

# Endogenous APOBEC3A DNA Cytosine Deaminase Is Cytoplasmic and Nongenotoxic\*

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**Background:** APOBEC3A is a potentially genotoxic DNA cytosine deaminase expressed in myeloid lineage cells.

**Results:** Exogenous APOBEC3A genotoxicity correlates with expression level and nuclear localization. Endogenous APOBEC3A is nontoxic and cytoplasmic, despite its capacity to be highly induced by interferon.

**Conclusion:** Cytoplasmic localization prevents endogenous APOBEC3A from accessing nuclear DNA.

**Significance:** Endogenous APOBEC3A is not genotoxic.

APOBEC3A (A3A) is a myeloid lineage-specific DNA cytosine deaminase with a role in innate immunity to foreign DNA. Previous studies have shown that heterologously expressed A3A is genotoxic, suggesting that monocytes may have a mechanism to regulate this enzyme. Indeed, we observed no significant cytotoxicity when interferon was used to induce the expression of endogenous A3A in CD14<sup>+</sup>-enriched primary cells or the monocytic cell line THP-1. In contrast, doxycycline-induced A3A in HEK293 cells caused major cytotoxicity at protein levels lower than those observed when CD14<sup>+</sup> cells were stimulated with interferon. Immunofluorescent microscopy of interferon-stimulated CD14<sup>+</sup> and THP-1 cells revealed that endogenous A3A is cytoplasmic, in stark contrast to stably or transiently transfected A3A, which has a cell-wide localization. A3A constructs engineered to be cytoplasmic are also nontoxic in HEK293 cells. These data combine to suggest that monocytic cells use a cytoplasmic retention mechanism to control A3A and avert genotoxicity during innate immune responses.

Myeloid lineage cells have critical roles in both innate and adaptive immune responses, including phagocytosis, antigen presentation, and cytokine production (reviewed in Ref. 1). Monocytes are produced by hematopoietic stem cells in the bone marrow and released into circulation. The monocytes migrate through the endothelium toward the tissues, where they differentiate into macrophages or dendritic cells. These professional phagocytic cells are stationed throughout the body to perform immune surveillance and thus come into contact with a variety of foreign molecules, including DNA.

APOBEC3A (A3A)<sup>5</sup> is part of the APOBEC3 family of DNA cytosine deaminases, which in humans is composed of seven members: A, B, C, D, F, G, and H. A3A is the most active of these deaminases (2), but its expression is limited to myeloid lineage cells and therefore likely has a specialized function in these cells (3–6). A3A is strongly induced by type I interferon (10–1000-fold) (4, 6–8) and is also induced by other immunostimulatory molecules such as cytosolic DNA (6). This expression profile is consistent with A3A functioning in myeloid lineage cells as part of a foreign DNA clearance mechanism (6). By deaminating DNA cytosines to uracils, A3A can trigger further DNA damage and subsequent clearance. A3A has also been observed to deaminate 5-methyl cytosines in single-stranded DNA (2, 9, 10). The physiological relevance of this additional biochemical activity is not known, but it may benefit the cell by allowing A3A to recognize a broader range of foreign DNA substrates.

A3A may also have a role in restricting the replication of several transposons and viruses. For instance, A3A has been shown to limit the retrotransposition of L1 and Alu elements (3, 11–15). A3A has also been implicated in inhibiting the replication of certain DNA viruses, including parvovirus (16), hepatitis B virus (17), and papillomavirus (18), as well as the retroviruses human T-lymphotropic virus type-I (19) and HIV (4, 7, 20, 21).

Beyond these restriction activities, A3A has been suggested to have a role in cancer, due to its genotoxic capacity (22, 23). Overexpressed A3A has been shown to cause chromosomal DNA damage and mutation (22–26) as well as disruption of the cell cycle (22, 26). Expression of A3A in a cell threatens genomic integrity, suggesting that uncontrolled activation of this immune defense molecule would have undesirable consequences.

Alternatively, we hypothesize that myeloid lineage cells, which inducibly express A3A following interferon stimulation, have a mechanism to control A3A activity. We describe the induction of A3A expression with interferon in primary CD14<sup>+</sup>

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<sup>5</sup> The abbreviations used are: A3A, APOBEC3A; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; hnRNP U, heterogeneous nuclear ribonucleoprotein U; TRITC, tetramethylrhodamine isothiocyanate; NES, nuclear export sequence; AID, activation-induced cytidine deaminase; eGFP, enhanced green fluorescent protein.

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monocytic cells and the monocytic cell line THP-1, with no evidence of decreased viability or DNA damage. Expression of A3A in HEK293 cells to lower levels than those observed in the CD14<sup>+</sup> cells, however, caused marked genotoxicity. We show that endogenous A3A in CD14<sup>+</sup> and THP-1 cells localized to the cytoplasm, in contrast to the cell-wide localization observed for exogenous A3A in a variety of heterologous cell types (3, 13, 14, 16, 21, 27–34). We further reveal that nuclear localization is necessary for A3A-mediated genotoxicity. We conclude that endogenous A3A is nongenotoxic, due to its retention in the cytoplasm. We suggest that endogenous A3A is unlikely to have a role in editing chromosomal DNA as its cytoplasmic localization would prohibit access to the nuclear DNA. This study supports a role for A3A in innate immunity by demonstrating that monocytic cells can safely express high levels of this potent DNA mutator.

### EXPERIMENTAL PROCEDURES

**Constructs**—pcDNA5TO-A3A-eGFP encodes A3A-GFP under the control of a CMV promoter that contains two tetracycline operator sites and has previously been reported (26). Standard PCR cloning and site-directed mutagenesis techniques were used to construct derivatives. pCI-neo-3×FLAG-TRB3 has been described (24).

