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# The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1

Nan Yan[1], Ashton D Regalado-Magdos[1], Bart Stiggelbout[1], Min Ae Lee-Kirsch[2] & Judy Lieberman[1]



**Viral infection triggers innate immune sensors to produce type I interferon. However, infection of T cells and macrophages with human immunodeficiency virus (HIV) does not trip those alarms. How HIV avoids activating nucleic acid sensors is unknown. Here we found that the cytosolic exonuclease TREX1 suppressed interferon triggered by HIV. In *Trex1*$^{-/-}$ mouse cells and human CD4$^+$ T cells and macrophages in which *TREX1* was inhibited by RNA-mediated interference, cytosolic HIV DNA accumulated and HIV infection induced type I interferon that inhibited HIV replication and spreading. TREX1 bound to cytosolic HIV DNA and digested excess HIV DNA that would otherwise activate interferon expression via a pathway dependent on the kinase TBK1, the adaptor STING and the transcription factor IRF3. HIV-stimulated interferon production in cells deficient in TREX1 did not involve known nucleic acid sensors.**

Human immunodeficiency virus (HIV) introduces its single-stranded RNA (ssRNA) genome into a cell in the reverse-transcription complex, which matures into the preintegration complex. This complex delivers reverse-transcribed HIV DNA to the nucleus for chromosomal integration. Few copies of HIV DNA integrate, leaving behind HIV DNA in the cytosol to be cleared by host enzymes. Although nucleic acids in the reverse-transcription complex might be shielded from nucleic acid sensors, viral DNA in the preintegration complex is accessible to exogenous endonucleases[1] and thus is potentially accessible to cytosolic sensors of innate immunity. The endoplasmic reticulum–associated SET complex, which contains three DNases (APE1, NM23-H1 and TREX1) and other proteins (SET, pp32 and HMGB2), binds to the HIV preintegration complex and protects the integrase-activated DNA ends from self attack in suicidal autointegration[2]. Suppressing the expression of any gene encoding a molecule of the SET complex increases autointegration and interferes with chromosomal integration. TREX1 is the most abundant 3′-5′ DNase in cells[2]. Treatment with *TREX1*-specific small interfering RNA (siRNA) inhibits HIV replication more profoundly than do siRNAs specific for other SET complex components, decreasing viral production by a log[2]. *TREX1* mutations are associated with inflammatory and autoimmune diseases, including Aicardi-Goutieres syndrome, chilblain lupus, and systemic lupus erythematosus, some of which have more type I interferon[3–5]. TREX1 binds to transfected immunostimulatory DNA, and *Trex1*$^{-/-}$ cells accumulate cytoplasmic DNA derived from endogenous retroelements, which activates interferon expression dependent on interferon-regulatory factor 3 (IRF3)[6–8]. Like HIV, endogenous retroelements undergo cytoplasmic reverse transcription. We therefore investigated whether HIV might use TREX1 to avoid triggering antiviral innate immunity.

## RESULTS

### TREX1 inhibits interferon production in response to HIV

We first compared the replication of HIV and the expression and secretion of type I interferons and inflammatory cytokines after infecting *Trex1*$^{+/+}$ (wild-type) and *Trex1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) with vesicular stomatitis virus G (VSV-G)-pseudotyped HIV-luciferase virus, a single-round virus that does not produce infectious virus and has a nearly full-length HIV genome (lacking the gene encoding the envelope protein and with replacement of the gene encoding negative factor (Nef) with a gene encoding luciferase). This virus can infect MEFs, integrate and execute long terminal repeat (LTR)-driven expression of the luciferase reporter[9]. After being infected with HIV-luciferase, *Trex1*$^{-/-}$ MEFs had luciferase activity one tenth that of wild-type MEFs (**Fig. 1a**). Uninfected *Trex1*$^{-/-}$ MEFs constitutively expressed slightly more interferon-β (IFN-β) mRNA than did wild-type MEFs (**Fig. 1b**). In *Trex1*$^{-/-}$ cells, HIV infection induced both IFN-β mRNA (~100-fold more than that of uninfected cells) and interleukin 6 (IL-6) mRNA (~10-fold more than that of uninfected cells; **Fig. 1b**,**c**). Neither IFN-β nor IL-6 was induced by HIV infection of wild-type MEFs. HIV did not induce IL-1β, IFN-α or IFN-γ in wild-type or *Trex1*$^{-/-}$ MEFs (data not shown). IFN-β was secreted, as assessed by enzyme-linked immunoassay (ELISA) of cultured supernatants (**Fig. 1d**). Nevirapine, which inhibits HIV

[1]Immune Disease Institute and Program in Cellular and Molecular Medicine, Children's Hospital, Boston, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA. [2]Children's Hospital, Technical University Dresden, Dresden, Germany. Correspondence should be addressed to J.L. (lieberman@idi.harvard.edu).

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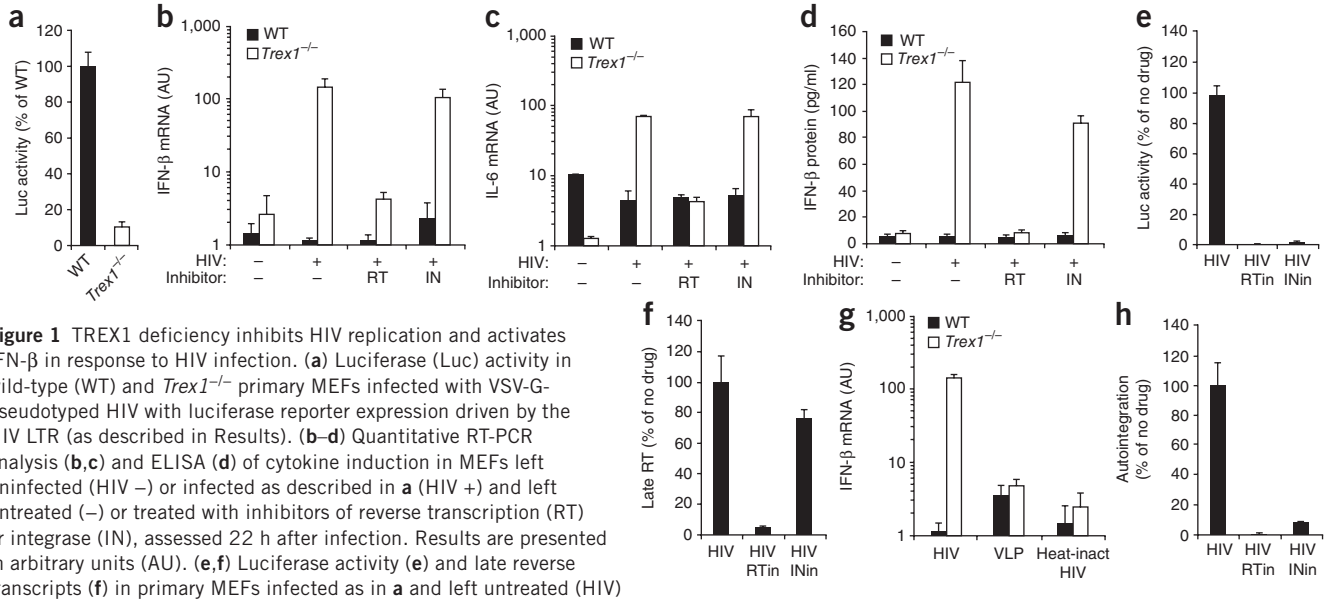


**Figure 1** TREX1 deficiency inhibits HIV replication and activates IFN-β in response to HIV infection. (**a**) Luciferase (Luc) activity in wild-type (WT) and *Trex1*$^{-/-}$ primary MEFs infected with VSV-G-pseudotyped HIV with luciferase reporter expression driven by the HIV LTR (as described in Results). (**b**–**d**) Quantitative RT-PCR analysis (**b**,**c**) and ELISA (**d**) of cytokine induction in MEFs left uninfected (HIV –) or infected as described in **a** (HIV +) and left untreated (–) or treated with inhibitors of reverse transcription (RT) or integrase (IN), assessed 22 h after infection. Results are presented in arbitrary units (AU). (**e**,**f**) Luciferase activity (**e**) and late reverse transcripts (**f**) in primary MEFs infected as in **a** and left untreated (HIV) or treated with inhibitors of reverse transcription (HIV RTin) or integrase (HIV INin), each added at the same time as HIV, assessed 48 h (**e**) or 10 h (**f**) after infection. (**g**) IFN-β induction in MEFs infected with HIV (far left) or equivalent amounts (based on p24 ELISA) of virus-like particles (VLP) or HIV inactivated by heating for 5 min at 95 °C (Heat-inact HIV); results are presented in arbitrary units (AU). (**h**) HIV autointegration in primary MEFs infected and treated with inhibitors as in **e**,**f**. Data are representative of at least three independent experiments (error bars, s.d.).



