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IFI16 DNA Sensor Is Required for Death of Lymphoid CD4 T-cells Abortively Infected with HIV

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Abstract

The progressive depletion of quiescent “bystander” CD4 T-cells, which are non-permissive to HIV infection, is a principal driver of the acquired immunodeficiency syndrome (AIDS). These cells undergo abortive infection characterized by the cytosolic accumulation of incomplete HIV reverse transcripts. These viral DNAs are sensed by an unidentified host sensor that triggers an innate immune response, leading to caspase-1 activation and pyroptosis. Using unbiased proteomic and targeted biochemical approaches as well as two independent methods of lentiviral shRNA-mediated gene knockdown in primary CD4 T-cells, we identify Interferon gamma Inducible protein 16 (IFI16) as a host DNA sensor required for CD4 T-cell death due to abortive HIV infection. These findings provide insights into a key host pathway that plays a central role in CD4 T-cell depletion during disease progression to AIDS.

HIV/AIDS is a devastating global epidemic with over 70 million infections and 35 million deaths (WHO). AIDS is primarily caused by loss of the quiescent “bystander” CD4 T-cells that populate lymphoid organs. These cells are not permissive for viral replication resulting in abortive infection and the accumulation of incomplete DNA transcripts (1). These cytosolic viral DNAs trigger an innate immune response that activates a highly inflammatory form of programmed cell death, pyroptosis (2). Here, we sought to identify the host DNA sensor that initiates pyroptosis in abortively infected CD4 T-cells.

An unbiased proteomic approach involving DNA affinity chromatography and mass spectrometry was utilized to identify potential viral DNA sensor candidates. Cytosolic fractions of tonsillar CD4 T-cell lysates were incubated with a biotinylated 500-bp HIV-1 Nef DNA fragment and subjected to strepavidin immunoprecipitation, SDS-PAGE, and silver staining (Fig. 1A). The Nef region is reverse transcribed early thus this DNA RT product is likely present during abortive HIV infection. Streptavidin immunoprecipitation

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samples incubated with biotinylated HIV DNA showed numerous bands (Fig. 1A). Nonspecific background binding was very low: protein was not detected when nonbiotinylated DNA was tested. The cytosolic lysates appeared free of nuclear contamination as immunoblotting showed no histone H3 (Fig. 1B). Mass spectrometry was employed to identify cytosolic proteins from the tonsillar CD4 T-cells that bound to HIV DNA. The top six hits, based on protein discriminant scores (30), correspond to Ku80, PARP-1, Ku70, RPA-1, IFI16, and IFIX (Fig. 1C) (see File S1 for the complete list).

A rational approach investigating biologically relevant DNA sensor candidates was pursued in parallel. Expression of various known innate immune sensors was assessed by immunoblotting cytosolic lysates from resting tonsillar CD4 T-cells, confirming the presence of IFI16 (3, 4), AIM2 (5-8), DAI (9), STING (10-12), DNPK-1 (13), NLRP3 (14-16) and IFIX (PYHIN-1) (17) (Fig. 1D). cGAS (18, 19) was neither detected at the protein level in tonsillar CD4 T-cells (Fig. S1D), nor in the affinity chromatography-mass spectrometry experiments (File S1). We were intrigued with IFI16 since it was identified in both approaches and shown to form an inflammasome (4, 17). Of the known inflammasome DNA sensors, IFI16, but not AIM2, bound HIV-1 DNA (Fig. 1D). Since AIM2 binds DNA in a non-sequence-specific manner, we had expected it would be a top candidate, but it was not identified by mass spectrometry (File S1). IFI16 mRNA levels are ~5-fold higher than AIM2 mRNA in resting tonsil CD4 T-cells (Fig. S1A). Of note, all three IFI16 isoforms were detected in the cytosol and nucleus of primary tonsillar CD4 T-cells (Fig. S1B).

RT of the HIV RNA genome initially generates single-stranded DNA (ssDNA) and then double-stranded DNA (dsDNA); either might be sensed during abortive infection. A biotinylated dsDNA probe was incubated with cytosolic extracts from tonsillar CD4 T-cells with 10-fold excess of unlabeled ssDNA as a competitor (Fig. 1E). IFI16 effectively bound dsDNA (Fig. 1F) as described (3, 20) and was competed by “cold” ssDNA. Biotinylated ssDNA was subjected to binding and competition with cold dsDNA, but IFI16 was not initially detected by immunoblotting. However, further analysis using higher protein input confirmed that IFI16 binds to ssDNA, albeit more weakly than dsDNA (Fig. 1G). RIG-I selectively bound dsRNA as a control (Fig. 1F, G).

