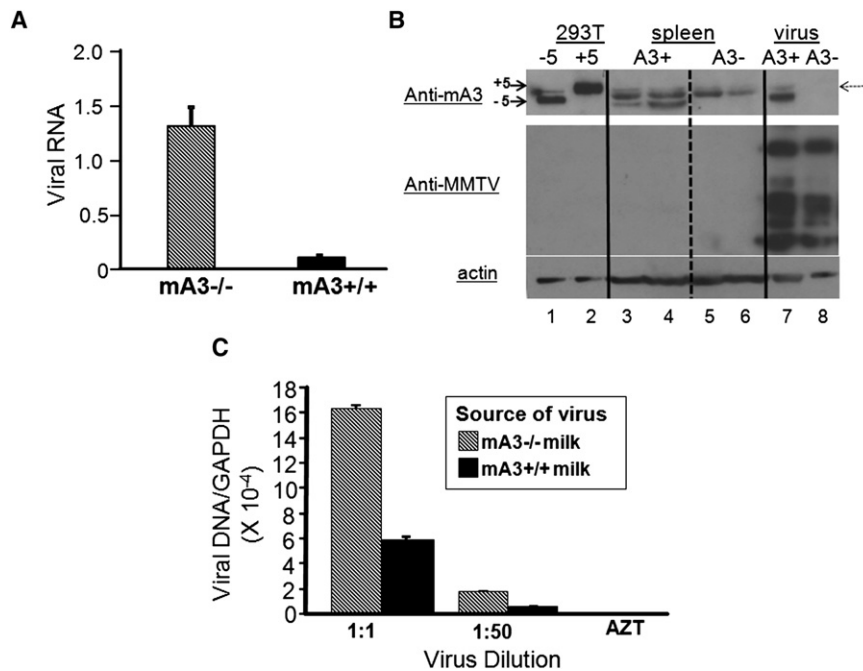


Figure 3. Virus Isolated from *mA3^{-/-}* Milk Is More Infectious Than that Isolated from *mA3^{+/+}* Milk

(A) Virions were isolated from the milk of MMTV-infected *mA3^{-/-}* and *mA3^{+/+}* mice and RNA isolated from equal amounts of milk was subjected to reverse-transcribed RT-qPCR. Error bars represent the SD of 11 *mA3^{-/-}* and 9 *mA3^{+/+}* milk samples.

(B) Western blot analysis of APOBEC3 in tissues and virions. Lanes 1 and 2, 293T cells transfected with expression vectors containing the BL/6 delta exon 5 (-5) or BALB/c + exon 5 (+5) cDNAs; lanes 3 and 4, splenic extracts from *mA3^{+/+}* mice; lanes 5 and 6, splenic extracts from *mA3^{-/-}* mice; lanes 7 and 8, virions isolated from the mammary tissue of *mA3^{+/+}* (A3+, lane 7) or *mA3^{-/-}* (A3-, lane 8) mice. The top panel was probed with rabbit anti-mouse APOBEC3 antisera, then stripped and reprobed with goat anti-MMTV antisera (middle panel); the bottom panel is a duplicate blot of the same extracts probed with anti- β -actin. A cross-reacting protein that is present in the tissue extracts of both +/+ and -/- mice is indicated by a dotted arrow; this protein was detected in some but not all virus preparations irrespective of the presence of APOBEC3 (data not shown).

(C) Various dilutions of virus isolated from *mA3^{-/-}* and *mA3^{+/+}* milk were used to infect 293-mTFR1 cells in triplicate, and DNA was isolated from the infected cells and subjected to RT-qPCR for MMTV and GAPDH. The numbers presented were normalized to the amount of virion RNA as measured in (A). Error bars represent SD from three replicate wells. $p \leq 0.001$ for both dilutions.

