APOBEC3A Is Upregulated by Human Cytomegalovirus (HCMV) in the Maternal-Fetal Interface, Acting as an Innate Anti-HCMV Effector

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ABSTRACT Human cytomegalovirus (HCMV) is the leading cause of congenital infection and is associated with a wide range of neurodevelopmental disabilities and intrauterine growth restriction. Yet our current understanding of the mechanisms modulating transplacental HCMV transmission is poor. The placenta, given its critical function in protecting the fetus, has evolved effective yet largely uncharacterized innate immune barriers against invading pathogens. Here we show that the intrinsic cellular restriction factor apolipoprotein B editing catalytic subunit-like 3A (APOBEC3A [A3A]) is profoundly upregulated following *ex vivo* HCMV infection in human decidual tissues—constituting the maternal aspect of the placenta. We directly demonstrated that A3A severely restricted HCMV replication upon controlled overexpression in epithelial cells, acting by a cytidine deamination mechanism to introduce hypermutations into the viral genome. Importantly, we further found that A3 editing of HCMV DNA occurs both *ex vivo* in HCMV-infected decidual organ cultures and *in vivo* in amniotic fluid samples obtained during natural congenital infection. Our results reveal a previously unexplored role for A3A as an innate anti-HCMV effector, activated by HCMV infection in the maternal-fetal interface. These findings pave the way to new insights into the potential impact of APOBEC proteins on HCMV pathogenesis.

IMPORTANCE In view of the grave outcomes associated with congenital HCMV infection, there is an urgent need to better understand the innate mechanisms acting to limit transplacental viral transmission. Toward this goal, our findings reveal the role of the intrinsic cellular restriction factor A3A (which has never before been studied in the context of HCMV infection and vertical viral transmission) as a potent anti-HCMV innate barrier, activated by HCMV infection in the authentic tissues of the maternal-fetal interface. The detection of naturally occurring hypermutations in clinical amniotic fluid samples of congenitally infected fetuses further supports the idea of the occurrence of A3 editing of the viral genome in the setting of congenital HCMV infection. Given the widely differential tissue distribution characteristics and biological functions of the members of the A3 protein family, our findings should pave the way to future studies examining the potential impact of A3A as well as of other A3s on HCMV pathogenesis.

Received 27 July 2017 Accepted 18 September 2017

Accepted manuscript posted online 27 September 2017

Citation Weisblum Y, Oiknine-Djian E, Zakay-Rones Z, Vorontsov O, Haimov-Kochman R, Nevo Y, Stockheim D, Yagel S, Panet A, Wolf DG. 2017. APOBEC3A is upregulated by human cytomegalovirus (HCMV) in the maternal-fetal interface, acting as an innate anti-HCMV effector. *J Virol* 91:e01296-17. <https://doi.org/10.1128/JVI.01296-17>.

Editor Jae U. Jung, University of Southern California

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KEYWORDS APOBEC, HCMV, antiviral cellular restrictions, congenital infection, decidua, intrinsic immunity, placental innate immunity, viral placental transmission

Human cytomegalovirus (HCMV), a ubiquitous betaherpesvirus, is the most common cause of congenital infection worldwide and the leading cause of congenital neurosensory disease of infectious origin (1). Yet our current understanding of the mechanisms modulating vertical HCMV transmission is poor, largely due to the lack of relevant animal models for this human-specific virus.

HCMV is transmitted from the mother to the fetus via the placenta (2, 3). The human placenta is a chimeric organ, containing both maternal structures (the maternal decidua) and fetal structures (the fetus-derived chorionic villus). The earliest events of HCMV maternal-to-fetal transmission are believed to occur in the maternal decidua, which constitutes a multicell-type tissue, cohabited by invasive fetal cells and maternal immune and nonimmune cells (2, 3). Given its critical function in protecting the developing fetus, the placenta has evolved effective and yet largely uncharacterized innate immune barriers against invading pathogens (3). Recent studies, further facilitated by the Zika virus epidemic, have begun to uncover the importance of these local-placental innate immune responses in the defense against congenital infections (4–7).

A central component of innate antiviral immunity, extensively studied in the context of HCMV infection, is the rapid activation of the interferon (IFN) signaling cascades and inflammatory cytokines upon viral sensing (6, 8–10). Intrinsic cellular restriction factors are also known to be key components of innate immunity to a variety of viruses (11, 12). Among the members of this arsenal of proteins are the seven members of the human apolipoprotein B editing catalytic subunit-like 3 (APOBEC3 [A3]) family of cytidine deaminases (A, B, C, DE, F, G, and H) (11, 13–15). These enzymes, which classically catalyze the deamination of cytidine nucleotides to uridine nucleotides in single-strand DNA substrates, have been recognized as fundamental players in the defense against various viral infections (13–16). Since the identification of A3G as a prototype antiretroviral host restriction factor, A3 subsets have been shown to restrict the replication of retroviruses, endogenous retroelements, and, more recently, DNA viruses such as hepatitis B virus (HBV), parvoviruses, and human papillomavirus (HPV) as well as of the human herpesviruses herpes simplex virus 1 (HSV-1) and Epstein-Barr virus (EBV) (13–20). In view of the growing range of A3-affected viruses, it is conceivable that HCMV may be vulnerable to these central restriction factors. Yet, thus far, the role of A3 proteins in HCMV infection has not been explored.

