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Self-cytoplasmic DNA upregulates the mutator enzyme APOBEC3A leading to chromosomal DNA damage

Rodolphe Suspène1, Bianka Mussil1,2, Hélène Laude1, Vincent Cavali1, Noémie Berry1, Mohamed S. Bouzidi1, Valérie Thiers1, Simon Wain-Hobson1 and Jean-Pierre Vartanian1,*

1Molecular Retrovirology Unit, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris cedex 15, France and 2Unit of Infection Models, German Primate Centre, Kellnerweg 4, 37077 Goettingen, Germany

ABSTRACT

Foreign and self-cytoplasmic DNA are recognized by numerous DNA sensor molecules leading to the production of type I interferons. Such DNA agonists should be degraded otherwise cells would be chronically stressed. Most human APOBEC3 cytidine deaminases can initiate catabolism of cytoplasmic mitochondrial DNA. Using the human myeloid cell line THP-1 with an interferon inducible APOBEC3A gene, we show that cytoplasmic DNA triggers interferon α and β production through the RNA polymerase III transcription/RIG-I pathway leading to massive upregulation of APOBEC3A. By catalyzing C→U editing in single stranded DNA fragments, the enzyme prevents them from re-annealing so attenuating the danger signal. The price to pay is chromosomal DNA damage in the form of CG→TA mutations and double stranded DNA breaks which, in the context of chronic inflammation, could drive cells down the path toward cancer.

INTRODUCTION

The APOBEC3 (A3) locus consists of seven genes (A3A-A3C, A3DE, A3F-A3H) (A3) on chromosome 22 that encode DNA cytidine deaminases (1). These enzymes preferentially deaminate single strand DNA (ssDNA), leading to a huge number of CG→TA transitions especially in the context of 5′TpC and 5′CpC dinucleotides (2–5). The cellular location of the A3A enzyme is both cytoplasmic and nuclear while A3B is exclusively nuclear (6,7). Despite this, A3A is the more active of the two enzymes (6,8,9). Their cellular location of the A3A enzyme is both cytoplasmic and nuclear while A3B is exclusively nuclear (6,7). Despite this, A3A is the more active of the two enzymes (6,8,9). Their

Epidemiological studies have shown that a 29.5 kb A3B deletion polymorphism, whereby all but the last exon of A3B is deleted, is correlated with a higher odds ratio of developing breast, ovarian and HBV-associated liver cancer (13,14). Breast cancer genomes from ΔA3B−/− patients harbor a higher mutation burden (15) while the chimeric A3A transcript resulting from the A3B deletion leads to greater intracellular steady state levels of A3A (6). In so doing, these studies indicate that A3A alone can generate human cancers, and show that the ΔA3B lesion is a causal cancer susceptibility marker (6,15).

Cancer emerges on a background of chronic inflammation (16), HBV, HCV, HPV, KSHV, HTLV-1, Merkel cell polyomavirus or Helicobacter pylori being well-known etiological agents. TLR9 recognizes CpG DNA resulting in IFNα (Interferon) production in plasmacytoid dendritic cells (17). Intracellular DNA can be captured by a myriad of DNA sensor molecules leading to the triggering of potent innate immune responses (18). The cytosolic DNA binding protein LRRFIP1 interacts with β-catenin and promotes its activation by phosphorylation (19). After binding to the C-terminal domain of the transcription factor IRF-3, IFNβ production is initiated (19). Recently, it was shown that the intracellular sensor cGAS can detect cytoplasmic DNA and induce type I IFN responses (20). Equally, AT-rich DNA can trigger type I IFN responses via intracytoplasmic transcription of double stranded DNA (dsDNA) by RNA polymerase III to form dsRNA intermediates that are sensed by RIG-I (21,22). Interestingly, these different pathways have in common STING (STimulator of INterferon Gene) that was shown to be pivotal for the production of type I IFNs (23). STING itself interacts with both MAVS and RIG-I, themselves crucial to interferon signaling (24).

As mitochondrial DNA fragments (mtDNA) resemble bacterial DNA in possessing unmethylated CpG motifs, they too can trigger cytoplasmic DNA sensor molecules leading to inflammatory responses (25). Mitochondrial...
DNA can induce TLR9-mediated inflammatory responses in cardiomyocytes and is even capable of inducing myocarditis (26). Cellular disruption and necrosis can release mtDNA into the circulation causing systemic inflammation (27), while extracellular DNA can be taken up into endosomes and sensed by specific TLRs (17).

Despite the wealth of knowledge concerning cytoplasmic DNA signaling, there is much less data on the fate of the DNA agonist, for the danger signal has to be countered, otherwise cells would be chronically stressed. This is suggested by the fact that germ line mutations in some endo- and exonuclease genes result in symptoms resembling chronic inflammation (28,29). Cytoplasmic mitochondrial DNA is deaminated by any of the six functional APOBEC3 enzymes. A3A and A3G are of special note as they are up-regulated by IFNα (4,30–32), by contrast, A3B is not. As the highly efficient enzyme uracil N-glycosylase and abasic pyrimidine/purine endonucleases mobilize around A3A edited DNA, the three enzymes effectively function as a cytidine specific endonuclease reducing ssDNA to very small fragments.

