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APOBEC3 proteins mediate the clearance of foreign DNA from human cells

Mark D Stenglein[1,2], Michael B Burns[1], Ming Li[1], Joy Lengyel[1] & Reuben S Harris[1]

Bacteria evolved restriction endonucleases to prevent interspecies DNA transmission and bacteriophage infection. Here we show that human cells possess an analogous mechanism. APOBEC3A is induced by interferon following DNA detection, and it deaminates foreign double-stranded DNA cytidines to uridines. These atypical DNA nucleosides are converted by the uracil DNA glycosylase UNG2 to abasic lesions, which lead to foreign DNA degradation. This mechanism is evident in cell lines and primary monocytes, where up to 97% of cytidines in foreign DNA are deaminated. In contrast, cellular genomic DNA appears unaffected. Several other APOBEC3s also restrict foreign gene transfer. Related proteins exist in all vertebrates, indicating that foreign DNA restriction may be a conserved innate immune defense mechanism. The efficiency and fidelity of genetic engineering, gene therapy, and DNA vaccination are likely to be influenced by this anti-DNA defense system.

Intracellular foreign DNA threatens the well-being and proper functioning of cells. If the DNA is pathogenic in origin, it could produce cytotoxic gene products or replicate to propagate an infection. Even nonpathogenic DNA, for instance DNA from dying cells internalized by phagocytosis, poses a threat to the cellular genome through insertional mutagenesis, which may disrupt gene expression.

Foreign DNA is sensed by Toll-like receptor (TLR)-dependent and TLR-independent mechanisms[1,2]. TLR9 senses single-stranded DNA (ssDNA) in endosomal compartments and signals to induce the production of type 1 interferons (IFNs) and pro-inflammatory cytokines and chemokines. Several double-stranded DNA (dsDNA) sensor(s) have been identified—DAI, AIM2 and, recently, RNA polymerase III—but only the latter seems to be required for IFN production[3–8]. Regardless, once IFN is produced, it stimulates the transcription of many genes whose products orchestrate a wide variety of innate immune responses[9]. For instance, BST2 (also known as tetherin) blocks viral budding, PKR inhibits translation, RNase L degrades intracellular RNA, and ADAR1 deaminates double-stranded RNA adenosines to inosines[10–15]. In contrast, comparatively little is known about cellular proteins that mediate the clearance of foreign intracellular DNA.

The enzymatic conversion of DNA cytidines to uridines currently has two established roles in immunity (**Supplementary Table 1**). Activation-induced deaminase (AID) deaminates antibody gene DNA and triggers antibody gene diversification by somatic hypermutation and class-switch recombination[16,17]. The APOBEC3s (A3s), A3A, A3B, A3C, A3DE, A3F, A3G and A3H, are DNA cytidine deaminases that have been shown to inhibit the replication of a diverse set of retroviruses and retrotransposons[18,19]. A3F and A3G, the best-studied members of this family, strongly inhibit the replication of Vif-defective HIV-1 by deaminating viral cDNA during retrovirus reverse transcription. In contrast, A3A does not restrict HIV-1 but instead has been shown to limit the retrotransposition of L1 and Alu elements as well as the replication of parvoviruses[20–26] (**Supplementary Fig. 1** and **Supplementary Tables 1** and **2**). Other DNA viruses such as hepatitis B virus (HBV) and human papillomavirus (HPV) may also be targets of A3 proteins, including A3A[27,28]. In all of these instances, the A3 proteins are thought to engage normal viral replication intermediates, which are intricate protein and nucleic acid complexes. Such intermediates are distinct from naked foreign DNA, which enters cells from multiple exogenous sources.

The fact that human cells have innate mechanisms to sense dsDNA and the fact that APOBEC3s are potent DNA deaminases with established roles in immunity led us to hypothesize that one or more of these proteins acts downstream of DNA-sensing molecules to mediate the clearance of foreign DNA. Here we show that A3A and several other human A3 proteins are potent foreign DNA restriction factors. We find that A3A is induced by DNA detection and IFN in phagocytes and that it triggers the degradation of foreign DNA by a cytidine-deamination and uracil-excision mechanism. These data reveal that foreign DNA restriction is a distinct and important physiological function of the APOBEC3 proteins.

RESULTS

A3A is expressed in phagocytes and induced by IFN

We reasoned that if A3 proteins were indeed DNA restriction factors, then at least one of these proteins ought to be expressed in cells frequently exposed to foreign DNA and induced by DNA detection. We focused on A3A because of reports suggesting that it is expressed predominantly in monocytes, macrophages and neutrophils[25,29].

[1]Department of Biochemistry, Molecular Biology and Biophysics, Institute for Molecular Virology, Center for Genome Engineering, University of Minnesota, Minneapolis, Minnesota, USA. [2]Present address: Howard Hughes Medical Institute, Departments of Medicine, Biochemistry and Microbiology, University of California, San Francisco, California, USA. Correspondence should be addressed to R.S.H. (rsh@umn.edu).

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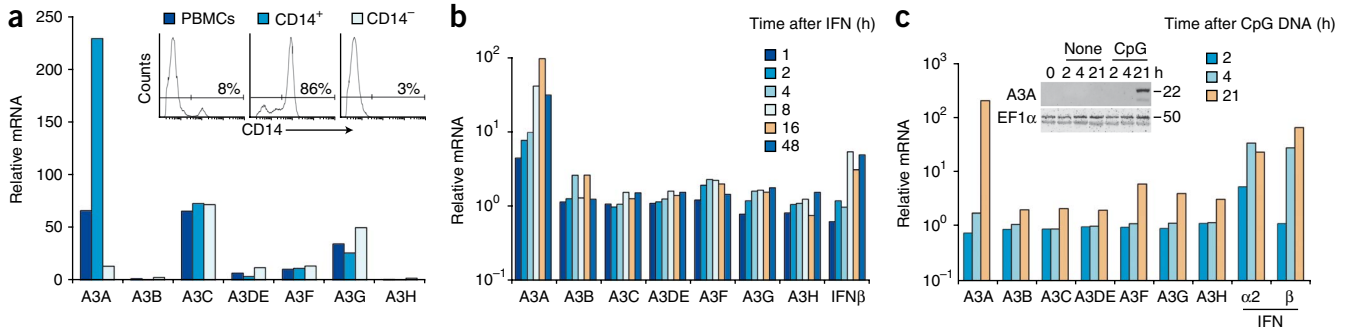

Figure 1 APOBEC3A is expressed in monocytes and macrophages and is induced by interferon and CpG DNA. (**a**) Relative basal A3 mRNA levels in PBMCs or the specified cell subpopulations, with a value of 1 assigned to the level of A3H mRNA in unsorted PBMCs. Inset, flow cytometry histograms showing the efficiency of CD14$^+$ cell enrichment. (**b**,**c**) A3 and IFNβ mRNA levels in PBMCs treated with recombinant IFN (**b**) or CpG DNA (**c**) oligonucleotide for the indicated time. mRNA levels are relative to those measured in untreated cells. Inset in **c**, immunoblot of A3A in PBMCs treated with a CpG DNA oligonucleotide. The same membrane probed with anti-eEF1α is shown as a loading control.