We have recently established a unique *ex vivo* model of HCMV infection in native human decidual tissues maintained as multicell-type organ cultures and demonstrated the robust decidual tissue innate immune response to HCMV infection (6, 9, 21). Employing our decidual tissue infection model, we hereby reveal a significant and specific upregulation of intrinsic immunity factor A3A in HCMV-infected decidual tissues. We directly show that A3A is a potent restrictive factor of HCMV replication, acting to introduce hypermutations into the viral genome both *ex vivo* in decidual organ cultures and *in vivo* during congenital infection. To the best of our knowledge, this is the first report of the role of A3 as an innate antiviral effector in the maternal-fetal interface.

RESULTS

HCMV infection upregulates A3A expression in decidual tissues. We have recently shown that HCMV infection triggers a distinct decidual tissue innate immune response characterized by predominant induction of immune cell activation, proliferation, and trafficking pathways (6, 9). To gain a global insight into these earliest tissue responses, we employed a genome-wide transcriptome analysis: decidual tissues were infected by HCMV, and transcriptome analysis of infected versus mock-infected tissues was conducted at 1 day postinfection (dpi). This time point was chosen based on our

TABLE 1 Profoundly upregulated genes in HCMV-infected decidual tissues^a

Gene symbol	Infected tissue/mock-infected tissue ratio	q value
IFN- γ	74.82	0.019
CXCL11	73.38	0.010
CXCL10	70.15	0.010
IFIT2	36.86	0.010
OASL	26.54	0.010
CCL8	23.55	0.010
GBP5	19.77	0.010
GBP4	16.99	0.010
XIRP1	16.98	0.010
IFIT1	16.45	0.010
GBP1	16.17	0.010
RBFOX1	15.01	0.010
IFIT3	14.38	0.010
APOBEC3A	13.45	0.010
UBD	13.36	0.010
RSAD2	12.66	0.010
NBR2	11.00	0.010
BATF2	10.87	0.010
NOS2	10.40	0.010

^aThe mean ratios of the levels of significantly differentially expressed genes in HCMV-infected versus mock-infected decidual cultures are shown for the genes which manifested the highest extent of upregulation (>10-fold) following HCMV infection.

previous demonstration that the innate immune response is already induced and reaches its peak at early times (1 dpi) (9). Focusing on the most profoundly upregulated genes in HCMV-infected decidual tissues, we found that HCMV most substantially upregulated the expression of gamma IFN (IFN- γ) and the chemokines CXCL11 and CXCL10, along with innate immunity genes related to the IFN signaling pathways (Table 1). Interestingly, we identified APOBEC3A (A3A), which was barely expressed in mock-infected tissues, among the genes which manifested the highest extent of upregulation (~13-fold) following HCMV infection in the decidua (Table 1). The finding that A3A came up as one of the most profoundly upregulated genes in HCMV-infected decidual tissues was especially notable since, to date, most of the information obtained about APOBEC inhibition of viruses has pertained to retroviruses, and although other viruses have recently emerged as potential targets (13, 15, 17–20), the role of A3 proteins in HCMV infection has not been previously explored. We therefore focused our attention on A3A. To confirm these results, we employed quantitative real-time reverse transcription-PCR (RT-PCR) on RNA extracted from infected and mock-infected decidual tissues, further expanding the analysis to include all seven human A3 family members (Fig. 1A). This analysis clearly corroborated findings that revealed the substantial and specific induction of A3A expression by HCMV infection in the decidua, whereas significant albeit much smaller (up to 6-fold) changes were found in the expression of other A3s (Fig. 1A). Notably, when we further examined the expression of A3A in chorionic villus tissues (the fetus-derived aspect of the placenta) and in individual cell cultures, we found that induction of A3A by HCMV was specific to the decidual tissues and was not observed in HCMV-infected chorionic villi maintained in the organ culture, despite comparable infection levels, as well as in primary fibroblasts (MRC-5 and human foreskin fibroblasts [HFF]) and epithelial (ARPE-19) cell cultures (Fig. 1B and C). APOBEC3A is also known to be an interferon-stimulated gene (ISG). Here we showed that IFN- β , but not IFN- γ (500 IU/well), significantly induced A3A expression in uninfected decidual tissues (Fig. 1D). These observations identify the cell type- and tissue-specific nature of A3A upregulation and its potential regulation as an interferon-stimulated gene following HCMV infection.

A3A restricts HCMV replication in a deaminase-dependent manner. Given the established role of A3A in intrinsic cellular immunity (13–16, 22–31), our finding that A3A expression was profoundly induced in the decidual tissues following HCMV infection suggested that A3A might act to restrict HCMV replication.