**Cells**—T-Rex 293 cells (Invitrogen), which stably express the tetracycline repressor, were transfected with the pcDNA5TO-A3A-eGFP construct and derivatives using TransIT-LT1 (Mirus). Stable clones were selected with hygromycin and blasticidin. Basal repression and doxycycline-induced expression of A3A were confirmed by flow cytometry for GFP fluorescence and by immunoblotting. T-Rex 293 A3A and T-Rex A3A-E72A stable clones were further engineered to stably express 3×FLAG-TRB3 or empty vector by transfection with pCI-neo-3×FLAG-TRB3 or pCI-neo-3×FLAG and selection with G418.

Primary human CD14<sup>+</sup> monocytes were purified by negative selection with Rosette Sep human monocyte enrichment mixture (Stemcell Technologies) from fresh whole blood obtained from Memorial Blood Center (St. Paul, MN). Purity (>90%) was confirmed by flow cytometry for CD14<sup>+</sup> cells with CD14-FITC (Miltenyi Biotec).

THP-1 cells (35) were obtained from Dr. Andrea Cimarelli (Ecole Normale Supérieure de Lyon). A3A knockdown clones were obtained by transduction with pLKO-based lentiviral constructs (Open Biosystems) followed by puromycin resistance selection. Specific A3A knockdown was verified by immunoblotting and quantitative PCR.

Endogenous A3A was up-regulated by treating CD14<sup>+</sup> or THP-1 cells with IFN $\alpha$  (300 units/ml Universal Type I IFN $\alpha$ ; R&D Systems). RNA was isolated 6 h after induction; whole cell lysates for immunoblotting were harvested 24 h after induction.

**Immunoblotting**— $\gamma$ -H2AX was detected with polyclonal rabbit anti- $\gamma$ -H2AX (Bethyl Laboratories), H2AX was detected with polyclonal rabbit anti-H2AX (Bethyl Laboratories), tubulin was detected with monoclonal mouse anti- $\alpha$ -tubulin (Covance), HSP90 was detected with mouse anti-HSP90 (BD Biosciences), 3×FLAG-TRB3 was detected with monoclonal mouse

anti-FLAG M2 (Sigma), and A3A was detected with rabbit polyclonal anti-A3A, as described previously (2).

**mRNA Quantification**—A3A mRNA was quantified by quantitative RT-PCR relative to the stable housekeeping transcript, TBP, using highly specific primers, as described previously (5).

**MTS Viability Assay**—Doxycycline was diluted in PBS in 96-well plates before adding to cells in appropriate growth medium. After 0, 24, 48, or 72 h, the medium was removed and replaced with 20  $\mu$ l of MTS/phenazine methosulfate solution, prepared as described (Promega), and 100  $\mu$ l of fresh growth medium. Absorbance was measured 2 h later at 490 nm in a Victor3 multilabel plate reader (PerkinElmer Life Sciences). The absorbance of the blank wells was subtracted from the experimental values, and the data were plotted relative to no doxycycline growth conditions.

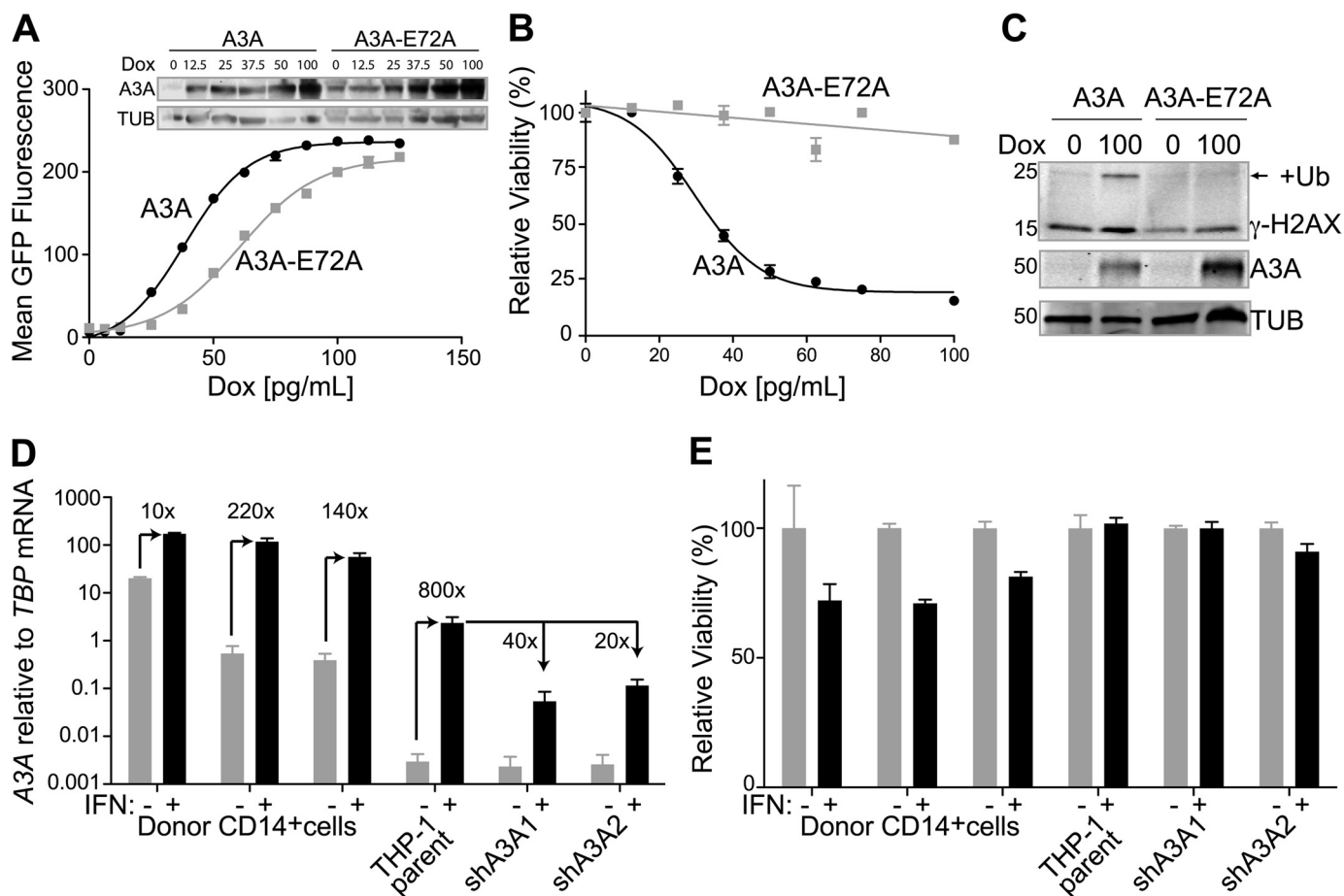
**Immunofluorescent Microscopy**—Endogenous A3A was up-regulated by treating cells with interferon (IFN) as described above. THP-1 cells were additionally treated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma) to promote adherence to the microscope slide. Cells were fixed with 4% paraformaldehyde 24 h after induction. A3A was detected with rabbit anti-A3A (described above) and goat anti-rabbit FITC (Jackson ImmunoResearch Laboratories). hnRNP U was detected with monoclonal mouse anti-hnRNP U (Santa Cruz Biotechnology) and goat anti-mouse FITC. Hoechst dye was used to visualize nuclei. Cells were imaged with a DeltaVision deconvolution microscope (Applied Precision).

