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Cell-specific Regulation of APOBEC3F by Interferons

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Abstract Human cytidine deaminase APOBEC3F (A3F) has broad anti-viral activity against hepatitis B virus and retroviruses including human immunodeficiency virus type 1. However, its regulation in viral natural target cells such $CD4^+$ T lymphocytes, macrophages, and primary liver cells has not been well studied. Here we showed that A3F was up-regulated by interferon (IFN)-α in primary hepatocytes and multiple liver cell lines as well as macrophages. Although the IFN-α signaling pathway was active in T lymphoid cells and induction of other IFN stimulated genes such as PKR was detected, A3F and APOBEC3G (A3G) were not induced by IFN-α in these cells. Thus, additional factors other than known IFN-stimulated genes also regulated IFN-α-induced A3F expression distinctly. A3F and A3G expression levels in primary hepatocytes, especially after IFN-α stimulation, were comparable to those in $CD4^+$ T lymphocytes in some individuals. Significant variations of A3F and A3G expression in primary hepatocytes from various subjects were observed. Individual variations in A3F and/or A3G regulation and expression might influence the clinical outcomes of hepatitis B infection.

Key words APOBEC3F; interferon; human immunodeficiency virus type 1; hepatitis B virus; cytidine deaminase

APOBEC3G (A3G) and APOBEC3F (A3F) are members of the APOBEC family proteins with homologous cytidine deaminase domains [1]. Although their cellular targets are not known, A3G and A3F are potent antiviral proteins that can suppress human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), and endogenous retroelements [2–8]. The antiviral mechanism of A3G against HIV-1 has been well studied. In the absence of the viral Vif protein, the A3G cytidine deaminase converts cytidines to uridines in single-stranded viral cDNA during reverse transcription resulting in lethal hypermutation of the virus genome. The HIV-1 Vif protein, however, can degrade A3G through a proteasomal-dependent pathway involving cullin 5 containing E3 ubiquitin ligase [9]. By targeting A3G for degradation in the virus-producing cell, Vif is able to prevent A3G molecules from incorporating into virions where they would otherwise be carried into the newly infected cell to inhibit productive infection. Although HBV is a DNA virus, it replicates through reverse transcription and can likewise be targeted by A3G and A3F [10].

Many studies have focused on the antiviral effects and post-translational regulation of A3G and A3F. However, little is known about how they are transcriptionally regulated. It is not known if A3G or A3F could be induced *in vivo* in response to inflammation or cytokines, or whether A3G or A3F participate in interferon (IFN)-mediated host defenses is unclear. One previous study found that A3G was induced by phorbol myristate acetate but not by IFNs in an immortalized $CD4^+$ T cell line H9 [11]. However, a recent report suggested that A3G could be induced by IFNs in macrophages [12]. The effects of IFNs on A3G transcription in primary $CD4^+$ T cells have not been reported.

Regulation of A3F by IFNs in relevant cells such as hepatocytes, macrophages, and primary $CD4^+$ T cells has not been well studied. In the present study, we observed

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that IFN-α can up-regulate A3F mRNA in macrophages, primary hepatocytes, and liver cell lines but not in primary $CD4^+$ T cells. We also observed a wide variation in the level of A3F induction by IFN in primary hepatocytes from different subjects, suggesting that individual variation in IFN responsiveness might account for differences in viral pathogenesis and clinical outcomes of HBV infections.