It is shown here for the human monocytic cell line THP-1, that transfected dsDNA is sensed and transcribed by RNA polymerase III leading to dsRNA intermediates that were captured by RIG-I ultimately leading to type I interferon induction, particularly A3A which resulted in deamination of cGAS and catabolism of the transfected DNA agonist. The data highlight the A3 cytidine deaminases as anti-inflammatory agents. The sting in the tail is A3A mediated chromosomal DNA damage.

**MATERIALS AND METHODS**

Reagents
dUTP was from Sigma, RNA polymerase III inhibitor (ML-60218) was from Merck Millipore, dNTP (Fermentas), type I IFNα were from PBL Biomedical Laboratories, Cpg (Invitrogen), polyIC and peptidoglycan (from Staphylococcus aureus, InvivoGen), Taq Polymerase (BIOTAQ DNA polymerase, Bioline), Pfu (Agilent Technologies), cGAS siRNA (sc-95512), pol III RPC39 siRNA (sc-36292), pol III RPC62 siRNA (sc-76188) were from Santa Cruz Biotechnology, STING (antibody #3337), phospho-IRF-3 (Ser396) (4D4, rabbit mAb #4947), IRF-3 (D64C, XP rabbit mAb #11904), β-catenin antibody (amino-terminal antigen, #9581), mouse anti-rabbit IgG (conformation specific, L27A9, mAb #3678), anti-rabbit IgG, HRP-linked (antibody #7074), cGAS (D1D3G, mAb#15102) antibodies were from Cell Signaling Technology. APOBEC3A antibody (SAB4500755) was from Sigma Aldrich. RIG-I (E-5, sc-376882), pol III RPC39 (C 39-2, sc-23913), pol III RPC62 (I-18, sc-69534) antibodies were from Santa Cruz Biotechnology, Inc. Anti-mouse IgG, (HRP-linked antibody #NA931V) was from GE Healthcare. Monoclonal anti-β-actin—peroxidase (antibody #A3854) was from Sigma Aldrich. Human IFNα (IgA, mba-hifnα-3), hIFNβ (IgG, mba-hifnβ-3), hIFNγ (IgA, mba-hifnγ-3) and Mouse IgG2a (mbg2a-ctrlm) antibodies used as control were from InVivogen.

**Cell culture and transfection**

THP-1 cells (ATCC® TIB-202™) were maintained in RPMI (Eurobio), supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Cells were grown in 75 cm² cell culture flasks at 37°C in a humidified atmosphere containing 5% CO₂. For transfection 1.5 x 10⁶ THP-1 cells were seeded in 12-well tissue culture plates and incubated for 24 h. DNA transfections were performed using jetPRIME (Polyplus transfection reagent). At 24 h post-transfection, supernatants were harvested and IFNα (VeriKine™ Human IFN Alpha Multi-subtype ELISA Kit) and IFNβ (VeriKine™ Human IFN Beta Multi-subtype ELISA Kit) were quantified and analyzed (PBL Assay Science). All DNAs were extracted using the MasterPure Complete DNA and RNA purification kit (Epicentre Biotechnologies).

2 x10⁶ THP-1 cells were plated in 6-well tissue culture plate in 1 ml antibiotic-free growth medium supplemented with fetal bovine serum (FBS). Cells were transfected according to standard protocol (Santa Cruz Biotechnology) with siRNA specific for RNA pol III (sc-76188) and RNA pol II (sc-36292), for cGAS (sc-95512) and control siRNA (sc-37007), using siRNA transfection reagent (sc-29528) and siRNA transfection medium (sc-36868). Six hours post-transfection with siRNA, cells were transfected with 500 ng of MT-COI DNA using jetPRIME. At 24 h, RNA was extracted, cDNA synthesized and A3A expression quantified by real time polymerase chain reaction (PCR). Data were normalized to the expression levels of the housekeeping reference gene RPL13A.

Approximately 1.5 x 10⁶ THP-1 cells were transfected using jetPRIME (Polyplus transfection reagent) with 500 ng of MT-COI DNA in quintuplicate. At 24 h post-transfection supernatants were grouped together and clarified by centrifugation. One ml of supernatant was incubated with 2 μg or 10 μg/ml of antibody against IFNα, or IFNβ, or IFNγ for 1 h and added to 1.5 x 10⁶ fresh THP-1 cells. At 24 h DNA was extracted, cDNA synthesized and A3A expression quantified by real time PCR. Data were normalized to the expression levels of the housekeeping reference gene RPL13A.

