In HIV-1-infected individuals, G-to-A hypermutation is found in HIV-1 DNA isolated from peripheral blood mononuclear cells (PBMCs). These mutations are thought to result from editing by one or more host enzymes in the APOBEC3 (A3) family of cytidine deaminases, which act on CC (APOBEC3G) and TC (other A3 proteins) dinucleotide motifs in DNA (edited cytidine underlined). Although many A3 proteins display high levels of deaminase activity in model systems, only low levels of A3 deaminase activity have been found in primary cells examined to date. In contrast, here we report high levels of deaminase activity at TC motifs when whole PBMCs or isolated primary monocyte-derived cells were treated with interferon-α (IFNα) or IFNα-inducing toll-like receptor ligands. Induction of TC-specific deaminase activity required new transcription and translation and correlated with the appearance of two APOBEC3A (A3A) isoforms. Knockdown of A3A in monocytes with siRNA abolished TC-specific deaminase activity, confirming that A3A isoforms are responsible for all TC-specific deaminase activity observed. Both A3A isoforms appear to be enzymatically active; moreover, our mutational studies raise the possibility that the smaller isoform results from internal translational initiation. In contrast to the high levels of TC-specific activity observed in IFNα-treated monocytes, CC-specific activity remained low in PBMCs, suggesting that A3G deaminase activity is relatively inhibited, unlike that of A3A. Together, these findings suggest that deaminase activity of A3A isoforms in monocytes and macrophages may play an important role in host defense against viruses.

A3 proteins are cytidine deaminases that are capable of inhibiting replication of retroviruses, DNA viruses, and retroviral elements (reviewed in Refs. 1–3). The seven A3 proteins in the human genome share a strong preference for deaminating cytidines that are in a CC or TC context in single-stranded DNA (edited cytidine underlined). Although APOBEC3G (A3G), the best understood A3 protein, acts preferentially on CC motifs, the six other human A3 proteins display varying degrees of preference for TC motifs (4–13). Because other cytidine deaminases, such as activation-induced deaminase and APOBEC1, either display a strong preference for editing deoxycytidines in other dinucleotide contexts or act on TC motifs in RNA (4), mutations that arise in a CC or TC context in DNA constitute signatures of editing by A3 proteins.

Much headway has been made in understanding how A3 proteins act on HIV-1 DNA, in part through transfection of A3 plasmids into human epithelium-derived cell lines, such as 293T or HeLa cells, which lack significant endogenous expression of most A3 proteins. From such studies, A3G and APOBEC3F (A3F) are known to be packaged into virus particles when the HIV-1 Vif protein is not expressed and to cause a substantial reduction in virus infectivity (5, 12, 14–16). Reduced virus infectivity results in part from non-enzymatic inhibition of reverse transcription by A3G and A3F (17–23); additionally, the HIV-1 reverse transcripts that are generated display a high frequency of G-to-A mutations in the plus strand due to deamination on the HIV-1 minus strand DNA. Expression of a functional HIV-1 Vif protein counteracts the antiviral effects of A3G and A3F by recruiting them to the proteasome for degradation, thereby preventing their incorporation into viral particles (14, 24–30). Although HIV-1 is the best studied A3 viral target, studies using A3 transfection systems have demonstrated that one or more human A3 proteins are also able to inhibit replication of other pathogenic human viruses, including HIV-2 (31), hepatitis B virus (32–35), and paroviruses (36, 37).

Although knowledge of A3G action has advanced rapidly with the use of transfection systems, large gaps remain in our understanding of the biology of A3 proteins in clinically relevant settings. Much of what we know about the interaction of A3 proteins with viruses during natural infection has been...
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inferred from mutational signatures in viral DNA from infected patients. For example, numerous studies have found signatures of A3-mediated mutations in HIV-1 proviral DNA from patient samples (38–44), providing clear evidence that A3 proteins edit HIV-1 DNA in vivo despite the opposing effects of HIV-1 Vif. Additionally, viral DNA sequences from tissues infected with human papillomavirus virus (HPV) (45) and hepatitis B virus (46) also contain mutations suggestive of A3 editing. Because mutations in the C or T context are highly characteristic of A3-mediated editing, the presence of these specific mutations in viral DNA provides strong evidence for editing by one or more A3 proteins. However, identifying the specific A3 protein(s) responsible for the observed mutations has proven challenging. C mutations indicate editing by A3G because A3G is the only A3 protein with a C preference. In contrast, one cannot definitively attribute mutations that arise in a T context in vivo to a particular human A3 protein because all six human A3 proteins besides A3G display a preference for editing at T motifs, and some primary human cell types express more than one A3 protein (47, 48). Moreover, little is known about in vivo regulation of A3 proteins, which could affect the ability of A3 proteins to interact with and edit viral DNA. Thus, for all of these reasons, the identity of the A3 proteins responsible for mutations that arise in a T context in viral DNA in vivo remains unclear.

To address some of the gaps in our understanding of how G-to-A mutation arises in vivo, we previously examined the deaminase activity of A3 proteins expressed endogenously in primary human T cells. We found that although C-specific deaminase activity was high in lysates of 293T cells transfected with A3G, deaminase activity was very low in resting and fully activated primary CD4+ T cells as well as in a wide variety of human T cell lines, despite abundant A3G expression (49). Additional experiments suggested that A3G deaminase activity in primary T cells and T cell lines is negatively regulated by an RNase-insensitive inhibitor that is not present in 293T cells (49). These observations raised the possibility that A3 proteins may be enzymatically inhibited in many primary cell types, leading to A3 deaminase activity being displayed only in specific circumstances.