A recent study showed that estrogen induces the transcription of several human *APOBEC3* genes (Pauklin et al., 2009). We also tested whether progesterone or the synthetic glucocorticoid dexamethasone induced expression of *APOBEC3G* in HS578T and T47D cells. HS578T cells are estrogen receptor (ER) and progesterone receptor (PR) negative, whereas T47D cells are both ER and PR positive; both cell lines have the glucocorticoid receptor (GR) (Buser et al., 2007; Keydar et al., 1979; Small et al., 1999). No hormones significantly changed the level of *APOBEC3G* RNA in HS578T cells (Figure 4B). In contrast, glucocorticoids, estrogen, and progesterone all induced expression of *APOBEC3G* in T47D cells. *APOBEC3A* was induced by dexamethasone in both HS578T and T47D cells, there was no effect by any hormone on *APOBEC3B* or -3F in either cell line, and *APOBEC3H* levels were low even in the presence of hormones (Figure S3).

We also examined *APOBEC3G* protein expression in the human cell lines and tissue. Using immunoprecipitation followed by western blot analysis, *APOBEC3G* protein was detected in untreated HS578T but not the other MEC lines (Figure 5A and data not shown), in agreement with the higher RNA levels in HS578T cells (Figure 4A). *APOBEC3G* protein was also detected in progesterone-induced T47D cells (Figure 5A). In both HS578T and T47D cells, *APOBEC3G* protein expression was at least 10-fold lower than that detected in H9 cells, consistent with the relative RNA levels in the different cell lines (Figure S1). We also performed indirect immunohistochemistry on HS578T and T47D cells. Abundant cytoplasmic staining, with occasional punctuate structures, was detected in HS578T but not T47D cells (Figure 5B). This pattern of staining is similar to that previ-

ously reported for *APOBEC3G* (Gallois-Montbrun et al., 2007, 2008; Mangeat et al., 2003; Wichroski et al., 2006). Although the rabbit antisera we used also recognizes *APOBEC3A* (Thielen et al., 2010), both HS578T and T47D cells had very low levels of *APOBEC3A* RNA (Figures S1 and S3), and we did not see a band of the appropriate molecular weight in the immunoprecipitation/western blot analyses of extracts from these cells (data not shown).

Additionally, three different primary human mammary gland samples were stained with anti-*APOBEC3G* antisera (Newman et al., 2005); Figure 6 shows a representative section from one of these samples. *APOBEC3G* protein was detected in the luminal epithelial cells of the intralobular mammary duct. This staining was not seen in sections incubated with normal rabbit serum and or with anti-*APOBEC3G* antisera in the presence of recombinant *APOBEC3G* (r3G) protein. In addition, occasional stromal cells showed reactivity with anti-*APOBEC3G* antisera which was also blocked by r3G protein. The immunostaining both in cultured mammary cells and primary tissue was predominantly cytoplasmic, indicating that the antisera were recognizing *APOBEC3G*. However, since we did detect *APOBEC3A* RNA in primary human mammary tissue (Figure S2), it is also possible that the epithelial or stromal cell staining in mammary tissue was due at least in part to *APOBEC3A* protein, which has predominantly been found in the nucleus (Chen et al., 2006).

Vif Increases Infectivity of HIV-1 Virions Produced in MECs

We next performed a proof-of-principle experiment to test whether MEC-expressed *APOBEC3* proteins could restrict virus