**FACS analysis for DNA double stranded breaks**

Twenty-four hours post-transfection, THP-1 cells were washed with PBS, fixed in 2% ice-cold paraformaldehyde (Electron Microscopy Sciences) for 15 min and permeabilized in 90% ice-cold methanol (Sigma) for 30 min. After two washes with PBS, cells were incubated for 1 h with 1:100 diluted Alexa Fluor 488-conjugated mouse monoclonal anti-γH2AX (N1-431) antibody (BD Pharmingen). All incubation steps were performed on ice. Cells were analyzed on MACSQuant Analyzer (Miltenyi Biotec) using
the MACSQuantify™ Software (Miltenyi Biotec) or FlowJo software (Tree Star, Inc., version 8.7.1). For each sample 10,000 cells were counted.

**PCR/3DPCR**

For amplification of human MT-CYB, the first-round reaction parameters were 95°C for 5 min, followed by 40 cycles (95°C for 30 s, 60°C for 30 s and 72°C for 2 min) and finally 10 min at 72°C. Second round 3DPCR were performed using the equivalent of 1 μL of the first round reaction as input. For second-round 3DPCR, the reaction parameters were 75–85°C for 5 min, followed by 40 cycles (75–85°C for 30 s, 60°C for 30 s and 72°C for 2 min) and finally 10 min at 72°C. All amplifications were carried out using 2.5 U Taq (Bioline) DNA polymerase per reaction. MT-COI DNA (511 bp) and inMT-COI DNA (247 bp) corresponding to the mitochondrial cytochrome oxidase gene were amplified. PCR conditions and primers were described before (4,33). HIV-1 V1V2 region amplification was already described (34). Upon migration on agarose gel in presence of protease inhibitor (Roche), Gels used were 4–12% NuPAGE 4–12% Bis-Tris Gel (Invitrogen). Western blot analysis was carried out according to standard procedures by using a mouse or rabbit monoclonal specific antibodies. The immunoassay used a membrane made of nitrocellulose (GE Healthcare Life science). After incubation with an anti-mouse IgG horseradish peroxidase-coupled secondary antibody (Amersham), the membrane was subjected to detection by enhanced chemiluminescence (Pierce). β-Actin was used as a loading control using 1/25000 diluted mouse monoclonal antibody specific for β-actin (Sigma Aldrich).

**RNA protein co-immunoprecipitation and RT-PCR**

Eight hours post-transfection of 500 ng of HIV-1 V1V2 DNA, 1.5 × 10⁶ THP-1 transfected cells were incubated with 37% formaldehyde (to a final concentration of 1%) for 15 min at room temperature under gentle shaking. Cross-linking was stopped by adding 2 M glycine to a final concentration of 0.2 M for 5 min at room temperature. Cells were then centrifuged at 1800 g for 5 min at 4°C and lysed in cell lysis buffer (#9803, Cell Signaling) containing 40 U/ml RNasin on ice for 5 min. Lysates were then sonicated on ice three times for 5 s each. After centrifugation at 14,000 g for 10 min at 4°C, supernatants were transferred to new tubes and incubated for 1 h at 4°C with 30 μl of 50% protein A agarose bead (#9863, Cell Signaling) under rotation. After 10 min centrifugation at 14,000 g and 4°C, supernatants were transferred to fresh tubes and incubated with 20 μl of anti-RIG-I antibody (200 μg/ml) under gentle shaking overnight at 4°C. Thirty microliters of protein A agarose beads were then added and incubated for 30 min at 4°C. After centrifugation at 14,000 g for 10 min at 4°C, pellets were washed five times with 500 μl of 1× cell lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 14 mM beta-glycerolphosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin) (Cell Signaling, #9803) containing 40 U/ml RNasin. Pellets were resuspended with 50 μl of water and heated to 95°C for 2 min. After centrifugation for 10 min at 4°C, 14,000 g RT-PCR was performed on the supernatant.

**RESULTS**

Transfected MT-COI DNA upregulates type I interferon and leads to A3A expression

THP-1 is an IFNα-inducible human acute monocytic leukemia cell line that is one of the very few where all A3 genes are either expressed or can be induced, notably A3A. THP-1 cells were transfected by DNA amplified by PCR, corresponding to the mitochondrial cytochrome oxidase gene (MT-COI, 511 bp). Supernatants were clarified 24 h post-transfection and IFNα and IFNβ production measured by ELISA. As can be seen in Figure 1A, 500 ng generated strong IFNα and β responses compared to the jetPRIME control. THP-1 cells incubated with 500 ng of MT-COI DNA (MT-COIP), but not transfected, failed to induce IFNα or IFNβ, indicating that intracellular DNA constitutes the trigger. As THP-1 cells are insensitive to unmethylated CpG and polyIC, IFN induction is not via TLR3 and TLR9 pathways (Figure 1A) (36). A transcription study of APOBEC3 genes showed that MT-COI DNA increased A3A, A3F, A3G and A3H expression at 24 h in a dose-dependent manner, even at concentrations that did not induce detectable IFNα or IFNβ levels in culture supernatants (Figure 1B). A3A was by far the most sensitive to transfected DNA being upregulated by almost four orders of magnitude and confirmed by western blot (Figure 1C) showing the presence of the two A3A isoforms (p1 and p2) (37,38). By contrast, A3F, A3G and A3H were upregulated at most 10-fold (Figure 1B).
Figure 1. Transfected MT-COI DNA upregulates type I interferon and A3A expression. (A) Interferon α and β production following transfection of THP-1 cells by dT containing PCR DNA fragments. MT-COIP indicates incubation with DNA but no transfection. (B) APOBEC3 transcription profiling of transfected THP-1 cells. Data were normalized to the expression levels of RPL13A housekeeping reference gene. Profiling was performed in duplicate and normalized to JetPRIME to facilitate comparison. (C) Western blot of A3A isoforms p1 and p2 from transfected THP-1 and compared to β-actin. Lanes 2, 3 and lanes 4, 5 represent duplicates. (D) Agarose gel of MT-COI PCR products (511 bp) amplified with Taq or Pfu polymerase in the presence of dTTP, dUTP, dTTP+dUTP. M, molecular weight markers. (E) Interferon α and β production following transfection of THP-1 cells by dU or 50:50 mixture dT+dU containing PCR DNA fragments. (F and G) Transcriptomes were established for DNA containing dU (F) or a 50:50 mixture of dT and dU (G). Duplicates were normalized to the expression levels of RPL13A housekeeping gene and normalized to JetPRIME to facilitate comparison.