reverse transcription, inhibited the expression of IFN-β and IL-6 in response to HIV in *Trex1*$^{-/-}$ cells, but the integrase inhibitor raltegravir, which acts after the synthesis of HIV DNA, did not (**Fig. 1b**–**f**); this suggested that reverse-transcribed HIV DNA, rather than genomic RNA, was triggering the response. Treatment of the virus with DNase did not alter the IFN-β response to HIV-luciferase in *Trex1*$^{-/-}$ cells (data not shown), which eliminated concerns that carryover of plasmid DNA was responsible for the induction of IFN-β. Virus-like particles lacking genomic RNA and heat-inactivated HIV also did not trigger IFN-β expression (**Fig. 1g**), which suggested that viral nucleic acid and an infectious virus were required. Because nonproductive autointegrated (autointegrant) DNA accumulates when *TREX1* is inhibited by siRNA[2], autointegrant DNA might have been triggering IFN-β. However, although only the reverse-transcription inhibitor suppressed IFN-β production, both the reverse-transcription inhibitor and integrase inhibitor blocked the production of autointegrants (**Fig. 1h**). These results suggest that the HIV-stimulated production of IFN-β in *Trex1*$^{-/-}$ MEFs was activated by HIV DNA other than autointegrant DNA.

## HIV-stimulated interferon expression is IRF3 dependent

IFN-β expression induced by transfected immunostimulatory DNA or endogenous retroelements in *Trex1*$^{-/-}$ cells is mediated by the transcription factor IRF3 (refs. 6,7). To investigate whether IRF3 also activates HIV-induced expression of IFN-β, we compared IFN-β mRNA and HIV infectivity in wild-type, *Trex1*$^{-/-}$ and *Trex1*$^{-/-}$*Irf3*$^{-/-}$ MEFs. Lack of IRF3 completely inhibited IFN-β induction (**Fig. 2a**). HIV-luciferase activity was also partially restored in *Trex1*$^{-/-}$*Irf3*$^{-/-}$ cells (**Fig. 2b**). Therefore, the IFN-β induction in response to HIV in *Trex1*$^{-/-}$ cells was mediated by IRF3. Autointegration in the absence of *Trex1* was indistinguishable in *Trex1*$^{-/-}$ and *Trex1*$^{-/-}$*Irf3*$^{-/-}$ cells (**Fig. 2c**), which suggested that autointegration is not altered by endogenous IFN-β production and that the two effects of TREX1 on HIV infection (blocking autointegration and inhibiting IFN-β induction) operate independently. Similarly, in human 293T embryonic kidney cells, *TREX1*-specific siRNA resulted in higher HIV-induced luciferase expression from the *IFNB* promoter, whereas siRNAs specific for some other SET complex genes had no effect on IFN-β expression (**Supplementary Fig. 1**). Therefore, protection from IFN-β activation involves TREX1 but not the entire SET complex.

Activation of IRF3 triggers its nuclear translocation. To verify the role of IRF3 in HIV-induced IFN-β expression in *Trex1*$^{-/-}$ cells, we monitored IRF3 localization by confocal microscopy. IRF3 was cytoplasmic in uninfected wild-type and *Trex1*$^{-/-}$ MEFs (**Fig. 2d**). After infection with VSV-G-pseudotyped HIV expressing green fluorescent protein (HIV-GFP) at a multiplicity of infection (MOI) of 1, 68% of wild-type MEFs were infected, as assessed by GFP expression. At the same MOI, viral entry was similar in *Trex1*$^{-/-}$ cells (assessed by quantitative RT-PCR for HIV genomic RNA; data not shown), but GFP expression was much lower (**Fig. 2e**). IRF3 remained cytoplasmic in 100% of wild-type cells but translocated into the nucleus in 44% of *Trex1*$^{-/-}$ cells (**Fig. 2d**). These data confirm that the HIV-stimulated IFN-β response is IRF3 dependent and indicate the involvement of a cytosolic detection pathway. Likewise, these findings suggest that HIV DNA is not sensed by Toll-like receptor 9, which is contained in endosomes and activates type I interferon through IRF7 rather than IRF3 (refs. 10,11).

## HIV reverse transcripts accumulate in *Trex1*$^{-/-}$ cells

To gain insight into how TREX1 suppresses HIV-stimulated IFN-β induction, we measured cytosolic HIV DNA and IFN-β mRNA in wild-type and *Trex1*$^{-/-}$ cells during infection with HIV-GFP (MOI = 1; **Fig. 3a**). We also measured incoming HIV genomic RNA at 2 h and 5 h after infection to verify that wild-type and *Trex1*$^{-/-}$ cells were infected with the same amount of virions (**Fig. 3b**). Cytosolic HIV DNA steadily increased for the first 20 h after infection and then achieved a three- to fourfold higher plateau in *Trex1*$^{-/-}$ cells than in wild-type cells. In wild-type cells, IFN-β mRNA remained at baseline. In *Trex1*$^{-/-}$ cells, IFN-β mRNA was induced but lagged behind the accumulation of HIV DNA, first increasing 12 h after infection.

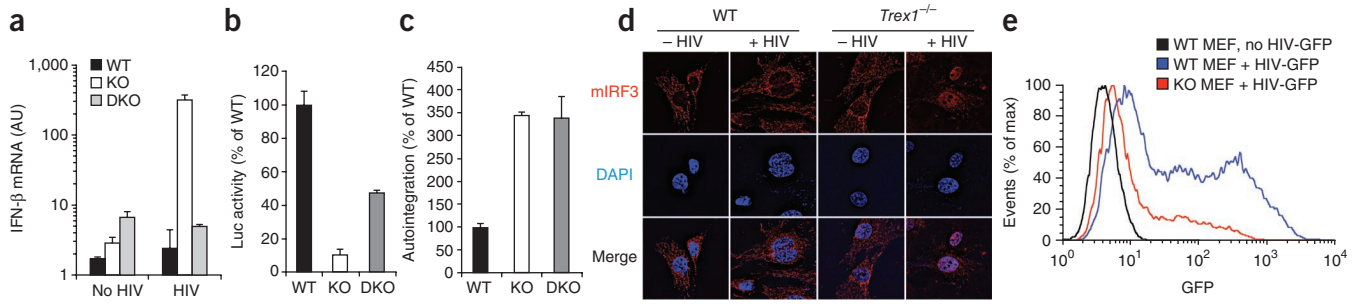


**Figure 2** HIV-stimulated expression of interferon is IRF3 dependent. (**a**) Quantitative RT-PCR analysis of IFN-β induction in wild-type (WT), *Trex1*$^{-/-}$ (KO) and *Trex1*$^{-/-}$*Irf3*$^{-/-}$ (DKO) primary MEFs infected with VSV-G-pseudotyped HIV-GFP, assessed 22 h after infection; results are presented in arbitrary units. (**b**) Luciferase activity of primary MEFs infected with HIV-luciferase, assessed 48 h after infection. (**c**) HIV autointegration in primary MEFs infected as in **a**. (**d**) Epifluorescence microscopy of the translocation of IRF3 to the nucleus in wild-type and *Trex1*$^{-/-}$ MEFs left uninfected (– HIV) or infected with HIV (+ HIV) and stained 22 h later for IRF3 (red) and with the DNA-intercalating dye DAPI (blue). Original magnification, ×63. (**e**) Flow cytometry analysis of GFP expression in wild-type and *Trex1*$^{-/-}$ MEFs infected as in **a**, assessed 24 h after infection. Data are representative of three experiments (**a**,**b**), at least three independent experiments (**c**; error bars (**a**–**c**), s.d.) or two experiments (**d**), or are from one of three independent experiments (**e**).

IFN-β mRNA peaked 20–24 h after infection and then plummeted to close to baseline, even though cytosolic HIV DNA remained high. The induction of IFN-β and accumulation of cytosolic HIV DNA increased in tandem in *Trex1*$^{-/-}$ cells as the amount of virus used for infection increased, but even the highest viral dose (MOI = 8) did not stimulate IFN-β expression in wild-type cells (**Fig. 3c**,**d**). Although cytosolic HIV DNA was about tenfold more abundant in *Trex1*$^{-/-}$ MEFs than in wild-type MEFs, HIV DNA integration was lower in *Trex1*$^{-/-}$ cells than in wild-type cells (**Fig. 3e**), which suggested that most of the HIV DNA that accumulated in *Trex1*$^{-/-}$ cells did not contribute to productive infection.