T-Rex 293 A3A-GFP cells were induced with 100 pg/ml doxycycline for 24 h. Cells were fixed with 4% paraformaldehyde. 3×FLAG-TRB3 was detected with monoclonal mouse anti-FLAG M2 (Sigma) and donkey anti-mouse-TRITC.

**Transfection Studies**—T-Rex 293 A3A and A3A-E72A cells were transfected with 3×FLAG-TRB3 or empty vector with TransIT-2020 (Mirus). After 24 h, the cells were counted, and (a) plated into doxycycline dilutions for the MTS viability assay, (b) plated into doxycycline or PBS for immunoblot analysis, or (c) plated into doxycycline or PBS for immunofluorescence microscopy.

### RESULTS

**Overexpressed Heterologous A3A Is Genotoxic, whereas Up-regulated Physiologic A3A Is Safely Expressed**—A3A overexpression has been reported to be genotoxic (22, 23, 26). To characterize the extent of A3A toxicity, we employed a doxycycline-inducible system to regulate A3A expression levels. Stable clones were established by transfection of eGFP-tagged A3A (and catalytic mutant A3A-E72A) in pcDNA-5TO into the T-Rex 293 cell line. Doxycycline allowed repression to be relieved in a dose-dependent manner, as seen by increasing mean GFP fluorescence by flow cytometry and by increasing band intensity by immunoblotting (Fig. 1A). We assessed the toxicity of A3A as compared with A3A-E72A in T-Rex 293 cells by the MTS cellular viability assay, which measures the metabolism of a colorimetric substrate (Fig. 1B). Increasing expression of the catalytic mutant (A3A-E72A) did not result in any significant changes in viability, whereas viability strongly decreased with increasing wild-type A3A expression. A similar



**FIGURE 1. Exogenously overexpressed A3A is toxic, whereas physiologically up-regulated A3A is not.** *A*, mean GFP fluorescence with increasing doxycycline (Dox) concentrations for T-REx 293 doxycycline-inducible A3A-GFP and A3A-E72A-GFP cell lines. *Inset*: anti-A3A and anti-tubulin (TUB) immunoblots. *B*, MTS assay for cell viability following induction of A3A and A3A-E72A by increasing doxycycline concentrations. *C*, immunoblots for the indicated proteins in T-REx 293 A3A and A3A-E72A cell lines, incubated in the presence and absence of doxycycline induction. Ub, ubiquitin. *D*, A3A mRNA induction with type I interferon in primary CD14<sup>+</sup> cells from three donors, THP-1 cell line, and two stable A3A shRNA knockdown THP-1 clones, relative to the housekeeping gene *TBP*. High base-line level of A3A expression in primary cells (especially the first donor) may be due to preexisting immune activation. *E*, MTS assay for the cell types used in *panel D*. Error bars in *D* and *E* indicate S.D.

dose-dependent relationship was seen with untagged A3A (data not shown).

To determine the cause of the observed toxicity, we examined the cells for  $\gamma$ -H2AX, an indicator of DNA damage (36). We observed induction of  $\gamma$ -H2AX, particularly the monoubiquitinated form, when A3A expression was induced in the T-REx 293 cells with doxycycline (Fig. 1C). In contrast,  $\gamma$ -H2AX was not induced with increased expression of the catalytic mutant.