Because deaminase activity probably contributes to the antiviral ability of A3 deaminases, we reasoned that enzymatically active endogenous A3 deaminases might only be expressed as part of a physiologic response to virus infection. Therefore, in the current study, we tested the hypothesis that exposing primary cells to signals that activate innate immune responses would lead to induction of A3 deaminase activity. We found that treatment with IFNα or with toll-like receptor (TLR) ligands that induce IFNα expression caused a large and prolonged increase in T-specific deaminase activity but not C-specific deaminase activity. This increase in T-specific deaminase activity occurred in primary monocytes and monocyte-derived macrophages (MDMs) but not in other peripheral blood mononuclear cells (PBMCs). Moreover, we demonstrated that this T-specific deaminase activity is due to de novo expression of two A3A isoforms, both of which are enzymatically active. These findings demonstrate that monocytes and MDMs respond to innate immune signaling pathways that induce IFNα by expressing enzymatically active A3A isoforms, whereas the deaminase activity of endogenous A3G in these cells remains largely inhibited.

EXPERIMENTAL PROCEDURES

Plasmids—To generate an A3A expression plasmid, total RNA was isolated from IFNα-treated human PBMCs from an HIV-1-seronegative donor using the RNaqueous microkit (Ambion) according to the manufacturer’s protocol and subjected to DNase I treatment. Reverse transcription reactions were performed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol, using oligo(dT) primers. Untagged A3A sequences containing the native translational start site (5′-NNNNNGCTAGCCGGAC-AAGCACATGGAAAGCCAGCCC-3′ and 5′-NNNNNGCT- TCCATCTTTCTTCCTGATTCGAG-3′) or an optimized Kozak consensus sequence (5′-NNNNNGCTAGCCGGACAACAGCATGGAAAG-3′ and 5′-NNNNNGCTAGTCGCCTATCCTCTCAGGG-3′) were amplified by PCR using Pfu polymerase (Promega) and the indicated primers. Amplicons were ligated into pCI-neo (Promega) for expression in mammalian cells. Constructs containing point mutations M1A, M13A, and E72Q were generated by site-directed mutagenesis (QuikChange, Stratagene). All coding regions were confirmed by sequencing, and the A3A sequence from PBMCs was found to match the A3A reference sequence (accession number NM_145699). The plasmid encoding untagged A3G was previously described (49).

Cell Lines, Transfections, and Harvests—293T cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). Transfections were typically performed by mixing 0.5 µg of plasmid DNA and 40 mg of polyethyleneimine (Polysciences) in serum-free DMEM and adding to cells plated in 60-mm dishes in antibiotic-free DMEM. For native harvests, cells were lysed in Nonidet P-40 buffer containing EDTA as described previously (49). All cell lines, including THP-1 and U937 cells, were obtained from the ATCC.

PBMC Culture and Harvest—Peripheral blood mononuclear cells were obtained from a Seattle Assay Controls study. Following informed consent in accordance with procedures approved at the Fred Hutchinson Cancer Research Center, cells from healthy human donors were separated by leukapheresis and immediately frozen. For the present study, frozen PBMCs from anonymous donors were thawed and cultured for 24 h in AIM-V serum-free medium (Invitrogen), except where noted, at a density of 10 × 10⁶ cells/well in a 6-well plate in the presence or absence of interferon-blocking antibodies: mouse anti-human IFNα (50 ng/ml; PBL Interferon Source 21385-1) and mouse anti-human IFNα/β receptor (0.5 µl/ml; Chemicon International MAB411). To obtain specific cell populations from PBMCs, cells were separated immediately after thawing using the EasySep negative selection kits directed against the following surface markers: 1) monocytes (CD2, CD3, CD19, CD20, CD56, CD66b, CD123, and glycoporin A); 2) CD4+ T cells (CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCRγ/δ, and glycoporin A); or 3) CD8+ T cells (CD4, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCRγ/δ, and glyco-
phosphor A). After the 24-h culture, drugs were added to the following final concentrations: phytohemagglutinin (PHA) (2 µg/ml; Fisher), phorbol 12-myristate 13-acetate (PMA) (500 ng/ml; Sigma-Aldrich), IL-2 (10 IU/ml; Peprotech), CD3 antibody (30 ng/ml; eBioscience), CD28 antibody (1 µg/ml; BD Pharmingen), IFNα 2a (1000 IU/ml; PBL Interferon Source), IFNγ (1 µg/ml; PBL Interferon Source), poly(I-C) (10 µg/ml; Invivogen), imiquimod (0.25 µg/ml; Invivogen), ODN2016 (0.5 µM; Invivogen), actinomycin (10 µg/ml; Sigma-Aldrich), cycloheximide (10 µg/ml; Sigma-Aldrich). For macrophage differentiation, monocytes were negatively selected, as described above, and cultured for 7 days with 40 ng/ml M-CSF (Peprotech) before treating with IFNα or neutralizing antibody against IFNα and the IFNα/β receptor, as described above.

To prepare cell lysates, cell suspensions were removed, and the remaining adherent cells were washed once with PBS and detached by treatment with trypsin-EDTA (Invitrogen) for 15 min at 37 °C. All cells were pooled, pelleted at 160 × g for 10 min in a Beckman Allegra 6r centrifuge using a GH-3.8 rotor, washed once in cold PBS, and then resuspended in 200 µl of Nonidet P-40 buffer (0.625% Nonidet P-40, 10 mM Tris acetate, pH 7.4, 50 mM potassium acetate, 100 mM NaCl, 10 mM EDTA) supplemented with protease inhibitor mixture for mammalian cells (Sigma) and incubated at 4 °C with shaking for 5 min. Lysates were spun for 45 s in a microcentrifuge at 18,000 × g, and the supernatants were removed. Lysates were then flash-frozen in liquid nitrogen and stored at −80 °C so that all lysates from a single experiment could be thawed and analyzed for deaminase activity in parallel.

**Fluorescence Dequenching Activity Assay (Previously Called FRET-based Activity Assay) from Cell Lysate**—Total protein in cell lysates was measured using the BCA protein assay kit (Pierce), and deaminase activity for each sample was measured in triplicate for 2-fold serial dilutions ranging from 0.625 to 5 µg of total protein, as described previously (49), with the modifications described below. To each well was added 10 µl of cell lysate in Nonidet P-40 buffer and 50 µl of a master mix containing 10 pmol of Taqman probe labeled with Fam (6-carboxy-fluorescein) and TAMRA (carboxytetramethylrhodamine), 0.4 unit of uracil DNA glycosylase (NEB), 50 mM Tris (pH 7.4), and 10 mM EDTA. For all assays, deaminase activity was assessed using the following oligonucleotides: 5’-GCAUCGUGACGAGAUAdTdT-3’ or the 3’-UTR of A3A mRNA (5’-GCAUCGUGACGAGAUAdTdT-3’). A control oligonucleotide that was chemically modified to prevent incorporation into the RISC-complex were chemically synthesized by Invitrogen. siRNAs were delivered into PBMC-derived monocytes using an AMAXA nucleofector apparatus (Lonza human monocytes nucleofector kit, program Y-001; 30 pmol of siRNA/3 × 10^6 cells).