### HIV-stimulated IFN-β from *Trex1*$^{-/-}$ cells inhibits HIV

Type I interferons inhibit the replication of most viruses by both cell-autonomous and non–cell-autonomous effects and block both early and late stages of the HIV life cycle[12–15]. To determine whether IFN-β (and potentially other cytokines) secreted during HIV infection of *Trex1*$^{-/-}$ cells suppressed new HIV infection, we infected wild-type MEFs with VSV-G-pseudotyped HIV-luciferase in the presence of conditioned medium collected from wild-type, *Trex1*$^{-/-}$ or *Trex1*$^{-/-}$*Irf3*$^{-/-}$ MEFs infected with HIV-GFP or mock infected. Only conditioned medium from HIV-infected *Trex1*$^{-/-}$ MEFs inhibited luciferase activity (by a factor of 4; **Fig. 3f**). Preincubating the conditioned medium from *Trex1*$^{-/-}$ cells with neutralizing antibody to mouse IFN-β abrogated the antiviral effect (**Fig. 3g**). Infection with HIV-luciferase was inhibited similarly in cells incubated with mouse IFN-β at a concentration of 100 pg/ml or with conditioned medium from HIV-infected *Trex1*$^{-/-}$ MEFs, which contained IFN-β at a concentration of 120 pg/ml (**Figs. 1d** and **3h**). These findings suggest that IFN-β is the main secreted antiviral factor.

To pinpoint which stage(s) of HIV replication IFN-β blocks, we treated wild-type MEFs with mouse IFN-β and measured HIV DNA synthesis, two-LTR circle formation and integration. To assess

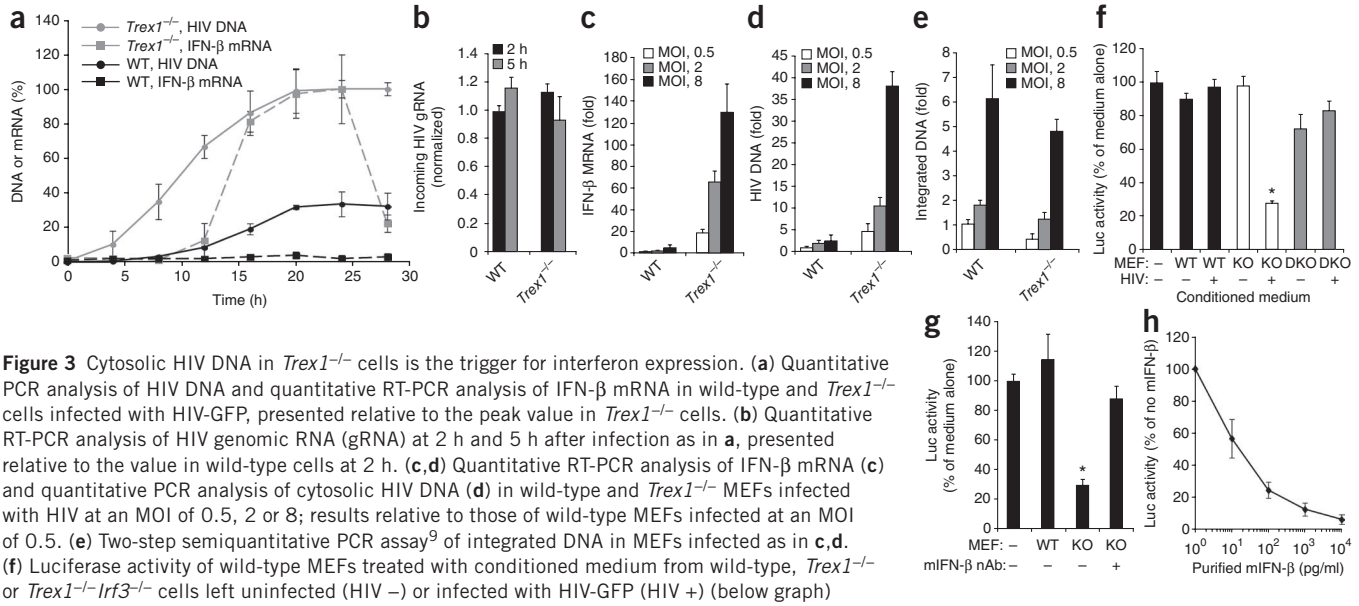


**Figure 3** Cytosolic HIV DNA in *Trex1*$^{-/-}$ cells is the trigger for interferon expression. (**a**) Quantitative PCR analysis of HIV DNA and quantitative RT-PCR analysis of IFN-β mRNA in wild-type and *Trex1*$^{-/-}$ cells infected with HIV-GFP, presented relative to the peak value in *Trex1*$^{-/-}$ cells. (**b**) Quantitative RT-PCR analysis of HIV genomic RNA (gRNA) at 2 h and 5 h after infection as in **a**, presented relative to the value in wild-type cells at 2 h. (**c**,**d**) Quantitative RT-PCR analysis of IFN-β mRNA (**c**) and quantitative PCR analysis of cytosolic HIV DNA (**d**) in wild-type and *Trex1*$^{-/-}$ MEFs infected with HIV at an MOI of 0.5, 2 or 8; results relative to those of wild-type MEFs infected at an MOI of 0.5. (**e**) Two-step semiquantitative PCR assay[9] of integrated DNA in MEFs infected as in **c**,**d**. (**f**) Luciferase activity of wild-type MEFs treated with conditioned medium from wild-type, *Trex1*$^{-/-}$ or *Trex1*$^{-/-}$*Irf3*$^{-/-}$ cells left uninfected (HIV –) or infected with HIV-GFP (HIV +) (below graph) and then infected with HIV-luciferase, assessed 48 h after infection and presented relative to the activity of cells incubated with medium alone (far left). (**g**) Luciferase activity of wild-type and *Trex1*$^{-/-}$ MEFs treated with medium alone (far left; MEF –, mIFN-β nAb –) or with conditioned medium (preincubated with (+) or without (–) neutralizing antibody to mouse IFN-β (mIFN-β nAb)) and infected with HIV-luciferase. (**h**) Luciferase activity of wild-type MEFs infected with HIV-luciferase and treated with various concentrations (horizontal axis) of purified recombinant mouse IFN-β (mIFN-β). $^{*}P < 0.01$ (Student's *t*-test). Data are representative of three independent experiments (error bars, s.d.).