Uracil in cytosolic DNA is not a danger signal

Given that hyperedited cytoplasmic mitochondrial DNA (cymtDNA) bears non-canonical uridine residues, they might constitute a novel danger signal. To explore this hypothesis, MT-COI DNA was amplified using either dUTP, an equimolar mix of dTTP and dUTP or just dTTP. To control for the incorporation of dUTP in the DNA, a second round of PCR was performed with internal primers and Pfu or Taq DNA polymerase. Like all archaeal DNA polymerases, Pfu is unable to amplify DNA templates bearing dU (39). As expected Pfu PCR failed to recover DNA when dUTP (MT-COIU) or mixed nucleotides dUTP+dTTP (MT-COIUT) were incorporated, in contrast to Taq PCR (Figure 1D). When transfected into THP-1 cells both MT-COIU and MT-COIUT DNA resulted in induction of IFNα and IFNβ in culture supernatants (Figure 1E) and upregulation of the A3A, A3F, A3G and A3H genes (Figure 1F and G), although, once again, A3A was upregulated by approximately four orders of magnitude. These data indicate that uracil in DNA does not constitute a novel danger signal.

Interferon β exerts a paracrine effect on APOBEC3A expression

In order to demonstrate that upregulation of A3A by several logs (Figure 1B, F and G) could be amplified by paracrine IFNα/β induction via IFNAR (IFNα/β receptor), THP-1 cells were transfected with 500 ng of MT-COIUT DNA. At 24 h post-transfection after clarification, cell supernatant was incubated with 2 or 10 µg/ml of antibody against IFNα or IFNβ or IFNγ and added to 1.5 × 10⁶ fresh THP-1 cells for 24 h. Interestingly, we observed that relative A3A expression was reduced in a dose-dependent manner by one–two orders of magnitude when the supernatant was incubated with anti-IFNβ antibodies (Figure 2A). This experiment suggests that A3A expression could be amplified by the paracrine effect of IFNβ.
Figure 2. Paracrine effect of interferon β and editing of endogenous MT-CYB. (A) A3A relative expression was amplified by paracrine IFNβ induction in THP-1 cells and analyzed with only JetPRIME or transfected with 500 ng of MT-COI DNA. Cell supernatant (SN-MT-COI) was incubated with 2 or 10 μg of antibody against IFNα, IFNβ or IFNγ for 1 h and added to 1.5 × 10^6 fresh THP-1 cells for 24 h. ND, not detectable. (B) THP-1 DNA transfection increases A3-editing of endogenous cytoplasmic MT-CYB DNA recovered by 3DPCR. The white line indicates the threshold between edited and unedited 3DPCR products. Asterisks refer to the samples cloned and sequenced. M: molecular weight markers. (C) Frequency distribution of C→T editing per clone as a function of the quantity of DNA transfected.

We have previously shown that transfected DNA amplified by PCR can be deaminated by endogenous A3 enzymes (34). In order to see if the induction of A3 genes by exogenous DNA could result in editing of endogenous cytDNA, THP-1 cells were transfected with MT-COI DNA and analyzed at 24 h. Cytochrome b mtDNA (MT-CYB) was amplified by 3DPCR as previously described (4). 3DPCR recovered DNA at denaturation temperatures lower than for the jetPRIME control, indicative of increased A3 editing (Figure 2B). 3DPCR products recovered at 81.7°C were cloned and sequenced. The sequences were peppered by C→T substitutions, the mean editing frequency per sequence increasing with the quantity of transfected DNA (Figure 2C).