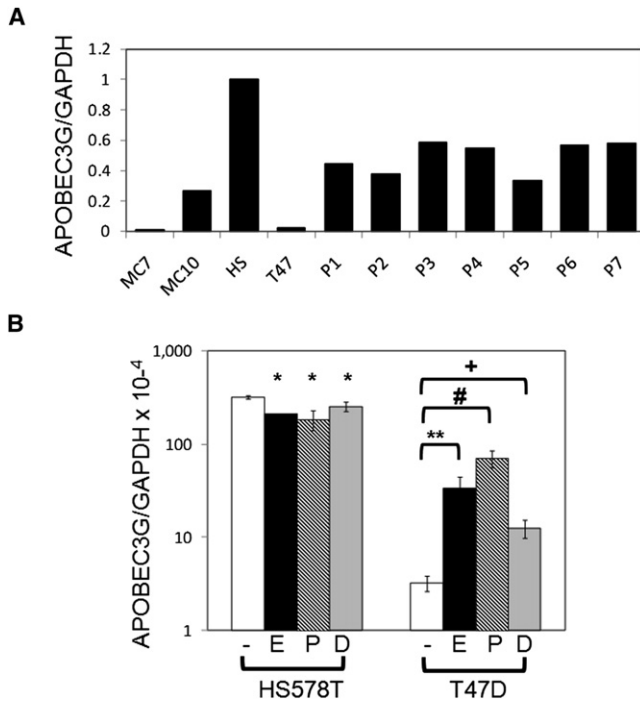


Figure 4. APOBEC3G Is Made in Human Mammary Epithelial Cells
(A) RNA was isolated from MCF7, MCF10, HS578T, and T47D mammary cell lines, and from seven different primary mammary gland samples from normal, nonlactating females (P1–P7) and subjected to reverse-transcribed RT-qPCR, using primers to APOBEC3G. All values were normalized to GAPDH. The values for the HS578T cell line were set at 1.0, and the results from the other cells and tissues are normalized to this value.
(B) Reverse-transcribed RT-qPCR analysis of RNA isolated from the indicated cell lines after 48 hr treatment with estrogen (E), progesterone (P), or dexamethasone (D); – indicates untreated cells. Error bars represent the SD of three replicate wells of each cell line/treatment. *NS; **p ≤ 0.04; #p ≤ 0.01; *p ≤ 0.02. See also Figures S1–S3.

infection. HS578T and T47D cells were cotransfected with the vectors PNL-luc+vif and PNL-luc-vif and a plasmid bearing the vesicular stomatitis virus (VSV) G protein. The transfected cells were grown in the presence and absence of progesterone to induce APOBEC3G expression. If APOBEC3G was packaged into HIV-1 virions in MECs, then vif+ virions should be more infectious than vif– virus when produced by these cells. Additionally, HIV produced by progesterone-treated T47D but not HS578T cells should be less infectious, since APOBEC3G levels were increased in response to hormone in the former and not the latter cell line. Virus was isolated from the supernatants of these cells, analyzed by western blot analysis and ELISA for Gag protein, and then used to infect NP2 cells.

First, we found that PNL-Luc+vif virus was more infectious than PNL-Luc-vif virus, when produced in HS578T cells (Figure 7A), indicating that endogenously produced APOBEC3G may be packaged into virions and that this was counteracted by Vif. No difference in infectivity was seen when virus was produced from HS578T cells grown in the presence or absence of progesterone (Figure 7A), consistent with the observation that progesterone does not induce APOBEC3G expression in this cell line (Figure 5A). However, virus produced by progesterone-

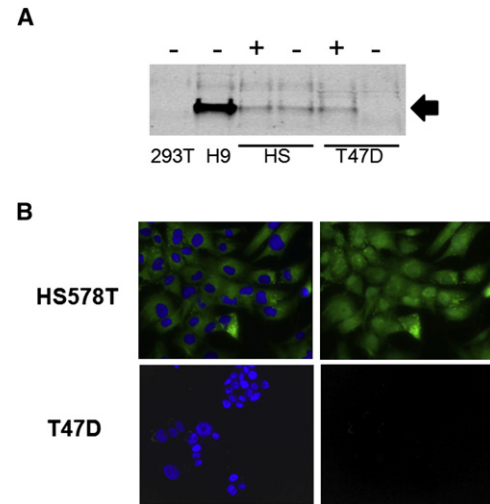


Figure 5. APOBEC3G Protein Is Made in Human MECs
(A) Western blots of lysates made from the different MECs following 48 hr treatment with (+) or without (–) progesterone. Also included are lysates prepared from 293T (APOBEC3G–) and H9 (APOBEC3+) cells. Arrow points to APOBEC3G.
(B) Indirect immunofluorescence of HS578T and T47D cells, using anti-APOBEC3G antisera. Cells were counterstained with DAPI. Left panel, IF plus DAPI; right panel, IF alone.