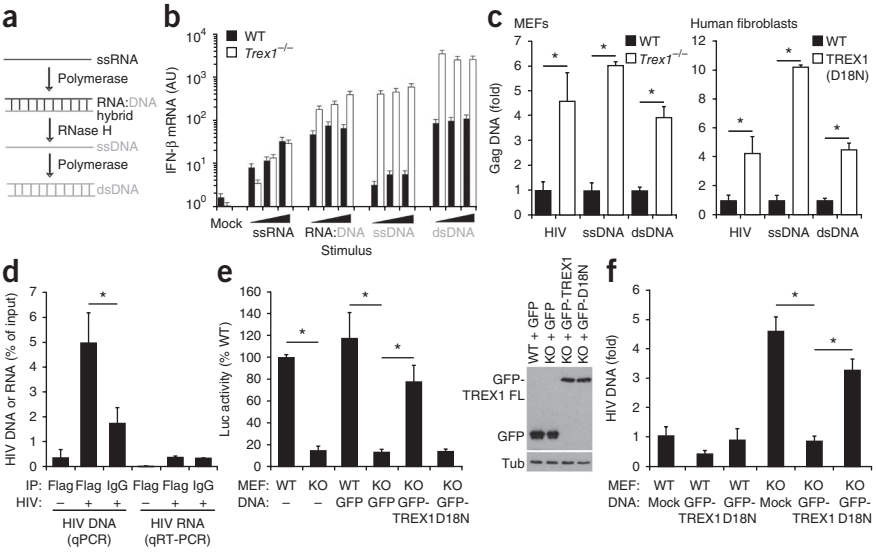


**Figure 4** Recognition of HIV products of reverse transcription by enzymatically active TREX1 suppresses interferon induction. (**a**) Synthetic nucleic acids used in **b** as generated during reverse transcription. (**b**) Quantitative RT-PCR analysis of IFN-β expression by wild-type and *Trex1*$^{-/-}$ cells mock-transfected (Mock) or transfected with the synthetic nucleic acids in **a** and assessed 6 h later. (**c**) Quantitative PCR analysis of cytosolic DNA in wild-type and *Trex1*$^{-/-}$ MEFs (left) or wild-type and TREX1-mutant (TREX1(D18N)) human fibroblasts (right) infected with HIV or transfected with ssDNA and dsDNA oligonucleotides of Gag-encoding sequence (100 base pairs), assessed with primers for sequence encoding Gag 10 h after infection or 3 h after transfection. *$P < 0.01$ (Student's *t*-test). (**d**) Immunoprecipitation (IP), with anti-Flag or immunoglobulin G (IgG), of cytosolic lysates of HeLa-CD4 cells expressing Flag-tagged TREX1, left uninfected (HIV –) or infected for 10 h with HIV IIIB (HIV +), followed by quantitative PCR (qPCR) or quantitative RT-PCR (qRT-PCR), respectively, of DNA and RNA extracted from the immunoprecipitates. *$P < 0.05$ (Student's *t*-test). (**e**) Luciferase activity (left) of wild-type and *Trex1*$^{-/-}$ MEFs left untransfected (–) or transfected to express GFP alone (GFP) or GFP-tagged TREX1, either wild-type (GFP-TREX1) or an enzymatically inactive mutant (GFP-D18N), and then infected with HIV-luciferase, assessed 48 h after infection. *$P < 0.01$ (Student's *t*-test). Right, immunoblot analysis of TREX1, GFP and tubulin (Tub; loading control). GFP-TREX1 FL, full-length TREX1 (wild type or D18N) fused to GFP. (**f**) Quantitative PCR analysis of HIV DNA in wild-type and *Trex1*$^{-/-}$ MEFs treated as in **e**; results are presented relative to those of mock-transfected wild-type MEFs. *$P < 0.01$ (Student's *t*-test). Data are representative of three independent experiments (**b**,**c**,**e**; error bars, s.d.) or two independent experiments (**d**,**f**; error bars, s.d. of triplicates).

LTR-mediated transcription, we incubated the well characterized cell line TZM-bl, which is derived from human HeLa-CD4 cervical cancer cells that have an integrated copy of an LTR-driven luciferase reporter gene, with human IFN-β. At the IFN-β concentration in conditioned medium from infected *Trex1*$^{-/-}$ cells, IFN-β substantially blocked HIV integration and LTR-mediated transcription (**Supplementary Fig. 2**). Higher doses of IFN-β blocked nearly all early stages of HIV replication in single-round infection. Thus, secreted IFN-β inhibits multiple steps in the early phase of HIV infection.

## TREX1 digestion of HIV DNA blocks interferon induction

To identify which HIV nucleic acids trigger IFN-β expression, we transfected wild-type and *Trex1*$^{-/-}$ MEFs with synthetic 100–base pair oligonucleotides containing sequences from the HIV gene encoding the group-associated antigen (Gag) protein, which corresponded to HIV nucleic acids in the cytosol during reverse transcription (ssRNA to represent genomic RNA, RNA-DNA hybrids, single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA)), and measured IFN-β mRNA 6 h later by quantitative RT-PCR (**Fig. 4a**,**b**). All DNA-containing oligonucleotides induced more IFN-β in *Trex1*$^{-/-}$ MEFs than in wild-type MEFs, but ssRNA did not. We found that ssDNA elicited the largest difference (~200-fold more IFN-β for ssDNA compared with 20- to 40-fold more for dsDNA and 2- to fourfold for RNA-DNA duplexes). None of the oligonucleotides induced IFN-β in *Trex1*$^{-/-}$*Irf3*$^{-/-}$ MEFs (data not shown), which supported the idea of IRF3's role in signaling the presence of these cytosolic nucleic acids. Although synthetic oligonucleotides may not completely mimic native HIV products of reverse transcription, these data are consistent with the known enzymatic preference of TREX1 for ssDNA substrates rather than dsDNA substrates[16]. To determine whether transfected oligonucleotides accumulated in *Trex1*$^{-/-}$ cells as HIV DNA does during infection, we quantified cytosolic DNA after transfection of ssDNA or dsDNA containing the sequence encoding Gag or after HIV infection. Cytosolic DNA was four- to sixfold more abundant in *Trex1*$^{-/-}$ MEFs than in wild-type MEFs when measured 3 h after transfection or 10 h after infection (**Fig. 4c**). We repeated this experiment with human fibroblasts derived from a patient with chilblain lupus that expressed either wild-type TREX1 or mutant TREX1 (with substitution of asparagine for aspartic acid at position 18 (D18N)). D18N is a substitution in a highly conserved $Mg^{2+}$-binding site in the Exo1 domain of TREX1 that eliminates exonuclease activity and interferes with the enzymatic activity of the wild-type–mutant TREX1 heterodimer[3,17]. Cells expressing the D18N mutant also accumulated all DNA species introduced by infection or transfection (**Fig. 4c**). The accumulation of HIV DNA in *Trex1*$^{-/-}$ or TREX1 mutant cells is consistent with a published report showing that the cytosol of *Trex1*$^{-/-}$ cells has more ssDNA derived from endogenous retroelements[7]. These results suggest that TREX1 suppresses IFN-β induction by digesting cytosolic DNA.

For further evidence that TREX1 is responsible for removing extraneous cytosolic HIV DNA, we assessed whether TREX1 interacted with HIV DNA during infection with wild-type HIV strain IIIB. We infected HeLa-CD4 cells expressing Flag-tagged TREX1 with HIV IIIB for 10 h, then obtained cytosolic extracts of these cells and immunoprecipitated proteins with antibody to Flag (anti-Flag) or immunoglobulin G (control antibody). We assessed enrichment for HIV DNA and RNA encoding Gag in the precipitates by quantitative PCR or quantitative RT-PCR, respectively. Anti-Flag immunoprecipitated threefold more HIV DNA than did the immunoglobulin G control, but there was no enrichment for HIV RNA (**Fig. 4d**), which confirmed that HIV DNA binds to TREX1 and is its 'preferred' target.

To determine whether the enzymatic activity of TREX1 is needed to enhance HIV infection, we assessed whether expression of wild-type or D18N TREX1 in *Trex1*$^{-/-}$ cells could restore HIV-luciferase infectivity (**Fig. 4e**). Wild-type TREX1 partially restored HIV infection, but the enzymatically inactive D18N mutant had no effect, which indicated that TREX1's exonuclease activity is needed for both inhibiting autointegration and blocking IFN-β induction. We also measured the accumulation of HIV DNA in wild-type and *Trex1*$^{-/-}$ cells transfected with GFP-tagged

wild-type or D18N TREX1. As shown above (**Fig. 3**), *Trex1*$^{-/-}$ cells accumulated about five times more cytosolic HIV DNA than did wild-type cells. The excess HIV DNA was completely eliminated by expression of GFP-tagged wild-type TREX1 but not by expression of GFP-tagged D18N TREX1 (**Fig. 4f**). These results suggest that TREX1 metabolizes cytosolic HIV DNA. Because overexpressing GFP-tagged wild-type TREX1 did not diminish HIV DNA abundance below that in an infected wild-type cell, TREX1 may not have access to all HIV DNA products.

### HIV activates interferon in *TREX1*-deficient human cells

Most experiments in this study reported above used mouse *Trex1*$^{-/-}$ cells to take advantage of their complete lack of TREX1, in contrast to the incomplete inhibition afforded by siRNAs. To investigate whether our findings were physiologically relevant during HIV infection of primary human immune cells, we used siRNA to suppress *TREX1* alone or both *TREX1* and *IRF3* in monocyte-derived macrophages (MDMs) from two human donors. At 3 d after siRNA treatment, we infected siRNA-treated cells, which had 60–80% less TREX1 and/or IRF3 mRNA than did cells treated with control siRNA, with the BaL strain of HIV (**Fig. 5a–c**). At 24 h after infection, cytosolic HIV DNA was about fourfold higher in samples treated with siRNA specific for *TREX1* or for both *TREX1* plus *IRF3* than in samples treated with control siRNA. HIV replication and spreading in cultures of macrophages treated with *TREX1*-specific siRNA was one fourth to one half that in control cells, as assessed by measurement of HIV Gag p24 antigen in the medium. When expression of both *IRF3* and *TREX1* was suppressed, HIV replication was partially restored (**Fig. 5d**), which suggested that IRF3-dependent induction of IFN-β contributed to the inhibition of HIV replication caused by *TREX1*-specific siRNA. Consistent with that idea, expression of both IFN-α and IFN-β mRNA increased up to tenfold in macrophages treated with *TREX1*-specific siRNA, but not in those treated with control siRNA or with *TREX1*-specific and *IRF3*-specific siRNAs (**Fig. 5e**). We obtained similar results after transfection with *TREX1*-specific siRNA into human peripheral blood CD4$^+$ T cells infected with HIV IIIB (**Fig. 5f–l**). In T cells treated with *TREX1*-specific siRNA, HIV DNA accumulated in the cytosol and the expression of both IFN-α and IFN-β was induced. *TREX1*-specific siRNA similarly resulted in less HIV production, as measured by flow cytometry analysis of intracellular p24. Both the number of p24$^+$ cells and p24 mean fluorescence intensity were lower. Therefore, *TREX1* deficiency resulted in less HIV replication and spreading in culture. The magnitude of these effects increased with the amount of siRNA transfected and the extent of *TREX1* suppression. Therefore, during infection of primary human cells with wild-type HIV, TREX1 suppresses HIV-induced IFN-β activation through IRF3 to promote HIV replication.