**RESULTS**

**TC- But Not CC-specific Deaminase Activity Is Induced in PBMCs by Treatment with IFNα or TLR Ligands That Induce IFNα Expression**—To test the hypothesis that physiologically relevant stimuli might induce A3 deaminase activity, we examined deaminase activity in PBMCs upon treatment with a variety of stimuli, including ligands that activate T cells and cytokines that promote adaptive and innate immune responses to viral infection. PBMCs from healthy human donors were thawed, rested for 24 h, subjected to treatments described below, and lysed at multiple time points after treatment. PBMC lysates were treated with RNase A to remove any inhibitory RNA that might be complexed with A3 proteins and then assayed for deaminase activity using a previously described fluorescence dequenching assay (49). In this assay, DNA oligonucleotide substrates that contain either CC or TC motifs are labeled on the 5’ end with a reporter fluorophore that is quenched by a 3’-fluorophore when the oligonucleotide is intact. When these oligonucleotides are incubated with lysates containing enzymatically active A3 proteins, cytidine deamination occurs. The resulting uridines are converted to abasic sites by uracil N-glycosylases present in cell lysates and by recombinant uracil DNA glycosylase that is added to the reaction. Cleavage occurs at abasic sites when the pH of the reaction is raised, leading to dequenching with fluorescence emission from the reporter fluorophore, measured as relative fluorescence units (RFU). We have previously shown that this assay reproducibly detects a dose-dependent increase in fluorescence upon incubation with lysates of 293T cells transfected with A3G (49), which are included as a positive control in all of the experiments described below.
FIGURE 1. INFγ treatment of PBMCs induces TC-specific deaminase activity. A, PBMCs from a healthy human donor were cultured overnight and either left untreated (Un), or treated with anti-CD3 and anti-CD28 antibodies (3/28), PMA + IL-2 (PMA), INFα (α), or INFγ (γ). Cells were lysed at the indicated time points and analyzed for deaminase activity in the presence of RNase A using oligonucleotides containing motifs for either ACCC (left graph) or ATTC (right graph). Lysates from 293T cells or 293T cells expressing A3G were analyzed in parallel (data not shown). Activity is graphed as RFU. Lysates shown in the activity graphs were also analyzed by Western blot (WB) with antisera against A3G, A3F, or calreticulin (cal), which served as a loading control. Lysates from 293T cells (−) or 293T cells transiently expressing untagged A3G, A3A, or A3F were analyzed in parallel (293T). Bar, lanes containing INFα-treated samples. B, PBMCs from the same donor as above were cultured in the presence of neutralizing antibodies against INFα and the INFα/β receptor (anti-INFα/β). Cells were then either left untreated or treated with αCD3/28, PMA + IL-2, INFα, or INFγ. Cells were lysed and analyzed for deaminase activity as in A using oligonucleotides containing motifs for either ACCC (left graph) or ATTC (right graph). These lysates were also analyzed by Western blot as in A. The arrows in both blot panels show the expected positions of A3G, A3A, A3F, and calreticulin. For deaminase assays, error bars represent S.E. of triplicate reactions. All samples in A and B are from the same experiment and were analyzed together. Graphs in this figure are presented in color to help with clarity.
When examined for deaminase activity using CC-containing oligonucleotides, lysates of untreated PBMCs displayed no significant activity (Fig. 1A, left graph). Only modest CC-specific deaminase activity was seen in PBMCs subjected to T cell activation using either a combination of antibodies to CD3 and CD28 (αCD3/CD28) or PMA + IL-2, and this activity was only observed 72 h after stimulation. When PBMCs were treated with IFNα and IFNγ, cytokines that induce antiviral adaptive and innate immune responses, similar modest increases in CC-specific deaminase activity were observed (Fig. 1A, left graph). As a positive control, lysates of 293T cells transfected with A3G were analyzed in parallel and exhibited high levels of CC-specific activity, as expected (data not shown), consistent with results obtained previously (49).

PBMC lysates were also examined for deaminase activity using an oligonucleotide containing a TC motif (Fig. 1A, right graph). No TC-specific deaminase activity was observed in untreated cells, and only modest activity was observed with the T cell activation protocols described above or upon treatment with IFNγ, as was the case with CC-specific deaminase activity. In contrast, strikingly high levels of TC-specific deaminase activity (13-fold above the untreated base line at 24 h) were observed when PBMCs were treated with IFNα (Fig. 1A, right graph). Thus, IFNα was the only treatment that resulted in high levels of deaminase activity in PBMCs, and the resulting activity was specific for a TC motif.

To determine which A3 protein(s) was responsible for the increase in TC-specific deaminase activity upon IFNα treatment, we analyzed PBMC lysates for expression of A3 proteins by Western blotting. A3F protein levels were analyzed because A3F is known to display a TC preference during deamination (5, 12, 14, 16). Although a signal was observed by Western blot in control 293T cells transfected with A3F, no significant A3F signal was observed in lysates of untreated or treated PBMCs analyzed in parallel (Fig. 1A, blots), indicating that expression of A3F in all of the PBMC groups was absent or very low. As a control, we also examined A3G levels because A3G is expressed at high levels in some PBMC subsets (although it does not display a preference for TC motifs). A3G protein levels were roughly equivalent in PBMCs from all treated groups and were comparable with or higher than levels in the A3G-transfected 293T control cells (Fig. 1A, blots). Western blots for calreticulin confirmed that loading was equivalent for all lanes (Fig. 1A, blots). Thus, neither A3F nor A3G levels correlated with differences in deaminase activity observed in PBMC lysates. Additionally, neither A3G nor A3F levels were increased significantly by IFNα treatment. However, in A3G Western blots, two proteins of ∼20 kDa were observed only in IFNα-treated lysates. These ∼20-kDa proteins (referred to below as the large and small ∼20-kDa proteins) appeared with a time course that roughly paralleled that of TC-specific deaminase activity in IFNα-treated lysates (Fig. 1A, blots, black bar). Thus, the appearance of the two ∼20-kDa proteins detected by A3G antiserum correlated closely with induction of TC-specific deaminase activity in PBMCs treated with IFNα. Notably, the larger of the two ∼20-kDa proteins seen in PBMCs had the same migration as A3A that was expressed in 293T cells from a plasmid that contains an optimized Kozak consensus at the A3A start codon and analyzed in parallel with PBMC samples (Fig. 1A, blots).