These phagocytic cells ingest large quantities of foreign materials, including nucleic acids. To confirm and extend these studies, we obtained fresh human peripheral blood mononuclear cells (PBMCs), purified CD14-positive monocytes and macrophages, and used a panel of specific PCR assays to quantify A3 expression. Several A3s were expressed in PBMCs, with A3A, A3C and A3G consistently showing the highest basal mRNA levels (**Fig. 1a**). The relative A3A expression level increased threefold in the CD14-enriched cell population and decreased proportionately in the CD14-depleted fraction, whereas A3C and A3G mRNA levels were similar in the different cell populations. These data indicated that A3A is expressed predominantly in CD14-positive phagocytic cells.

The mRNA levels of several A3s are inducible by IFN[29–34]. To systematically quantify the relative magnitudes and kinetics of this IFN response, we treated fresh PBMCs with IFN and isolated RNA at multiple time points for quantification by real-time PCR. A3A was strongly responsive to IFN, with mRNA levels consistently peaking 100-fold above those in untreated cells (**Fig. 1b**). Other A3s showed modest two- to threefold increases. We also found that incubating PBMCs with the TLR9 ligand CpG ssDNA caused a 200-fold increase in A3A levels, and a two- to fourfold increase in the expression of other A3s (**Fig. 1c**). This dramatic level of induction places A3A among the most IFN-responsive genes (**Supplementary Fig. 2**). A3A induction by IFN was also observed both by immunoblotting using an A3A-specific polyclonal antibody and by monitoring DNA cytidine deaminase activity in cell extracts (**Fig. 1c**, inset, and **Supplementary Fig. 3**).

A3A reduces gene transfer efficiency

The finding that A3A is expressed in cells that ingest pathogens and extracellular debris, and the fact that its expression is induced by DNA detection, suggested that it might mediate the clearance of foreign DNA. To directly test this hypothesis, we quantified the impact of A3A expression on several measures of foreign DNA integrity and stability. First, we measured the impact of A3A on stable gene transfer efficiency (**Fig. 2a–c**). HEK 293 cells, which lack detectable A3A mRNA (data not shown), were co-transfected with a neomycin-resistance (Neo^R) reporter plasmid and an expression construct for A3A or A3A$_{E72A}$ (a catalytically inactive mutant; **Supplementary Fig. 4**), or a GFP control. We allowed 2 d for integration of the reporter plasmid into the chromosomal DNA and for expression of the Neo^R gene product. We then plated the cells into neomycin (G418)-containing medium, allowed time for drug-resistant colonies to grow, and then stained and counted the resulting Neo^R colonies.

A3A expression resulted in a dose-dependent decrease in gene transfer efficiency in comparison to either control condition (**Fig. 2b**,**c**). This effect was potent: transfecting as little as 16 ng of A3A expression plasmid decreased the frequency of G418^R colonies to 40% of controls (**Fig. 2c**). We obtained similar data with HeLa cells or with a more efficient DNA transposon-mediated gene transfer system (**Supplementary Fig. 5** and see below for a comparison with other human A3s). A3A expression did not decrease the number of colony-forming cells (assessed by plating in drug-free medium), nor was there any indication of altered proliferation or increased apoptosis in A3A-expressing cells (**Figs. 2d–f**). Identical results were obtained in experiments with transfection efficiencies ranging from 25–75%.

A3A Glu72 is part of a conserved zinc-coordinating catalytic motif, H-X-E-X$_{28}$-C-X$_{2–4}$-C, in which X represents other residues[35,36]. A3A$_{E72A}$ was catalytically defective *in vitro*, in agreement with prior studies[25,26] (**Supplementary Fig. 4**). The fact that this mutant did not decrease the efficiency of stable gene transfer suggested that this effect requires the DNA cytidine deaminase activity of A3A (addressed further below).

A3A inhibits transient gene expression

As a second measure of foreign DNA stability, we asked whether A3A affects transient gene expression. We transfected GFP-encoding plasmid DNA into HEK 293 cells and monitored GFP fluorescence by flow cytometry (**Fig. 2g**). After 2 d of incubation, the higher A3A expression levels caused a small decrease in GFP fluorescence. By 5 d, all A3A levels caused significant reductions in GFP fluorescence, and a clear dose response was apparent. The rate of GFP fluorescence decay was also accelerated in A3A-expressing cells compared to control cells (~4% decline in GFP-positive cells per day versus 2% in the control cells). Thus, A3A expression caused a more rapid disappearance of transient reporter gene expression.

A3A destabilizes foreign plasmid DNA

Because reduced gene transfer efficiency and diminished reporter gene expression could result from a reduction in either the quantity or the integrity of the transfected DNA itself, we used quantitative PCR to directly measure plasmid DNA levels. These experiments revealed a 60% reduction in plasmid DNA levels in A3A-expressing cells in comparison to control cells 2 d after transfection (**Fig. 2h**). It is noteworthy that this is likely an underestimate of the potency of A3A, because transfected DNA may resist DNase digestion (done before cell lysis to remove free DNA) or may become sequestered in an A3A-impermeable compartment (for example, endosomes). Nevertheless, these data showed that A3A compromises