### HIV DNA signals via STING, TBK1 and IRF3

To investigate the pathway triggered by HIV DNA, we treated *Trex1*$^{-/-}$ MEFs with siRNAs targeting selected genes linked to DNA-stimulated induction of interferon and examined the effect on HIV-stimulated IFN-β expression (**Fig. 6a**,**b**). Inhibiting a gene

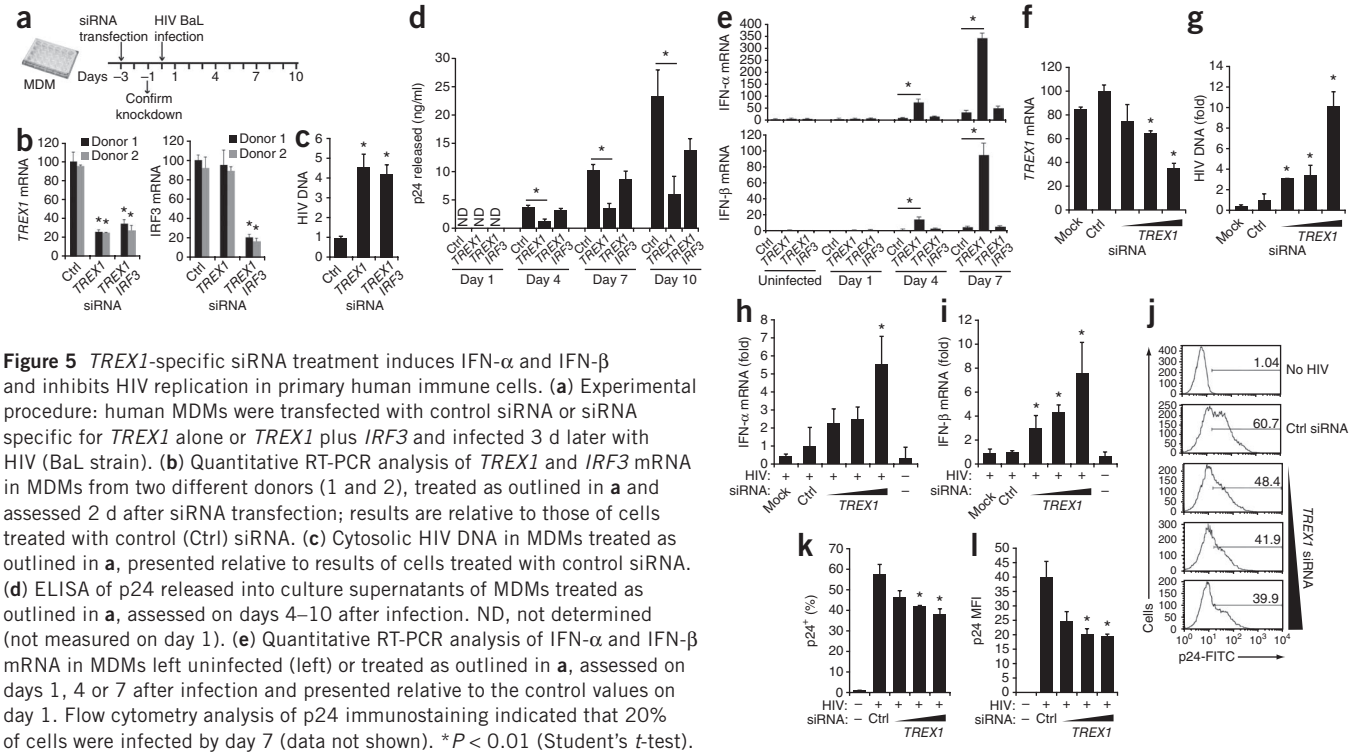


**Figure 5** *TREX1*-specific siRNA treatment induces IFN-α and IFN-β and inhibits HIV replication in primary human immune cells. (**a**) Experimental procedure: human MDMs were transfected with control siRNA or siRNA specific for *TREX1* alone or *TREX1* plus *IRF3* and infected 3 d later with HIV (BaL strain). (**b**) Quantitative RT-PCR analysis of *TREX1* and *IRF3* mRNA in MDMs from two different donors (1 and 2), treated as outlined in **a** and assessed 2 d after siRNA transfection; results are relative to those of cells treated with control (Ctrl) siRNA. (**c**) Cytosolic HIV DNA in MDMs treated as outlined in **a**, presented relative to results of cells treated with control siRNA. (**d**) ELISA of p24 released into culture supernatants of MDMs treated as outlined in **a**, assessed on days 4–10 after infection. ND, not determined (not measured on day 1). (**e**) Quantitative RT-PCR analysis of IFN-α and IFN-β mRNA in MDMs left uninfected (left) or treated as outlined in **a**, assessed on days 1, 4 or 7 after infection and presented relative to the control values on day 1. Flow cytometry analysis of p24 immunostaining indicated that 20% of cells were infected by day 7 (data not shown). *$P < 0.01$ (Student's *t*-test). (**f**) *TREX1* mRNA in CD4$^+$ T cells positively selected from peripheral blood mononuclear cells, transfected with no siRNA (Mock), control siRNA (600 pmol) or *TREX1*-specific siRNA (200, 400 or 600 pmol per 3 × 10$^6$ cells) and infected 3 d later with HIV IIIB; mRNA measured 2 d after transfection is presented relative to that in cells treated with control siRNA. (**g**) Cytosolic HIV DNA in the cells in **f**, assessed 10 h after infection. (**h**,**i**) IFN-α mRNA (**h**) and IFN-β mRNA (**i**) in the cells in **f**, measured 22 h after infection and presented relative to results obtained with control cells. (**j**–**l**) Flow cytometry analysis of HIV IIIB replication in CD4$^+$ T cells treated with control or *TREX1*-specific siRNA and then left uninfected (No HIV) or infected with HIV IIIB, and assessed 3 d after infection as percent p24$^+$ cells (**j**; numbers above bracketed lines), frequency of p24$^+$ cells (**k**) and the mean fluorescence intensity (MFI) of p24 (**l**). FITC, fluorescein isothiocyanate. *$P < 0.05$ (Student's *t*-test). Data are representative of two experiments ((**b**–**e**); error bars, s.d. (**b**) or average and s.d. of two replicates from two independent donors (**d**,**e**)) or 2 experiments (**d**; error bars, s.d.) or are from two independent experiments (**f**–**l**; error bars, s.d. of six replicates).

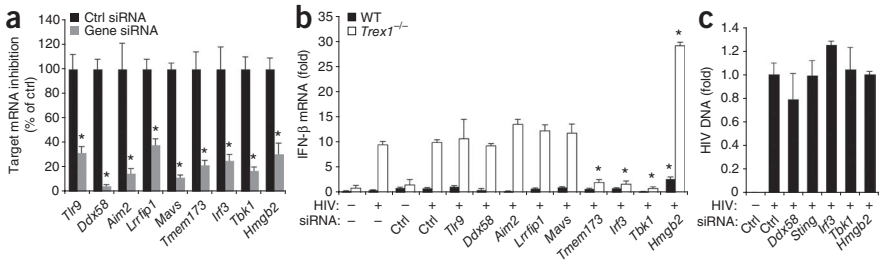


**Figure 6** HIV-stimulated interferon induction requires IRF3, TBK1 and STING. (**a**) Quantitative RT-PCR analysis of genes related to innate immunity in *Trex1*$^{-/-}$ MEFs 48 h after transfection with control or gene-specific siRNA. *Ddx58* encodes RIG-I; *Tmem173* encodes STING. *$P < 0.01$ (Student's *t*-test). (**b**) Quantitative RT-PCR analysis of IFN-β mRNA in wild-type and *Trex1*$^{-/-}$ MEFs left untransfected (siRNA –) or transfected with control or gene-specific siRNA (siRNA +), and left uninfected (HIV –) or infected with VSV-G-pseudotyped HIV (HIV +), assessed 24 h after infection; results are presented relative to those of untransfected wild-type MEFs. *$P < 0.01$ (Student's *t*-test). (**c**) Quantitative PCR analysis of cytosolic HIV DNA in *Trex1*$^{-/-}$ MEFs transfected with control or gene-specific siRNA, assessed 10 h after infection with HIV-GFP; results are presented relative to those of infected *Trex1*$^{-/-}$ MEFs transfected with control siRNA. Data are representative of three independent experiments (error bars, s.d.).