Materials and Methods

Cell culture and IFN treatment

Freshly isolated primary hepatocytes from anonymous donors were obtained from BD Biosciences (San Jose, USA). Hepatocytes were received within 24–48 h of isolation as adherent cultures in 6-well plates in Hepato-STIM medium (BD Biosciences) and used immediately for induction studies. Hep3B, HepG2, and QSG7701 are hepatocellular carcinoma cell lines maintained in DMEM (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS). Primary $CD4^+$ T cells were purified from freshly isolated PBMC by incubation with CD4-conjugated magnetic microbeads (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions and cultured in RPMI 1640 with 10% FBS. To obtain macrophages, freshly isolated PBMC were plated in 6-well plates overnight at 2×10^7 cells/ml in RPMI 1640 with 10% FBS, after which non-adherent cells were removed and the medium replaced every 2 d. Macrophages, differentiated by adherence to the plastic, were used on day 12 after isolation for induction studies. All cytokines were obtained from EMD Biosciences. IFN-α and IFN-γ were dissolved in phosphate-buffered saline with 0.5% bovine serum albumin (control medium) and stored in single-use aliquots at −70 ºC. Unless otherwise stated, IFN-α was used at 1000 IU/ml and IFN-γ at 10 IU/ml. In IFN induction experiments, cells were treated with equal volumes of IFN or control medium.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was carried out according to standard protocols [13]. Briefly, total RNA from cells was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including an on-column DNase digestion step using the RNase-free DNase set (Qiagen). One-fifth of the RNA was reverse transcribed using random primers and the Multiscribe reverse transcriptase (Applied Biosystems, Foster City, USA). The cDNA was amplified using TaqMan universal PCR master mix (Applied Biosystems) and an ABI Prism 7000 sequence detection system (Applied Biosystems). The primer/probe sets were pre-designed TaqMan gene expression assays specific for A3G, A3F, PKR and IRF-1 (Hs00222415_m1, Hs00736570_m1, Hs00169345_m1 and Hs00233698_m1, respectively). Amplification of target genes was normalized using amplification levels of β-actin as an endogenous control (human ACTB endogenous control FAM/MGB probe; Applied Biosystems). The efficiency of the PCR was tested by amplification of the target from serially diluted cDNA generated from reverse transcription of a stock set of human RNA. Data analysis and calculations were carried out using the $2^{-\Delta\Delta CT}$ comparative method as previously described [13]. Gene expression is expressed as a fold induction of a gene measured in IFN-treated samples relative to samples treated with control media (phosphate-buffered saline plus 0.5% bovine serum albumin).

Results

A3F is induced by IFN-α in liver cell lines and primary hepatocytes

A3F was reported to have potent anti-HBV activity [10]. However, whether A3F expression is regulated by IFNs in liver cells is not known [14]. To examine the effect of IFNs on A3F transcription, we treated the various cell types with IFN-α, IFN-γ, or control media for various times. Cells were then collected to isolate RNA for measuring A3F mRNA by qRT-PCR. PKR and IRF-1 mRNA, inducible primarily by IFN-α and IFN-γ, respectively, were also measured as positive controls for IFN treatment. mRNA expression in IFN treated cells were expressed as a fold induction over control treated cells after normalizing to β-actin.

We observed that A3F mRNA was expressed in liver cell line HepG2 and, more importantly, its expression in these cells was up-regulated by IFN-α in a dose-dependent fashion [**Fig. 1(A)**]. As positive controls for IFN stimulation, we detected other known IFN stimulated genes (ISG) such as PKR, which was mainly induced by IFN-α [**Fig. 1(B)**], and IRF-1, which was primarily induced by IFN-γ [**Fig. 1(C)**]. The time courses of IFN-α and IFN-γ induced A3F expression was also examined. IFN-α induced A3F expression peaked 4–12 h after treatment in HepG2 cells [**Fig. 1(D)**]. IFN-γ induced A3F expression peaked 8–24 h after treatment in these cells [**Fig. 1(D)**]. A similar time-dependent pattern of IFN-α induced PKR expression [**Fig. 1(E)**] or IFN-γ induced IRF-1 expres-