treated T47D cells was less infectious than that produced in untreated cells (Figure 7A). The presence of vif in the vector did not alter the infectivity of virus produced in T47D cells that received no progesterone (Figure 7A), most likely because there

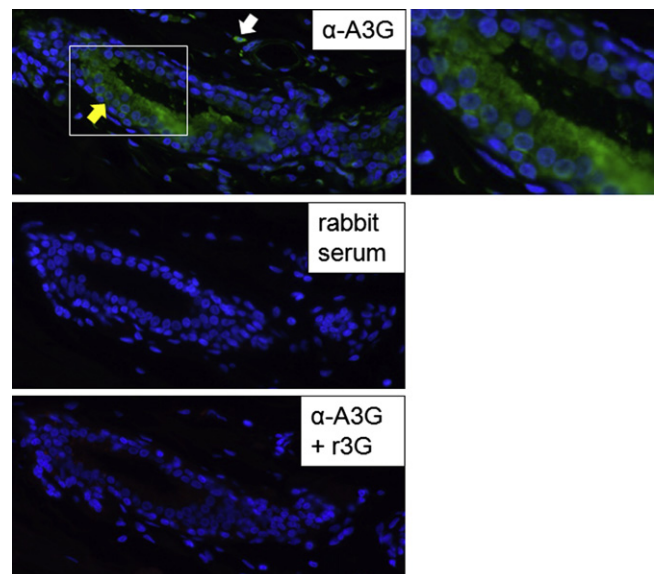


Figure 6. APOBEC3G Protein Is Made in Primary Human Mammary Tissue
Indirect IF of primary mammary tissue was performed, using rabbit anti-APOBEC3G antisera in the absence (α-A3G) or presence (α-A3G +r3G) of recombinant APOBEC3G protein. An enlargement of the outlined area of the lumen is shown to the right of the top panel. Yellow arrow, luminal epithelial cells; white arrow, stromal cell.

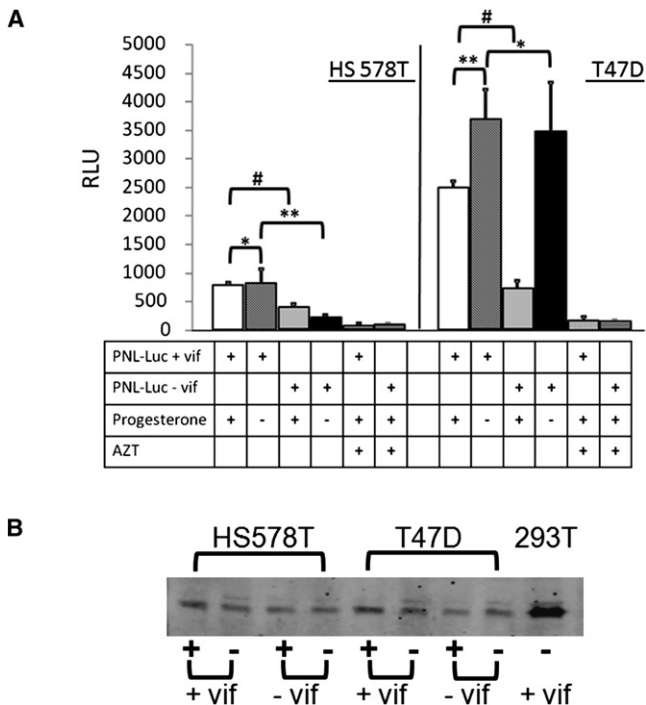


Figure 7. APOBEC3G Made in MECs Is Packaged into HIV

(A) HS578T (left) and T47D (right) cells were transduced with PNL-Luc that contained (+vif) or lacked (-vif) the *vif* gene and a vector bearing the VSV G protein. Twenty-four hours posttransduction, progesterone was added to the media as indicated (+) or left untreated (-), and 24 hr later virus was harvested from the cell supernatants and used to infect NP2 cells, in the absence or presence of AZT, as indicated. In addition, p24 levels were measured by ELISA. Twenty-four hours after infection, luciferase assays were performed on lysates prepared from the NP2 cells. RLU were normalized to the p24 levels in the virus preparations. Error bars represent SD from three replicate infections. *NS; ** $p \leq 0.05$; # $p \leq 0.001$. This experiment was performed three times with similar results.