Cytosolic dsDNA induces APOBEC3A leading to double-stranded DNA breaks

In order to distinguish whether ssDNA or dsDNA was the IFN agonist, four pairs of complementary oligonucleotides corresponding to a small region of MT-COI were synthesized, WT1+2 (43 bp, 51% GC) and WT3+4 (39 bp, 56% GC) while oligos Hyp1+2 (43 bp, 7% GC) and Hyp3+4 (39 bp, 13% GC) correspond to hypermutated forms of WT1+2 and WT3+4 respectively (Supplementary Table S1). THP-1 cells were transfected with DNA duplexes and IFNα induction measured (Figure 3A). WT1+2 and WT3+4 (dsDNA) were potent inducers of IFNα, and on a par with the 511 bp MT-COI DNA fragment, whereas the mismatched pairs of oligos, WT1+3 or WT2+4, effectively ssDNA, failed to induce IFNα (Figure 3A). As the complementary oligonucleotides Hyp1+2 and Hyp3+4 did not induce IFN expression, it is probable that they did not form stable heteroduplexes at 37°C given their low GC content, only 7–13%.
MT-COI DNA transfection of THP-1 cells led to significantly increased DSBs (Figure 3B) as expected from A3A upregulation (Figure 1B). To focus on the transfected cells alone, THP-1 cells were transfected with pairs of oligonucleotides one being labeled with the Fluorescein (FAM)-fluorophore i.e. WT1+2FAM (dsDNA) or WT3+2FAM (ssDNA). Twenty-four hours post-transfection, addition of FAM to the DNA oligos did not induce DSBs (WT3+2 versus WT3+2FAM and WT1+2 versus WT1+2FAM, Figure 3C). However, as FAM-fluorescence decreased over time, FAM+ positive cells were sorted 6 h post-transfection, cultured for a further 18 h and fixed for DSB FACS analysis. WT1+2FAM led to ~40% of γH2AX positive THP-1 cells (Figure 3D) which was correlated with A3A upregulation (Figure 3E).

Cytosolic dsDNA is transcribed by RNA polymerase III impacting RIG-I

Which of the cytosolic dsDNA sensing pathway is upstream of IFN induction? At 24 h post-transfection of THP-1 cells by MT-COI DNA, western blot analysis showed increased levels of the phosphorylated forms of TBK1 (TBK1-P) and IRF3 (IRF3-P) as well as DNA dependent increases in RIG-I and MDA5 (Figure 4A). Steady state levels of STING, IKKe, MAVS were unchanged. Dose dependent increases of TBK1-P and IRF3-P, key regulators of IFN production, suggest signaling via STING and/or MAVS. As the RNA polymerase III and cGAS DNA sensor molecules are upstream of STING we explored these possibilities.

As RNA polymerase III has been implicated in the transcription of cytosolic dsDNA (21,22), THP-1 cells were transfected with MT-COI or MT-COIU DNA along with ML-60218, an inhibitor of RNA polymerase III (40) (Figure 4B). There was an inverse correlation between IFN production and inhibitor concentration. To exclude drug toxicity, 20 µg/ml of peptidoglycans (PGN) known to stimulate IFNα via TLR2, was used with ML-60218. IFN production was insensitive to all inhibitor concentrations used (Figure 4B). The inhibitor abolished transfected MT-COI.
DNA induced A3 mediated hyperediting of endogenous cytoplasmic MT-CYB DNA (Figure 4C versus Figure 2B), again something that treatment with PGN and ML-60218 alone or in combination failed to do (Figure 4C).

To correlate RNA polymerase III activity with A3A expression, 500 ng of V1V2 DNA were transfected in THP1 cells. Two hours post-transfection, cells were incubated with different amounts of RNA polymerase III inhibitor (25, 50 or 75 μM, or DMSO used as negative control). The level of A3A mRNA was quantified by TaqMan PCR (Figure 4D) and V1V2 DNA by SYBR Green (Figure 4E). The increase of V1V2 quantification was inversely proportional to the relative A3A expression. This experiment demonstrated that A3A expression could not be detected when RNA polymerase III activity was inhibited (Figure 4D) leading to maintain V1V2 DNA (Figure 4E).

To confirm the implication of RNA polymerase III in the transcription of cytosolic dsDNA knockdown experiments were performed using siRNAs to each of the subunit transcripts notably pol III1 and pol III2. When compared to control siRNA in THP-1 cells, the two RNA polymerase III siRNAs knockdown restricted A3A relative expression by ~4–5-fold (Figure 5A). Efficiency of cGAS, and RNA polymerases III siRNAs were confirmed by western blotting (Figure 5B). When cGAS siRNA were used, we observed a slight decrease of A3A expression but remained similar to control siRNA (Figure 5A), suggesting that in THP-1 cells, cGAS played a minor role in interferon type I production.