FIG 1 Effect of HCMV infection on the expression of human APOBEC3 (A3) family members in decidua and chorionic villus organ cultures and in individual cell cultures. RNA from HCMV- and mock-infected cultures was extracted at 1 dpi and analyzed for the indicated A3 mRNA expression by quantitative RT-PCR, normalized to the β actin housekeeping gene. (A) Expression of the indicated A3s in decidua organ cultures. (B) A3A expression in the indicated cell and organ cultures. (C) Expression of HCMV immediate early 1 (IE1) gene in infected decidua and chorionic villus organ cultures. (D) A3A expression in uninfected decidua organ cultures subjected to mock treatment or treated with IFN- β or IFN- γ . The results shown are representative of results of at least 3 independent experiments that included tissues from different individuals. Significant ($P < 0.05$) changes from mock treatment results are indicated by an asterisk.

To directly examine this assumption, ARPE-19 epithelial cells (which basically lack detectable A3A expression, as shown in Fig. 1B) were transduced by a lentiviral vector expressing A3A under the control of an inducible Tet-on system. In this system, addition of doxycycline (DOX) to the cell culture medium is required to initiate transcription of A3A, generating ARPE-19 cells expressing A3A. Expression of A3A in the transduced cells following DOX addition was validated by RT-PCR, Western blotting (WB), and deaminase activity assays (data not shown). Of note, DOX was added to the cell layer once completely confluent, and no evidence of A3A-associated cellular toxicity was found by microscopic examination and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay. Cells were infected with HCMV

FIG 2 Inhibition of HCMV replication by A3A overexpression in ARPE-19 cells. ARPE-19 cells expressing A3A or the A3A catalytic mutant (E72Q) under the control of an inducible Tet-on system were infected with HCMV (MOI of 0.1) with doxycycline (+DOX) or without doxycycline (–DOX) for 7 days. (A to C) mRNA levels of representative HCMV immediate early (A) and delayed early (B) genes and a late (C) transcript were analyzed by quantitative RT-PCR and normalized to the β actin housekeeping gene. (D) HCMV DNA levels were quantified by quantitative real-time PCR and normalized to RNase P. (E) Expression of HaloTag-A3A and HCMV immediate early (IE p86 and IE p72) and late (pp28) proteins was detected by WB. Cellular GAPDH was used as a loading control. (F) Viral titers in the supernatants of infected cells were determined by a standard plaque assay. Significant changes ($P < 0.05$) between the results seen with and without DOX are indicated by an asterisk.

(at a multiplicity of infection [MOI] of 0.1), and the impact of A3A expression on HCMV replication was quantified at 7 dpi by several measures. As shown in Fig. 2A to C, A3A expression resulted in a significant decrease in the expression levels of the viral major immediate early 1 (IE1) and delayed early (UL89) genes and in R160461 (a genome locus expressed exclusively with late kinetics [32]) and HCMV DNA accumulation (Fig. 2D) compared to the results seen with infected cells incubated in the absence of DOX (–DOX; not expressing A3A). In accordance, a significant decrease in the expression levels of viral IE and late (pp28) proteins was specifically observed in cells expressing A3A (+DOX; Fig. 2E). We also examined the effect of A3A on the yield of infectious progeny virus. As shown in Fig. 2F, the titer of the virus released from A3A-expressing cells was significantly reduced (by 10-fold) compared with the results seen with control (–DOX) cells, reflecting impaired virus production in the presence of A3A overexpression.

APOBEC proteins have been shown to exert their antiviral effect on HIV mainly by deaminase-dependent but also by deaminase-independent mechanisms (13–16, 33). To examine if A3A cytosine deaminase activity was required for the observed HCMV inhibition, we generated ARPE-19 cells expressing a catalytically inactive A3A E72Q mutant (under Tet control as described above). Deaminase activity assay confirmed the abrogation of cytosine deaminase activity in A3A E72Q-expressing cells (data not shown). In contrast to the inhibitory effect of wild-type (WT) A3A, the catalytically inactive A3A mutant did not affect any of the measured parameters of HCMV replication and progeny-virus yield (Fig. 2A to F), despite levels of cellular expression of WT and E72Q A3A that were comparable overall in independent experiments. While their relative expression levels slightly differed (in both directions) in individual experiments

FIG 3 A3A inhibits HCMV infection during the first viral replication cycle. Cells expressing A3A or the A3A catalytic mutant (E72Q) under the control of an inducible Tet-on system were infected with HCMV (MOI of 0.1) with doxycycline (+DOX) or without doxycycline (−DOX) for 3 days, in the presence of the viral DNA cleavage-packaging inhibitor BDCRB. (A to C) mRNA levels of HCMV immediate early (A), delayed early (B), and late (C) genes were analyzed by quantitative RT-PCR and normalized to the housekeeping gene β actin. (D) HCMV DNA was quantified by quantitative real-time PCR and normalized to RNase P. Significant changes ($P < 0.05$) between the results seen with and without DOX are indicated by an asterisk.

(with, e.g., a 1.7-fold difference in the results shown in Fig. 2E), a significant and specific viral restriction effect of WT A3A was consistently found. In contrast, none of the changes seen with the E72Q samples under the different conditions (with and without DOX; Fig. 2A to D and F) were found to be significant.