(B) The virion preparations were analyzed by western blots, using anti-p24 antisera. Virions prepared in PNL-Luc+vif-transduced 293T cells were included as positive controls.

was little or no APOBEC3G expression in untreated cells (Figures 5A and 5B). However, PNL-Luc+vif virus was more infectious than PNL-Luc-vif virus when produced in T47D cells treated with progesterone (Figure 7A), indicating that the increased APOBEC3G produced in hormone-treated cells was counteracted by Vif. We also performed western blots on the virions produced by MECs, but although we could detect Gag protein (Figure 7B) and APOBEC3G protein was detectable in cell extracts (Figure 5A), the low levels of virus produced precluded detection of APOBEC3G in virions. Although both T47D and HS578T cells also expressed APOBEC3B RNA, which could play a role in restricting HIV produced in these cells, expression of this factor was not induced by progesterone. Moreover, APOBEC3B has weaker anti-HIV activity than APOBEC3G (Rose et al., 2005), and it is not highly inhibited by Vif (Doehle et al., 2005; Marin et al., 2008). Thus, our data indicate that APOBEC3G expressed in MECs may be packaged in HIV-1 virions and has antiviral activity that can be counteracted by Vif.

DISCUSSION

A large number of intrinsic immune factors that restrict virus infection in a variety of cell types have been identified in the past few years, in large part through study of viral proteins such as the HIV-1 Vif protein that counteract their action (Goff, 2004). Although APOBEC3G was first identified as an antiviral factor in HIV-1 infection, APOBEC3 proteins have been shown to function as anti-viral factors against not only retroviruses but also against diverse viruses such as HBV, foamy viruses, HPV, and parvoviruses and there is much interest in developing antiviral therapies that increase the ability of APOBEC3 to inhibit viral infection. However, while much has been learned about how this family of antiviral factors functions using cell culture models, less is known about how APOBEC3 proteins function in vivo. Recent studies in which mice with targeted deletion of the *Apobec3* gene were infected with different mouse retroviruses have begun to increase our understanding of how APOBEC3 proteins decrease the pathogenic effects of in vivo infection.

Here we show that MECs express APOBEC3 that is packaged into virions and restricts infectivity. In mice, APOBEC3 is expressed in lymph nodes (T, B, and DCs) at the highest levels, followed by adipose and epithelial tissues such as the mammary gland (Miki et al., 2005; Okeoma et al., 2007; Su et al., 2004). Our studies demonstrate that APOBEC3 limits MMTV infection of the lymphoid compartment in vivo and that this restriction results in lower levels of virus infection in mammary tissue, accompanied by a decreased incidence in mammary tumorigenesis. These data are consistent with our previous studies showing that MEC infection in mammary tissue depends on the level of lymphoid cell infection (Golovkina et al., 1998). Although APOBEC3 is made in MECs, its absence in this cell type in knockout mice reconstituted with wild-type bone marrow did not alter in vivo mammary gland infection.