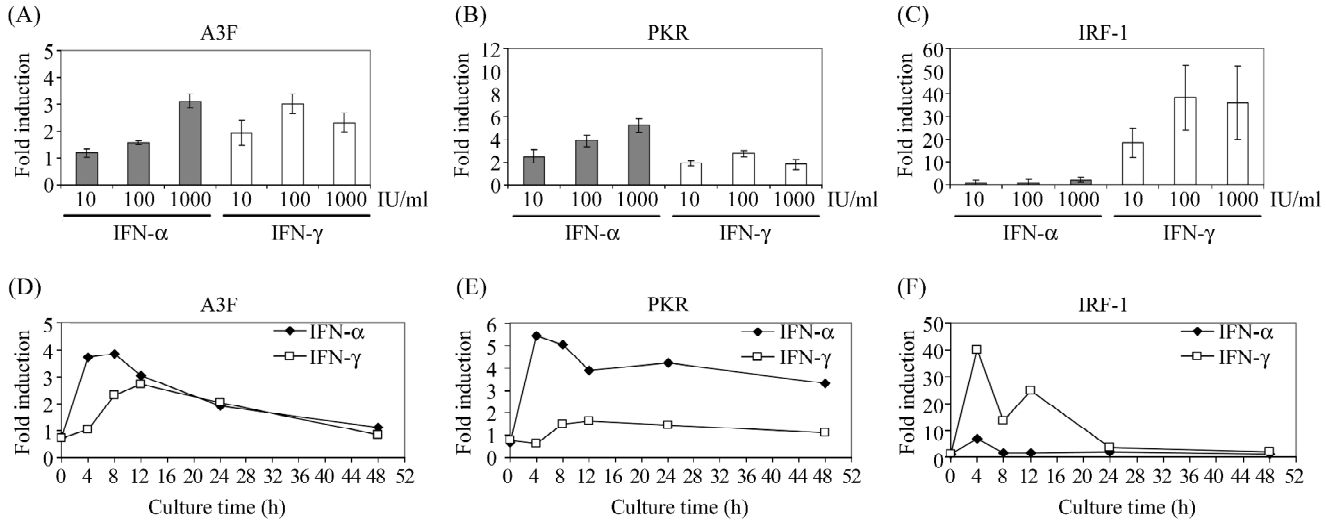

Fig. 1 Induction of human cytidine deaminase APOBEC3F (A3F) by interferons (IFNs) in HepG2 liver cells

(A–C) HepG2 cells were treated with IFN-α or IFN-γ at doses of 10, 100, or 1000 IU/ml or control medium (phosphate-buffered saline plus 0.5% bovine serum albumin) for 8 h. Induction of A3F, PKR, or IRF-1 by IFN was measured by quantitative real-time reverse transcription-polymerase chain reaction. Error bars represent standard deviations of a fold induction from triplicate cell samples. (D–F) HepG2 cells were treated with IFN-α (1000 IU/ml), IFN-γ (10 IU/ml), or control medium for 0, 4, 8, 12, 24 and 48 h. Relative mRNA expression of A3F, PKR, and IRF-1 was measured by the same method as in (A–C).

sion [**Fig. 1(F)**] was observed in HepG2 cells.

Our data differed from a recent report which did not detect any IFN induced A3F expression in HepG2 cells [14]. To determine if IFN induced A3F expression in liver cells is a common feature, we studied A3F expression in another liver cell line, Hep3B. Similar to HepG2 cells, IFN-α induced A3F expression in Hep3B cells in a dose- [**Fig. 2(A)**] and time-dependent fashion [**Fig. 2(D)**]. A3F induction was more sustained by IFN-γ than IFN-α in Hep3B cells [**Fig. 2(D)**]. PKR [**Fig. 2(B,E)**] as well as IRF-1 [**Fig. 2(C,F)**] mRNA were induced, indicating that the IFN doses used were effective for inducing known ISG. We also

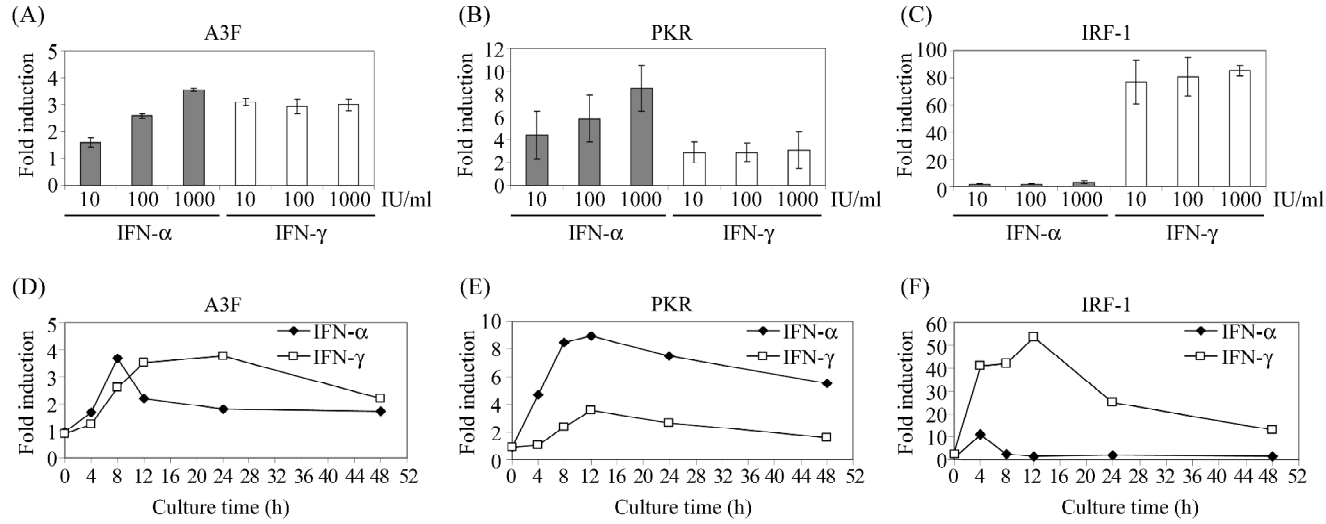

Fig. 2 Induction of human cytidine deaminase APOBEC3F (A3F) by interferons (IFNs) in Hep3B liver cells