To determine whether endogenous A3A is similarly cytotoxic, we utilized the monocytic cell line THP-1, in which A3A has been described to be strongly up-regulated upon type I IFN treatment (2). For comparison, we generated stable A3A shRNA knockdown clones, which expressed 20–40-fold lower A3A upon IFN treatment (Fig. 1D). Analysis of cell viability by the MTS assay revealed no significant changes between the parental line and the A3A knockdown clones, in either the presence or the absence of IFN (Fig. 1E).

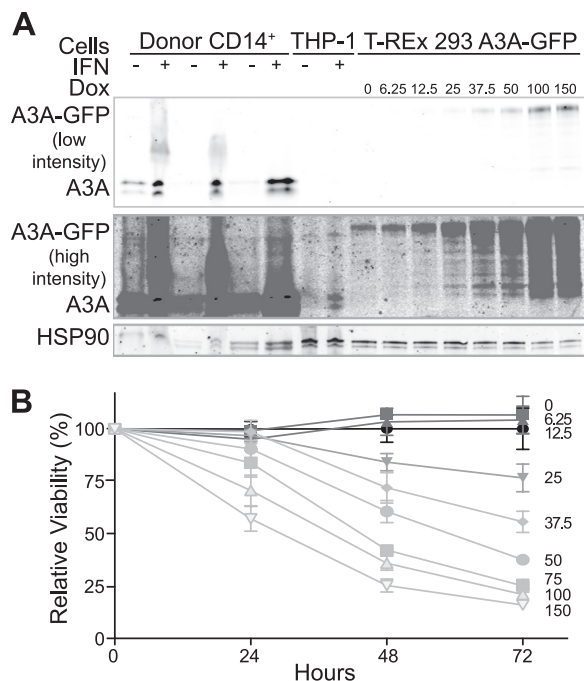
To extend these endogenous A3A results to primary cells, we performed similar experiments with primary CD14<sup>+</sup> cells purified from the whole blood of three donors. These cells have been shown to express the highest levels of A3A (3–6). IFN treatment caused a further induction of A3A expression, as

seen by increased mRNA (Fig. 1D) and protein levels (Fig. 2A). Cellular viability, as measured by the MTS assay, only changed modestly (Fig. 1E).

*Near Physiologic Levels of Overexpressed A3A Are Still Toxic*—To determine why endogenous A3A is not genotoxic, we performed a doxycycline titration on T-REx 293 cells inducibly expressing A3A and blotted for A3A with a recently developed rabbit anti-A3A polyclonal antibody, comparing doxycycline induced A3A with endogenously expressed A3A from THP-1 cells and primary monocytes (Fig. 2A). To our surprise, the induced level of A3A in THP-1 cells corresponded to a low level of doxycycline induction of A3A in the T-REx 293 cells (~6.25–12.5 pg/ml). In contrast, the induced level of A3A in CD14<sup>+</sup> cells was higher than the highest levels of A3A induction in the T-REx 293 cells. We repeated the MTS viability assay for our inducible cells and determined that at THP-1 physiologic levels of A3A, viability was constant, even after 72 h of induction (lower doxycycline concentrations in Fig. 2B). In contrast, at the highest levels of A3A induction in the T-REx 293 cells, which is still lower than what was observed in the CD14<sup>+</sup> cells, dramatic cytotoxicity was observed (higher doxycycline concentrations in Fig. 2B).



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**FIGURE 2. Heterologously induced A3A is toxic even at near physiologic levels.** *A*, immunoblot of endogenous A3A after interferon induction of primary CD14<sup>+</sup> cells and the THP-1 cell line, alongside the T-REx 293 A3A cell line treated with the indicated doxycycline (*Dox*) concentrations (pg/ml) for comparison. A low intensity LI-COR scan is shown to assess A3A levels in CD14<sup>+</sup> cells, whereas a higher intensity scan is shown to visualize the level of A3A in the induced THP-1 cells. HSP90 is a loading control. *B*, MTS viability assay of T-REx 293 cells expressing A3A through a range that spans the maximal interferon-induced levels in primary CD14<sup>+</sup> cells or the THP-1 line. Doxycycline concentration (pg/ml) is indicated. Error bars indicate S.D.

**Endogenous A3A Is Cytoplasmic**—To further explore the mechanism of protection from endogenous A3A, we investigated cellular localization using a rabbit anti-A3A polyclonal antibody (2). Surprisingly, in both CD14<sup>+</sup> and THP-1 cells, A3A was observed to be cytoplasmic upon IFN induction (Fig. 3, *A* and *B*). A3A levels in the absence of induction were markedly lower with a faint cytoplasmic localization still detectable (not shown). This is in contrast to the observed cell-wide localization of overexpressed A3A in a variety of nonmyeloid lineage cell lines (3, 13, 14, 16, 21, 27–34) (see Fig. 4*A* below). As a control for nuclear permeabilization and staining, hnRNP U was seen to localize to the nucleus of the same cells (Fig. 3, *C* and *D*).

**Cytoplasmic A3A Is Not Toxic**—We created a series of doxycycline-inducible A3A-eGFP localization mutants and stably cloned them into the T-REx 293 cells to test the effect of A3A localization on cytotoxicity. GFP alone showed cell-wide localization, as did wild-type A3A, as observed previously (3, 13, 14, 16, 21, 27–34) (Fig. 4*A*). Fusion of the nuclear export sequence (NES) of the related protein AID (AID positions 186–198) onto the C terminus of A3A caused the localization to become primarily cytoplasmic, presumably by enabling active CRM1-dependent export of the protein from the nucleus, as seen with AID (37–39). A single point mutation in the NES (AID position L198S) abolishes NES activity and reverts the protein localization to cell-wide (38). The addition of the SV40 nuclear localization sequence (NLS) (×3) to the N terminus of the A3A-GFP fusion protein targeted the construct to the nucleus, causing

the localization to become predominantly nuclear. In contrast, adding the large (116-kDa)  $\beta$ -galactosidase protein (encoded by *lacZ*) caused localization to become predominantly cytoplasmic, likely by making the construct too large to passively enter the nucleus. DiNoia and colleagues (40) used a similar strategy to block the active import of AID into the nuclear compartment.