Although virus infection levels in MECs themselves were not affected by the loss of APOBEC3, APOBEC3 protein produced in mammary tissue was packaged into virions. While there are numerous studies using transfected and primary cells as well as knockout mice to study the effect of APOBEC3 proteins on virus infection and spread, the current study provides direct demonstration that endogenous APOBEC3 protein is packaged into virions in vivo. The presence of APOBEC3 in MMTV virions resulted in a significant decrease in the relative infectivity of MMTV shed in the milk of wild-type versus knockout mice. Our data thus suggest that APOBEC3 expression in MECs is retained in mice as a way of limiting milk-borne transmission of MMTV, and perhaps other mouse viruses; several studies have indicated that the natural transmission route of MLV, which is also restricted by APOBEC3, is through milk (Duggan et al., 2006; Jenson et al., 1976). Milk-borne virus transmission in mice may also be affected by genetic variations in *Apobec3*. C57BL/6-derived and BALB/c mice have a number of polymorphic differences in both the coding and noncoding regions of *Apobec3*, and the BL/6 allele has been shown to be expressed at higher levels in vivo, as well as more effective at restricting infection by F-MLV, AK virus, and MMTV (Langlois et al., 2009; Okeoma et al., 2009; Takeda

et al., 2008). Indeed, we showed previously that MMTV virions isolated from the mammary tumors or milk of C57BL/6 mice were less infectious than those produced in BALB/c mice (Okeoma et al., 2009).

We also show here that APOBEC proteins expressed in human MECs have the potential to restrict virus infectivity. Human APOBEC3F, -3G, and -3C are expressed at high levels in spleen, lymphocytes, DCs, and monocytes, while APOBEC3B is highly expressed in cancer cells, B lymphoblasts, and endothelial cells and APOBEC3A in immature monocytes (Aguilar and Peterlin, 2008; Koning et al., 2009; Refsland et al., 2010; Su et al., 2004). We detected APOBEC3A, -3B, -3F, and -3G RNA in primary mammary tissue (Figure S3), but only APOBEC3B and APOBEC3G were highly expressed in cultured MECs (Figure 4A and Figure S1). This difference may be due to the presence of diverse cell types in the primary tissue (MECs, stromal cells, vascular cells, lymphoid cells, etc.); indeed, we detected APOBEC3G protein in both ductal epithelial and stromal cells in primary tissue (Figure 6). This differential expression may play a role in how APOBEC3 proteins restrict infection by diverse viruses in different tissues. The most potent inhibitors of HIV infection, APOBEC3G and -3F, are highly expressed in HIV-target lymphoid cells, while APOBEC3A, -3B, and -3H, also expressed in keratinocytes and skin, could play a role in restricting human papilloma virus infection (Vartanian et al., 2008).

In contrast to MMTV, it is unclear whether milk-borne transmission of lentiviruses and delta viruses is cell-free or cell associated and whether lymphoid or mammary cells or both are the virus reservoir (Becquart et al., 2002; Bobat et al., 1997; Henderson et al., 2004; Hino et al., 1994; Koulinska et al., 2006; Lawrence and Lawrence, 2004; Leroy et al., 1998; Lewis et al., 1998; Li et al., 2004; Miotti et al., 1999; Pandrea et al., 2008; Rousseau et al., 2003, 2004; Rychert et al., 2006; Ziegler et al., 1985). Cultured human MECs can be productively infected with HTLV1 (LeVasseur et al., 1998), and several studies have shown that human MECs express low levels of CXCR4 and can be infected by CXCR4-tropic HIV-1; at least in tissue culture cells, these cells produce infectious virus (El Messaoudi et al., 2000; Moriuchi and Moriuchi, 2004; Su et al., 2004; Toniolo et al., 1995). A more recent study has suggested that while primary MECs express CD4, CCR5, and CXCR4, they are not productively infected with HIV-1 but instead sequester the virus in endosomal compartments which could then facilitate infection of CD4+ T cells in the mammary gland (Dorosko and Connor, 2010).

Although it is unclear whether APOBEC3G in mammary tissue plays a role in restricting milk-borne HIV infection, it is interesting that this factor, the major anti-HIV family member, as well as APOBEC3A and -3B, were all highly expressed in primary mammary tissue. It may be that these factors are important for restricting milk-borne transmission of other viruses. For example, APOBEC3A has also been implicated in the restriction of papillomavirus infection, which some studies have shown to be present in human MECs and mammary tumors (de Villiers et al., 2005). That APOBEC3 RNA and protein are found in human mammary tissue suggests that this intrinsic antiviral factor may have been retained as a means of limiting milk-borne virus transmission in humans as well as in mice.

