Supplementary Materials for

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

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Other Supplementary Materials for this manuscript includes the following:

Data file S1: Complete mass spectrometry protein list

Materials and Methods

Cell culture and Tissues

Human tonsillar and splenic tissue was purchased from Cooperative Human Tissue Network and processed as previously described(*30*). HEK293T cells were cultured in DMEM supplemented with %10 FBS, 2mM L-glutamine, 100uM streptomycin, and 100U/ml penicillin.

Biotinylation of Nucleic Acid Probes

5' biotinylated double stranded (ds) HIV DNA was generated by PCR: forward primer (5' biotin-AAGGCAGCTGTAGATCTTAG-3') and reverse primer (5'-CAGTACAGGCAAAAAGCAGC-3'). 5' biotinylated single stranded (ss) HIV DNA was purchased from IDT DNA Technologies: 5' biotin-CAGTACAGGCAAAAAG CAGCTGCTTATATGCAGCATCTGAGGGCTCGCCACTCCCCAGTCCCGCCCAG GCCACGCCTCCCTGGAAAGTCCCCAGCGGAAAGTCCCTTGTAGCAAGCTCGA TGTCAGCAGTTCTTGAAGTACTCCGGATGCAGCTCTCGGGCCACGTGATGAA ATGCTAGGCGGCTGTCAAACCTCCACTC. Poly(I:C) (InvivoGen) was biotinylated using 5' EndTag Nucleic Acid Labeling System (Vector Labs, Burlingame, CA) according to the manufacturer's instructions.

Immunoprecipitation of Biotinylated Nucleic Acid

20 million tonsillar $CD4^+$ T cells were lysed in digitonin lysis buffer (0.5% digitonin, 20mM Tris-HCl, pH7.4, and 150 mM NaCl) on ice for 10 minutes. The cell lysates were centrifuged at 10,000xg for 10 minutes at 4C. Lysates were pre-cleared with

Dynabeads followed by the addition of 5'-biotinylated DNA or RNA pre-coupled to Dynabeads (MyOne Streptavidin T1, Invitrogen). Samples were incubated at 4^{o}C for 1 hour. Precipitated DNA-protein complexes were washed in digitonin lysis buffer. Proteins were eluted by boiling in 2% SDS loading buffer and resolved by 12.5% (Fig 1A) and 10% (Fig 1E) SDS-PAGE followed by silver staining or western blotting. For mass spectrometry, proteins were eluted with 2M KCl buffer w/ 0.05% Rapigest (Waters Corporation) at RT for 20 minutes.

Mass Spectrometry Analysis.

Eluted proteins were digested with trypsin prior to LC-MS/MS analysis. Samples were denatured and reduced in 2M urea, 10 mM NH_4HCO_3, 2 mM DTT for 30 minutes at 60C, then alkylated with 2 mM iodoacetamide for 45 minutes at room temperature. Trypsin (Promega) was added at a 1:100 enzyme:substrate ratio and digested overnight at 37C. Following digestion, samples were concentrated using C18 ZipTips (Millipore) according to the manufacturer's specifications. Digested peptide mixtures were analyzed by LC-MS/MS on a Thermo Scientific LTQ Orbitrap Elite mass spectrometry system equipped with a Proxeon Easy nLC 1000 ultra high-pressure liquid chromatography and autosampler system. Samples were injected onto a pre-column (2 cm x 100 um I.D. packed with ReproSil Pur C18 AQ 5um particles) in 0.1% formic acid and then separated with a one-hour gradient from 5% to 30% ACN in 0.1% formic acid on an analytical column (10 cm x 75 um I.D. packed with ReproSil Pur C18 AQ 3 um particles). The mass spectrometer collected data in a data-dependent fashion, collecting one full scan in the Orbitrap at 120,000 resolution followed by 20 collision-induced dissociation MS/MS scans in the dual linear ion trap for the 20 most intense peaks from the full scan. Dynamic