required for HIV-stimulated interferon signaling in *Trex1*$^{-/-}$ cells should result in lower IFN-β expression. As expected, treatment with *Irf3*-specific siRNA resulted in less HIV-stimulated IFN-β mRNA. Similarly, siRNA targeting *Tbk1*, which encodes an IRF3 kinase, also inhibited HIV-stimulated expression of IFN-β (**Fig. 6b**). We found that siRNA specific for genes encoding the DNA sensors TLR9 (ref. 18), AIM2 (ref. 19), LRRFIP1 (ref. 20) or HMGB2 (ref. 21), or encoding RIG-I (which recognizes RNA transcribed from cytosolic DNA[22,23]) or its adaptor MAVS (also known as IPS-1, VISA and CARDIF)[24–27], did not suppress HIV-stimulated expression of IFN-β, which suggested that an unknown sensor detects HIV DNA or that multiple known DNA sensors might function redundantly. Additional experiments confirmed that the RNA polymerase III–RIG-I–MAVS DNA-detection pathway[22,23] was not involved in HIV-stimulated induction of interferon (**Supplementary Fig. 3**). However, siRNA specific for the gene encoding another membrane-associated adaptor, STING (also known as MITA and ERIS[28–30]), which has been identified as mediating innate immune responses to cytosolic DNA[28,29], inhibited HIV-stimulated expression of IFN-β (**Fig. 6b**). STING is phosphorylated by the kinase TBK1 (refs. 29,31). We found that siRNA specific for the genes encoding STING, IRF3 or TBK1 did not affect cytosolic accumulation of HIV DNA in *Trex1*$^{-/-}$ MEFs (**Fig. 6c**), which suggested that they act downstream of DNA sensing. Thus, HIV DNA is detected by a pathway that signals through STING, TBK1 and IRF3 and does not involve any known DNA sensor (**Supplementary Fig. 4**).

HMGB2, but not its homolog HMGB1, associates with TREX1 in the cytosolic SET complex[32]. Treatment of HIV-infected wild-type and *Trex1*$^{-/-}$ MEFs with *Hmgb2*-specific siRNA enhanced IFN-β expression (**Fig. 6b**), which suggested that HMGB2 inhibits the response to cytosolic HIV DNA. However, treatment with *Hmgb2*-specific siRNA did not result in more cytosolic HIV DNA in *Trex1*$^{-/-}$ MEFs than in those treated with control siRNA (**Fig. 6c**), which suggested that HMGB2 might act downstream of the recognition of HIV DNA. HMGB proteins repress the transcription of many genes, including proinflammatory genes, such as *TNF*[33–35]. To determine whether HMGB2 regulates *IFNB* transcription, we transfected *HMGB2*-specific siRNA into 293T cells expressing a luciferase reporter plasmid driven by the *IFNB* promoter. IFN-β–luciferase expression was about twofold higher in cells treated with *HMGB2*-specific siRNA than in those treated with control siRNA in response to poly(dA:dT) or MAVS overexpression (**Supplementary Fig. 5**), which suggested that HMGB2 inhibits *IFNB* expression, either directly or indirectly, though its promoter, but acts downstream of DNA sensing. To determine whether HMGB2 has a role in the removal of cytosolic HIV DNA or HIV-stimulated expression of interferon in primary human MDMs, we suppressed *HMGB2* alone or together with *TREX1*. *HMGB2*-specific siRNA on its own did not result in more cytosolic HIV DNA. However, unlike results obtained with *Trex1*$^{-/-}$ MEFs, treatment of human macrophages with *HMGB2*- and *TREX1*-specific siRNA resulted in significantly more accumulation of cytosolic HIV DNA than did treatment with *TREX1*-specific siRNA alone ($P < 0.05$). Treatment with *HMGB2*-specific siRNA led to HIV-stimulated expression of IFN-α and IFN-β, but did not induce interferon expression in uninfected cells (data not shown); this was significantly greater than that induced by *TREX1*-specific siRNA alone and was not enhanced by treatment with both siRNAs ($P < 0.05$). These results suggest that HMGB2 may act together with TREX1 to remove HIV DNA from the cytoplasm (model, **Supplementary Fig. 4**). Further work is needed to elucidate the role of HMGB2 in suppressing HIV DNA–stimulated induction of interferon, but these results suggest that HMGB2 might act at multiple points (by recognizing and/or helping eliminate cytosolic DNA and suppressing the *IFNB* promoter).

## DISCUSSION

HIV infection of its main target cells, macrophages and CD4$^+$ T cells, does not induce cell-autonomous interferons[36]. We have shown here that the host cytoplasmic exonuclease TREX1 helps HIV evade innate immunity by digesting reverse transcripts that are not imported into the nucleus and would otherwise induce interferons. When *TREX1* was inhibited by RNAi, HIV infection of primary cells triggered expression and secretion of type I interferon. The HIV-stimulated interferon response in cells deficient in TREX1, like the response to endogenous retroelement DNA and transfected DNA[7], was IRF3 dependent. We found that interferon induction in *TREX1*-deficient cells was blocked by the expression of enzymatically active TREX1 or by cosuppression of IRF3 expression to interrupt interferon signaling. HIV-stimulated innate immune signaling also required STING and TBK1. On the basis of our siRNA experiments, we conclude that none of the known DNA sensors was involved. Therefore, our working model of the innate immune pathway activated by cytosolic HIV DNA starts with an unknown sensor (that may 'preferentially' recognize ssDNA) that signals through STING, TBK1 and IRF3 to activate interferon expression.

Type I interferons inhibit HIV replication at multiple steps in the early phase of its life cycle and thereby suppress viral spreading. Failure to induce antiviral interferons in infected T cells and macrophages may promote transmission by allowing the virus to spread from the initial nidus of infection to neighboring cells in genital tissue. However, assessing the importance of TREX1 in transmission will require efficient methods for inhibiting TREX1 expression *in vivo* in the immune cells that HIV infects. Such methods are not yet available but are being developed. We used an MOI of 1 to achieve a reasonable frequency of infected cells. As cytoplasmic accumulation

of and interferon triggering by HIV DNA depend on the MOI, the physiological relevance of our results to HIV transmission hinges on what viral concentrations are achieved *in vivo*, which is unknown. Viral concentrations may reach a high MOI locally during the replicative burst that occurs in the genital tract during transmission, when a strong intrinsic antiviral immune response might prevent dissemination[37]. Other settings of high viral concentration might be activated lymph nodes or gut-associated lymphoid tissues.

We did not determine whether TREX1 affects interferon production by plasmacytoid DCs, the main source of type I interferon during HIV infection. HIV replication is inefficient in DCs. Interferon stimulation in plasmacytoid DCs seems to be triggered mostly by endocytosed virus, whose genomic RNA is recognized by Toll-like receptor 7 in endosomes[38]. Productive HIV infection of macrophages and T cells, however, involves fusion of the viral membrane with the cell membrane and direct uncoating of the viral capsid into the cytosol, bypassing the endosomal compartment and Toll-like receptor signaling. Nonetheless, it will be important to determine whether TREX1 modulates interferon signaling in plasmacytoid DCs.

We found that HIV-stimulated activation of interferon in *Trex1*$^{-/-}$ cells was eliminated by treatment of infected cells with an inhibitor of reverse transcription but not by treatment with an integrase inhibitor, which suggests that HIV DNA, not genomic RNA, is the nucleic acid that triggers innate immunity. The nucleic acid most sensitive to TREX1 activity is ssDNA, and therefore ssDNA is probably its main substrate. IFN-β mRNA is normally detected 6–8 h after transfection of immunostimulatory DNA or infection with a DNA virus[6,22,39]. After HIV infection, interferon mRNA is not detected until 12 h after infection; the lag in IFN-β expression is probably due to the time needed to complete reverse transcription. The rapid decrease in IFN-β mRNA expression after it reaches its peak value suggests that a cell-autonomous secondary mechanism tempers the innate immune response that, if unchecked, could be harmful to the host. Some HIV DNA accumulated in the cytoplasm of HIV-infected cells, even when TREX1 was normally expressed, but it did not activate interferon expression. A cytoplasmic DNA threshold, which might vary in different cell types, may need to be exceeded to trigger innate immunity.