We tested the effect of A3A localization on cell viability with the MTS assay. Constructs that could access the nucleus (either nuclear or cell-wide localization, *i.e.* A3A, A3A-NES-L198S and A3A-NLS) showed comparable, high levels of toxicity (Fig. 4*B*). In contrast, constructs that did not localize to the nucleus (A3A-NES and A3A-LacZ) showed little to no toxicity. As expected, the catalytic mutant, A3A-E72A, also showed no toxicity. These data demonstrate that nuclear localization is required for A3A to cause cytotoxicity and that this activity is completely dependent upon catalytic activity.

**TRB3 Does Not Decrease A3A Cytotoxicity**—TRB3 was recently implicated in negative regulation of A3A through direct binding (24). To determine whether TRB3 decreases A3A-mediated cellular toxicity in our experimental system, 3×FLAG-TRB3 was transfected into T-REx 293 A3A and A3A-E72A cells. Viability was assessed by the MTS assay over a range of doxycycline-induced A3A expression levels (Fig. 5*A*). As expected, TRB3 expression had no effect on the viability of cells expressing the catalytic mutant A3A-E72A. However, unexpectedly, TRB3 expression failed to attenuate the toxicity of wild-type A3A.

To determine whether TRB3 modulated the levels of A3A expression, we assayed protein levels by immunoblotting (Fig. 5*B*). Similar levels of A3A were expressed in the presence and absence of TRB3. The localization of A3A in the presence of TRB3 was assessed by immunofluorescent microscopy. As has been reported previously, TRB3 was observed in the nucleus of transfected cells (Fig. 5*C*) (24). A3A showed cell-wide localization in 293 cells, both in cells transfected with TRB3 and in adjacent nontransfected cells.

To confirm our findings, we also engineered T-REx 293 A3A and A3A-E72A cells to stably express 3×FLAG-TRB3. We again tested for modulation of A3A-mediated toxicity by TRB3 but observed no difference between TRB3-overexpressing clones and vector control-expressing clones (Fig. 6*A*), nor did TRB3 alter A3A protein levels (Fig. 6*B*). Thus, in our hands, TRB3 does not appear to be a negative regulator of A3A. We note that this negative result is consistent with the cytoplasmic localization of endogenous A3A described above.

## DISCUSSION

A3A is a powerful DNA cytosine deaminase that has been identified as an interferon-inducible restrictor of foreign DNA, including plasmid DNA (6), methyl-C-containing sequences (2, 9, 10), retrotransposons (3, 11–15), and several viruses (4, 7, 16–21). However, A3A has also been shown to be genotoxic under conditions of overexpression (22, 23, 26) and to mutate genomic DNA (24, 25). CD14<sup>+</sup> cells are known to express high levels of A3A upon type I IFN induction (4, 6, 7), suggesting that these cells must either have a mechanism for regulating A3A activity or be subject to its genotoxic effects.