(A–C) Hep3B cells were treated with IFN-α or IFN-γ at doses of 10, 100, or 1000 IU/ml or control medium (phosphate-buffered saline with 0.5% bovine serum albumin) for 8 h. Induction of A3F, PKR, or IRF-1 by IFN was measured by quantitative real-time reverse transcription-polymerase chain reaction. Error bars represent standard deviations of a fold induction from triplicate cell samples. (D–F) Hep3B cells were treated with IFN-α (1000 IU/ml), IFN-γ (10 IU/ml), or control medium for 0, 4, 8, 12, 24 and 48 h. Induction of A3F, PKR, and IRF-1 was measured by the same method as in (A–C).

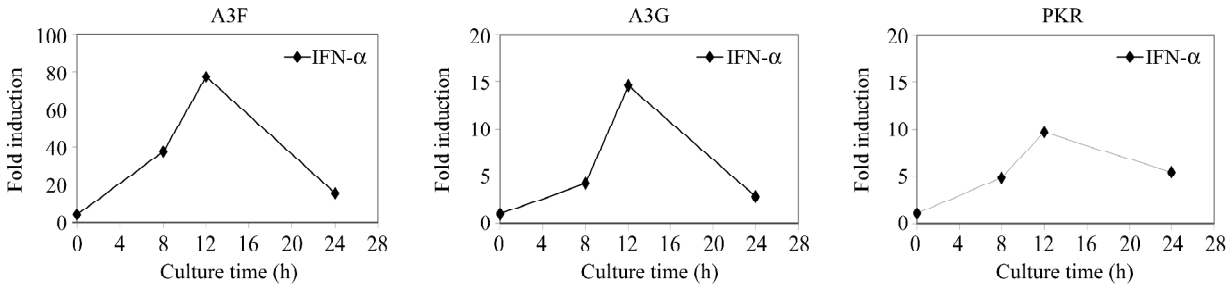

Fig. 3 Induction of human cytidine deaminase APOBEC3F (A3F) by interferon (IFN)-α in QSG7701 liver cells

QSG7701 cells were treated with IFN-α (1000 IU/ml) or control medium for 0, 8, 12, and 24 h. Relative mRNA expression of A3F, A3G, and PKR was measured by quantitative real-time reverse transcription-polymerase chain reaction.

observed A3F up-regulation by IFN-α in another liver cell line, QSG7701 (**Fig. 3**).

IFN-α only induced A3F expression moderately (3- to 4-fold) in HepG2 and Hep3B cell lines

It has been reported that A3F could be detected in liver tissues [15,16]. However, little is known about its regulation by IFNs in primary human hepatocytes. Therefore, we determined the effect of IFN treatment on A3F expression in cell-cultured primary hepatocytes from several healthy donors. We observed that A3F was detectable in primary hepatocytes from multiple donors and, more importantly, IFN-α induced A3F mRNA expression in these primary human hepatocytes [**Fig. 4(A)**]. However, A3F induction by IFN-α varied widely between 2- to 9-fold among various donors [**Fig. 4(A)**]. A previous study detected A3G induction by IFN-α in primary hepatocytes from a single donor [14]. Therefore, it is not known whether IFN-α induced A3G expression also varies among various donors. A3G induction by IFN-α in primary hepatocytes varied among various donors ranging from approximately 5- to 19-fold [**Fig. 4(A)**]. IFN-γ was less effective than IFN-α in up-regulating A3F in the primary hepatocytes [**Fig. 4(B)**] although IRF-1 was efficiently induced by IFN-γ in these primary cells [**Fig. 4(B)**].