Standard methods, including liposome-mediated delivery of siRNAs or infection with VSV-G pseudotyped lentiviruses encoding shRNAs, are ineffective for targeted gene knockdown in resting CD4 T-cells (21, 22). siRNA nucleofection is highly variable, often toxic, and associated with extensive cell death in tonsillar cultures. To overcome these challenges and to test whether specific DNA sensor candidates are required for cell death in primary lymphoid CD4 T-cells undergoing abortive HIV infection, we used an “activation-rest” strategy. Splenic CD4 T-cells were activated with PHA and cultured in 100U/ml of IL-2, which rendered cells permissive for infection with VSV-G-pseudotyped lentiviruses encoding shRNA and mCherry. mCherry-positive cells were isolated by cell sorting (Fig. S2), expanded by two rounds of activation with anti-CD3/anti-CD28 antibody-conjugated beads, and then rested by reducing IL-2 levels to 10 U/ml for 3-4 days (23). IFI16 protein expression was markedly decreased in the mCherry-positive splenic CD4 T-cells receiving the lentivirus encoding shIFI16-A compared to cells receiving the lentivirus encoding the control scramble shRNA (Fig. 2A). Next, the rested mCherry-positive $CD4^+$ T-cells were

co-cultured with tonsil or spleen CD4 T cells infected with an HIV-GFP reporter virus (NLENG1). In cells expressing the scramble-shRNA, marked depletion of CD4 T-cells occurred (Fig. 2B); this death was rescued by adding a non-nucleoside reverse-transcriptase inhibitor (NNRTI) efavirenz (EFV), implicating abortive HIV infection as previously described (1). In sharp contrast, introduction of shIFI16-A resulted in survival of the mCherry-positive $CD4^+$ T-cells. In the same experiments, mCherry-negative $CD4^+$ T cells were markedly depleted, suggesting that they had returned to a sufficient state of rest to undergo abortive infection.

To exclude more formally the possibility that the “activated and rested” CD4 T-cells were dying as a result of productive infection, we assessed the activation status of these cells. Flow cytometry analysis revealed that CD4 T-cells cultured in reduced IL-2 levels had lower levels of CD25 and CD69 than cells activated with 100 U/ml IL-2 and 10 μg/ml PHA (Fig. 2C). However, CD25 levels were higher than found in unactivated cells, indicating that these cells had not fully returned to a resting state. This finding likely relates in part to the upregulation of CD25 expression by IL-2 (24). To directly test the permissivity of these cells to productive HIV infection, we utilized an HIV-1-GFP reporter virus. In cells expressing shScramble or shIFI16-A, only ~1-2% of the mCherry-positive cells, and ~1-2% of $mCherry^-$negative cells, were productively infected as indicated by GFP expression (Fig. 2D). Thus, the 60-70% depletion of CD4 T-cells observed was not due to high levels of productive viral infection.

To confirm IFI16 as an HIV-1 DNA sensor and to test a broader array of potential candidates, a second, more rapid shRNA knockdown strategy was employed. Virus-like particles (VLPs) were packaged with the SIV accessory protein Vpx that degrades the SAMHD1 restriction factor and render cells susceptible to lentiviral infection (25, 26). This method was adapted for use in resting CD4 T-cells based on prior success in monocyte-derived dendritic cells (27). Twenty-four hours after VLP-Vpx spinoculation, complete tonsillar HLACs were spinoculated with shRNA-mCherry lentiviral vectors pseudotyped with HIV gp160 Env (Fig S3, *30*). Cells were co-cultured 3 days later with HEK293T cells producing or not producing HIV-1 virions. CD4 T-cell death was assessed 2 days later in mCherry-positive $CD4^+$ T-cells expressing the shRNA and mCherry-negative $CD4^+$ T-cells lacking the shRNA. In parallel, EFV was added to select wells.

Three independent shRNAs targeting IFI16 reduced IFI16 protein expression in mCherry-positive $CD4^+$ T-cells, compared to the shScramble control (Fig. 3A, C). All three IFI16 shRNAs prevented depletion of mCherry-positive $CD4^+$ T cells, while shScramble did not (Fig. 3B, D). EFV rescued depletion of scramble-shRNA-expressing cells, supporting the notion that the CD4 T-cell depletion resulted from abortive infection (Fig. 3B). Moreover, mCherry negative $CD4^+$ T-cells were depleted regardless of the shRNA demonstrating that experimental conditions were sufficient for abortive infection in all infected samples (Fig. 3B, D). Thus, using an independent method for shRNA knockdown, we confirmed that IFI16 is required for lymphoid CD4 T-cell depletion by HIV following abortive HIV infection.