To confirm that IFNα treatment was responsible for the appearance of TC-specific deaminase activity as well as the induction of the two ∼20-kDa proteins, we pretreated PBMCs with antibodies to both IFNα and the IFNα/β receptor (anti-IFNα/β) before treating with IFNα. PBMCs pretreated with anti-IFNα/β failed to display the large increase in TC-specific deaminase activity in response to IFNα treatment (Fig. 1B, right graph). Additionally, pretreatment with anti-IFNα/β eliminated the appearance of the ∼20-kDa proteins detected by Western blot with A3G antiserum (Fig. 1B, blots). These findings confirmed that both the large increase in TC-specific deaminase activity and the induction of the ∼20-kDa proteins are due to IFNα. Notably, pretreatment of PBMCs with anti-IFNα/β did not inhibit the modest increases in CC- and TC-specific deaminase activity observed upon IFNγ treatment (Fig. 1B), indicating that IFNγ acts through an IFNα-independent pathway.

We also examined whether the IFNα effect is observed in PBMCs from other donors. In three different donors, IFNα treatment caused large increases in TC-specific deaminase activity but not CC-specific activity. In all three donors, the same time course was observed, with TC-specific deaminase activity rising within 12 h after IFNα treatment, peaking at 12–24 h, and remaining high for at least another 48 h in the presence of IFNα (Fig. 2A). Additionally, treatment with PHA, a lectin that activates T cells, only caused modest increases in CC activity in all three donors (Fig. 2A), consistent with results shown in Fig. 1A. We also examined the effect of TLR ligands that induce IFNα (52). Treatment with ligands for TLR3, TLR7, TLR8, or TLR9 induced TC-specific deaminase activity and the appearance of the ∼20-kDa proteins in A3G blots with a time course and magnitude similar to that produced by IFNα (Fig. 2B). Similar results were seen using PBMCs from other donors (data not shown). Thus, the effect of IFNα was very similar in PBMCs from multiple donors and was also observed upon treatment with TLR ligands that act through signaling pathways to induce IFNα production.

Having demonstrated that TC-specific deaminase activity can be induced by IFNα production in PBMCs, we wondered if this TC-specific activity could be down-regulated by other stimuli. When we examined the effect of combined stimuli, we observed that treatment of PBMCs with both IFNα and PHA resulted in only a transient increase in TC activity, with a rise at 12 h followed by a drop after 24 h, reaching base line at 48 h (Fig. 3, graph). This transient increase in TC-specific deaminase activity upon treatment with PHA and IFNα contrasted sharply with the prolonged increase in TC-specific deaminase activity observed with IFNα alone in the same experiment (Fig. 3, graph) and was also observed in PBMCs from other donors (data not shown). In cells treated with IFNα + PHA, Western blots showed that the two ∼20-kDa proteins were induced only transiently (Fig. 3, blots), with a time course corresponding closely to the short term induction of deaminase activity. In contrast, treatment with IFNα alone led to a sustained increase in expression of the two ∼20-kDa proteins (Fig. 3, blots, compare 24 and 72 h). Additionally, treatment with PHA plus IFNγ
inhibited the increase in TC-specific deaminase activity observed upon treatment with IFNγ alone, with a corresponding loss of the two ~20-kDa proteins (Fig. 3). Together, these data suggest that, in addition to being up-regulated by IFNα, TC-specific deaminase activity and expression of the two ~20-kDa proteins in PBMCs can be negatively regulated by other signaling pathways.

Appearance of TC-specific Deaminase Activity Correlates with Induction of Two A3A Isoforms—The A3G antiserum that was utilized to detect A3G as well as the ~20-kDa IFNα-induced proteins was generated against 29 amino acids in the C terminus of A3G (23, 49). Others have noted that use of this antiserum results in detection of two ~20-kDa A3 proteins in dendritic cells and monocytes (47, 53). Although these proteins were initially described as possible A3G degradation products by negative selection (as confirmed by flow cytometry; see supplemental Fig. 1) and measured deaminase activity in each subset following 24 h of IFNα treatment. Although isolated CD4+ and CD8+ T cells treated with IFNα displayed no induction of deaminase activity, isolated monocytes treated with IFNα displayed a 15-fold increase in TC-specific deaminase activity relative to untreated cells (Fig. 5A, graph), with no significant change in CC-specific activity (data not shown). Additionally, Western blots revealed that both A3A isoforms were induced in the IFNα-treated monocytes but not in the IFNα-treated T cell subsets (Fig. 5A, blot). The time course of induction of TC-specific deaminase activity and the A3A isoforms in isolated monocytes (Fig. 5B) was the same as that observed previously in PBMCs (Figs. 1–3). In contrast to the striking increase in A3A protein levels, only a modest
increase in A3G levels was observed in isolated monocytes following IFNα treatment (Figs. 5, A and B). Thus, IFNα treatment causes a large increase in both TC-specific deaminase activity and expression of A3A isoforms in isolated monocytes but not in isolated CD4+ or CD8+ T cells.