HMGB proteins have been proposed as innate immune sentinel proteins that facilitate nucleic acid recognition by sensors of RNA and DNA[21]. Here, experiments with *HMGB2*-specific siRNA demonstrated the opposite effect: HMGB2 helped suppress interferon induction by HIV in human cells. Although RNAi of *Hmgb2* in *Trex1*$^{-/-}$ MEFs did not result in more cytosolic HIV DNA, RNAi of *HMGB2* in human macrophages that were also treated with *TREX1*-specific siRNA resulted in enhanced cytosolic HIV DNA and induction of IFN-α and IFN-β. Therefore, HMGB proteins may have a more complex role in innate immunity than originally suggested. In their role as sentinels for foreign nucleic acids, they may facilitate the recognition of nucleic acids both by sensors that trigger innate immune responses and by proteins, such as TREX1, that inhibit interferon induction. Therefore, the net effect of a lower abundance of HMGB proteins could be either to inhibit interferon induction (as reported before[21]) or to enhance it, as shown here. We also found that HMGB2 acted downstream of nucleic acid sensing at the *IFNB* promoter to suppress *IFNB* transcription, which adds another layer of complexity. This transcriptional effect extended to non-HIV innate immune stimuli (the synthetic dsDNA poly(dA:dT) and overexpression of MAVS). In the study noted above[21], IFN-β expression stimulated by poly(dA:dT) was lower in *Hmgb2*$^{-/-}$ MEFs than in wild-type MEFs, whereas we found the opposite effect with RNAi of *Hmgb2*. The apparent discrepancy between those results[21] and ours could be due to a difference in the consequences of complete or partial *Hmgb2* elimination, especially if HMGB2 operates at multiple steps in interferon induction.

In many of our experiments we used genetically deficient mouse cells to demonstrate that TREX1 helps HIV evade detection by the innate immune system and to define the HIV DNA–stimulated interferon signaling pathway. Knockout mouse cells are powerful tools for HIV research[9,40,41]. Once the block in the entry into mouse cells is overcome by VSV-G pseudotyping, most early steps of HIV replication, including reverse transcription, integration and LTR-driven transcription, are similar in human and mouse cells. Furthermore, human TREX1 is 73.3% identical to its mouse homolog in amino acid sequence, and is 71.4%, 100% and 86.7% identical to its mouse homolog in its three exonuclease motifs[16]. Human and mouse TREX1 have the same enzymatic activity and can substitute for each other[16]. Therefore, mouse cells are well suited for the study of TREX1 function in HIV replication. Nonetheless, human immune cells susceptible to HIV can differ from MEFs in their ability to activate innate immune pathways. For example, IFN-α was induced by HIV in human immune cells but not in MEFs. The differences in the role of HMGB2 in the accumulation of HIV DNA in human macrophages and MEFs may be another case in point. We confirmed all key findings in primary human cells, including HIV DNA accumulation, interferon induction and inhibition of HIV replication when *TREX1* was inhibited by RNAi, and efficient rescue by cosuppression of *IRF3* expression.

Our data shed light on the fate of nonproductive or nonintegrated HIV DNA in the cell. At an MOI of 1, HIV infection produced many reverse transcripts (although only one per incoming genomic RNA), but very few copies managed to integrate into the host chromosome. The remaining HIV DNA was cleared by TREX1, as cytosolic HIV DNA built up when TREX1 function was deficient or inhibited and was removed after expression of enzymatically active TREX1. As a consequence, wild-type TREX1 fully restored HIV infectivity in *Trex1*$^{-/-}$ MEFs, and the D18N mutant failed to do so. Other host nucleases might also help digest cytosolic HIV DNA. It is unclear why the excess HIV DNA that accumulated in *Trex1*$^{-/-}$ cells did not lead to more chromosomal integration. Sequencing these excess HIV DNAs may show whether they are able to integrate and what prevents them from integrating. These excess HIV DNAs may be mostly nonproductive products of reverse transcription.

TREX1 promotes HIV replication in the following two ways: it inhibits autointegration[2], and it suppresses the interferon response. Several models might explain the dual effects of TREX1 on HIV DNA. One possibility is that TREX1 might sort productive versus nonproductive HIV products of reverse transcription. Reverse transcription of HIV is error prone and often produces incomplete products. TREX1 recognizes ssDNA or dsDNA with single-strand overhangs, the kind of DNA in failed products of reverse transcription. TREX1 might bind to HIV DNA nonspecifically in the cytosol but as an exonuclease can only efficiently digest HIV DNA that contains broken ends or single-strand overhangs. HIV integrase binds to the ends of reverse transcripts that are capable of chromosomal integration and might protect them from digestion by TREX1. Incomplete products of reverse transcription, however, would not bind integrase and therefore would be susceptible to degradation by TREX1. Autointegration requires the full-length product of reverse transcription and active DNA ends bound by integrase, which catalyzes autointegration. Although TREX1 probably binds to full-length integration-competent products, as well as to transcripts that are not competent for integration, its exonuclease activity might be inhibited

in the full-length transcript by lack of some DNA feature that facilitates digestion (such as shielding by integrase). Another possibility is that TREX1 is inhibited by components of the SET complex that also bind to the HIV preintegration complex[2]. Another DNase in the SET complex, NM23-H1, is inhibited by SET protein and is activated only when granzyme A cleaves SET[42]. TREX1 is an abundant protein that is not exclusively present in the SET complex. Two subpopulations of TREX1 could be involved in different actions: the SET complex–associated TREX1 inhibits autointegration, whereas TREX1 outside the SET complex is enzymatically active and removes excess HIV DNA. This model would also explain why siRNAs directed against genes encoding most other molecules of the SET complex do not induce interferon but do protect against autointegration. Further studies are needed to test these ideas.

Mutations in *TREX1* that interfere with the enzymatic function or localization of TREX1 are associated with systemic lupus erythematosus and other autoimmune and/or inflammatory diseases[3–5]. Patients with systemic lupus erythematosus are underrepresented in HIV-infected populations[43]. It would be worth evaluating whether TREX1 polymorphisms or autoimmune diseases are associated with less HIV transmission or a more benign disease course. The innate immune pathway identified in this study will improve understanding of how HIV intersects with innate immunity and may also shed light on autoimmune and inflammatory syndromes linked to *TREX1* mutation.

## METHODS

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

*Note: Supplementary information is available on the Nature Immunology website.*

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We thank D. Stetson (University of Washington) for wild-type, *Trex1*$^{-/-}$ and *Trex1*$^{-/-}$*Irf3*$^{-/-}$ primary MEFs, under agreement with D. Barnes and T. Lindahl (Cancer Research UK); J. Jung (University of Southern California) for RIG-I-deficient (*Ddx58*$^{-/-}$) MEFs; S. Harvey and F. Perrino (Wake Forest University) for wild-type and *Trex1*$^{-/-}$ transformed MEFs; A. Engelman (Dana-Farber Cancer Institute) for the HIV-luciferase plasmid (pNL4-3/Env$^{-}$); D. Gabuzda (Dana-Farber Cancer Institute) for the HIV-GFP plasmid (pNL4-3/Env$^{-}$); T. Fujita (Kyoto University) for antiserum to mouse IRF3; S. Nagata (Kyoto University) for the hemagglutinin-tagged MAVS plasmid; K. Fitzgerald (University of Massachusetts) for siRNA and inhibitors of RNA polymerase III; J. Hiscott (McGill University) for *IFNB*-luciferase plasmid; L. Gehrke (Harvard Medical School) for CMV–renilla luciferase plasmid; and members of the Lieberman lab for discussions. Supported by the US National Institutes of Health (AI45587 to J.L., and T32 HL066987 to N.Y.), the Harvard Center for AIDS Research (N.Y.), Harvard Summer Honors Undergraduate Research Program (A.D.R.-M.) and Deutsche Forschungsgemeinschaft (Le 1074/3-1 to M.A.L.-K.).

### AUTHOR CONTRIBUTIONS

N.Y. conceived of the study, designed and did most experiments and helped write the paper; A.D.R.-M. and B.S. helped do the experiments; M.A.L.-K. provided human cell lines and scientific advice; and J.L. conceived of and supervised the study and helped write the paper.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Cells.** Wild-type, *Trex1*$^{-/-}$ and *Trex1*$^{-/-}$*Irf3*$^{-/-}$ MEFs[7,44] were provided by D. Stetson under an agreement with D. Barnes and T. Lindahl. RIG-I-deficient (*Ddx58*$^{-/-}$) MEFs were provided by J. Jung. The human fibroblast line heterozygous for the mutation in *TREX1* resulting in the D18N substitution was derived from a patient with chilblain lupus. Primary human macrophages and CD4$^+$ T cells were isolated from peripheral blood mononuclear cells and were maintained by standard protocols. HeLa-CD4 and 293T cells have been described[2]. HeLa-CD4 cells, TZM-bl cells, 293T cells and human fibroblasts were grown in DMEM (Invitrogen), and MEFs were grown in DMEM F12 (Invitrogen), both supplemented with 10% (vol/vol) heat-inactivated FBS. Primary CD4$^+$ T cells were grown in RPMI medium (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated FBS, activated in phytohemagglutinin (2 μg/ml) and maintained in recombinant human IL-2 (30 U/ml). MDMs were grown in RPMI medium supplemented with 10% (vol/vol) heat-inactivated human serum. Experiments involving human and mouse materials were approved by the institutional review boards of the Immune Disease Institute, Harvard Medical School and the Children's Hospital, Technical University Dresden.