To confirm that shScramble mCherry-positive CD4 T-cells die via abortive infection, which requires RT but not integration (1), cells were co-cultured with 293T cells producing single round HIV-1(ΔEnv with gp160 coexpressed) or HIV containing a disabling integrase mutation, D116N (Fig. 3E). These replication-defective, non-spreading viruses induced depletion of mCherry-positive CD4 T-cells expressing shScramble. In contrast, introduction of shIFI16-A rescued cells from HIV-1-mediated depletion. Thus, neither productive infection nor HIV integration is required for cell death. Knockdown of IFI16 decreased caspase-1 activation in the mCherry positive cells, while IFNβ was induced in HIV-infected cells with the shScramble control, but not in cells expressing shIFI16-A (Fig. 3G). These findings suggest that IFI16 is required to sense incomplete DNA reverse transcripts that accumulate in abortively infected cells, leading to caspase-1 activation, which results in the subsequent death of these cells via pyroptosis (2). IFI16 sensing also leads to IFNβ induction.

Although IFI16 shRNAs consistently rescued death of lymphoid CD4 T-cells during abortive infection, other DNA sensor candidates were also evaluated. The VLP-Vpx method was used to render resting lymphoid CD4 T-cells permissive to infection with lentiviruses encoding shRNAs directed against AIM2 and STING. Although effective in inhibiting expression of AIM2 and STING protein in THP-1 cells (Fig. 4A), neither of these shRNAs rescued the mCherry positive cells from depletion (Fig. 4B). Validated shRNAs targeting IFIX (Fig. 4C) or DNPK-1 (Fig. 4E) also did not rescue mCherry-positive CD4 T-cell depletion (Fig. 4D, F). Moreover, small-molecule inhibitors of DNPK-1, Nu7026 and Nu7441 (13), did not rescue cells from abortive infection and pyroptosis (Fig 4G). These findings and a recent publication suggest that DNPK-1 may play a role in DNA sensing only within the small fraction of cells (5% in tonsil) that are permissive for productive HIV infection and trigger noninflammatory apoptosis (13). In contrast, IFI16 appears to be required to detect abortive infection and induction of highly inflammatory pyroptosis in nonpermissive CD4 T-cells (Fig. 4H). These cells form the majority of HIV-1 cellular targets in most lymphoid tissues (95% in tonsil cultures). Both mechanisms likely contribute to HIV-1-induced AIDS, but at different frequencies determined by the number of permissive versus nonpermissive cellular targets residing within various lymphoid tissues.

IFI16 evolved as an anti-viral DNA sensor (3, 4). In addition, IFI16 exerts novel antiviral activity, including restriction of herpesvirus replication by inhibiting viral gene expression (28). That IFI16 is targeted for degradation by herpesviruses (29) further highlights an evolutionary pressure to counteract its activity. Our studies reveal that IFI16 initiates an innate immune response that, rather than protecting the host, drives the debilitating CD4 T-cell depletion that underlies progression to AIDS in untreated HIV-infected individuals. The cycle of abortive infection, inflammatory death, and recruitment of new cells likely explains how this innate host response is undermined and, in fact, centrally contributes to HIV pathogenesis. Our findings now identify IFI16 as a critical DNA sensor required for cell death during abortive HIV-1 infection. Therapies directed against this host pathway might preserve CD4 T-cells and reduce chronic inflammation—two signature pathologies in HIV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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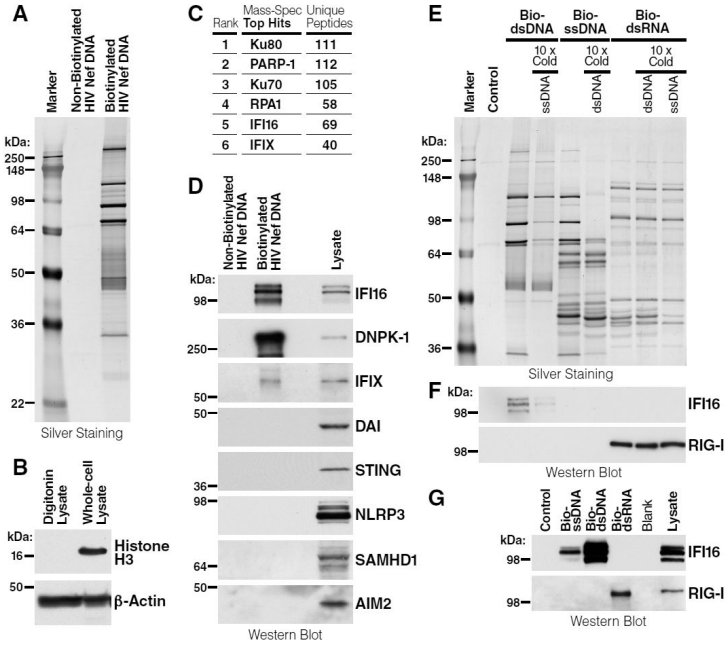