When untreated primary monocytes were differentiated into MDMs using M-CSF, no CC-specific deaminase activity was observed with or without IFNα treatment (Fig. 5C). However, treatment with IFNα led to a large increase in TC-specific deaminase activity in MDMs (Fig. 5C), similar to that seen upon IFNα treatment of undifferentiated monocytes (Fig. 5, A and B). Western blots revealed large increases in both A3A and A3G levels in IFNα-treated MDMs (Fig. 5C, blot). Interestingly, although both A3A and A3G protein levels are significantly higher in MDMs following IFNα treatment, only TC-specific deaminase activity was observed. These data suggest that a deaminase(s) with a TC preference is enzymatically active in MDMs upon IFNα treatment, whereas A3G in MDMs remains enzymatically inhibited by an RNase-insensitive inhibitor, as is the case for A3G in CD4+ T cells (49).

We also examined deaminase activity in two monocytic cell lines, Thp-1 and U937, derived from hematologic malignancies (58, 59). Although IFNα induces TC-specific activity in primary monocytes and MDMs, no significant deaminase activity was observed against oligonucleotides containing either a TC (supplemental Fig. 2, graph) or CC (data not shown) motif when Thp-1 and U937 cells were treated with IFNα for 24 or 48 h. Consistent with the activity data, only faint traces of A3A isoforms were observed in IFNα-treated samples upon long exposures of Western blots (supplemental Fig. 2, blots). From these data, we concluded that primary monocytes and MDMs display large increases in TC-specific deaminase activity as well as de novo induction of A3A isoforms upon IFNα treatment, but the monocytic cell lines we examined do not.

TC-specific Deaminase Activity in Monocytes Requires de novo Transcription and Translation—Because IFNα causes de novo transcription and translation of interferon-stimulated genes (reviewed in Ref. 60), we asked whether transcription and translation are required for induction of TC-specific deaminase activity upon IFNα treatment. Isolated monocytes were treated for 24 h with either IFNα or anti-IFNα/β, in the presence of either actinomycin (an inhibitor of RNA synthesis), cyclohexamide (an inhibitor of protein synthesis), or a vehicle control. Both actinomycin and cyclohexamide completely inhibited the IFNα-mediated increase in TC-specific deaminase activity as well as the expression of the A3A isoforms (supplemental Fig. 3). Thus, upon IFNα treatment of monocytes, de novo transcription and translation of one or more factors is required for induction of TC-specific deaminase activity and A3A isoforms. Additionally, Western blots revealed that calreticulin levels were reduced significantly upon cyclohexamide treatment (supplemental Fig. 3), suggesting that calreticulin undergoes relatively rapid turnover. In contrast, a more modest decrease

FIGURE 3. Treatment with PHA plus IFNα results in a transient increase in deaminase activity. PBMCs from a healthy human donor were cultured overnight and then treated with either anti-IFNα/β, PHA alone, IFNα alone, IFNγ alone, IFNα + PHA, or IFNγ + PHA. Cells were lysed at the indicated time points and analyzed for deaminase activity in the presence of RNase A using oligonucleotides containing ATTCC motif. Activity is graphed as RFU, and error bars represent S.E. of triplicate reactions. Lysates from all time points were analyzed by Western blot (WB) with antiserum against A3G or calreticulin (cal) as a loading control. The arrows show the expected positions of A3G, A3A, and calreticulin. All samples are from the same experiment and were analyzed together.

FIGURE 4. Western blots with A3A/B antiserum confirm that the ~20-kDa proteins likely represent A3A isoforms. Lysates containing 10 μg of total cellular protein from untreated PBMCs, IFNα-treated PBMCs (PBMC + IFNα), and 293T cells transiently transfected with an A3A plasmid engineered to contain an optimized Kozak consensus (A3A-Koz) were analyzed by Western blot (WB) with a commercial antiserum directed against A3A and -B (A3A/B) and an antiserum directed against A3G. Markers show the expected positions of A3B, A3G, and A3A, all of which share significant sequence homology, accounting for cross-reactivity of antiserum.
IFNα induces TC-specific deaminase activity and A3A isoforms in monocytes and macrophages but not in T cells. A, PBMCs from a healthy human donor were cultured overnight and then subjected to negative selection to obtain three populations enriched for either CD4+ T cells, CD8+ T cells, or monocytes (for subset analysis, see supplemental Fig. 1). Cell subsets were either left untreated or treated with IFNα for 24 h. The cells were then lysed and analyzed for deaminase activity in the presence of RNase A using the ATT-C-containing oligonucleotide. Activity is graphed as RFU. Lysates from these cells were analyzed by Western blot (WB) with antiserum against A3G or calreticulin (cal) as a loading control.

B, PBMCs from a healthy human donor were cultured overnight and then subjected to negative selection for monocytes. Both the monocytes and the positively selected cells were treated with either IFNα or anti-IFNα/β, as described above. Cells were lysed at the indicated time points and analyzed for deaminase activity as described in A. Activity is graphed as RFU. Lysates from monocytes (mono) or positively selected cells (other) treated with IFNα (α) or anti-IFNα/β (ab) were analyzed by Western blot with antiserum against A3G or calreticulin as a loading control. C, PBMCs from a healthy human donor were cultured overnight and then subjected to negative selection for isolation of monocytes, which were differentiated into MDMs by treating with M-CSF for 7 days. After differentiation, MDMs were treated with either IFNα or anti-IFNα/β, as described above. Cells were lysed at the indicated time points and analyzed for deaminase activity in the presence of RNase A using either the ACCC- or ATT-C-containing oligonucleotide. Activity is graphed as RFU. Lysates from these cells were analyzed by Western blot with antiserum against A3G, or calreticulin as a loading control. In all blots, arrows show expected positions of A3G, A3A, and calreticulin. In each panel, samples are from one experiment and were analyzed together. For deaminase assays, error bars represent S.E. of triplicate reactions.
The bottom arrow reactions. Lysates were also analyzed by Western blot (WB) for IFN-induced deaminase activity. PBMCs from a healthy human donor were cultured overnight. The following day, the monocytes were transfected with either a control siRNA oligonucleotide (control) or siRNAs targeting specific for A3G (G) or A3A (A) using the Amaxa Nucleofector system and then allowed to rest in serum-containing recovery medium for 4 h. The cells were then either left untreated or treated with either IFNα or the IFNα receptor (anti-IFNα/β). Cells were lysed at 48 h post-transfection and analyzed for deaminase activity in the presence of RNase A using oligonucleotides containing the ATTCC motif. Activity is graphed as RFU. Error bars represent S.E. of triplicate reactions. Lysates were also analyzed by Western blot (WB) with antisera against A3G or calreticulin (cal) as a loading control (bottom). The arrows show expected positions of A3G, A3A, and calreticulin. All samples are from the same experiment and were analyzed together.