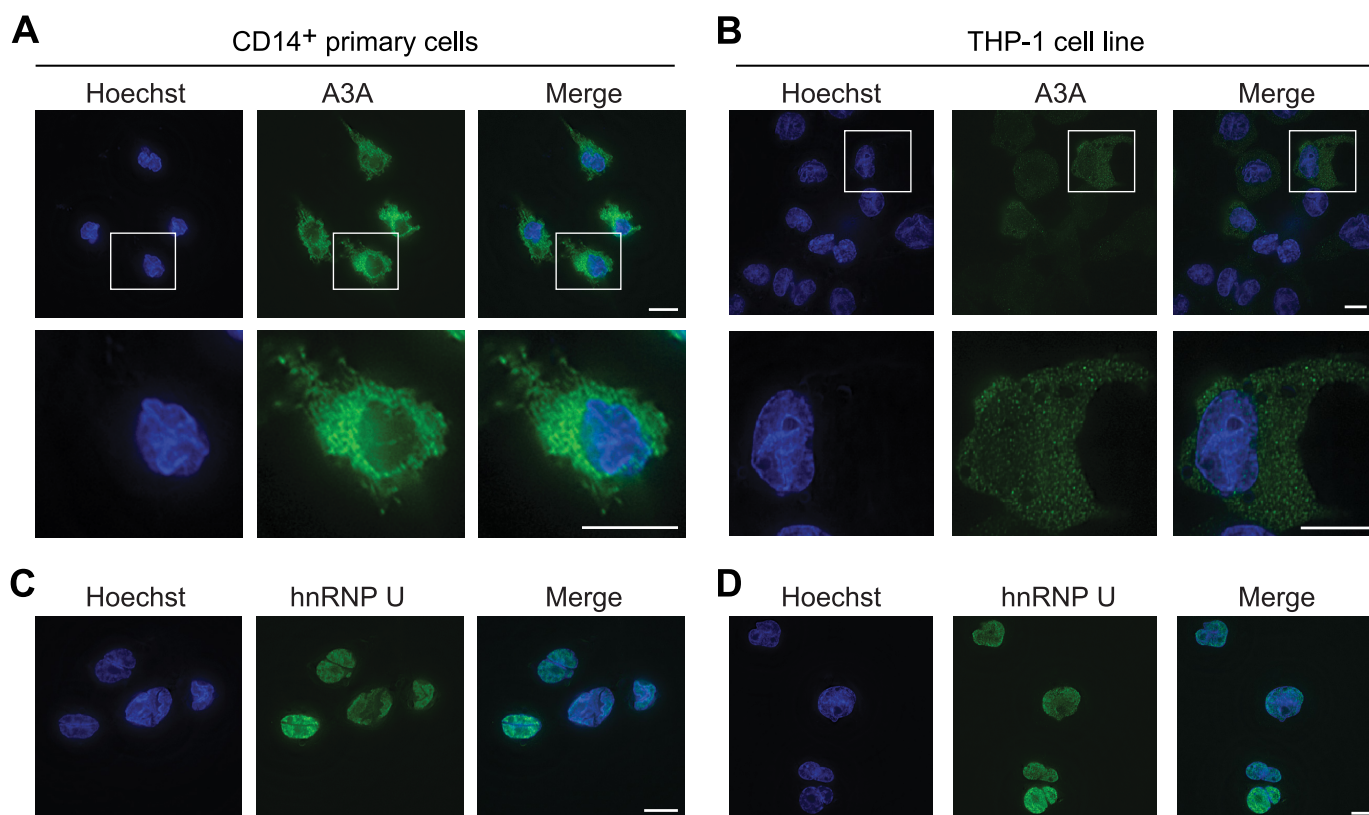


FIGURE 3. **Endogenous A3A is predominantly cytoplasmic.** *A* and *B*, representative immunofluorescent microscopy images of endogenous A3A in primary CD14<sup>+</sup> cells and THP-1 cells, respectively, following treatment with IFN. Individual cells are shown enlarged below. Nuclear DNA is stained blue with Hoechst for compartment identification. Cells were imaged at a 600 $\times$  magnification; the length of the white bar is 10  $\mu$ m. *C* and *D*, representative immunofluorescent microscopy images of hnRNP U as a control for nuclear permeabilization and staining procedures. Nuclear DNA is stained blue with Hoechst for compartment identification. Cells were imaged at a 600 $\times$  magnification; the length of the white bar is 10  $\mu$ m.

Genotoxicity was determined to be proportional to A3A expression level by using the doxycycline-inducible T-REx 293 system to titrate A3A expression. As A3A levels increased, cell viability decreased. The catalytic mutant, however, did not decrease viability, demonstrating that DNA cytosine deamination activity is responsible for toxicity. A3A expression also caused increased levels of the DNA damage marker  $\gamma$ -H2AX. Interestingly, the increase was most obvious for the monoubiquitinated form of  $\gamma$ -H2AX, which is an important mediator of the DNA damage response (41–43). In contrast, endogenous A3A was not toxic, despite the high levels of A3A induction by type I IFN seen in primary CD14<sup>+</sup> cells and the model monocytic cell line, THP-1.

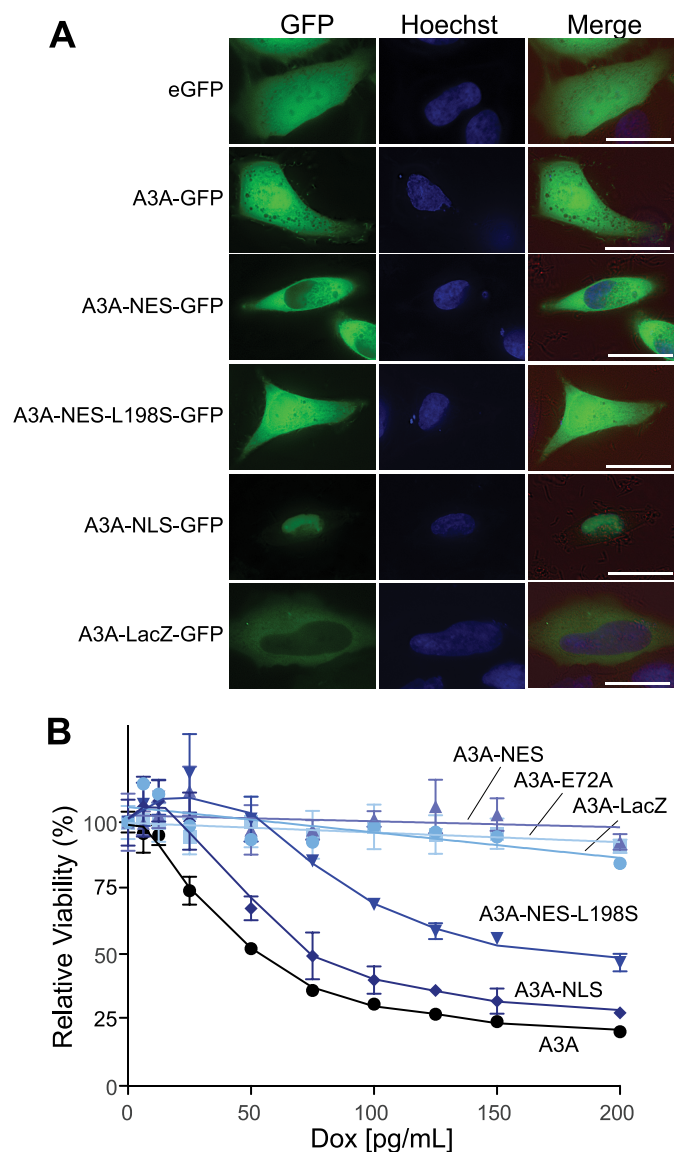
The physiological level of induction seen in THP-1 cells and primary CD14<sup>+</sup> cells was mimicked by inducing A3A with doxycycline in our T-REx 293 system to similar levels, as determined by immunoblot. A3A induction in the model monocytic cell line THP-1 corresponded to a low, sublethal level of A3A induction in our T-REx 293 cell line. In contrast, the level of A3A expression in primary CD14<sup>+</sup> cells was higher than the highest level of induction in our T-REx 293 cells, corresponding to highly toxic heterologous A3A levels. However, no evidence of cytotoxicity was observed in primary cells.