EXPERIMENTAL PROCEDURES

Ethics Statement

All human tissues were received as unidentified archived biopsied tissue from the Tumor Tissue and Biospecimen bank from the University of Pennsylvania. The use of these specimens received exemption from the University of Pennsylvania Institutional Research Board. All mice were housed according to the policies of the Institutional Animal Care and Use Committee of the University of Pennsylvania, and all experiments performed with mice were approved by this committee.

Cell Lines and Human Tissue

293T, HS578T, MCF 7, MCF 10A (nontumorigenic MEC line), and T47D cell lines were purchased from American Type Culture Collection. H9 and NP2 cells were obtained from Robert Doms and Jim Hoxie, respectively. 293T-mTfR1 cells have been previously described (Zhang et al., 2003); MMTV infection of these cells was analyzed by RT-qPCR for viral DNA sequences (Okeoma et al., 2009). All cells were maintained according to the suppliers' instructions. Primary human mammary tissues were obtained from the University of Pennsylvania's tissue bank in accordance with IRB guidelines and after pathologic review.

Foster Nursing and Tumorigenesis Studies

The $mA3^{-/-}$ and matched control ($mA3^{+/+}$) mice were previously described (Okeoma et al., 2007). Age-matched pups from $mA3^{-/-}$ and $mA3^{+/+}$ MMTV⁻ females were foster nursed on C57BL/6 MMTV(RIIII)⁺ females. The foster-nursed pups were sacrificed at the indicated times. Different tissues were harvested for DNA isolation and analysis of infection by real-time quantitative polymerase chain reaction (RT-qPCR) for integrated MMTV proviruses; all values were normalized to GAPDH, as previously described (Courreges et al., 2007). Foster-nursed $mA3^{+/+}$ and $mA3^{-/-}$ female mice were kept in constant mating and palpated weekly for mammary tumors; all mice were sacrificed at the time of tumor detection.

Adoptive Transfers

Five MMTV⁻ age-matched (6 weeks old) $mA3^{+/+}$ and $mA3^{-/-}$ female mice each were lethally irradiated using a Cs-137 irradiator (γ cell 40 Extractor; MDS Nordion). Four hours after irradiation, the mice were reconstituted with bone marrow stem cells from MMTV⁻ $mA3^{+/+}$ mice. One week after reconstitution, the mice were inoculated with MMTV(RIIII) isolated from milk and then sacrificed 9 weeks after irradiation. Prior to sacrifice, peripheral blood lymphocytes from each mouse were subjected to FACs analysis with conjugated anti-mouse CD8, CD4, CD11c, and B220 (BD-PharMingen) to test for uniform reconstitution. At sacrifice, RNA was isolated from lymph nodes, spleens, and mammary glands and subjected to mouse reverse transcribed PCR with primers specific to mouse APOBEC3, and DNA was isolated and analyzed by RT-qPCR with primers specific to MMTV and GAPDH (Okeoma et al., 2007).

Mouse APOBEC3 RNA and Protein Analysis

RNA was isolated from milk-borne virions and subjected to reverse-transcribed RT-qPCR as previously described (Okeoma et al., 2009). Virions, as well as extracts from tissues and 293T cells transiently transfected with mouse APOBEC3 expression vectors (+ and - exon 5; Takeda et al., 2008), were subjected to western blot analysis with the indicated antibodies. Rabbit anti-mouse APOBEC3 antisera were generated by immunizing rabbits with a hapten-coupled peptide unique to the mouse protein (CSHRKCYSPIRNLISQE) (Lampire Biological Laboratories) followed by affinity purification of the antisera. The goat anti-MMTV antisera were originally obtained from the National Cancer Institute Biochemical Carcinogenesis Branch Repository, Bethesda, MD. Detection was accomplished using the species-appropriate horseradish peroxidase-conjugated secondary antibody followed by ECL (Amersham Biosciences, Inc.).