Intracellular A3G was observed in Western blots in the presence of cycloheximide (supplemental Fig. 3), suggesting that A3G in monocytes has a relatively long half-life.

IFN-induced Deaminase Activity Is Eliminated by siRNA Knockdown of A3A—The close correlation between TC-specific deaminase activity and the appearance of the A3A isoforms in the experiments described above suggest that A3A is responsible for the TC-specific deaminase activity induced in monocytes by IFNα treatment. However, because six human A3 proteins display preferences for TC-containing substrates, it is possible that another A3 protein(s), besides A3A, is responsible for or contributes to the observed TC-specific deaminase activity. To determine whether A3A is solely responsible for all of the TC-specific deaminase activity observed, monocytes were subjected to nucleofection with either a control siRNA oligonucleotide, or siRNAs oligonucleotides directed against A3A or A3G. Following knockdown, monocytes were treated with IFNα or anti-IFNα/β for 48 h or left untreated (Fig. 6). Cells nucleofected with control oligonucleotide displayed the expected increase in TC-specific deaminase activity upon IFNα treatment relative to untreated or anti-IFNα/β controls. A similar induction of TC-specific deaminase activity was observed in cells that received the A3G knockdown oligonucleotide. In contrast, knockdown of A3A prevented the IFNα-induced increase in A3A deaminase activity that was observed in the other IFNα-treated groups (Fig. 6). Western blots confirmed that expression of both A3A isoforms was dramatically reduced in cells treated with the siRNA oligonucleotide directed against A3A (Fig. 6). Thus, these data indicate that A3A isoforms alone are responsible for the vast majority of TC-specific deaminase activity observed in monocytes upon IFNα treatment. Interestingly, in cells treated with siRNA oligonucleotide directed against A3G, only a small decrease in A3G expression was observed. This is consistent with the observation that monocytes contain a long lived pool of A3G that undergoes little turnover, as demonstrated in supplemental Fig. 3.

**A3A Enzymatic Activity Is Detected Even in the Absence of RNase A Treatment**—Because it has been reported that a significant fraction of A3A is nuclear (36) and that A3 deaminase activity can pose a threat to host DNA (61), we wondered whether the enzymatic activity of A3A in monocytes is inhibited by the formation of RNA-containing complexes, as is the case for deaminase activity of A3G from 293T cells (49, 50). Because RNase treatment releases A3G from 293T cells (49, 50), we wondered whether the enzymatic activity of A3A in monocytes is inhibited by the formation of RNA-containing complexes, as is the case for deaminase activity of A3G from 293T cells (49, 50). Because RNase treatment releases A3G from 293T cells from this inhibition (49, 50), all deaminase assays shown thus far were performed on RNase A-treated lysates. To examine whether treatment of lysates with RNase A is required for detection of A3A deaminase activity in monocytes, we measured deaminase activity in lysates of IFNα-treated and untreated monocytes with and without RNase A treatment. A significant amount of deaminase activity was observed in lysates of IFNα-treated monocytes that were not treated with RNase A (Fig. 7). These data indicate that at least some of the A3A in IFNα-treated monocytes is free of inhibitory RNA and is therefore enzymatically active in the form in which it is expressed endogenously in monocytes.

Both A3A Isoforms Appear to Be Enzymatically Active—As seen in Figs. 1–6, IFNα treatment of PBMCs results in expression of two ~20-kDa proteins that are immunoreactive to the A3A/B antiserum (Fig. 4), the larger of which probably corresponds to the human A3A sequence recorded in the NCBI database (NP_663745; also called phorbolin 1). We noticed that the A3A sequence contains a methionine at position 13 (Met13). This could allow both A3A isoforms to be produced from one transcript, with internal translation initiation at Met13 resulting in production of a protein with approximately the same molecular weight as the smaller A3A isoform. To test the hypothesis that both A3A isoforms could be produced from a single A3A
mRNA, we engineered an A3A plasmid in which the sequence preceding the position 1 start codon matched the sequence found in the A3A cDNA amplified from PBMCs (native translational start context; A3A-nat in Fig. 8). Transfection of this A3A plasmid into 293T cells resulted in nearly equivalent quantities of both A3A isoforms (Fig. 8A, blot), in contrast to transfection of the A3A plasmid engineered to have an optimized Kozak consensus at position 1, which produced mainly the larger A3A isoform (A3A-Koz in Fig. 8; also shown in Figs. 1A and 4). Thus, when A3A was expressed from a transcript containing the native translational start context found in PBMCs, both A3A isoforms are produced. Moreover, manipulation of the Kozak consensus at position 1 altered the proportion of large versus small A3A isoform produced.

To test the hypothesis that the small A3A isoform could result from internal initiation at Met\(^1\), we engineered point mutations at Met\(^1\) and Met\(^{13}\) in the A3A plasmid containing the native translational start context. When the methionine in position 1 (Met\(^1\)) was mutated to an alanine (M1A), only the small A3A isoform was synthesized (Fig. 8A, blot). Furthermore, when Met\(^{13}\) was mutated to an alanine (M13A), only the large A3A isoform was produced (Fig. 8A, blot). Notably, the migration of the M13A and M1A proteins on SDS gels closely paralleled the migrations of the large and small A3A isoforms expressed in IFN\(\gamma\)-treated PBMCs, respectively (Fig. 8A, blot). Although we cannot rule out the possibility that some or all of the smaller A3A isoform seen in monocytes constitutes a degradation product, these findings demonstrate that the smaller isoform can be produced by internal initiation at Met\(^{13}\) and is expressed in significant quantities when the native sequence context at the A3A start site is maintained.