Having shown the significant inhibition of HCMV infection by A3A at 7 dpi (reflecting the outcome of potentially two or more cycles of viral replication), we further sought to delineate the inhibition stage of HCMV replication and to determine whether viral restriction already occurs during the first cycle of viral replication. To this end, we treated the A3A-expressing infected cells with the well-known HCMV inhibitor BDCRB (2-bromo-5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole) (34, 35), which blocks the HCMV DNA cleavage packaging step and inhibits progeny virus formation and further viral spread. Under these conditions, A3A overexpression had no effect on viral IE gene expression but instead significantly inhibited the accumulation of viral DNA and the expression of delayed-early and late viral genes (Fig. 3). Taken together, these results indicate that A3A inhibits HCMV infection via its cytidine deaminase activity during the first cycle of viral replication.

A3A edits the HCMV genome in A3A-expressing cells. Having demonstrated the requirement for A3A catalytic activity, we next sought to determine if A3A indeed edits HCMV DNA. It is expected that DNA editing by A3A would yield C/G-to-T/A hypermutations. Another prediction, based on previous reports (27, 36, 37), is that the immediate product of deamination—DNA containing uracil residues—is a substrate for excision by cellular and potentially viral uracil DNA glycosylase (UNG). In this case, inhibiting UNG should allow the accumulation of the A3A-edited DNA intermediates. To examine these predictions, we combined the use of UNG inhibitor (UGI) with the differential DNA three-dimensional denaturation (3D-PCR) detection technique, proven useful in analyzing A3A-edited DNA intermediates (20, 27, 28, 38). The 3D-PCR, in which a temperature gradient is used in the denaturation step, is based on the fact that DNA with fewer interstrand hydrogen bonds (i.e., A/T-rich DNA) preferentially amplifies at lower denaturation temperatures, allowing its enrichment among excess amounts of WT DNA. We therefore cotransduced the A3A (or A3A E72Q)-expressing cells by the use of a lentiviral vector expressing UGI. The cotransduced cells were validated for the expression of both A3A and UGI by RT-PCR. These cells were then infected with HCMV (MOI of 0.1) with or without DOX for 7 days. We noticed that the expression of UGI resulted in some inhibition of HCMV replication in both A3A-expressing (+DOX) and A3A-nonexpressing (−DOX) cells (Fig. 4A). This inhibitory effect could have resulted from the inhibition of the HCMV-encoded UNG—known to play a role in viral replication (39). The presence of A3A-edited viral DNA intermediates was tested by 3D-PCR

FIG 4 A3A edits the HCMV genome in A3A-expressing cells. ARPE-19 cells expressing A3A or the A3A catalytic mutant (E72Q) under the control of an inducible Tet-on system were transduced with a lentiviral vector expressing the uracil DNA glycosylase inhibitor UGI or with empty vector as a control (–UGI). The cells were infected with HCMV (MOI of 0.1) with doxycycline (+DOX) or without doxycycline (–DOX) for 7 days. (A) mRNA levels of HCMV immediate early and late genes were analyzed by quantitative RT-PCR and normalized to the β actin housekeeping gene. Significant changes ($P < 0.05$) between DOX and UGI conditions (indicated below the x axis) and –DOX –UGI conditions are indicated by an asterisk. (B) DNA from the infected cells was extracted and subjected to 3D-PCR analysis of the viral DNA polymerase gene region by the use of a denaturation temperature gradient of 85 to 87°C. (C) PCR products from the 85°C denaturation temperature reaction whose results are shown in panel B were excised from agarose gel and cloned into pGEM-T vector. Five randomly selected clones were sequenced. The HCMV TB40/E sequence (GenBank accession number [EF999921](#)) is shown at the top as a reference (ref). Dots in the alignment represent identity to the reference sequence. For clarity, only 100 bases are shown. Data corresponding to the total number and type of mutations found are shown in panel D. nt, nucleotides.

amplification of the HCMV DNA polymerase (UL54) gene region. Interestingly, at the lower denaturation temperatures, PCR amplicons from A3A-expressing cells (+DOX) were apparent only in the presence of the UNG inhibitor (+UGI; Fig. 4B, third row from the top). We did not detect PCR products at lower denaturation temperatures in cells not expressing A3A (−DOX) or in A3A-expressing cells in the absence of UGI (Fig. 4B). No low-denaturation-temperature PCR products were detected in the control cells expressing A3A E72Q in the presence of UGI (Fig. 4B), indicating that the expression of UGI alone without catalytically active A3A was insufficient for the recovery of low-denaturation-temperature PCR products.

To verify that the observed low-denaturation-temperature PCR products represented A3A-edited viral DNA, we sequenced several randomly selected DNA clones of these PCR products (Fig. 4C). These analyses revealed high frequencies of C-to-T transitions along with some G-to-A transitions (Fig. 4D). Sequencing of the cloned high-denaturation-temperature PCR amplicons from –DOX samples (in which no A3A is expressed) revealed no mutations, indicating that the mutations found in the lower-temperature PCR amplicons in the presence of A3A expression (+DOX samples) represented true enrichment by the 3D-PCR. Together, these results indicated that A3A induces hypermutations in HCMV DNA and that UNG processes the edited viral DNA.