Human APOBEC3 RNA and Protein Analysis

Cells were cultured for 48 hr in the presence or absence of 1 μ M 17- β -estradiol, dexamethasone, or progesterone (all purchased from Sigma-Aldrich). RNA was isolated from MCF7, MCF10, HS578T, and T47D cells and primary tissues using RNeasy kits (QIAGEN, Inc.) and subjected to reverse-transcribed

RT-qPCR, with primers to APOBEC3A, -B, -F, -G, and -H (Table S1). To ensure that the reverse-transcribed RT-qPCR products were specific to the corresponding *ApoBec3* gene, the cDNA amplicons were sequenced and the identity of the transcript was confirmed (data not shown). APOBEC3G protein expression was analyzed by immunoprecipitation of extracts prepared from HS578T and T47D cells with anti-APOBEC3G monoclonal antibody (murine anti-human APOBEC3G (CEM15) #10069), followed by western blot analysis with a rabbit anti-APOBEC3G antisera (#10201; Thielen et al., 2007); both were obtained from the AIDS Research and Reference Reagent Program.

Packaging of hA3G into HIV-1 Virions Produced in Human MECs

The PNL LUC-vif and PNL LUC-Δvif plasmids were kind gifts from Matija Peterlin (Connor et al., 1995). The PNL-Luc vectors (+/- vif) viruses pseudotyped with VSV-G were generated by plasmid cotransfection of 293T, HS578T, and T47D cells using Lipofectamine 2000 (Invitrogen, Inc.). Twenty-four hours after transfection, progesterone was added to the media and 48 hr later virus was harvested from the supernatants. Virions were purified from culture supernatant by centrifugation at 500 × g, followed by filtration through a 0.45 μ sterile membrane filter (Millex-HV; Millipore), and pelleting through a 20% sucrose cushion by centrifugation at 105,000 × g for 1 hr. Purified virions were used to infect NP2 cells in the absence or presence of AZT. Forty-eight hours after infection, cells were harvested and extracts subjected to luciferase assays. Luciferase relative light units (RLUs) were normalized to the p24 levels in the virus preparations. The p24 ELISAs were performed by the University of Pennsylvania Center for AIDS Research Immunology Core. The virions were also analyzed by western blot analysis with anti-p24 antisera, a kind gift from Robert Doms.

Immunofluorescence

HS578T and T47D cells were cultured on poly-L-lysine (Sigma)-coated coverslips. Twenty-four hours later, cells were fixed with paraformaldehyde. Mammary specimens (formalin-fixed and paraffin-embedded) were cut into 4 micron thick sections using a microtome and mounted on charged slides. Sections were deparaffinized and rehydrated, and then the antigen binding sites were unmasked (antigen unmasking solution; Vector Labs) at 98°C for 20 min. All samples were permeabilized with 0.1% Triton X-100, and nonspecific binding sites were blocked with normal goat serum in the presence of avidin (Vector Labs) for 1 hr at room temperature. Samples were washed and reacted with polyclonal rabbit anti-APOBEC3G antibody in the absence and presence of recombinant APOBEC3G (NIH AIDS Research and Reference Reagent Program) or normal rabbit serum (Santa Cruz Biotechnology) in the presence of biotin (Vector Labs) for 1 hr at room temperature, washed, and then incubated with biotin-labeled goat anti-rabbit IgG for 30 min at 37°C. The APOBEC3G-positive signal was detected with FITC-streptavidin. Images were taken with a LEICA DM1000 fluorescent microscope using a Nuance multispectral imaging camera and Nuance software (Cambridge Research Instrumentation Inc, Woburn, MA).

Statistical Analysis

Statistical analysis of significant differences between experimental groups was tested using paired two-tailed Student's t test. Error bars represent standard deviations. For the Kaplan-Meier survival curve (Figure 1D), statistical analyses were done using SAS/STAT software, Version 9 of the SAS System for Windows.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one table and can be found with this article online at doi:10.1016/j.chom.2010.11.003.

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