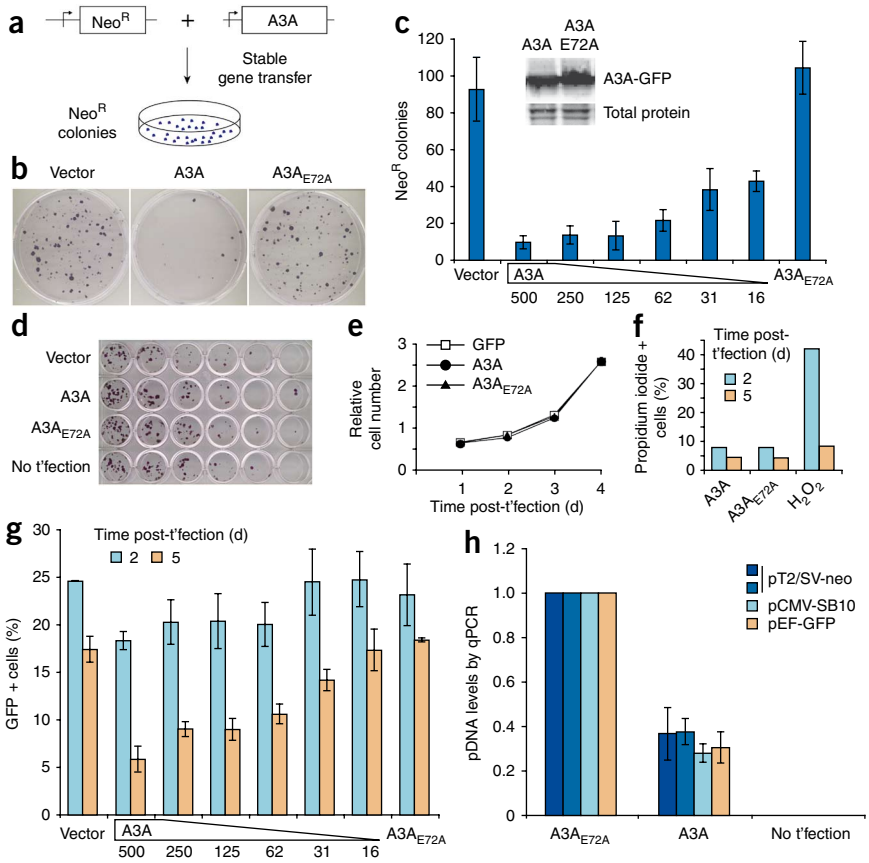

Figure 2 Foreign DNA restriction by APOBEC3A. (**a**) Schematic of plasmid-based stable gene transfer experiments. (**b**) Representative plates of Neo^R colonies obtained in a stable gene transfer experiment in HEK 293 cells transfected with a Neo^R plasmid and A3A-expression or control plasmids. (**c**) Quantification of the data in **b** and two additional experiments. The ng amount of A3A-expression plasmid was decreased as indicated. Inset, immunoblot of A3A-GFP and $A3A_{E72A}$-GFP and the same membrane stained with Ponceau S. (**d**) The number of colony-forming cells in transfected cell populations in **b** and **c** was determined by diluting and plating cells into drug-free medium. (**e**) The proliferative capacity of transfected cells. (**f**) The percentage of apoptotic cells in transfected-cell populations. Hydrogen peroxide (H_2O_2) was used as a cytotoxic control. (**g**) Transient expression of a GFP reporter plasmid in HEK 293 cells. (**h**) pDNA persistence in HeLa cells transfected with A3A or A3A-E72A was determined by quantitative PCR on DNA recovered 48 h post-transfection. For each pDNA, the $A3A_{E72A}$ data were normalized to one. No t'fection, nontransfected controls. In **c**, **e**, **g** and **h**, the mean and s.d. of three replica experiments is shown.

the physical integrity of foreign plasmid DNA, and that this effect requires the conserved catalytic glutamate.

A3A deaminates foreign DNA, creating UDG substrates

If A3A indeed triggers the clearance of foreign DNA by a mechanism involving DNA deamination, then in addition to a requirement for catalytic activity, two major predictions follow. First, the immediate products of deamination, DNA uracils, might be substrates for excision by cellular uracil DNA glycosylases. A likely candidate is the major cellular uracil DNA glycosylase, UNG2, which functions normally in error-free excision repair of genomic uracils but also processes these lesions during error-prone antibody gene diversification events[16,37] (**Supplementary Table 1**). Second, because DNA uridines base-pair like thymidines and template the insertion of adenosines, they ought to amplify by PCR to result in C/G-to-T/A transition mutations. Furthermore, if A3A were the source of these mutations, then the mutated cytidines should be biased toward 5′-TC and 5′-CC dinucleotides, which are the reported targets for A3A deamination (the underlined C is deaminated and the 5′ nucleotide is a specificity determinant)[25,27,38,39] (**Supplementary Table 2**).

To test these predictions, we used a uracil DNA glycosylase inhibitor (UGI) to inhibit UNG2 and we used a technique called differential DNA denaturation (3D)-PCR to recover edited intermediates[40,41]. If UNG2 excises A3A-generated DNA uracils, then inhibiting UNG2 should cause an accumulation of uridine-containing intermediates. 3D-PCR is based on the fact that DNA with fewer interstrand hydrogen bonds will amplify preferentially at lower denaturation temperatures (for example, duplex DNA with G/U mismatches or A/T rich DNA). The combined use of these techniques allows for the detection of potentially short-lived uridine-containing DNA intermediates.

We co-transfected UGI-expressing HEK 293T cells with a reporter plasmid and A3A or $A3A_{E72A}$ expression plasmids. Two days later, we recovered transfected DNA from cells and analyzed it by 3D-PCR. At the highest denaturation temperatures, PCR products were apparent from both A3A- and $A3A_{E72A}$-expressing cells (**Fig. 3a**). However, at lower denaturation temperatures, we only obtained PCR products from A3A-expressing cells. We transfected UNG2-proficient (UGI-negative) cells similarly, and we did not detect PCR products at lower denaturation temperatures (data not shown). These results indicate that A3A edits transfected plasmid DNA and that UNG2 processes the edited molecules.

To confirm that UNG2 excises uracil from DNA molecules edited by A3A, we transfected HEK 293 cells with a reporter plasmid, A3A or $A3A_{E72A}$ expression plasmid, and a range of UGI expression plasmids. In support of the prior experiments, higher doses of UGI (greater UNG2 inhibition) led to an increased abundance of PCR products at lower denaturation temperatures (**Fig. 3b**). As controls, UNG2 inhibition was alone insufficient to enable the recovery of low-denaturation-temperature PCR amplicons, and again A3A catalytic activity was required.

To unambiguously determine whether the low-denaturation-temperature amplicons corresponded to A3A-edited molecules, we cloned and sequenced a number of amplicons from several independent PCRs (**Fig. 3c**). These analyses revealed high levels of C-to-T transition mutations in molecules recovered from A3A-expressing cells. DNA cytidines within a 5′-TC dinucleotide context were preferred, consistent with prior studies[25,27,38,39] (see below and **Supplementary Table 2**). Mutations were even apparent in the majority of molecules amplified at the highest denaturation temperature (94.4 °C), indicating that selective amplification by 3D-PCR was not required to detect edited sequences. In contrast, we found no mutations in molecules recovered from $A3A_{E72A}$-expressing cells. Overall, these data demonstrate that A3A deaminates foreign DNA in human cells and that the resulting uridines are substrates for UNG2-mediated excision.