**Viruses, infection and antiviral compounds.** HIV (IIIB and BaL strains) were propagated as described[45]. The HIV-GFP plasmid (pNL4-3/Env$^-$)[46] was provided by D. Gabuzda. The HIV-luciferase plasmid (pNL4-3/Env$^-$)[47] was provided by A. Engelman. Viral supernatants were produced from transfected 293T cells as described[2]. Virus-like particles were produced by transfection of 293T cells with VSV-G plasmid and a plasmid that encodes only the Gag and polymerase proteins of HIV. Virus was collected in three batches every 12 h at 48–72 h after transfection and was concentrated approximately tenfold with Centricon filters (UFC910024; Millipore) according to the manufacturer's instructions.

All HIV viruses were titered on wild-type MEFs (for mouse cell experiments) and on 293T cells (for human cell experiments) by flow cytometry analysis of p24 antigen 24 h after infection. Cells were stained with fluorescein isothiocyanate–anti-p24 (KC57-FITC; Beckman Coulter) or were not stained, for HIV-GFP. The MOI was calculated by the following formula: percent infected = $1 - e^{-MOI}$, so that 63.2% positive would be an MOI of 1. This was determined separately for mouse and human cells with pseudotyped viruses. Cells were infected with virus at an MOI of 1, unless indicated otherwise, for 6–8 h before replacement of viral supernatants with fresh medium. Primary CD4$^+$ T cells at a density of $3 \times 10^5$ cells per well in 24-well plates were infected with HIV IIIB (400 ng/ml of p24) and MDMs were similarly infected with HIV BaL (200 ng/ml of p24). The infectivity of HIV-GFP and HIV-luciferase was measured as described[2,46]. For experiments measuring stage-specific HIV DNA, viral stocks were pretreated at 37 °C with Turbo DNase (40 U/ml; Ambion) for 30 min before infection. Cytosolic HIV DNA was isolated at various times after infection by separation of nuclear and cytoplasmic fractions by lysis for 10 min on ice in lysis buffer (20 mM Tris, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.3% (vol/vol) Nonidet P-40 and Complete Mini-Protease Inhibitor Cocktail (EDTA-free; Roche)) followed by centrifugation at 10,000*g* for 10 min. Subgenomic DNA in the supernatant (cytoplasmic fraction) was then isolated by a published method[48]. CD4$^+$ T cells were infected by spin-inoculation at 1,500*g* for 2 h.

To obtain conditioned medium, wild-type, *Trex1*$^{-/-}$ and *Trex1*$^{-/-}$*Irf3*$^{-/-}$ cells were infected for 6–8 h with VSV-G-pseudotyped HIV-GFP. Viruses were then removed and replaced with fresh medium and were 'conditioned' overnight. Conditioned medium was collected the next morning, filtered (0.45-μm pores) and added to wild-type cells with HIV-luciferase. Luciferase activity was then measured 48 h after infection. A different reporter virus was used at the second step to eliminate any concerns about virus carryover. For neutralizing-antibody experiment, conditioned medium was preincubated for 1 h at 25 °C with antibody to mouse IFN-β (5 μg/ml; 7F-D3; ab24324; Abcam) before being added to wild-type cells together with HIV-luciferase.

Drugs to inhibit reverse transcription and integrase (nevirapine and raltegravir) were obtained from the National Institutes of Health AIDS Reagent and Reference Program and were used at a concentration of 5 μM. The RNA polymerase III inhibitor ML-60218 was from Calbiochem.

**Plasmids, siRNA, DNA and RNA oligonucleotides and transfection.** The GFP-tagged TREX1 plasmid has been described[4]. The GFP-tagged TREX1 D18N mutant was generated by site-directed mutagenesis. For 'rescue' experiments, MEFs were transfected with the Amaxa Nucleofector kit (VPD-1004) and were sorted for GFP$^+$ cells 24 h after transfection. Cells expressing GFP or GFP fusion proteins were then infected with VSV-G-pseudotyped HIV. *IFNB*-luciferase and CMV–renilla luciferase plasmids were provided by J. Hiscott (McGill University, Canada) and L. Gehrke (Harvard Medical School)[49,50]. The dual luciferase assay was done according to standard protocols (Invitrogen). Primary MDMs were transfected with control siRNA (600 pmol per $3 \times 10^6$ cells) or *TREX1*- or *IRF3*-specific siRNA (600 pmol per $3 \times 10^6$ cells) with the Amaxa Nuclefector kit (VPA-1008). CD4$^+$ T cells were transfected with similar concentrations of siRNA (200–600 pmol per $3 \times 10^6$ cells for *TREX1*-specific siRNA) with the Amaxa Nucleofector kit (VPA-1002). The hemagglutinin-MAVS plasmid was provided by S. Nagata.

The ssDNA oligonucleotide was Gag-100 forward (sequence, **Supplementary Table 1**). The dsDNA was generated by hybridization of the primers Gag-100 forward and Gag-100 reverse at equimolar concentrations (by heating for 3 min at 100 °C, followed by slow cooling to 25 °C); ssRNA was generated by *in vitro* transcription with the T7 transcription kit (1354; Ambion) and Gag-100-T7 oligonucleotide as a template. RNA-DNA hybrids were generated by hybridization of ssDNA and ssRNA at equimolar concentrations. Poly(dA:dT) was from Sigma. Nucleic acids were transfected with Lipofectamine 2000 (Invitrogen). Cytosolic ssDNA and dsDNA were quantified by quantitative PCR with Gag primers.

Cells were transfected with 2 μl Lipofectamine 2000 (Invitrogen) and 100 nM siRNA (sequences, **Supplementary Table 2**; Dharmacon) in 24-well plates or were transfected by nucleofection (Amaxa) for primary cells.

**HIV DNA, cytokine mRNA and protein analysis.** DNA primer sequences are in **Supplementary Table 1**. Stage-specific HIV DNA in mouse cells was measured as described[2,9] with mouse mitochondrial DNA for normalization; integrated DNA in mouse cells was measured by a similar nested PCR design with two mouse long interspersed nuclear element primers[9] instead of one Alu universal primer, and results were normalized to mouse *Gapdh* DNA (encoding glyceraldehyde phosphate dehydrogenase). Cytokine mRNA was extracted with TRIzol reagent (Invitrogen) and was measured by quantitative RT-PCR with specific gene primers (**Supplementary Table 1**) and is presented relative to *Gapdh* mRNA. Mouse IFN-β protein in culture supernatants was measured by ELISA (42400-1; PBL).

**Immunoblot analysis and immunostaining.** Antibody to mouse TREX1 (mouse; 1:1,000 dilution; 29; 611987; BD Biosciences), anti-SET (rabbit; 1:1,000 dilution; antiserum produced in-house)[2], anti-APE1 (rabbit; 1:1,000 dilution; antiserum produced in-house)[2], anti-NM23-H1 (rabbit; 1:1,000 dilution; sc-343; Santa Cruz), anti-HMGB1 (rabbit; 1:2,000 dilution; ab18256; Abcam), anti-HMGB2 (rabbit; 1:2,000 dilution; ab67282; Abcam), anti-RIG-I (rabbit; 1:1000; ab65588; Abcam), anti-GFP (rabbit; 1:500 dilution; ab290; Abcam) and anti-tubulin (mouse; 1:1,000 dilution; B-5-1-2; Sigma) were used for immunoblot analysis according to standard protocols. Anti-Flag (mouse; M2; Sigma) and mouse immunoglobulin G (Jackson Laboratories) were used for immunoprecipitation as described[2]. Rabbit antiserum to mouse IRF3 (1:100 dilution; T. Fujita) was used for staining of endogenous mouse IRF3 in MEFs. MEFs grown on coverslips were fixed in 4% (wt/vol) paraformaldehyde and were made permeable and stained by standard protocols. Samples mounted in Vectashield mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories) were imaged with a Zeiss 200M inverted epifluorescence microscope (Carl Zeiss MicroImaging) equipped with SlideBook software (Intelligent Imaging Innovations).

**Statistical methods.** Statistical significance was determined by Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

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