If cymtDNA was signaling via the RIG-I pathway downstream of RNA polymerase III, it should be possible to identify the pol III RNA transcripts. In the previous experiments a strong background would come from endogenous mtRNA transcripts. To overcome this, THP-1 cells were transfected by PCR products corresponding to a segment of HIV-1 envelope (V1V2, 685 bp, 38% GC (34)). THP-1 cells transfected with 500 ng of V1V2 DNA in-
Figure 5. Cytoplasmic RNA synthesis by DNA-dependent RNA polymerase III binds to RIG-I. (A) THP-1 cells were transfected with control siRNA or 2 RNA polymerase III siRNA (siRNA pol III1 and siRNA pol III2) or cGAS siRNA. Six hours post-transfection with siRNA, cells were transfected with 500 ng of MT-COI DNA. At 24 h post-transfection, total RNA was extracted and quantitative PCR was performed on A3A. (B) WB control of the experiment performed in 5A, using cGAS, pol III1 and pol III2 antibodies. The β-actin loading controls are shown below. (C) Interferon alpha and beta production by THP-1 following transfection by 500 ng V1V2 HIV-1 DNA. JetPRIME was used as control. (D) A3A quantification by THP-1 following transfection by 500 ng inMT-COI0mut. and inMT-COI32mut. DNA. (E) V1V2 RNA transcripts from DNA transfected THP-1 cells. Total RNA was extracted at 24 h and a cDNA corresponding to V1V2 was produced in presence or absence of RT and amplified by PCR. T1 and T2 refer to independent transfections. (F) Immunoprecipitation with an anti-RIG-I monoclonal antibody performed at 8 h along with an anti-HA as control. V1V2 specific RT-PCR products were recovered only from anti-RIG-I immunoprecipitation. RT, T1 and T2 as for 5E. * indicates statistically significant difference between two observed values (P < 0.05, Student’s t-test).

DISCUSSION

The findings highlight a mechanism by which cytoplasmic DNA induces IFNα/β to a degree comparable to MT-COI (Figure 5C versus Figure 1A). When comparing a shorter region of MT-COI DNA, (inMT-COI DNA, 247 bp, 50% GC), inMT-COI0mut. (inMT-COI DNA without mutation) to inMT-COI32mut. (inMT-COI DNA with 32 mutations, 37% GC), it transpires that transfected dsDNA needed to be thermodynamically stable to detect A3A expression (Figure 5D).

To show that cytoplasmic RNA synthesis by DNA-dependent RNA polymerase III binds to RIG-I, total RNA was extracted from two independent transfections (T1 and T2), treated with DNase and RT-PCR performed. V1V2 RNA intermediates were recovered only when a reverse transcriptase step was performed (Figure 5E). Cell lysates were made and immunoprecipitation with an anti-RIG-I monoclonal antibody was performed along with an anti-HA as control. V1V2 specific RT-PCR products were recovered only from the anti-RIG-I immunoprecipitates (Figure 5F).
Figure 6. Cytosolic DNA mediated innate immune signaling. Upon cellular stress (1), mtDNA is released into the cytoplasm (2) and recognized by RNA polymerase III and transcribed into RNA which anneals to form dsRNA duplexes (3). These activate RIG-I signaling leading to IRF-3 phosphorylated by TBK1 (4-5) and induction of interferon (6). Through IFNAR1/2, signaling IFN production leads to A3A upregulation (7), which initiates catabolism of cytoplasmic ssDNA. As A3A can translocate to the nucleus, it can cause hypermutation of nuDNA and formation of double stranded DNA breaks DSB (8).

catabolism of single stranded DNA generated by cytoplasmic exonucleases (Figure 6).

DNA heteroduplexes as small as 39 bp can be transcribed for IFNα/β induction is almost as efficient as for the 511 bp MT-COI fragment (Figure 3A). By comparison, single stranded DNA signaling was weak (Figure 3A). These findings do not exclude other DNA sensing pathways in primary cells for THP-1 cells do not mount TLR3- and TLR9-dependent responses. However, as the ML-60218 inhibitor (Figure 4B, D and E) and RNA polymerase III siRNAs knockdown (Figure 5A) completely blocks IFNα/β induction and A3A expression, RNA polymerase III is the major DNA sensing pathway following transfection of THP-1 cells.

Although A3 enzymes catalyze oxidation of cytidine to uracil in ssDNA, the uracil bases do not constitute a novel danger signal per se (Figure 1F, G and E). However, as AU-rich edited DNA is thermodynamically less stable, it represents a mechanism to prevent annealing of complementary ssDNA fragments (Figures 3A and 5D). This is particularly relevant for cymtDNA. If hundreds of mtDNA genomes can leak out to the cytoplasm and be processed by dsDNA-dependent endonucleases and exonucleases, there is the possibility that complementary single stranded mtDNA molecules could re-anneal to dsDNA and re-signal danger via DNA sensor molecules. A recent paper highlighted a parallel phenomenon concerning ADAR1 editing of dsRNA. Edited molecules were thermodynamically less stable due to lower stacking energy of inosine:thymine pairs—inosine is the product of adenine deamination—and failed to trigger dsRNA sensors (42).