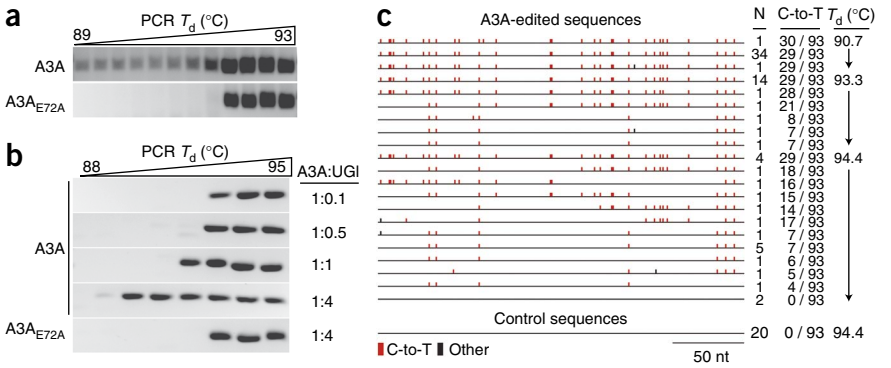

Figure 3 APOBEC3A deaminates transfected plasmid DNA and generates lesions for uracil DNA glycosylase. (**a**) Agarose gel analysis of 3D-PCR products from stable UGI-expressing HEK 293T cells. Cells were transfected with plasmids encoding A3A or $A3A_{E72A}$ and a mCherry (pTre2-mCherry) reporter construct. Total DNA was recovered 48 h post-transfection and analyzed by 3D-PCR at the indicated denaturation temperatures (T_d). (**b**) Agarose gel analysis of 3D-PCR products from HEK 293 cells transiently transfected with increasing amounts of UGI plasmid, a GFP reporter construct (pEGFP-N3) and a plasmid encoding A3A or $A3A_{E72A}$. Total DNA was recovered 48 h post-transfection and analyzed by 3D-PCR at the indicated T_d ranges. (**c**) Summary of the plasmid DNA sequences (nucleotides 1170–1426 of pEGFP-N3) recovered from the experiment in **b**. C-to-T hypermutations are indicated as red tics along the consensus sequence, and all other base substitutions are shown as black tics. The control sequences were obtained from cells expressing $A3A_{E72A}$. The number of times each sequence was recovered, the number of C-to-T conversions in each sequence (out of 93 total cytidines), and the PCR T_d used to amplify the populations of molecules from which the sequences are derived are indicated.

Extensive foreign DNA editing occurs in human monocytes

We next wanted to assess the impact of foreign DNA editing in A3A's normal physiological context, which is in primary human phagocytes. We took advantage of the fact that a 100-fold difference in A3A expression exists between CpG-treated and mock-treated PBMCs (**Fig. 1c**). We prepared and transfected these two cell populations with GFP-encoding plasmid DNA or mock-transfected them with buffer alone. We found that the introduction of plasmid DNA into cells caused A3A to be induced, regardless of whether the cells had been pretreated with CpG DNA (**Fig. 4a**). These data are consistent with prior studies showing that the introduction of dsDNA into leukocytes triggers strong TLR-independent IFN responses[42,43].

Next, to determine whether endogenous A3A mutates transfected plasmid DNA, we recovered DNA from cells at 8 and 24 h post-transfection and used 3D-PCR to amplify a region of the *GFP* gene. For a control template, we mixed GFP plasmid with DNA prepared from mock-transfected cells. This control only amplified at the highest denaturation temperatures (**Fig. 4b**, pDNA). In contrast, we were able to detect plasmid DNA amplification at lower denaturation temperatures using the DNA recovered from transfected monocytes. Cloning and sequencing these PCR products showed that they resulted from amplification of extensively edited plasmid DNA, with some molecules having as many as 72 of the 93 cytidines edited in the 257-nucleotide amplicon (**Fig. 4c**,**d**).

We also noted that considerably more amplification occurred at lower denaturation temperatures using DNA recovered from the CpG-pretreated cells than from the nonpretreated cells at 8 h post-transfection (**Fig. 4b**). This suggests that the plasmid DNA had been more extensively mutated in the cells that were expressing higher levels of A3A at the time of transfection. At 24 h post-transfection, weak bands appeared at the lowest denaturation temperatures in the mock-pretreated cells, likely corresponding to mutations catalyzed by A3A that had been induced by plasmid DNA transfection itself (**Fig. 4b**).

In addition to cloning and sequencing individual molecules, we also directly sequenced populations of PCR products (**Fig. 4c** and **Supplementary Fig. 6**). Remarkably, these analyses revealed evidence for C-to-U conversion of 70 of the 72 cytidines (97%) within