Cytidine deamination editing of the HCMV genome occurs *ex vivo* in organ-cultured decidual tissues and during natural *in vivo* infection in the maternal-fetal interface. To determine whether the upregulated A3A in HCMV-infected decidual tissues is physiologically functional in editing the HCMV genome, DNA extracted from *ex vivo* infected decidual tissues at 7 dpi was subjected to 3D-PCR analysis as explained above. As shown in Fig. 5A, low-denaturation-temperature PCR products of HCMV DNA were recovered from the infected decidual tissues. Sequence analysis of the cloned PCR products (6 randomly selected clones) revealed that the majority of the nucleotide substitutions were G/C-to-A/T transitions (Fig. 5B), consistent with cytidine deaminase activity in the infected decidual tissues.

FIG 5 Detection of cytidine deamination of the HCMV genome in *ex vivo* infected decidual tissues and during natural *in vivo* infection in the maternal-fetal interface. DNA was extracted from *ex vivo* infected decidual tissues and from 3 amniotic fluid samples obtained from congenitally infected cases and subjected to 3D-PCR of the viral DNA polymerase gene region, using a denaturation temperature gradient of 85 to 87°C. The PCR products are shown in panel A. PCR products from the 85°C denaturation temperature reaction (A) were excised from the agarose gel and cloned into pGEM-T vector. Six randomly selected clones from the infected decidual tissues and 15 randomly selected clones from the HCMV-positive amniotic fluid samples were sequenced, and data corresponding to the total number and type of mutations found are shown in panel B. Six of the amniotic fluid HCMV DNA sequences are shown in panel C. The HCMV TB40/E sequence (GenBank accession number [EF999921](#)) is shown at the top as a reference. Dots in the alignment represent identity to the reference sequence. For clarity, only 100 bases are shown.

Finally, we asked whether A3A editing of the HCMV genome occurs *in vivo* during congenital infection. We analyzed the presence of mutations in clinical amniotic fluid specimens obtained from 3 congenitally infected fetuses. Clearly, all 3 samples yielded low-denaturation-temperature PCR products (Fig. 5A). Analysis of 15 randomly selected clones derived from the PCR products from amniotic fluids confirmed the presence of hypermutated HCMV DNA, with G/C-to-A/T transitions constituting the vast majority of the identified mutations (Fig. 5B and C). Analysis of the high-denaturation-temperature amplicons from the same amniotic fluid samples showed only a few mutations (3 among 1,800 bases analyzed). Interestingly, all three identified mutations were G-to-A transitions, supporting the validity of the 3D-PCR analysis and further suggesting that at least some of the polymorphism in these natural strains could be driven by APOBEC editing. Together, these findings directly imply that A3 editing of the HCMV genome occurs during natural congenital infection.

DISCUSSION

HCMV has been shown to induce a broad innate immune response (8, 10, 40–42), and yet little is known about the individual effector proteins mediating the host defense against HCMV infection. In view of the grave outcome associated with congenital HCMV infection, there is an urgent need to better understand the innate mechanisms acting to limit transplacental viral transmission. Toward this goal, our findings reveal the role of the intrinsic cellular restriction factor A3A as a potent anti-HCMV innate barrier, activated by HCMV infection in the maternal-fetal interface. Employing a global transcriptome analysis of *ex vivo* infected human decidual tissues, we found that the A3A gene was one of the most profoundly upregulated innate immune genes following HCMV infection in the decidua (Table 1). Importantly, the induction of A3A by HCMV appeared to be tissue and cell type specific, as it was distinctively detected in the decidual tissues, constituting the maternal aspect of the placenta, and was not observed in similarly infected chorionic villi, constituting the fetal aspect of the placenta, or in fibroblasts and epithelial cell cultures (Fig. 1). The decidua is a complex tissue, cohabited by invasive fetal cytotrophoblasts, the uterine microvas-

culature, and maternal epithelial and stromal cells, as well as by a multitude of maternal immune cells (constituting ~40% of the total cells in the decidua during the first trimester) which include a dominant population of decidual NK cells, macrophages, and dendritic cells and a limited number of T cells (3). Hence, one or more of these heterogeneous cell types might be responsible for the increased expression of A3A.

The lack of A3A induction in fibroblasts cell cultures, most commonly used for the *in vitro* studies of HCMV, could have accounted at least in part for the fact that the role of A3—widely recognized as a pivotal arm of innate antiviral immunity—has so far been overlooked in the context of HCMV infection. This finding underscores the importance of the analysis of the innate immune response within authentic multicellular integral tissues (that also contain myeloid cells—known to express A3A), which closely recapitulate the *in vivo* diversity of HCMV-infected target cells and complex tissue responses. APOBEC3A is also known to be an interferon-stimulated gene (ISG). In accordance, we showed the upregulation of decidual A3A in uninfected tissues by IFN- β (but not IFN- γ ; Fig. 1D). While we have previously demonstrated that HCMV infection affects the expression and secretion of type I IFNs in the decidua only minimally (6, 9), suggesting the involvement of other (viral or virus-induced) factors in A3A upregulation, it could be still mediated by the (slightly) induced IFNs.