exclusion was enabled for 30 seconds with a repeat count of 1. Charge state screening was employed to reject analysis of singly charged species or species for which a charge could not be assigned. The raw data was matched to protein sequences by the Protein Prospector algorithm(*31*). Data were searched against a database containing SwissProt Human protein sequences (downloaded March 6, 2012) sequences, concatenated to a decoy database where each sequence was randomized in order to estimate the false positive rate. The searches considered a precursor mass tolerance of 20 ppm and fragment ion tolerances of 0.8 da, and considered variable modifications for protein N-terminal acetylation, protein N-terminal acetylation and oxidation, glutamine to pyroglutamate conversion for peptide N-terminal glutamine residues, protein N-terminal methionine loss, protein N-terminal acetylation and methionine loss, and methionine oxidation, and constant modification for carbamidomethyl cysteine. Prospector data was filtered using a maximum protein expectation value of 0.01 and a maximum peptide expectation value of 0.05.

Raw mass spectrometry data was matched to peptide sequences using the Protein Prospector algorithm(*31*). The Prospector algorithm uses the MOWSE method to score each mass spectrum to database sequences meeting mass tolerance and in silico digestion criteria(*32*). An expectation value was calculated comparing the top-scoring match to a distribution of all other matches for each individual spectrum. A peptide discriminant score combined the MOWSE score and the expectation value into one score using a formula that was determined to best differentiate between true and false positive matches(*33*). Peptide discriminant scores were summed for all peptides detected within a

protein sequence to generate a protein discriminant score. The protein list was ranked by this protein discriminant score.

Immunoblotting Antibodies

The following commercial antibodies were used: anti-IFI16 (Santa Cruz Biotechnology, cat# sc-8023, 1:1000), anti-RIG-I (Cell Signaling Technology, cat#3743, 1:1000), anti-SAMHD1 (Sigma-Aldrich, cat#SAB1101454, 1:1000) and anti-beta-actin (Sigma-Aldrich, cat#A5316, 1:5000), anti-NLRP3 (Abcam, cat# ab17267, 1:1000), anti-DAI (Abcam, cat# ab81526, 1:1000), anti-histone H3 (Abcam, cat# ab1791, 1:1000), anti-AIM2 (Novus Biologicals, cat#H00009447-B01P, 1:1000), and anti-DNAPK-1 (Proteintech, cat#19983-1-AP, 1:1000). We thank Drs. D. Burdette and R. Vance (University of California, Berkeley) for providing anti-STING(*11*) antibody.

shRNA Constructs, Viruses, and Knockdown

shRNA target sequences from the RNAi consortium (TRC) shRNA library were imported into pSicoOligomaker 1.5 (http://web.mit.edu/jacks-lab/protocols/pSico.html) to generate sense and antisense oligos. Annealed shRNA oligos were ligated into pSicoR-mCherry after HpaI/XhoI digestion. Resulting constructs were either pseudotyped with VSV-G or HIV gp160 and packaged into the pCMVΔR8.91 lentivirus delivery system(*34*), concentrated at 20,000 rpm for 2 hours at 4C in a Beckman Coulter ultracentrifuge with a SW28 rotor, resuspended in cell culture media, and quantified via p24 ELISA (Perkin Elmer). See Supplementary Table 1 for a complete list of shRNA oligo sequences. shRNA knockdown was assessed by western blot and quantitative RT-PCR using TaqMan primer/probes (Applied Biosystems) on an ABI Prism 7900HT (Applied Biosystems).

Reagents

Caspase-1 inhibitor II (Ac-YVAD-CMK, EMD Millipore) and DNPK-1 inhibitors Nu7026 and Nu7441 (Tocris Biosciences) were solubilized in DMSO and used at a final concentration of 100uM, 10uM or 20uM, 1uM or 2uM, respectively. Intracellular detection of activated caspase-1 and IFNβ was performed 16 hours post co-culture with HIV-1 producing 293Ts. FLICA-660-Caspase-1 (YVAD-FMK, Immunochemistry Technologies) was used according to manufacturer's protocol. Anti-human-IFNβ FITC (Antigenix America) was used in conjunction with the intracellular fixation and permeabilization kit (eBioscience) according to manufacturer's protocol (without addition of protein transport inhibitors).