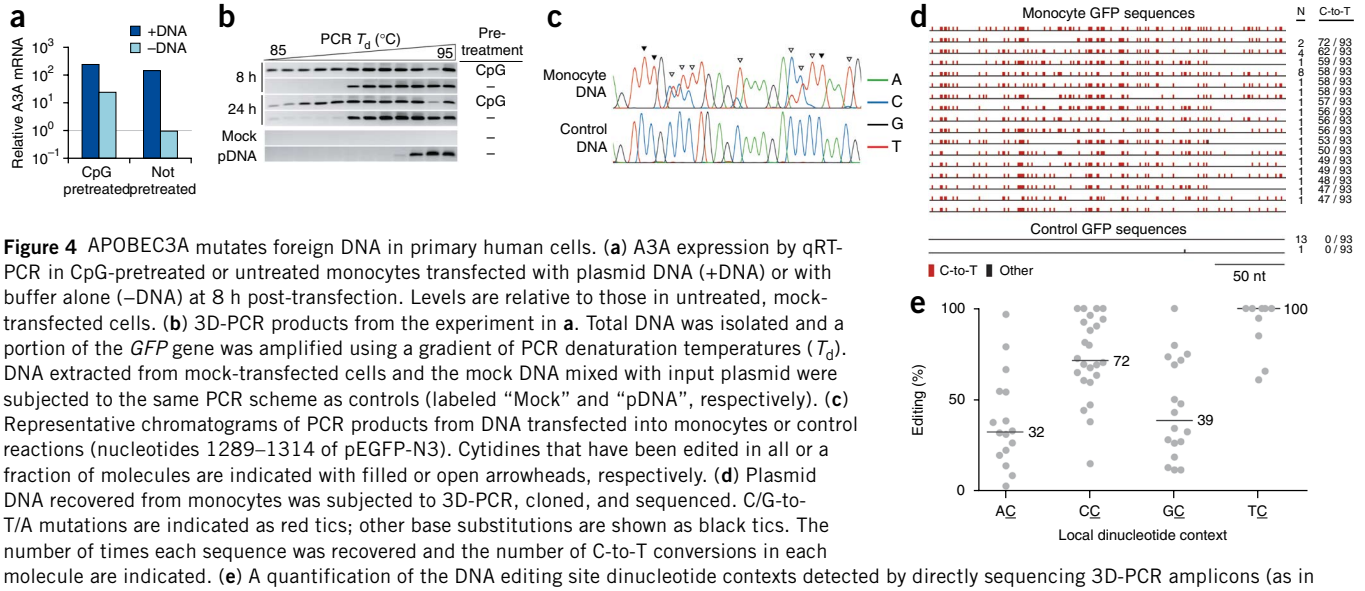

Figure 4 APOBEC3A mutates foreign DNA in primary human cells. (**a**) A3A expression by qRT-PCR in CpG-pretreated or untreated monocytes transfected with plasmid DNA (+DNA) or with buffer alone (–DNA) at 8 h post-transfection. Levels are relative to those in untreated, mock-transfected cells. (**b**) 3D-PCR products from the experiment in **a**. Total DNA was isolated and a portion of the *GFP* gene was amplified using a gradient of PCR denaturation temperatures (T_d). DNA extracted from mock-transfected cells and the mock DNA mixed with input plasmid were subjected to the same PCR scheme as controls (labeled "Mock" and "pDNA", respectively). (**c**) Representative chromatograms of PCR products from DNA transfected into monocytes or control reactions (nucleotides 1289–1314 of pEGFP-N3). Cytidines that have been edited in all or a fraction of molecules are indicated with filled or open arrowheads, respectively. (**d**) Plasmid DNA recovered from monocytes was subjected to 3D-PCR, cloned, and sequenced. C/G-to-T/A mutations are indicated as red tics; other base substitutions are shown as black tics. The number of times each sequence was recovered and the number of C-to-T conversions in each molecule are indicated. (**e**) A quantification of the DNA editing site dinucleotide contexts detected by directly sequencing 3D-PCR amplicons (as in **c**). The editing percentages were grouped by dinucleotide context and the median percentage for each group is indicated. For instance, five of the nine cytidines within 5′-TC dinucleotides were edited in 100% of the molecules amplified (in other words, only a T peak was evident in the chromatogram).

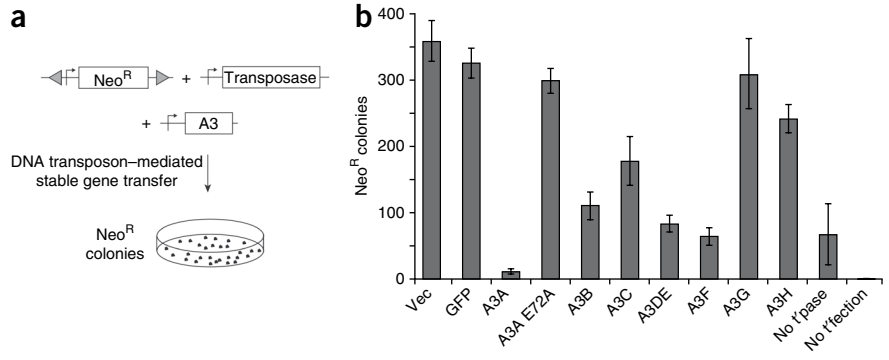

Figure 5 Foreign DNA restriction by multiple human APOBEC3 proteins. (**a**) Schematic of the DNA transposon-mediated stable gene transfer experiments. (**b**) A histogram showing the mean and s.d. of three independent transposon-mediated stable gene transfer experiments done in the presence of the indicated A3 expression constructs. Controls included the number of Neo^R colonies resulting from random integration (no transposase, No t'pase) and from no transfection (no foreign DNA, No t'fectection). Flow cytometry analysis confirmed that all of the A3-GFP constructs were expressed similarly (data not shown).

a 200-nucleotide region of the *GFP* gene (in other words, these cytidines had been edited in at least some of the molecules amplified; **Supplementary Fig. 6**). We observed a strong bias toward deamination at 5′-TC and 5′-CC dinucleotides, fully consistent with catalysis by A3A (**Fig. 4e** and **Supplementary Table 2**; compare with prior reports[25,27,38,39]). There was a high degree of correspondence between the most frequently edited sites in these primary cell experiments and those observed in plasmid DNA recovered from A3A-expressing HEK 293 cells (**Figs. 3**,**4** and **Supplementary Fig. 6**). We noted that the editing appeared to be strand-specific (**Figs. 3c** and **4d**). However, additional experiments using partly degenerate primer sets revealed that both DNA strands were edited (data not shown, but see Online Methods for assay details).

Interestingly, in contrast to experiments in HEK 293 cells, we detected hyperedited DNA molecules recovered from primary monocytes without inhibiting UNG2. This suggested that editing is more extensive in primary monocytes (consistent with the dramatic induction of A3A), but many other alternatives such as lower UNG2 activity may also be tenable. We obtained essentially identical results using PBMCs or CD14-enriched phagocytes from three independent donors and analyzed them with additional sets of PCR primers (for example, **Supplementary Fig. 7** and data not shown). These results clearly show that foreign DNA is hypermutated extensively in primary human cells. The main enzyme responsible is likely A3A, because its expression is highly induced (making it the best-expressed A3 family member), the kinetics of induction correlate with the appearance of 3D-PCR amplicons at low denaturation temperatures, and the primary-cell foreign DNA hypermutation patterns are similar to those observed in cell-culture experiments with only A3A present[25,27,38,39] (**Figs. 1**,**3**,**4** and **Supplementary Table 2**). RNA interference experiments intended to knock-down A3A were not successful, likely because the A3A induction was too strong (both the siRNA itself and the foreign DNA are immunostimulatory; data not shown and **Fig. 4a**).

Foreign DNA restriction by multiple human A3 proteins

Humans have six A3 proteins in addition to A3A, and most of these have much broader expression profiles[32,44–46]. To test whether any of these proteins are capable of inhibiting foreign DNA, we used an efficient DNA-transfer system based on the cut-and-paste transposon Sleeping Beauty[47] (**Fig. 5a**). Here, stable gene transfer results from both random and transposase-mediated integration events. As expected, A3A nearly eliminated DNA transfer, and restriction depended on catalytic activity (**Fig. 5b**). Several other human A3 proteins also inhibited DNA transfer but to different degrees: A3B, A3DE and A3F were most potent, A3C and A3H showed intermediate levels and A3G had essentially no activity. These restriction profiles contrast with those for HIV-1, of which A3G and A3F are the most potent inhibitors[18,19] (**Supplementary Table 1** and **Supplementary Fig. 1**). Similar results were obtained in stable gene transfer experiments analogous to those described in **Figure 2** (data not shown). Taken together with the aforementioned results, it is possible that A3C and A3F make minor contributions to foreign DNA editing in primary human phagocytes, and further, that multiple A3s may contribute to foreign DNA restriction in other cell types (**Supplementary Table 2**).