A3F is not induced by IFN-α in primary CD4$^+$ T lymphocytes

Phorbol myristate acetate activates A3G in CD4$^+$ H9 T cells through protein kinase C activation [11]. However, IFN-α did not induce A3G expression in H9 cells [11]. We have also observed that IFN-α did not induce the expression of A3F in H9 cells (data not shown). The ability of IFN-α to induce A3F expression in primary CD4$^+$ T lymphocytes was evaluated. In contrast to liver cells, we observed that A3F was not induced by IFN-α or IFN-γ in primary CD4$^+$ T cells over a time course of 16 h [**Fig. 5 (A)**] or in primary CD4$^+$ T cells from several other individuals stimulated with IFNs for 8 h [**Fig. 5(E,F)**]. However, other ISG, including PKR and IRF-1, were readily induced by IFN-α and IFN-γ, respectively, in all primary CD4$^+$ T cells [**Fig. 5(B,C,E,F)**]. These data indi-

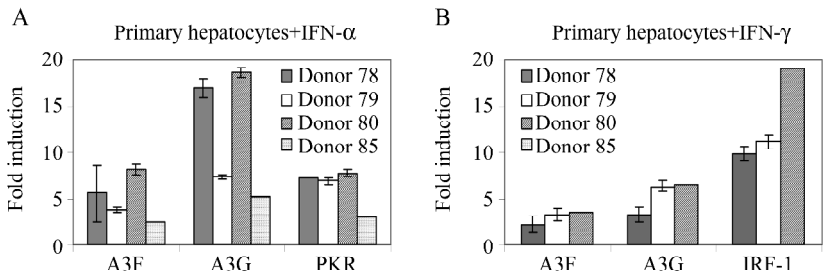

Fig. 4 Human cytidine deaminase APOBEC3F (A3F) is induced by interferons (IFNs) in primary hepatocytes

(A) Primary hepatocytes from four donors (78, 79, 80 and 85) were treated with IFN-α (1000 IU/ml) or control medium (phosphate-buffered saline with 0.5% bovine serum albumin) for 16 h. Induction of A3F, A3G, and PKR was measured by quantitative real-time reverse transcription-polymerase chain reaction. Error bars represent standard deviations of a fold induction from triplicate cell samples for each treatment from each donor (except for donor 85 in which only one cell sample was tested). (B) Primary hepatocytes from three donors (79, 80 and 85) were treated with IFN-γ (10 IU/ml) or control medium for 16 h, and induction of A3F, A3G, and IRF-1 was measured by the same method as in (A). Error bars represent standard deviations of a fold induction from triplicate cell samples for each treatment from each donor (except for donor 85 in which only one cell sample was tested).

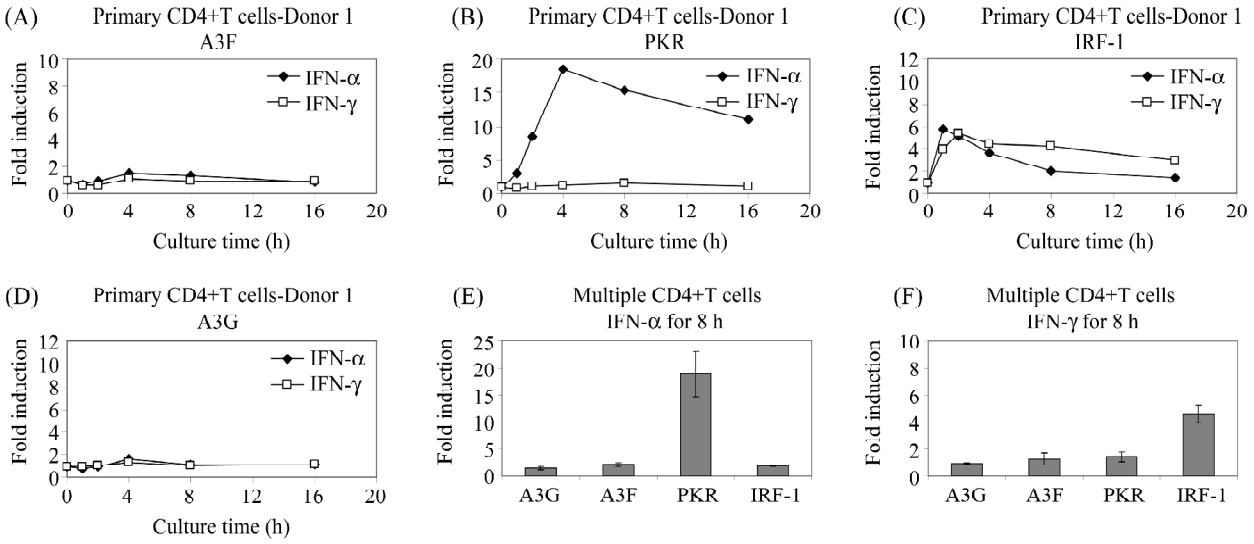

Fig. 5 Human cytidine deaminase APOBEC3F (A3F) is not up-regulated by interferons (IFNs) in T cells