While most of the information about APOBEC inhibition of viruses pertains to retroviruses and retroelements, DNA viruses—including the human herpesviruses HSV-1 and EBV—have emerged as potential targets for restriction by A3s (13–16, 18, 29, 43–45). Specifically, APOBEC3C overexpression was reported to inhibit HSV-1 replication in cell culture, and HSV-1 and EBV sequence mutations consistent with cytidine deamination were detected in patient samples and immortalized cell lines, respectively (20). Different viruses have been shown to be differentially restricted by subsets of A3 proteins, and it is noteworthy that HCMV induced much higher decidual tissue upregulation of A3A than of other A3 family members (Fig. 1). A3A has been reported to inhibit retrotransposons and foreign DNA as well as parvoviruses, HBV, and HPV (13–16, 22–31). Hence, the profound and differential levels of upregulation of A3A in the maternal decidua suggest that A3A could be functional in the protection against HCMV transmission. This assumed role is supported by our finding that controlled overexpression of A3A in transduced epithelial cell cultures resulted in early and potent restriction of HCMV replication, with inhibition of viral DNA accumulation, early late gene expression, and infectious virus production (Fig. 2 and 3). Ideally, complementary data showing that silencing of A3A enhances HCMV replication could have strengthened these conclusions; however, the tissue used in our study, along with the demonstrated absence of significant A3A expression in the HCMV-susceptible cell cultures (Fig. 1), precluded this experimental approach. Additionally, A3A could be one of several ISGs that exert anti-CMV activity in decidual tissue.

The reported mechanisms for viral inhibition by A3s are diverse and include deamination-dependent and -independent processes (13–16, 25, 33, 44). Here we show that A3A anti-HCMV activity was mediated by its cytidine-deaminase activity: (i) the catalytically inactive A3A E72Q mutant did not inhibit HCMV replication, and (ii) cytidine deaminase-edited viral DNA intermediates containing C/G-to-T/A hypermutations were exclusively detected in cells expressing the catalytically active A3A (Fig. 2 to 4). Notably, edited viral DNA could be recovered from A3A-expressing cells in culture only in the presence of ectopically expressed uracil DNA glycosylase inhibitor, indicating that the A3A-edited HCMV DNA intermediates are subject to excision-degradation by cellular and/or potentially viral UNGs. This combination of deamination- and uracil excision-mediated processes of viral DNA clearance is reminiscent of the mechanism by which A3A has been reported to restrict foreign DNA and L1 retrotransposition (27, 46). During HCMV DNA synthesis, A3A may act on the transiently exposed single-strand regions, giving rise to dead-end degradation-prone intermediates and to disrupted coding potential, accounting for the impaired viral replication.

The implications of the findings in a cellular overexpression model system (in which A3A levels are ~100-fold higher than in the maternal decidua) should be regarded with

caution. Yet our finding of A3-edited viral DNA in *ex vivo* infected decidual tissues (Fig. 5) supports the idea of the physiological relevance and impact of the virus-induced A3A in the decidua. Moreover, the occurrence of A3 editing of the viral genome in the actual setting of congenital HCMV infection is implied by the direct detection of naturally occurring hypermutations in viral DNA recovered from clinical amniotic fluid samples of congenitally infected fetuses. Of note, some of these mutations, identified in the essential HCMV DNA polymerase gene, were nonsynonymous and led to premature stop codons. While the most substantially upregulated A3 transcript in the decidua was A3A, we also noted the upregulation of other A3s (to a lower extent and yet reaching similar expression levels) following HCMV infection (Fig. 1A), and we therefore cannot exclude the possibility of their contribution to viral DNA editing. Additionally, one should bear in mind that the use of the 3D-PCR technique, which preferentially amplifies hypermutated sequences, might have resulted in an overestimation of their occurrence. The true frequency and pattern of A3-related mutations across the viral genome remain to be studied in unbiased high-fidelity deep sequencing analyses in multiple clinical specimens from congenital HCMV cases.

The impact of A3A on viral restriction *in vivo* is likely to be defined by the balance between the levels of cell and tissue expression of A3A and viral countermeasures (11, 13, 47–49). It is plausible that HCMV, known as “a master of immune evasion,” has evolved a mechanism(s) to avoid A3A restriction. Studies aimed to identify HCMV-encoded anti-A3A functions are under way in our laboratory.

Given the widely differential tissue distribution characteristics and biological functions of A3 protein family, our findings—beyond their implications for maternal-fetal HCMV transmission—bring to light intriguing new issues pertaining to the possible role of A3A (as well as of other A3 family members) in the cellular tropism and spread of HCMV, in the increasingly recognized genetic variability of HCMV strains, and maybe even in the oncomodulating activity attributed to HCMV.

In summary, we have shown that HCMV profoundly upregulates A3A in *ex vivo* infected human decidual tissues, that A3A restricts HCMV infection by a cytidine deamination mechanism, and that cytidine deaminase editing of the viral genome occurs *in vivo* during natural congenital infection. Our findings reveal a previously unexplored role for A3A as an innate anti-HCMV effector in the maternal-fetal interface and pave the way for new insights into the potential impact of APOBEC proteins on HCMV pathogenesis.