Mutation of nuclear genomic DNA is undetectable

The extraordinary level of A3A-dependent foreign DNA mutation prompted us to ask whether genomic DNA may also be susceptible to attack. We first established stable HEK 293 clones expressing an HSV *thymidine kinase* (*TK*) reporter gene (**Fig. 6a** and Online Methods). *TK*-positive cells are sensitive to gancyclovir (Gan^S),

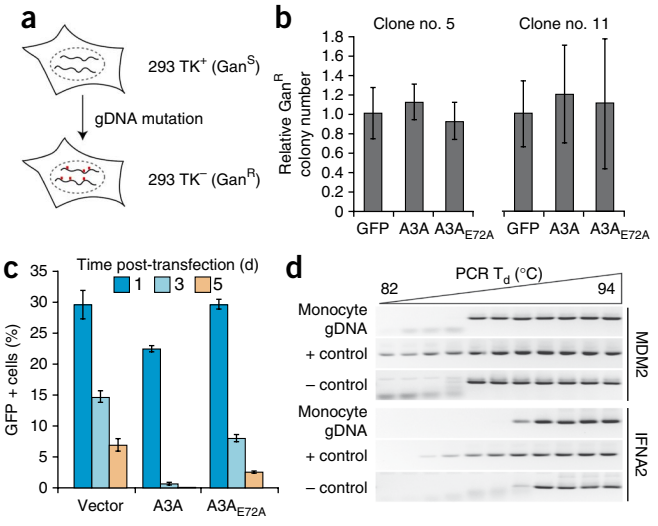

Figure 6 Lack of detectable genomic DNA mutation in cells that restrict foreign DNA. (**a**) Schematic of the genomic *TK* mutation assay. (**b**) Two independent clonal HEK 293 cell lines harboring integrated *TK* genes were transfected with the indicated expression plasmids and selected with gancyclovir-containing medium. The relative mean number of Gan^R (TK^-) colonies and s.d. from duplicate or quadruplicate experiments is shown. (**c**) GFP (or A3A-GFP) expression in transfected HEK 293 TK cells at the indicated times (clone #5 in **b**). (**d**) 3D-PCR results with primers specific to *MDM2* or *IFNA2* genomic loci (as described in **Fig. 4b**). For a positive control (+), genomic DNA was heat denatured and incubated with purified A3A before PCR. For a negative control (–), total DNA recovered from untransfected HEK 293 cells was subjected to the same PCR amplification procedures.

and genetic inactivation of *TK* confers resistance to this drug (Gan^R). We used this sensitive assay to ask whether transient A3A expression caused an elevated genomic mutation frequency. Although the level of A3A expressed in these experiments caused a strong reduction in foreign gene expression, it had no effect on the frequency of Gan^R mutation (**Fig. 6b**,**c**). We obtained similar results for an independently established cell line with *TK* integrated elsewhere in the genome (**Fig. 6b**).

To generalize these findings to primary human cells, we used 3D-PCR to try to detect genomic DNA mutation in monocytes expressing high levels of A3A (in other words, in cells where foreign DNA is edited; **Fig. 4**). Two distinct genomic loci, *IFNA2* and *MDM2*, were analyzed, and no evidence for genomic hypermutation was observed (**Fig. 6d**). The 3D-PCR profiles were indistinguishable for DNA derived from A3A-induced cells versus control DNA templates. As a positive control, the DNA recovered from monocytes was heat denatured and treated with recombinant A3A *in vitro*, and as expected, amplification occurred at much lower denaturation temperatures. It is also notable that, as anticipated, each 3D-PCR amplicon has a unique amplification threshold that correlates with its overall guanosine/cytidine content (*MDM2*, 38%; *IFNA2*, 49%; *GFP*, 59%).

Both *TK* mutation and 3D-PCR datasets were consistent with control experiments showing that transient A3A transfection into HEK 293 or HeLa cells does not cause overt signs of cell cycle arrest, growth defects, or apoptosis (**Fig. 2d–f** and data not shown). Thus, using several measures, we did not observe genomic-DNA editing in the same cells in which foreign DNA mutation is readily detectable. We conclude that, in contrast to foreign DNA, endogenous nuclear DNA is much less susceptible and possibly even completely resistant to A3A-dependent DNA deamination.

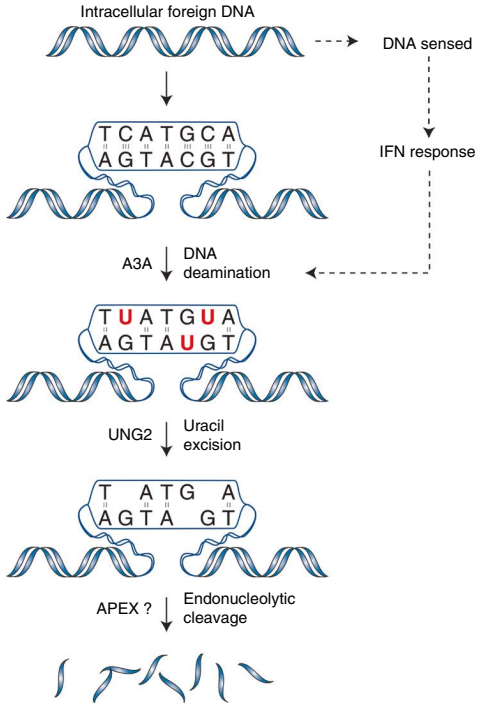

Figure 7 A model for foreign DNA restriction. Foreign DNA enters the cell by endocytosis, phagocytosis, infection, or other means. TLR-dependent or -independent DNA sensing initiates signaling cascades that result in production of IFN, which in turn induces A3A expression. A3A engages the foreign DNA, deaminating multiple cytidines in a molecule. The resulting uracils are excised by UNG2, creating nuclease-sensitive abasic sites. Cleavage of the backbone by APEX1 or other nucleases results in fragmentation and degradation of the foreign DNA.