(A–D) Primary CD4+ cells purified from fresh PBMC of donor 1 was treated with IFN-α (1000 IU/ml) or IFN-γ (10 IU/ml) or control medium for various times up to 16 h. Fold induction of A3F, PKR, IRF-1 and A3G in IFN treated over control cells was measured by quantitative real-time reverse transcription-polymerase chain reaction. (E,F) Primary CD4+ cells purified from fresh PBMC from 3 healthy donors were stimulated with IFN-α, IFN-γ, or control medium for 8 h, and induction of A3G, PKR, and IRF-1 was measured by the same method as in (A–D). Error bars represent standard deviations of a fold induction of single samples from each of the three individual PBMC donors.

cate that IFN mediated signaling pathway is functional in primary CD4+ T lymphocytes. Therefore, the transcriptional regulation of A3F by IFNs appears to be cell type-dependent and might be distinct from those of PKR and IRF-1. In addition to A3F, we have also observed that the related cytidine deaminase A3G was also not induced by IFN-α or IFN-γ in primary CD4+ T cells [**Fig. 5(D)**], an extension from a previous report [11].

A3F is induced by IFN-α in macrophages

A3G was not induced by IFN-α in H9 cells (data not shown and previous report [11]) or primary CD4+ T lymphocytes [**Fig. 5(D)**], but it was efficiently induced by IFN in macrophages from the same donor [**Fig. 6(A)**]. Similarly, IFN-α also induced A3F expression in macrophages from the same donor [**Fig. 6(B)**] from which A3F was not induced by IFN-α in primary CD4+ T lymphocytes [**Fig. 5(A)**]. Thus, A3F and A3G were not induced by IFN-α in primary CD4+ T lymphocytes but both were efficiently induced by IFN-α in macrophages. IFN-α induced consistent A3F expression in macrophages from various donors [**Fig. 6(B–D)**]. Interestingly, IFN-γ induced A3F expression in macrophages was more donor-dependent (**Fig. 6**). In one individual IFN-γ induced A3F expression in macrophages as efficiently as IFN-α [**Fig. 6(C)**], whereas in others IFN-γ induced no appreciable A3F expression [**Fig. 6(B,D)**].

Relative A3F and A3G expression in untreated or IFN-α treated primary liver cells versus CD+ T lymphocytes

Using qRT-PCR, we compared the relative levels of A3F and A3G mRNA in various liver cell lines, primary liver cells, and T cells that were either untreated (control) or treated with IFN-α (1000 IU/ml for 16 h). The relative expression of the target genes was normalized to the endogenous control gene β-actin. The expression levels of A3F and A3G, both lowest in the QSG liver cell line, were set as unitless values of 1 and A3F or A3G expression in all other cells were calculated as a relative value above 1. The A3G expression varied over four logs of magnitude between these cell types, and the order of expression in control (untreated) cells from lowest to highest were: liver cell lines (QSG, Huh7, Hep3B, HepG2), primary hepatocytes, primary CD4+ T cells (**Fig. 7**). IFN-α up-regulated A3G in the liver cell lines and primary liver cells, but not in primary CD4+ T cells. Although A3G was not up-regulated by IFN-α in T cells, A3G expression was relatively high in these cells. Interestingly, A3G expression in some of the primary liver cells after IFN induction approached the levels seen in T cells. Although IFN could induce A3G significantly in liver cell lines, the basal levels