shRNA Knockdown in Primary CD4 T Cells

VLP-Vpx Method: Introduction of gp160 pseudotyped shRNA lentiviruses into tonsillar CD4 T cells was facilitated by incubation of 100ng viral like particles containing Vpx (VLP-Vpx) per 1 million cells at 4C for 20-30 minutes followed by spinoculation at 1,200xg for 2 hours at 4C in a V-bottom 96-well plate. 24 hours later the media was changed and shRNA lentiviral spinoculation was performed under the same conditions. Cells were incubated for 48 hours at 37C, and then treated with 5uM AZT. The same day HEK293Ts were plated at 50% confluency in 24-well plates and transfected using Fugene HD (Promega) with 50ng per well of NL4-3 HIV DNA or 5ng gp160 plus 45ng delta Env NL4-3 (single round HIV-1) where noted. Twenty-four hours post-transfection, 4 $x10^6$ tonsillar cells were co-cultured with HEK293T producing virus. Cell populations were visualized with CD4-APC (BD Biosciences, clone RPA-T4) and CD8-FITC (BD Biosciences, clone SK1) FACS antibodies incubated on ice for 30 minutes 24, 36, and 48

hours post-co-culture. Flow cytometry was performed on a FACS Calibur (B.D. Biosciences) and data were analyzed with FlowJo software (Tree Star). $CD4^+$ and mCherry postive populations were normalized based on $CD8^+$ T cell counts.

Activation-Rest Method: Splenic CD4 T cells were isolated by negative selection (Stem Cell Technologies) and activated with 100U/ml IL-2 (R&D Systems) and 10 ug/ml PHA (Sigma Aldrich) for 24–48 hours. Activated CD4 T cells were spinoculated with VSV-G pseudotyped shRNA lentiviruses at an MOI of 200 for 2 hours at 32C. Cells were cultured for 6–7 days in 100U/ml IL-2, and then mCherry positive cells were sorted on a FACSAria-II (B.D. Biosciences). Cells were expanded with CD3/CD28 Dynabeads (Life Technologies) 48 hours later, according to the manufacturer's protocol. Dynabeads were removed and cells were cultured in 30U/ml for 24 hours, then 10U/ml IL-2 for 3 days followed by co-culture with either donor-matched mCherry negative splenic CD4 T cells or tonsillar HLACs spinoculated with HIV-GFP reporter virus. Co-culture was performed 3 days post-spinoculation with either uninfected, infected, or infected plus 10uM efavirenz treated mCherry positive $CD4^+$ cells. Efavirenz was added to mCherry positive $CD4^+$ cells one hour before co-culture. mCherry positive $CD4^+$ versus mCherry negative $CD4^+$ T cell depletion was assessed by flow cytometry on a FACSCalibur (B.D. Biosciences) and normalized to fluorescent beads (Beckman Coulter). Activation status of mCherry positive cells was determined by CD25 expression (CD25-FITC, B.D. Biosciences, clone 2A3) and analyzed by flow cytometry on the day of co-culture.