Additionally, A3 deaminase assays were performed using lysates expressing each of the transfected A3A constructs (Fig. 8A, graph). Included as controls were lysates of 293T cells expressing either A3A-nat or A3A-Koz or an A3A protein containing a mutation in the active site (E72Q) that is known to abolish deaminase activity (36, 37) as well as PBMCs treated with IFN\(\gamma\). The M1A plasmid, which produces exclusively the small isoform, displayed high levels of TC-specific deaminase activity. Unexpectedly, the M13A mutant, which produces only the large isoform, albeit with a conservative substitution at Met\(^{13}\), was enzymatically inactive (Fig. 8A, graph). However, A3A-Koz, which is engineered to produce predominantly the larger of the two A3A isoforms, displayed activity equivalent to that seen upon expression of A3A-nat, which produces roughly equivalent amounts of both isoforms. These data suggest that both the large and small wild-type isoforms of A3A are enzymatically active and contribute to the TC-specific deaminase activity observed in primary monocytes and MDMs and that conversion of methionine to alanine at position 13 (which is outside of the deaminase domain) abrogates activity. However, it is still possible that the smaller isoform is highly enzymatically active and accounts for most of the activity even
when it is expressed poorly. Interestingly, in at least two other species that express A3A homologs (sheep and cows), the predicted A3A protein sequence begins at the methionine equivalent to Met13 in human A3A (Fig. 8B). Additionally, the predicted sequence for macaque A3A also begins at a downstream methionine relative to full-length human A3A. Thus, other species may rely entirely on the equivalent of the small, enzymatically active A3A isoform.

**DISCUSSION**

The data presented here demonstrate that treatment with IFNα induces A3 deaminase activity in primary monocytes and MDMs. This deaminase activity is directed against TC motifs, is sustained at high levels for at least 60 h in the presence of IFNα, is induced by TLR signaling pathways that produce IFNα, and is down-regulated when PBMCs are treated simultaneously with IFNα and the T cell activator PHA. Additionally, this deaminase activity in monocytes requires new transcription and translation, is solely due to A3A isoforms without significant contribution from other TC-specific A3 deaminases, and is not inactivated by inhibitory RNAs. From these findings, we conclude that enzymatically active A3A isoforms in primary monocytes and MDMs are induced by IFNα and down-regulated by other signaling pathways. Thus, to date, A3A is the only A3 protein that has been demonstrated to be enzymatically active in lysates of primary cells. Moreover, the enzymatic activity of A3A isoforms in monocytes and MDMs contrasts strikingly with the inhibition of A3G deaminase activity observed in primary monocytes and MDMs in this report and previously demonstrated for A3G in resting and activated primary CD4+ T cells (49).

Although we found that both IFNα and IFNγ induced TC-specific deaminase activity in PBMCs, IFNα had a much larger effect. IFNα is the key component of the type 1 interferon response pathway, which induces a few hundred interferon-stimulated genes and can cause profound inhibition of viral replication (reviewed in Ref. 62). Although interferon-stimulated genes include a few well studied enzymes, such as protein kinase R, 2′,5′-oligoadenylate synthetase, RNase L, and the Mx GTPases, the actions of most interferon-stimulated genes are not well understood (52). Based on data reported here, the deaminase activity of A3A, but not A3G, can be added to the list of interferon-stimulated enzymatic responses. Moreover, our studies demonstrate that the pathways that govern deaminase activity and A3A induction in primary monocytes appear to be complex. In addition to identifying two cytokines that act as positive regulators of deaminase activity in PBMCs (IFNα and, to a lesser extent, IFNγ), we also demonstrated the existence of a negative regulatory pathway. Moreover, because viruses have developed numerous ways to counteract and subvert the type 1 interferon host response (63), it is possible that some viruses have evolved mechanisms for down-regulating A3A deaminase activity, either directly or by co-opting host regulatory pathways.

Exactly how A3A functions in primary cells in physiologically relevant settings remains somewhat unclear. While our work was under review, Stengelein et al. (57) published the finding that A3A acts enzymatically in primary monocytes to reduce stability of foreign DNA introduced by transient transfection. These findings indicate that one role of A3A in primary cells may be to recognize and clear foreign DNA. The striking induction of TC-specific deaminase activity that we observed in monocytes and MDMs in response to IFNα-inducing signaling pathways raises the possibility that A3A deaminase activity may also play an important role in the host defense against viral pathogens. However, the exact pathogen target(s) of A3A deaminase activity in vivo has not yet been determined. Our data suggest that the A3A target is likely to be a pathogenic virus that induces IFNα, infects monocytes and/or MDMs, and exists as single-stranded DNA at some point in its life cycle. One intriguing potential viral target of A3A is HPV. A3A is induced by PMA in primary keratinocytes (64, 65), which are targets of HPV infection in vivo (reviewed in Ref. 66), and G-to-A hypermutation has been identified in HPV DNA from plantar warts (45). Although it remains unclear which A3 protein causes TC mutation of HPV in vivo, A3A has been a candidate based on its tissue distribution, along with two other APOBEC proteins, APOBEC3C and APOBEC3H (reviewed in Ref. 57). The finding that endogenous A3A is highly enzymatically active in at least one primary cell type (monocytes/MDMs), reported here and by Stengelein et al. (57), provides yet another reason why A3A is an attractive candidate for causing HPV mutations in vivo.

It is also known that A3A inhibits LTR-containing and non-LTR retroelements (36, 68, 69) and is capable of inhibiting parvoviruses (36, 37), although it is not known if it encounters these agents or other viruses in vivo. Although the human parvoviruses examined in A3A studies are not human pathogens, other parvoviruses are pathogenic, including parvovirus B19, which causes a childhood disease called erythema infectiosum (slapped cheek syndrome; reviewed in Ref. 70). B19 typically infects erythroid precursors, but evidence of B19 infection has been reported in macrophages from patient samples (71), raising the possibility that MDMs may act as a first line of defense against this infection. Moreover, studies of transfected 293T and HeLa cells demonstrate that a significant fraction of A3A is localized to the nucleus (36, 69), where parvoviruses replicate. However, data suggesting that A3A acts by a deaminase-independent mechanism against the two parvoviruses that have been examined to date (37) as well as an LTR retrotransposon (68) argue that parvoviruses and retroelements might not be targets of the TC-specific deaminase activity of A3A isoforms.