There could be two steps in the catabolism of cytoplasmic ssDNA. A3C, A3F and A3G, which can edit cymtDNA, are expressed in most cells (1,4) and presumably function as constitutive catabolic enzymes. By contrast, A3A (nucleo-cytoplasmic) is massively upregulated by IFNα/β as is A3G (cytoplasmic) albeit to a lesser extent (4,30–32).
When THP-1 cells are triggered by high levels of cytoplasmic DNA, cymtDNA is more edited (Figure 2B and C). The findings tie in well with the massive egress of mtDNA to the cytosol resulting from a mitochondrial gene lesions or stress following herpesvirus infection (20). Here, the signaling pathway was the DNA sensor cGAS that promoted STING-IRF3-dependent signaling resulting in IFN production. The difference here is that finding pertains to the mouse, which unusually for mammals, does not encode an A3A ortholog (43). Although different DNA sensors probably overlap and converge on induction of IFN and a vast array of downstream effector molecules (44) catabolism of the DNA agonist in the mouse may proceed by a different mechanism for we were unable to detect cytosine deaminated mtDNA in mouse tissues (4).

There is a price to pay. Cancer genomes are characterized by tens of thousands to more than a hundred thousand CG→TA transitions, frequently in the tell-tale 5TpC signature, deamination of 5-methylcytosine residues and large numbers of DNA rearrangements (10,12,34,45–48). The A3A DNA mutator enzyme can reproduce these three mutational hallmarks experimentally (34,37). The link between A3A and cancer has been established epidemiologically (9,10,47,49,50). While both A3A and A3B contribute to mutation of chromosomal DNA (47,51–54), from an experimental setting, A3A is the more active of the two enzymes, A3B activity being greatly attenuated (6,8,9). The major difference is that A3A is massively upregulated by IFNα in hematopoietic cells while A3B is not (4,30–32) and the emergence of cancer on a background of chronic inflammation is well established (55).

Chronic inflammation, autoimmune diseases such as systemic lupus erythematosus and interferonopathies are associated with an increased risk for cancer. All show type I interferon signatures of which A3A is a part. While a powerful catabolic enzyme in the cytoplasm, A3A can locate to the nucleus and mutate nuclear DNA setting up a pathway for DNA catabolism. Proc. Natl. Acad. Sci. U.S.A., 108, 4858–4863.

Vartanian, J.P., Henry, M., Marchio, A., Suspène, R., Aynaud, M.M., Guétard, D., Cervantes-Gonzalez, M., Battiston, C., Mazzaferrero, V., Pineau, P. et al. (2010) Massive APOBEC3 editing of hepatitis B viral DNA in cirrhosis. PLoS Pathog., 6, e1000928.

Caval, V., Suspène, R., Shapira, M., Vartanian, J.P. and Wain-Hobson, S. (2014) A prevalent cancer susceptibility APOBEC3A hybrid allele bearing APOBEC3B 3′UTR enhances chromosomal DNA damage. Nat. Commun., 5, 5129.

Landry, S., Narvaiza, J.L., Linstedt, D.C. and Weitzman, M.D. (2011) APOBEC3A can activate the DNA damage response and cause cell-cycle arrest. EMBO Rep., 12, 444–450.

Caval, V., Bouzidi, M.S., Suspène, R., Laude, H., Dumargne, M.C., Bashamboo, A., Krey, T., Vartanian, J.P. and Wain-Hobson, S. (2015) Molecular basis of the attenuated phenotype of human APOBEC3B DNA mutator enzyme. Nucleic Acids Res., 43, 9340–9349.

Chan, K., Roberts, S.A., Klimczak, L.J., Sterling, J.F., Saini, N., Male, E.P., Kim, J., Kwiatkowski, D.J., Fargo, D.C., Miezczkowski, P.A. et al. (2015) An APOBEC3A hypermutation signature is distinguishable from the signature of background mutagenesis by APOBEC3B in human cancers. Nat. Genet., 47, 1067–1072.

Alexandroff, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A., Behjati, S., Blankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Borresen-Dale, A.L. et al. (2013) Signatures of mutational processes in human cancer. Nature, 500, 415–421.

BURNS, M.B., TEMIZ, N.A. and HARRIS, R.S. (2013) Evidence for APOBEC3B mutagenesis in multiple human cancers. Nat. Genet., 45, 977–983.

Stephens, P.J., Tarpey, P.S., Davies, H., Van Loo, P., Greenman, C., Wedge, D.C., Nik-Zainal, S., Martin, S., Varela, I., Bignell, G.R. et al. (2012) The landscape of cancer genes and mutational processes in breast cancer. Nature, 486, 400–404.

Komatsu, A., Nagasaki, K., Fujimori, M., Amano, J. and Miki, Y. (2000) Cytidine deamination of retroviral DNA by APOBEC3B and Malim, M.H. (2004) Cytidine deamination of retroviral DNA by diverse APOBEC proteins. Proc. Natl. Acad. Sci. U.A., 108, 4858–4863.

Bolli, N., Davies, H.R., Knappskog, S., Martin, S., Papaemmanuil, E. et al. (2014) Association of a germline copy number polymorphism of APOBEC3A and APOBEC3B with burden of putative APOBEC-dependent mutations in breast cancer. Nat. Genet., 46, 487–491.