Figure 1. Biochemical analysis of cytosolic DNA binding proteins in CD4 T-cells

(A) Tonsillar CD4 T-cell lysates were incubated with a 500-bp biotinylated HIV Nef DNA probe or control non-biotinylated DNA and immunoprecipitated with streptavidin-coated beads. Samples were separated by SDS-PAGE and silver stained. **(B)** Western blot analysis of nuclear histone H3 and beta-actin in whole or digitonin lysis buffer prepared CD4 T-cell lysates. **(C)** Top ranked hits (rank based on protein discriminant scores described in Materials and Methods) from MS samples prepared as in (A). **(D)** Western blot analysis of candidate DNA sensors. **(E)** SDS-PAGE and silver stain analysis of biotinylated dsDNA or ssDNA samples prepared as in (A) and competed with a 10-fold excess of ssDNA or dsDNA **(F)** Western blot analysis of IFI16 and RIG-I binding samples in (E). **(G)** Western blots with high levels of protein input showing IFI16 binding to biotinylated ssDNA and dsDNA and RIG-I-RNA controls.

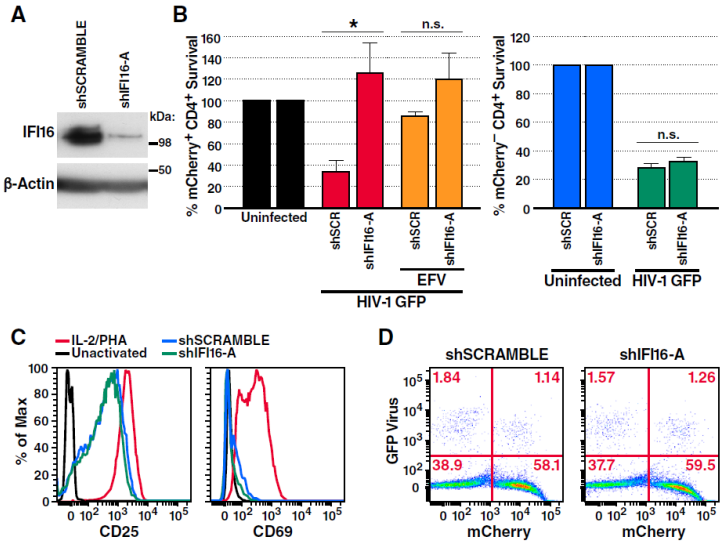

Figure 2. IFI16 shRNA knockdown rescues activated and rested splenic CD4 T-cells from depletion following abortive HIV infection

(A) Western blot analysis of IFI16 and beta-actin expression in shRNA expressing mCherry^{+} CD4^{+} T-cells after activation and rest in reduced IL-2. **(B)** Flow cytometry analysis of mCherry-positive CD4 T-cell survival after knockdown with shSCR or shIFI16-A and co-culture with either donor-matched mCherry^{-} CD4^{+} T-cells or tonsillar HLAC spinoculated with an HIV-1-GFP reporter virus. Cells were co-cultured in the presence or absence of 100 nM efavirenz, or with uninfected cells. Data represent the average of three independent experiments from three different donors. Error bars indicate standard error of the mean, * $p<0.05$ (Student's t-test), n.s.=not significant, $p>0.1$. **(C)** Flow cytometry analysis of CD25 and CD69 expression after IL-2 reduction. **(D)** Flow cytometry analysis of mCherry^{+} GFP^{+} populations in shRNA-expressing spleen cells post-co-culture.