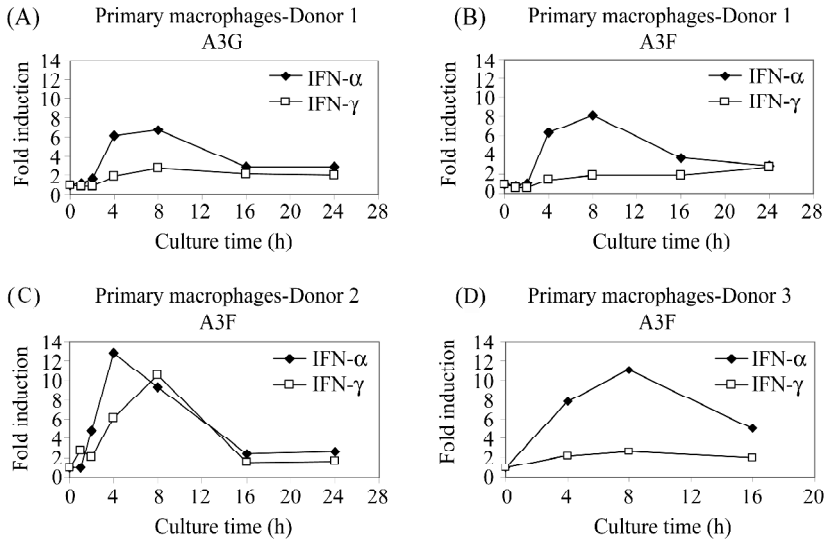

Fig. 6 Interferon (IFN)-α induces human cytidine deaminase APOBEC3G (A3G) and APOBEC3F (A3F) in primary macrophages

(A,B) Primary macrophages from donor 1 were stimulated with IFN-α (1000 IU/ml), IFN-γ (10 IU/ml), or control medium for 0,1, 2, 4, 8, or 16 h. Fold inductions of A3G and A3F were measured by quantitative real-time reverse transcription-polymerase chain reaction. (C,D) Primary macrophages from two other donors (donors 2 and 3) were stimulated with IFN-α (1000 IU/ml), IFN-γ (10 IU/ml), or control medium for various times up to 24 h (donor 3 was tested only up to 16 h). Fold inductions of A3F were measured by the same method as in (A,B).

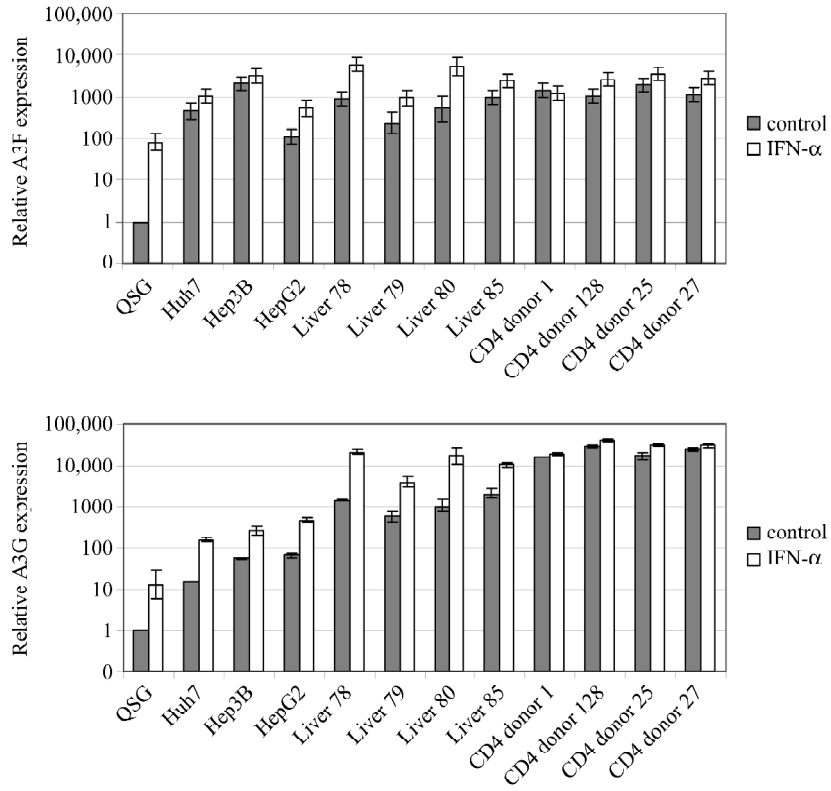

Fig. 7 Human cytidine deaminase APOBEC3F (A3F) and APOBEC3G (A3G) relative expression in interferon (IFN)-α treated primary liver cells versus CD⁺ T lymphocytes

mRNA expression of A3F or A3G was measured by quantitative real-time reverse transcription-polymerase chain reactionand normalized to β-actin in untreated and IFN-α-stimulated (16 h) liver cell lines (QSG, Huh7, Hep3B, HepG2), primary hepatocytes (liver donors 78, 79, 80, 85), and primary $CD4^+$ T cells ($CD4^+$ donors 1, 25, 27 and 128). The graphs show A3F or A3G expression relative to the calibrator, untreated QSG cells (value=1) which expressed the lowest amount of both A3F and A3G among the cells tested.

of A3G in those cells were low and remained relatively low even after IFN treatment as compared to the other cells tested.