DISCUSSION

The recognition and clearance of foreign intracellular DNA has been postulated to be a fundamental arm of the innate immune response[42,43,48]. Although both TLR-dependent and -independent pathways sense foreign DNA and trigger robust IFN production, little is known about the downstream effector proteins, some of which presumably must function to mediate DNA clearance. Here we show that several A3 proteins, particularly A3A, are such foreign DNA restriction factors. A3A is expressed predominantly in phagocytic cells and it is strongly inducible by IFN, consistent with a front-line role in foreign DNA restriction. A3A is also induced by CpG ssDNA or plasmid dsDNA, presumably through the induction of IFN by the TLR9 pathway or the TLR-independent DNA sensing pathway(s), respectively. A3A reduces foreign DNA stability and integrity by three measures: stable gene transfer, transient gene expression, and DNA persistence. The extraordinary levels of foreign DNA C-to-T hypermutation, a requirement for the A3A active-site glutamate, and the strong influence of UGI combine to show that foreign DNA restriction is a deamination- and uracil excision-dependent process (**Supplementary Table 1**).

Our data suggest a model in which foreign DNA clearance is initiated by A3A-dependent deamination of DNA cytidines to uridines (**Fig. 7**). The subcellular location in which this occurs is presently unclear. Additionally, it is possible that the naked plasmid dsDNA substrates used in our experiments are rendered transiently single stranded by cellular enzymes such as a polymerase or helicase. Regardless, the uridine lesions in foreign DNA are subsequently converted by the cellular uracil DNA glycosylase UNG2 to nuclease-susceptible abasic sites. It is likely that these abasic sites are processed by the endonuclease APEX, which cleaves the DNA phosphodiester backbone[37], but many other cellular nucleases such as TREX1 could also be involved[49]. Additionally, DNases 1 and 2 may contribute to foreign DNA clearance, but it is notable that these enzymes are ubiquitously expressed and not considered part of the IFN response[48,50].

Prior to this study, physiologically relevant DNA deamination substrates were thought to be limited to antibody gene DNA and to viral and retroelement ssDNA replication intermediates (**Supplementary Table 1**). Here we show that naked plasmid dsDNA from *Escherichia coli* is subject to restriction by A3A and other human A3 proteins. It is reasonable to assume that B-form, dsDNA from other types of bacteria (in other words, all other bacterial DNA) will be similarly restricted. Restriction-susceptible DNA may also originate from other microbes (fungi or viruses; foreign DNA) and/or from apoptotic or necrotic cells (self DNA). Indeed, DNA released from apoptotic cells has been shown to trigger an IFN response[51]. However, it is also possible that most restriction-susceptible viruses will have evolved a counter-defense mechanism such as those of lentivirus Vif or foamy virus Bet[18,19]. Further experiments with natural and synthetic DNA substrates will shed light on the types and characteristics of foreign DNA subject to A3-mediated restriction.

Our studies raise important questions about how A3 proteins discriminate between foreign and self DNA. It is possible that the foreign DNA restriction mechanism is compartmentalized to help mitigate the obvious risk to the normal genomic DNA of the cell. However, epitope-tagged versions of A3A distribute cell-wide and penetrate the nuclear compartment[25] (data not shown), and yet they do not elevate genomic-mutation frequencies (**Fig. 6**). Therefore, presumably at least one

additional mechanism exists to ensure the integrity of cellular genomic DNA. The issue of target specificity may be analogous to prokaryotic restriction and modification systems, in which self DNA is protected from restriction by nucleic-acid modifications (usually methylation). Analogous DNA modifications or epigenetic markings may serve to protect genomic DNA from A3-mediated DNA editing. Perhaps the relevant imprints are removed from the DNA of dying cells. Again, experiments with synthetic DNA substrates are likely to be informative.

An ancestral *AID* gene duplicated and diverged to root the *APOBEC3* locus, which has expanded significantly in several mammalian lineages, including that defined by modern primates[36,52]. It is therefore not surprising that the foreign DNA restriction mechanism described here shares some features with the mechanism of AID-mediated antibody-gene diversification and others with the mechanisms of A3-mediated retroelement restriction (**Supplementary Table 1**). However, the foreign DNA restriction mechanism described here is clearly distinct, targeting exogenous dsDNA (not chromosomal or replication-associated substrates), requiring both DNA deamination and uracil excision and occurring in different cell types. The A3A-dependent foreign DNA restriction mechanism described here appears to be the result of evolutionary and functional specialization. The fact that other A3s are to varying extents also capable of foreign DNA restriction, and yet are much less responsive to IFN, suggests that the mechanism described here may be both constitutively operative in most cells (a housekeeping function) and strongly inducible in specific cells, such as phagocytes that frequently encounter foreign DNA. Along these lines, it is notable that mouse A3 expression is induced in dendritic cells by DNA transfection or CpG DNA incubation[43]. Further studies with a variety of cell types and tissues from humans, mice, and other species will help address these important regulatory and evolutionary points. Nevertheless, despite many new questions, our current studies establish foreign DNA deamination as a new fundamental effector function within the broader innate immune response.

Finally, the existence of a foreign DNA restriction system in human cells and possibly in other vertebrates has major implications. First, the outcomes of experiments involving transfection of A3-expressing cells may be confounded by restriction of the transfected DNA. Such studies, including virus or transposon co-transfection experiments, should be carefully (re)interpreted in light of the mechanism described here. Second, for the basic biomedical sciences, it is likely that transient or stable gene delivery will be influenced by the A3 repertoire of a cell. For instance, the T-cell line CEM and its derivative CEM-SS are differentially amenable to stable gene transfection, with the latter A3-deficient line more efficient at stably incorporating foreign DNA (data not shown). We suggest that the difficulty in transfecting many cell lines and primary cells may be due significantly to their expressed A3 repertoire. Third, for the applied biomedical sciences, such as nonviral gene therapy, it is possible that endogenous A3 proteins undermine gene transfer efficiency and/or mutate therapeutically intended DNA. Fourth, the efficacy of DNA-based vaccines or adjuvants could be dramatically impacted by the foreign DNA restriction mechanism described here. Future studies defining the range of cell types and species in which this foreign DNA defense operates will undoubtedly serve to broaden these implications.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

M.D.S. worked together with R.S.H. on all aspects of these studies and did most of the experiments; M.B.B. and M.L. established the TK mutation assay and the *in vitro* DNA deamination assays, respectively; J.L. performed the virus infectivity experiments.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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ONLINE METHODS

DNA constructs. We obtained an A3A cDNA (NM_145699) from B. Cullen[22]. The construction of A3A-expressing derivatives and all other constructs is described in **Supplementary Methods**.