Baikwill, F. and Mantovani, A. (2001) Inflammation and cancer: back to Virchow? Lancet, 357, 539–545.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsunoto, M., Hoshino, K., Wagner, H., Takeda, K. et al. (2000) A Toll-like receptor recognizes bacterial DNA. Nature, 408, 740–745.

Atianand, M.K. and Fitzgerald, K.A. (2013) Molecular basis of DNA recognition in the immune system. J. Immunol., 190, 1911–1918.

Yang, P., An, H., Liu, X., Wen, M., Zheng, Y., Rui, Y. and Cao, X. (2010) The cytosolic nucleic acid sensor LRFRIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. Nat. Immunol., 11, 487–494.
20. West,A.P., Khoury-Hanford,W., Staron,M., Tal,M.C., Pineda,C.M., Lang,S.M., Bestwick,M., Duguay,B.A., Raimundo,N., MacDuff,D.A. et al. (2015) Mitochondrial DNA stress primes the antiviral innate immune response. *Nature*, **520**, 553–557.

21. Ablasser,A., Bauerfeind,F., Hartmann,G., Latz,E., Fitzgerald,K.A. and Hornung,V. (2009) RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat. Immunol.*, **10**, 1065–1072.

22. Chiu,Y.H., Macmillan,J.B. and Chen,Z.J. (2009) RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell*, **138**, 576–591.

23. Ishikawa,H., Ma,Z. and Barber,G.N. (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature*, **461**, 788–792.

24. Zhong,B., Yang,Y., Li,S., Wang,Y.Y., Li,Y., Diao,F., He,X., Zhang,L., Tien,P. et al. (2008) The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity*, **29**, 538–550.

25. Fernandes-Alnemri,T., Yu,J.W., Datta,P., Wu,J. and Alnemri,E.S. (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, **458**, 509–513.

26. Oka,T., Hikoso,S., Yamaguchi,O., Taneike,M., Takeda,T., Tamai,T., Oyabu,J., Murakawa,T., Nakayama,H., Nishida,K. et al. (2012) Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature*, **485**, 251–255.

27. Zhang,Q., Raouf,M., Chen,Y., Sumi,Y., Sursal,T., Junger,W., Brohi,K., Itagaki,K. and Hauser,C.J. (2010) Circulating mitochondrial DAMPS cause inflammatory responses to injury. *Nature*, **464**, 104–107.

28. Crow,Y.J., Hayward,B.E., Parmar,R., Robins,P., Leitch,A., Ali,M., Black,D.N., van Bokhoven,H., Brunner,H.G., Hamel,B.C. et al. (2006) Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. *Nat. Genet.*, **38**, 917–920.

29. Yasutomo,K., Horiiuchi,T., Kagami,S., Tsukamoto,H., Hashimura,C., Uruishihara,M. and Kuroda,Y. (2001) Mutation of *TREX1* causes Aicardi-Goutieres syndrome at the AGS1 locus. *Nucleic Acids Res.*, **38**, 4274–4284.

30. Bonvin,M., Achermann,F., Greve,I., Stroka,D., Keogh,A., Inderbitzin,D., Candinas,D., Sommer,P., Wain-Hobson,S. and Vartanian,J.P. (2006) Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. *Hepatology*, **43**, 1364–1374.

31. Stenglein,M.D., Burns,M.B., Li,M., Lengyel,J. and Harris,R.S. (2010) APOBEC3 proteins mediate the clearance of foreign DNA from human cells. *Nat. Struct. Mol. Biol.*, **17**, 222–229.

32. Koning,F.A., Newman,E.N., Kim,E.Y., Kunstman,K.J., Wolinsky,S.M. and Malim,M.H. (2009) Defining APOBEC3 editing pathways in human tissues and hematopoietic cell subsets. *J. Virol.*, **83**, 9474–9495.

33. Suspène,R., Henry,M., Guillot,S., Wain-Hobson,S. and Vartanian,J.P. (2005) Recovery of APOBEC3-edited human immunodeficiency virus type 1- a heterozygous human by differential DNA denaturation PCR. *J. Gen. Virol.*, **86**, 125–129.

34. Suspène,R., Aynaud,M.M., Vartanian,J.P. and Wain-Hobson,S. (2013) Efficient demethylation of 5-methylcytidine and 5-substituted cytidine residues in DNA by human APOBEC3A cytidine deaminase. *PLoS One*, **8**, e63461.

35. Refsland,E.W., Stenglein,M.D., Shindo,K., Albin,J.S., Brown,W.L. and Harris,R.S. (2010) Quantitative profiling of the full APOBEC3 mRNA repertoire in lymphocytes and tissues: implications for HIV-1 restriction. *Nucleic Acids Res.*, **38**, 4274–4284.

36. Zarembra,K.A. and Godowski,P.J. (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J. Immunol.*, **168**, 554–561.

37. Müssel,B., Suspène,R., Aynaud,M.M., Gauvrit,A., Vartanian,J.P. and Wain-Hobson,S. (2013) Human APOBEC3A isoforms translocate to the nucleus and induce DNA double strand breaks leading to cell stress and death. *PLoS One*, **8**, e73641.