MATERIALS AND METHODS

Cells and viruses. Primary human foreskin fibroblasts (HFF; provided by A. Lifshitz, the Hadassah Medical Center Clinical Virology Laboratory) were used for HCMV propagation. The HCMV TB40/E strain expressing IE2-fused enhanced yellow fluorescent protein (EYFP) (generously provided by M. Winkler, Germany) was propagated as previously described (21). For determination of viral titers, infected cell supernatants were collected, centrifuged to remove cellular debris, and stored at -80°C until assayed by a standard plaque assay.

ARPE-19 cells (human retinal pigmented epithelial cells), MRC-5 cells (human fetal lung fibroblasts), and 293T cells were obtained from the ATCC and maintained according to ATCC instructions.

Preparation and infection of decidual and chorionic villus organ cultures. Decidual and chorionic villus organ cultures were prepared and infected as previously described (21). In brief, first-trimester decidual and chorionic villus tissues were obtained from consenting women undergoing first-trimester elective pregnancy terminations. The decidual tissue contained the maternal tissue from the basal plate and placental bed encompassing the decidua with interstitial trophoblastic invasion as described previously (21). The tissues were sectioned by a microtome into thin slices and incubated at 37°C and 5% CO_2 in the following decidual medium: Dulbecco's modified Eagle's medium (DMEM) with 25% Ham's F12, 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B (Biological Industries). For infection of the organ cultures, the tissues were placed in 48-well plates and inoculated with 100 μl of the virus (5×10^4 PFU/well) for 12 h to allow effective viral adsorption. While the exact MOI in the tissues cannot be calculated, this inoculum typically resulted in an MOI of 0.05 or lower.

DNA and RNA purification and quantification. Infected and mock-infected organ and cell cultures were washed and stored at -80°C until assayed. RNA and DNA were extracted using a NucleoSpin RNA isolation kit and a NucleoSpin tissue kit (Macherey-Nagel), respectively, and were subjected (RNA) to reverse transcription (GoScript; Promega), followed by quantitative real-time PCR (7900HT; Applied Biosystems), using TaqMan Fast Advanced master mix (ABI) and FastStart Universal Sybr green Master

(Roche), according to the instructions of the manufacturers. Primers used for A3 mRNA quantification were from qPrimerDepot (50). Primer and probe sequences for HCMV DNA and RNA and for β actin were previously described (21, 32).

cDNA library preparation, deep sequencing, and bioinformatics analysis. Transcriptome libraries were prepared using an Illumina TruSeq RNA library preparation kit (Illumina catalog no. RS-122-2001), according to the manufacturer's recommended protocol, starting with around 450 ng of total RNA. mRNA was purified using poly(A) selection, and then pooled libraries were sequenced on a HiSeq 2500 instrument in a single-end configuration, reading 50 bases. Raw reads (fastq files) were inspected for quality issues with FastQC (v0.11.2; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). According to the FastQC report, reads were trimmed from their base at position 51, using the `fastx_trimmer` program of the FASTX package (version 0.0.14; http://hannonlab.cshl.edu/fastx_toolkit/), and were then subjected to quality control and adapter trimming at the 3' end, using in-house perl scripts with a quality threshold of 35. In short, the script uses a sliding window of 5 bases from the read's end and trims one base at a time until the average quality value corresponding to the window passes the given threshold. Following quality trimming, adapter sequences were removed with an in-house perl script, allowing up to 10% mismatches and requiring at least 6 bases that match the adapter sequence for trimming to occur. Reads that became shorter than 15 bases were filtered out. Following trimming, the remaining reads were further filtered to remove low-quality reads, using the `fastq_quality_filter` program of the FASTX package (version 0.0.14; http://hannonlab.cshl.edu/fastx_toolkit/), with a quality threshold of 30 at 95% or more of the read's positions.

The processed fastq files were mapped to the human transcriptome and genome using TopHat (v2.0.13) (51). The genome version was GRCh37, with annotations from Ensembl release 75. Mapping allowed up to 3 mismatches per read, a maximum gap of 5 bases, and a total edit distance of 8 (full command: `tophat -G genes.gtf -N 3 -read-gap-length 5 -read-edit-dist 8 -segment-length 20 -read-realign-edit-dist 4 genome.processed.fastq`). Quantification, normalization, and differential expression procedures were done with the Cufflinks package (v2.2.1) (52, 53). Quantification was done with cuffquant, using the genome bias correction (`-b` parameter) and the multimapped read assignment algorithm (`-u` parameter). Normalization was done with cuffnorm (using the output format of Cuffdiff), and results were visualized in R, using the `cummeRbund` package (version 2.8.2) and in-house R scripts. Counts and distributions of numbers of fragments per kilobase per million (FPKM), as well as multidimensional scaling (MDS) analysis, were used for comparing levels of global expression between samples, for evaluation of outliers (none found), and for background expression level estimations. Differential expression levels were calculated with cuffdiff, using the default minimal count threshold (`-c 10` parameter). Significantly differentially expressed genes were defined as ones with a FPKM value of at least 1 corresponding to expression under at least one set of the conditions and a false-discovery-rate value (q value) of less than 0.05. Pseudogenes and transcripts known to lack poly(A) were removed from the analysis. Data sets related to this study have been deposited in GEO (see below).