In contrast to A3G expression, A3F expression between untreated cells was less variable. Except for the QSG cell line, where A3F expression was just above detection, A3F expression varied over just one log between the other cells tested. In general, A3F expression in untreated $CD4^+$ T cells was higher than in primary liver cells or liver cell lines, with the exception of Hep3B liver cells which had relatively high A3F expression at baseline. In all primary liver cells, IFN-α significantly up-regulated A3F. IFN-α also significantly up-regulated A3F in the QSG and HepG2 cell lines but less significantly in Huh7 or Hep3B cells at the same time point.

Discussion

Like A3G, A3F has potent antiviral activity against diverse retroviruses [3–8] and HBV [10]. A3F and the related cytidine deaminase A3G can now be classified among other well-known ISG, such as PKR, ISG15 and MX1 that mediate a variety of antiviral effects. Macrophages and liver cells are primary targets of lentiviruses and HBV, respectively. It is therefore plausible that up-regulation of A3F by IFNs could contribute to innate antiviral defenses against HBV in liver cells and lentiviruses in macrophages.

Our results differed considerably from a recent report which did not observe IFN-α induced A3F expression in HepG2 cells [14]. Plausible explanations for this divergence might be the differences in cell culture conditions, differences in the sources of IFN-α used in these studies, or differences in A3F quantifications. Induction of A3F in macrophages by IFN-α observed in this study was consistent with another recent report [12].

In addition to HepG2 cells, we have observed that A3F could also be induced by IFN-α in other liver cell lines Hep3B (**Fig. 2**) and QSG7701 (**Fig. 3**). More importantly, IFN-α efficiently induced A3F expression in primary hepatocytes from multiple healthy donors [**Fig. 4(B)**]. This is the first report showing that A3F was induced by IFN-α in primary hepatocytes and macrophages but not $CD4^+$ T cells. Although A3G could also be induced by IFN-α in primary hepatocytes (**Fig. 4** and previous report [14]), our data suggests that the IFN-α inducible anti-HBV A3F could contribute to HBV suppression *in vivo*. This argument would be consistent with the *in vivo* observation of HBV G-to-A mutation patterns characteristics of APOBEC3 cytidine deaminases [16–18]. A3G primarily mediates GG-to-GA mutations, whereas A3F mostly generates GA-to-AA mutations [3–8]. GA-to-AA mutations are highly represented in viral sequences recovered from HBV-infected individuals [17,18]. Therefore, A3F or other related APOBEC3 proteins in addition to A3G might represent a major selection force against HBV *in vivo*.

The abilities of spontaneous clearance of viral infection after HBV exposure differ significantly among various subjects [19–21]. Variations in the magnitudes of A3F induction by IFN-α differed among various healthy donors. We noted significant variations in IFN-induced A3F expression (**Fig. 4**) in primary hepatocytes from various subjects. Using primary hepatocytes from multiple donors, we also observed that expression of IFN induction of A3G in primary hepatocytes also varied significantly among individual donors (**Fig. 4**). This finding raises the interesting possibility that individual differences in APOBEC3 protein expression and interferon inducibility in liver cells might contribute to different courses of viral pathogenesis in infected individuals.

A3F was regulated differently than other IFN-α-responsive genes, such as PKR. PKR was induced by IFN-α in all cells tested: T cells, macrophages, and liver cells. A3F was induced in macrophages and liver cells but not H9 T cells or primary $CD4^+$ T lymphocytes. Similarly, A3G was also not induced by IFN-α in $CD4^+$ T lymphocytes (**Fig. 5**) or H9 cells [11]. H9 cells are relatively resistant to certain IFN induced responses including cell growth properties and down-regulation of c-myc [22–25]. However, we observed normal IFN-α-induced PKR expression in H9 cells as well as primary $CD4^+$ T lymphocytes, suggesting at least some IFN-α-mediated signaling pathways are functional in these T cells. It is conceivable that IFN-α-induced A3F or A3G expression requires an additional factor/s which is/are absent in T cells. Therefore, A3F and A3G appeared to be uniquely regulated by IFNs in a cell type-dependent manner. Consistent with previous reports [15,16], our data in multiple liver cell lines, primary hepatocytes, and macrophages also indicate that A3F and A3G expressions are coordinately regulated.

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