Cell culture. We maintained cell lines in 10% (v/v) FBS- and pen/strep-supplemented DMEM at 37 °C and 5% CO_2. Cells were transfected with TransIT-LT1 (Mirus Bio Corporation). Cell viability was monitored by propidium iodide staining and flow cytometry, and proliferation by Cell Titer 96 aqueous reagent (Promega).

HEK 293T clones stably expressing uracil DNA glycosylase were created by transfecting cells with pEF-UGI and screening puromycin resistant clones for UNG2 inhibition[40]. Clonal HEK 293 cell lines harboring a stably integrated TK-neo cassette were generated by transfecting cells with pCMV-SB-10 and pT2-TK-neo and screening G418-resistant clones for sensitivity to gancyclovir.

Primary cell experiments. We obtained blood from healthy donors (Memorial Blood Centers, Minneapolis, Minnesota, USA). We isolated PBMCs by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare Life Sciences). We enriched monocytes by negative selection of nonmonocytes using magnetic separation (MACS separation, Miltenyi Biotec) or centrifugation (RosetteSep, Stem Cell Technologies). $CD14^+$ cells were stained with CD14-FITC (Miltenyi Biotec). Some experiments included 2 ng ml^{-1} universal type 1 interferon (R&D Systems) or 3 μM CpG oligonucleotide.

Immunoblotting. We extracted cellular proteins with 25 mM HEPES (pH 7.4), 10% (v/v) glycerol, 150 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ and protease inhibitors and clarified by centrifugation (10 min at 20,800*g* at 4 °C). We fractionated 15 μg of protein by SDS-PAGE, transferred it to PVDF (Millipore), and probed it with an anti-A3A polyclonal antiserum, an anti-GFP monoclonal antibody (Clontech) or an anti-eEF1α monoclonal antibody (Upstate). We generated the anti-A3A polyclonal serum by immunizing a rabbit with a peptide corresponding to A3A residues 171–199 (CPFQPWDGLEEHSQALSGRLRAILQNQGN) mixed with TiterMax Gold adjuvant (Sigma). We detected Primary antibodies with fluorescently labeled secondary antibodies and imaging (LI-COR Biosciences).

Quantitative reverse-transcription PCR assays. We made cDNA from total RNA (Qiagen) by reverse transcription with random hexamers and AMV reverse transcriptase (Roche) and used the cDNA to template qPCR reactions (Lightcycler 480, Roche). We normalized data to the geometric mean of at least two of the following three reference genes: *TBP*, *RPL13A* or *HPRT*. **Supplementary Table 3** lists all primer and probe sequences. A detailed description of these quantitative assays, as applied to multiple cell types and tissues, will be reported elsewhere.

Gene transfer experiments. We plated 250,000 HEK 293 or HeLa cells into 6-well plates. After 24 h of incubation, we transfected the cells with pcDNA3.1 (Neo^R, Invitrogen) and pEF-A3A-GFP or pEF-$A3A_{E72A}$-GFP. Two days after transfection, we plated 100,000 cells into 10-cm dishes in medium containing 1 mg ml^{-1} G418 (Cellgro). We monitored colony-forming efficiency simultaneously by plating serial cell dilutions into drug-free medium. After 12–14 d of additional incubation, we fixed the colonies and stained them with crystal violet. We performed Sleeping Beauty DNA transposon-mediated gene-transfer assays as described[47].

Plasmid DNA qPCR assays. We transfected HEK 293 or HeLa cells as above and, at the indicated times after transfection, extracted total DNA (Qiagen). We used 50 ng total DNA as template for each qPCR reaction. We performed reactions on an iCycler instrument using SYBR Green I (BioRad). We normalized plasmid DNA levels to genomic DNA levels as measured by a qPCR assay specific for the β actin locus. Primers are listed in **Supplementary Table 3**.

3D-PCR and DNA sequencing assays to detect hyperediting in cell lines. We transfected HEK 293 cells, HEK 293T cells, or HEK 293T cells stably expressing UGI with reporter plasmids pTRE2-Δpuro-mCherry or pEGFP-N3, pcDNA3.1-UGI or pcDNA3.1 and pEF-A3A-GFP or pEF-$A3A_{E72A}$-GFP. Twenty-four hours after transfection, we treated cells with DNase I (Roche) to remove extracellular input plasmid. Twenty-four hours later, we extracted total DNA (Qiagen). We used 50 ng DNA as input to PCR using primers listed in **Supplementary Table 4** and Taq DNA polymerase (Roche), which amplifies uracil-containing DNA (25 cycles: 94 °C, 15 s; 50 °C, 30 s; and 68 °C, 2 min). We used 0.25 μl of this PCR as template for nested PCR with Phusion DNA polymerase (Finnzymes) and primers listed in **Supplementary Table 4** (25 cycles: T_d gradient as indicated; 52 °C, 30 s; and 72 °C, 15 s). PCR products were visualized on agarose gels.

Sequencing to detect edited DNA. We recovered PCR products from agarose gels (Qiagen). We directly sequenced 20 ng PCR product (Genewiz) or cloned and sequenced individual amplicons. The amplicon contained nucleotides 1170–1426 of pEGFP-N3 (Genbank U57609.1). We analyzed PCR product sequences using phred software version 0.071220.b (http://www.phrap.org[53]). During the course of these studies, we observed that the level of A3A-induced deamination was so high that primer design was difficult. For instance, because almost every DNA cytidine is a potential A3A substrate, an ideal PCR primer set must be devoid of guanosines. As this is not feasible, we elected to minimize the number of guanosines and, at unavoidable positions, to have the primers synthesized with R's (guanosine or adenosine). This approach enabled us to detect C/G-to-T/A transitions in both strands of plasmid substrates recovered from A3A-overexpressing HEK 293 cells or from A3A-induced (IFN or CpG-treated) CD14-positive monocytes (data not shown).

Primary cell transfection. We incubated monocytes for 20–24 h with 3 μM CpG DNA or else mock-treated them. We transfected 2×10^7 monocytes with 2 μg endotoxin-free pEGFP-N3 (Qiagen) using nucleofection (Amaxa). Three hours after transfection, we treated cells with DNase I to remove extracellular DNA. We harvested cells periodically for RNA and protein preparation. We monitored gene expression using qRT-PCR, and we analyzed recovered DNA as above.

***TK* selection experiments.** We used the frequency of cells converting from TK^+ to TK^- as a measure of genomic DNA mutation. Inactivating mutations of the integrated *TK* gene confer Gan^R. We transfected TK-neo clones, as above, with A3A or control expression plasmids. Two days later, we plated 1×10^6 cells into Gan-containing (5 μM) medium. We allowed Gan^R colonies to grow for 12–14 days, and then stained them with crystal violet.

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