The likely reason for the lack of cytotoxicity became apparent when we determined the localization of endogenous A3A. Previous studies of A3A localization have shown that untagged protein in HeLa cells (27), GFP-tagged protein in HeLa (28–30)

and HEK293T cells (29), or HA-tagged protein in HeLa (13, 14, 31, 32), HEK293 (3), HEK293T (21, 33, 34), and U2OS cells (16) has a cell-wide localization. A3A was determined to enter the nucleus of HeLa cells by passive diffusion (29). Our group has recently developed antibodies that can detect endogenous A3A by immunofluorescence, enabling visualization of the endogenous protein. In contrast to the cell-wide localization of transiently or stably transfected A3A in nonphysiological cell types, endogenous A3A, both in THP-1 cells and in primary CD14<sup>+</sup> cells, showed cytoplasmic localization. Exclusion from the nucleus would presumably prevent A3A from accessing the cellular DNA and thus protect the DNA from A3A-induced damage. We tested this hypothesis by creating A3A localization mutants and indeed determined that only the constructs that could localize to the nucleus (*i.e.* nuclear or cell-wide localization) showed similar cellular toxicity to wild-type A3A. In contrast, both A3A tagged with a nuclear export sequence and A3A tagged with a large protein to prevent entry into the nucleus showed cytoplasmic localization, similar to endogenous A3A, and both had negligible effects on cell viability. This was evident even at the highest levels of protein expression.

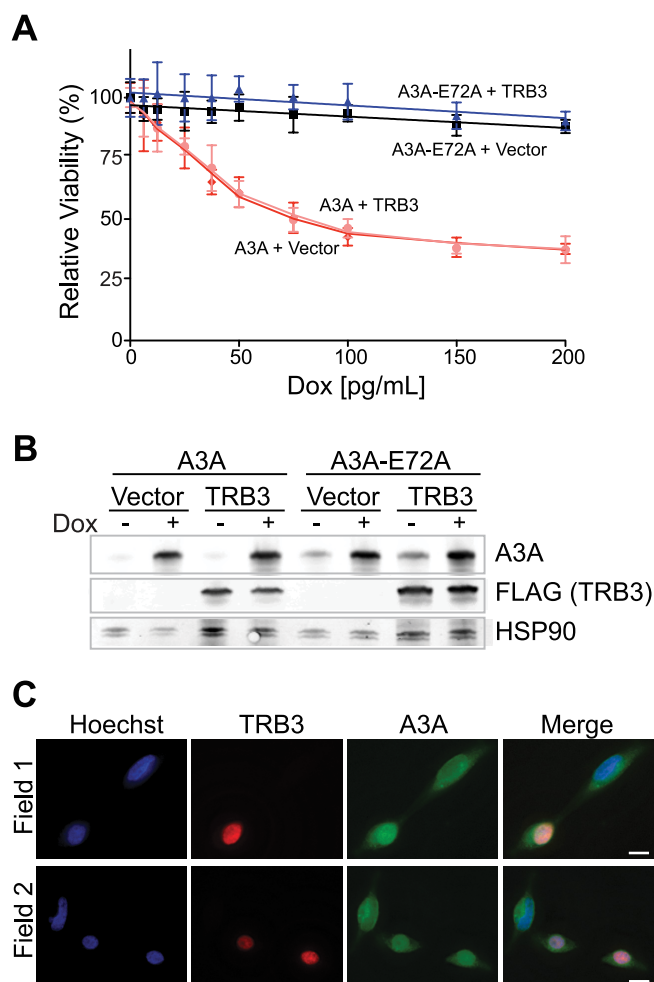
The other APOBEC3 proteins show a variety of steady-state localization patterns. A3B is observed to be nuclear in the majority of tested cell lines, including HeLa (14, 28, 29, 44, 45), U2OS (29), TR146 (29), JSQ3 (29), MCF10A (29), HuH-7 (46), HepG2 (46), MDA-MB-453 (26), MDA-MB-468 (26), and

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**FIGURE 4. Heterologously induced cytoplasmic A3A is not toxic.** *A*, representative live cell microscopy images of the indicated A3A-GFP constructs in HEK293 cells. Nuclear DNA is stained blue with Hoechst for compartment identification. Bright field view (red) is shown in the merge, with GFP and Hoechst. Cells were imaged at a 600 $\times$  magnification; the length of the white bar is 10  $\mu$ m. *B*, MTS assay for viability of T-Rex 293 cell lines expressing the indicated A3A-eGFP constructs by induction with increasing concentrations of doxycycline (Dox). Error bars indicate S.D.