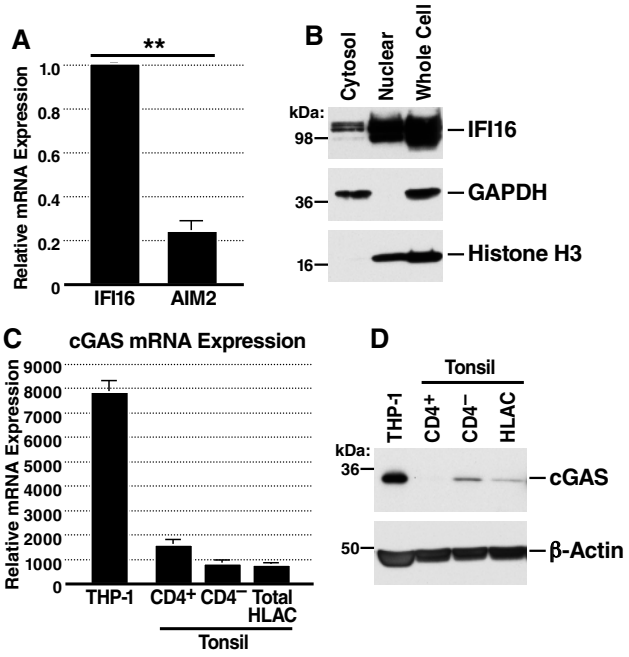

Fig. S1. Expression of innate immune nucleic acid sensors in tonsil cells. ***(A)*** *IFI16* mRNA is expressed at approximately four to five-fold higher levels than *AIM2* mRNA in tonsillar CD4 T cells. Isolated CD4 T cells were analyzed by quantitative RT-PCR for relative *IFI16* and *AIM2* mRNA levels. Data were normalized to *GAPDH*. (* indicates $p<0.05$, Student's t test). **(B)** Western blot analysis of IFI16, histone H3, and GAPDH in cytosol and nuclear fractions compared to whole cell lysate from tonsillar cultures. **(C)** Quantitative RT-PCR assessing cGAS mRNA levels in THP-1 cells, complete tonsillar HLAC cultures, or isolated $CD4^+$ or $CD4^-$ populations relative to levels in HEK293T cells. Data were normalized to *GAPDH*. **(D)** Cell types from **(C)** were analyzed by immunoblotting for cGAS and β-actin expression.

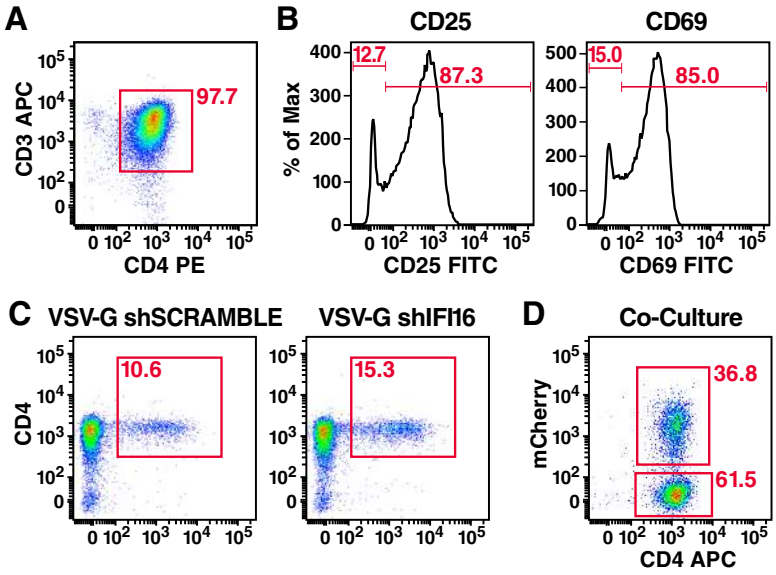

Fig. S2. Analysis of activated-rested splenic CD4 T cells. (A) Flow cytometry analysis of the purity of $CD3^+$ $CD4^+$ T cells following negative selection of spleen cells. **(B)** Activation markers CD25 and CD69 were analyzed by flow cytometry 48 hours following activation with 10ug/ml PHA and 100U/ml IL-2. **(C)** Activated and shRNA-lentivirus-infected CD4 T cells were analyzed by flow cytometry for mCherry expression and then sorted. **(D)** Flow cytometry analysis showing gating strategy for $mCherry^+$ $CD4^+$ T cells and $mCherry^-$ $CD4^+$ T cell populations post co-culture with productively infected HIV-1-GFP reporter $mCherry^-$ cells.