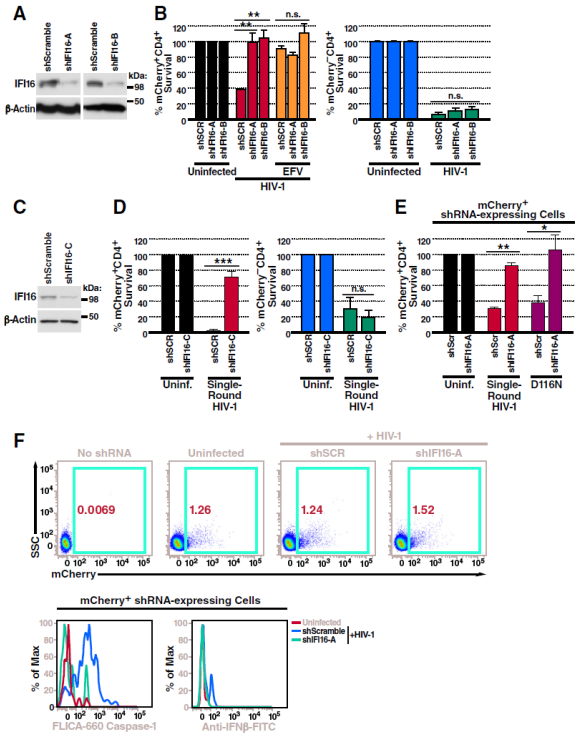

Figure 3. shRNA knockdown of IFI16 rescues HIV-induced tonsillar CD4 T-cell depletion

(A) Western blot analysis of IFI16 and beta-actin expression in mCherry^{+} tonsil CD4 T cells receiving shScramble, shIFI16-A or shIFI16-B. **(B)** Quantitation of flow cytometry of HLAC infected with VLP-Vpx, followed by shScramble, shIFI16-A, B lentiviruses pseudotyped with HIV gp160 Env then co-cultured with 293T cells producing HIV-1. ** p≥0.01 (Student's t-test), n.s.=not significant, p>0.1. **(C)** Western blot analysis of shIFI16-C knockdown. **(D)** Quantitation of flow cytometry results as in (B). *** p<0.001. **(E)** Quantitation of mCherry^{+} gate of HLAC treated as in (B) with single round HIV-1ΔEnv pseudotyped with gp160 envelope or HIV-1 D116N integrase mutant. ** p<0.01, * p<0.05. **(F)** Flow cytometry analysis of FLICA-660 Caspase-1 and IFNβ intracellular staining in mCherry^{+} cells, histograms are representative of results obtained with two donors.

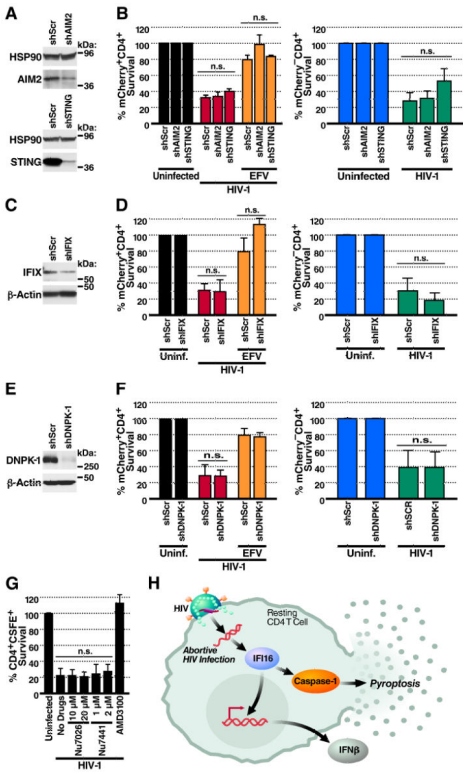

Figure 4. VLP-Vpx-facilitated shRNA knockdown of other candidate DNA sensors does not rescue cells from depletion following abortive HIV infection

(A) Western blot analysis of AIM2, STING, and HSP90 in shRNA expressing mCherry^{+} THP-1 cells. **(B)** Quantitation of flow cytometry results for HLAC infected with shScramble, shAIM2, or shSTING. n.s.=not significant, p>0.1 (Student's t-test). **(C)** Western blot analysis of IFIX in mCherry^{+} SupT1 cells. **(D)** Quantitation of flow cytometry analysis as in (B) of shScramble and shIFIX. **(E)** Western blot analysis of DNPK-1 in shRNA expressing mCherry^{+} Jurkat T-cells. **(F)** Flow cytometry analysis as in (B) with shDNPK-1. **(G)** CFSE labeled HLAC were pre-treated with DMSO alone in uninfected and no drug conditions, 10 or 20 μM Nu7026 or 1 or 2 μM Nu7441 or 250 nM AMD3100. CFSE^{+} cells were co-cultured with donor-matched HLAC productively infected with HIV and analyzed 3 days post co-culture. Quantified data represent the average of three independent experiments from three different donors. Error bars represent the standard errors of the mean. **(H)** Summary model.