HIV-1 is another virus that infects monocyte-derived primary cells and induces IFNα. However, to date, it remains unclear whether A3A has an inhibitory effect on HIV-1. Numerous studies have found that wild-type A3A expressed in 293T cells displays no antiretroviral activity against HIV-1 (5, 12, 36, 54, 68, 72, 73). Our preliminary studies using transiently transfected 293T cells concur with these reports.4 Consistent with A3A lacking antiretroviral activity is the finding that transfected A3A in 293T cells is not packaged into the core of HIV-1 particles (54, 72, 73). However, two studies in primary cells are

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4 B. K. Thielen and J. R. Lingappa, unpublished observations.
at odds with findings in 293T cells. One reported that A3A inhibits HIV-1 replication in monocytes (51), whereas the other reported that A3A expression correlates with inhibition of HIV-1 replication in dendritic cells (53). Neither study addressed the mechanism by which A3A could inhibit HIV-1 replication in these primary cell types, given that A3A is not properly packaged into the HIV-1 core. Interestingly, in the case of Rous sarcoma virus, an α-retrovirus, transfected A3A is packaged into virus particles, edits viral DNA, and inhibits replication (74). Thus, although the weight of evidence currently argues against A3A being able to inhibit HIV-1 replication, discrepancies remain to be resolved.

It is worth noting that our initial attempts to maintain untreated PBMCs or isolated monocytes in culture were complicated by sporadic, unintentional induction of TC-specific deaminase activity, with corresponding appearance of A3A isoforms. We were eventually able to eliminate this problem by altering our cell culture methods. We observed that many lots of FBS appeared to induce IFNα production by primary cells and that we could completely eliminate the unintentional induction of deaminase activity using anti-IFNα/β pretreatment. Interestingly, our siRNA transfection with control oligonucleotide also appeared to stimulate low levels of IFNα production, with resulting low levels of A3A induction that could be largely abolished by anti-IFNα/β pretreatment (see Fig. 6). Thus, in studying deaminase activity in primary cells, it is important to assess potential confounding factors, such as inadvertent IFNα production, which can have very significant undesired effects.

Another observation worth discussing concerns A3G protein levels in the primary cells we studied. We found that treatment with IFNα for 72 h did not cause a significant increase in A3G protein levels in whole PBMCs (Figs. 1 and 2B, blot) or in isolated CD4+ T cells and CD8+ T cells (Fig. 5A, blot). Similarly, TLR ligands that induce IFNα also failed to induce A3G protein levels in PBMCs (Fig. 2B, blot). In contrast, IFNα treatment of MDMs resulted in a large increase in A3G protein levels (Fig. 5C, blot). These findings are in very close agreement with those reported recently by Koning et al. (47), who found that A3G protein levels did not increase in IFNα-treated CD4+ T cells although they displayed an IFNα-mediated induction of A3G mRNA, whereas A3G protein levels did increase significantly in MDMs treated with IFNα. Thus, our data are consistent with previous reports that IFNα leads to higher steady state A3G protein levels in MDMs (47, 75, 76) but not in CD4+ T cells (47, 76).

One important unanswered question raised by our data concerns the mechanism by which the host genome is protected from A3 deaminase activity. We find that upon induction by IFNα, A3A in monocytes is not completely inactivated by inhibitory RNAs. Moreover, in contrast to the profound RNAse-independent inhibition of A3G that we have observed in T cells, monocytes, and MDMs, our data demonstrate that A3A is capable of being enzymatically active for days at a time in monocytes exposed to IFNα. These findings are puzzling given that A3G expressed in yeast (61) as well as ectopically expressed, unregulated activation-induced deaminase and APOBEC1 in humans (77–80) are capable of mutating human DNA. One possible explanation is suggested by our observation that treatment of PBMCs with IFNα and PHA leads to down-regulation of A3A synthesis and deaminase activity, rendering A3A active for only a short window of time. Although the details of this inhibitory pathway remain to be determined, it is possible that the induction of enzymatically active A3A may be only transient in vivo during the course of natural infection because of down-regulation by such inhibitory pathways. Thus, the extent of A3A deaminase activity in vivo may be determined by a complex dynamic between stimulatory and inhibitory pathways. Disruption of the balance between such pathways could explain the lack of A3A deaminase activity in monocyte cell lines derived from hematopoietic malignancies (supplemental Fig. 2).

We began this study by searching for situations in which the nearly global inhibition of A3 deaminase activity observed in primary cells of various types (49) (Fig. 1) is altered in response to physiologically relevant stimuli. In principle, this broad inhibition could be overcome in at least two ways. Either the RNAse-independent inhibition we have described for A3G could be reversed, resulting in activation of A3G and possibly other A3 proteins, or alternatively, a new A3 protein that is not subject to this inhibition could be induced. The findings reported here are consistent with the second mechanism because newly induced A3A is enzymatically active in the same cells in which A3G is abundantly expressed but enzymatically inhibited. However, it is still plausible that physiologically relevant scenarios exist in which A3G or another A3 protein in primary cells is relieved of inhibition, although this has yet to be demonstrated.

Finally, our studies raise the possibility that expression of other A3 deaminases, in addition to A3A and A3G, may be under the control of complex regulatory pathways when expressed endogenously in primary cells. Such regulation may not be recapitulated when A3 proteins are expressed in immortalized cell lines, as was the case in this study, emphasizing the importance of studying A3 proteins in primary cells. The finding that IFNα induces enzymatically active A3A in primary monocytes, reported here, supports a role for A3A-mediated deamination in innate immunity. Similarly, studying the regulation of other A3 deaminases in physiologically relevant systems could lead to insights concerning the function of other A3 proteins in vivo.

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**Innate Immunity and APOBEC3A Deaminase Activity in Monocytes**

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