Generation of ARPE-19 cells expressing A3A and A3A E72Q and the UNG inhibitor UGI. A3A cDNA was obtained by reverse transcription-PCR of mRNA isolated from infected decidual cultures. The cDNA was first cloned to pFC27A vector (Promega), and the A3A-HaloTag open reading frame (ORF) was then cloned into pEF1 α -Tet3G vector (Clontech) expressing monomeric red fluorescent protein (mRFP). For the generation of A3A catalytic mutant E72Q (24), site-directed mutagenesis was performed using a Q5 site-directed mutagenesis kit (NEB). Plasmids were sequenced prior to transfection. Lentiviruses were propagated in 293T cells prior to infection of ARPE-19 cells. mRFP-expressing ARPE-19 cells were sorted by fluorescence-activated cell sorter (FACS) analysis (FACSARIA III; BD Biosciences). Expression of A3A was verified by reverse transcription real-time PCR and Western blot (WB) analysis, and A3A activity was measured by fluorescence resonance energy transfer (FRET)-based deamination assay as described below. For the generation of UGI-expressing cells, codon-optimized UGI was synthesized as gBlock gene fragments (Integrated DNA Technologies) and cloned into pLVX-EF1 α vector (Clontech) expressing the neomycin gene. Transduced cells underwent selection with G418 until only G418-resistant cells remained. Cells expressing pLVX-EF1 α vector without UGI were used as a control.

FRET-based *in vitro* deamination assay in cell lysates. Cells were lysed using a 10 mM Tris (pH 7.5) buffer containing 1% IGEPAL CA-630, 150 mM NaCl, and protease inhibitor cocktail (Sigma). Protein quantity was assessed by the Bradford assay. The protein concentration was normalized and an A3A activity assay was performed as published by Thielen et al. (54) with some modifications. A 10- μ l volume of normalized lysates was mixed with 70 μ l master mix containing 10 pmol TaqMan probe (6-carboxyfluorescein [FAM]-AAATCTAATAGATAATGTGA-black hole quencher [BHQ]) (55), 0.4 units of UDG (NEB), 50 mM Tris-HCl (pH 7.4), and 10 mM EDTA. Plates were incubated at 37°C. After 1.5 h, 4 μ l of 4 N NaOH was added and the plates were incubated for 30 min at 37°C followed by addition of 4 μ l 4 N HCl and 36 μ l of 2 M Tris-HCl (pH 7.9). Endpoint fluorescence was measured using a Spark 10M (Tecan) microplate reader set to 490 nm.

Protein purification and WB. Cells were lysed using a buffer containing 1% IGEPAL CA-630, 10 mM Tris (pH 7.5), and protease inhibitors (Sigma). Lysates were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were used at the concentrations recommended by the manufacturers for protein detection: anti-IE1 and anti-IE2 (anti-IE1/2) and anti-pp28 (Virusys Corporation); anti-GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase; Santa Cruz); and anti-HaloTag (Promega). Horseradish peroxidase (HRP)-conjugated secondary antibodies (Promega) were used for detection.

Differential DNA denaturation (3D)-PCR. Amplification primers were derived from the HCMV DNA polymerase (UL54) gene. For the first PCR, the primers used were as follows: forward, 5'CTRCCTCAT

RTTCTATC3'; reverse, 5'ATRCACCTCRATATCRAA3'. The reaction parameters were as follows: 95°C for 5 min, followed by 34 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 1 min, and finally 10 min at 72°C. The PCR products were diluted by 10-fold, and 1 μ l of the diluted product was used as a template for the second round of PCR. The nested PCR primers were as follows: forward, 5'CRTTTTACACCTACRATC3'; reverse, 5'ACCTCACRCARCTATC3'. The reaction parameters were as follows: 85 to 87°C for 5 min; 34 cycles consisting of 85 to 87°C for 1 min, 54°C for 30 s, and 72°C for 1 min; finally, 10 min at 72°C. The lengths of the outer and inner fragments were 722 and 212 bp, respectively. Reactions were carried out using DreamTaq green PCR master mix (ThermoFisher Scientific). Specific bands from the low-denaturation-temperature reaction were excised from 2% agarose gel and ligated into pGEMT-Easy vector (Promega), and the cloned DNA fragments were subjected to DNA sequencing (Hylabs).

Statistical analysis. All data (means $\pm$ standard errors of the means [SEM]) were analyzed using unpaired, two-tailed *t* tests for comparisons between two groups; *P* values of <0.05 were considered significant. Statistical analysis of the transcriptome data was done as described under "cDNA library preparation, deep sequencing, and bioinformatics analysis" above.

Ethics statement. The study was approved by the Hadassah Medical Center Institutional Review Board (0138-08-HMO) and was performed according to the Declaration of Helsinki, good clinical practice guidelines, and the Human-Experimentation Guidelines of the Israeli Ministry of Health. All participants provided written informed consent.

Accession number(s). Data sets related to this study have been deposited in GEO under accession number [GSE94724](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94724).

ACKNOWLEDGMENTS

We thank Orit Caplan for technical assistance.

This work was supported by grants from the Israel Science Foundation, European Union Seventh Framework Programme 562 FP7/2012-2016 (grant agreement number 316655), and the Israeli Ministry of Health.

We declare that we have no competing financial interests.

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