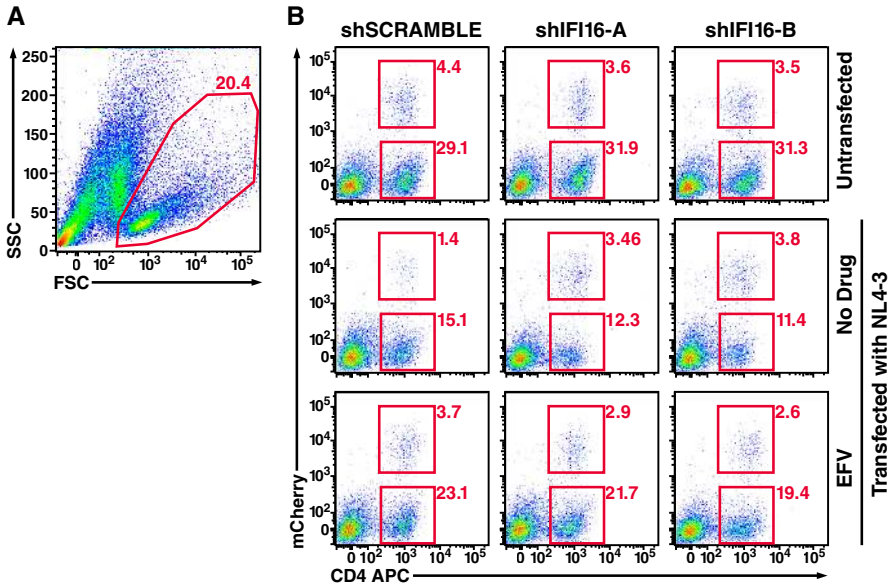

Fig. S3. Primary flow cytometry data for VLP-Vpx mediated lentiviral shRNA knockdown of IFI16. (A) Flow cytometry analysis showing live cell gates. **(B)** Flow cytometry analysis of mCherry^{+} CD4^{+} T cells and mCherry^{-} CD4^{+} T cells from HLAC spinoculated with VLP-Vpx pseudotyped with gp160 Env followed by spinoculation with shRNA lentiviruses pseudotyped with gp160 Env and packaged with shScramble, shIFI16-A, or shIFI16-B. Cells were co-cultured with 293Ts either untransfected or transfected with HIV-1 (NL4-3) in the presence or absence of the NNRTI efavirenz. Cells were analyzed 48 hours post-co-culture. All flow cytometry data are representative of three independent experiments from three different donors. Quantitation of these representative flow plots, including standard errors of the mean, and statistical analyses are provided in Figure 3B.

Target	Target Sequence 5′– 3′	Oligo Sequence 5′– 3′
SCRAMBLE	GTCAAGTCTCACTTGCGT	TGTCAAGTCTCACTTGCGTCTTCAAGAGAGACGCAAGTGAGACTTGACTTTTTTC
IFI16-A	CCACAATCTACGAAATTCA	TCCACAATCTACGAAATTCATTCAAGAGATGAATTTCGTAGATTGTGGTTTTTTC
IFI16-B	AAGAACATTGTTCTACTAA	TAAGAACATTGTTCTACTAATTCAAGAGATTAGTAGAACAATGTTCTTTTTTTTC
IFI16-C	GGGGTGAATTCACTTATTA	TGGGGTGAATTCACTTATTATTCAAGAGATAATAAGTGAATTCACCCCTTTTTTC
AIM2	TTGTATTGCTTATCAACTT	TTTGTATTGCTTATCAACTTTTCAAGAGAAAGTTGATAAGCAATACAATTTTTTC
IFIX	ATTCCTTTGACTGGAATGG	TATTCCTTTGACTGGAATGGTTCAAGAGACCATTCCAGTCAAAGGAATTTTTTTC
STING	GCAGAGCTATTTCTTCCAT	TGCAGAGCTATTTCTTCCATTCAAGAGATGGAAGGAAATAG CTCTGCTTTTTTC
DNPK-1	TACATTAGCATGTAAGCTC	TTACATTAGCATGTAAGCTCTTCAAGAGAGAGCTTACATGCTAATGTATTTTTTC

Table S1. shRNA target sequence and oligonucleotide sequences.

Supplementary Data File S1 (separate file)
Complete mass spectrometry protein list