HCC1569 (26), whereas it is cell-wide in 293T (29, 33) cells, which are believed to have an import defect (29). A3C is considered cell-wide in both HeLa (14, 28, 30) and 293T cells (31, 33, 34). A3D, A3F, and A3G are cytoplasmic in HeLa (14, 28, 30, 44, 45, 48–51) and 293T (3, 31, 33, 34, 48, 52) cells, with A3G additionally shown to be cytoplasmic in MDA-MB-453 (26), MDA-MB-468 (26), HCC1569 (26), HuH-7 (46), CEM-SS (51), H9 (48, 51), primary peripheral blood mononuclear cells (51), and primary T cells (48). The localization of A3H, interestingly, appears haplotype-dependent, with haplotype I being cell-wide in 293T (31) and HeLa (14, 30) cells and haplotype II being cytoplasmic in 293T and HeLa cells (28, 53). Indeed, Li and Emerman (53) suggest that the more active form of A3H, haplotype II, is actively retained in the cytoplasm.

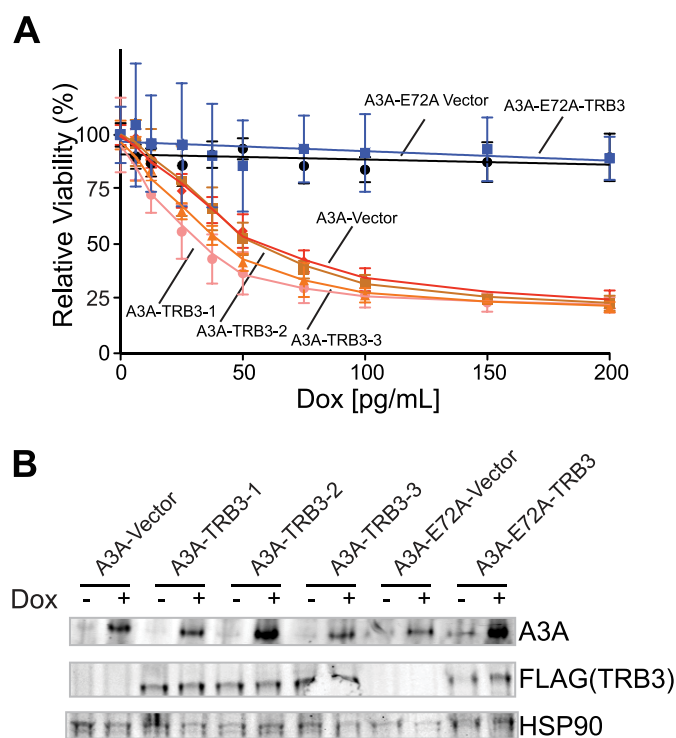


**FIGURE 5. Transient overexpression of TRB3 does not affect A3A toxicity or localization.** *A*, MTS assay for viability of T-Rex 293 A3A and A3A-E72A cell lines transiently transfected with 3 $\times$ FLAG-TRB3 or empty vector. Error bars indicate S.D. *B*, immunoblots of A3A (anti-A3A) and TRB3 (anti-FLAG) from the cells in panel *A* treated with 0 and 100 pg/ml doxycycline (Dox). HSP90 is a loading control. *C*, representative immunofluorescent microscopy images of T-Rex 293 cells induced with doxycycline for A3A and transfected with 3 $\times$ FLAG-TRB3, alongside non-TRB3 transfected cells. Nuclear DNA is stained blue with Hoechst. Cells were imaged at a 600 $\times$  magnification; the length of the white bar is 10  $\mu$ m.

A recent study reported that TRB3 is a negative regulator of A3A (24). However, in our inducible system, neither transient nor stable TRB3 overexpression had an effect on A3A-mediated cellular toxicity. Furthermore, TRB3 did not divert A3A localization from the nucleus, which, based on the evidence presented in this study, might have been a reasonable function for this putative negative regulator. Thus, because endogenous A3A is cytoplasmic (this study) and TRB3 is nuclear (Refs. 24, 54, and 55 and this study), we favor a model in which the inferred mechanism responsible for the cytoplasmic retention of A3A is TRB3-independent and remains to be discovered.

We conclude that cells that endogenously express A3A, *i.e.* CD14<sup>+</sup> monocytic cells, are able to control its activity and protect their DNA from this powerful DNA cytosine deaminase by a cytoplasmic retention mechanism. This mechanism may be fundamentally distinct from that previously inferred for A3G as even heterologously expressed full-length A3G and the N-terminal half of A3G are cytoplasmic with the latter being of sim-





**FIGURE 6. Stable expression of TRB3 does not affect A3A toxicity or localization.** A, MTS assay for viability of three T-REx 293 A3A TRB3 stable clones as compared with vector stable clone and T-REx 293 A3A-E72A TRB3 and vector stable clones. Dox, doxycycline. Error bars indicate S.D. B, immunoblots of A3A (anti-A3A) and TRB3 (anti-FLAG) from the cells in panel A treated with 0 and 100 pg/ml doxycycline. HSP90 is a loading control.

ilar size to A3A (45, 47, 52). The mechanism may also be distinct from that of A3H haplotype II as A3A is cell-wide in HeLa and 293T cells, in contrast to A3H-hapII (53), suggesting that in these cells, the A3A cytoplasmic retention mechanism may be either lacking or defective. Cellular DNA is protected from A3A largely, and perhaps exclusively, based on the localization of A3A to the cytoplasm, as the cytoplasmic localization of exogenous A3A in the T-REx 293 system was sufficient to prevent cytotoxicity. Thus, CD14<sup>+</sup> monocytic cells are able to safely express high levels of A3A and mediate innate immune functions such as foreign DNA clearance. Further work will identify the cellular machinery responsible for cytoplasmic localization and A3A regulation and determine how this regulation influences the innate immune activity of A3A. Together, the restricted expression profile of A3A in myeloid lineage cell types and the cytoplasmic localization of endogenous A3A in these cell types combine to strongly indicate that this protein is not normally genotoxic or a major factor in carcinogenesis.

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